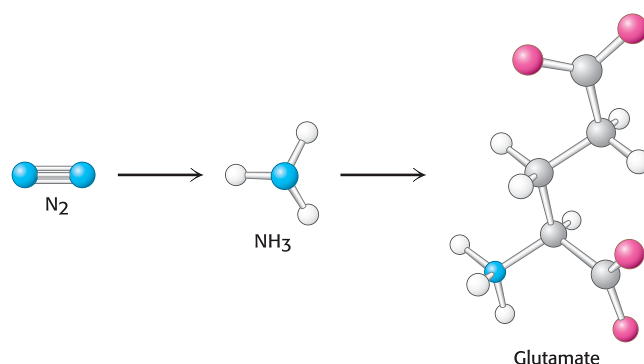
The Biosynthesis of Amino Acids



Nitrogen is a key component of amino acids. The atmosphere is rich in nitrogen gas (N_2), a very unreactive molecule. Certain organisms, such as bacteria that live in the root nodules of yellow clover, can convert nitrogen gas into ammonia (NH_3), which can then be used to synthesize, first, glutamate and then other amino acids. [(Left) Runk/Schoenburg/Grant Heilman Photography.]

The assembly of biological molecules, including proteins and nucleic acids, requires the generation of appropriate starting materials. We have already considered the assembly of carbohydrates in discussions of the Calvin cycle and the pentose phosphate pathway (Chapter 20). The present chapter and the next two examine the assembly of the other important building blocks—namely, amino acids, nucleotides, and lipids.

The pathways for the biosynthesis of these molecules are extremely ancient, going back to the last common ancestor of all living things. Indeed, these pathways probably predate many of the pathways of energy transduction discussed in Part II and may have provided key selective advantages in early evolution. Many of the intermediates in energy-transduction pathways play a role in biosynthesis as well. These common intermediates allow efficient interplay between energy-transduction (catabolic) and biosynthetic (anabolic) pathways. Thus, cells are able to balance the degradation of compounds for energy mobilization and the synthesis of starting materials for macromolecular construction.

We begin our consideration of biosynthesis with amino acids—the building blocks of proteins and the nitrogen source for many other important molecules, including nucleotides, neurotransmitters, and prosthetic groups such as porphyrins. Amino acid biosynthesis is intimately connected with nutrition because many higher organisms, including human beings, have

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Anabolism

Biosynthetic processes.

Catabolism

Degradative processes.

Derived from the Greek *ana,* "up"; *kata,* "down"; *ballein,* "to throw."

lost the ability to synthesize some amino acids and must therefore obtain adequate quantities of these essential amino acids in their diets. Furthermore, because some amino acid biosynthetic enzymes are absent in mammals but present in plants and microorganisms, they are useful targets for herbicides and antibiotics.

Amino acid synthesis requires solutions to three key biochemical problems

Nitrogen is an essential component of amino acids. Earth has an abundant supply of nitrogen, but it is primarily in the form of atmospheric nitrogen gas, a remarkably inert molecule. Thus, a fundamental problem for biological systems is to obtain nitrogen in a more usable form. This problem is solved by certain microorganisms capable of reducing the inert $N{\equiv}N$ molecule of nitrogen gas to two molecules of ammonia in one of the most amazing reactions in biochemistry. Nitrogen in the form of ammonia is the source of nitrogen for all the amino acids. The carbon backbones come from the glycolytic pathway, the pentose phosphate pathway, or the citric acid cycle.

In amino acid production, we encounter an important problem in biosynthesis—namely, stereochemical control. Because all amino acids except glycine are chiral, biosynthetic pathways must generate the correct isomer with high fidelity. In each of the 19 pathways for the generation of chiral amino acids, the stereochemistry at the α-carbon atom is established by a transamination reaction that includes pyridoxal phosphate (PLP). Almost all the transaminases that catalyze these reactions descend from a common ancestor, illustrating once again that effective solutions to biochemical problems are retained throughout evolution.

Biosynthetic pathways are often highly regulated such that building blocks are synthesized only when supplies are low. Very often, a high concentration of the final product of a pathway inhibits the activity of enzymes that function early in the pathway. Often present are allosteric enzymes capable of sensing and responding to concentrations of regulatory species. These enzymes are similar in functional properties to aspartate transcarbamylase and its regulators (Section 10.1). Feedback and allosteric mechanisms ensure that all 20 amino acids are maintained in sufficient amounts for protein synthesis and other processes.

24.1 Nitrogen Fixation: Microorganisms Use ATP and a Powerful Reductant to Reduce Atmospheric Nitrogen to Ammonia

The nitrogen in amino acids, purines, pyrimidines, and other biomolecules ultimately comes from atmospheric nitrogen, N_2. The biosynthetic process starts with the reduction of N_2 to NH_3 (ammonia), a process called *nitrogen fixation.* The extremely strong $N{\equiv}N$ bond, which has a bond energy of 940 kJ mol^{-1} (225 kcal mol^{-1}), is highly resistant to chemical attack. Indeed, Antoine Lavoisier named nitrogen gas "azote," *from Greek words* meaning "without life," because it is so unreactive. Nevertheless, the conversion of nitrogen and hydrogen to form ammonia is thermodynamically favorable; the reaction is difficult kinetically because intermediates along the reaction pathway are unstable.

Although higher organisms are unable to fix nitrogen, this conversion is carried out by some bacteria and archaea. Symbiotic *Rhizobium* bacteria invade the roots of leguminous plants and form root nodules in which they

fix nitrogen, supplying both the bacteria and the plants. The importance of nitrogen fixation by *diazotrophic (nitrogen-fixing) microorganisms* to the metabolism of all higher eukaryotes cannot be overstated: the amount of N_2 fixed by these species has been estimated to be 10^{11} kilograms per year, about 60% of Earth's newly fixed nitrogen. Lightning and ultraviolet radiation fix another 15%; the other 25% is fixed by industrial processes. The industrial process for nitrogen fixation devised by Fritz Haber in 1910 is still being used in fertilizer factories.

$$N_2 + 3\,H_2 \rightleftharpoons 2\,NH_3$$

The fixation of N_2 is typically carried out by mixing with H_2 gas over an iron catalyst at about 500°C and a pressure of 300 atmospheres.

To meet the kinetic challenge, the biological process of nitrogen fixation requires a complex enzyme with multiple redox centers. The *nitrogenase complex,* which carries out this fundamental transformation, consists of two proteins: a *reductase,* which provides electrons with high reducing power, and *nitrogenase,* which uses these electrons to reduce N_2 to NH_3. The transfer of electrons from the reductase to the nitrogenase component is coupled to the hydrolysis of ATP by the reductase (Figure 24.1). The nitrogenase complex is exquisitely sensitive to inactivation by O_2. Leguminous plants maintain a very low concentration of free O_2 in their root nodules by binding O_2 to *leghemoglobin,* a homolog of hemoglobin.

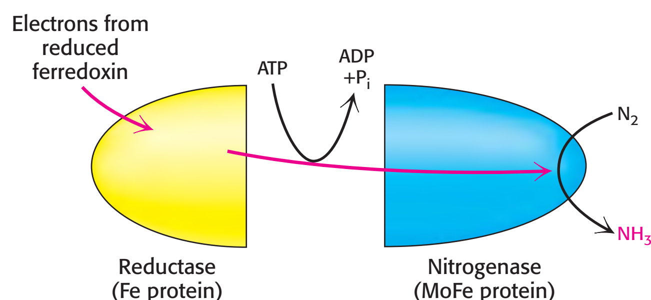
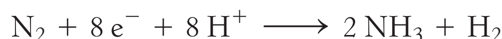


Figure 24.1 Nitrogen fixation. Electrons flow from ferredoxin to the reductase (iron protein, or Fe protein) to nitrogenase (molybdenum–iron protein, or MoFe protein) to reduce nitrogen to ammonia. ATP hydrolysis within the reductase drives conformational changes necessary for the efficient transfer of electrons.

In principle, the reduction of N_2 to NH_3 is a six-electron process.

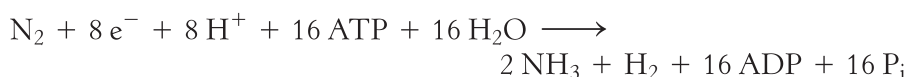
$$N_2 + 6\,e^- + 6\,H^+ \longrightarrow 2\,NH_3$$

However, the biological reaction always generates at least 1 mol of H_2 in addition to 2 mol of NH_3 for each mol of N≡N. Hence, an input of two additional electrons is required.



$$N_2 + 8\,e^- + 8\,H^+ \longrightarrow 2\,NH_3 + H_2$$

In most nitrogen-fixing microorganisms, *the eight high-potential electrons come from reduced ferredoxin,* generated by photosynthesis or oxidative processes. Two molecules of ATP are hydrolyzed for each electron transferred. Thus, *at least 16 molecules of ATP are hydrolyzed for each molecule of N_2 reduced.*



$$N_2 + 8\,e^- + 8\,H^+ + 16\,ATP + 16\,H_2O \longrightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,P_i$$

Again, ATP hydrolysis is not required to make nitrogen reduction favorable thermodynamically. Rather, it is essential to reduce the heights of activation barriers along the reaction pathway, thus making the reaction kinetically feasible.

The iron–molybdenum cofactor of nitrogenase binds and reduces atmospheric nitrogen

Both the reductase and the nitrogenase components of the complex are *iron–sulfur proteins,* in which iron is bonded to the sulfur atom of a cysteine residue and to inorganic sulfide. Recall that iron–sulfur clusters act as electron carriers (Section 18.3). The *reductase* (also called the *iron protein* or the *Fe protein*) is a dimer of identical 30-kd subunits bridged by a 4Fe-4S cluster (Figure 24.2).

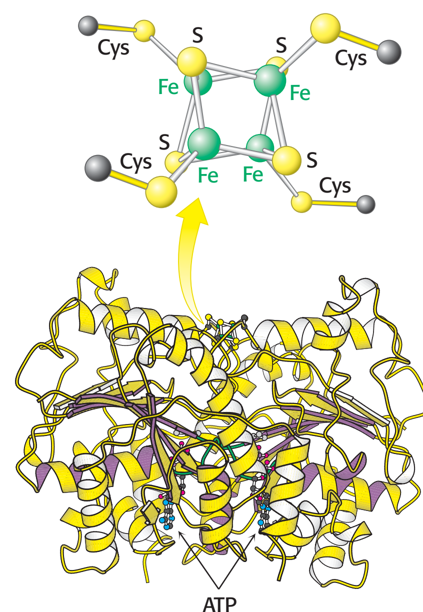


Figure 24.2 Fe Protein. This protein is a dimer composed of two polypeptide chains linked by a 4Fe-4S cluster. *Notice* that each monomer is a member of the P-loop NTPase family and contains an ATP-binding site. [Drawn from 1N2C.pdb.]

The role of the reductase is to transfer electrons from a suitable donor, such as reduced ferredoxin, to the nitrogenase component. The 4Fe-4S cluster carries the electrons, one at a time, to nitrogenase. The binding and hydrolysis of ATP triggers a conformational change that moves the reductase closer to the nitrogenase component, whence it is able to transfer its electron to the center of nitrogen reduction. The structure of the ATP-binding region reveals it to be a member of the P-loop NTPase family (Section 9.4) that is clearly related to the nucleotide-binding regions found in G proteins and related proteins. Thus, we see another example of how this domain has been recruited in evolution because of its ability to couple nucleoside triphosphate hydrolysis to conformational changes.

The nitrogenase component is an $\alpha_2\beta_2$ tetramer (240 kd), in which the α and β subunits are homologous to each other and structurally quite similar (Figure 24.3). Because molybdenum is present in this cluster, the nitrogenase component is also called the *molybdenum–iron protein* (MoFe protein). The FeMo cofactor consists of two M-3Fe-3S clusters, in which molybdenum occupies the M site in one cluster and iron occupies it in the other. The two clusters are joined by three sulfide ions and a central atom, the identity of which has not yet been conclusively established. The FeMo cofactor is also coordinated to a homocitrate moiety and to the α subunit through one histidine residue and one cysteinate residue. This cofactor is distinct from apparently all other molybdenum-containing enzymes.

Electrons from the reductase enter at the *P clusters,* which are located at the α–β interface. The role of the P clusters is to store electrons until they can be used productively to reduce nitrogen at the FeMo cofactor. *The FeMo cofactor is the site of nitrogen fixation.* One face of the FeMo cofactor is likely to be the site of nitrogen reduction. The electron-transfer reactions from the P cluster take place in concert with the binding of hydrogen ions to

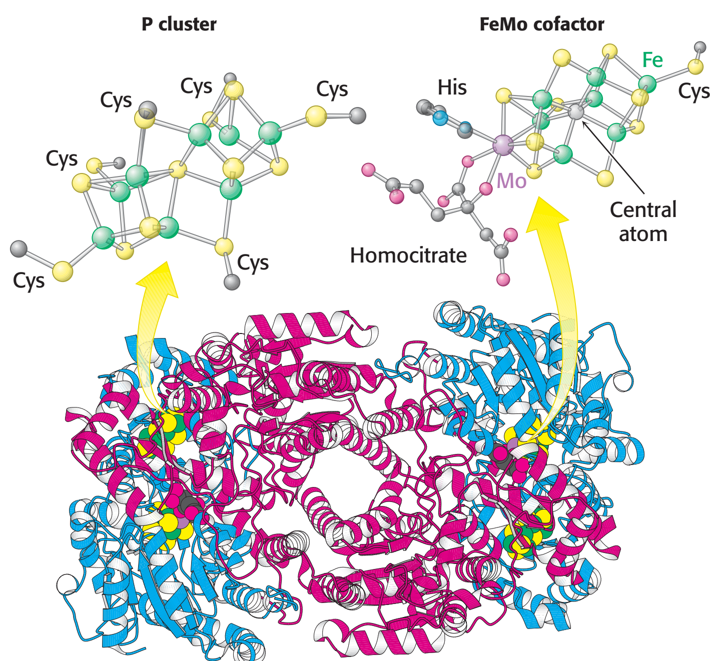


Figure 24.3 MoFe protein. This protein is a heterotetramer composed of two α subunits (red) and two β subunits (blue). *Notice* that the protein contains two copies each of two types of clusters: P clusters and FeMo cofactors. Each P cluster contains eight iron atoms (green) and seven sulfides linked to the protein by six cysteinate residues. Each FeMo cofactor contains one molybdenum atom, seven iron atoms, nine sulfides, a central atom, and a homocitrate, and is linked to the protein by one cysteinate residue and one histidine residue. [Drawn from 1M1N.pdb.]

nitrogen as it is reduced. Further studies are under way to elucidate the mechanism of this remarkable reaction.

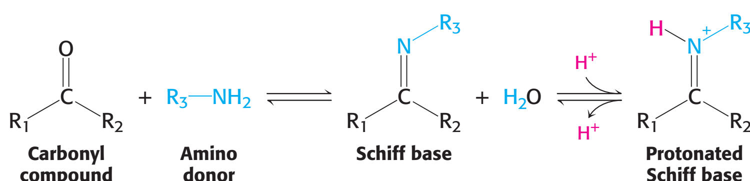
Ammonium ion is assimilated into an amino acid through glutamate and glutamine

The next step in the assimilation of nitrogen into biomolecules is the entry of NH_4^+ into amino acids. The amino acids *glutamate* and *glutamine* play pivotal roles in this regard, acting as nitrogen donors for most amino acids. The α-amino group of most amino acids comes from the α-amino group of glutamate by transamination (Section 23.3). Glutamine, the other major nitrogen donor, contributes its side-chain nitrogen atom in the biosynthesis of a wide range of important compounds, including the amino acids tryptophan and histidine.

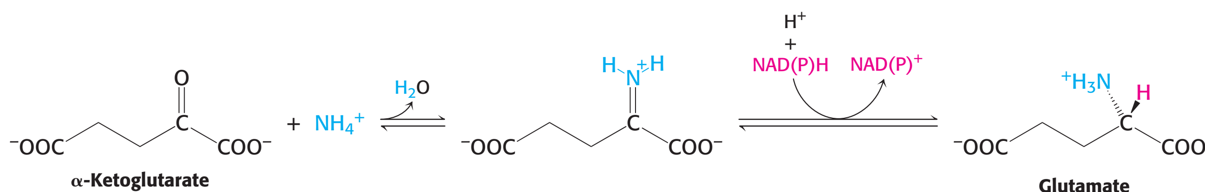
Glutamate is synthesized from NH_4^+ and α-ketoglutarate, a citric acid cycle intermediate, by the action of *glutamate dehydrogenase.* We have already encountered this enzyme in the degradation of amino acids (Section 23.3). Recall that NAD^+ is the oxidant in catabolism, whereas NADPH is the reductant in biosyntheses. Glutamate dehydrogenase is unusual in that it does not discriminate between NADH and NADPH, at least in some species.

$$NH_4^+ + \alpha\text{-ketoglutarate} + NADPH + H^+ \rightleftharpoons \text{glutamate} + NADP^+ + H_2O$$

The reaction proceeds in two steps. First, a Schiff base forms between ammonia and α-ketoglutarate. The formation of a Schiff base between an amine and a carbonyl compound is a key reaction that takes place at many stages of amino acid biosynthesis and degradation.



Schiff bases are easily protonated. In the second step, the protonated Schiff base is reduced by the transfer of a hydride ion from NADPH to form glutamate.



This reaction is crucial because it establishes the stereochemistry of the α-carbon atom (*S* absolute configuration) in glutamate. The enzyme binds the α-ketoglutarate substrate in such a way that hydride transferred from NAD(P)H is added to form the L isomer of glutamate (Figure 24.4). As we shall see, this stereochemistry is established for other amino acids by transamination reactions that rely on pyridoxal phosphate.

A second ammonium ion is incorporated into glutamate to form glutamine by the action of *glutamine synthetase.* This amidation is driven by the hydrolysis of ATP. ATP participates directly in the reaction by

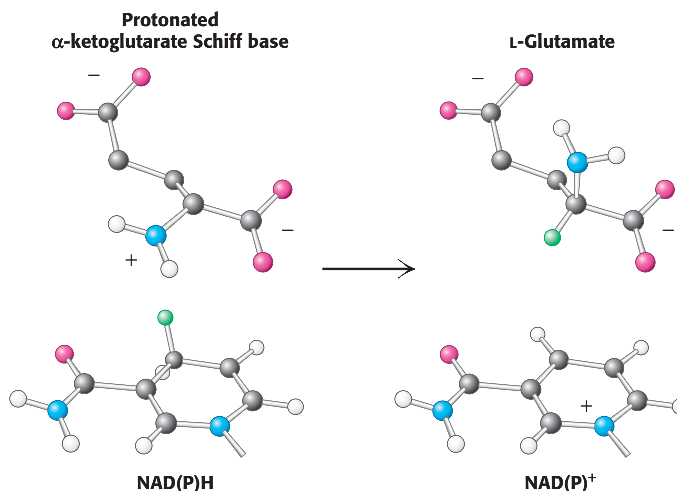
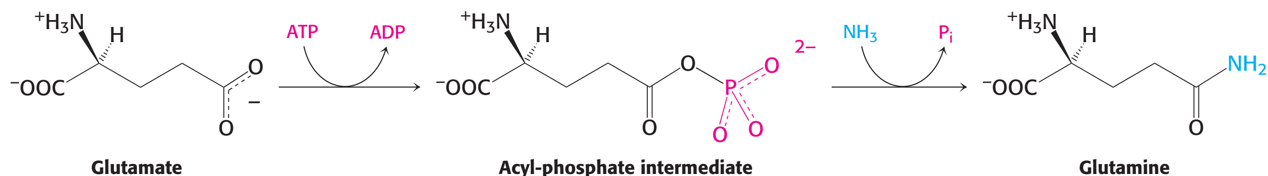


Figure 24.4 Establishment of chirality. In the active site of glutamate dehydrogenase, hydride transfer (green) from NAD(P)H to a specific face of the achiral protonated Schiff base of α-ketoglutarate establishes the L configuration of glutamate.

phosphorylating the side chain of glutamate to form an acyl-phosphate intermediate, which then reacts with ammonia to form glutamine.



A high-affinity ammonia-binding site is formed in the enzyme only after the formation of the acyl-phosphate intermediate. A specific site for ammonia binding is required to prevent attack by water from hydrolyzing the intermediate and wasting a molecule of ATP. The regulation of glutamine synthetase plays a critical role in controlling nitrogen metabolism (Section 24.3).

Glutamate dehydrogenase and glutamine synthetase are present in all organisms. Most prokaryotes also contain an evolutionarily unrelated enzyme, *glutamate synthase,* which catalyzes the reductive amination of α-ketoglutarate to glutamate. Glutamine is the nitrogen donor.

$$\alpha\text{-Ketoglutarate} + \text{glutamine} + \text{NADPH} + H^+ \rightleftharpoons 2\ \text{glutamate} + \text{NADP}^+$$

The side-chain amide of glutamine is hydrolyzed to generate ammonia within the enzyme, a recurring theme throughout nitrogen metabolism. *When NH_4^+ is limiting, most of the glutamate is made by the sequential action of glutamine synthetase and glutamate synthase.* The sum of these reactions is

$$NH_4^+ + \alpha\text{-ketoglutarate} + \text{NADPH} + \text{ATP} \longrightarrow \text{glutamate} + \text{NADP}^+ + \text{ADP} + P_i$$

Note that this stoichiometry differs from that of the glutamate dehydrogenase reaction in that ATP is hydrolyzed. Why do prokaryotes sometimes use this more expensive pathway? The answer is that the value of K_M of glutamate dehydrogenase for NH_4^+ is high (~1 mM), and so this enzyme is not saturated when NH_4^+ is limiting. In contrast, glutamine synthetase has very high affinity for NH_4^+. Thus, ATP hydrolysis is required to capture ammonia when it is scarce.

24.2 Amino Acids Are Made from Intermediates of the Citric Acid Cycle and Other Major Pathways

Thus far, we have considered the conversion of N_2 into NH_4^+ and the assimilation of NH_4^+ into glutamate and glutamine. We turn now to the biosynthesis of the other amino acids, the majority of which obtain their nitrogen from glutamate or glutamine. The pathways for the biosynthesis of amino acids are diverse. However, they have an important common feature: *their carbon skeletons come from intermediates of glycolysis, the pentose phosphate pathway, or the citric acid cycle.* On the basis of these starting materials, amino acids can be grouped into six biosynthetic families (Figure 24.5).

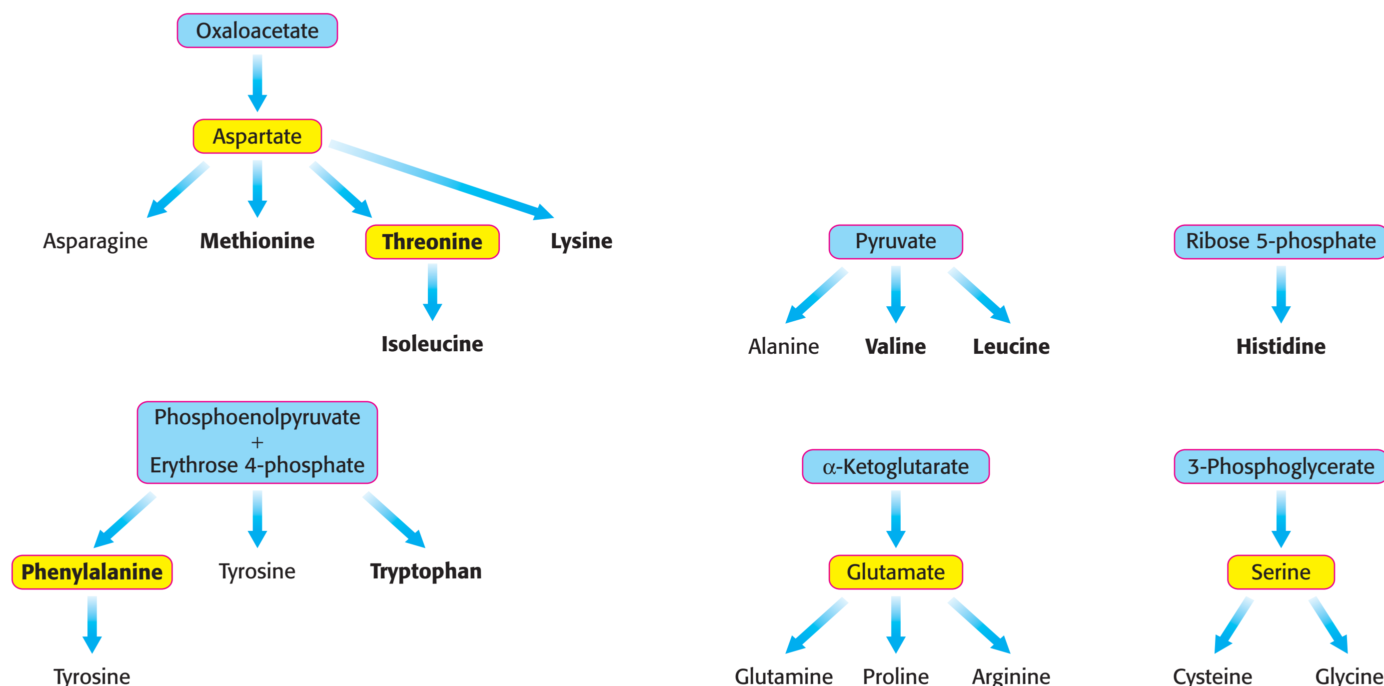


Figure 24.5 Biosynthetic families of amino acids in bacteria and plants. Major metabolic precursors are shaded blue. Amino acids that give rise to other amino acids are shaded yellow. Essential amino acids are in boldface type.

Human beings can synthesize some amino acids but must obtain others from the diet

Most microorganisms, such as *E. coli,* can synthesize the entire basic set of 20 amino acids, whereas human beings cannot make 9 of them. The amino acids that must be supplied in the diet are called *essential amino acids,* whereas the others are termed *nonessential amino acids* (Table 24.1). These designations refer to the needs of an organism under a particular set of conditions. For example, enough arginine is synthesized by the urea cycle to meet the needs of an adult but perhaps not those of a growing child. A deficiency of even one amino acid results in a *negative nitrogen balance.* In this state, more protein is degraded than is synthesized, and so more nitrogen is excreted than is ingested.

The nonessential amino acids are synthesized by quite simple reactions, whereas the pathways for the formation of the essential amino acids are quite complex. For example, the nonessential amino acids *alanine* and *aspartate* are synthesized in a single step from pyruvate and oxaloacetate, respectively. In contrast, the pathways for the essential amino acids require from 5 to 16 steps (Figure 24.6). The sole exception to this pattern is

Table 24.1 Basic set of 20 amino acids

Nonessential	Essential
Alanine	Histidine
Arginine	Isoleucine
Asparagine	Leucine
Aspartate	Lysine
Cysteine	Methionine
Glutamate	Phenylalanine
Glutamine	Threonine
Glycine	Tryptophan
Proline	Valine
Serine	
Tyrosine	

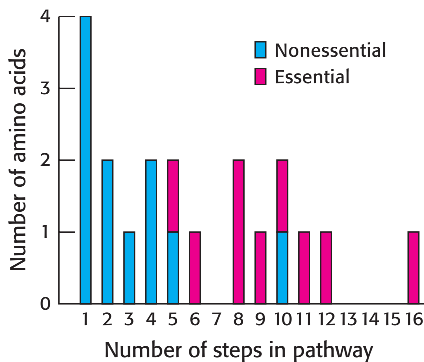
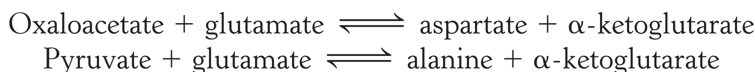


Figure 24.6 Essential and nonessential amino acids. Some amino acids are nonessential to human beings because they can be biosynthesized in a small number of steps. Those amino acids requiring a large number of steps for their synthesis are essential in the diet because some of the enzymes for these steps have been lost in the course of evolution.

arginine, inasmuch as the synthesis of this nonessential amino acid de novo requires 10 steps. Typically, though, it is made in only 3 steps from ornithine as part of the urea cycle. Tyrosine, classified as a nonessential amino acid because it can be synthesized in 1 step from phenylalanine, requires 10 steps to be synthesized from scratch and is essential if phenylalanine is not abundant. We begin with the biosynthesis of nonessential amino acids.

Aspartate, alanine, and glutamate are formed by the addition of an amino group to an alpha-ketoacid

Three α-ketoacids—α-ketoglutarate, oxaloacetate, and pyruvate—can be converted into amino acids in one step through the addition of an amino group. We have seen that α-ketoglutarate can be converted into glutamate by reductive amination (p. 709). The amino group from glutamate can be transferred to other α-ketoacids by transamination reactions. Thus, aspartate and alanine can be made from the addition of an amino group to oxaloacetate and pyruvate, respectively.

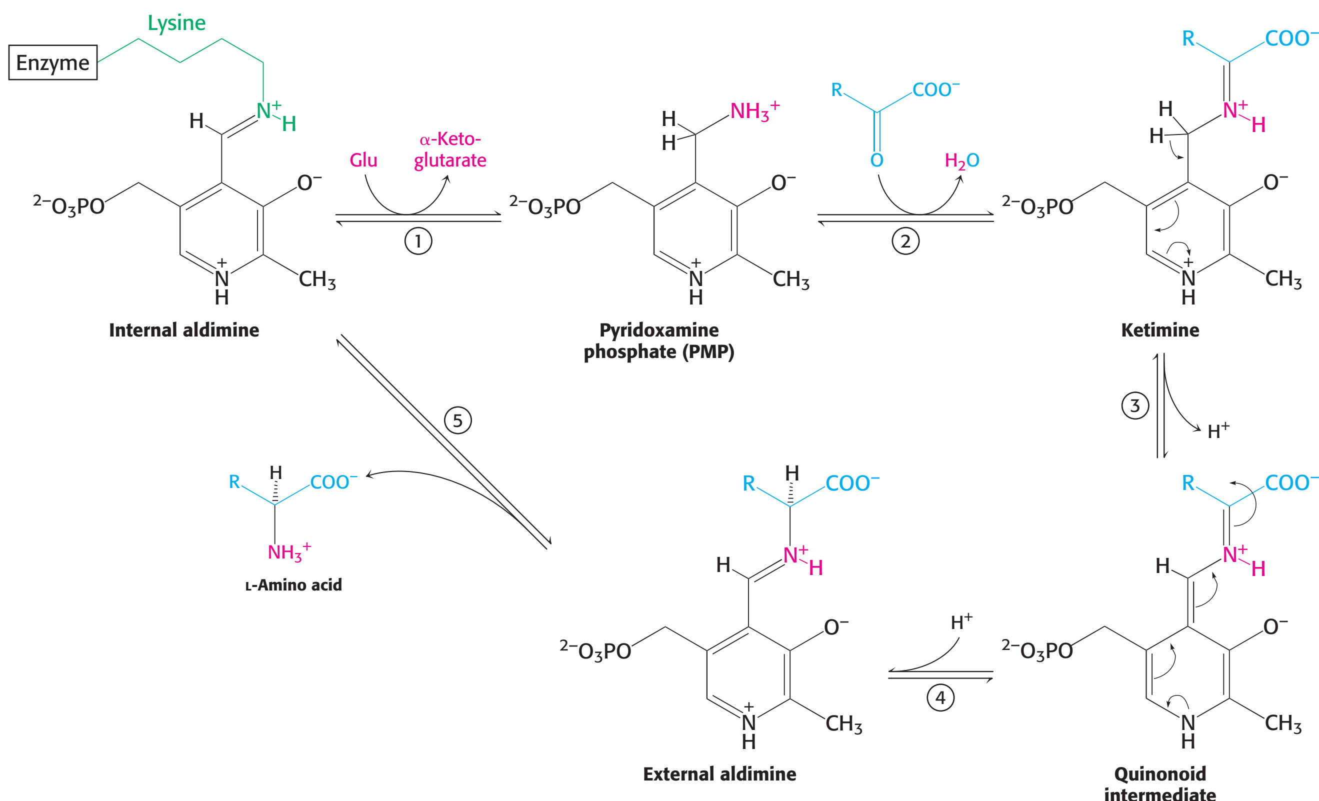


$$\text{Oxaloacetate} + \text{glutamate} \rightleftharpoons \text{aspartate} + \alpha\text{-ketoglutarate}$$
$$\text{Pyruvate} + \text{glutamate} \rightleftharpoons \text{alanine} + \alpha\text{-ketoglutarate}$$

These reactions are carried out by *pyridoxal phosphate-dependent transaminases.* Transamination reactions are required for the synthesis of most amino acids.

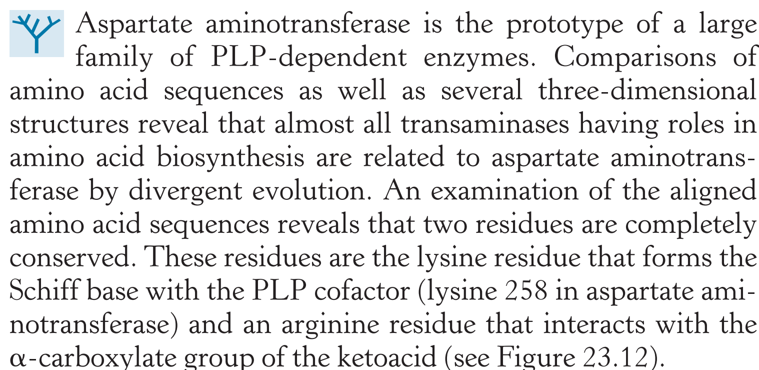
In Section 23.3, we considered the mechanism of transaminases as applied to the metabolism of amino acids. Let us review the transaminase mechanism as it operates in the *biosynthesis* of amino acids (see Figure 23.11). The reaction pathway begins with *pyridoxal phosphate* in a Schiff-base linkage with lysine at the transaminase active site, forming an internal aldimine (Figure 24.7). An amino group is transferred from glutamate to form pyridoxamine phosphate (PMP), the actual amino donor, in a multistep process. PMP then reacts with an incoming α-ketoacid to form a ketimine. Proton

Figure 24.7 Amino acid biosynthesis by transamination. (1) Within a transaminase, the internal aldimine is converted into pyridoxamine phosphate (PMP) by reaction with glutamate in a multistep process not shown. (2) PMP then reacts with an α-ketoacid to generate a ketimine. (3) This intermediate is converted into a quinonoid intermediate (4), which in turn yields an external aldimine. (5) The aldimine is cleaved to release the newly formed amino acid to complete the cycle.



loss forms a quinonoid intermediate that then accepts a proton at a different site to form an external aldimine. The newly formed amino acid is released with the concomitant formation of the internal aldimine.

A common step determines the chirality of all amino acids



Aspartate aminotransferase is the prototype of a large family of PLP-dependent enzymes. Comparisons of amino acid sequences as well as several three-dimensional structures reveal that almost all transaminases having roles in amino acid biosynthesis are related to aspartate aminotransferase by divergent evolution. An examination of the aligned amino acid sequences reveals that two residues are completely conserved. These residues are the lysine residue that forms the Schiff base with the PLP cofactor (lysine 258 in aspartate aminotransferase) and an arginine residue that interacts with the α-carboxylate group of the ketoacid (see Figure 23.12).

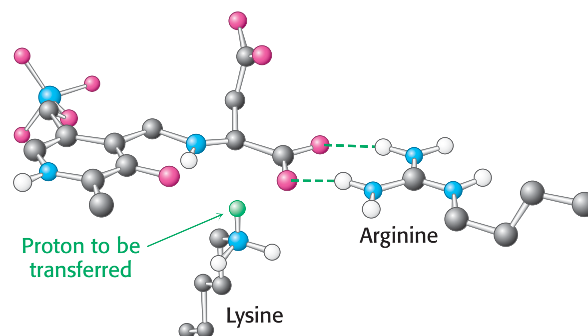
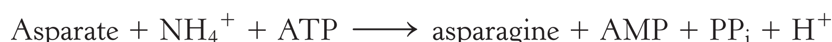


Figure 24.8 Stereochemistry of proton addition. In a transaminase active site, the addition of a proton from the lysine residue to the bottom face of the quinonoid intermediate determines the L configuration of the amino acid product. The conserved arginine residue interacts with the α-carboxylate group and helps establish the appropriate geometry of the quinonoid intermediate.

An essential step in the transamination reaction is the protonation of the quinonoid intermediate to form the external aldimine. *The chirality of the amino acid formed is determined by the direction from which this proton is added to the quinonoid form* (Figure 24.8). The interaction between the conserved arginine residue and the α-carboxylate group helps orient the substrate so that the lysine residue transfers a proton to the bottom face of the quinonoid intermediate, generating an aldimine with an L configuration at the C_α center.

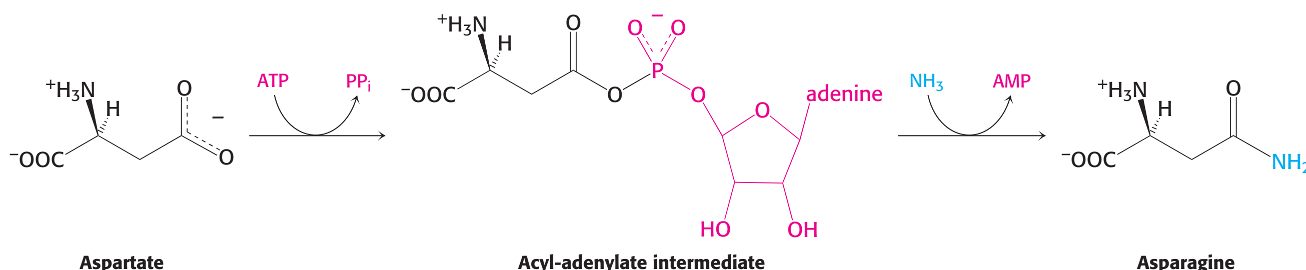
The formation of asparagine from aspartate requires an adenylated intermediate

The formation of asparagine from aspartate is chemically analogous to the formation of glutamine from glutamate. Both transformations are amidation reactions and both are driven by the hydrolysis of ATP. The actual reactions are different, however. In bacteria, the reaction for the asparagine synthesis is



$$\text{Asparate} + NH_4^+ + \text{ATP} \longrightarrow \text{asparagine} + \text{AMP} + PP_i + H^+$$

Thus, the products of ATP hydrolysis are AMP and PP_i rather than ADP and P_i. Aspartate is activated by adenylation rather than by phosphorylation.

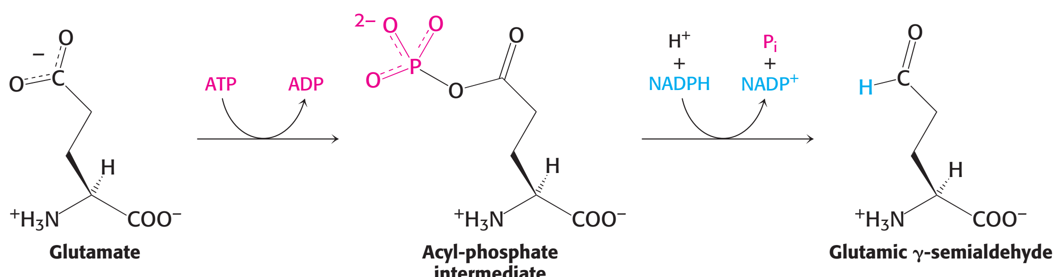


We have encountered this mode of activation in fatty acid degradation and will see it again in lipid and protein synthesis.

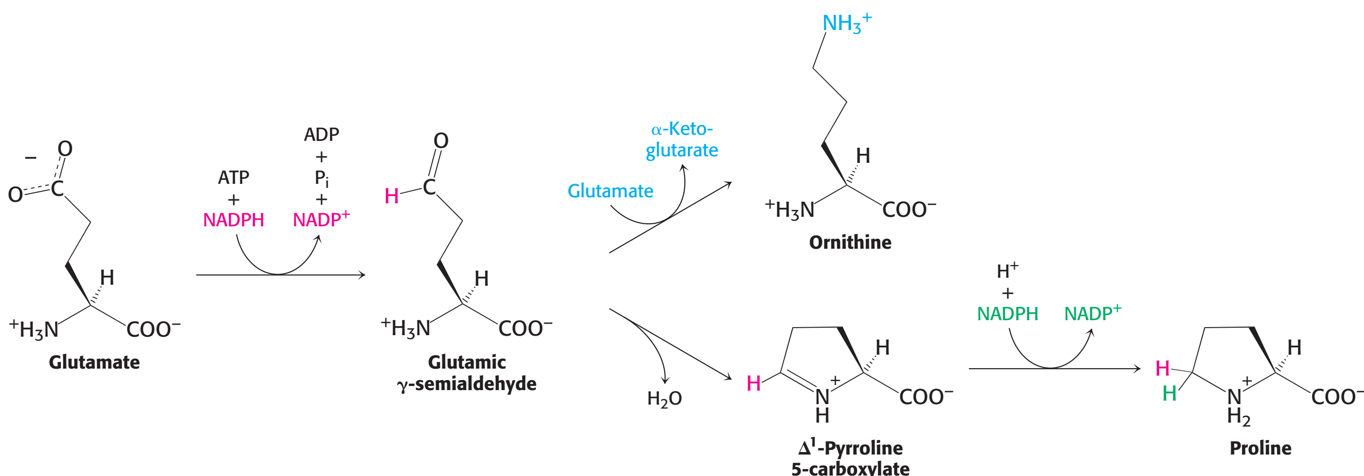
In mammals, the nitrogen donor for asparagine is glutamine rather than ammonia as in bacteria. Ammonia is generated by hydrolysis of the side chain of glutamine and directly transferred to activated aspartate, bound in the active site. An advantage is that the cell is not directly exposed to NH_4^+, which is toxic at high levels to human beings and other mammals. *The use of glutamine hydrolysis as a mechanism for generating ammonia for use within the same enzyme is a motif common throughout biosynthetic pathways.*

Glutamate is the precursor of glutamine, proline, and arginine

The synthesis of glutamate by the reductive amination of α-ketoglutarate has already been discussed, as has the conversion of glutamate into glutamine (p. 710). Glutamate is the precursor of two other nonessential amino acids: *proline* and *arginine.* First, the γ-carboxyl group of glutamate reacts with ATP to form an acyl phosphate. This mixed anhydride is then reduced by NADPH to an aldehyde.

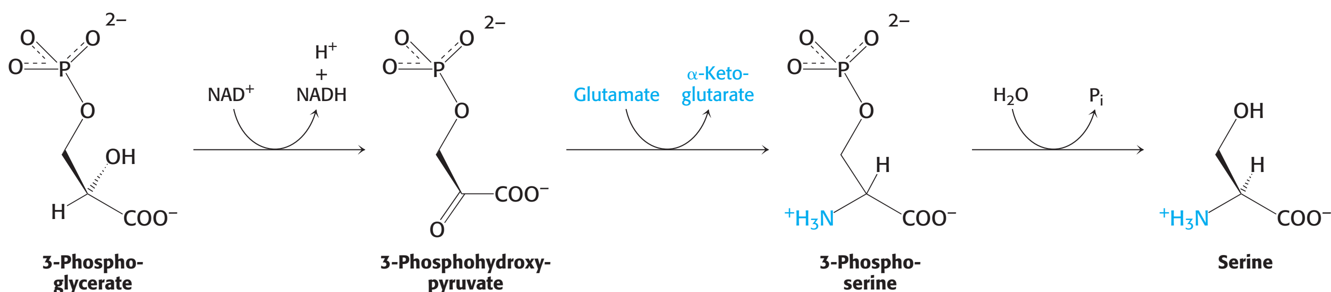


Glutamic γ-semialdehyde cyclizes with a loss of H_2O in a nonenzymatic process to give Δ^1-pyrroline 5-carboxylate, which is reduced by NADPH to proline. Alternatively, the semialdehyde can be transaminated to ornithine, which is converted in several steps into arginine (see Figure 23.17).



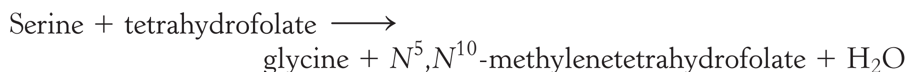
3-Phosphoglycerate is the precursor of serine, cysteine, and glycine

Serine is synthesized from 3-phosphoglycerate, an intermediate in glycolysis. The first step is an oxidation to 3-phosphohydroxypyruvate. This α-ketoacid is transaminated to 3-phosphoserine, which is then hydrolyzed to serine.



Serine is the precursor of *cysteine* and *glycine.* As we shall see, the conversion of serine into cysteine requires the substitution of a sulfur atom derived

from methionine for the side-chain oxygen atom. In the formation of glycine, the side-chain methylene group of serine is transferred to *tetrahydrofolate,* a carrier of one-carbon units that will be discussed shortly.



$$\text{Serine} + \text{tetrahydrofolate} \longrightarrow \text{glycine} + N^5,N^{10}\text{-methylenetetrahydrofolate} + H_2O$$

This interconversion is catalyzed by *serine hydroxymethyltransferase,* a PLP enzyme that is homologous to aspartate aminotransferase. The formation of the Schiff base of serine renders the bond between its α- and β-carbon atoms susceptible to cleavage, enabling the transfer of the β-carbon to tetrahydrofolate and producing the Schiff base of glycine.

Tetrahydrofolate carries activated one-carbon units at several oxidation levels

Tetrahydrofolate (also called *tetrahydropteroylglutamate*) is a highly versatile carrier of activated one-carbon units. This cofactor consists of three groups: a substituted pteridine, *p*-aminobenzoate, and a chain of one or more glutamate residues (Figure 24.9). Mammals can synthesize the pteridine ring, but they are unable to conjugate it to the other two units. They obtain tetrahydrofolate from their diets or from microorganisms in their intestinal tracts.

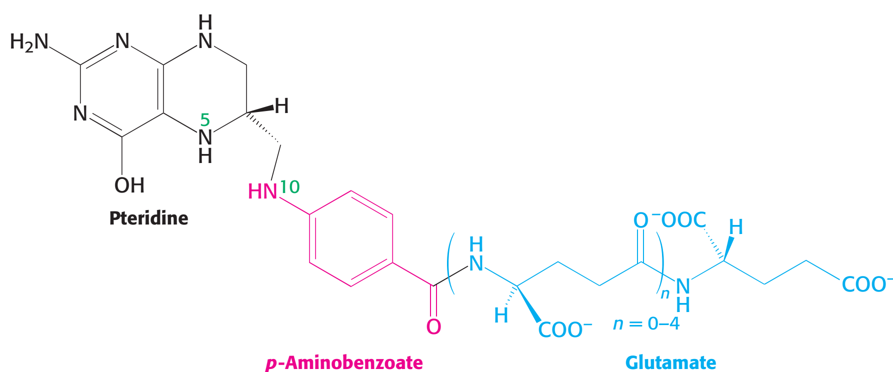
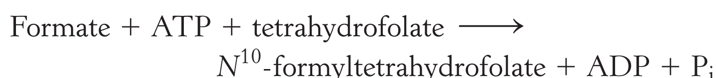


Figure 24.9 Tetrahydrofolate. This cofactor includes three components: a pteridine ring, *p*-aminobenzoate, and one or more glutamate residues.

The one-carbon group carried by tetrahydrofolate is bonded to its N-5 or N-10 nitrogen atom (denoted as N^5 and N^{10}) or to both. This unit can exist in three oxidation states (Table 24.2). The most-reduced form carries a *methyl* group, whereas the intermediate form carries a *methylene* group. More-oxidized forms carry a *formyl, formimino,* or *methenyl* group. The fully oxidized one-carbon unit, CO_2, is carried by biotin rather than by tetrahydrofolate.

The one-carbon units carried by tetrahydrofolate are interconvertible (Figure 24.10). N^5,N^{10}-*Methylene*tetrahydrofolate can be reduced to N^5-*methyl*tetrahydrofolate or oxidized to N^5,N^{10}-*methenyl*tetrahydrofolate. N^5,N^{10}-*Methenyl*tetrahydrofolate can be converted into N^5-*formimino*tetrahydrofolate or N^{10}-*formyl*tetrahydrofolate, both of which are at the same oxidation level. N^{10}-*Formyl*tetrahydrofolate can also be synthesized from tetrahydrofolate, formate, and ATP.



$$\text{Formate} + \text{ATP} + \text{tetrahydrofolate} \longrightarrow N^{10}\text{-formyltetrahydrofolate} + \text{ADP} + P_i$$

N^5-*Formyl*tetrahydrofolate can be reversibly isomerized to N^{10}-*formyl*tetrahydrofolate or it can be converted into N^5,N^{10}-*methenyl*tetrahydrofolate.

Table 24.2 One-carbon groups carried by tetrahydrofolate

	Group	
Oxidation state	Formula	Name
Most reduced (= methanol)	$—CH_3$	Methyl
Intermediate (= formaldehyde)	$—CH_2—$	Methylene
Most oxidized (= formic acid)	—CHO	Formyl
	—CHNH	Formimino
	—CH=	Methenyl

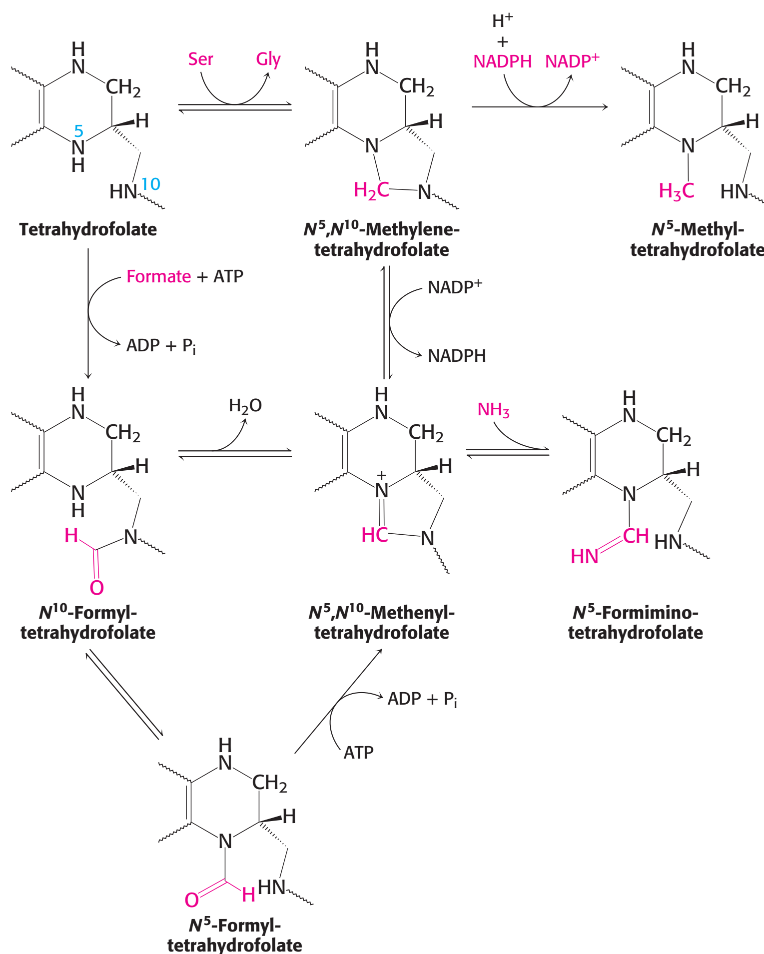


Figure 24.10 Conversions of one-carbon units attached to tetrahydrofolate.

These tetrahydrofolate derivatives serve as donors of one-carbon units in a variety of biosyntheses. Methionine is regenerated from homocysteine by transfer of the methyl group of N^5-methyltetrahydrofolate, as will be discussed shortly. We shall see in Chapter 25 that some of the carbon atoms of *purines* are acquired from derivatives of N^{10}-formyltetrahydrofolate. The methyl group of *thymine,* a pyrimidine, comes from N^5, N^{10}-methylenetetrahydrofolate. This tetrahydrofolate derivative can also donate a one-carbon unit in an alternative synthesis of *glycine* that starts with CO_2 and NH_4^+, a reaction catalyzed by *glycine synthase* (called the *glycine cleavage enzyme* when it operates in the reverse direction).

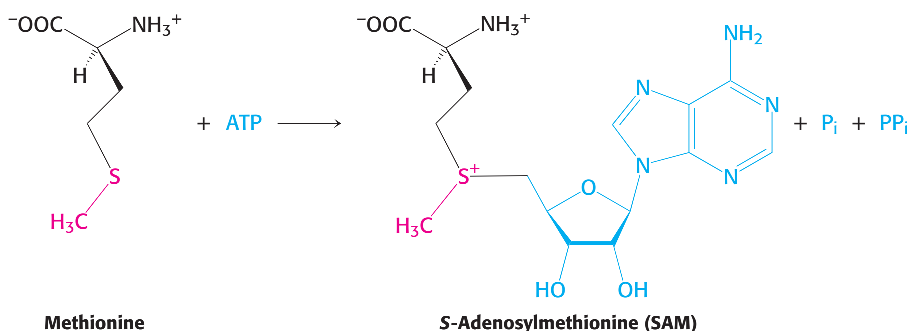
$$CO_2 + NH_4^+ + N^5,N^{10}\text{-methylenetetrahydrofolate} + NADH \rightleftharpoons \text{glycine} + \text{tetrahydrofolate} + NAD^+$$

Thus, one-carbon units at each of the three oxidation levels are utilized in biosyntheses. Furthermore, *tetrahydrofolate serves as an acceptor of one-carbon units in degradative reactions.* The major source of one-carbon units is the facile conversion of serine into glycine by serine hydroxymethyltransferase (p. 715), which yields N^5,N^{10}-methylenetetrahydrofolate. Serine can be derived from 3-phosphoglycerate, and so *this pathway enables one-carbon units to be formed de novo from carbohydrates.*

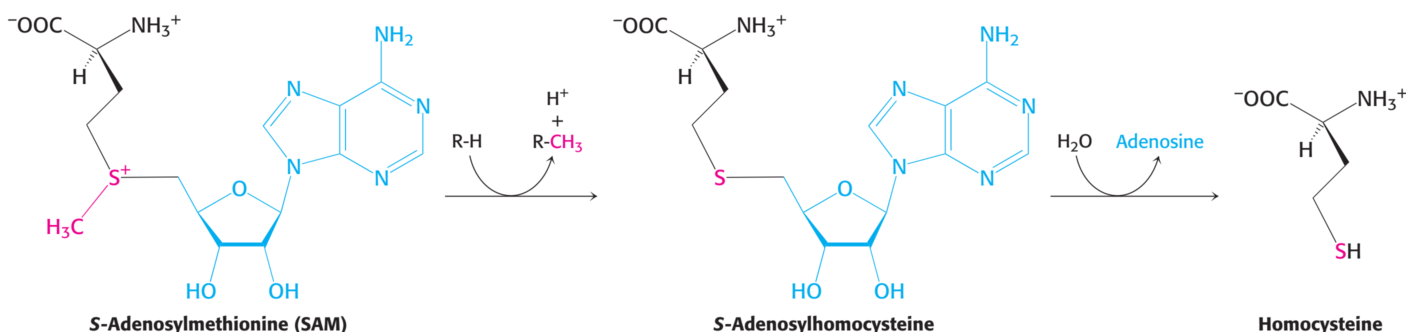
S-Adenosylmethionine is the major donor of methyl groups

Tetrahydrofolate can carry a methyl group on its N-5 atom, but its transfer potential is not sufficiently high for most biosynthetic methylations. Rather, the activated methyl donor is usually S-*adenosylmethionine* (SAM), which is

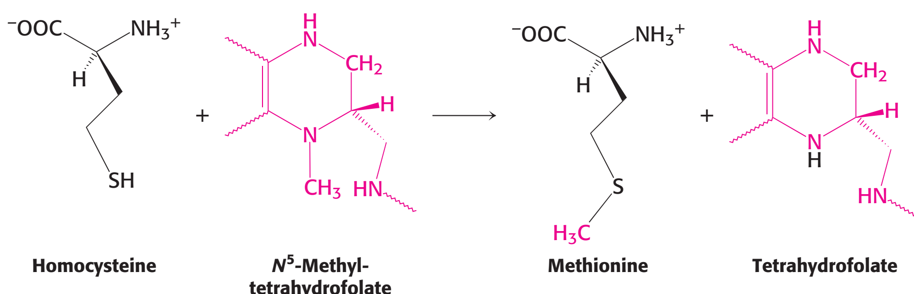
synthesized by the transfer of an adenosyl group from ATP to the sulfur atom of methionine.



The methyl group of the methionine unit is activated by the positive charge on the adjacent sulfur atom, which makes the molecule much more reactive than N^5-methyltetrahydrofolate. The synthesis of *S*-adenosylmethionine is unusual in that the triphosphate group of ATP is split into pyrophosphate and orthophosphate; the pyrophosphate is subsequently hydrolyzed to two molecules of P_i. S-*Adenosylhomocysteine* is formed when the methyl group of *S*-adenosylmethionine is transferred to an acceptor. *S*-Adenosylhomocysteine is then hydrolyzed to *homocysteine* and adenosine.



Methionine can be regenerated by the transfer of a methyl group to homocysteine from N^5-methyltetrahydrofolate, a reaction catalyzed by *methionine synthase* (also known as *homocysteine methyltransferase*).



The coenzyme that mediates this transfer of a methyl group is *methylcobalamin,* derived from vitamin B_{12}. In fact, this reaction and the rearrangement of L-methylmalonyl CoA to succinyl CoA (p. 650), catalyzed by a homologous enzyme, are the only two B_{12}-dependent reactions known to take place in mammals. Another enzyme that converts homocysteine into methionine without vitamin B_{12} also is present in many organisms.

These reactions constitute the *activated methyl cycle* (Figure 24.11). Methyl groups enter the cycle in the conversion of homocysteine into

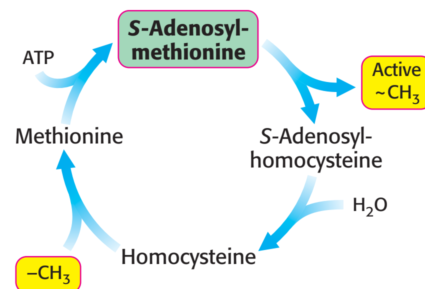


Figure 24.11 Activated methyl cycle. The methyl group of methionine is activated by the formation of *S*-adenosylmethionine.

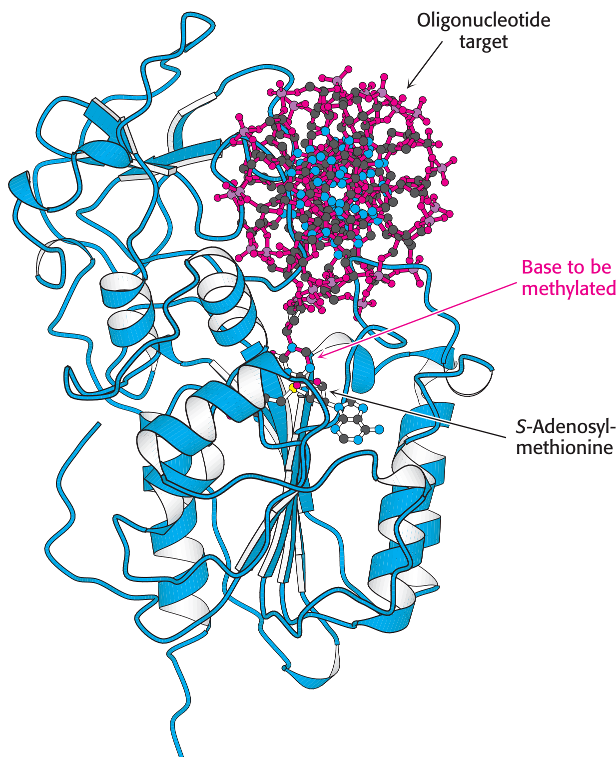
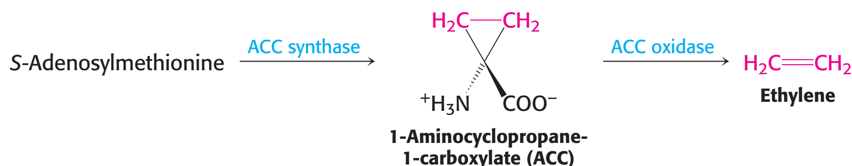


Figure 24.12 DNA methylation. The structure of a DNA methylase bound to an oligonucleotide target shows that the base to be methylated is flipped out of the DNA helix into the active site of a SAM-dependent methylase. [Drawn from 10MH.pdb.]

methionine and are then made highly reactive by the addition of an adenosyl group, which makes the sulfur atoms positively charged and the methyl groups much more electrophilic. The high transfer potential of the *S*-methyl group enables it to be transferred to a wide variety of acceptors.

Among the acceptors modified by *S*-adenosylmethionine are specific bases in DNA. The methylation of DNA protects bacterial DNA from cleavage by restriction enzymes (Section 9.3). The base to be methylated is flipped out of the DNA double helix into the active site of a DNA methylase, where it can accept a methyl group from *S*-adenosylmethionine (Figure 24.12). A recurring *S*-adenosylmethionine-binding domain is present in many SAM-dependent methylases.

S-Adenosylmethionine is also the precursor of *ethylene,* a gaseous plant hormone that induces the ripening of fruit. *S*-Adenosylmethionine is cyclized to a cyclopropane derivative that is then oxidized to form ethylene. The Greek philosopher Theophrastus recognized more than 2000 years ago that sycamore figs do not ripen unless they are scraped with an iron claw. The reason is now known: *wounding triggers ethylene production, which in turn induces ripening.* Much effort is being made to understand this biosynthetic pathway because ethylene is a culprit in the spoilage of fruit.



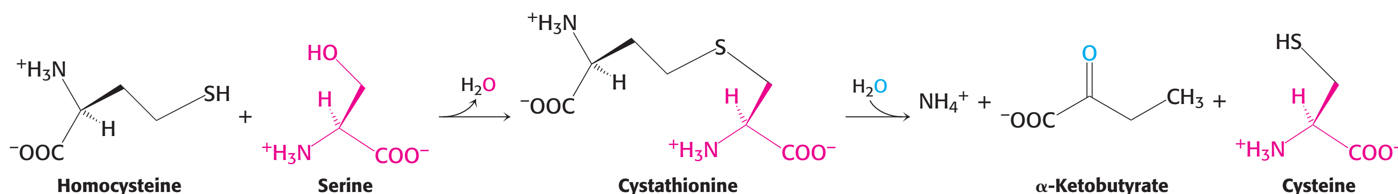
Cysteine is synthesized from serine and homocysteine

In addition to being a precursor of methionine in the activated methyl cycle, homocysteine is an intermediate in the synthesis of cysteine. Serine and homocysteine condense to form *cystathionine.* This reaction is catalyzed by *cystathionine β-synthase.* Cystathionine is then deaminated and cleaved to

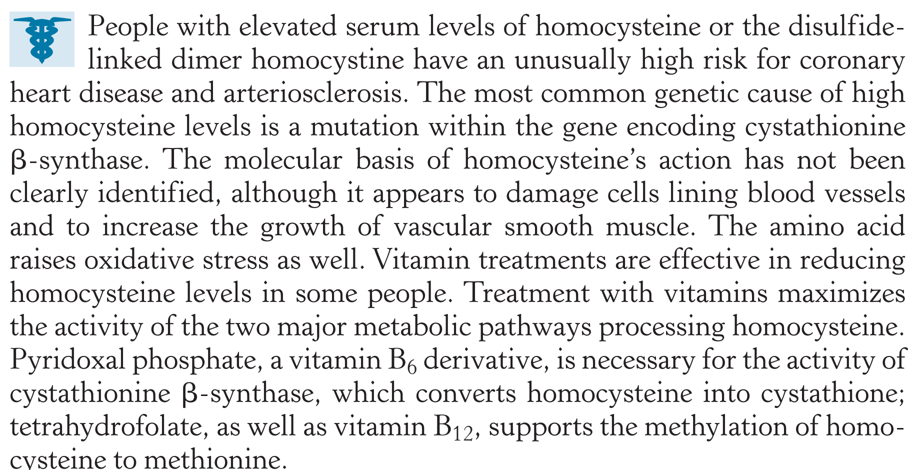
cysteine and α-ketobutyrate by *cystathionine γ-lyase*, or *cystathionase*. Both of these enzymes utilize PLP and are homologous to aspartate aminotransferase. The net reaction is

$$\text{Homocysteine} + \text{serine} \rightleftharpoons \text{cysteine} + \alpha\text{-ketobutyrate} + NH_4^+$$

Note that the sulfur atom of cysteine is derived from homocysteine, whereas the carbon skeleton comes from serine.



High homocysteine levels correlate with vascular disease



People with elevated serum levels of homocysteine or the disulfide-linked dimer homocystine have an unusually high risk for coronary heart disease and arteriosclerosis. The most common genetic cause of high homocysteine levels is a mutation within the gene encoding cystathionine β-synthase. The molecular basis of homocysteine's action has not been clearly identified, although it appears to damage cells lining blood vessels and to increase the growth of vascular smooth muscle. The amino acid raises oxidative stress as well. Vitamin treatments are effective in reducing homocysteine levels in some people. Treatment with vitamins maximizes the activity of the two major metabolic pathways processing homocysteine. Pyridoxal phosphate, a vitamin B_6 derivative, is necessary for the activity of cystathionine β-synthase, which converts homocysteine into cystathione; tetrahydrofolate, as well as vitamin B_{12}, supports the methylation of homocysteine to methionine.

Shikimate and chorismate are intermediates in the biosynthesis of aromatic amino acids

We turn now to the biosynthesis of essential amino acids. These amino acids are synthesized by plants and microorganisms, and those in the human diet are ultimately derived primarily from plants. The essential amino acids are formed by much more complex routes than are the nonessential amino acids. The pathways for the synthesis of aromatic amino acids in bacteria have been selected for discussion here because they are well understood and exemplify recurring mechanistic motifs.

Phenylalanine, tyrosine, and tryptophan are synthesized by a common pathway in *E. coli* (Figure 24.13). The initial step is the condensation of phosphoenolpyruvate (a glycolytic intermediate) with erythrose 4-phosphate (a pentose phosphate pathway intermediate). The resulting seven-carbon open-chain sugar is oxidized, loses its phosphoryl group, and cyclizes to 3-dehydroquinate. Dehydration then yields 3-dehydroshikimate, which is reduced by NADPH to shikimate. The phosphorylation of shikimate by ATP gives shikimate 3-phosphate, which condenses with a second molecule of phosphoenolpyruvate. The resulting 5-enolpyruvyl intermediate loses its phosphoryl group, yielding chorismate, the common precursor of all three aromatic amino acids. The importance of this pathway is revealed by the effectiveness of glyphosate (commercially known as Roundup), a broad-spectrum herbicide. This compound is an uncompetitive inhibitor of the enzyme that produces 5-enolpyruvylshikimate 3-phosphate. It blocks

Figure 24.13 Pathway to chorismate. Chorismate is an intermediate in the biosynthesis of phenylalanine, tyrosine, and tryptophan.

aromatic amino acid biosynthesis in plants but is fairly nontoxic in animals because they lack the enzyme.

Glyphosate
(Roundup)

The pathway bifurcates at chorismate. Let us first follow the *prephenate branch* (Figure 24.14). A mutase converts chorismate into prephenate, the immediate precursor of the aromatic ring of phenylalanine and tyrosine. This fascinating conversion is a rare example of an electrocyclic reaction in biochemistry, mechanistically similar to the well-known Diels–Alder reaction in organic chemistry. Dehydration and decarboxylation yield *phenylpyruvate.* Alternatively, prephenate can be oxidatively decarboxylated to p-*hydroxyphenylpyruvate.* These α-ketoacids are then transaminated to form *phenylalanine* and *tyrosine.*

The branch starting with *anthranilate* leads to the synthesis of tryptophan (Figure 24.15). Chorismate acquires an amino group derived from the hydrolysis of the side chain of glutamine and releases pyruvate to form anthranilate. Then anthranilate condenses with *5-phosphoribosyl-1-pyrophosphate* (PRPP), *an activated form of ribose phosphate.* PRPP is also an important intermediate in the synthesis of histidine, pyrimidine nucleotides, and purine nucleotides (Sections 25.1 and 25.2). The C-1 atom of ribose 5-phosphate becomes bonded to the nitrogen atom of anthranilate in

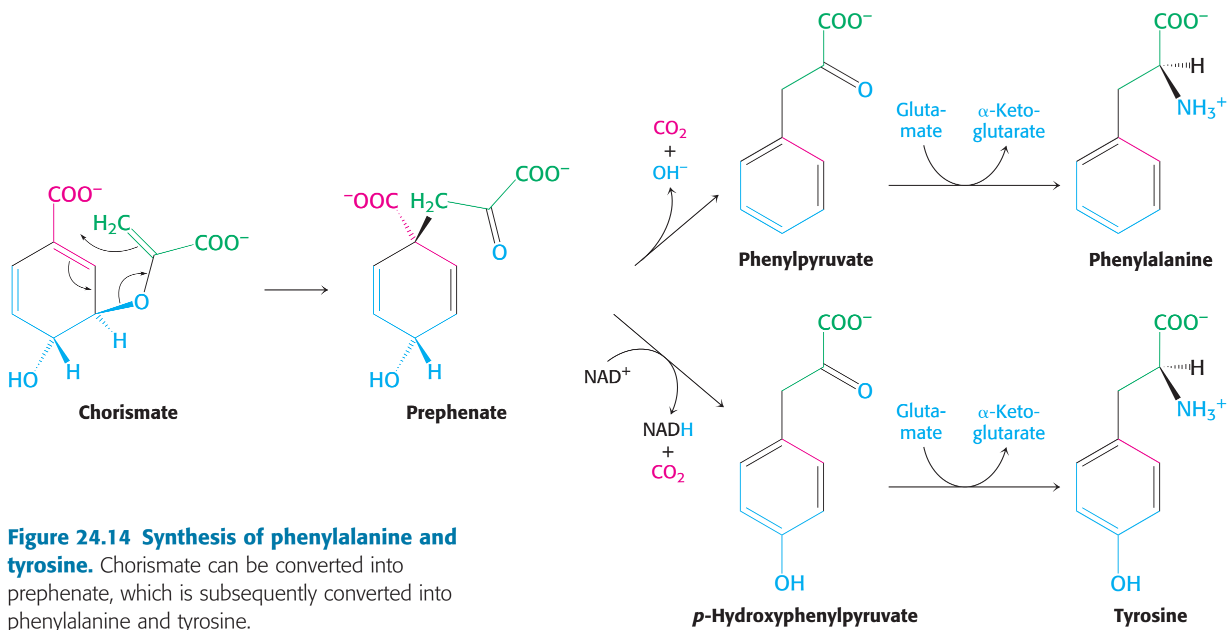
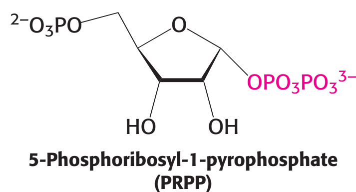


Figure 24.14 Synthesis of phenylalanine and tyrosine. Chorismate can be converted into prephenate, which is subsequently converted into phenylalanine and tyrosine.

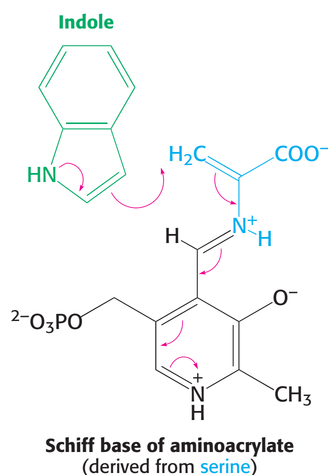
COO⁻
H₂C
COO
O
H
HO
H
Chorismate
Glutamine
Glutamate
+
Pyruvate
H_2N
COO⁻
Anthranilate
PRPP
PP_i
$^{2-}O_3PO$
O
HN
⁻OOC
HO
OH
N-(5′-Phosphoribosyl)-anthranilate
$^{2-}O_3PO$
H
OH
⁻OOC
OH
H
OH
N
H
1-(*o*-Carboxyphenylamino)-1-deoxyribulose 5-phosphate
OH^- + CO_2
$^{2-}O_3PO$
H
OH
H
HO
N
H
Indole-3-glycerol phosphate
Glyceraldehyde 3-phosphate
N
H
Indole
Serine
H_2O
⁻OOC
H
NH_3^+
N
H
Tryptophan

Figure 24.15 Synthesis of tryptophan. Chorismate can be converted into anthranilate, which is subsequently converted into tryptophan.



5-Phosphoribosyl-1-pyrophosphate (PRPP)

a reaction that is driven by the release and hydrolysis of pyrophosphate. The ribose moiety of phosphoribosylanthranilate undergoes rearrangement to yield 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate. This intermediate is dehydrated and then decarboxylated to indole-3-glycerol phosphate, which is cleaved to indole. Then indole reacts with serine to form tryptophan. In these final steps, which are catalyzed by tryptophan synthase, the side chain of indole-3-glycerol phosphate is removed as glyceraldehyde 3-phosphate and replaced by the carbon skeleton of serine.



Schiff base of aminoacrylate
(derived from serine)

Tryptophan synthase illustrates substrate channeling in enzymatic catalysis

The *E. coli* enzyme *tryptophan synthase,* an $\alpha_2\beta_2$ tetramer, can be dissociated into two α subunits and a β_2 dimer (Figure 24.16). The α subunit catalyzes the formation of indole from indole-3-glycerol phosphate, whereas each β subunit has a PLP-containing active site that catalyzes the condensation of indole and serine to form tryptophan. Serine forms a Schiff base with this PLP, which is then dehydrated to give the *Schiff base of aminoacrylate.* This reactive intermediate is attacked by indole to give tryptophan. The overall three-dimensional structure of this enzyme is distinct from that of aspartate aminotransferase and the other PLP enzymes already discussed.

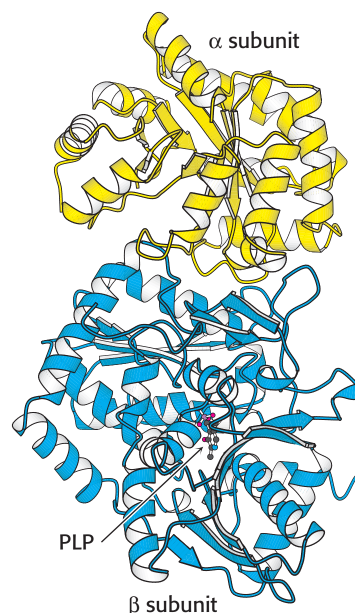


Figure 24.16 Structure of tryptophan synthase. The structure of the complex formed by one α subunit (yellow) and one β subunit (blue). *Notice* that pyridoxal phosphate (PLP) is bound deeply inside the β subunit, a considerable distance from the α subunit. [Drawn from 1BKS.pdb.]

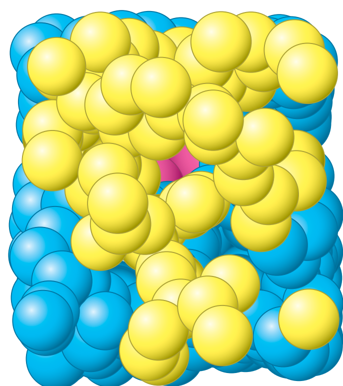


Figure 24.17 Substrate channeling. A 25-Å tunnel runs from the active site of the α subunit of tryptophan synthase (yellow) to the PLP cofactor (red) in the active site of the β subunit (blue).

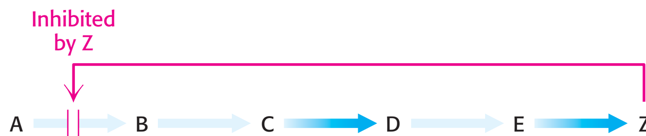
The synthesis of tryptophan poses a challenge. Indole, a hydrophobic molecule, readily traverses membranes and would be lost from the cell if it were allowed to diffuse away from the enzyme. This problem is solved in an ingenious way. A 25-Å-long channel connects the active site of the α subunit with that of the adjacent β subunit in the $\alpha_2\beta_2$ tetramer (Figure 24.17). Thus, indole can diffuse from one active site to the other without being released into bulk solvent. Isotopic-labeling experiments showed that indole formed by the α subunit does not leave the enzyme when serine is present. Furthermore, the two partial reactions are coordinated. Indole is not formed by the α subunit until the highly reactive aminoacrylate is ready and waiting in the β subunit. We see here a clear-cut example of *substrate channeling* in catalysis by a multienzyme complex. Channeling substantially increases the catalytic rate. Furthermore, a deleterious side reaction—in this

case, the potential loss of an intermediate—is prevented. We shall encounter other examples of substrate channeling in Chapter 25.

24.3 Feedback Inhibition Regulates Amino Acid Biosynthesis

The rate of synthesis of amino acids depends mainly on the *amounts* of the biosynthetic enzymes and on their *activities*. We now consider the control of enzymatic activity. The regulation of enzyme synthesis will be discussed in Chapter 31.

In a biosynthetic pathway, the first irreversible reaction, called the *committed step*, is usually an important regulatory site. The final product of the pathway (*Z*) often inhibits the enzyme that catalyzes the committed step ($A \longrightarrow B$).



This kind of control is essential for the conservation of building blocks and metabolic energy. Consider the biosynthesis of serine (p. 714). The committed step in this pathway is the oxidation of 3-phosphoglycerate, catalyzed by the enzyme *3-phosphoglycerate dehydrogenase.* The *E. coli* enzyme is a tetramer of four identical subunits, each comprising a catalytic domain and a serine-binding regulatory domain (Figure 24.18). The binding of serine to a regulatory site reduces the value of V_{max} for the enzyme; an enzyme bound to four molecules of serine is essentially inactive. Thus, if serine is abundant in the cell, the enzyme activity is inhibited, and so 3-phosphoglycerate, a key building block that can be used for other processes, is not wasted.

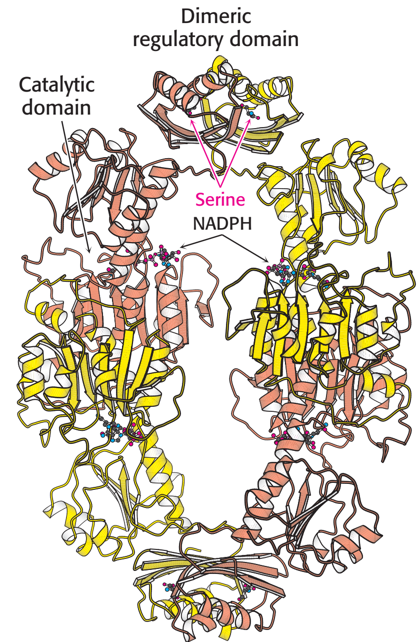


Figure 24.18 Structure of 3-phosphoglycerate dehydrogenase. This enzyme, which catalyzes the committed step in the serine biosynthetic pathway, is inhibited by serine. *Notice* the two serine-binding dimeric regulatory domains—one at the top and the other at the bottom of the structure. [Drawn from 1PSD.pdb.]

Branched pathways require sophisticated regulation

The regulation of branched pathways is more complicated because the concentration of two products must be accounted for. In fact, several intricate feedback mechanisms have been found in branched biosynthetic pathways.

Feedback inhibition and activation. Two pathways with a common initial step may each be inhibited by its own product and activated by the product of the other pathway. Consider, for example, the biosynthesis of the amino acids valine, leucine, and isoleucine. A common intermediate, hydroxyethyl thiamine pyrophosphate (hydroxyethyl-TPP; Section 17.1), initiates the pathways leading to all three of these amino acids. Hydroxyethyl-TPP reacts with α-ketobutyrate in the initial step for the synthesis of isoleucine. Alternatively, hydroxyethyl-TPP reacts with pyruvate in the committed step for the pathways leading to valine and leucine. Thus, the relative concentrations of α-ketobutyrate and pyruvate determine how much isoleucine is produced compared with valine and leucine. *Threonine deaminase,* the PLP enzyme that catalyzes the formation of α-ketobutyrate, is allosterically inhibited by isoleucine (Figure 24.19). This enzyme is also allosterically activated by valine. Thus, this enzyme is inhibited

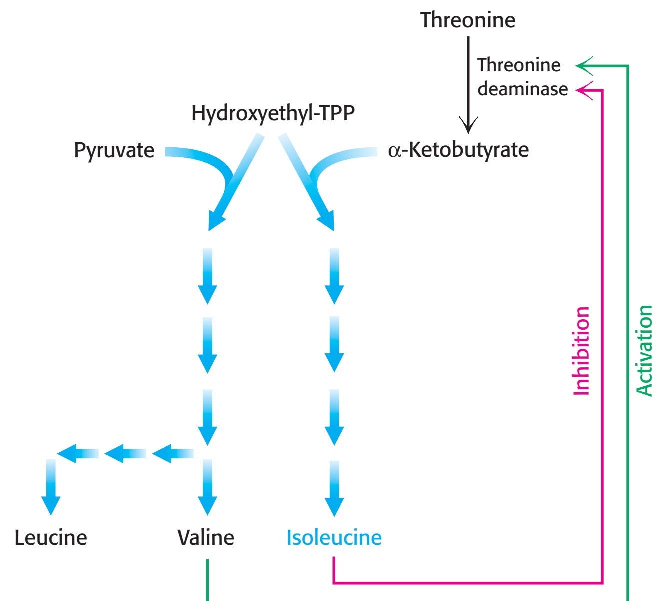


Figure 24.19 Regulation of threonine deaminase. Threonine is converted into α-ketobutyrate in the committed step, leading to the synthesis of isoleucine. The enzyme that catalyzes this step, threonine deaminase, is inhibited by isoleucine and activated by valine, the product of a parallel pathway.

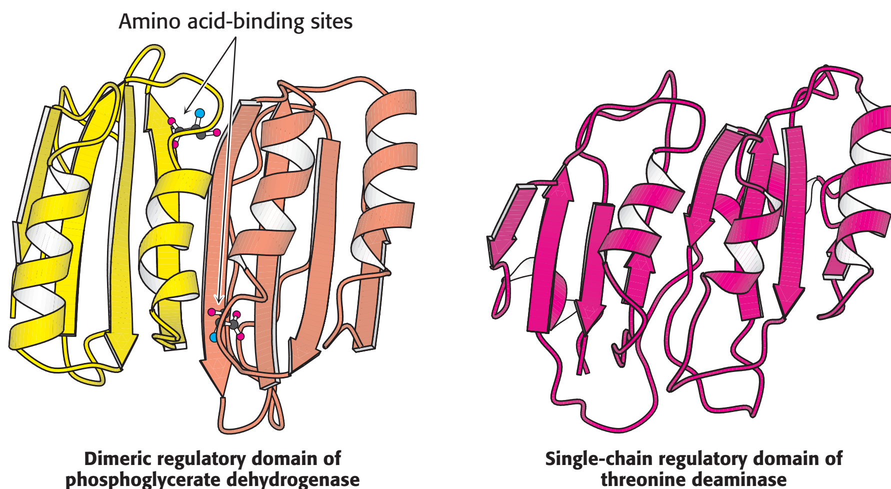
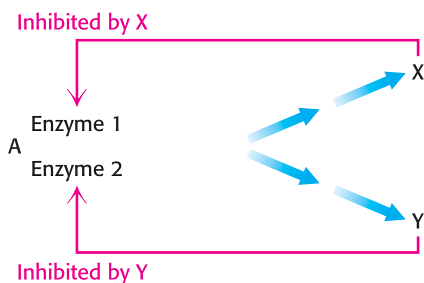


Figure 24.20 A recurring regulatory domain. The regulatory domain formed by two subunits of 3-phosphoglycerate dehydrogenase is structurally related to the single-chain regulatory domain of threonine deaminase. *Notice* that both structures have four α helices and eight β strands in similar locations. Sequence analyses have revealed this amino acid-binding regulatory domain to be present in other enzymes as well. [Drawn from 1PSD and 1TDJ.pdb.]

by the end product of the pathway that it initiates and is activated by the end product of a competitive pathway. This mechanism balances the amounts of different amino acids that are synthesized.

The regulatory domain in threonine deaminase is very similar in structure to the regulatory domain in 3-phosphoglycerate dehydrogenase (Figure 24.20). In the latter enzyme, regulatory domains of two subunits interact to form a dimeric serine-binding regulatory unit, and so the tetrameric enzyme contains two such regulatory units. Each unit is capable of binding two serine molecules. In threonine deaminase, the two regulatory domains are fused into a single unit with two differentiated amino acid-binding sites, one for isoleucine and the other for valine. Sequence analysis shows that similar regulatory domains are present in other amino acid biosynthetic enzymes. *The similarities suggest that feedback-inhibition processes may have evolved by the linkage of specific regulatory domains to the catalytic domains of biosynthetic enzymes.*

Enzyme multiplicity. The committed step can be catalyzed by two or more enzymes with different regulatory properties. For example, the phosphorylation of aspartate is the committed step in the biosynthesis of threonine, methionine, and lysine. Three distinct aspartokinases catalyze this reaction in *E. coli* (Figure 24.21). The catalytic domains of these enzymes show approximately 30% sequence identity. Although the mechanisms of catalysis are essentially identical, their activities are regulated differently: one enzyme is not subject to feedback inhibition, another is inhibited by threonine, and the third is inhibited by lysine. Thus, sophisticated regulation can also evolve by duplication of the genes encoding the biosynthetic enzymes.



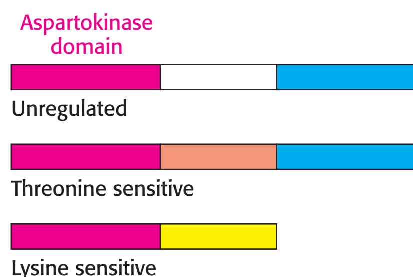


Figure 24.21 Domain structures of three aspartokinases. Each catalyzes the committed step in the biosynthesis of a different amino acid: (top) methionine, (middle) threonine, and (bottom) lysine. They have a catalytic domain in common but differ in their regulatory domains.

Cumulative feedback inhibition. A common step is partly inhibited by each of the final products, acting independently. The regulation of glutamine synthetase in *E. coli* is a striking example of cumulative feedback inhibition. Recall that glutamine is synthesized from glutamate, NH_4^+, and ATP. *Glutamine synthetase* consists of 12 identical 50-kd subunits arranged in two hexagonal rings that face each other. Earl Stadtman showed that this enzyme regulates the flow of nitrogen and hence plays a key role in controlling bacterial metabolism. The amide group of glutamine is a source of nitrogen in the biosyntheses of a variety of compounds, such as tryptophan, histidine, carbamoyl phosphate, glucosamine 6-phosphate, cytidine triphosphate, and adenosine monophosphate. Glutamine synthetase is cumulatively inhibited by each of these final products of glutamine metabolism, as well as by alanine and glycine. *In cumulative inhibition, each inhibitor can reduce the activity of the enzyme, even when other inhibitors are bound at saturating levels.* The enzymatic activity of glutamine synthetase is switched off almost completely when all final products are bound to the enzyme.

An enzymatic cascade modulates the activity of glutamine synthetase

The activity of glutamine synthetase is also controlled by *reversible covalent modification*—the attachment of an *AMP unit* by a phosphodiester bond to the hydroxyl group of a specific tyrosine residue in each subunit (Figure 24.22). *This adenylylated enzyme is less active and more susceptible to cumulative feedback inhibition than is the de-adenylylated form.* The covalently attached AMP unit is removed from the adenylylated enzyme by phosphorolysis. The attachment of an AMP unit is the final step in an enzymatic cascade that is initiated several steps back by reactants and immediate products in glutamine synthesis.

The adenylylation and phosphorolysis reactions are catalyzed by the same enzyme, *adenylyl transferase*. Sequence analysis indicates that this adenylyl transferase comprises two homologous halves, suggesting that one half catalyzes the adenylation reaction and the other half the phospholytic de-adenylylation reaction. What determines whether an AMP unit is added or removed? The specificity of adenylyl transferase is controlled by a *regulatory protein* (designated P or P_{II}), a trimeric protein that can exist in two forms, P_A and P_D. The complex of P_A and adenylyl transferase catalyzes the attachment of an AMP unit to glutamine synthetase, which reduces its activity. Conversely, the complex of P_D and adenylyl transferase removes AMP from the adenylylated enzyme.

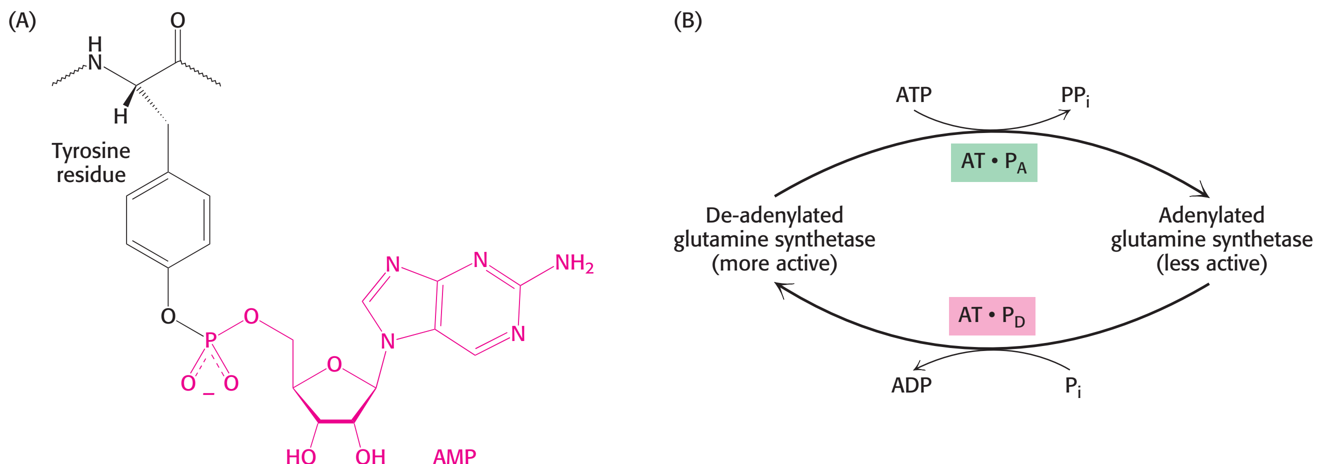


Figure 24.22 Regulation by adenylation. (A) A specific tyrosine residue in each subunit in glutamine synthetase is modified by adenylation. (B) Adenylation of tyrosine is catalyzed by a complex of adenylyl transferase (AT) and one form of a regulatory protein (P_A). The same enzyme catalyzes de-adenylation when it is complexed with the other form (P_D) of the regulatory protein.

This brings us to another level of reversible covalent modification. P_A is converted into P_D by the attachment of uridine monophosphate to a specific tyrosine residue (Figure 24.23). This reaction, which is catalyzed by *uridylyl transferase,* is stimulated by ATP and α-ketoglutarate, whereas it is inhibited by glutamine. In turn, the UMP units on P_D are removed by hydrolysis, a reaction promoted by glutamine and inhibited by α-ketoglutarate. These opposing catalytic activities are present on a single polypeptide chain, homologous to adenylyl transferase, and are controlled so that the enzyme does not simultaneously catalyze uridylylation and hydrolysis.

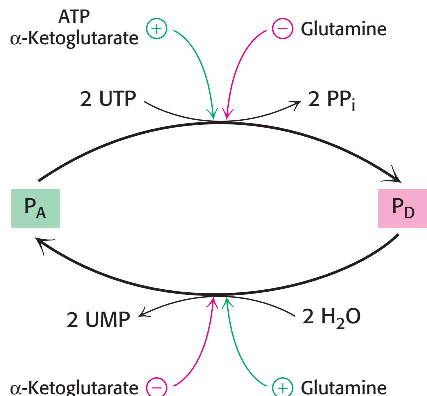


Figure 24.23 A higher level in the regulatory cascade of glutamine synthetase. P_A and P_D, the regulatory proteins that control the specificity of adenylyl transferase, are interconvertible. P_A is converted into P_D by uridylylation, which is reversed by hydrolysis. The enzymes catalyzing these reactions are regulated by the concentrations of metabolic intermediates.

Why is an enzymatic cascade used to regulate glutamine synthetase? One advantage of a cascade is that it *amplifies signals,* as in blood clotting and the control of glycogen metabolism. Another advantage is that the *potential for allosteric control is markedly increased when each enzyme in the cascade is an independent target for regulation.* The integration of nitrogen metabolism in a cell requires that a large number of input signals be detected and processed. In addition, the regulatory protein P also participates in regulating the transcription of genes for glutamine synthetase and other enzymes taking part in nitrogen metabolism. The evolution of a cascade provided many more regulatory sites and made possible a finer tuning of the flow of nitrogen in the cell.

24.4 Amino Acids Are Precursors of Many Biomolecules

In addition to being the building blocks of proteins and peptides, amino acids serve as precursors of many kinds of small molecules that have important and diverse biological roles. Let us briefly survey some of the biomolecules that are derived from amino acids (Figure 24.24).

Purines and *pyrimidines* are derived largely from amino acids. The biosynthesis of these precursors of DNA, RNA, and numerous coenzymes will be discussed in detail in Chapter 25. The reactive terminus of *sphingosine,* an intermediate in the synthesis of sphingolipids, comes from serine. *Histamine,* a potent vasodilator, is derived from histidine by decarboxylation. Tyrosine is a precursor of the hormones *thyroxine* (tetraiodothyronine) and *epinephrine* and of *melanin,* a complex polymeric pigment. The neurotransmitter *serotonin* (5-hydroxytryptamine) and the *nicotinamide ring* of NAD^+ are synthesized from tryptophan. Let us now consider in more detail three particularly important biochemicals derived from amino acids.

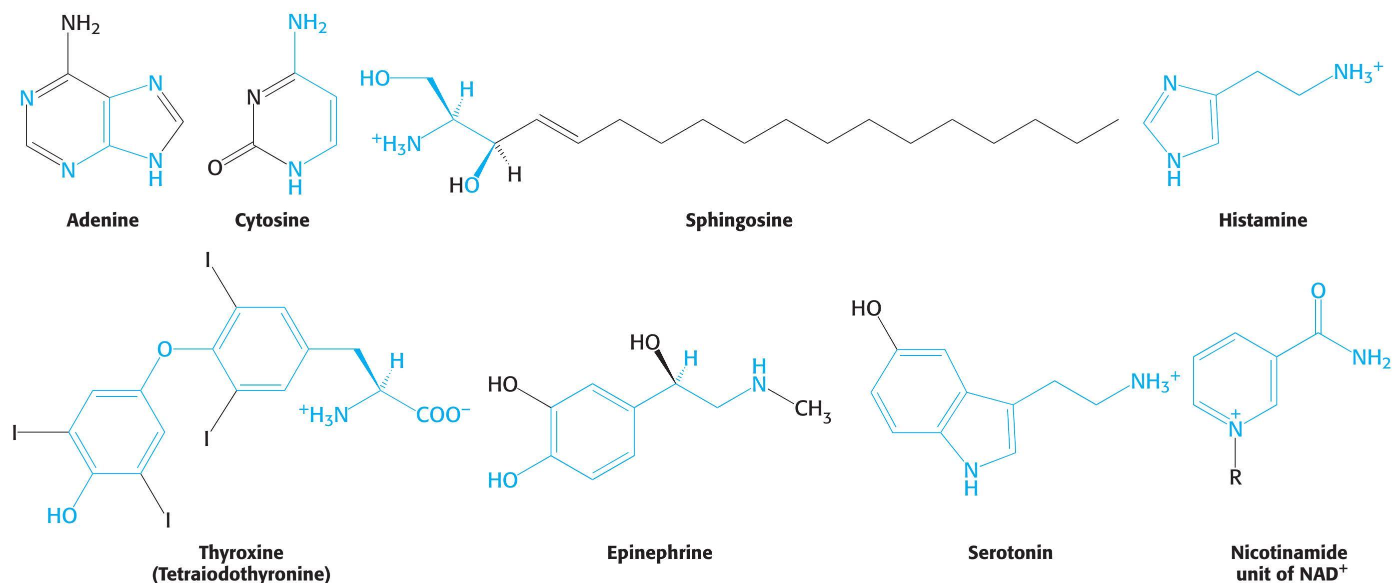


Figure 24.24 Selected biomolecules derived from amino acids. The atoms contributed by amino acids are shown in blue.

Glutathione, a gamma-glutamyl peptide, serves as a sulfhydryl buffer and an antioxidant

Glutathione, a tripeptide containing a sulfhydryl group, is a highly distinctive amino acid derivative with several important roles (Figure 24.25).

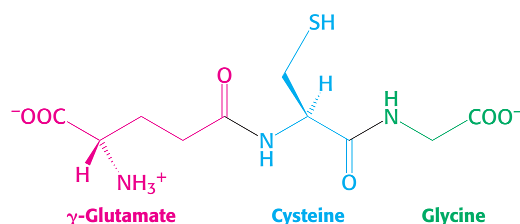


Figure 24.25 Glutathione. This tripeptide consists of a cysteine residue flanked by a glycine residue and a glutamate residue that is linked to cysteine by an isopeptide bond between glutamate's side-chain carboxylate group and cysteine's amino group.

For example, glutathione, present at high levels (~5 mM) in animal cells, protects red blood cells from oxidative damage by serving as a sulfhydryl buffer (Section 20.5). It cycles between a reduced thiol form (GSH) and an oxidized form (GSSG) in which two tripeptides are linked by a disulfide bond.

$$2\ \text{GSH} + \text{RO—OH} \rightleftharpoons \text{GSSG} + \text{H}_2\text{O} + \text{ROH}$$

GSSG is reduced to GSH by *glutathione reductase,* a flavoprotein that uses NADPH as the electron source. The ratio of GSH to GSSG in most cells is greater than 500. *Glutathione plays a key role in detoxification by reacting with hydrogen peroxide and organic peroxides, the harmful by-products of aerobic life.*

Glutathione peroxidase, the enzyme catalyzing this reaction, is remarkable in having a modified amino acid containing a *selenium* (Se) atom (Figure 24.26). Specifically, its active site contains the selenium analog of cysteine, in which selenium has replaced sulfur. The selenolate (E-Se$^-$) form of this residue reduces the peroxide substrate to an alcohol and is in turn oxidized to selenenic acid (E-SeOH). Glutathione then comes into action by forming a selenosulfide adduct (E-Se-S-G). A second molecule of glutathione then regenerates the active form of the enzyme by attacking the selenosulfide to form oxidized glutathione (Figure 24.27).

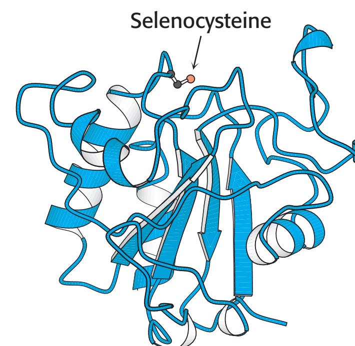


Figure 24.26 Structure of glutathione peroxidase. This enzyme, which has a role in peroxide detoxification, contains a selenocysteine residue in its active site. [Drawn from 1GP1.pdb.]

Nitric oxide, a short-lived signal molecule, is formed from arginine

Nitric oxide (NO) is an important messenger in many vertebrate signal-transduction processes. For instance, NO stimulates mitochondrial biogenesis.

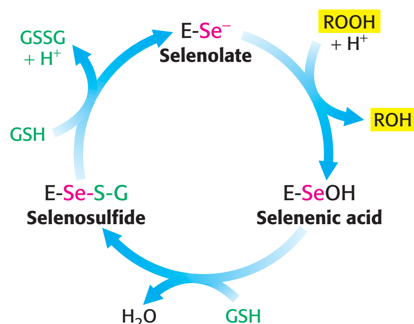
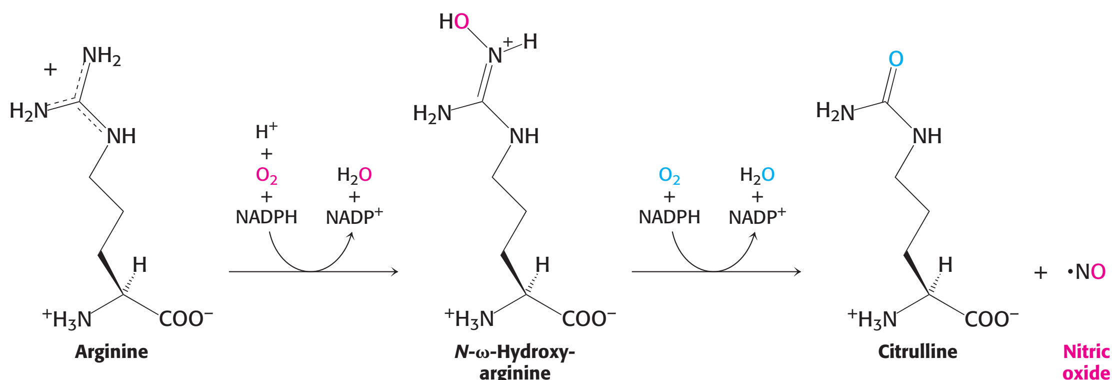


Figure 24.27 Catalytic cycle of glutathione peroxidase. [After O. Epp, R. Ladenstein, and A. Wendel. *Eur. J. Biochem.* 133(1983):51–69.]

Figure 24.28 Formation of nitric oxide. NO is generated by the oxidation of arginine.



> **^{15}N labeling: A pioneer's account**
>
> "Myself as a Guinea Pig
>
> . . . in 1944, I undertook, together with David Rittenberg, an investigation on the turnover of blood proteins of man. To this end I synthesized 66 g of glycine labeled with 35 percent ^{15}N at a cost of $1000 for the ^{15}N. On 12 February 1945, I started the ingestion of the labeled glycine. Since we did not know the effect of relatively large doses of the stable isotope of nitrogen and since we believed that the maximum incorporation into the proteins could be achieved by the administration of glycine in some continual manner, I ingested 1 g samples of glycine at hourly intervals for the next 66 hours . . . . At stated intervals, blood was withdrawn and after proper preparation the ^{15}N concentrations of different blood proteins were determined."
>
> —David Shemin
> *Bioessays* 10(1989):30

This free-radical gas is produced endogenously from *arginine* in a complex reaction that is catalyzed by *nitric oxide synthase.* NADPH and O_2 are required for the synthesis of nitric oxide (Figure 24.28). Nitric oxide acts by binding to and activating soluble guanylate cyclase, an important enzyme in signal transduction (Section 32.3). This enzyme is homologous to adenylate cyclase but includes a heme-containing domain that binds NO.

Porphyrins are synthesized from glycine and succinyl coenzyme A

The participation of an amino acid in the biosynthesis of the porphyrin rings of hemes and chlorophylls was first revealed by isotope-labeling experiments carried out by David Shemin and his colleagues. In 1945, they showed that the nitrogen atoms of heme were labeled after the feeding of [^{15}N]glycine to human subjects (of whom Shemin was the first), whereas the ingestion of [^{15}N]glutamate resulted in very little labeling.

Using ^{14}C, which had just become available, they discovered that 8 of the carbon atoms of heme in nucleated duck erythrocytes are derived from the α-carbon atom of glycine and none from the carboxyl carbon atom. Subsequent studies demonstrated that the other 26 carbon atoms of heme can arise from acetate. Moreover, the ^{14}C in methyl-labeled acetate emerged in 24 of these carbon atoms, whereas the ^{14}C in carboxyl-labeled acetate appeared only in the other 2 (Figure 24.29).

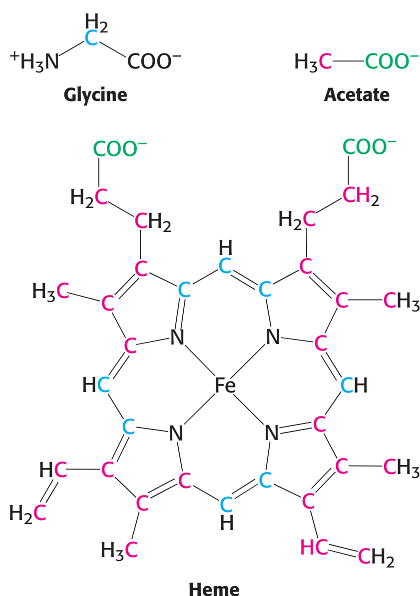
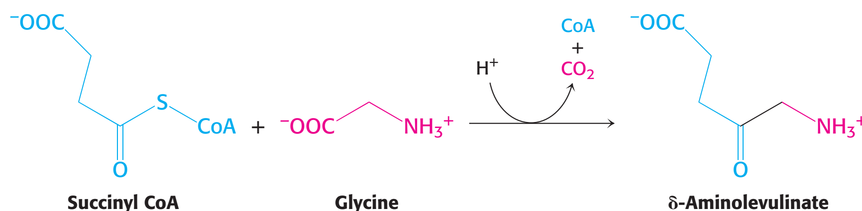


Figure 24.29 Heme labeling. The origins of atoms in heme revealed by the results of isotopic labeling studies.

This highly distinctive labeling pattern led Shemin to propose that prior to incorporation into heme, acetate is converted to succinyl-CoA through enzymes from the citric acid cycle (Section 17.2). Shemin further posited that a heme precursor is formed by the condensation of glycine with succinyl-CoA. In fact, *the first step in the biosynthesis of porphyrins in mammals is the condensation of glycine and succinyl CoA to form δ-aminolevulinate.*



This reaction is catalyzed by *δ-aminolevulinate synthase,* a PLP enzyme present in mitochondria. Consistent with the labeling studies performed by Shemin and his coworkers, the carbon atom from the carboxyl group of glycine is lost as carbon dioxide, while the α-carbon remains in δ-aminolevulinate.

Two molecules of δ-aminolevulinate condense to form *porphobilinogen,* the next intermediate. Four molecules of porphobilinogen then condense head to tail to form a linear *tetrapyrrole* in a reaction catalyzed by *porpho-*

bilinogen deaminase. The enzyme-bound linear tetrapyrrole then cyclizes to form uroporphyrinogen III, which has an asymmetric arrangement of side chains. This reaction requires a *cosynthase.* In the presence of synthase alone, uroporphyrinogen I, the nonphysiological symmetric isomer, is produced. Uroporphyrinogen III is also a key intermediate in the synthesis of vitamin B_{12} by bacteria and that of chlorophyll by bacteria and plants (Figure 24.30).

The porphyrin skeleton is now formed. Subsequent reactions alter the side chains and the degree of saturation of the porphyrin ring (see Figure 24.29). *Coproporphyrinogen III* is formed by the decarboxylation of the acetate side chains. The desaturation of the porphyrin ring and the conversion of two of the propionate side chains into vinyl groups yield *protoporphyrin IX.* The chelation of iron finally gives *heme,* the prosthetic group of

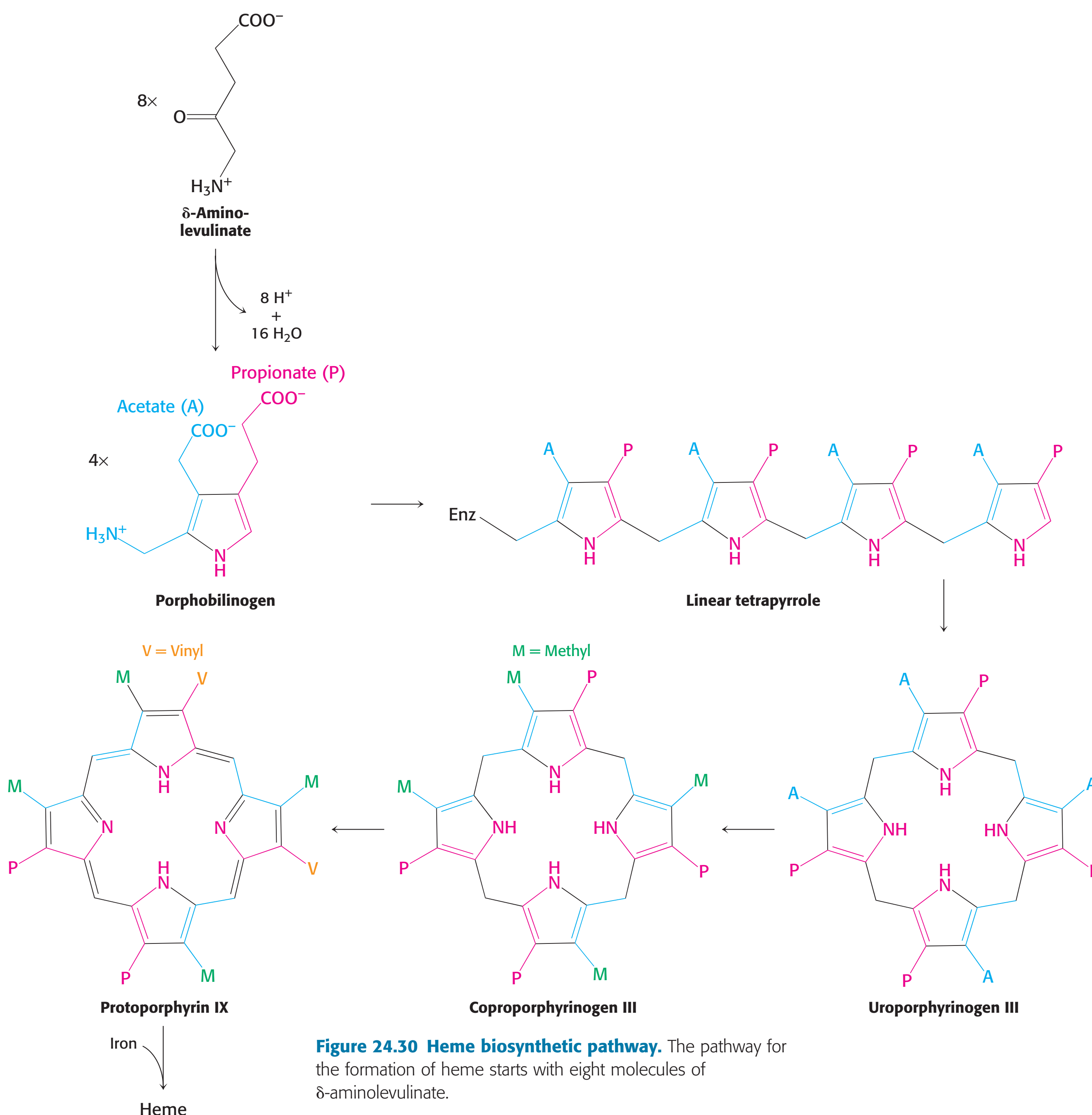


Figure 24.30 Heme biosynthetic pathway. The pathway for the formation of heme starts with eight molecules of δ-aminolevulinate.

proteins such as myoglobin, hemoglobin, catalase, peroxidase, and cytochrome *c*. The insertion of the *ferrous* form of iron is catalyzed by *ferrochelatase*. Iron is transported in the plasma by *transferrin,* a protein that binds two ferric ions, and is stored in tissues inside molecules of *ferritin*. The large internal cavity (~80 Å in diameter) of ferritin can hold as many as 4500 ferric ions (Section 32.4).

The normal human erythrocyte has a life span of about 120 days, as was first shown by the time course of ^{15}N in Shemin's own hemoglobin after he ingested ^{15}N-labeled glycine. The first step in the degradation of the heme group is the cleavage of its α-methine bridge to form the green pigment *biliverdin,* a linear tetrapyrrole. The central methine bridge of biliverdin is then reduced by *biliverdin reductase* to form *bilirubin,* a red pigment (Figure 24.31). The changing color of a bruise is a highly graphic indicator of these degradative reactions.

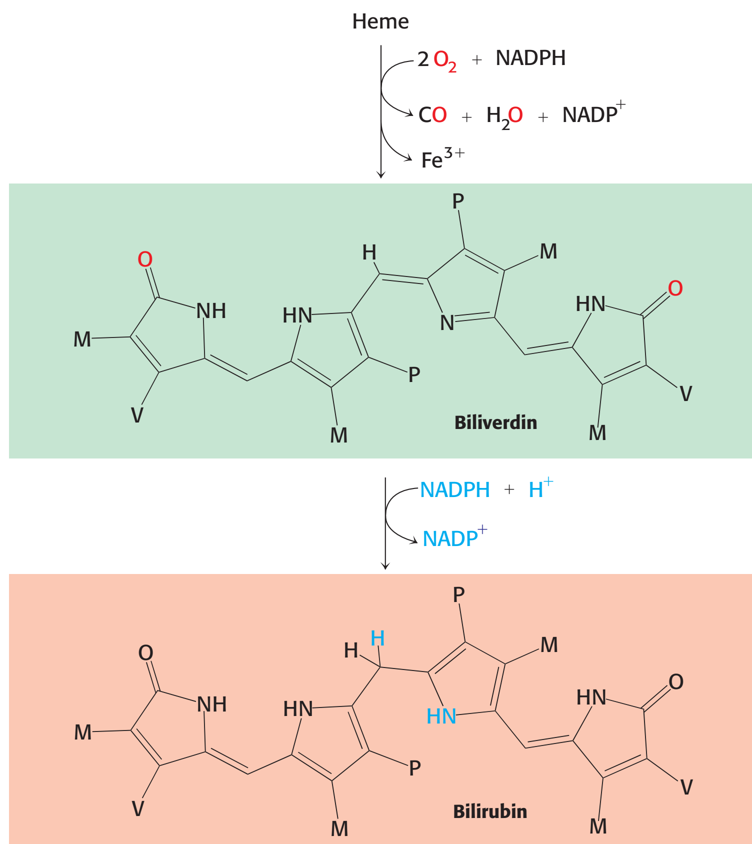


Figure 24.31 Heme degradation. The formation of the heme-degradation products biliverdin and bilirubin is responsible for the color of bruises. Abbreviations: M, methyl; V, vinyl.

Porphyrins accumulate in some inherited disorders of porphyrin metabolism

Porphyrias are inherited or acquired disorders caused by a deficiency of enzymes in the heme biosynthetic pathway. Porphyrin is synthesized in both the erythroblasts and the liver, and either one may be the site of a disorder. *Congenital erythropoietic porphyria,* for example, prematurely destroys eythrocytes. This disease results from insufficient cosynthase. In this porphyria, the synthesis of the required amount of uroporphyrinogen III is accompanied by the formation of very large quantities of uroporphyrinogen I, the useless symmetric isomer. Uroporphyrin I, coproporphyrin I, and other symmetric derivatives also accumulate. The urine of patients having this disease is red because of the excretion of large amounts of uroporphyrin

I. Their teeth exhibit a strong red fluorescence under ultraviolet light because of the deposition of porphyrins. Furthermore, their skin is usually very sensitive to light because photoexcited porphyrins are quite reactive. *Acute intermittent porphyria* is the most prevalent of the porphyrias affecting the liver. This porphyria is characterized by the overproduction of porphobilinogen and δ-aminolevulinate, which results in severe abdominal pain and neurological dysfunction. The "madness" of George III, king of England during the American Revolution, is believed to have been due to this porphyria.

Summary

24.1 Nitrogen Fixation: Microorganisms Use ATP and a Powerful Reductant to Reduce Atmospheric Nitrogen to Ammonia

Microorganisms use ATP and reduced ferredoxin, a powerful reductant, to reduce N_2 to NH_3. An iron–molybdenum cluster in nitrogenase deftly catalyzes the fixation of N_2, a very inert molecule. Higher organisms consume the fixed nitrogen to synthesize amino acids, nucleotides, and other nitrogen-containing biomolecules. The major points of entry of NH_4^+ into metabolism are glutamine or glutamate.

24.2 Amino Acids Are Made from Intermediates of the Citric Acid Cycle and Other Major Pathways

Human beings can synthesize 11 of the basic set of 20 amino acids. These amino acids are called nonessential, in contrast with the essential amino acids, which must be supplied in the diet. The pathways for the synthesis of nonessential amino acids are quite simple. Glutamate dehydrogenase catalyzes the reductive amination of α-ketoglutarate to glutamate. A transamination reaction takes place in the synthesis of most amino acids. At this step, the chirality of the amino acid is established. Alanine and aspartate are synthesized by the transamination of pyruvate and oxaloacetate, respectively. Glutamine is synthesized from NH_4^+ and glutamate, and asparagine is synthesized similarly. Proline and arginine are derived from glutamate. Serine, formed from 3-phosphoglycerate, is the precursor of glycine and cysteine. Tyrosine is synthesized by the hydroxylation of phenylalanine, an essential amino acid. The pathways for the biosynthesis of essential amino acids are much more complex than those for the nonessential ones.

Tetrahydrofolate, a carrier of activated one-carbon units, plays an important role in the metabolism of amino acids and nucleotides. This coenzyme carries one-carbon units at three oxidation states, which are interconvertible: most reduced—methyl; intermediate—methylene; and most oxidized—formyl, formimino, and methenyl. The major donor of activated methyl groups is *S*-adenosylmethionine, which is synthesized by the transfer of an adenosyl group from ATP to the sulfur atom of methionine. *S*-Adenosylhomocysteine is formed when the activated methyl group is transferred to an acceptor. It is hydrolyzed to adenosine and homocysteine, and the latter is then methylated to methionine to complete the activated methyl cycle.

24.3 Feedback Inhibition Regulates Amino Acid Biosynthesis

Most of the pathways of amino acid biosynthesis are regulated by feedback inhibition, in which the committed step is allosterically inhibited by the final product. The regulation of branched pathways requires extensive interaction among the branches that includes both negative and positive regulation. The regulation of glutamine synthetase

in *E. coli* is a striking demonstration of cumulative feedback inhibition and of control by a cascade of reversible covalent modifications.

24.4 Amino Acids Are Precursors of Many Biomolecules

Amino acids are precursors of a variety of biomolecules. Glutathione (γ-Glu-Cys-Gly) serves as a sulfhydryl buffer and detoxifying agent. Glutathione peroxidase, a selenoenzyme, catalyzes the reduction of hydrogen peroxide and organic peroxides by glutathione. Nitric oxide, a short-lived messenger, is formed from arginine. Porphyrins are synthesized from glycine and succinyl CoA, which condense to give δ-aminolevulinate. Two molecules of this intermediate become linked to form porphobilinogen. Four molecules of porphobilinogen combine to form a linear tetrapyrrole, which cyclizes to uroporphyrinogen III. Oxidation and side-chain modifications lead to the synthesis of protoporphyrin IX, which acquires an iron atom to form heme.

Key Terms

nitrogen fixation (p. 706)
nitrogenase complex (p. 707)
essential amino acids (p. 711)
nonessential amino acids (p. 711)
pyridoxal phosphate (p. 712)
tetrahydrofolate (p. 715)
S-adenosylmethionine (SAM) (p. 716)
activated methyl cycle (p. 717)
substrate channeling (p. 722)
committed step (p. 723)
enzyme multiplicity (p. 724)
cumulative feedback inhibition (p. 725)
glutathione (p. 727)
nitric oxide (NO) (p. 727)
porphyria (p. 730)

Problems

1. *Out of thin air.* Define nitrogen fixation. What organisms are capable of nitrogen fixation?

2. *From few, many.* What are the seven precursors of the 20 amino acids?

3. *Vital, in the truest sense.* Why are certain amino acids defined as essential for human beings?

4. *From sugar to amino acid.* Write a balanced equation for the synthesis of alanine from glucose.

5. *From air to blood.* What are the intermediates in the flow of nitrogen from N_2 to heme?

6. *The fix is in.* "The mechanistic complexity of nitrogenase is necessary because nitrogen fixation is a thermodynamically unfavorable process." True or false? Explain.

7. *Common component.* What cofactor is required by all transaminases (aminotransferases)?

8. *Here, hold this.* In this chapter, we considered three different cofactors/cosubstrates that act as carriers of one-carbon units. Name them.

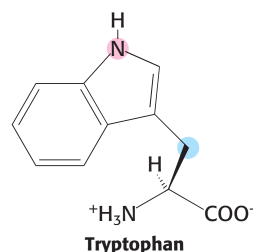
9. *One-carbon transfers.* Which derivative of folate is a reactant in the conversion of (a) glycine into serine? (b) homocysteine into methionine?

10. *Telltale tag.* In the reaction catalyzed by glutamine synthetase, an oxygen atom is transferred from the side chain of glutamate to orthophosphate, as shown by the results of ^{18}O-labeling studies. Account for this finding.

11. *Telltale tag, redux.* In contrast to the production of glutamine by glutamine synthetase (see Problem 10), the generation of asparagine from ^{18}O-labeled aspartate does not result in the transfer of an ^{18}O atom to orthophosphate. In what molecule do you expect to find one of the ^{18}O atoms?

12. *Therapeutic glycine.* Isovaleric acidemia is an inherited disorder of leucine metabolism caused by a deficiency of isovaleryl CoA dehydrogenase. Many infants having this disease die in the first month of life. The administration of large amounts of glycine sometimes leads to marked clinical improvement. Propose a mechanism for the therapeutic action of glycine.

13. *Lending a hand.* The atoms from tryptophan shaded below are derived from two other amino acids. Name them.



Tryptophan

14. *Deprived bacteria.* Blue-green algae (cyanobacteria) form *heterocysts* when deprived of ammonia and nitrate. In this form, the cyanobacteria lack nuclei and are attached to

adjacent vegetative cells. Heterocysts have photosystem I activity but are entirely devoid of photosystem II activity. What is their role?

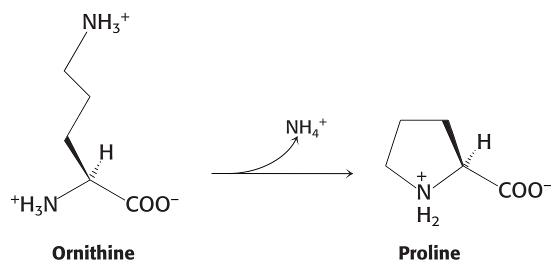
15. *Cysteine and cystine.* Most cytoplasmic proteins lack disulfide bonds, whereas extracellular proteins usually contain them. Why?

16. *Through the looking-glass.* Suppose that aspartate aminotransferase were chemically synthesized with the use of D-amino acids only. What products would you expect if this mirror-image enzyme were treated with (a) L-asparate and α-ketoglutarate; (b) D-aspartate and α-ketoglutarate?

17. *To and fro.* The synthesis of δ-aminolevulinate takes place in the mitochondrial matrix, whereas the formation of porphobilinogen takes place in the cytoplasm. Propose a reason for the mitochondrial location of the first step in heme synthesis.

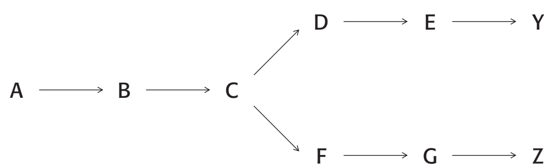
18. *Direct synthesis.* Which of the 20 amino acids can be synthesized directly from a common metabolic intermediate by a transamination reaction?

19. *Alternative route to proline.* Certain species of bacteria possess an enzyme, ornithine cyclodeaminase, that can catalyze the conversion of L-ornithine into L-proline in a single catalytic cycle.



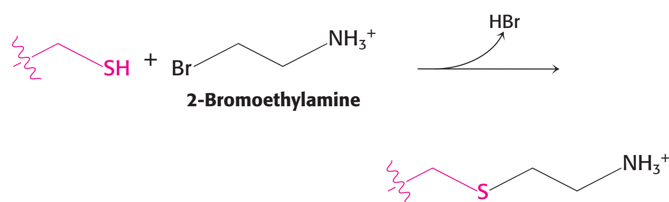
The enzyme *lysine* cyclodeaminase has also been identified. Predict the product of the reaction catalyzed by lysine cyclodeaminase.

20. *Lines of communication.* For the following example of a branched pathway, propose a feedback inhibition scheme that would result in the production of equal amounts of Y and Z.



21. *Cumulative feedback inhibition.* Consider the branched pathway in Problem 20. The first common step (A ⟶ B) is partly inhibited by both of the final products, each acting independently of the other. Suppose that a high level of Y alone decreased the rate of the A ⟶ B step from 100 to 60 s^{-1} and that a high level of Z alone decreased the rate from 100 to 40 s^{-1}. What would the rate be in the presence of high levels of both Y and Z?

22. *Recovered activity.* Free sulfhydryl groups can be alkylated with 2-bromoethylamine to the corresponding thioether.



Researchers prepared a mutant form of aspartate aminotransferase in which lysine 258 was replaced by cysteine (Lys258Cys). This mutant protein has no observable catalytic activity. However, treatment of Lys258Cys with 2-bromoethylamine yielded a protein with ~7% activity relative to the wild-type enzyme. Explain why alkylation recovered some enzyme activity.

Mechanism Problems

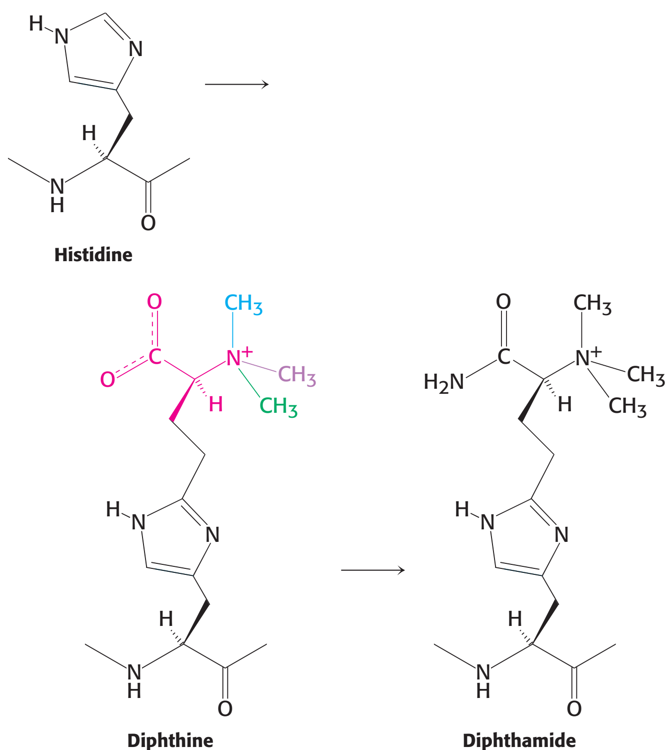
23. *Ethylene formation.* Propose a mechanism for the conversion of S-adenosylmethionine into 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase, a PLP enzyme. What is the other product?

24. *Mirror-image serine.* Brain tissue contains substantial amounts of D-serine, which is generated from L-serine by serine racemase, a PLP enzyme. Propose a mechanism for the interconversion of L- and D-serine. What is the equilibrium constant for the reaction L-serine $\rightleftharpoons$ D-serine?

25. *An unusual amino acid.* Elongation factor-2 (eEF-2), a protein taking part in translation, contains a histidine residue that is modified posttranslationally in several steps to a complex side chain known as diphthamide. An intermediate along this pathway is referred to as diphthine.

(a) Labeling experiments indicate that the diphthine intermediate is formed by the modification of histidine with four molecules of S-adenosylmethionine (indicated by the four colors on page 734). Propose a mechanism for the formation of diphthine.

(b) The final conversion of diphthine into diphthamide is known to be ATP dependent. Propose two possible mechanisms for the final amidation step.



Chapter Integration Problems

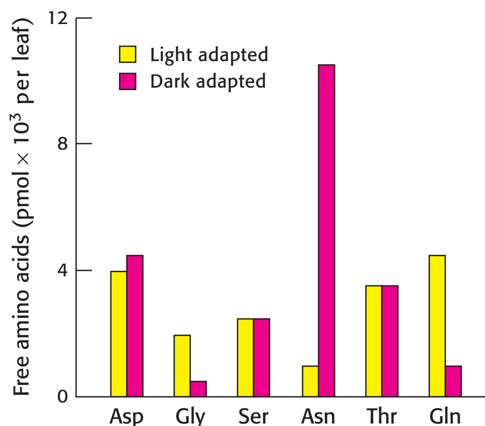
26. *Connections.* How might increased synthesis of aspartate and glutamate affect energy production in a cell? How would the cell respond to such an effect?

27. *Protection required.* Suppose that a mutation in bacteria resulted in the diminished activity of methionine adenosyltransferase, the enzyme responsible for the synthesis of SAM from methionine and ATP. Predict how this diminished activity might affect the stability of the mutated bacteria's DNA.

28. *Heme biosynthesis.* Shemin and coworkers used acetate-labeling experiments to conclude that succinyl-CoA is a key intermediate in the biosynthesis of heme. Identify the intermediates in the conversion of acetate into succinyl-CoA.

Chapter Integration and Data Interpretation Problem

29. *Light effects.* The adjoining graph shows the concentration of several free amino acids in light- and dark-adapted plants.



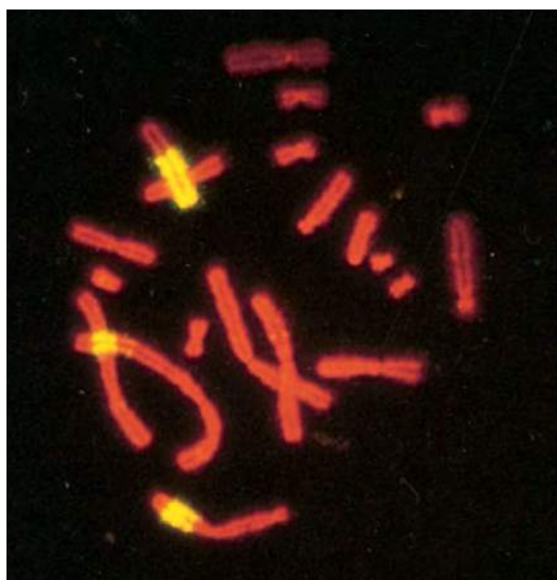
[After B. B. Buchanan, W. Gruissem, and R. L. Jones, *Biochemistry and Molecular Biology of Plants* (American Society of Plant Physiology, 2000), Fig. 8.3, p. 363.]

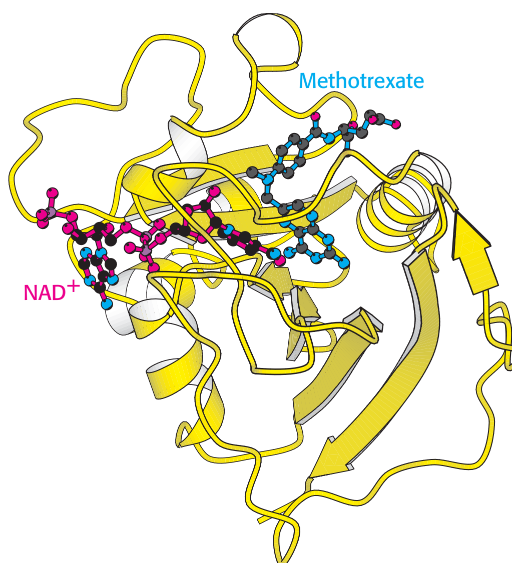
(a) Of the amino acids shown, which are most affected by light–dark adaptation?

(b) Suggest a plausible biochemical explanation for the difference observed.

(c) White asparagus, a culinary delicacy, is the result of growing asparagus plants in the dark. What chemical might you think enhances the taste of white asparagus?

Nucleotide Biosynthesis





Nucleotides are required for cell growth and replication. A key enzyme for the synthesis of one nucleotide is dihydrofolate reductase (right). Cells grown in the presence of methotrexate, a reductase inhibitor, respond by increasing the number of copies of the reductase gene. The bright yellow regions visible on three of the chromosomes in the fluorescence micrograph (left), which were grown in the presence of methotrexate, contain hundreds of copies of the reductase gene. [(Left) Courtesy of Dr. Barbara Trask and Dr. Joyce Hamlin.]

Nucleotides are key biomolecules required for a variety of life processes. First, nucleotides are the *activated precursors of nucleic acids,* necessary for the replication of the genome and the transcription of the genetic information into RNA. Second, an adenine nucleotide, ATP, is *the universal currency of energy.* A guanine nucleotide, GTP, also serves as an energy source for a more select group of biological processes. Third, nucleotide derivatives such as UDP-glucose *participate in biosynthetic processes* such as the formation of glycogen. Fourth, nucleotides are *essential components of signal-transduction pathways.* Cyclic nucleotides such as cyclic AMP and cyclic GMP are second messengers that transmit signals both within and between cells. Furthermore, ATP acts as the donor of phosphoryl groups transferred by protein kinases in a variety of signaling pathways and, in some cases, ATP is secreted as a signal molecule.

In this chapter, we continue along the path begun in Chapter 24, which described the incorporation of nitrogen into amino acids from inorganic sources such as nitrogen gas. The amino acids glycine and aspartate are the scaffolds on which the ring systems present in nucleotides are assembled. Furthermore, aspartate and the side chain of glutamine serve as sources of NH_2 groups in the formation of nucleotides.

OUTLINE

25.1 The Pyrimidine Ring Is Assembled de Novo or Recovered by Salvage Pathways

25.2 Purine Bases Can Be Synthesized de Novo or Recycled by Salvage Pathways

25.3 Deoxyribonucleotides Are Synthesized by the Reduction of Ribonucleotides Through a Radical Mechanism

25.4 Key Steps in Nucleotide Biosynthesis Are Regulated by Feedback Inhibition

25.5 Disruptions in Nucleotide Metabolism Can Cause Pathological Conditions

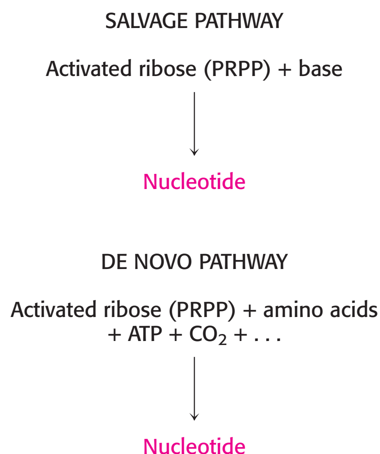


Figure 25.1 Salvage and de novo pathways. In a salvage pathway, a base is reattached to a ribose, activated in the form of 5-phosphoribosyl-1-pyrophosphate (PRPP). In de novo synthesis, the base itself is synthesized from simpler starting materials, including amino acids. ATP hydrolysis is required for de novo synthesis.

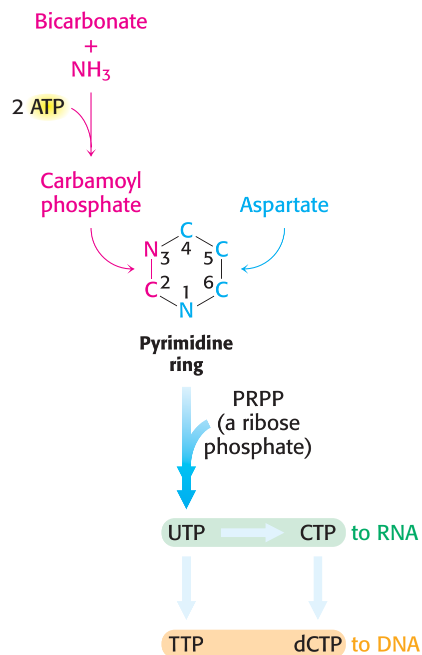


Figure 25.2 De novo pathway for pyrimidine nucleotide synthesis. The C-2 and N-3 atoms in the pyrimidine ring come from carbamoyl phosphate, whereas the other atoms of the ring come from aspartate.

Table 25.1 Nomenclature of bases, nucleosides, and nucleotides

RNA		
Base	Ribonucleoside	Ribonucleotide (5′-monophosphate)
Adenine (A)	Adenosine	Adenylate (AMP)
Guanine (G)	Guanosine	Guanylate (GMP)
Uracil (U)	Uridine	Uridylate (UMP)
Cytosine (C)	Cytidine	Cytidylate (CMP)
DNA		
Base	Deoxyribonucleoside	Deoxyribonucleotide (5′-monophosphate)
Adenine (A)	Deoxyadenosine	Deoxyadenylate (dAMP)
Guanine (G)	Deoxyguanosine	Deoxyguanylate (dGMP)
Thymine (T)	Thymidine	Thymidylate (TMP)
Cytosine (C)	Deoxycytidine	Deoxycytidylate (dCMP)

Nucleotide biosynthetic pathways are tremendously important as intervention points for therapeutic agents. Many of the most widely used drugs in the treatment of cancer block steps in nucleotide biosynthesis, particularly steps in the synthesis of DNA precursors.

Nucleotides can be synthesized by de novo or salvage pathways

The pathways for the biosynthesis of nucleotides fall into two classes: *de novo* pathways and *salvage* pathways (Figure 25.1). In de novo (from scratch) pathways, the nucleotide bases are assembled from simpler compounds. The framework for a *pyrimidine* base is assembled first and then attached to ribose. In contrast, the framework for a *purine* base is synthesized piece by piece directly onto a ribose-based structure. These pathways each comprise a small number of elementary reactions that are repeated with variation to generate different nucleotides, as might be expected for pathways that appeared very early in evolution. In salvage pathways, preformed bases are recovered and reconnected to a ribose unit.

De novo pathways lead to the synthesis of *ribo*nucleotides. However, DNA is built from *deoxyribo*nucleotides. Consistent with the notion that RNA preceded DNA in the course of evolution, all deoxyribonucleotides are synthesized from the corresponding ribonucleotides. The deoxyribose sugar is generated by the reduction of ribose within a fully formed nucleotide. Furthermore, the methyl group that distinguishes the thymine of DNA from the uracil of RNA is added at the last step in the pathway.

The nomenclature of nucleotides and their constituent units was presented in Chapter 4. Recall that a *nucleoside* is a purine or pyrimidine base linked to a sugar and that a *nucleotide* is a phosphate ester of a nucleoside. The names of the major bases of RNA and DNA, and of their nucleoside and nucleotide derivatives, are given in Table 25.1.

25.1 The Pyrimidine Ring Is Assembled de Novo or Recovered by Salvage Pathways

In de novo synthesis of pyrimidines, the ring is synthesized first and then it is attached to a ribose phosphate to form a *pyrimidine nucleotide* (Figure 25.2). Pyrimidine rings are assembled from bicarbonate, aspartate, and ammonia. Although an ammonia molecule already present in solution can be used, the ammonia is usually produced from the hydrolysis of the side chain of glutamine.

Bicarbonate and other oxygenated carbon compounds are activated by phosphorylation

The first step in de novo pyrimidine biosynthesis is the synthesis of *carbamoyl phosphate* from bicarbonate and ammonia in a multistep process, requiring the cleavage of two molecules of ATP. This reaction is catalyzed by *carbamoyl phosphate synthetase* (CPS; Section 23.4). Analysis of the structure of CPS reveals two homologous domains, each of which catalyzes an ATP-dependent step (Figure 25.3).

In the first step, bicarbonate is phosphorylated by ATP to form carboxyphosphate and ADP. Ammonia then reacts with carboxyphosphate to form carbamic acid and inorganic phosphate.

$$\text{Bicarbonate} \xrightarrow[\;]{\text{ATP} \;\to\; \text{ADP}} \text{Carboxyphosphate} \xrightarrow{NH_3 \;\to\; P_i} \text{Carbamic acid}$$

The active site for this reaction lies in a domain formed by the amino-terminal third of CPS. This domain forms a structure called an *ATP-grasp fold,* which surrounds ATP and holds it in an orientation suitable for nucleophilic attack at the γ phosphoryl group. Proteins containing ATP-grasp folds catalyze the formation of carbon–nitrogen bonds through acyl-phosphate intermediates. Such ATP-grasp folds are widely used in nucleotide biosynthesis.

In the second step catalyzed by carbamoyl phosphate synthetase, carbamic acid is phosphorylated by another molecule of ATP to form carbamoyl phosphate.

$$\text{Carbamic acid} \xrightarrow{\text{ATP} \;\to\; \text{ADP}} \text{Carbamoyl phosphate}$$

This reaction takes place in a second ATP-grasp domain within the enzyme. The active sites leading to carbamic acid formation and carbamoyl phosphate formation are very similar, revealing that this enzyme evolved by a gene duplication event. Indeed, duplication of a gene encoding an ATP-grasp domain followed by specialization was central to the evolution of nucleotide biosynthetic processes (p. 741).

The side chain of glutamine can be hydrolyzed to generate ammonia

Glutamine is the primary source of ammonia for carbamoyl phosphate synthetase. In this case, a second polypeptide component of the enzyme hydrolyzes glutamine to form ammonia and glutamate. The active site of the glutamine-hydrolyzing component contains a catalytic dyad comprising a cysteine and a histidine residue. Such a catalytic dyad, reminiscent of the active site of cysteine proteases (see Figure 9.16), is conserved in a family of amidotransferases, including CTP synthetase and GMP synthetase.

Intermediates can move between active sites by channeling

Carbamoyl phosphate synthetase contains three different active sites (see Figure 25.3), separated from one another by a total of 80 Å. Intermediates generated at one site move to the next without leaving the enzyme. These intermediates move within the enzyme by means of substrate channeling,

Figure 25.3 Structure of carbamoyl phosphate synthetase. *Notice* that the enzyme contains sites for three reactions. This enzyme consists of two chains. The smaller chain (yellow) contains a site for glutamine hydrolysis to generate ammonia. The larger chain includes two ATP-grasp domains (blue and red). In one ATP-grasp domain (blue), bicarbonate is phosphorylated to carboxyphosphate, which then reacts with ammonia to generate carbamic acid. In the other ATP-grasp domain, the carbamic acid is phosphorylated to produce carbamoyl phosphate. [Drawn from 1JDB.pdb.]

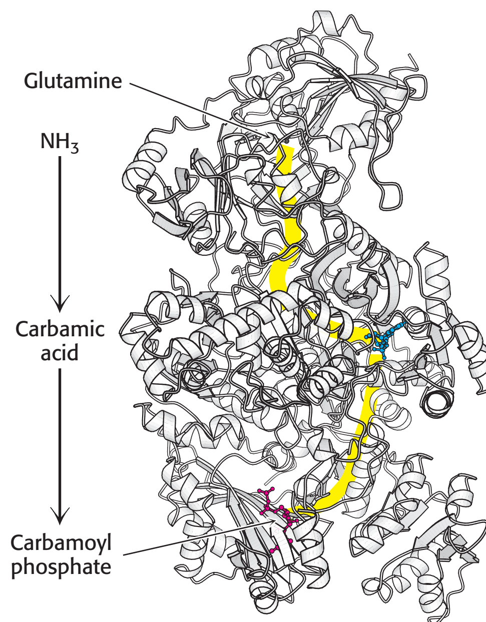
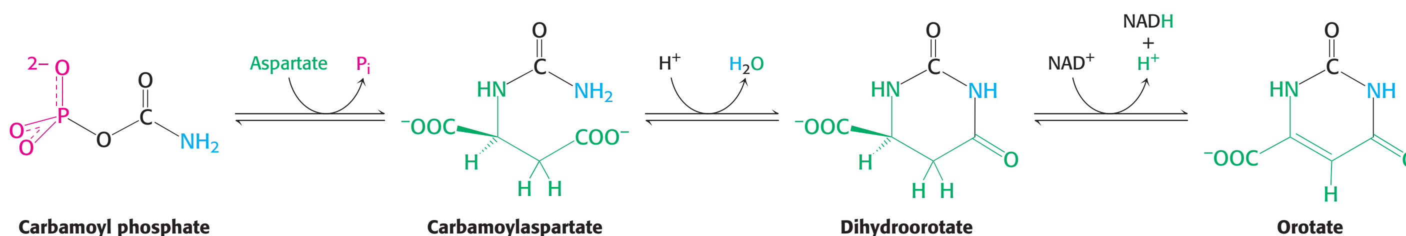


Figure 25.4 Substrate channeling. The three active sites of carbamoyl phosphate synthetase are linked by a channel (yellow) through which intermediates pass. Glutamine enters one active site, and carbamoyl phosphate, which includes the nitrogen atom from the glutamine side chain, leaves another 80 Å away. [Drawn from 1JDB.pdb.]

similar to the process described for tryptophan synthetase (Figure 25.4; also Figure 24.17). The ammonia generated in the glutamine-hydrolysis active site travels 45 Å through a channel within the enzyme to reach the site at which carboxyphosphate has been generated. The carbamic acid generated at this site diffuses an additional 35 Å through an extension of the channel to reach the site at which carbamoyl phosphate is generated. This channeling serves two roles: (1) intermediates generated at one active site are captured with no loss caused by diffusion and (2) labile intermediates, such as carboxyphosphate and carbamic acid (which decompose in less than 1 s at pH 7), are protected from hydrolysis. We will see additional examples of substrate channeling later in this chapter.

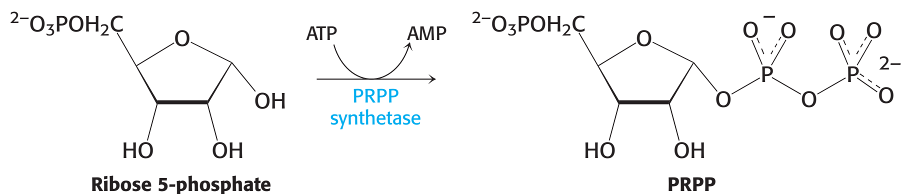
Orotate acquires a ribose ring from PRPP to form a pyrimidine nucleotide and is converted into uridylate

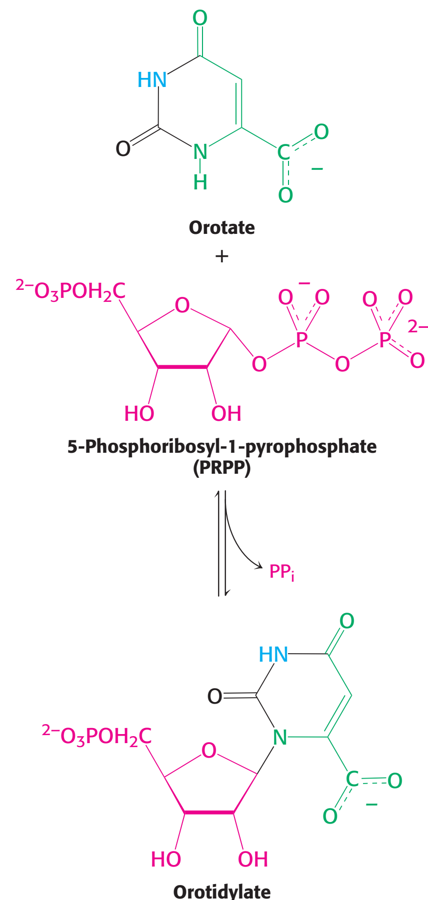
Carbamoyl phosphate reacts with aspartate to form carbamoylaspartate in a reaction catalyzed by *aspartate transcarbamoylase* (Section 10.1). Carbamoylaspartate then cyclizes to form dihydroorotate, which is then oxidized by NAD^+ to form orotate.



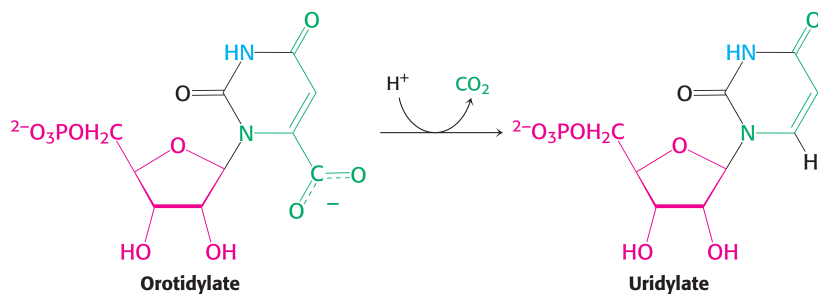
In mammals, the enzymes that form orotate are part of single large polypeptide chain called CAD, for *c*arbamoyl phosphate synthetase, *a*spartate transcarbamoylase and *d*ihydroorotase.

At this stage, orotate couples to ribose, in the form of *5-phosphoribosyl-1-pyrophosphate* (PRPP), a form of ribose activated to accept nucleotide bases. *5-Phosphoribosyl-1-pyrophosphate synthetase* synthesizes PRPP by adding a pyrophosphate from ATP to ribose-5-phosphate, which is formed by the pentose phosphate pathway.





Orotate reacts with PRPP to form *orotidylate,* a pyrimidine nucleotide. This reaction is driven by the hydrolysis of pyrophosphate. The enzyme that catalyzes this addition, *pyrimidine phosphoribosyltransferase,* is homologous to a number of other phosphoribosyltransferases that add different groups to PRPP to form the other nucleotides. Orotidylate is then decarboxylated to form *uridylate* (UMP), a major pyrimidine nucleotide that is a precursor to RNA. This reaction is catalyzed by *orotidylate decarboxylase.*



Orotidylate decarboxylase is one of the most proficient enzymes known. In its absence, decarboxylation is extremely slow and is estimated to take place once every 78 million years; with the enzyme present, it takes place approximately once per second, a rate enhancement of 10^{17}-fold.

Nucleotide mono-, di-, and triphosphates are interconvertible

How is the other major pyrimidine ribonucleotide, cytidine, formed? It is synthesized from the uracil base of UMP, but the synthesis can take place only after UMP has been converted into UTP. Recall that the diphosphates and triphosphates are the active forms of nucleotides in biosynthesis and energy conversions. Nucleoside monophosphates are converted into nucleoside triphosphates in stages. First, nucleoside monophosphates are converted into diphosphates by specific *nucleoside monophosphate kinases* that utilize ATP as the phosphoryl-group donor. For example, UMP is phosphorylated to UDP by *UMP kinase.*

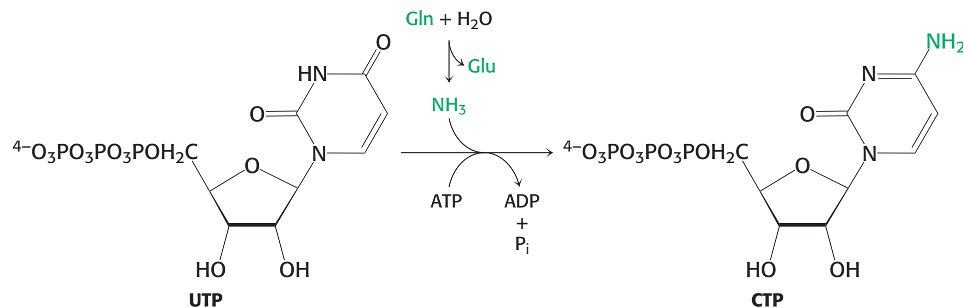
$$\text{UMP} + \text{ATP} \rightleftharpoons \text{UDP} + \text{ADP}$$

Nucleoside diphosphates and triphosphates are interconverted by *nucleoside diphosphate kinase,* an enzyme that has broad specificity, in contrast with the monophosphate kinases. X and Y represent any of several ribonucleosides or even deoxyribonucleosides:

$$\text{XDP} + \text{YTP} \rightleftharpoons \text{XTP} + \text{YDP}$$

CTP is formed by amination of UTP

After uridine triphosphate has been formed, it can be transformed into *cytidine triphosphate* by the replacement of a carbonyl group by an amino group, a reaction catalyzed by *cytidine triphosphate synthetase.*



Like the synthesis of carbamoyl phosphate, this reaction requires ATP and uses glutamine as the source of the amino group. The reaction proceeds through an analogous mechanism in which the O-4 atom is phosphorylated to form a reactive intermediate, and then the phosphate is displaced by ammonia, freed from glutamine by hydrolysis. CTP can then be used in many biochemical processes, including lipid and RNA synthesis.

Salvage pathways recycle pyrimidine bases

Pyrimidine bases can be recovered from the breakdown products of DNA and RNA by the use of *salvage pathways*. In these pathways, a preformed base is reincorporated into a nucleotide. We will consider the salvage for the pyrimidine base thymine. Thymine is found in DNA and base-pairs with adenine in the DNA double helix. Thymine released from degraded DNA is salvaged in two steps. First, thymine is converted into nucleoside thymidine by *thymidine phosphorylase*.

Thymine + deoxyribose-1-phosphate $\rightleftharpoons$ thymidine + P_i

Thymidine is then converted into a nucleotide by *thymidine kinase*.

Thymidine + ATP $\rightleftharpoons$ TMP + ADP

The activity of thymidine kinase fluctuates with the cell cycle, displaying peak activity during S phase when DNA synthesis is occurring. Viral thymidine kinase differs from the mammalian enzyme and thus provides a therapeutic target. For instance, herpes simplex infections are treated with acyclovir, which viral thymidine kinase converts into a suicide inhibitor that terminates DNA synthesis. As we will see shortly, thymidine kinase also plays a role in the de novo synthesis of thymidylate.

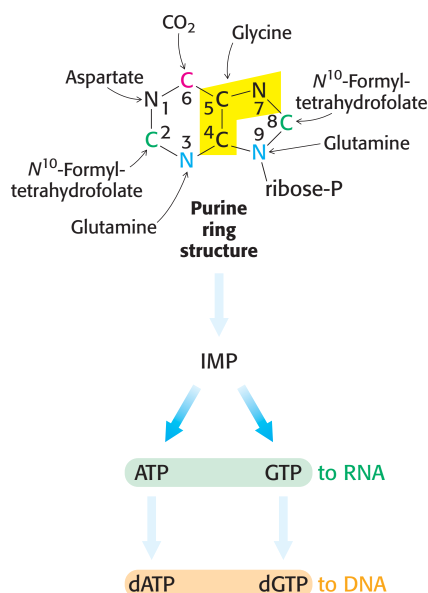


Figure 25.5 De novo pathway for purine nucleotide synthesis. The origins of the atoms in the purine ring are indicated.

25.2 Purine Bases Can Be Synthesized de Novo or Recycled by Salvage Pathways

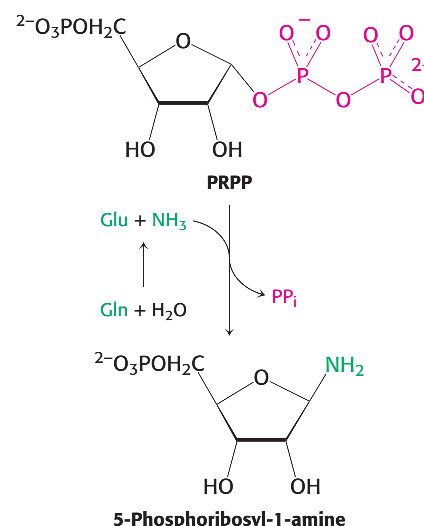
Like pyrimidine nucleotides, *purine nucleotides* can be synthesized de novo or by a salvage pathway. When synthesized de novo, purine synthesis begins with simple starting materials such as amino acids and bicarbonate (Figure 25.5). Unlike the bases of pyrimidines, the purine bases are assembled already attached to the ribose ring. Alternatively, purine bases, released by the hydrolytic degradation of nucleic acids and nucleotides, can be salvaged and recycled. Purine salvage pathways are especially noted for the energy that they save and the remarkable effects of their absence (p. 752).

The purine ring system is assembled on ribose phosphate

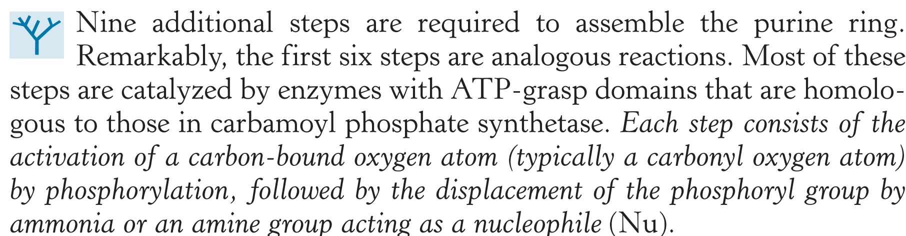
De novo purine biosynthesis, like pyrimidine biosynthesis, requires PRPP but, for purines, PRPP provides the foundation on which the bases are

constructed step by step. The initial committed step is the displacement of pyrophosphate by ammonia, rather than by a preassembled base, to produce *5-phosphoribosyl-1-amine,* with the amine in the β configuration.

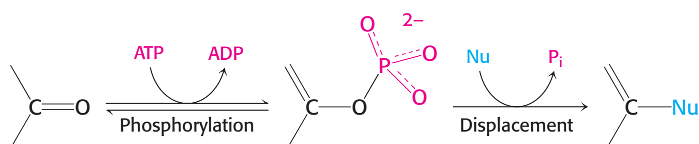
Glutamine phosphoribosyl amidotransferase catalyzes this reaction. This enzyme comprises two domains: the first is homologous to the phosphoribosyltransferases in purine salvage pathways (p. 744), whereas the second produces ammonia from glutamine by hydrolysis. However, this glutamine-hydrolysis domain is distinct from the domain that performs the same function in carbamoyl phosphate synthetase. In glutamine phosphoribosyl amidotransferase, a cysteine residue located at the amino terminus facilitates glutamine hydrolysis. To prevent wasteful hydrolysis of either substrate, the amidotransferase assumes the active configuration only on binding of both PRPP and glutamine. As is the case with carbamoyl phosphate synthetase, the ammonia generated at the glutamine-hydrolysis active site passes through a channel to reach PRPP without being released into solution.



The purine ring is assembled by successive steps of activation by phosphorylation followed by displacement



Nine additional steps are required to assemble the purine ring. Remarkably, the first six steps are analogous reactions. Most of these steps are catalyzed by enzymes with ATP-grasp domains that are homologous to those in carbamoyl phosphate synthetase. *Each step consists of the activation of a carbon-bound oxygen atom (typically a carbonyl oxygen atom) by phosphorylation, followed by the displacement of the phosphoryl group by ammonia or an amine group acting as a nucleophile* (Nu).



De novo purine biosynthesis proceeds as shown in Figure 25.6. Table 25.2 lists the enzymes that catalyze each step of the reaction.

1. The carboxylate group of a glycine residue is activated by phosphorylation and then coupled to the amino group of phosphoribosylamine. A new amide bond is formed, and the amino group of glycine is free to act as a nucleophile in the next step.

2. Formate is activated and then added to this amino group to form formylglycinamide ribonucleotide.

Table 25.2 The enzymes of de novo purine synthesis

Step	Enzyme
1	Glycinamide ribonucleotide (GAR) synthetase
2	GAR transformylase
3	Formylglycinamidine synthase
4	Aminoimidazole ribonucleotide synthetase
5	Carboxyaminoimidazole ribonucleotide synthase
6	Succinylaminoimidazole carboxamide ribonucleotide synthetase
7	Adenylosuccinate lyase
8	Aminoimidazole carboxamide ribonucleotide transformylase
9	Inosine monophosphate cyclohydrolyase

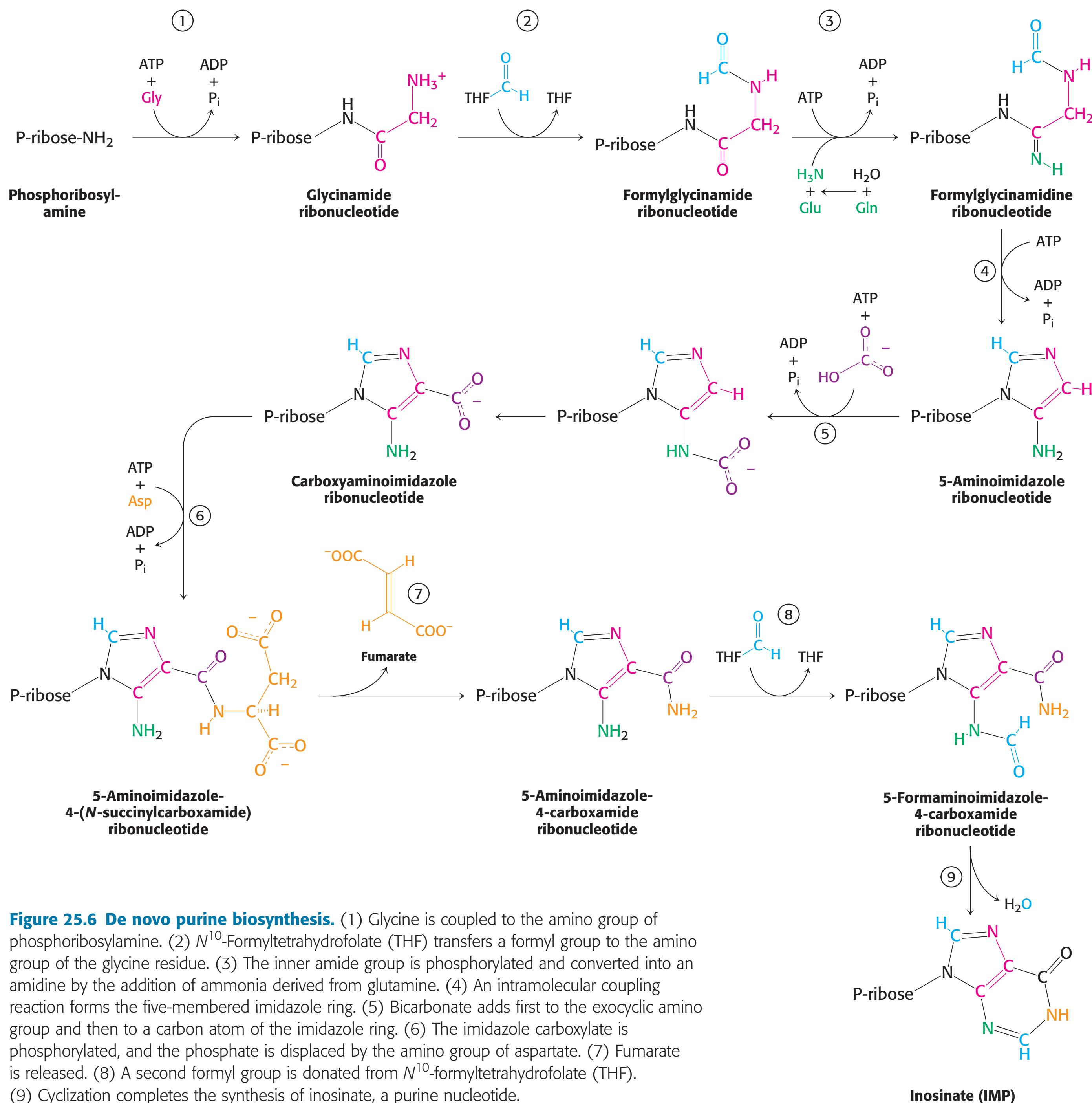


Figure 25.6 De novo purine biosynthesis. (1) Glycine is coupled to the amino group of phosphoribosylamine. (2) N^{10}-Formyltetrahydrofolate (THF) transfers a formyl group to the amino group of the glycine residue. (3) The inner amide group is phosphorylated and converted into an amidine by the addition of ammonia derived from glutamine. (4) An intramolecular coupling reaction forms the five-membered imidazole ring. (5) Bicarbonate adds first to the exocyclic amino group and then to a carbon atom of the imidazole ring. (6) The imidazole carboxylate is phosphorylated, and the phosphate is displaced by the amino group of aspartate. (7) Fumarate is released. (8) A second formyl group is donated from N^{10}-formyltetrahydrofolate (THF). (9) Cyclization completes the synthesis of inosinate, a purine nucleotide.

3. The inner carbonyl group is activated by phosphorylation and then converted into an amidine by the addition of ammonia derived from glutamine.

4. The product of this reaction, formylglycinamidine ribonucleotide, cyclizes to form the five-membered imidazole ring found in purines. Although this cyclization is likely to be favorable thermodynamically, a molecule of ATP is consumed to ensure irreversibility. The familiar pattern is repeated: a phosphoryl group from the ATP molecule activates the carbonyl group and is displaced by the nitrogen atom attached to the ribose molecule. Cyclization is thus an intramolecular reaction in which the nucleophile and phosphate-activated carbon atom are present within the same molecule. In higher eukaryotes, the enzymes catalyzing steps 1, 2, and 4 (see Table 25.2) are components of a single polypeptide chain.

5. Bicarbonate is activated by phosphorylation and then attacked by the exocyclic amino group. The product of the reaction in step 5 rearranges to transfer the carboxylate group to the imidazole ring. Interestingly, mammals do not require ATP for this step; bicarbonate apparently attaches directly to the exocyclic amino group and is then transferred to the imidazole ring.

6. The imidazole carboxylate group is phosphorylated again and the phosphate group is displaced by the amino group of aspartate. Once again, in higher eukaryotes, the enzymes catalyzing steps 5 and 6 (see Table 25.2) share a single polypeptide chain.

7. Fumarate, an intermediate in the citric acid cycle, is eliminated, leaving the nitrogen atom from aspartate joined to the imidazole ring. The use of aspartate as an amino-group donor and the concomitant release of fumarate are reminiscent of the conversion of citrulline into arginine in the urea cycle, and these steps are catalyzed by homologous enzymes in the two pathways (Section 23.4).

8. A formyl group from N^{10}-formyltetrahydrofolate is added to this nitrogen atom to form a final 5-formaminoimidazole-4-carboxamide ribonucleotide.

9. 5-Formaminoimidazole-4-carboxamide ribonucleotide cyclizes with the loss of water to form inosinate.

Many of the intermediates in the de novo purine biosynthesis pathway degrade rapidly in water. Their instability in water suggests that the product of one enzyme must be channeled directly to the next enzyme along the pathway. Recent evidence shows that the enzymes do indeed form complexes when purine synthesis is required.

AMP and GMP are formed from IMP

A few steps convert inosinate into either AMP or GMP (Figure 25.7). *Adenylate* is synthesized from inosinate by the substitution of an amino group for the carbonyl oxygen atom at C-6. Again, the addition of aspartate followed by the elimination of fumarate contributes the amino group. GTP, rather than ATP, is the phosphoryl-group donor in the synthesis of the

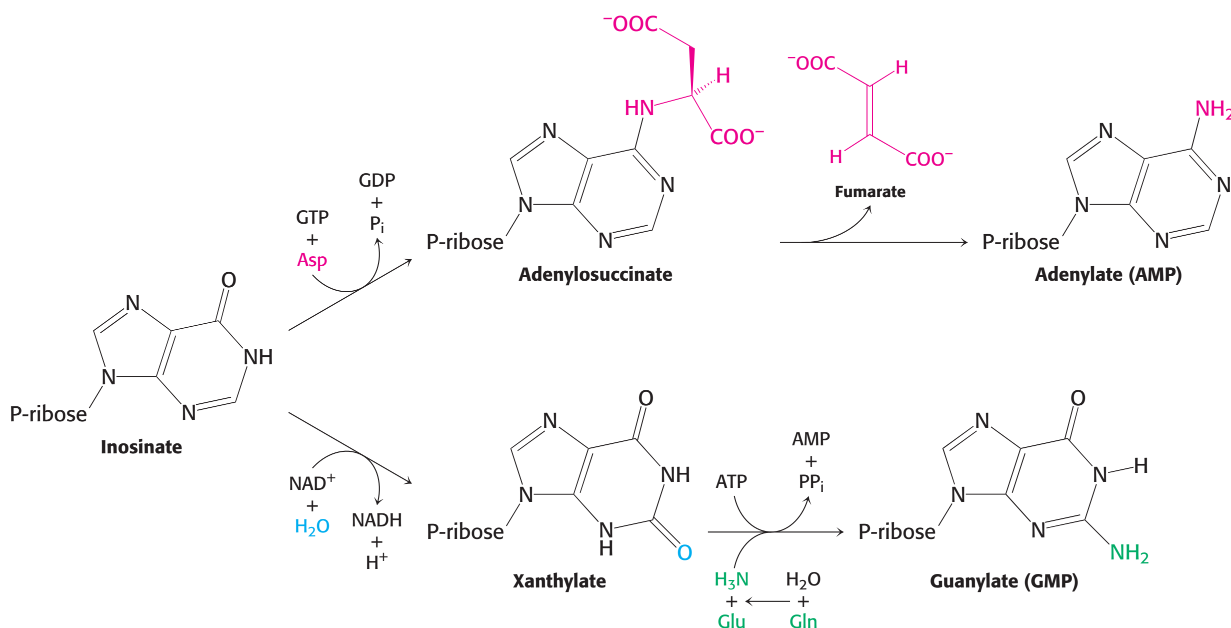


Figure 25.7 Generating AMP and GMP. Inosinate is the precursor of AMP and GMP. AMP is formed by the addition of aspartate followed by the release of fumarate. GMP is generated by the addition of water, dehydrogenation by NAD^+, and the replacement of the carbonyl oxygen atom by —NH_2 derived by the hydrolysis of glutamine.

adenylosuccinate intermediate from inosinate and aspartate. In accord with the use of GTP, the enzyme that promotes this conversion, *adenylosuccinate synthase*, is structurally related to the G-protein family and does not contain an ATP-grasp domain.

Guanylate is synthesized by the oxidation of inosinate to xanthylate (XMP), followed by the incorporation of an amino group at C-2. NAD^+ is the hydrogen acceptor in the oxidation of inosinate. Xanthylate is activated by the transfer of an AMP group (rather than a phosphoryl group) from ATP to the oxygen atom in the newly formed carbonyl group. Ammonia, generated by the hydrolysis of glutamine, then displaces the AMP group to form guanylate, in a reaction catalyzed by *GMP synthetase.* Note that the synthesis of adenylate requires GTP, whereas the synthesis of guanylate requires ATP. This reciprocal use of nucleotides by the pathways creates an important regulatory opportunity (Section 25.4).

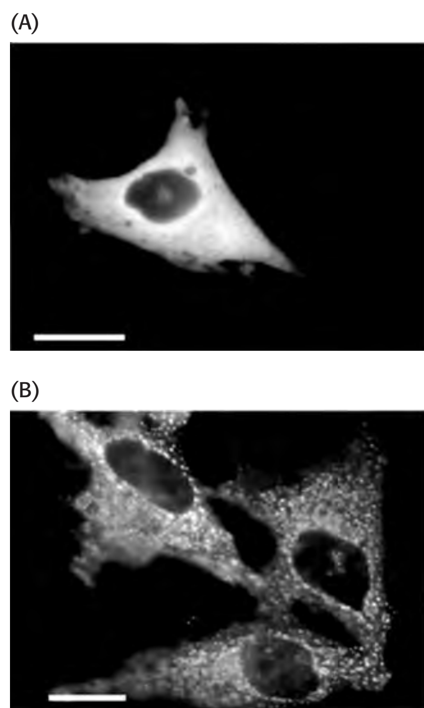
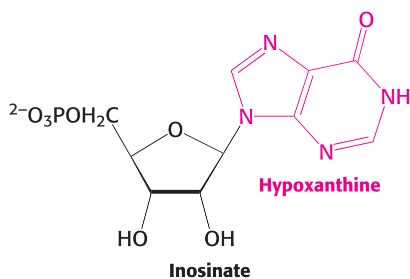


Figure 25.8 Formation of purinosomes. A gene construct encoding a fusion protein consisting of formylglycinamidine synthase and GFP was transfected into and expressed in Hela cells, a human cell line. (A) In the presence of purines (the absence of purine synthesis), the GFP was seen as a diffuse stain throughout the cytoplasm. (B) When the cells were shifted to a purine-free medium, purinosomes formed, seen as cytoplasmic granules, and purine synthesis occurred. [An, S., Kumar, R., Sheets, E. D., and Benkovic, S. J. 2008. *Science* 320: 103–106. Figure 2, C and D.]

Enzymes of the purine synthesis pathway associate with one another in vivo

Biochemists believe that the enzymes of many metabolic pathways, such as glycolysis and the citric acid cycle, are physically associated with one another. Such associations would increase the efficiency of pathways by facilitating the movement of the product of one enzyme to the active site of the next enzyme in the pathway. The evidence for such associations comes primarily from experiments in which one component of a pathway, carefully isolated from the cell, is found to be bound to other components of the pathway. However, these observations raise the question, do enzymes associate with one another in vivo or do they spuriously associate during the isolation procedure? Recent in vivo evidence shows that the enzymes of the purine synthesis pathway associate with one another when purine synthesis is required. Various enzymes of the pathway were fused with the green fluorescent protein (see Figure 2.65) and transfected into cells. When cells were grown in the presence of purine, the GFP was spread diffusely throughout the cytoplasm (Figure 25.8A). When the cells were switched to growth media without purines, purine synthesis began and the enzymes became associated with one another, forming complexes dubbed *purinosomes* (Figure 25.8B). The experiments were repeated with other enzymes of the purine synthesis pathway bearing the GFP, and the results were the same: purine synthesis occurs when the enzymes form the purinosomes. What actually causes complex formation? While the results are not yet established, it appears that a phosphatase, presumably somehow responding to the absence of purines, instigates complex formation, while a kinase, responding to the presence of purines, causes disassembly of the purinosome.

Salvage pathways economize intracellular energy expenditure



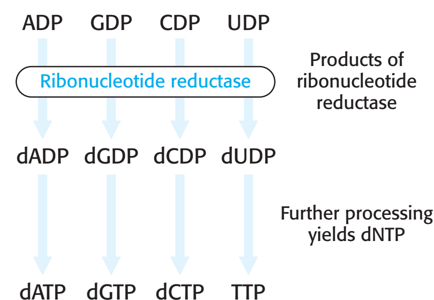
As we have seen, the de novo synthesis of purines requires a substantial investment of ATP. Purine salvage pathways provide a more economical means of generating purines. Free purine bases, derived from the turnover of nucleotides or from the diet, can be attached to PRPP to form purine nucleoside monophosphates, in a reaction analogous to the formation of orotidylate. Two salvage enzymes with different specificities recover purine bases. *Adenine phosphoribosyltransferase* catalyzes the formation of adenylate (AMP):

$$\text{Adenine} + \text{PRPP} \longrightarrow \text{adenylate} + PP_i$$

whereas *hypoxanthine-guanine phosphoribosyltransferase* (HGPRT) catalyzes the formation of guanylate (GMP) as well as *inosinate* (inosine monophosphate, IMP), a precursor of guanylate and adenylate.

25.3 Deoxyribonucleotides Are Synthesized by the Reduction of Ribonucleotides Through a Radical Mechanism

We turn now to the synthesis of deoxyribonucleotides. These precursors of DNA are formed by the reduction of ribonucleotides; specifically, the 2′-hydroxyl group on the ribose moiety is replaced by a hydrogen atom. The substrates are ribonucleoside diphosphates, and the ultimate reductant is NADPH. The enzyme *ribonucleotide reductase* is responsible for the reduction reaction for all four ribonucleotides. The ribonucleotide reductases of different organisms are a remarkably diverse set of enzymes. Yet detailed studies have revealed that they have a common reaction mechanism, and their three-dimensional structural features indicate that these enzymes are homologous. We will focus on the best understood of these enzymes, that of *E. coli* living aerobically.



Mechanism: A tyrosyl radical is critical to the action of ribonucleotide reductase

The ribonucleotide reductase of *E. coli* consists of two subunits: R1 (an 87-kd dimer) and R2 (a 43-kd dimer). The R1 subunit contains the active site as well as two allosteric control sites (Section 25.4). This subunit includes three conserved cysteine residues and a glutamate residue, all four of which participate in the reduction of ribose to deoxyribose (Figure 25.9). The R2 subunit's role in catalysis is to generate a remarkable free radical in each of its two chains. Each R2 chain contains a stable *tyrosyl radical* with

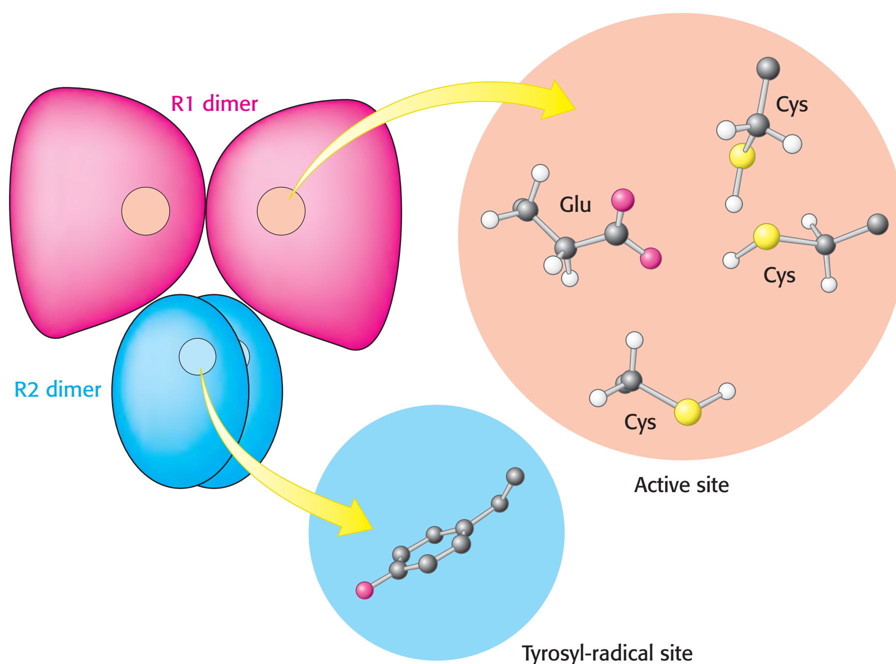


Figure 25.9 Ribonucleotide reductase. Ribonucleotide reductase reduces ribonucleotides to deoxyribonucleotides in its active site, which contains three key cysteine residues and one glutamate residue. Each R2 subunit contains a tyrosyl radical that accepts an electron from one of the cysteine residues in the active site to initiate the reduction reaction. Two R1 subunits come together to form a dimer as do two R2 subunits.

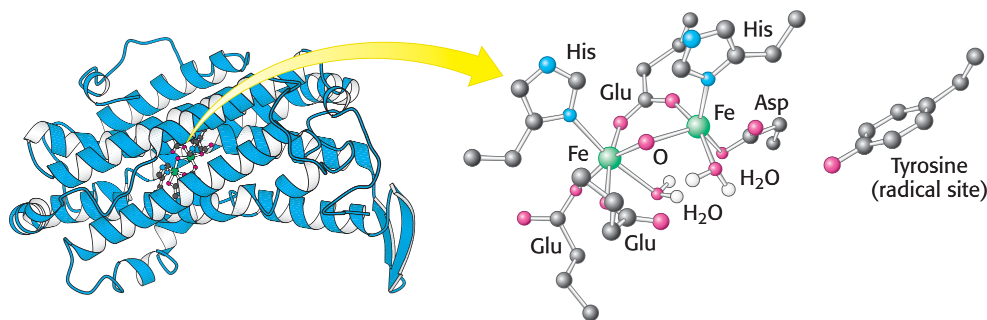


Figure 25.10 Ribonucleotide reductase R2 subunit. The R2 subunit contains a stable free radical on a tyrosine residue. This radical is generated by the reaction of oxygen (not shown) at a nearby site containing two iron atoms. Two R2 subunits come together to form a dimer. [Drawn from 1RIB.pdb.]

an unpaired electron delocalized onto its aromatic ring (Figure 25.10). This very unusual free radical is generated by a nearby *iron center* consisting of two ferric (Fe^{3+}) ions bridged by an oxide (O^{2-}) ion.

In the synthesis of a deoxyribonucleotide, the OH bonded to C-2' of the ribose ring is replaced by H, with retention of the configuration at the C-2' carbon atom (Figure 25.11).

Figure 25.11 Ribonucleotide reductase mechanism. (1) An electron is transferred from a cysteine residue on R1 to a tyrosine radical on R2, generating a highly reactive cysteine thiyl radical. (2) This radical abstracts a hydrogen atom from C-3′ of the ribose unit. (3) The radical at C-3′ releases OH^- from the C-2′ carbon atom. Combined with a proton from a second cysteine residue, the OH^- is eliminated as water. (4) A hydride ion is transferred from a third cysteine residue with the concomitant formation of a disulfide bond. (5) The C-3′ radical recaptures the originally abstracted hydrogen atom. (6) An electron is transferred from R2 to reduce the thiyl radical, which also accepts a proton. The deoxyribonucleotide is free to leave R1. The disulfide formed in the active site must be reduced to begin another cycle.

1. The reaction begins with the transfer of an electron from a cysteine residue on R1 to the tyrosyl radical on R2. The loss of an electron generates a highly reactive *cysteine thiyl radical* within the active site of R1.

2. This radical then abstracts a hydrogen atom from C-3' of the ribose unit, generating a radical at that carbon atom.

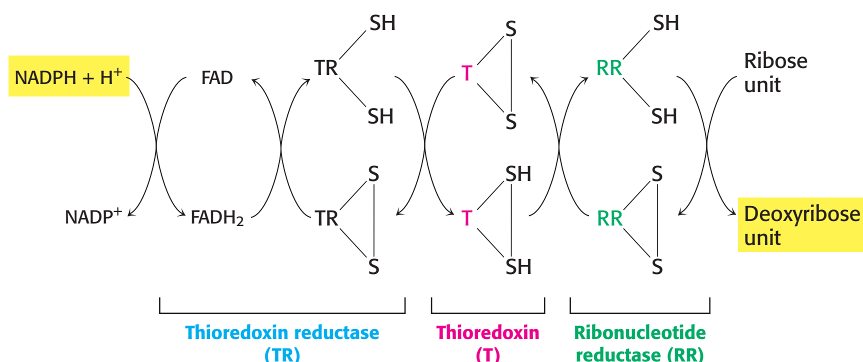
3. The radical at C-3' promotes the release of the OH^- from the C-2' carbon atom. Protonated by a second cysteine residue, the departing OH^- leaves as a water molecule.

4. A hydride ion (a proton with two electrons) is then transferred from a third cysteine residue to complete the reduction of the position, form a disulfide bond, and re-form a radical.

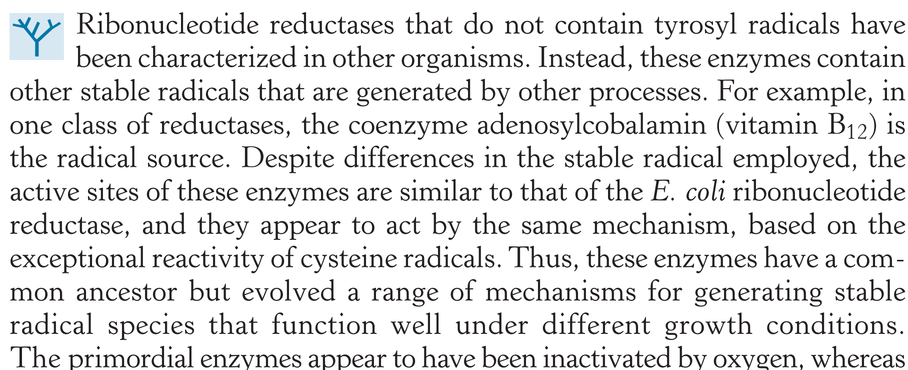
5. This C-3' radical recaptures the same hydrogen atom originally abstracted by the first cysteine residue, and the deoxyribonucleotide is free to leave the enzyme.

6. R2 provides an electron to reduce the thiyl radical. The disulfide bond generated in the enzyme's active site must then be reduced to regenerate the active enzyme.

The electrons for this reduction come from NADPH, but not directly. One carrier of reducing power linking NADPH with the reductase is *thioredoxin,* a 12-kd protein with two exposed cysteine residues near each other. These sulfhydryls are oxidized to a disulfide in the reaction catalyzed by ribonucleotide reductase itself. In turn, reduced thioredoxin is regenerated by electron flow from NADPH. This reaction is catalyzed by *thioredoxin reductase,* a flavoprotein. Electrons flow from NADPH to bound FAD of the reductase, to the disulfide of oxidized thioredoxin, and then to ribonucleotide reductase and finally to the ribose unit.



Stable radicals other than tyrosyl radical are employed by other ribonucleotide reductases



Ribonucleotide reductases that do not contain tyrosyl radicals have been characterized in other organisms. Instead, these enzymes contain other stable radicals that are generated by other processes. For example, in one class of reductases, the coenzyme adenosylcobalamin (vitamin B_{12}) is the radical source. Despite differences in the stable radical employed, the active sites of these enzymes are similar to that of the *E. coli* ribonucleotide reductase, and they appear to act by the same mechanism, based on the exceptional reactivity of cysteine radicals. Thus, these enzymes have a common ancestor but evolved a range of mechanisms for generating stable radical species that function well under different growth conditions. The primordial enzymes appear to have been inactivated by oxygen, whereas

enzymes such as the *E. coli* enzyme make use of oxygen to generate the initial tyrosyl radical. Note that the reduction of ribonucleotides to deoxyribonucleotides is a difficult reaction chemically, likely to require a sophisticated catalyst. The existence of a common protein enzyme framework for this process strongly suggests that proteins joined the RNA world before the evolution of DNA as a stable storage form for genetic information.

Thymidylate is formed by the methylation of deoxyuridylate

Uracil, produced by the pyrimidine synthesis pathway, is not a component of DNA. Rather, DNA contains *thymine,* a methylated analog of uracil. Another step is required to generate thymidylate from uracil. *Thymidylate synthase* catalyzes this finishing touch: deoxyuridylate (dUMP) is methylated to thymidylate (TMP). Recall that thymidylate synthase also functions in the thymine salvage pathways. As will be described in Chapter 28, the methylation of this nucleotide marks sites of DNA damage for repair and, hence, helps preserve the integrity of the genetic information stored in DNA. The methyl donor in this reaction is N^5,N^{10}-methylenetetrahydrofolate rather than *S*-adenosylmethionine (Section 24.2).

The methyl group becomes attached to the C-5 atom within the aromatic ring of dUMP, but this carbon atom is not a good nucleophile and cannot itself attack the appropriate group on the methyl donor. Thymidylate synthase promotes methylation by adding a thiolate from a cysteine side chain to this ring to generate a nucleophilic species that can attack the methylene group of N^5,N^{10}-methylenetetrahydrofolate (Figure 25.12). This methylene group, in turn, is activated by distortions imposed by the enzyme that favor opening the five-membered ring. The activated dUMP's attack on the methylene group forms the new carbon–carbon bond. The intermediate formed is then converted into product: a hydride ion is transferred from the tetrahydrofolate ring to transform the methylene group into a methyl group, and a proton is abstracted from the carbon atom bearing the methyl group to eliminate the cysteine and regenerate the aromatic ring. The tetrahydrofolate derivative loses both its methylene group and a hydride ion and, hence, is oxidized to dihydrofolate. For the synthesis of more thymidylate, tetrahydrofolate must be regenerated.

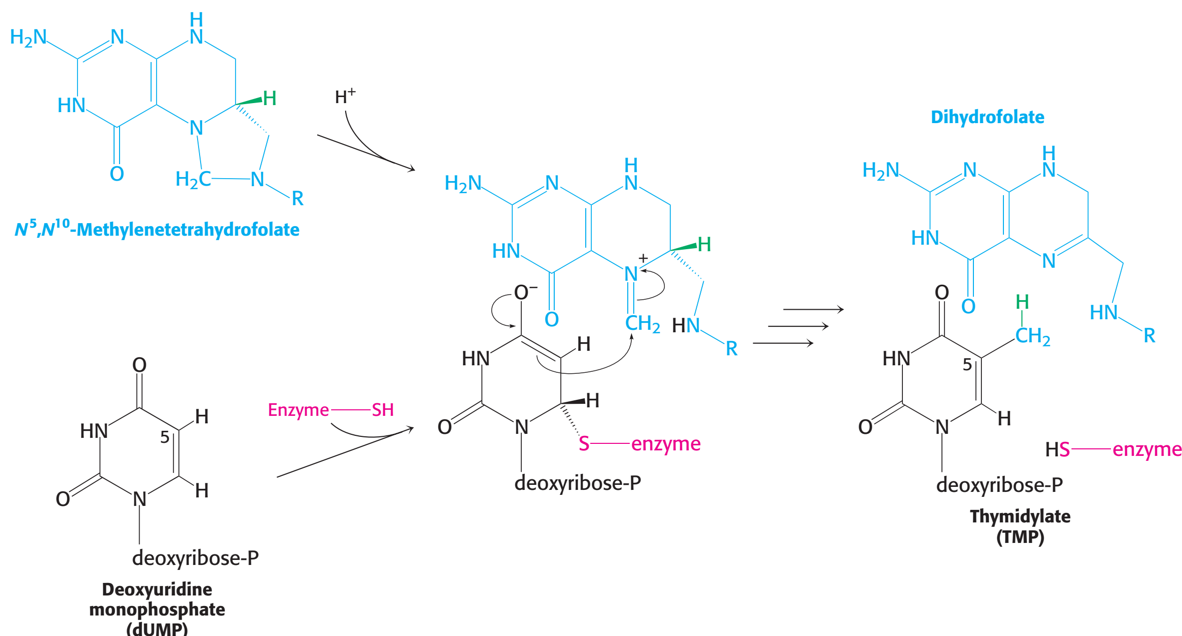
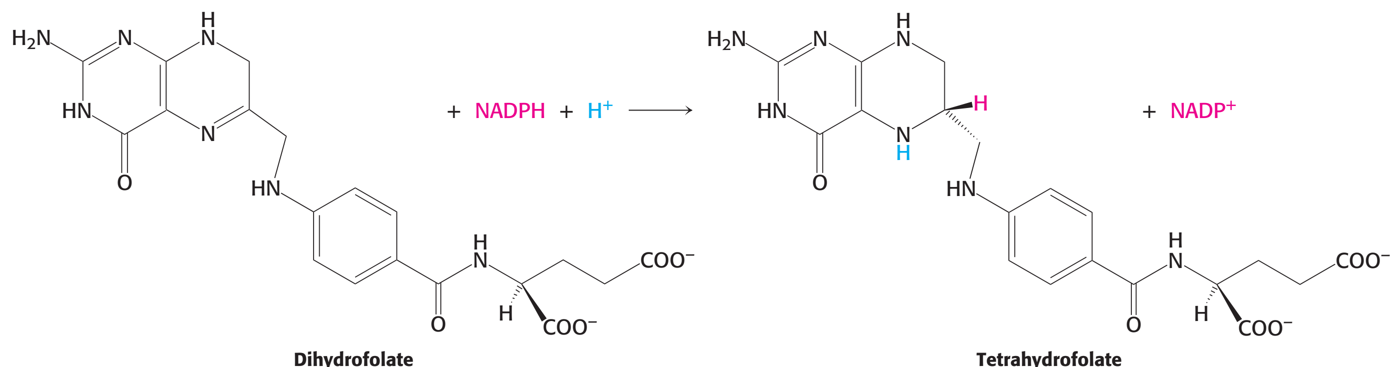


Figure 25.12 Thymidylate synthesis. Thymidylate synthase catalyzes the addition of a methyl group (derived from N^5,N^{10}-methylenetetrahydrofolate) to dUMP to form TMP. The addition of a thiolate from the enzyme activates dUMP. Opening the five-membered ring of the THF derivative prepares the methylene group for nucleophilic attack by the activated dUMP. The reaction is completed by the transfer of a hydride ion to form dihydrofolate.

Dihydrofolate reductase catalyzes the regeneration of tetrahydrofolate, a one-carbon carrier

Tetrahydrofolate is regenerated from the dihydrofolate that is produced in the synthesis of thymidylate. This regeneration is accomplished by *dihydrofolate reductase* with the use of NADPH as the reductant.



A hydride ion is directly transferred from the nicotinamide ring of NADPH to the pteridine ring of dihydrofolate. The bound dihydrofolate and NADPH are held in close proximity to facilitate the hydride transfer.

Several valuable anticancer drugs block the synthesis of thymidylate

Rapidly dividing cells require an abundant supply of thymidylate for the synthesis of DNA. The vulnerability of these cells to the inhibition of TMP synthesis has been exploited in the treatment of cancer. Thymidylate synthase and dihydrofolate reductase are choice targets of chemotherapy (Figure 25.13).

Fluorouracil, an anticancer drug, is converted in vivo into *fluorodeoxyuridylate* (F-dUMP). This analog of dUMP irreversibly inhibits thymidylate synthase after acting as a normal substrate through part of the catalytic

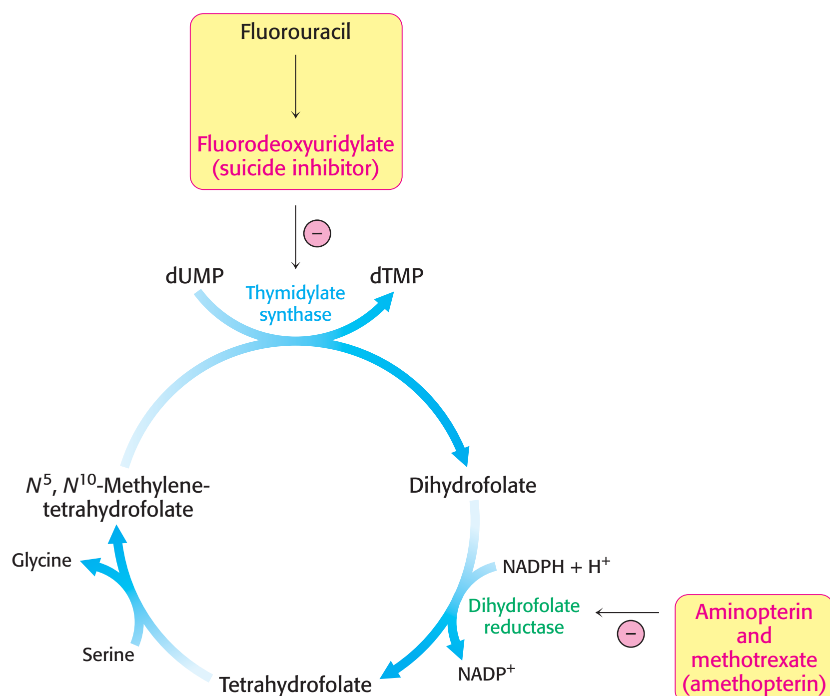


Figure 25.13 Anticancer drug targets. Thymidylate synthase and dihydrofolate reductase are choice targets in cancer chemotherapy because the generation of large quantities of precursors for DNA synthesis is required for rapidly dividing cancer cells.

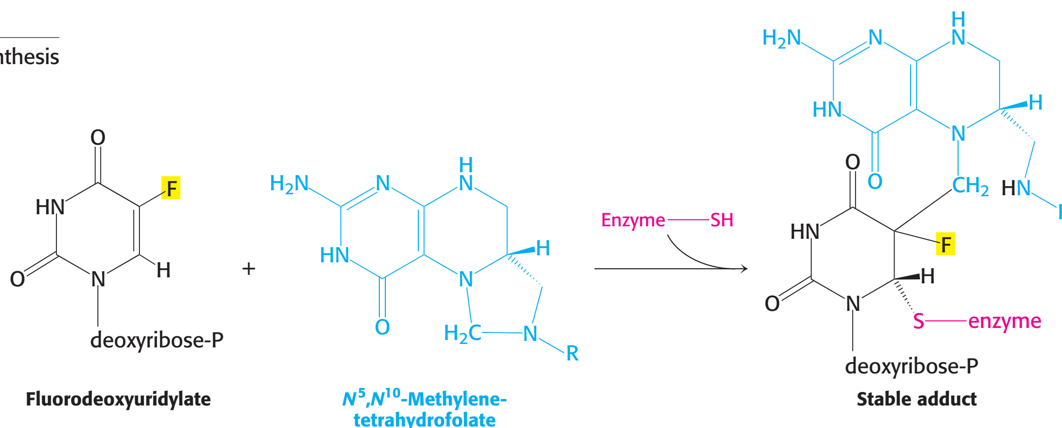
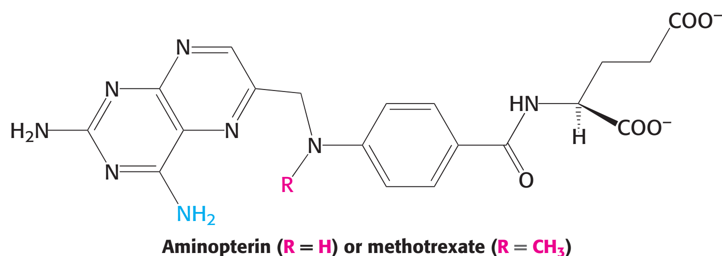


Figure 25.14 Suicide inhibition. Fluorodeoxyuridylate (generated from fluorouracil) traps thymidylate synthase in a form that cannot proceed down the reaction pathway.

cycle. Recall that the formation of TMP requires the removal of a proton (H^+) from C-5 of the bound nucleotide (see Figure 25.12). However, the enzyme cannot abstract F^+ from F-dUMP, and so catalysis is blocked at the stage of the covalent complex formed by F-dUMP, methylenetetrahydrofolate, and the sulfhydryl group of the enzyme (Figure 25.14). We see here an example of *suicide inhibition,* in which an enzyme converts a substrate into a reactive inhibitor that halts the enzyme's catalytic activity (Section 8.5).

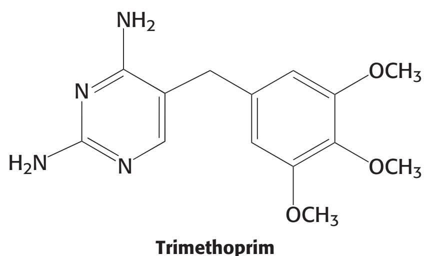
The synthesis of TMP can also be blocked by inhibiting the regeneration of tetrahydrofolate. Analogs of dihydrofolate, such as *aminopterin* and *methotrexate* (amethopterin), are potent competitive inhibitors ($K_i < 1$ nM) of dihydrofolate reductase.



Aminopterin (R = H) or methotrexate (R = CH_3)

Methotrexate is a valuable drug in the treatment of many rapidly growing tumors, such as those in acute leukemia and choriocarcinoma, a cancer derived from placental cells. However, methotrexate kills rapidly replicating cells whether they are malignant or not. Stem cells in bone marrow, epithelial cells of the intestinal tract, and hair follicles are vulnerable to the action of this folate antagonist, accounting for its toxic side effects, which include weakening of the immune system, nausea, and hair loss.

Folate analogs such as *trimethoprim* have potent antibacterial and antiprotozoal activity. Trimethoprim binds 10^5-fold less tightly to mammalian dihydrofolate reductase than it does to reductases of susceptible microorganisms. Small differences in the active-site clefts of these enzymes account for the highly selective antimicrobial action. The combination of trimethoprim and sulfamethoxazole (an inhibitor of folate synthesis) is widely used to treat infections.



Trimethoprim

25.4 Key Steps in Nucleotide Biosynthesis Are Regulated by Feedback Inhibition

Nucleotide biosynthesis is regulated by feedback inhibition in a manner similar to the regulation of amino acid biosynthesis (Section 24.3). These

regulatory pathways ensure that the various nucleotides are produced in the required quantities.

Pyrimidine biosynthesis is regulated by aspartate transcarbamoylase

Aspartate transcarbamoylase, one of the key enzymes for the regulation of pyrimidine biosynthesis in bacteria, was described in detail in Chapter 10. Recall that *ATCase is inhibited by CTP, the final product of pyrimidine biosynthesis,* and stimulated by ATP.

Carbamoyl phosphate synthetase is also a site of feedback inhibition in both prokaryotes and eukaryotes.

The synthesis of purine nucleotides is controlled by feedback inhibition at several sites

The regulatory scheme for purine nucleotides is more complex than that for pyrmidine nucleotides (Figure 25.15).

1. The committed step in purine nucleotide biosynthesis is the conversion of PRPP into phosphoribosylamine by *glutamine phosphoribosyl amidotransferase.* This important enzyme is feedback-inhibited by many purine ribonucleotides. It is noteworthy that AMP and GMP, the final products of the pathway, are synergistic in inhibiting the amidotransferase.

2. Inosinate is the branch point in the synthesis of AMP and GMP. *The reactions leading away from inosinate are sites of feedback inhibition.* AMP inhibits the conversion of inosinate into adenylosuccinate, its immediate precursor. Similarly, GMP inhibits the conversion of inosinate into xanthylate, its immediate precursor.

3. As already noted, GTP is a substrate in the synthesis of AMP, whereas ATP is a substrate in the synthesis of GMP. This *reciprocal substrate relation* tends to balance the synthesis of adenine and guanine ribonucleotides.

Note that the synthesis of PRPP by PRPP synthetase is highly regulated even though it is not the committed step in purine synthesis. Mutations have been identified in PRPP synthetase that result in a loss of allosteric response to nucleotides without any effect on catalytic activity of the enzyme. A consequence of this mutation is an overabundance of purine nucleotides that can result in gout, a pathological condition discussed later in the chapter.

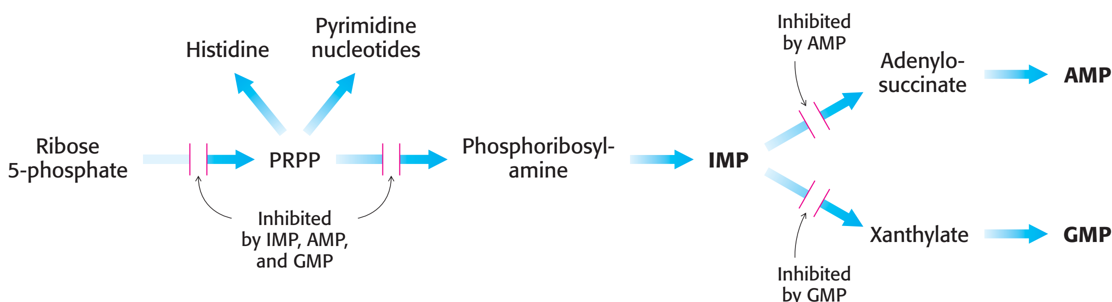


Figure 25.15 Control of purine biosynthesis. Feedback inhibition controls both the overall rate of purine biosynthesis and the balance between AMP and GMP production.

The synthesis of deoxyribonucleotides is controlled by the regulation of ribonucleotide reductase

The reduction of ribonucleotides to deoxyribonucleotides is precisely controlled by allosteric interactions. Each polypeptide of the R1 subunit of the aerobic *E. coli* ribonucleotide reductase contains two allosteric sites: one of them controls the *overall activity* of the enzyme, and the other regulates *substrate specificity* (Figure 25.16). The overall catalytic activity of ribonucleotide reductase is diminished by the binding of dATP, which signals an abundance of deoxyribonucleotides. The binding of ATP reverses this feedback inhibition. The binding of dATP or ATP to the substrate-specificity control site enhances the reduction of UDP and CDP, the pyrimidine nucleotides. The binding of thymidine triphosphate (TTP) promotes the reduction of GDP and inhibits the further reduction of pyrimidine ribonucleotides. The subsequent increase in the level of dGTP stimulates the reduction of ATP to dATP. This complex pattern of regulation supplies the appropriate balance of the four deoxyribonucleotides needed for the synthesis of DNA.

25.5 Disruptions in Nucleotide Metabolism Can Cause Pathological Conditions

Nucleotides are vital to a host of biochemical processes. It is not surprising, then, that disruption of nucleotide metabolism would have a variety of physiological effects. The nucleotides of a cell undergo continual turnover. Nucleotides are hydrolytically degraded to nucleosides by *nucleotidases*. The phosphorolytic cleavage of nucleosides to free bases and ribose 1-phosphate (or deoxyribose 1-phosphate) is catalyzed by *nucleoside phosphorylases*. Ribose 1-phosphate is isomerized by *phosphoribomutase* to ribose 5-phosphate, a substrate in the synthesis of PRPP. Some of the bases are reused to form nucleotides by salvage pathways. Others are degraded to products that are excreted (Figure 25.17). A deficiency of an enzyme can disrupt these pathways, leading to a pathological condition.

The loss of adenosine deaminase activity results in severe combined immunodeficiency

The pathway for the degradation of AMP includes an extra step because adenosine is not a substrate for nucleoside phosphorylase. First, the phosphate is removed by a nucleotidase to yield the nucleoside

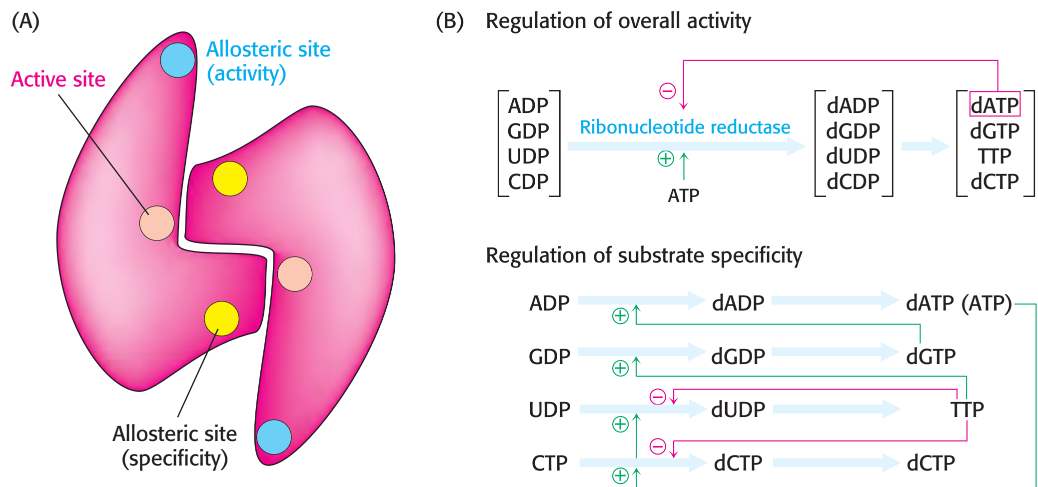


Figure 25.16 Regulation of ribonucleotide reductase. (A) Each subunit in the R1 dimer contains two allosteric sites in addition to the active site. One site regulates the overall activity and the other site regulates substrate specificity. (B) The patterns of regulation with regard to different nucleoside diphosphates demonstrated by ribonucleotide reductase.

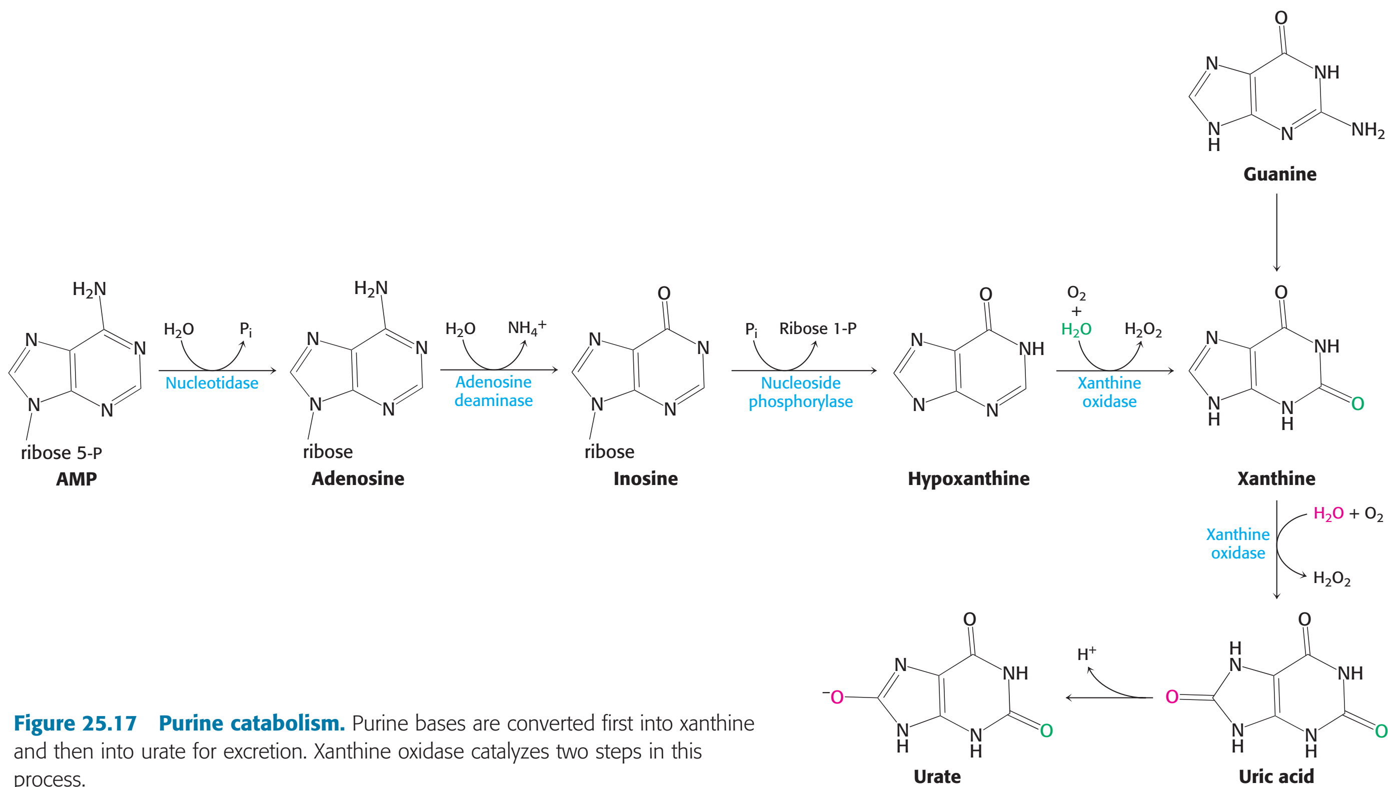
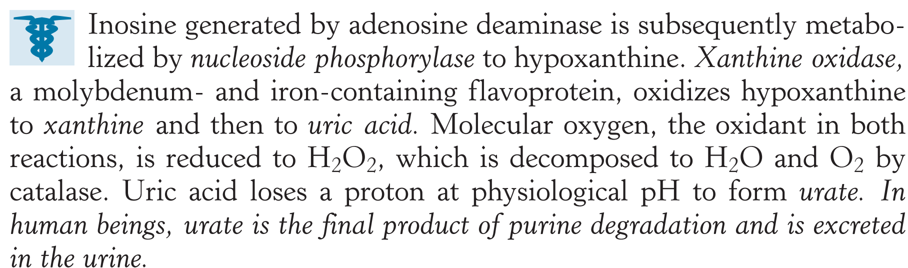


Figure 25.17 Purine catabolism. Purine bases are converted first into xanthine and then into urate for excretion. Xanthine oxidase catalyzes two steps in this process.

adenosine (see Figure 25.17). In the extra step, adenosine is deaminated by *adenosine deaminase* to form inosine.

A deficiency in adenosine deaminase activity is associated with some forms of *severe combined immunodeficiency* (SCID), an immunological disorder. Persons with the disorder have severe recurring infections, often leading to death at an early age. SCID is characterized by a loss of T cells, which are crucial to the immune response (Section 34.5). Although the biochemical basis of the disorder is not clearly established, a lack of adenosine deaminase results in an increase of 50 to 100 times the normal level of dATP, which inhibits ribonucleotide reductase and, consequently, DNA synthesis. Moreover, adenosine itself is a powerful signal molecule with a role in a number of regulatory pathways. Disruption in the levels of adenosine may also be deleterious. SCID is often called the "bubble boy disease" because its treatment may include complete isolation of the patient from the environment. Adenosine deaminase deficiency has been successfully treated by gene therapy.

Gout is induced by high serum levels of urate



Inosine generated by adenosine deaminase is subsequently metabolized by *nucleoside phosphorylase* to hypoxanthine. *Xanthine oxidase,* a molybdenum- and iron-containing flavoprotein, oxidizes hypoxanthine to *xanthine* and then to *uric acid.* Molecular oxygen, the oxidant in both reactions, is reduced to H_2O_2, which is decomposed to H_2O and O_2 by catalase. Uric acid loses a proton at physiological pH to form *urate. In human beings, urate is the final product of purine degradation and is excreted in the urine.*

High serum levels of urate (hyperuricemia) induce the painful joint disease *gout*. In this disease, the sodium salt of urate crystallizes in the fluid and lining of the joints (Figure 25.18). The small joint at the base of the big toe is a common site for sodium urate buildup, although the salt accumulates

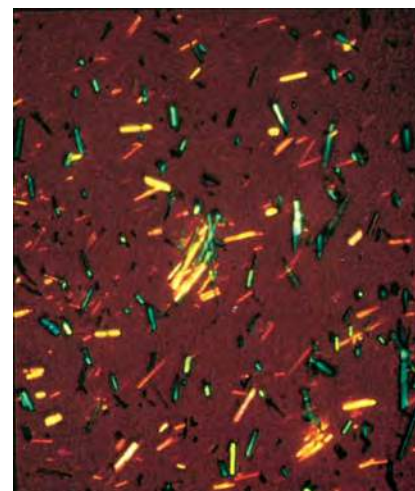
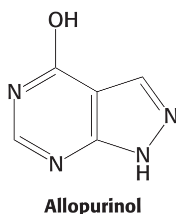


Figure 25.18 Micrograph of sodium urate crystals. The accumulation of these crystals damages joints and kidneys. [Courtesy of Dr. James McGuire.]

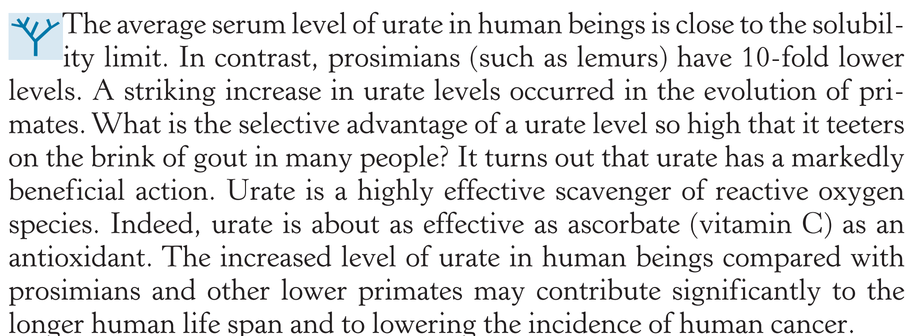
at other joints also. Painful inflammation results when cells of the immune system engulf the sodium urate crystals. The kidneys, too, may be damaged by the deposition of urate crystals. Gout is a common medical problem, affecting 1% of the population of Western countries. It is nine times as common in men as in women.

Administration of *allopurinol,* an analog of hypoxanthine, is one treatment for gout. The mechanism of action of allopurinol is interesting: it acts *first as a substrate and then as an inhibitor* of xanthine oxidase. The oxidase hydroxylates allopurinol to *alloxanthine (oxipurinol),* which then remains tightly bound to the active site. The binding of alloxanthine keeps the molybdenum atom of xanthine oxidase in the +4 oxidation state instead of it returning to the +6 oxidation state as in a normal catalytic cycle. We see here another example of *suicide inhibition.*



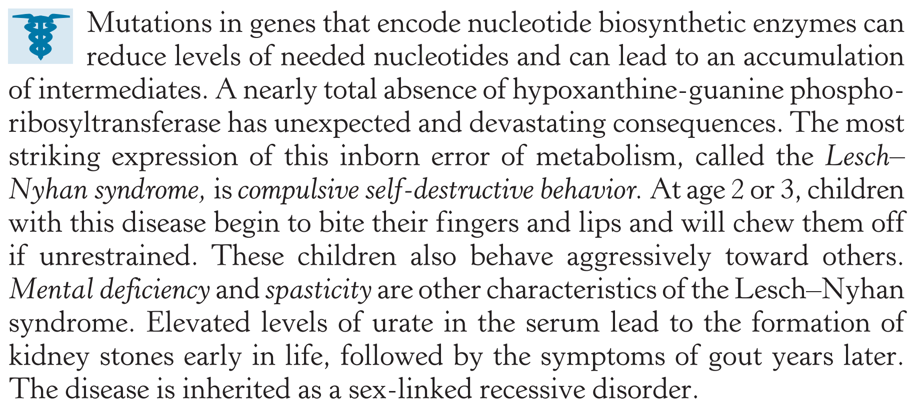
Allopurinol

The synthesis of urate from hypoxanthine and xanthine decreases soon after the administration of allopurinol. The serum concentrations of hypoxanthine and xanthine rise, and that of urate drops.



The average serum level of urate in human beings is close to the solubility limit. In contrast, prosimians (such as lemurs) have 10-fold lower levels. A striking increase in urate levels occurred in the evolution of primates. What is the selective advantage of a urate level so high that it teeters on the brink of gout in many people? It turns out that urate has a markedly beneficial action. Urate is a highly effective scavenger of reactive oxygen species. Indeed, urate is about as effective as ascorbate (vitamin C) as an antioxidant. The increased level of urate in human beings compared with prosimians and other lower primates may contribute significantly to the longer human life span and to lowering the incidence of human cancer.

Lesch–Nyhan syndrome is a dramatic consequence of mutations in a salvage-pathway enzyme



Mutations in genes that encode nucleotide biosynthetic enzymes can reduce levels of needed nucleotides and can lead to an accumulation of intermediates. A nearly total absence of hypoxanthine-guanine phosphoribosyltransferase has unexpected and devastating consequences. The most striking expression of this inborn error of metabolism, called the *Lesch–Nyhan syndrome,* is *compulsive self-destructive behavior.* At age 2 or 3, children with this disease begin to bite their fingers and lips and will chew them off if unrestrained. These children also behave aggressively toward others. *Mental deficiency* and *spasticity* are other characteristics of the Lesch–Nyhan syndrome. Elevated levels of urate in the serum lead to the formation of kidney stones early in life, followed by the symptoms of gout years later. The disease is inherited as a sex-linked recessive disorder.

The biochemical consequences of the virtual absence of hypoxanthine-guanine phosphoribosyl transferase are *an elevated concentration of PRPP, a marked increase in the rate of purine biosynthesis by the de novo pathway, and an overproduction of urate.* The relation between the absence of the transferase and the bizarre neurological signs is an enigma, although recent evidence suggests that the lack of hypoxanthine-guanine phosphoribosyltransferase results, in some undetermined fashion, in an imbalance of key neurotransmitters. The Lesch–Nyhan syndrome demonstrates that the salvage pathway for the synthesis of IMP and GMP is not gratuitous. Moreover, the Lesch–Nyhan syndrome reveals that *abnormal behavior such as self-mutilation and extreme hostility can be caused by the absence of a single enzyme.* Psychiatry will no doubt benefit from the unraveling of the molecular basis of such mental disorders.

Folic acid deficiency promotes birth defects such as spina bifida

Spina bifida is one of a class of birth defects characterized by the incomplete or incorrect formation of the neural tube early in development. In the United States, the prevalence of *neural-tube defects* is approximately 1 case per 1000 births. A variety of studies have demonstrated that the prevalence of neural-tube defects is reduced by as much as 70% when women take folic acid as a dietary supplement before and during the first trimester of pregnancy. One hypothesis is that more folate derivatives are needed for the synthesis of DNA precursors when cell division is frequent and substantial amounts of DNA must be synthesized.

Summary

25.1 The Pyrimidine Ring Is Assembled de Novo or Recovered by Salvage Pathways

The pyrimidine ring is assembled first and then linked to ribose phosphate to form a pyrimidine nucleotide. 5-Phosphoribosyl-1-pyrophosphate is the donor of the ribose phosphate moiety. The synthesis of the pyrimidine ring starts with the formation of carbamoylaspartate from carbamoyl phosphate and aspartate, a reaction catalyzed by aspartate transcarbamoylase. Dehydration, cyclization, and oxidation yield orotate, which reacts with PRPP to give orotidylate. Decarboxylation of this pyrimidine nucleotide yields UMP. CTP is then formed by the amination of UTP.

25.2 Purine Bases Can Be Synthesized de Novo or Recycled by Salvage Pathways

The purine ring is assembled from a variety of precursors: glutamine, glycine, aspartate, N^{10}-formyltetrahydrofolate, and CO_2. The committed step in the de novo synthesis of purine nucleotides is the formation of 5-phosphoribosylamine from PRPP and glutamine. The purine ring is assembled on ribose phosphate, in contrast with the de novo synthesis of pyrimidine nucleotides. The addition of glycine, followed by formylation, amination, and ring closure, yields 5-aminoimidazole ribonucleotide. This intermediate contains the completed five-membered ring of the purine skeleton. The addition of CO_2, the nitrogen atom of aspartate, and a formyl group, followed by ring closure, yields inosinate, a purine ribonucleotide. AMP and GMP are formed from IMP. Purine ribonucleotides can also be synthesized by a salvage pathway in which a preformed base reacts directly with PRPP.

25.3 Deoxyribonucleotides Are Synthesized by the Reduction of Ribonucleotides Through a Radical Mechanism

Deoxyribonucleotides, the precursors of DNA, are formed in *E. coli* by the reduction of ribonucleoside diphosphates. These conversions are catalyzed by ribonucleotide reductase. Electrons are transferred from NADPH to sulfhydryl groups at the active sites of this enzyme by thioredoxin. A tyrosyl free radical generated by an iron center in the reductase initiates a radical reaction on the sugar, leading to the exchange of H for OH at C-2'. TMP is formed by the methylation of dUMP. The donor of a methylene group and a hydride in this reaction is N^5,N^{10}-methylenetetrahydrofolate, which is converted into dihydrofolate. Tetrahydrofolate is regenerated by the reduction of dihydrofolate by NADPH. Dihydrofolate reductase, which catalyzes this reaction, is inhibited by folate analogs such as aminopterin and

methotrexate. These compounds and fluorouracil, an inhibitor of thymidylate synthase, are used as anticancer drugs.

25.4 Key Steps in Nucleotide Biosynthesis Are Regulated by Feedback Inhibition

Pyrimidine biosynthesis in *E. coli* is regulated by the feedback inhibition of aspartate transcarbamoylase, the enzyme that catalyzes the committed step. CTP inhibits and ATP stimulates this enzyme. The feedback inhibition of glutamine-PRPP amidotransferase by purine nucleotides is important in regulating their biosynthesis.

25.5 Disruptions in Nucleotide Metabolism Can Cause Pathological Conditions

Severe combined immunodeficiency results from the absence of adenosine deaminase, an enzyme in the purine degradation pathway. Purines are degraded to urate in human beings. Gout, a disease that affects joints and leads to arthritis, is associated with an excessive accumulation of urate. The Lesch–Nyhan syndrome, a genetic disease characterized by self-mutilation, mental deficiency, and gout, is caused by the absence of hypoxanthine-guanine phosphoribosyltransferase. This enzyme is essential for the synthesis of purine nucleotides by the salvage pathway. Neural-tube defects are more frequent when a pregnant woman is deficient in folate derivatives early in pregnancy, possibly because of the important role of these derivatives in the synthesis of DNA precursors.

Key Terms

pyrimidine nucleotide (p. 736)
carbamoyl phosphate synthetase (CPS) (p. 737)
ATP-grasp fold (p. 737)
5-phosphoribosyl-1-pyrophosphate (PRPP) (p. 738)
orotidylate (p. 739)
salvage pathway (p. 740)
purine nucleotide (p. 740)
glutamine phosphoribosyl amidotransferase (p. 741)
ribonucleotide reductase (p. 745)
thymidylate synthase (p. 748)
dihydrofolate reductase (p. 749)
severe combined immunodeficiency (SCID) (p. 753)
gout (p. 753)
Lesch–Nyhan syndrome (p. 754)
spina bifida (p. 755)
neural-tube defect (p. 755)

Problems

1. *From the beginning or extract and save and reuse*. Differentiate between the de novo synthesis of nucleotides and salvage pathway synthesis.

2. *Finding their roots 1*. Identify the source of the atoms in the pyrimidine ring

3. *Finding their roots 2*. Identify the source of the atoms in the purine ring.

4. *Multifaceted*. List some of the biochemical roles played by nucleotides.

5. *An s instead of a t?* Differentiate between a nucleoside and a nucleotide.

6. *Associate 'em.*

(a) Excessive urate
(b) Lack of adenosine deaminase
(c) lack of HGPRT
(d) Carbamoyl phosphate
(e) Inosinate
(f) Ribonucleotide reductase
(g) Lack of folic acid
(h) Glutamine phosphoribosyl transferase
(i) Single ring
(j) Bicyclic ring
(k) Precursor to CTP

1. Spina bifida
2. Precursor to both ATP and GTP
3. Purine
4. Deoxynucleotide synthesis
5. UTP
6. Lesch-Nyhan disease
7. Immunodeficiency
8. Pyrimidine
9. Gout
10. First step in pyrimidine synthesis
11. Committed step in purine synthesis

7. *Safe passage*. What is substrate channeling? How does it affect enzyme efficiency?

8. *Activated ribose phosphate.* Write a balanced equation for the synthesis of PRPP from glucose through the oxidative branch of the pentose phosphate pathway.

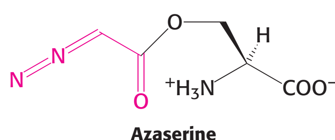
9. *Making a pyrimidine.* Write a balanced equation for the synthesis of orotate from glutamine, CO_2, and aspartate.

10. *Identifying the donor.* What is the activated reactant in the biosynthesis of each of the following compounds?

(a) Phosphoribosylamine (c) Orotidylate (from orotate)

(b) Carbamoylaspartate (d) Phosphoribosylanthranilate

11. *Inhibiting purine biosynthesis.* Amidotransferases are inhibited by the antibiotic azaserine (*O*-diazoacetyl-L-serine), which is an analog of glutamine.

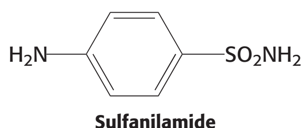


Azaserine

Which intermediates in purine biosynthesis would accumulate in cells treated with azaserine?

12. *The price of methylation.* Write a balanced equation for the synthesis of TMP from dUMP that is coupled to the conversion of serine into glycine.

13. *Sulfa action.* Bacterial growth is inhibited by sulfanilamide and related sulfa drugs, and there is a concomitant accumulation of 5-aminoimidazole-4-carboxamide ribonucleotide. This inhibition is reversed by the addition of *p*-aminobenzoate.



Sulfanilamide

Propose a mechanism for the inhibitory effect of sulfanilamide.

14. *HAT medium.* Mutant cells unable to synthesize nucleotides by salvage pathways are very useful tools in molecular and cell biology. Suppose that cell A lacks thymidine kinase, the enzyme catalyzing the phosphorylation of thymidine to thymidylate, and that cell B lacks hypoxanthine-guanine phosphoribosyl transferase.

(a) Cell A and cell B do not proliferate in a HAT medium containing *h*ypoxanthine, *a*minopterin or *a*methopterin (methotrexate), and *t*hymine. However, cell C, formed by the fusion of cells A and B, grows in this medium. Why?

(b) Suppose that you want to introduce foreign genes into cell A. Devise a simple means of distinguishing between cells that have taken up foreign DNA and those that have not.

15. *Bringing equilibrium.* What is the reciprocal substrate relation in the synthesis of ATP and GTP?

16. *Find the label.* Suppose that cells are grown on amino acids that have all been labeled at the α carbons with ^{13}C. Identify the atoms in cytosine and guanine that will be labeled with ^{13}C.

17. *Different strokes.* Human beings contain two different carbamoyl phosphate synthetase enzymes. One uses glutamine as a substrate, whereas the other uses ammonia. What are the functions of these two enzymes?

18. *Adjunct therapy.* Allopurinol is sometimes given to patients with acute leukemia who are being treated with anticancer drugs. Why is allopurinol used?

19. *A hobbled enzyme.* Both side-chain oxygen atoms of aspartate 27 at the active site of dihydrofolate reductase form hydrogen bonds with the pteridine ring of folates. The importance of this interaction was assessed by studying two mutants at this position, Asn 27 and Ser 27. The dissociation constant of methotrexate was 0.07 nM for the wild type, 1.9 nM for the Asn 27 mutant, and 210 nM for the Ser 27 mutant, at 25°C. Calculate the standard free energy of the binding of methotrexate by these three proteins. What is the decrease in binding energy resulting from each mutation?

20. *Correcting deficiencies.* Suppose that a person is found who is deficient in an enzyme required for IMP synthesis. How might this person be treated?

21. *Labeled nitrogen.* Purine biosynthesis is allowed to take place in the presence of [^{15}N]aspartate, and the newly synthesized GTP and ATP are isolated. What positions are labeled in the two nucleotides?

22. *On the trail of carbons.* Tissue culture cells were incubated with glutamine labeled with ^{15}N in the amide group. Subsequently, IMP was isolated and found to contain some ^{15}N. Which atoms in IMP were labeled?

23. *Mechanism of action.* What is the biochemical basis of allopurinol treatment for gout?

24. *Changed inhibitor.* Xanthine oxidase treated with allopurinol results in the formation of a new compound that is an extremely potent inhibitor of the enzyme. Propose a structure for this compound.

25. *Calculate the ATP footprint.* How many molecules of ATP are required to synthesize one molecule of CTP from scratch?

26. *Blockages.* What intermediate in purine synthesis will accumulate if a strain of bacteria is lacking each of the following biochemicals?

(a) Aspartate (c) Glycine
(b) Tetrahydrofolate (d) Glutamine

Mechanism Problems

27. *The same and not the same.* Write out mechanisms for the conversion of phosphoribosylamine into glycinamide ribonucleotide and of xanthylate into guanylate.

28. *Closing the ring.* Propose a mechanism for the conversion of 5-formamidoimidazole-4-carboxamide ribonucleotide into inosinate.

Chapter Integration Problems

29. *A generous donor.* What major biosynthetic reactions utilize PRPP?

30. *They're everywhere!* Nucleotides play a variety of roles in the cell. Give an example of a nucleotide that acts in each of the following roles or processes.

(a) Second messenger
(b) Phosphoryl-group transfer
(c) Activation of carbohydrates
(d) Activation of acetyl groups
(e) Transfer of electrons
(f) DNA sequencing
(g) Chemotherapy
(h) Allosteric effector

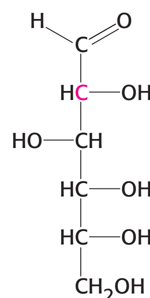
31. *Pernicious anemia.* Purine biosynthesis is impaired by vitamin B_{12} deficiency. Why? How might fatty acid and amino acid metabolism also be affected by a vitamin B_{12} deficiency?

32. *Folate deficiency*. Suppose someone was suffering from a folate deficiency. What cells would you think might be most affected? Symptoms may include diarrhea and anemia.

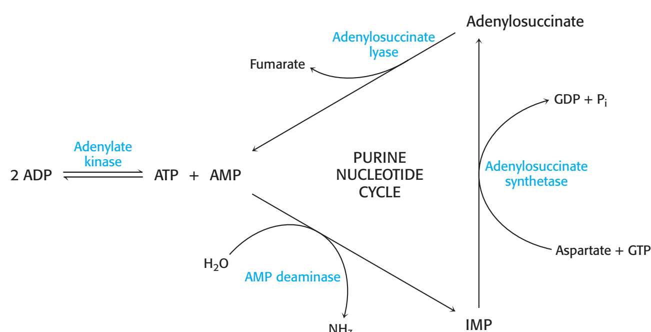
33. *Hyperuricemia.* Many patients with glucose 6-phosphatase deficiency have high serum levels of urate. Hyperuricemia can be induced in normal people by the ingestion of alcohol or by strenuous exercise. Propose a common mechanism that accounts for these findings.

34. *Labeled carbon.* Succinate uniformly labeled with ^{14}C is added to cells actively engaged in pyrimidine biosynthesis. Propose a mechanism by which carbon atoms from succinate could be incorporated into a pyrimidine. At what positions is the pyrimidine labeled?

35. *Something funny going on here.* Cells were incubated with glucose labeled with ^{14}C in carbon 2, shown in red in the structure at the top of the next column. Later, uracil was isolated and found to contain ^{14}C in carbons 4 and 6. Account for this labeling pattern.



36. *Exercising muscle.* Some interesting reactions take place in muscle tissue to facilitate the generation of ATP for contraction.



In muscle contraction, ATP is converted into ADP. Adenylate kinase converts two molecules of ADP into a molecule of ATP and AMP.

(a) Why is this reaction beneficial to contracting muscle?

(b) Why is the equilibrium for the adenylate kinase approximately equal to 1?

Muscle can metabolize AMP by using the purine nucleotide cycle. The initial step in this cycle, catalyzed by AMP deaminase, is the conversion of AMP into IMP.

(c) Why might the deamination of AMP facilitate ATP formation in muscle?

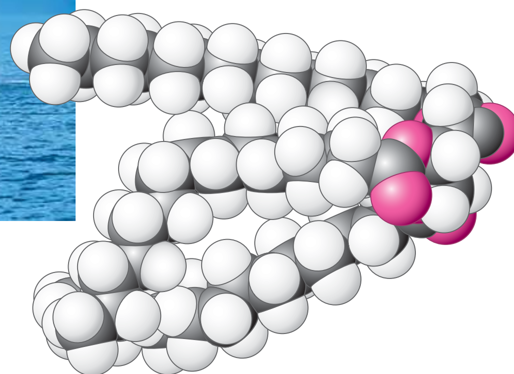
(d) How does the purine nucleotide cycle assist the aerobic generation of ATP?

37. *A common step*. What three reactions transfer an amino group from aspartate to yield the aminated product and fumarate?

38. *Your pet duck*. You suspect that your pet duck has gout. Why should you think twice before administering a dose of allopurinol-laced bread?

The Biosynthesis of Membrane Lipids and Steroids

Fats such as the triacylglycerol molecule (below) are widely used to store excess energy for later use and to fulfill other purposes, illustrated by the insulating blubber of whales. The natural tendency of fats to exist in nearly water free forms makes these molecules well suited to these roles. [(Left) François Cohier/Photo Researchers.]



This chapter examines the biosynthesis of three important components of biological membranes—phospholipids, sphingolipids, and cholesterol (Chapter 12). Triacylglycerols also are considered here because the pathway for their synthesis overlaps that of phospholipids. Cholesterol is of interest both as a membrane component and as a precursor of many signal molecules, including the steroid hormones progesterone, testosterone, estrogen, and cortisol.

The transport and uptake of cholesterol vividly illustrate a recurring mechanism for the entry of metabolites and signal molecules into cells. Cholesterol is transported in blood by the low-density lipoprotein (LDL) and taken up into cells by the LDL receptor on the cell surface. The LDL receptor is absent in people with *familial hypercholesterolemia*, a genetic disease. People lacking the receptor have markedly elevated cholesterol levels in the blood and cholesterol deposits on blood vessels, and they are prone to childhood heart attacks. Indeed, cholesterol is implicated in the development of atherosclerosis in people who do not have genetic defects. Thus, the regulation of cholesterol synthesis and transport can be a source of especially clear insight into the role that our understanding of biochemistry plays in medicine.

OUTLINE

26.1 Phosphatidate Is a Common Intermediate in the Synthesis of Phospholipids and Triacylglycerols

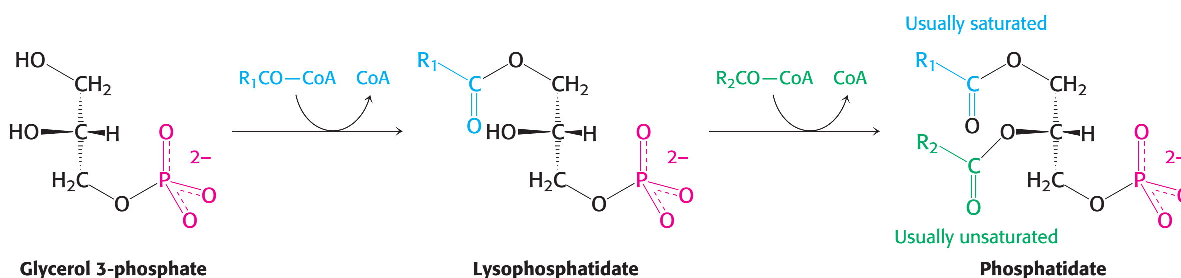
26.2 Cholesterol Is Synthesized from Acetyl Coenzyme A in Three Stages

26.3 The Complex Regulation of Cholesterol Biosynthesis Takes Place at Several Levels

26.4 Important Derivatives of Cholesterol Include Bile Salts and Steroid Hormones

26.1 Phosphatidate Is a Common Intermediate in the Synthesis of Phospholipids and Triacylglycerols

Lipid synthesis requires the coordinated action of gluconeogenesis and fatty acid metabolism, as illustrated in Figure 26.1. The first step in the synthesis of both phospholipids for membranes and triacylglycerols for energy storage is the synthesis of *phosphatidate* (diacylglycerol 3-phosphate). In mammalian cells, phosphatidate is synthesized in the endoplasmic reticulum and the outer mitochondrial membrane. The pathway begins with *glycerol 3-phosphate,* which is formed primarily by the reduction of dihydroxyacetone phosphate (DHAP) synthesized by the gluconeogenic pathway, and to a lesser extent by the phosphorylation of glycerol. The addition of two fatty acids to glycerol-3-phosphate yields phosphatidate. First, acyl coenzyme A contributes a fatty acid chain to form *lysophosphatidate* and, then, a second acyl CoA contributes a fatty acid chain to yield phosphatidate.



These acylations are catalyzed by *glycerol phosphate acyltransferase.* In most phosphatidates, the fatty acid chain attached to the C-1 atom is saturated, whereas the one attached to the C-2 atom is unsaturated. Phosphatidate can also be synthesized from diacylglycerol, in what is essentially a salvage pathway, by the action of *diacylglycerol kinase*:

$$\text{Diacylglycerol} + \text{ATP} \longrightarrow \text{phosphatidate} + \text{ADP}$$

The phospholipid and triacylglycerol pathways diverge at phosphatidate. In the synthesis of triacylglycerols, a key enzyme in the regulation of

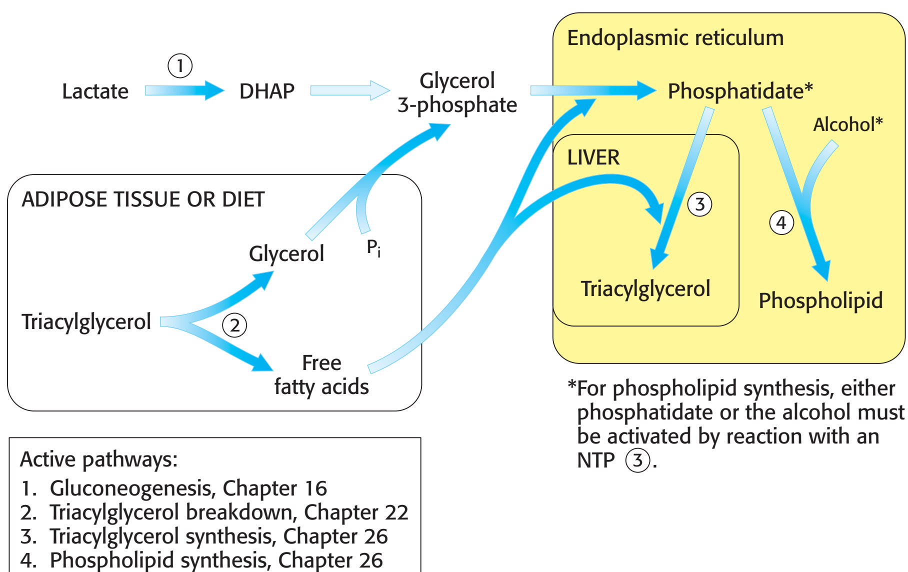
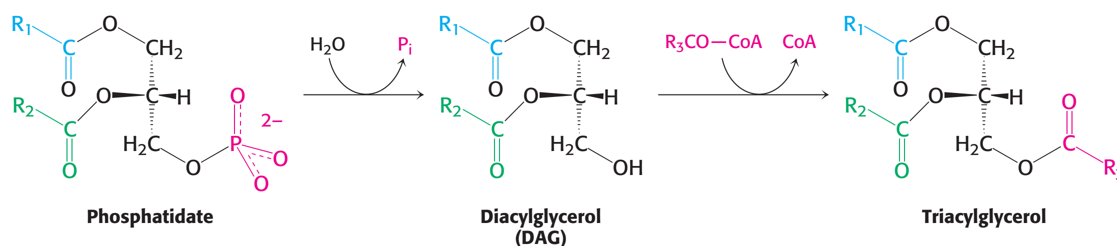


Figure 26.1 PATHWAY INTEGRATION: Sources of intermediates in the synthesis of triacylglycerols and phospholipids. Phosphatidate, synthesized from dihydroxyacetone phosphate (DHAP) produced in gluconeogenesis and fatty acids, can be further processed to produce triacylglycerol or phospholipids. Phospholipids and other membrane lipids are continuously produced in all cells.

lipid synthesis, *phosphatidic acid phosphatase* hydrolyzes phosphatidate to give a *diacylglycerol* (DAG). This intermediate is acylated to a *triacylglycerol* through the addition of a third fatty acid chain in a reaction that is catalyzed by *diglyceride acyltransferase*. Both enzymes are associated in a *triacylglycerol synthetase complex* that is bound to the endoplasmic reticulum membrane.



The liver is the primary site of triacylglycerol synthesis. From the liver, the triacylglycerols are transported to the muscles for energy conversion or to the adipose cells for storage.

The synthesis of phospholipids requires an activated intermediate

Membrane-lipid synthesis continues in the endoplasmic reticulum and in the Golgi apparatus. *Phospholipid* synthesis requires the combination of a diacylglycerol with an alcohol. As in most anabolic reactions, one of the components must be activated. In this case, either the diacylglycerol or the alcohol may be activated, depending on the source of the reactants.

Synthesis from an activated diacylglycerol. The de novo pathway starts with the reaction of phosphatidate with cytidine triphosphate (CTP) to form the activated diacylglycerol, *cytidine diphosphodiacylglycerol* (CDP-diacylglycerol; Figure 26.2). This reaction, like those of many biosyntheses, is driven forward by the hydrolysis of pyrophosphate.

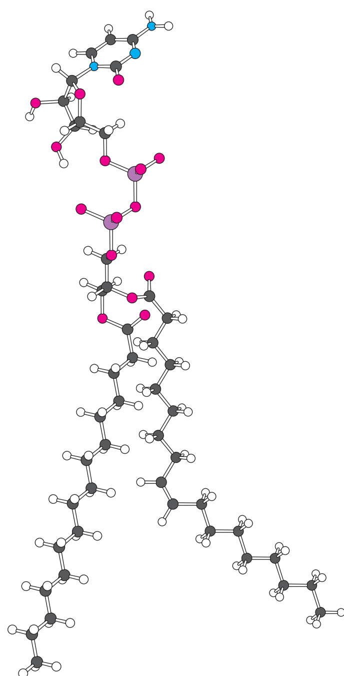
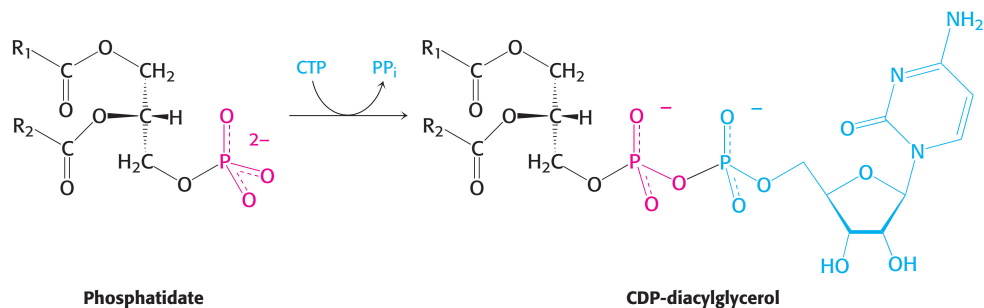
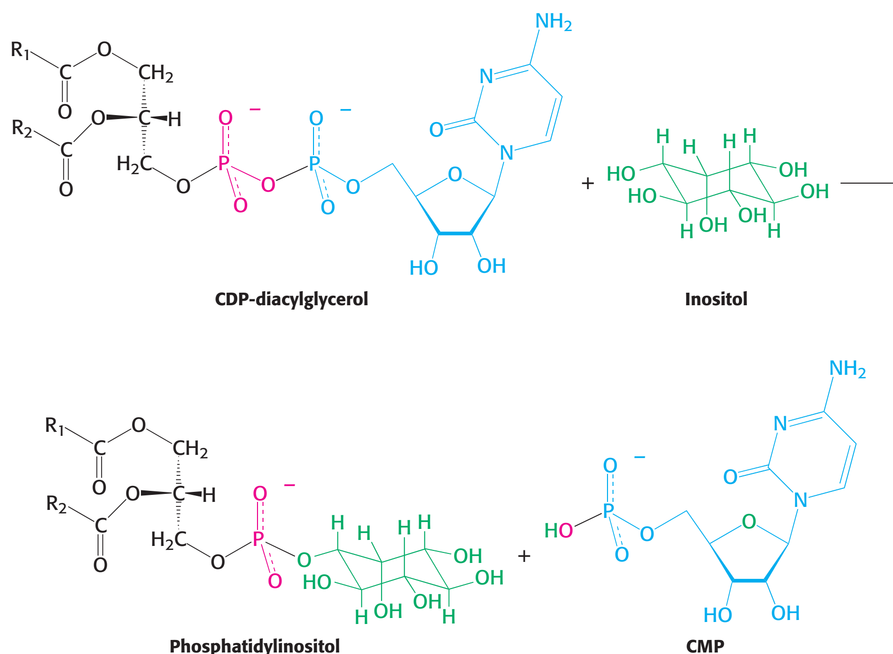


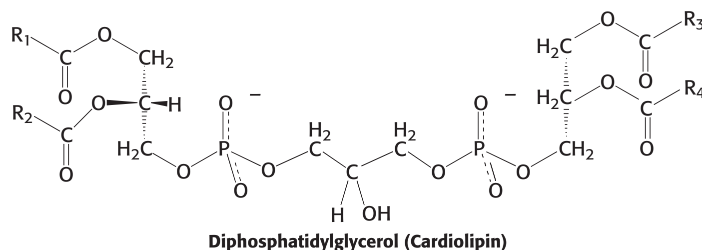
Figure 26.2 Structure of CDP-diacylglycerol. A key intermediate in the synthesis of phospholipids consists of phosphatidate and cytidine monophosphate joined by a pyrophosphate linkage.



The activated phosphatidyl unit then reacts with the hydroxyl group of an alcohol to form a phosphodiester linkage. If the alcohol is inositol, the products are *phosphatidylinositol* and cytidine monophosphate (CMP).



Subsequent phosphorylations catalyzed by specific kinases lead to the synthesis of *phosphatidylinositol 4,5-bisphosphate,* the precursor of two intracellular messengers—diacylglycerol and inositol 1,4,5-trisphosphate (Section 14.2). If the alcohol is phosphatidylglycerol, the products are diphosphatidylglycerol (cardiolipin) and CMP. In eukaryotes, cardiolipin is located exclusively in inner mitochondrial membranes and plays an important role in the organization of the protein components of oxidative phosphorylation. For example, cardiolipin is required for the full activity of cytochrome oxidase.



The fatty acid components of phospholipids may vary, and thus cardiolipin, as well as most other phospholipids, represents a class of molecules

rather than a single species. As a result, a single mammalian cell may contain thousands of distinct phospholipids. Phosphatidylinositol is unusual in that it has a nearly fixed fatty acid composition. Stearic acid usually occupies the C-1 position and arachidonic acid the C-2 position.

Synthesis from an activated alcohol. Phosphatidylethanolamine, the major phospholipid of the inner leaflet of cell membranes, is synthesized from the alcohol ethanolamine. To activate the alcohol, ethanolamine is phosphorylated by ATP to form the precursor, *phosphorylethanolamine.* This precursor then reacts with CTP to form the activated alcohol, *CDP-ethanolamine.* The phosphorylethanolamine unit of CDP-ethanolamine is transferred to a diacylglycerol to form *phosphatidylethanolamine.*

The most common phospholipid in mammals is phosphatidylcholine. In this case, dietary choline is activated in a series of reactions analogous to those in the activation of ethanolamine. Interestingly, the liver possesses an enzyme, *phosphatidylethanolamine methyltransferase,* which synthesizes phosphatidylcholine from phosphatidylethanolamine when dietary choline is insufficient. The amino group of this phosphatidylethanolamine is methylated three times to form *phosphatidylcholine.* S-*Adenosylmethionine* is the methyl donor.

Thus, phosphatidylcholine can be produced by two distinct pathways in mammals, ensuring that this phospholipid can be synthesized even if the components for one pathway are in limited supply.

Phosphatidylserine makes up 10% of the phospholipids in mammals. This phospholipid is synthesized in a *base-exchange reaction* of serine with phosphatidylcholine or phosphatidylethanolamine. In the reaction, serine replaces choline or ethanolamine.

$$\text{Phosphatidylcholine} + \text{serine} \longrightarrow \text{choline} + \text{phosphatidylserine}$$

$$\text{Phosphatidylethanolamine} + \text{serine} \longrightarrow \text{ethanolamine} + \text{phosphatidylserine}$$

Phosphatidylserine is normally located in the inner leaflet of the plasma membrane bilayer but is moved to the outer leaflet in apoptosis (Section 18.6). There, it may serve to attracted phagocytes to consume the cell remnants after apoptosis is complete.

Note that a cytidine nucleotide plays the same role in the synthesis of these phosphoglycerides as a uridine nucleotide does in the formation of glycogen (Section 21.4). In all of these biosyntheses, an activated intermediate (UDP-glucose, CDP-diacylglycerol, or CDP-alcohol) is formed from a phosphorylated substrate (glucose 1-phosphate, phosphatidate, or a phosphorylalcohol) and a nucleoside triphosphate (UTP or CTP). The activated intermediate then reacts with a hydroxyl group (the terminus of glycogen, the side chain of serine, or a diacylglycerol).

Sphingolipids are synthesized from ceramide

We now turn from glycerol-based phospholipids to another class of membrane lipid—the *sphingolipids.* These lipids are found in the plasma

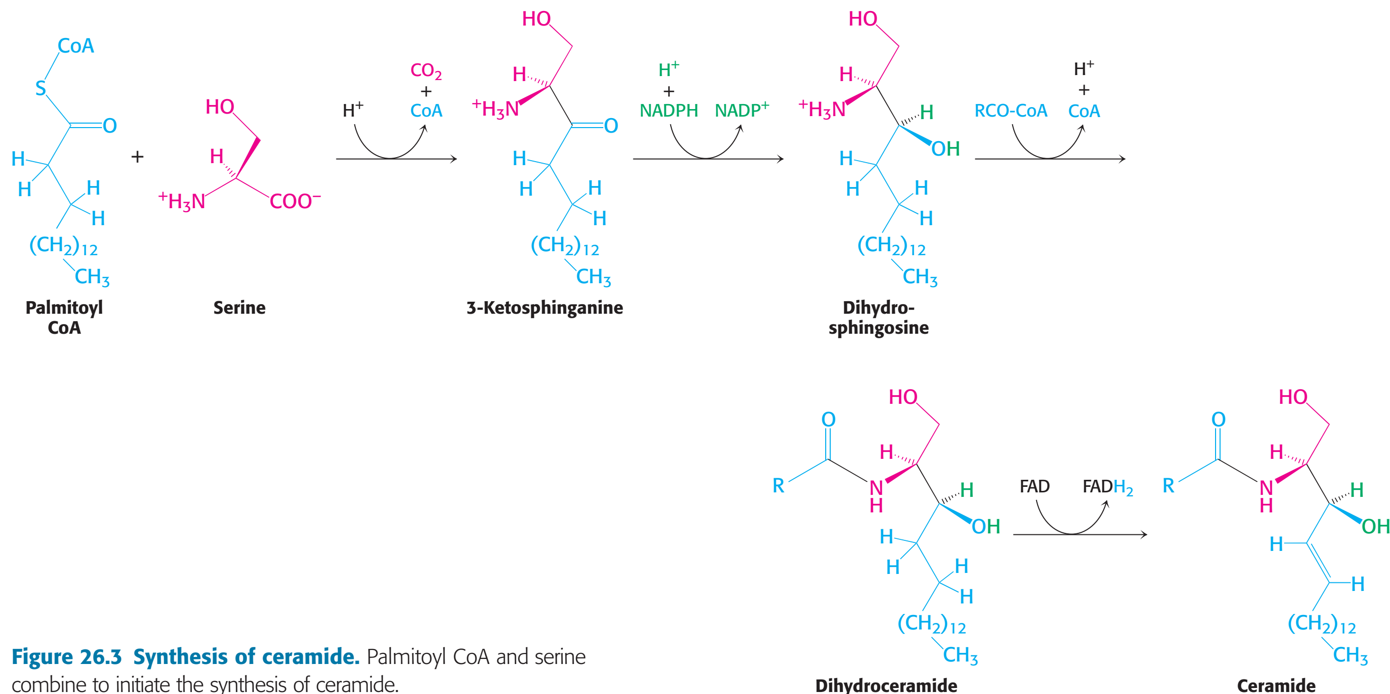
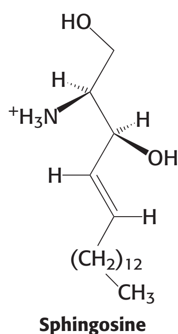


Figure 26.3 Synthesis of ceramide. Palmitoyl CoA and serine combine to initiate the synthesis of ceramide.



membranes of all eukaryotic cells, although the concentration is highest in the cells of the central nervous system. The backbone of a sphingolipid is *sphingosine,* rather than glycerol. Palmitoyl CoA and serine condense to form 3-ketosphinganine. The serine–palmitoyl transferase catalyzing this reaction is the rate-limiting step in the pathway and requires pyridoxal phosphate, revealing again the dominant role of this cofactor in transformations that include amino acids. Ketosphinganine is then reduced to dihydrosphingosine before conversion into *ceramide,* a lipid consisting of a fatty acid chain attached to the amino group of a sphingosine backbone (Figure 26.3).

In all sphingolipids, the amino group of ceramide is acylated (Figure 26.4). The terminal hydroxyl group also is substituted. In *sphingomyelin,* a component of the myelin sheath covering many nerve fibers, the substituent is phosphorylcholine, which comes from phosphatidylcholine. In a *cerebroside,* the substituent is glucose or galactose. UDP-glucose or UDP-galactose is the sugar donor.

Gangliosides are carbohydrate-rich sphingolipids that contain acidic sugars

Gangliosides are the most complex sphingolipids. In a *ganglioside,* an *oligosaccharide chain* is linked to the terminal hydroxyl group of ceramide by a glucose residue (Figure 26.5). This oligosaccharide chain contains at least one acidic sugar, either N-*acetylneuraminate* or N-*glycolylneuraminate.* These acidic sugars are called *sialic acids.* Their nine-carbon backbones are synthesized from phosphoenolpyruvate (a three-carbon unit) and *N*-acetylmannosamine 6-phosphate (a six-carbon unit).

Gangliosides are synthesized by the ordered, step-by-step addition of sugar residues to ceramide. The synthesis of these complex lipids requires the activated sugars UDP-glucose, UDP-galactose, and UDP-*N*-acetylgalactosamine, as well as the CMP derivative of *N*-acetylneuraminate. CMP-*N*-acetylneuraminate is synthesized from CTP and *N*-acetylneuraminate. The sugar composition of the resulting ganglioside is determined by the specificity of the glycosyltransferases in the cell. More than 60 different

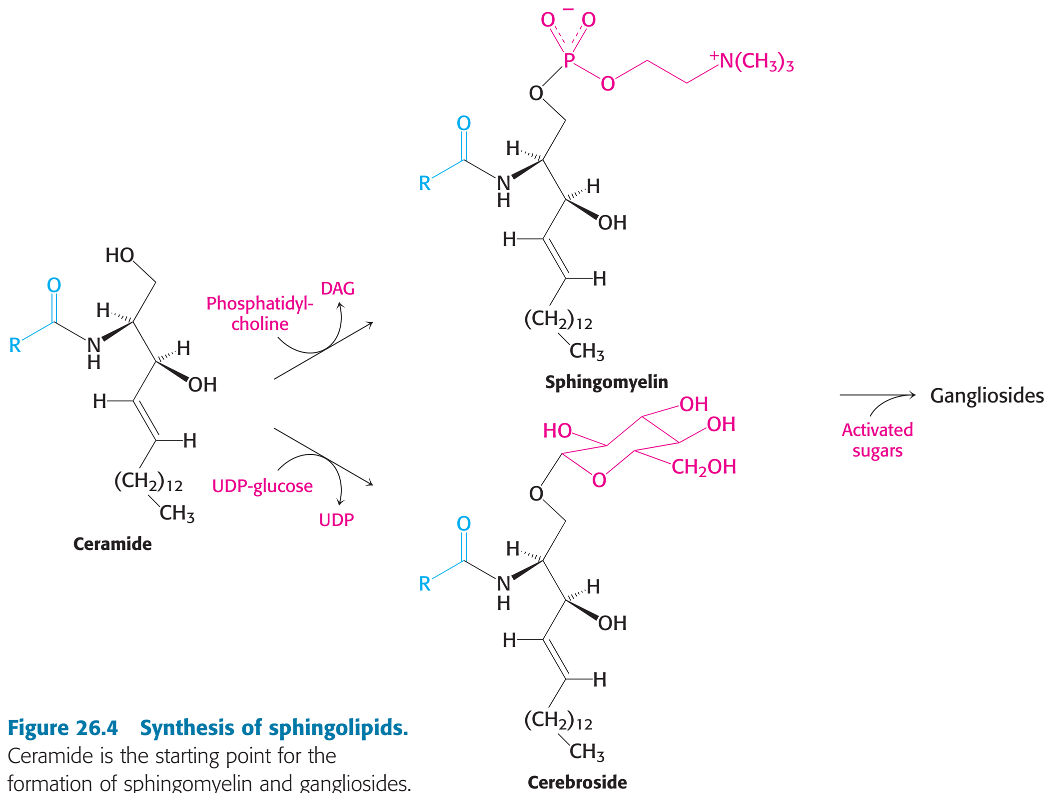


Figure 26.4 Synthesis of sphingolipids. Ceramide is the starting point for the formation of sphingomyelin and gangliosides.

gangliosides have been characterized (see Figure 26.5 for the composition of ganglioside G_{M1}). Ganglioside-binding by cholera toxin is the first step in the development of cholera, a pathological condition characterized by severe diarrhea. Gangliosides are also crucial for binding immune-system cells to sites of injury in the inflammatory response.

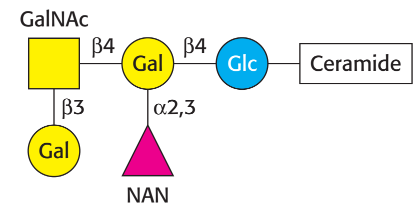
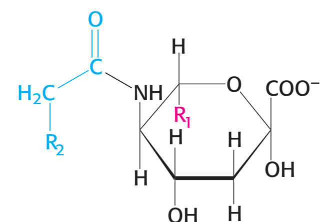
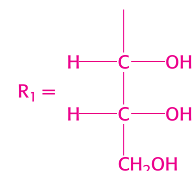


Figure 26.5 Ganglioside G_{M1}. This ganglioside consists of five monosaccharides linked to ceramide: one glucose (Glc) molecule, two galactose (Gal) molecules, one *N*-acetylgalactosamine (GalNAc) molecule, and one *N*-acetylneuraminate (NAN) molecule.

Sphingolipids confer diversity on lipid structure and function

The structures of sphingolipids and the more abundant glycerophospholipids are very similar. Given the structural similarity of these two types of lipids, why are sphingolipids required at all? Indeed, the prefix "sphingo" was applied to capture the "sphinxlike" properties of this enigmatic class of lipids. Although the precise role of sphingolipids is not firmly established, progress toward solving the riddle of their function is being made. As discussed in Chapter 12, sphingolipids are important components of lipid rafts, highly organized regions of the plasma membrane that are important in signal transduction. Sphingosine, sphingosine 1-phosphate, and ceramide serve as second messengers in the regulation of cell growth, differentiation, and death. For instance, ceramide derived from a sphingolipid initiates programmed cell death in some cell types and may contribute to the development of type 2 diabetes (Chapter 27).





Respiratory distress syndrome and Tay–Sachs disease result from the disruption of lipid metabolism

Respiratory distress syndrome is a pathological condition resulting from a failure in the biosynthetic pathway for dipalmitoyl phosphatidylcholine. This phospholipid, in conjunction with specific proteins and other phospholipids, is found in the extracellular fluid that surrounds the alveoli of the lung. Its function is to decrease the surface tension of the fluid to prevent lung collapse at the end of the expiration phase of breathing. Premature

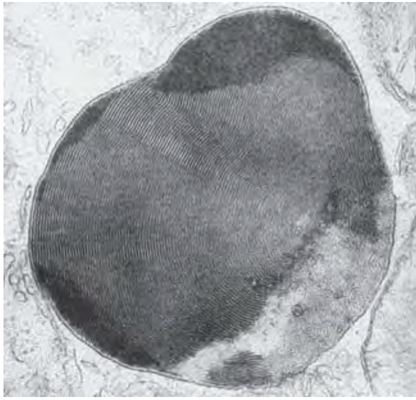
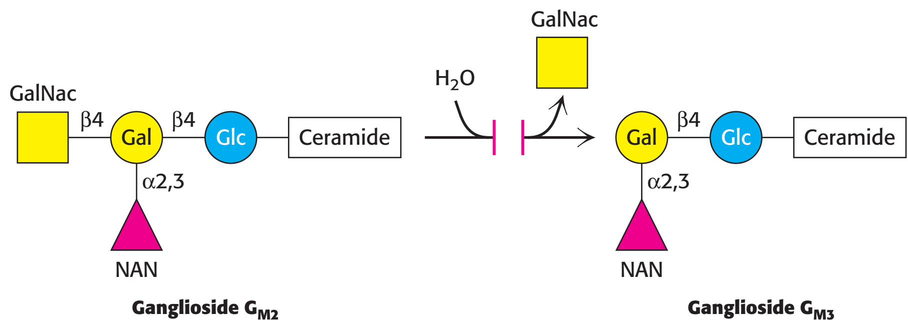


Figure 26.6 Lysosome with lipids. An electron micrograph of a lysosome containing an abnormal amount of lipid. [Courtesy of Dr. George Palade.]

infants may suffer from respiratory distress syndrome because their immature lungs do not synthesize enough dipalmitoyl phosphatidylcholine.

Tay–Sachs disease is caused by a failure of lipid degradation: an inability to degrade gangliosides. Gangliosides are found in highest concentration in the nervous system, particularly in gray matter, where they constitute 6% of the lipids. Gangliosides are normally degraded inside lysosomes by the sequential removal of their terminal sugars but, in Tay–Sachs disease, this degradation does not take place. As a consequence, neurons become significantly swollen with lipid-filled lysosomes (Figure 26.6). An affected infant displays weakness and retarded psychomotor skills before 1 year of age. The child is demented and blind by age 2 and usually dies before age 3.

The ganglioside content of the brain of an infant with Tay–Sachs disease is greatly elevated. *The concentration of ganglioside G_{M2} is many times higher than normal because its terminal* N-*acetylgalactosamine residue is removed very slowly or not at all.* The missing or deficient enzyme is a specific *β-N-acetylhexosaminidase.*



Tay–Sachs disease can be diagnosed in the course of fetal development. Amniotic fluid is obtained by amniocentesis and assayed for β-*N*-acetylhexosaminidase activity.

Phosphatiditic acid phosphatase is a key regulatory enzyme in lipid metabolism

Although the details of the regulation of lipid synthesis remain to be elucidated, evidence suggests that *phosphatidic acid phosphatase* (PAP), works in concert with diacylglycerol kinase, playing a key role in the regulation of lipid synthesis. PAP, also called lipin 1 in mammals, controls the extent to which triacylglycerols are synthesized relative to phospholipids and regulates the type of phospholipid synthesized (Figure 26.7). For instance, when PAP activity is high, phosphatidate is dephosphorylated and diacylglycerol is produced, which can react with the appropriate activated alcohols to yield phosphatidylethanolamine, phosphatidylserine or phosphatidylcholine. Diacylglycerol can also be converted into triacylglycerols, and evidence

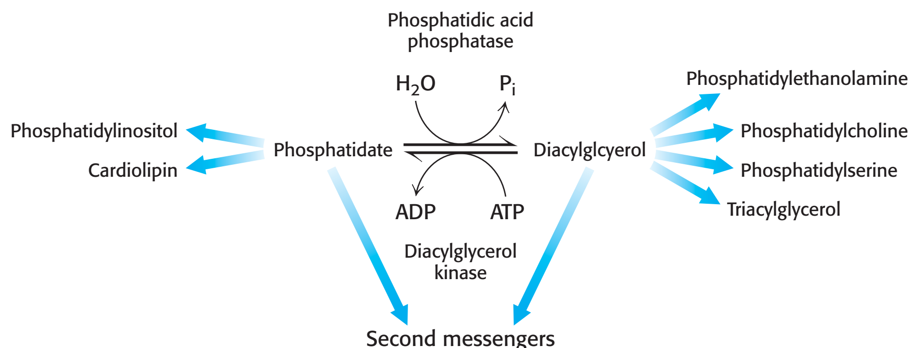


Figure 26.7 Regulation of lipid synthesis. Phosphatidic acid phosphatase is the key regulatory enzyme in lipid synthesis. When active, PAP generates diacylglycerol, which can react with activated alcohols to form phospholipids or with fatty acyl CoA to form triacylglycerols. When PAP is inactive, phosphatidate is converted into CMP-DAG for the synthesis of different phospholipids. PAP also controls the amount of DAG and phosphatidate, both of which function as second messengers.

suggests that the formation of triacylglycerols may act as a fatty acid buffer, which helps to regulate the levels of diacylglycerol and sphingolipids, which serve signaling functions.

When PAP activity is lower, phosphatidate is used as a precursor for different phospholipids, such as phosphatidylinositol and cardiolipin. Moreover, phosphatidate is a signal molecule itself. Phosphatidate regulates the growth of endoplasmic reticulum and nuclear membranes and acts as a cofactor that stimulates the expression of genes in phospholipid synthesis.

What are the signal molecules that regulate the activity of PAP? CDP-diacylglycerol, phosphatidylinositol, and cardiolipin enhance PAP activity, and sphingosine and dihydrosphingosine inhibit it.

Studies in mice clearly show the importance of PAP for the regulation of fatty acid synthesis. The loss of PAP function prevents normal adipose-tissue development, leading to lipodystrophy (severe loss of body fat) and insulin resistance. Excess PAP activity results in obesity. Understanding the regulation of phospholipid synthesis is an exciting area of research that will be active for some time to come.

26.2 Cholesterol Is Synthesized from Acetyl Coenzyme A in Three Stages

We now turn our attention to the synthesis of the fundamental lipid *cholesterol*. This steroid modulates the fluidity of animal cell membranes (Section 12.5) and is the precursor of steroid hormones such as progesterone, testosterone, estradiol, and cortisol. *All 27 carbon atoms of cholesterol are derived from acetyl CoA* in a three-stage synthetic process (Figure 26.8).

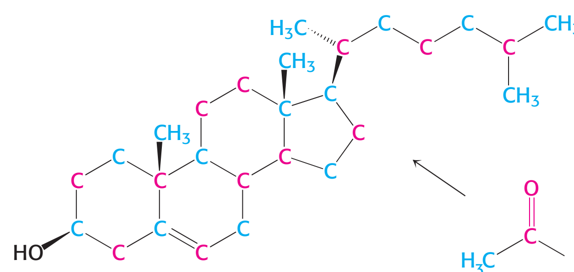


Figure 26.8 Labeling of cholesterol. Isotope-labeling experiments reveal the source of carbon atoms in cholesterol synthesized from acetate labeled in its methyl group (blue) or carboxylate atom (red).

1. Stage one is the synthesis of isopentenyl pyrophosphate, an activated isoprene unit that is the key building block of cholesterol.

2. Stage two is the condensation of six molecules of isopentenyl pyrophosphate to form squalene.

3. In stage three, squalene cyclizes and the tetracyclic product is subsequently converted into cholesterol.

The first stage takes place in the cytoplasm, and the second two in the endoplasmic reticulum.

Cholesterol

"Cholesterol is the most highly decorated small molecule in biology. Thirteen Nobel Prizes have been awarded to scientists who devoted major parts of their careers to cholesterol. Ever since it was isolated from gallstones in 1784, cholesterol has exerted an almost hypnotic fascination for scientists from the most diverse areas of science and medicine. . . . Cholesterol is a Janus-faced molecule. The very property that makes it useful in cell membranes, namely its absolute insolubility in water, also makes it lethal."

—Michael Brown and Joseph Goldstein, on the occasion of their receipt of a Nobel Prize for elucidating the control of blood levels of cholesterol. Nobel Lectures (1985)
© The Nobel Foundation, 1985

The synthesis of mevalonate, which is activated as isopentenyl pyrophosphate, initiates the synthesis of cholesterol

The first stage in the synthesis of cholesterol is the formation of isopentenyl pyrophosphate from acetyl CoA. This set of reactions starts with the formation of 3-hydroxy-3-methylglutaryl CoA (HMG CoA) from acetyl CoA and acetoacetyl CoA. This intermediate is reduced to *mevalonate* for the synthesis of cholesterol (Figure 26.9). Recall that, alternatively, 3-hydroxy-3-methylglutaryl CoA may be generated in the mitochondria and processed to form ketone bodies, which are subsequently secreted to provide fuel for other tissues, notably the brain under starvation conditions (Section 22.3).

The synthesis of mevalonate is the committed step in cholesterol formation. The enzyme catalyzing this irreversible step, *3-hydroxy-3-methylglutaryl CoA reductase* (HMG-CoA reductase), is an important control site in cholesterol biosynthesis, as will be discussed shortly.

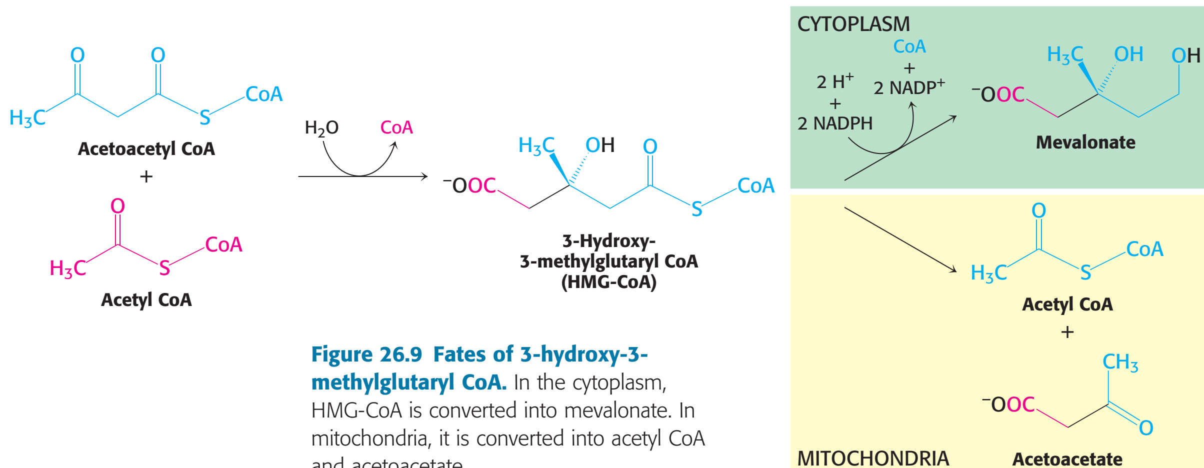
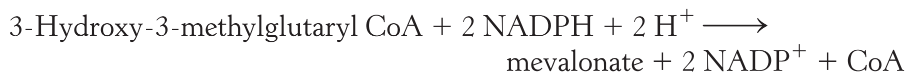
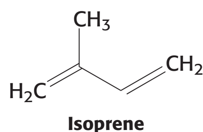


Figure 26.9 Fates of 3-hydroxy-3-methylglutaryl CoA. In the cytoplasm, HMG-CoA is converted into mevalonate. In mitochondria, it is converted into acetyl CoA and acetoacetate.



$$\text{3-Hydroxy-3-methylglutaryl CoA} + 2\ \text{NADPH} + 2\ \text{H}^+ \longrightarrow \text{mevalonate} + 2\ \text{NADP}^+ + \text{CoA}$$



HMG-CoA reductase is an integral membrane protein in the endoplasmic reticulum.

Mevalonate is converted into *3-isopentenyl pyrophosphate* in three consecutive reactions requiring ATP (Figure 26.10). In the last step, the release of CO_2 yields isopentenyl pyrophosphate, an activated isoprene unit that is a key building block for many important biomolecules throughout the kingdoms of life.

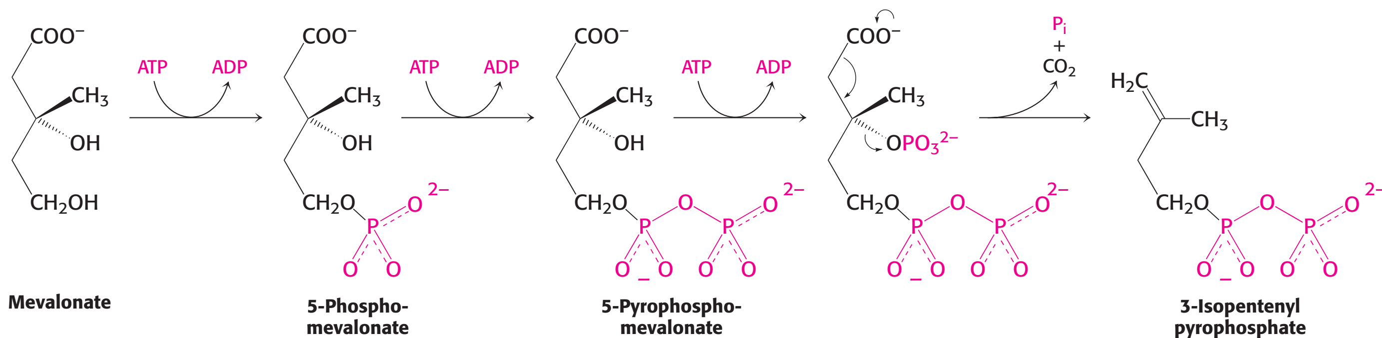


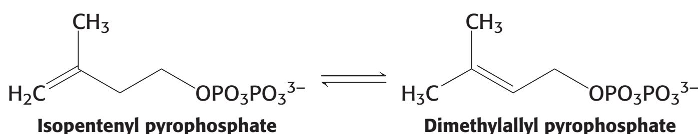
Figure 26.10 Synthesis of isopentenyl pyrophosphate. This activated intermediate is formed from mevalonate in three steps requiring ATP; followed by a decarboxylation.

Squalene (C_{30}) is synthesized from six molecules of isopentenyl pyrophosphate (C_5)

Squalene is synthesized from isopentenyl pyrophosphate by the reaction sequence

$$C_5 \longrightarrow C_{10} \longrightarrow C_{15} \longrightarrow C_{30}$$

This stage in the synthesis of cholesterol starts with the isomerization of *isopentenyl pyrophosphate* to *dimethylallyl pyrophosphate.*



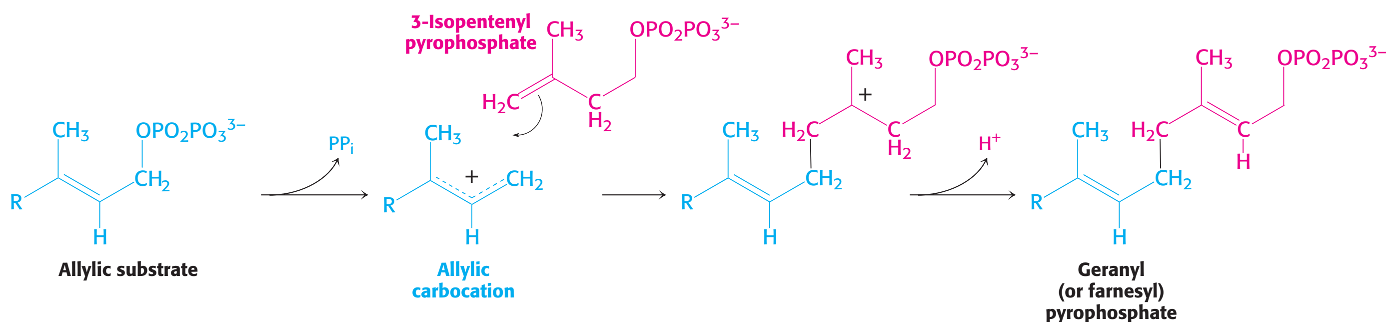
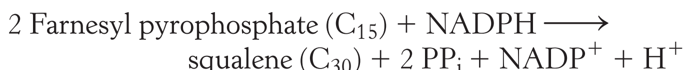


Figure 26.11 Condensation mechanism in cholesterol synthesis. The mechanism for joining dimethylallyl pyrophosphate and isopentenyl pyrophosphate to form geranyl pyrophosphate. The same mechanism is used to add an additional isopentenyl pyrophosphate to form farnesyl pyrophosphate.

These two isomeric C_5 units (one of each type) condense to form a C_{10} compound: isopentenyl pyrophosphate attacks an allylic carbocation ion formed from dimethylallyl pyrophosphate to yield *geranyl pyrophosphate* (Figure 26.11). The same kind of reaction takes place again: geranyl pyrophosphate is converted into an allylic carbonium ion, which is attacked by isopentenyl pyrophosphate. The resulting C_{15} compound is called *farnesyl pyrophosphate*. The same enzyme, *geranyl transferase*, catalyzes each of these condensations.

The last step in the synthesis of *squalene* is a reductive tail-to-tail condensation of two molecules of farnesyl pyrophosphate catalyzed by the endoplasmic reticulum enzyme *squalene synthase*.



$$2\text{ Farnesyl pyrophosphate } (C_{15}) + \text{NADPH} \longrightarrow \text{squalene } (C_{30}) + 2\,PP_i + \text{NADP}^+ + H^+$$

The reactions leading from C_5 units to squalene, a C_{30} isoprenoid, are summarized in Figure 26.12.

Squalene cyclizes to form cholesterol

The final stage of cholesterol biosynthesis starts with the cyclization of squalene (Figure 26.13). Squalene is first activated by conversion into squalene epoxide (2,3-oxidosqualene) in a reaction that uses O_2 and NADPH. Squalene epoxide is then cyclized to *lanosterol* by *oxidosqualene cyclase*. This remarkable transformation proceeds in a concerted fashion. The enzyme holds squalene epoxide in an appropriate conformation and initiates the reaction by protonating the epoxide oxygen. The carbocation formed spontaneously rearranges to produce lanosterol. Lanosterol is converted into cholesterol in a multistep process by the removal of three methyl groups, the reduction of one

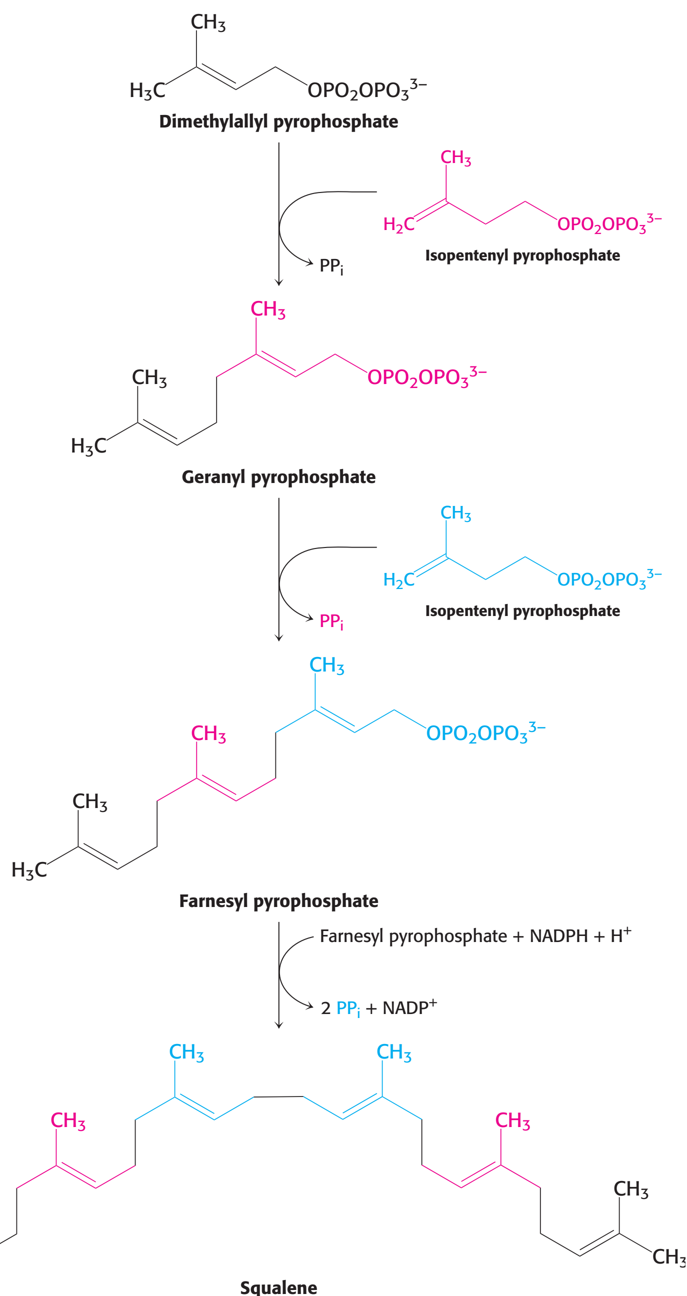


Figure 26.12 Squalene synthesis. One molecule of dimethyallyl pyrophosphate and two molecules of isopentenyl pyrophosphate condense to form farnesyl pyrophosphate. The tail-to-tail coupling of two molecules of farnesyl pyrophosphate yields squalene.

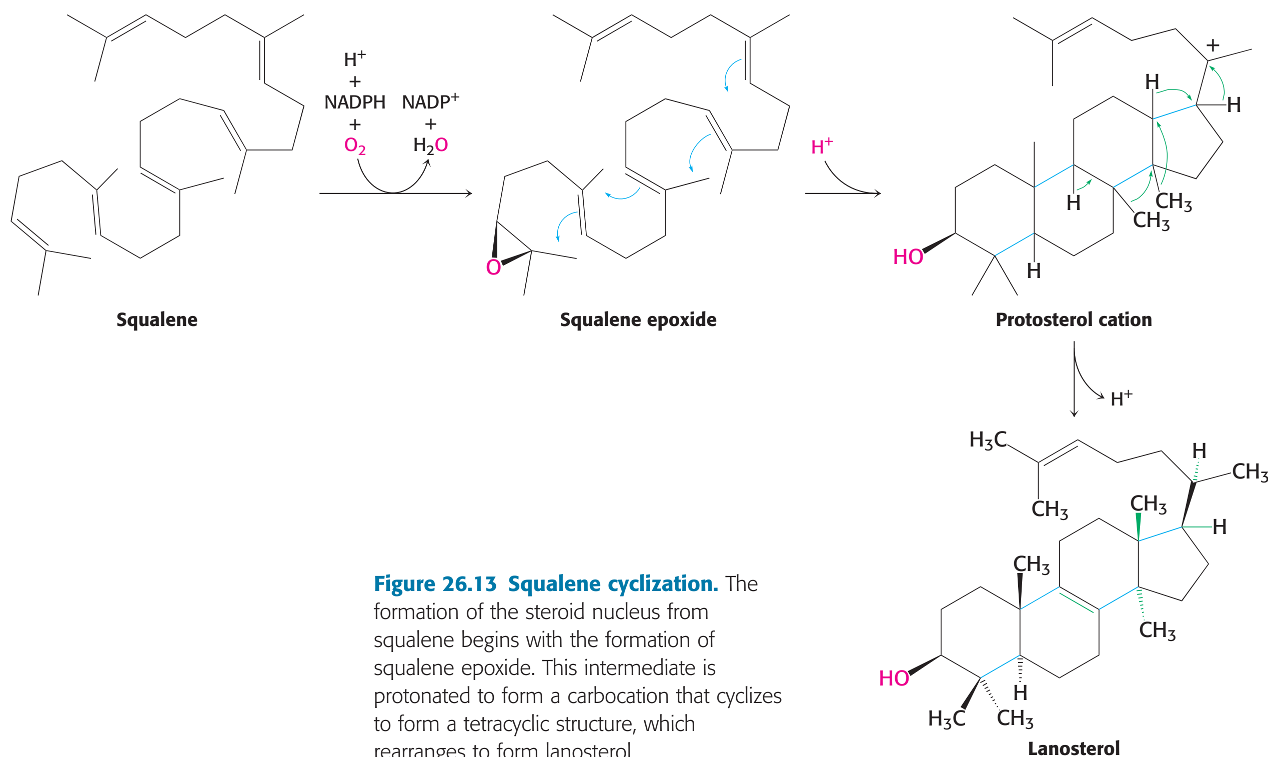


Figure 26.13 Squalene cyclization. The formation of the steroid nucleus from squalene begins with the formation of squalene epoxide. This intermediate is protonated to form a carbocation that cyclizes to form a tetracyclic structure, which rearranges to form lanosterol.

double bond by NADPH, and the migration of the other double bond (Figure 26.14).

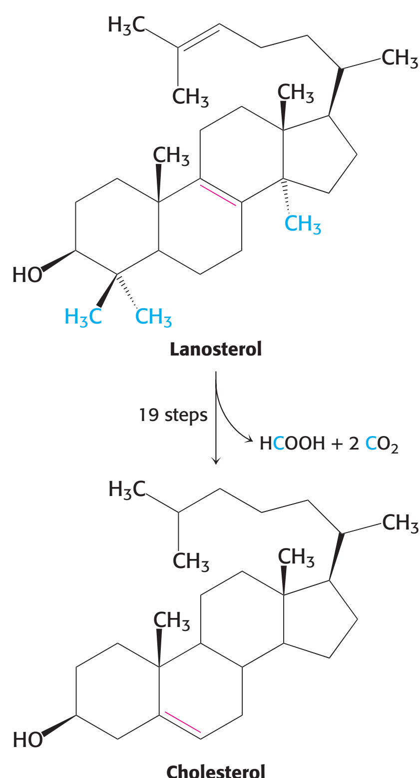


Figure 26.14 Cholesterol formation. Lanosterol is converted into cholesterol in a complex process.

26.3 The Complex Regulation of Cholesterol Biosynthesis Takes Place at Several Levels

Cholesterol can be obtained from the diet or it can be synthesized de novo. Cholesterol biosynthesis is one of the most highly regulated metabolic pathways known. Biosynthetic rates may vary several hundredfold, depending on how much cholesterol is consumed in the diet. An adult on a low-cholesterol diet typically synthesizes about 800 mg of cholesterol per day. The liver is the major site of cholesterol synthesis in mammals, although the intestine also forms significant amounts. The rate of cholesterol formation by these organs is highly responsive to the cellular level of cholesterol. *This feedback regulation is mediated primarily by changes in the amount and activity of 3-hydroxy-3-methylglutaryl CoA reductase*. As described earlier (p. 767), this enzyme catalyzes the formation of mevalonate, the committed step in cholesterol biosynthesis. HMG CoA reductase is controlled in multiple ways:

1. The rate of *synthesis of reductase mRNA* is controlled by the *sterol regulatory element binding protein* (SREBP). This transcription factor binds to a short DNA sequence called the *sterol regulatory element* (SRE) on the 5′ side of the reductase gene. It binds to the SRE when cholesterol levels are low and enhances transcription. In its inactive state, the SREBP resides in the endoplasmic reticulum membrane, where it is associated with the SREBP cleavage activating protein (SCAP), an integral membrane protein. SCAP is the cholesterol sensor. When cholesterol levels fall, SCAP escorts SREBP in small membrane vesicles to the Golgi complex, where it is released from the membrane by two specific proteolytic cleavages (Figure 26.15). The first cleavage frees a fragment of SREBP from SCAP, whereas the second

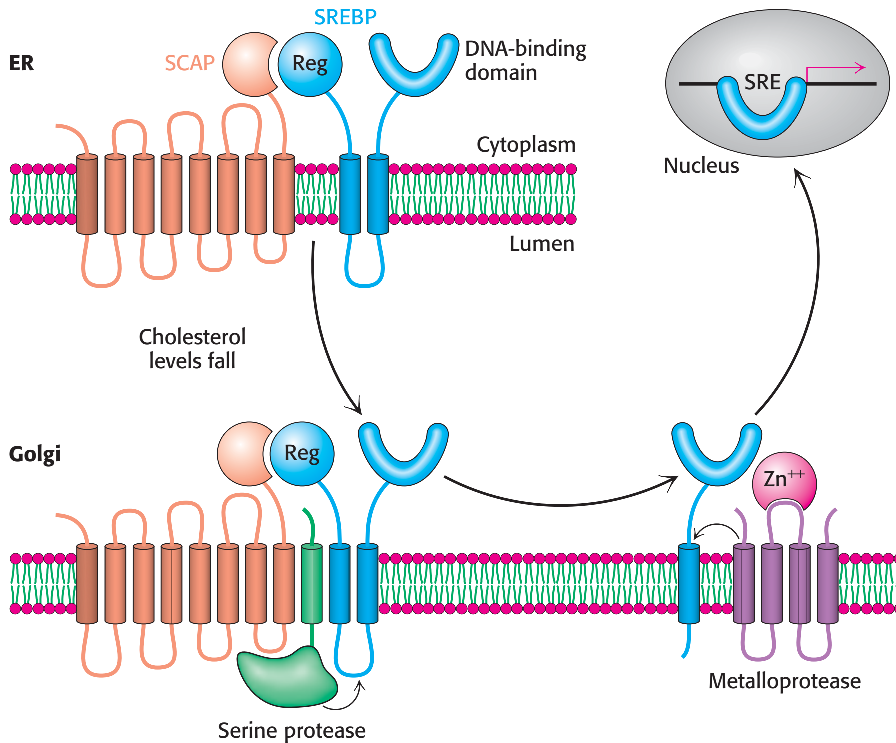


Figure 26.15 The SREBP pathway. SREBP resides in the endoplasmic reticulum, where it is bound to SCAP by its regulatory (Reg) domain. When cholesterol levels fall, SCAP and SREBP move to the Golgi complex, where SREBP undergoes successive proteolytic cleavages by a serine protease and a metalloprotease. The released DNA-binding domain moves to the nucleus to alter gene expression. [After an illustration provided by Dr. Michael Brown and Dr. Joseph Goldstein.]

cleavage releases the regulatory domain from the membrane. The released protein migrates to the nucleus and binds the SRE of the HMG-CoA reductase gene, as well as several other genes in the cholesterol biosynthetic pathway, to enhance transcription. When cholesterol levels rise, the proteolytic release of the SREBP is blocked, and the SREBP in the nucleus is rapidly degraded. These two events halt the transcription of genes of the cholesterol biosynthetic pathways.

What is the molecular mechanism that retains SCAP–SREBP in the ER when cholesterol is present but allows movement to the Golgi complex when cholesterol concentration is low? When cholesterol is low, SCAP binds to vesicular proteins that facilitate the transport of SCAP–SREBP to the Golgi apparatus, as heretofore described. When cholesterol is present, SCAP binds cholesterol, which causes a structural change in SCAP so that it binds to another endoplasmic reticulum protein called Insig (Figure 26.16). Insig is the anchor that retains SCAP and thus SREBP in the endoplasmic reticulum in the presence of cholesterol. The interactions between SCAP and Insig can also be forged when Insig binds 25-hydroxycholesterol, a metabolite of cholesterol. Thus, two distinct steroid–protein interactions serve to prevent the inappropriate movement of SCAP–SREBP to the Golgi complex.

2. The rate of *translation of reductase mRNA* is inhibited by nonsterol metabolites derived from mevalonate.

3. The *degradation of the reductase* is stringently controlled. The enzyme is bipartite: its cytoplasmic domain carries out catalysis and *its membrane*

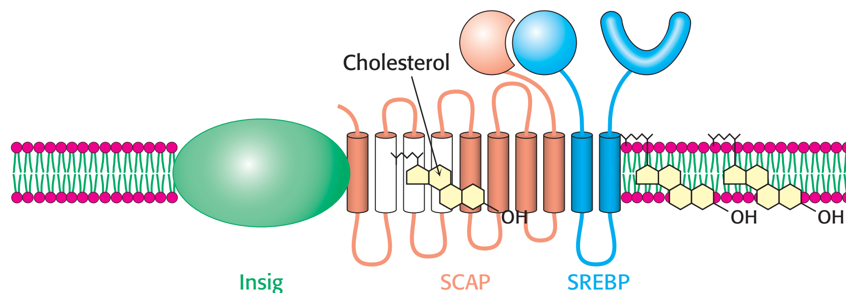


Figure 26.16 Insig regulates SCAP–SREBP movement. (A) In the presence of cholesterol, Insig interacts with SCAP–SREBP and prevents the activation of SREBP. Cholesterol binding to SCAP or 25-hydroxycholesterol binding to Insig facilitates the interaction of Insig and SCAP, retaining SCAP–SREBP in the endoplasmic reticulum. (B) In the absence of cholesterol or its derivatives, SCAP interacts with transport proteins and shepherds SREBP to the Golgi apparatus for activation. [After M. S. Brown and J. L. Goldstein. Cholesterol feedback: From Schoenheimer's bottle to Scap's MELADL. *J. Lipid Res.* 50:S15–S27, 2009.]

domain senses signals that lead to its degradation. The membrane domain may undergo structural changes in *response to increasing concentrations of sterols such as lanosterol and 25-hydroxycholesterol.* Under these conditions, the reductase appears to bind to a subset of Insigs that are also associated with the ubiquitinating enzymes (Figure 26.17). The reductase is polyubiquitinated and subsequently extracted from the membrane in a process that requires gerenylgeraniol. The extracted reductase is then degraded by the proteasome. The combined regulation at the levels of transcription, translation, and degradation can alter the amount of enzyme in the cell more than 200-fold.

4. *Phosphorylation decreases the activity of the reductase.* This enzyme, like acetyl CoA carboxylase (which catalyzes the committed step in fatty acid synthesis, Section 22.5), is switched off by an AMP-activated protein kinase. Thus, cholesterol synthesis ceases when the ATP level is low.

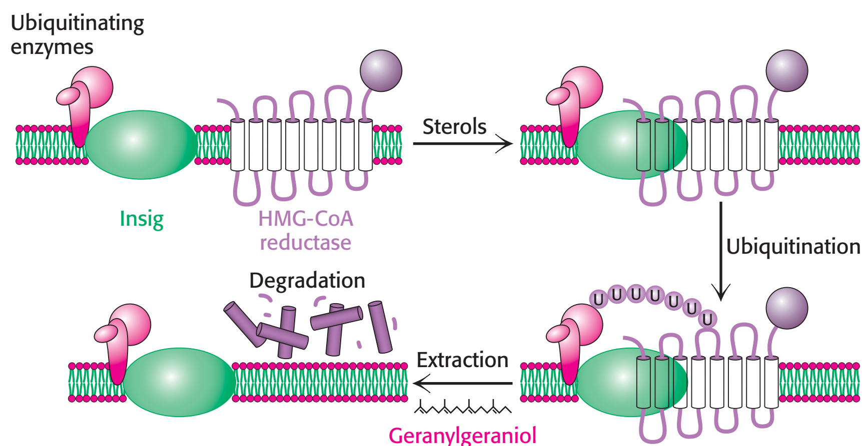


Figure 26.17 Insig facilitates the degradation of HMG-CoA reductase. In the presence of sterols, a subclass of Insig associated with ubquitinating enzymes binds HMG-CoA reductase. This interaction results in the ubquitination of the enzyme. This modification and the presence of geranylgeraniol results in extraction of the enzyme from the membrane and degradation by the proteasome. [After R. A. DeBose-Boyd. Feedback regulation of cholesterol synthesis: Sterol-accelerated ubiquitination and degradation of HMG CoA reductase. *Cell Res.* 18:609–621, 2008.]

As we will see shortly, all four regulatory mechanisms are modulated by receptors that sense the presence of cholesterol in the blood.

Lipoproteins transport cholesterol and triacylglycerols throughout the organism

Cholesterol and triacylglycerols are transported in body fluids in the form of *lipoprotein particles.* This transport is important for a number of reasons. First, lipoprotein particles are the means by which triacylglycerols are delivered to tissues, from the intestine or liver, for use as fuel or for storage. Second, the fatty acid constituents of the triacylglycerol components of the lipoprotein particles are incorporated into phospholipids for membrane synthesis. Likewise, cholesterol is a vital component of membranes and is a precursor to the powerful signal molecules, the steroid hormones. Finally, cells are not able to degrade the steroid nucleus. Consequently, the cholesterol must be used biochemically or excreted by the liver. Excess cholesterol plays a role in the development of atherosclerosis. Lipoprotein particles function in cholesterol homeostasis, transporting the molecule from sites of synthesis to sites of use, and finally to the liver for excretion.

Each lipoprotein particle consists of a core of hydrophobic lipids surrounded by a shell of more-polar lipids and proteins. The protein components of these macromolecular aggregates, called apoproteins, have two roles: *they solubilize hydrophobic lipids and contain cell-targeting signals.* Apolipoproteins are synthesized and secreted by the liver and the intestine. Lipoprotein particles are classified according to increasing density (Table 26.1): *chylomicrons, chylomicron remnants, very low density lipoproteins* (VLDLs), *intermediate-density lipoproteins* (IDLs), *low-density lipoproteins* (LDLs), and *high-density lipoproteins* (HDLs). These classes have numerous subtypes. Moreover, lipoprotein particles can shift between classes as they release or pick up cargo, thereby changing their density.

Triacylglycerols, cholesterol, and other lipids obtained from the diet are carried away from the intestine in the form of large *chylomicrons* (Section 22.1). These particles have a very low density because triacylglycerols constitute about 90% of their content. Apolipoprotein B-48 (apo B-48), a large protein (240 kd), forms an amphipathic spherical shell around the fat globule; the external face of this shell is hydrophilic. The triacylglycerols in chylomicrons are released through hydrolysis by *lipoprotein lipases.* These enzymes are located on the lining of blood vessels in muscle and other tissues that use fatty acids as fuels or in the synthesis of lipids. The liver then takes up the cholesterol-rich residues, known as *chylomicron remnants.*

Lipoprotein particles are also crucial for the transport of lipids from the liver, which is a major site of triacylglycerol and cholesterol synthesis, to

Table 26.1 Properties of plasma lipoproteins

					Composition (%)				
Plasma lipoproteins	Density (g ml^{-1})	Diameter (nm)	Apolipoprotein	Physiological role	TAG	CE	C	PL	P
Chylomicron	<0.95	75–1200	B-48, C, E	Dietary fat transport	86	3	1	8	2
Very low density lipoprotein	0.95–1.006	30–80	B-100, C, E	Endogenous fat transport	52	14	7	18	8
Intermediate-density lipoprotein	1.006–1.019	15–35	B-100, E	LDL precursor	38	30	8	23	11
Low-density lipoprotein	1.019–1.063	18–25	B-100	Cholesterol transport	10	38	8	22	21
High-density lipoprotein	1.063–1.21	7.5–20	A	Reverse cholesterol transport	5–10	14–21	3–7	19–29	33–57

Abbreviations: TAG, triacylglyerol; CE, cholesteryl ester; C, free cholesterol; PL, phospholipid; P, protein.

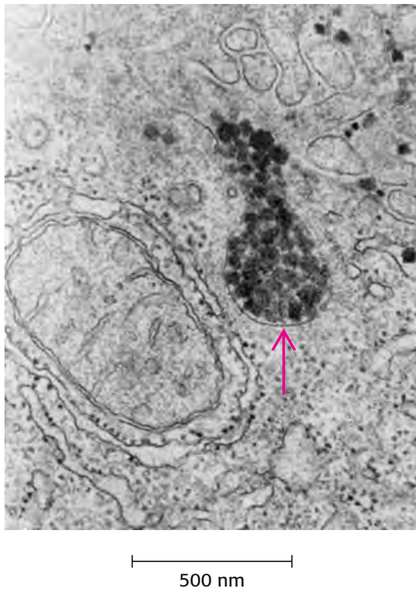


Figure 26.18 Site of cholesterol synthesis. Electron micrograph of a part of a liver cell actively engaged in the synthesis and secretion of very low density lipoprotein (VLDL). The arrow points to a vesicle that is releasing its content of VLDL particles. [Courtesy of Dr. George Palade.]

other tissues in the body (Figure 26.18). Triacylglycerols and cholesterol in excess of the liver's own needs are exported into the blood in the form of very low density lipoproteins. These particles are stabilized by two apolipoproteins—apo B-100 and apo E (34 kd). Apo B-100, one of the largest proteins known (513 kd), is a longer version of apo B-48. Both apo B proteins are encoded by the same gene and produced from the same initial RNA transcript. In the intestine, RNA editing (Section 29.3) modifies the transcript to generate the mRNA for apo B-48, the truncated form. Triacylglycerols in very low density lipoproteins, as in chylomicrons, are hydrolyzed by lipases on capillary surfaces, with the released fatty acids being taken up by the muscle and other tissues. The resulting remnants, which are rich in cholesteryl esters, are called *intermediate-density lipoproteins*. These particles have two fates. Half of them are taken up by the liver for processing, and half are converted into low-density lipoprotein by the removal of more triacylglycerol by tissue lipases that absorb the released fatty acids.

Low-density lipoprotein is the major carrier of cholesterol in blood (Figure 26.19). It contains a core of some 1500 cholesterol molecules esterified to fatty acids; the most common fatty acid chain in these esters is linoleate, a polyunsaturated fatty acid. A shell of phospholipids and unesterified cholesterol molecules surrounds this highly hydrophobic core. The shell also contains a single copy of apo B-100, which is recognized by target cells. *The role of LDL is to transport cholesterol to peripheral tissues and regulate de novo cholesterol synthesis at these sites,* as described on page 775. A different purpose is served by *high-density lipoprotein*, which picks up cholesterol released into the plasma from dying cells and from membranes undergoing turnover and delivers the cholesterol to the liver for excretion. An acyltransferase in HDL esterifies these cholesterols, which are then returned by HDL to the liver (Figure 26.20).

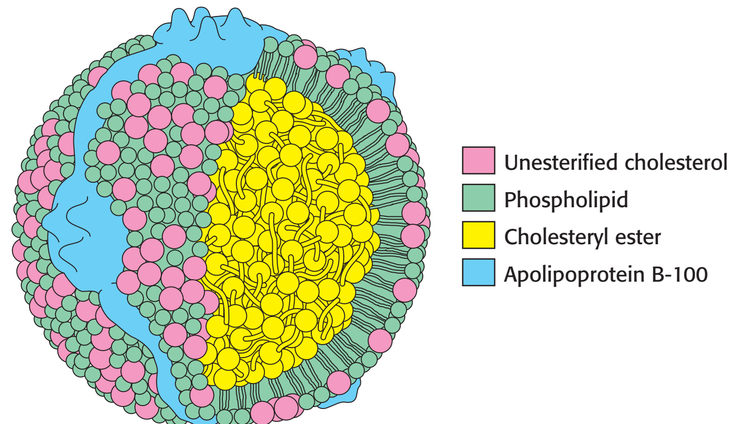


Figure 26.19 Schematic model of low-density lipoprotein. The LDL particle is approximately 22 nm (220 Å) in diameter.

The blood levels of certain lipoproteins can serve diagnostic purposes

High serum levels of cholesterol cause disease and death by contributing to the formation of atherosclerotic plaques in arteries throughout the body. This excess cholesterol is present in the form of the low-density lipoprotein particle, so-called bad cholesterol.

High-density lipoprotein is sometimes referred to as "good cholesterol." HDL functions as a shuttle that moves cholesterol throughout the body. HDL binds and esterifies cholesterol released from macrophages and the peripheral tissues and then transfers cholesteryl esters to tissues that use

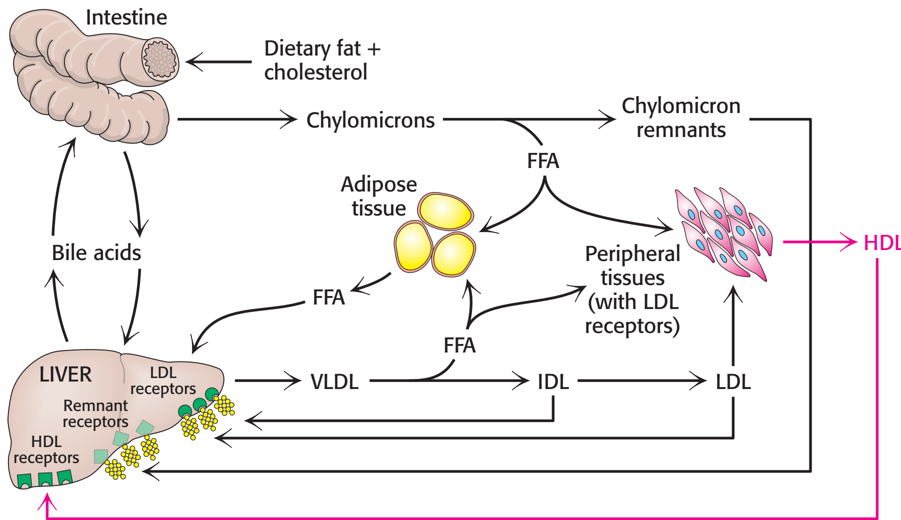


Figure 26.20 An overview of lipoprotein particle metabolism. Fatty acids are abbreviated FFA. [After J. G. Hardman (Ed.), L. L. Limbird (Ed.), and A. G. Gilman (Consult. Ed.), *Goodman and Gilman's The Pharmacological Basis of Therapeutics,* 10th ed. (McGraw-Hill, 2001), p. 975, Fig. 36.1.]

cholesterol to synthesize steroid hormones or to the liver, where the cholesterol is converted into bile salts or excreted. A specific receptor mediates the docking of the HDL to these tissues. The exact nature of the protective effect of HDL levels is not known; however, possible mechanisms are examined on page 776.

The ratio of cholesterol in the form of LDL to that in the form of HDL can be used to evaluate susceptibility to the development of heart disease. For a healthy person, the HDL/LDL ratio is 3.5.

Low-density lipoproteins play a central role in cholesterol metabolism

Cholesterol metabolism must be precisely regulated to prevent atherosclerosis. The mode of control in the liver, the primary site of cholesterol synthesis, has already been considered: dietary cholesterol reduces the activity and amount of 3-hydroxy-3-methylglutaryl CoA reductase, the enzyme catalyzing the committed step. Studies by Michael Brown and Joseph Goldstein are sources of insight into the control of cholesterol metabolism in nonhepatic cells. In general, cells outside the liver and intestine obtain cholesterol from the plasma rather than synthesizing it de novo. Specifically, *their primary source of cholesterol is the low-density lipoprotein.* The process of LDL uptake, called *receptor-mediated endocytosis,* serves as a paradigm for the uptake of many molecules (Figure 26.21).

Endocytosis begins when apolipoprotein B-100 on the surface of an LDL particle binds to a specific receptor protein on the plasma membrane of nonhepatic cells. The receptors for LDL are localized in specialized regions called *coated pits,* which contain a specialized protein called *clathrin.* The receptor–LDL complex is then internalized by *endocytosis;* that is, the plasma membrane in the vicinity of the complex invaginates and then fuses to form an endocytic vesicle called an *endosome* (Figure 26.22). The endosome is acidified, which causes the receptor to release its cargo. The receptor is returned to the cell membrane in a recycling vesicle. The round-trip time for a receptor is about 10 minutes;

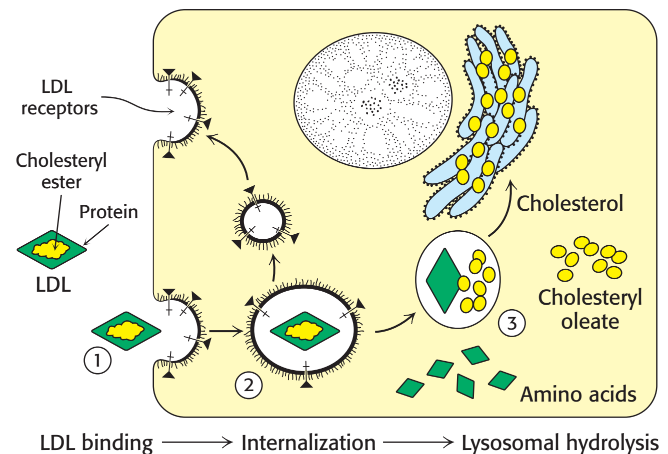
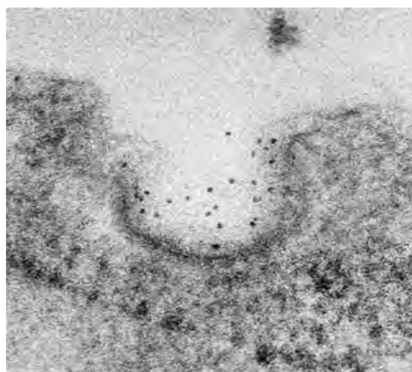
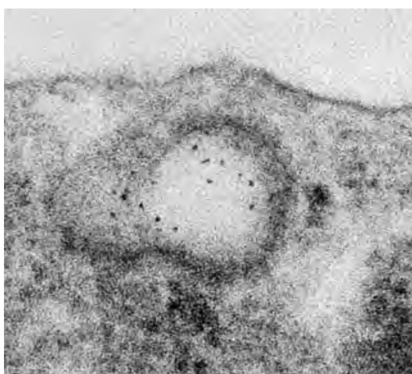


Figure 26.21 Receptor-mediated endocytosis. The process of receptor-mediated endocytosis is illustrated for the cholesterol-carrying complex, low-density lipoprotein (LDL): (1) LDL binds to a specific receptor, the LDL receptor; (2) this complex invaginates to form an internal vesicle; (3) after separation from its receptor, the LDL-containing vesicle fuses with a lysosome, leading to the degradation of the LDL and the release of the cholesterol.

in its lifetime of about a day, each receptor may bring hundreds of LDL particles into the cell. The vesicles containing LDL subsequently fuse with *lysosomes,* acidic vesicles that carry a wide array of degradative enzymes. The protein component of LDL is hydrolyzed to free amino acids. The cholesteryl esters in LDL are hydrolyzed by a lysosomal acid lipase. *The released unesterified cholesterol can then be used for membrane biosynthesis.* Alternatively, it can be *reesterified for storage inside the cell.* In fact, free cholesterol activates *acyl CoA:cholesterol acyltransferase* (ACAT), the enzyme catalyzing this reaction. Reesterified cholesterol contains mainly oleate and palmitoleate, which are monounsaturated fatty acids, in contrast with the cholesteryl esters in LDL, which are rich in linoleate, a polyunsaturated fatty acid (see Table 12.1). It is imperative that the cholesterol be reesterified. High concentrations of unesterified cholesterol disrupt the integrity of cell membranes.



(A)

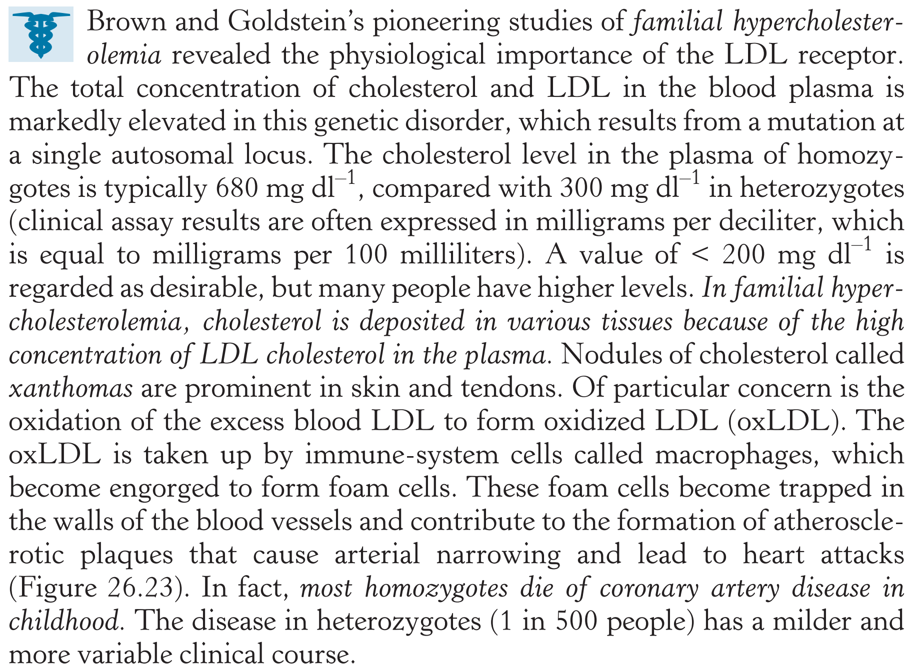


(B)

Figure 26.22 Endocytosis of LDL bound to its receptor. (A) Electron micrograph showing LDL (conjugated to ferritin for visualization, dark spots) bound to a coated-pit region on the surface of a cultured human fibroblast cell. (B) Micrograph showing this region invaginating and fusing to form an endocytic vesicle. [From R. G. W. Anderson, M. S. Brown, and J. L. Goldstein. *Cell* 10:351–364, 1977.]

The synthesis of the LDL receptor is itself subject to feedback regulation. Studies of cultured fibroblasts show that, *when cholesterol is abundant inside the cell, new LDL receptors are not synthesized, and so the uptake of additional cholesterol from plasma LDL is blocked.* The gene for the LDL receptor, like that for the reductase, is regulated by SREBP, which binds to a sterol regulatory element that controls the rate of mRNA synthesis.

The absence of the LDL receptor leads to hypercholesterolemia and atherosclerosis



Brown and Goldstein's pioneering studies of *familial hypercholesterolemia* revealed the physiological importance of the LDL receptor. The total concentration of cholesterol and LDL in the blood plasma is markedly elevated in this genetic disorder, which results from a mutation at a single autosomal locus. The cholesterol level in the plasma of homozygotes is typically 680 mg dl^{-1}, compared with 300 mg dl^{-1} in heterozygotes (clinical assay results are often expressed in milligrams per deciliter, which is equal to milligrams per 100 milliliters). A value of $<$ 200 mg dl^{-1} is regarded as desirable, but many people have higher levels. *In familial hypercholesterolemia, cholesterol is deposited in various tissues because of the high concentration of LDL cholesterol in the plasma.* Nodules of cholesterol called *xanthomas* are prominent in skin and tendons. Of particular concern is the oxidation of the excess blood LDL to form oxidized LDL (oxLDL). The oxLDL is taken up by immune-system cells called macrophages, which become engorged to form foam cells. These foam cells become trapped in the walls of the blood vessels and contribute to the formation of atherosclerotic plaques that cause arterial narrowing and lead to heart attacks (Figure 26.23). In fact, *most homozygotes die of coronary artery disease in childhood.* The disease in heterozygotes (1 in 500 people) has a milder and more variable clinical course.

The molecular defect in most cases of familial hypercholesterolemia is an absence or deficiency of functional receptors for LDL. Receptor mutations that disrupt each of the stages in the endocytotic pathway have been identified. Homozygotes have almost no functional receptors for LDL, whereas heterozygotes have about half the normal number. Consequently, the entry of LDL into liver and other cells is impaired, leading to an increased level of LDL in the blood plasma. Furthermore, less IDL enters liver cells because IDL entry, too, is mediated by the LDL receptor. Consequently, IDL stays in the blood longer in familial hypercholesterolemia, and more of it is converted into LDL than in normal people. All deleterious consequences of an absence or deficiency of the LDL receptor can be attributed to the ensuing elevated level of LDL cholesterol in the blood.

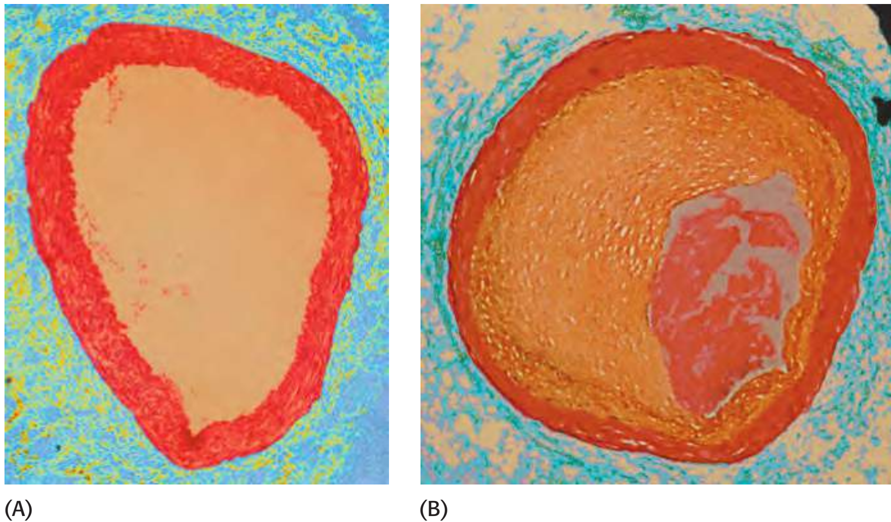


Figure 26.23 The effects of excess cholesterol. Cross section of (A) a normal artery and (B) an artery blocked by a cholesterol-rich plaque. [SPL/Photo Researchers.]

Mutations in the LDL receptor prevent LDL release and result in receptor destruction

One class of mutations that results in familial hypercholesterolemia generates receptors that are reluctant to give up the LDL cargo. Let us begin by examining the makeup of the receptor. The human LDL receptor is a 160-kd glycoprotein, which is composed of six different types of domains (Figure 26.24A). The amino-terminal region of the receptor, which is the site of LDL binding, consists of seven homologous LA domains, with domains 4 and 5 most important for LDL binding. A second type of domain is homologous to one found in the epidermal growth factor (EGF). This domain is repeated three times, and in between the second and third repeat is a propeller structure consisting of six bladelike domains. This

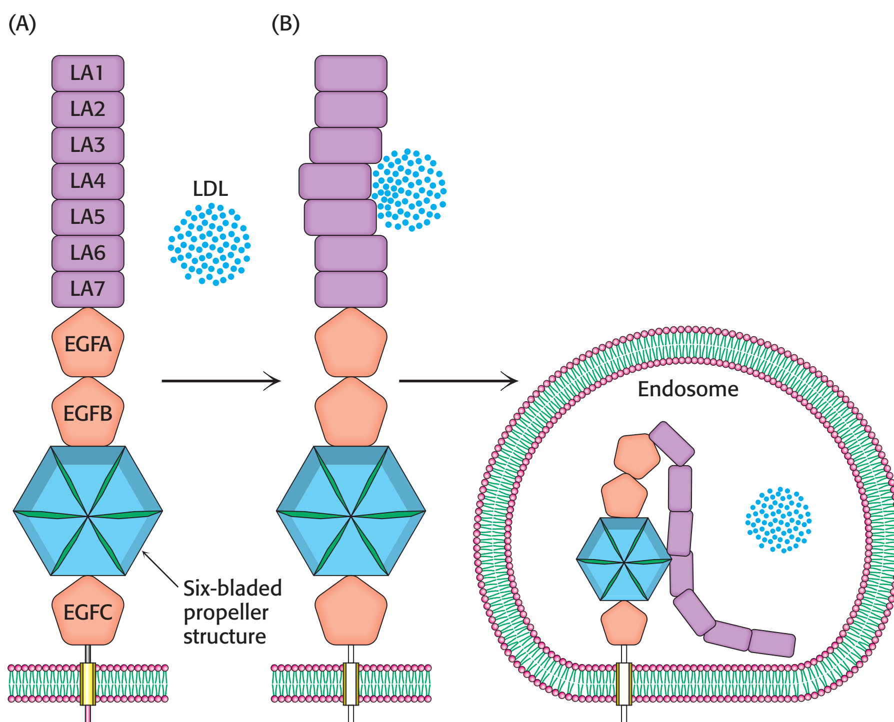


Figure 26.24 LDL receptor releases LDL in the endosome. (A) A schematic representation of the domain structure of the LDL receptor. (B) In the endosome, the receptor converts from the open structure into the closed structure, resulting in the release of the LDL into the endosome. [After I. D. Campbell, *Biochem. Soc. Trans.* 31(pt. 6p):1107–1114, 2003, Fig. 1A.]

part of the receptor is crucial in releasing LDL in the endosome. The fourth domain, which is very rich in serine and threonine residues, contains *O*-linked sugars. These oligosaccharides function as struts to keep the receptor extended from the membrane so that the LDL-binding domain is accessible to LDL. The fifth type of domain consists of 22 hydrophobic residues that span the plasma membrane. The sixth and final domain consists of 50 residues and emerges on the cytoplasmic side of the membrane, where it controls the interaction of the receptor with coated pits and participates in endocytosis.

How does the LDL receptor relinquish its cargo on entering the endosome? The receptor exists in two interconvertible states: an extended or open state, capable of binding LDL, and a closed state that results in release of the LDL in the endosome. The receptor maintains the open state while in the plasma membrane, on binding LDL, and throughout its journey to the endosome. The conversion from the open state into the closed state takes place on exposure to the acidic environment of the endosome (Figure 26.24B). Three contiguous modules LA7, EGFA, and EGFB rigidly position the propeller module to facilitate displacement of the LDL as the closed state is formed. Under neutral pH, aspartate residues of the propeller blades form hydrogen bonds that hold each blade to the rest of the propeller structure. Exposure to the low-pH environment of the endosome causes the propeller-like structures to interact with the LDL-binding domain. This interaction displaces the LDL, which is then digested by the lysosome. Under normal circumstances, the receptor is returned to the plasma membrane to again bind LDL. The importance of this process is highlighted by the fact that more than half of the point mutations that result in familial hypercholesterolemia are due to disruptions in the interconversion between the open state and the closed state. These mutations result in a failure to release the LDL cargo and loss of the receptor by degradation.

HDL appears to protect against artheriosclerosis

Although the events that result in atherosclerosis take place rapidly in familial hypercholesterolemia, a similar sequence of events take place in people who develop atherosclerosis over decades. In particular, the formation of foam cells and plaques are especially hazardous occurrences. HDL and its role in returning cholesterol to the liver appear to be important in mitigating these life-threatening circumstances.

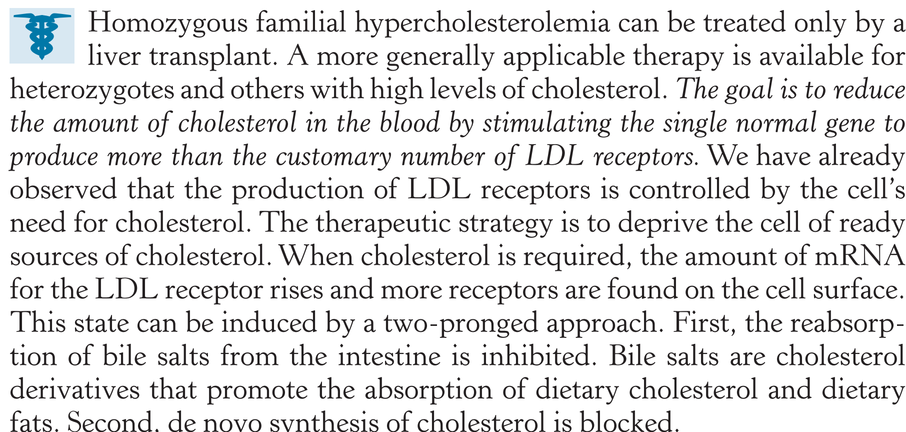
HDL has a number of antiatherogenic properties, including the inhibition of LDL oxidation, but the best-characterized property is the removal of cholesterol from cells, especially macrophages. Earlier, we learned that HDL retrieves cholesterol from other tissues in the body to return the cholesterol to the liver for excretion as bile or in the feces. This transport, called *reverse cholesterol transport,* is especially important in regard to macrophages. Indeed, when the transport fails, macrophages become foam cells and facilitate the formation of plaques. Macrophages that collect cholesterol from LDL normally transport the cholesterol to HDL particles. The more HDL, the more readily this transport takes place and the less likely that the macrophages will develop into foam cells. Presumably, this robust reverse cholesterol transport accounts for the observation that higher HDL levels confer protection against arthrosclerosis. Indeed, efforts are underway to develop drugs that will boost the levels of HDL in the blood.

The importance of reverse cholesterol transport is illustrated by the occurrence of mutations that inactivate one of the cholesterol-transport proteins in macrophages. This transport is facilitated by members of the ABC class of transport proteins (see Figure 13.7) present in macrophage

membranes. Loss of activity of cholesterol-transport protein ABAC1 results in a very rare condition called *Tangier disease*, which is characterized by HDL deficiency, accumulation of cholesterol in macrophages, and premature atherosclerosis.

Another antiatherogenic property of HDL is due to the association of a serum esterase, *paraoxanase*, with HDL. Paraoxanase may destroy oxLDL, accounting for some of HDL's ability to protect against coronary disease.

The clinical management of cholesterol levels can be understood at a biochemical level



Homozygous familial hypercholesterolemia can be treated only by a liver transplant. A more generally applicable therapy is available for heterozygotes and others with high levels of cholesterol. *The goal is to reduce the amount of cholesterol in the blood by stimulating the single normal gene to produce more than the customary number of LDL receptors.* We have already observed that the production of LDL receptors is controlled by the cell's need for cholesterol. The therapeutic strategy is to deprive the cell of ready sources of cholesterol. When cholesterol is required, the amount of mRNA for the LDL receptor rises and more receptors are found on the cell surface. This state can be induced by a two-pronged approach. First, the reabsorption of bile salts from the intestine is inhibited. Bile salts are cholesterol derivatives that promote the absorption of dietary cholesterol and dietary fats. Second, de novo synthesis of cholesterol is blocked.

The reabsorption of bile is impeded by oral administration of positively charged polymers, such as cholestyramine, that bind negatively charged bile salts and are not themselves absorbed. Cholesterol synthesis can be effectively blocked by a class of compounds called *statins.* A well-known example of such a compound is lovastatin, which is also called mevacor (Figure 26.25). These compounds are potent competitive inhibitors ($K_i = 1$ nM) of HMG-CoA reductase, the essential control point in the biosynthetic pathway. Plasma cholesterol levels decrease by 50% in many patients given both lovastatin and inhibitors of bile-salt reabsorption. Lovastatin and other inhibitors of HMG-CoA reductase are widely used to lower the plasma-cholesterol level in people who have atherosclerosis, which is the leading cause of death in industrialized societies. The development of statins as effective drugs is further described in Chapter 36.

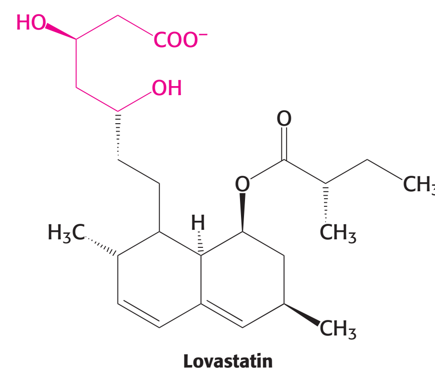


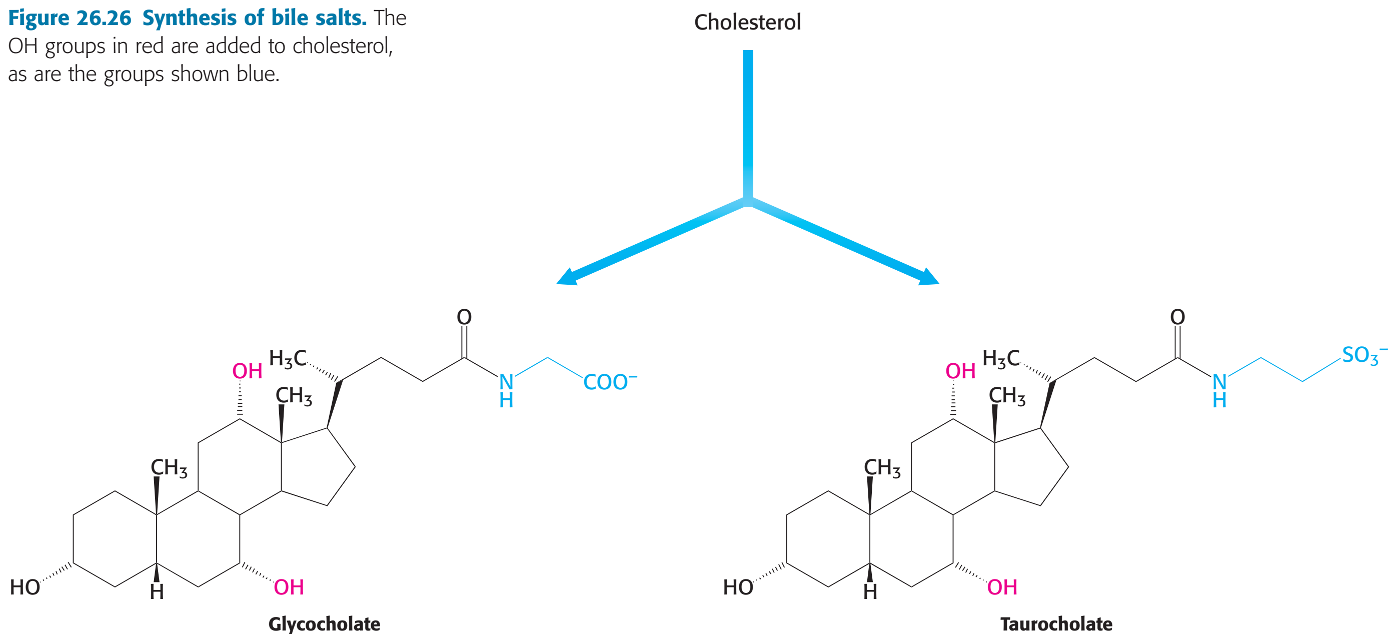
Figure 26.25 Lovastatin, a competitive inhibitor of HMG-CoA reductase. The part of the structure that resembles the 3-hydroxy-3-methylglutaryl moiety is shown in red.

26.4 Important Derivatives of Cholesterol Include Bile Salts and Steroid Hormones

Although cholesterol is well known in its own right as a contributor to the development of heart disease, metabolites of cholesterol—the steroid hormones—also appear in the news frequently. Indeed, steroid-hormone abuse seems to be as prominent in the sports pages as any athlete is. In addition to steroid hormones, cholesterol is a precursor for two other important molecules: bile salts and vitamin D. We begin with a look at the bile salts, molecules crucial for the uptake of lipids in the diet.

Bile salts. *Bile salts* are polar derivatives of cholesterol. These compounds are highly effective *detergents* because they contain both polar and nonpolar regions. Bile salts are synthesized in the liver, stored and concentrated in the gall bladder, and then released into the small intestine. Bile salts, the major constituent of bile, *solubilize dietary lipids*. Solubilization increases the effective surface area of lipids with two consequences: (1) more surface

Figure 26.26 Synthesis of bile salts. The OH groups in red are added to cholesterol, as are the groups shown blue.



area is exposed to the digestive action of lipases and (2) lipids are more readily absorbed by the intestine. Bile salts are also the major breakdown products of cholesterol. The bile salts glycocholate, the primary bile salt, and taurocholate are shown in Figure 26.26.

Steroid hormones. Cholesterol is the precursor of the five major classes of *steroid hormones:* progestagens, glucocorticoids, mineralocorticoids, androgens, and estrogens (Figure 26.27). These hormones are powerful signal molecules that regulate a host of organismal functions. *Progesterone, a progestogen,* prepares the lining of the uterus for the implantation of an ovum. Progesterone is also essential for the maintenance of pregnancy. *Androgens* (such as *testosterone*) are responsible for the development of male secondary sex characteristics, whereas *estrogens* (such as *estradiol*) are required for the development of female secondary sex characteristics. Estrogens, along with progesterone, also participate in the ovarian cycle. *Glucocorticoids* (such as *cortisol*) promote gluconeogenesis and the formation of glycogen, enhance the degradation of fat and protein, and inhibit the inflammatory response. They enable animals to respond to stress; indeed, the absence of glucocorticoids can be fatal. *Mineralocorticoids* (primarily *aldosterone*) act on the distal tubules of the kidney to increase the reabsorption of Na^+ and the excretion of K^+ and H^+, which leads to an increase in blood volume and blood pressure. The major sites of synthesis of these classes of hormones are the corpus luteum, for progestogens; the testes, for androgens; the ovaries, for estrogens; and the adrenal cortex, for glucocorticoids and mineralocorticoids.

Steroid hormones bind to and activate receptor molecules that serve as transcription factors to regulate gene expression (Section 32.2). These small similar molecules are able to have greatly differing effects because the slight structural differences among them allow interactions with specific receptor molecules.

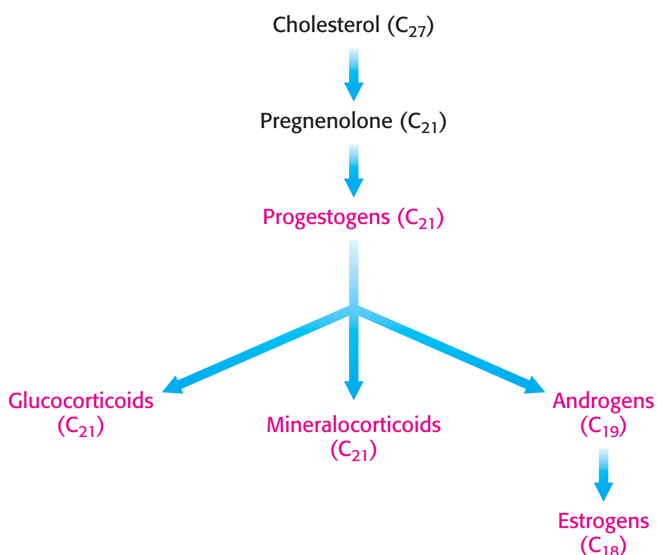


Figure 26.27 Biosynthetic relations of classes of steroid hormones and cholesterol.

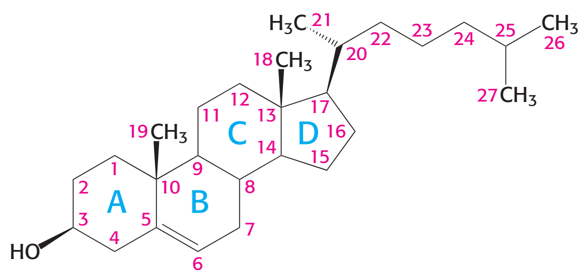
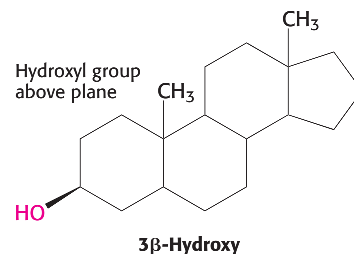
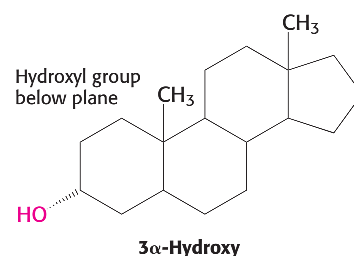


Figure 26.28 Cholesterol carbon numbering. The numbering scheme for the carbon atoms in cholesterol and other steroids.

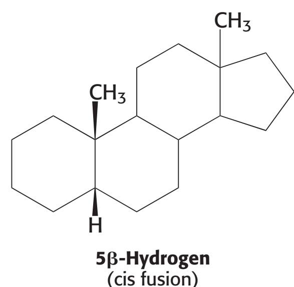
Letters identify the steroid rings and numbers identify the carbon atoms

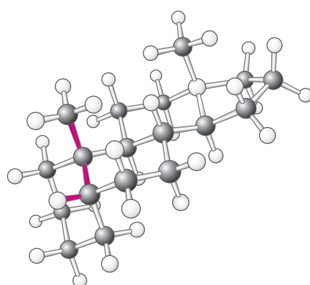


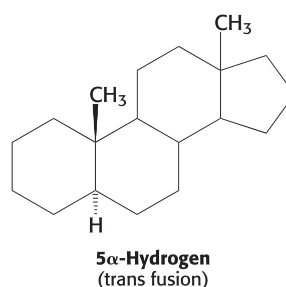


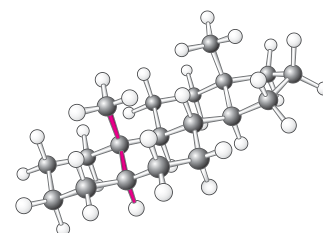
Carbon atoms in steroids are numbered, as shown for cholesterol in Figure 26.28. The rings in steroids are denoted by the letters A, B, C, and D. Cholesterol contains two angular methyl groups: the C-19 methyl group is attached to C-10, and the C-18 methyl group is attached to C-13. The C-18 and C-19 methyl groups of cholesterol lie *above* the plane containing the four rings. A substituent that is above the plane is termed *β oriented,* whereas a substituent that is below the plane is *α oriented.*

If a hydrogen atom is attached to C-5, it can be either α or β oriented. The A and B steroid rings are fused in a *trans* conformation if the C-5 hydrogen is *α oriented,* and *cis* if it is *β oriented.* The absence of a Greek letter for the C-5 hydrogen atom on the steroid nucleus implies a trans fusion. The C-5 hydrogen atom is α oriented in all steroid hormones that contain a hydrogen atom in that position. In contrast, bile salts have a β-oriented hydrogen atom at C-5. Thus, *a cis fusion is characteristic of the bile salts, whereas a trans fusion is characteristic of all steroid hormones that possess a hydrogen atom at C-5.* A trans fusion yields a nearly planar structure, whereas a cis fusion gives a buckled structure.









Steroids are hydroxylated by cytochrome P450 monooxygenases that use NADPH and O_2

The addition of OH groups plays an important role in the synthesis of cholesterol from squalene and in the conversion of cholesterol into steroid hormones and bile salts. All these hydroxylations require *NADPH* and O_2. The oxygen atom of the incorporated hydroxyl group comes from O_2 rather than from H_2O. Whereas one oxygen atom of the O_2 molecule goes into the substrate, the other is reduced to water. The enzymes catalyzing these reactions are called *monooxygenases* (or *mixed-function oxygenases*). Recall that a monooxygenase also participates in the hydroxylation of aromatic amino acids (Section 23.5).

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+$$

Hydroxylation requires the activation of oxygen. In the synthesis of steroid hormones and bile salts, activation is accomplished by members of the *cytochrome P450* family, a family of cytochromes that absorb light maximally at 450 nm when complexed in vitro with exogenous carbon monoxide. These membrane-anchored proteins (~50 kd) contain a heme prosthetic group. Oxygen is activated through its binding to the iron atom in the heme group.

Because the hydroxylation reactions promoted by P450 enzymes are oxidation reactions, it is at first glance surprising that they also consume the reductant NADPH. NADPH transfers its high-potential electrons to a flavoprotein, which transfers them, one at a time, to *adrenodoxin,* a nonheme iron protein. Adrenodoxin transfers one electron to reduce the ferric (Fe^{3+}) form of P450 to the ferrous (Fe^{2+}) form (Figure 26.29).

Without the addition of this electron, P450 will not bind oxygen. Recall that only the ferrous form (Fe^{2+}) of myoglobin binds oxygen (Section 7.1). The binding of O_2 to the heme is followed by the acceptance of a second electron from adrenodoxin. The acceptance of this second electron leads to cleavage of the O—O bond. One of the oxygen atoms is then protonated and released as water. The remaining oxygen atom forms a highly reactive ferryl Fe═O intermediate. This intermediate abstracts a hydrogen atom from the substrate RH to form R•. This transient free radical captures the OH group from the iron atom to form ROH, the hydroxylated product, returning the iron atom to the ferric state.

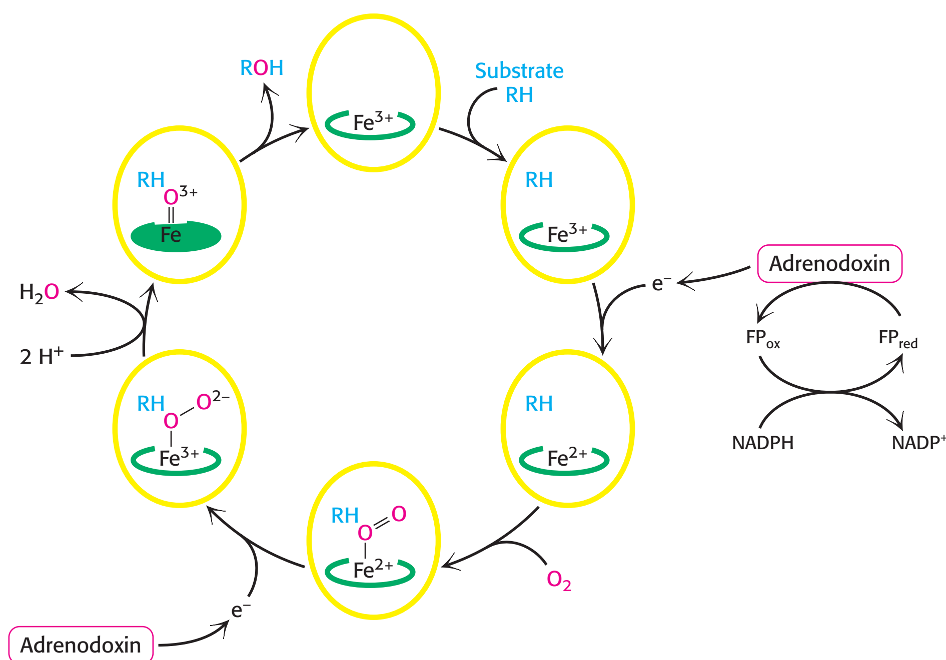


Figure 26.29 Cytochrome P450 mechanism. These enzymes bind O_2 and use one oxygen atom to hydroxylate their substrates.

The cytochrome P450 system is widespread and performs a protective function

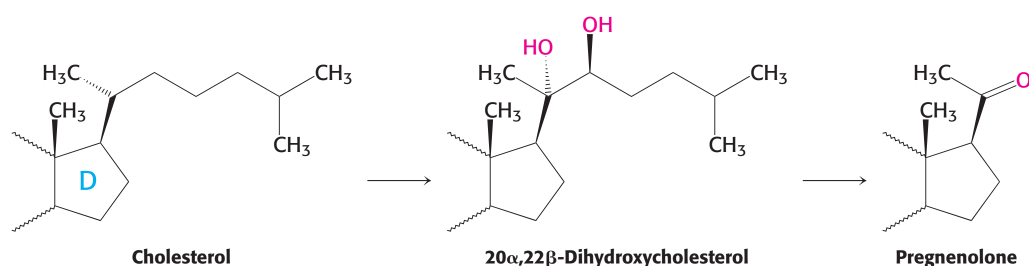
The cytochrome P450 system, which in mammals is located primarily in the endoplasmic reticulum of the liver and small intestine, is also important in the *detoxification of foreign substances* (xenobiotic compounds). For example, the hydroxylation of phenobarbital, a barbiturate, *increases its solubility* and *facilitates its excretion.* Likewise, polycyclic aromatic hydrocarbons that are ingested by drinking contaminated water are hydroxylated by P450, pro-

viding sites for conjugation with highly polar units (e.g., glucuronate or sulfate) that markedly increase the solubility of the modified aromatic molecule. One of the most relevant functions of the cytochrome P450 system to human beings is its role in metabolizing drugs such as caffeine and ibuprofen (Chapter 36). Some members of the cytochrome P450 system also metabolize ethanol (Section 27.6). The duration of action of many medications depends on their rate of inactivation by the P450 system. Despite its general protective role in the removal of foreign chemicals, the action of the P450 system is not always beneficial. *Some of the most powerful carcinogens are generated from harmless compounds by the P450 system in vivo* in the process of *metabolic activation.* In plants, the cytochrome P450 system plays a role in the synthesis of toxic compounds as well as the pigments of flowers.

The cytochrome P450 system is a ubiquitous superfamily of monooxygenases that is present in plants, animals, and prokaryotes. The human genome encodes more than 50 members of the family, whereas the genome of the plant *Arabidopsis* encodes more than 250 members. All members of this large family arose by gene duplication followed by subsequent divergence, which generated a range of substrate specificity. The specificity of these enzymes is encoded in delimited regions of the primary structure, and the substrate specificity of closely related members is often defined by a few critical residues or even a single amino acid.

Pregnenolone, a precursor of many other steroids, is formed from cholesterol by cleavage of its side chain

Steroid hormones contain 21 or fewer carbon atoms, whereas cholesterol contains 27. Thus, the first stage in the synthesis of steroid hormones is the removal of a six-carbon unit from the side chain of cholesterol to form *pregnenolone.* The side chain of cholesterol is hydroxylated at C-20 and then at C-22, and the bond between these carbon atoms is subsequently cleaved by *desmolase*. Three molecules of NADPH and three molecules of O_2 are consumed in this remarkable six-electron oxidation.



Progesterone and corticosteroids are synthesized from pregnenolone

Progesterone is synthesized from pregnenolone in two steps. The 3-hydroxyl group of pregnenolone is oxidized to a 3-keto group, and the Δ^5 double bond is isomerized to a Δ^4 double bond (Figure 26.30). *Cortisol,* the major glucocorticoid, is synthesized from progesterone by hydroxylations at C-17, C-21, and C-11; C-17 must be hydroxylated before C-21 is hydroxylated, whereas C-11 can be hydroxylated at any stage. The enzymes catalyzing these hydroxylations are highly specific, as shown by some inherited disorders. The initial step in the synthesis of *aldosterone,* the major mineralocorticoid, is the hydroxylation of progesterone at C-21. The resulting deoxycorticosterone is hydroxylated at C-11. The oxidation of the C-18 angular methyl group to an aldehyde then yields aldosterone.

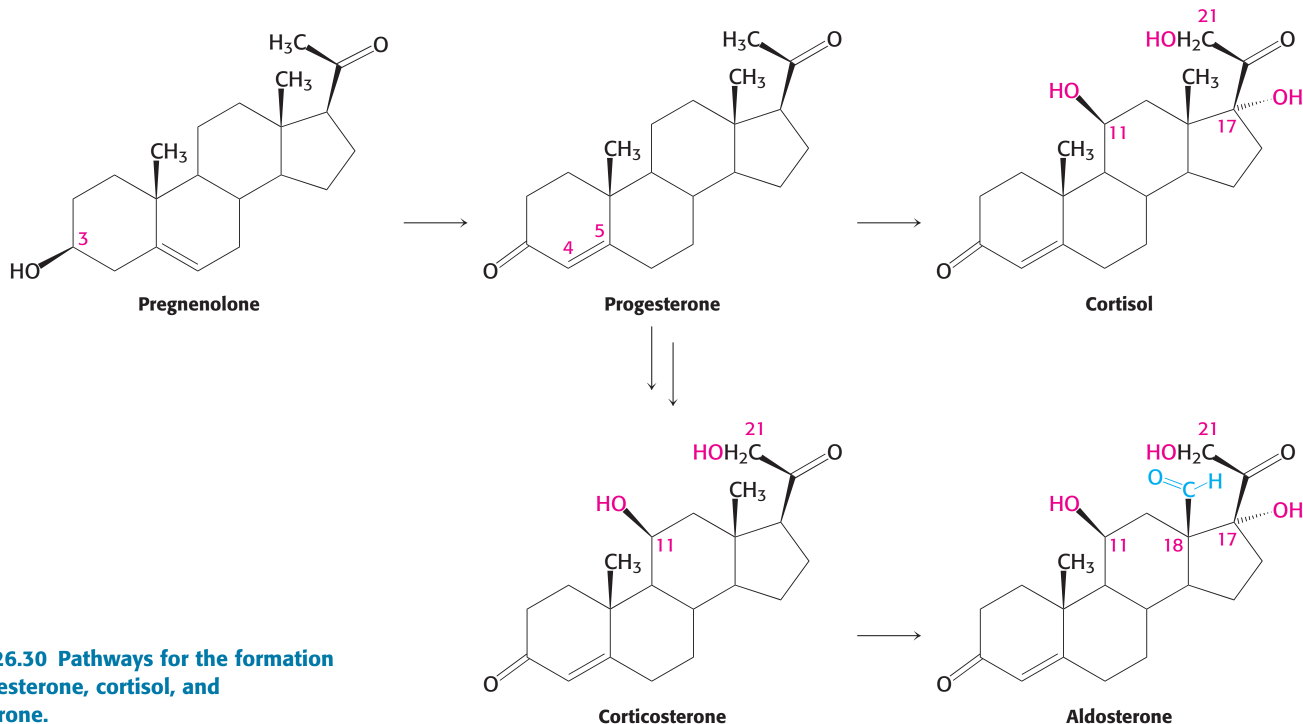


Figure 26.30 Pathways for the formation of progesterone, cortisol, and aldosterone.

Androgens and estrogens are synthesized from pregnenolone

Androgens and estrogens also are synthesized from pregnenolone through the intermediate progesterone. Androgens contain 19 carbon atoms. The synthesis of androgens starts with the hydroxylation of progesterone at C-17 (Figure 26.31). The side chain consisting of C-20 and C-21 is then cleaved to yield *androstenedione,* an androgen. *Testosterone,* another androgen, is formed by the reduction of the 17-keto group of androstenedione. Testosterone, through its actions in the brain, is paramount in the develop-

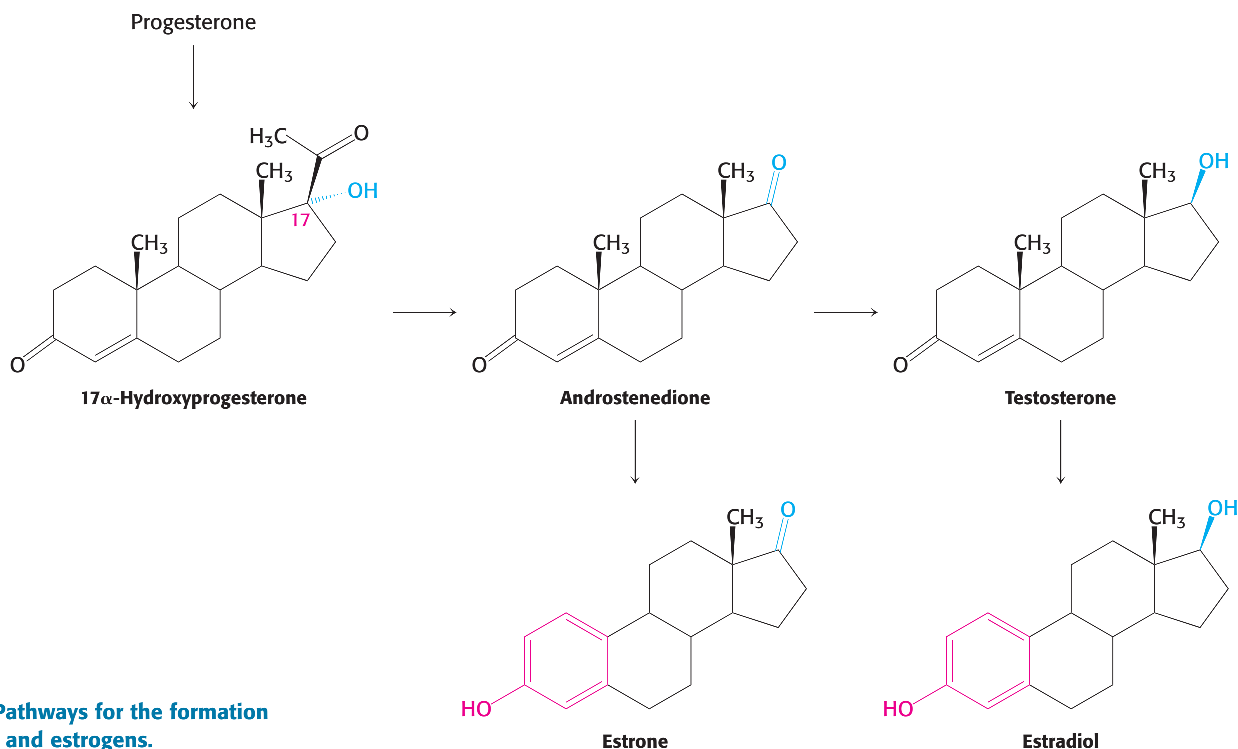
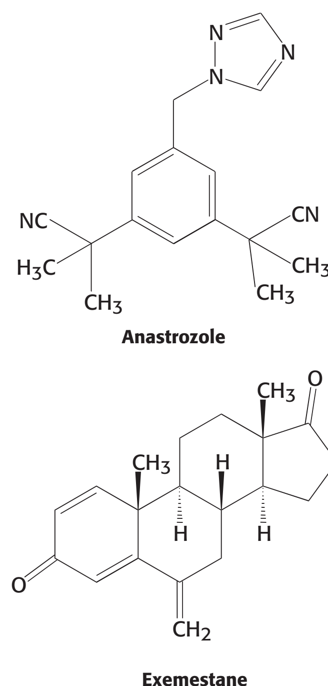


Figure 26.31 Pathways for the formation for androgens and estrogens.

ment of male sexual behavior. It is also important for the maintenance of the testes and the development of muscle mass. Owing to the latter activity, testosterone is referred to as an *anabolic steroid*. Testosterone is reduced by *5α-reductase* to yield *dihydrotestosterone* (DHT), a powerful embryonic androgen that instigates the development and differentiation of the male phenotype. Estrogens are synthesized from androgens by the loss of the C-19 angular methyl group and the formation of an aromatic A ring. *Estrone,* an estrogen, is derived from androstenedione, whereas *estradiol*, another estrogen, is formed from testosterone. The formation of the aromatic A ring is catalyzed by the P450 enzyme *aromatase.*

Because breast and ovarian cancers frequently depend on estrogens for growth, aromatase inhibitors are often used as a treatment for these cancers. Anastrazole is a competitive inhibitor of the enzyme, whereas exemestane is a suicide inhibitor that covalently modifies and inactivates the enzyme.



Vitamin D is derived from cholesterol by the ring-splitting activity of light

Cholesterol is also the precursor of vitamin D, which plays an essential role in the control of calcium and phosphorus metabolism. *7-Dehydrocholesterol (provitamin D_3) is photolyzed by the ultraviolet light of sunlight to previtamin D_3, which spontaneously isomerizes to vitamin D_3* (Figure 26.32). Vitamin D_3 (cholecalciferol) is converted into *calcitriol* (1,25-dihydroxycholecalciferol), the active hormone, by hydroxylation reactions in the liver and kidneys. Although not a steroid, vitamin D acts in an analogous fashion. It binds to a receptor, structurally similar to the steroid receptors, to form a complex that functions as a transcription factor, regulating gene expression.

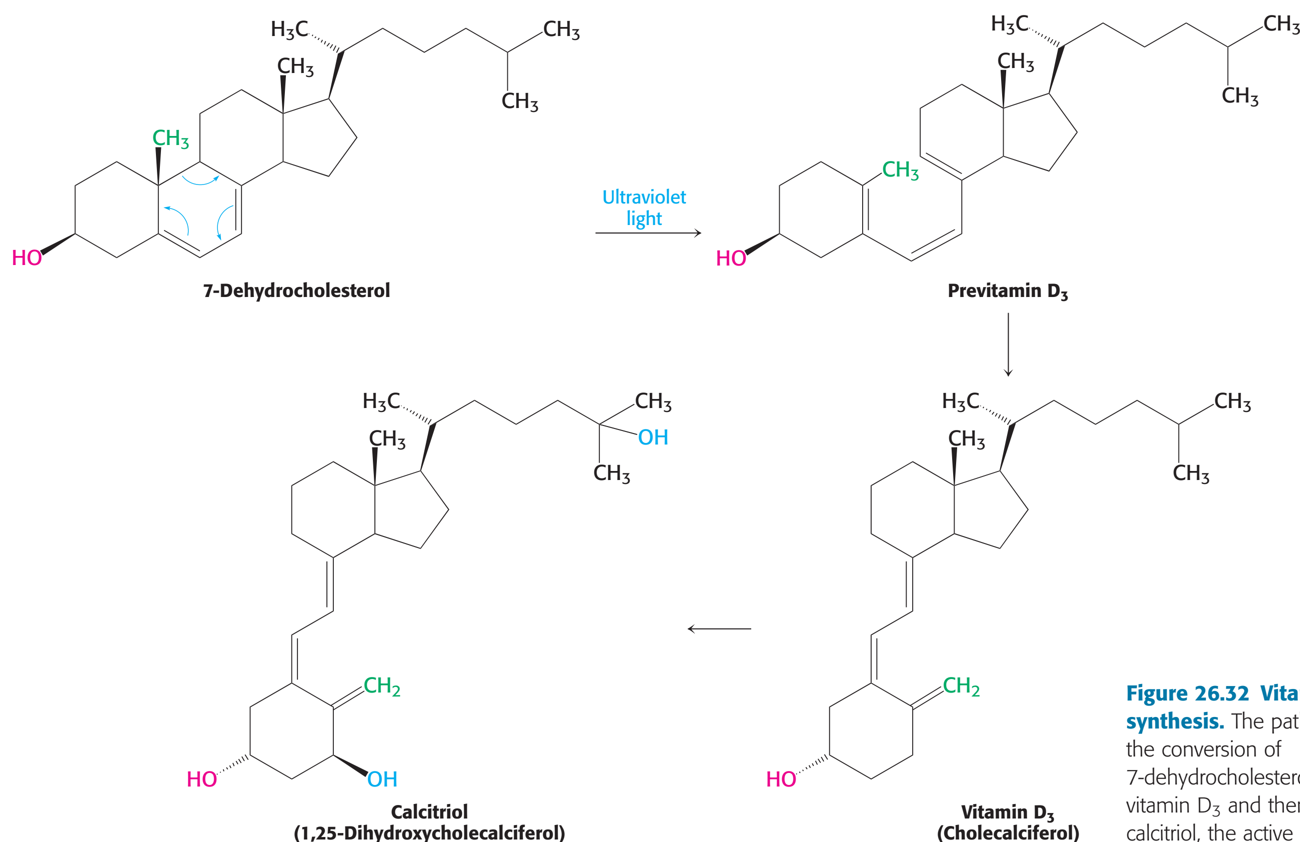


Figure 26.32 Vitamin D synthesis. The pathway for the conversion of 7-dehydrocholesterol into vitamin D_3 and then into calcitriol, the active hormone.

Vitamin D deficiency in childhood produces *rickets,* a disease characterized by inadequate calcification of cartilage and bone. Rickets was so common in seventeenth-century England that it was called the "children's disease of the English." The 7-dehydrocholesterol in the skin of these children was not photolyzed to previtamin D_3, because there was little sunlight for many months of the year. Furthermore, their diets provided little vitamin D, because most naturally occurring foods have a low content of this vitamin. Fish-liver oils are a notable exception. Cod-liver oil, abhorred by generations of children because of its unpleasant taste, was used in the past as a rich source of vitamin D. Today, the most reliable dietary sources of vitamin D are fortified foods. Milk, for example, is fortified to a level of 400 international units per quart (10 μg per quart). The recommended daily intake of vitamin D is 200 international units until age 50, after which it increases with age. In adults, vitamin D deficiency leads to softening and weakening of bones, a condition called *osteomalacia.* The occurrence of osteomalacia in Muslim women who are clothed so that only their eyes are exposed to sunlight is a striking reminder that vitamin D is needed by adults as well as by children.

Research over the past few years indicates that vitamin D may play a much larger biochemical role than simply the regulation of bone metabolism. Muscle seems to be a target of vitamin D action. In muscle, vitamin D appears to affect a number of biochemical processes with the net effect being enhanced muscle performance. Studies also suggest that vitamin D prevents cardiovascular disease, reduces the incidence of a variety of cancers, and protects against autoimmune diseases including diabetes. Moreover, vitamin D deficiency appears to be more common than thought. In the United States, 90% of Blacks and many Hispanics and Asians have insufficient blood levels of vitamin D. This recent research on vitamin D shows again the dynamic nature of biochemical investigations. Vitamin D, a chemical whose biochemical role was believed to be well established, now offers new frontiers of biomedical research.

Summary

26.1 Phosphatidate Is a Common Intermediate in the Synthesis of Phospholipids and Triacylglycerols

Phosphatidate is formed by successive acylations of glycerol 3-phosphate by acyl CoA. Hydrolysis of its phosphoryl group followed by acylation yields a triacylglycerol. CDP-diacylglycerol, the activated intermediate in the de novo synthesis of several phospholipids, is formed from phosphatidate and CTP. The activated phosphatidyl unit is then transferred to the hydroxyl group of a polar alcohol, such as inositol, to form a phospholipid such as phosphatidylinositol. In mammals, phosphatidylethanolamine is formed by CDP-ethanolamine and diacylglycerol. Phosphatidylethanolamine is methylated by *S*-adenosylmethionine to form phosphatidylcholine. In mammals, this phosphoglyceride can also be synthesized by a pathway that utilizes dietary choline. CDP-choline is the activated intermediate in this route.

Sphingolipids are synthesized from ceramide, which is formed by the acylation of sphingosine. Gangliosides are sphingolipids that contain an oligosaccharide unit having at least one residue of *N*-acetylneuraminate or a related sialic acid. They are synthesized by the step-by-step addition of activated sugars, such as UDP-glucose, to ceramide.

26.2 Cholesterol Is Synthesized from Acetyl Coenzyme A in Three Stages

Cholesterol is a steroid component of animal membranes and a precursor of steroid hormones. The committed step in its synthesis is the formation of mevalonate from 3-hydroxy-3-methylglutaryl CoA (derived from acetyl CoA and acetoacetyl CoA). Mevalonate is converted into isopentenyl pyrophosphate (C_5), which condenses with its isomer, dimethylallyl pyrophosphate (C_5), to form geranyl pyrophosphate (C_{10}). The addition of a second molecule of isopentenyl pyrophosphate yields farnesyl pyrophosphate (C_{15}), which condenses with itself to form squalene (C_{30}). This intermediate cyclizes to lanosterol (C_{30}), which is modified to yield cholesterol (C_{27}).

26.3 The Complex Regulation of Cholesterol Biosynthesis Takes Place at Several Levels

In the liver, cholesterol synthesis is regulated by changes in the amount and activity of 3-hydroxy-3-methylglutaryl CoA reductase. Transcription of the gene, translation of the mRNA, and degradation of the enzyme are stringently controlled. In addition, the activity of the reductase is regulated by phosphorylation.

Triacylglycerols exported by the intestine are carried by chylomicrons and then hydrolyzed by lipases lining the capillaries of target tissues. Cholesterol and other lipids in excess of those needed by the liver are exported in the form of very low density lipoprotein. After delivering its content of triacylglycerols to adipose tissue and other peripheral tissue, VLDL is converted into intermediate-density lipoprotein and then into low-density lipoprotein. IDL and LDL carry cholesteryl esters, primarily cholesteryl linoleate. Liver and peripheral tissue cells take up LDL by receptor-mediated endocytosis. The LDL receptor, a protein spanning the plasma membrane of the target cell, binds LDL and mediates its entry into the cell. Absence of the LDL receptor in the homozygous form of familial hypercholesterolemia leads to a markedly elevated plasma level of LDL cholesterol and the deposition of cholesterol on blood-vessel walls, which in turn may result in childhood heart attacks. Apolipoprotein B, a very large protein, is a key structural component of chylomicrons, VLDL, and LDL. High-density lipoproteins transport cholesterol from the peripheral tissues to the liver.

26.4 Important Derivatives of Cholesterol Include Bile Salts and Steroid Hormones

In addition to bile salts, which facilitate the digestion of lipids, five major classes of steroid hormones are derived from cholesterol: progestogens, glucocorticoids, mineralocorticoids, androgens, and estrogens. Hydroxylations by P450 monooxygenases that use NADPH and O_2 play an important role in the synthesis of steroid hormones and bile salts from cholesterol. P450 enzymes, a large superfamily, also participate in the detoxification of drugs and other foreign substances.

Pregnenolone (C_{21}) is an essential intermediate in the synthesis of steroids. This steroid is formed by scission of the side chain of cholesterol. Progesterone (C_{21}), synthesized from pregnenolone, is the precursor of cortisol and aldosterone. Hydroxylation of progesterone and cleavage of its side chain yields androstenedione, an androgen (C_{19}). Estrogens (C_{18}) are synthesized from androgens by the loss of an angular methyl group and the formation of an aromatic A ring. Vitamin D, which is important in the control of calcium and phosphorus metabolism, is formed from a derivative of cholesterol by the action of light.

Key Terms

phosphatidate (p. 760)
triacylglycerol (p. 761)
phospholipid (p. 761)
cytidine diphosphodiacylglycerol (CDP-diacylglycerol) (p. 761)
sphingolipid (p. 763)
ceramide (p. 764)
cerebroside (p. 764)
ganglioside (p. 764)
cholesterol (p. 767)
mevalonate (p. 767)
3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase) (p. 767)
3-isopentenyl pyrophosphate (p. 768)
sterol regulatory element binding protein (SREBP) (p. 770)
lipoprotein particles (p. 773)
low-density lipoprotein (LDL) (p. 773)
high-density lipoprotein (HDL) (p. 773)
receptor-mediated endocytosis (p. 775)
reverse cholesterol transport (p. 778)
bile salt (p. 779)
steroid hormone (p. 780)
cytochrome P450 monooxygenase (p. 782)
pregnenolone (p. 783)

Problems

1. *Different roles.* Describe the roles of glycerol 3-phosphate, phosphatidate, and diacylglycerol in triacylglycerol and phospholipid synthesis.

2. *Needed supplies*. How is the glycerol 3-phosphate required for phosphatidate synthesis generated?

3. *Making fat.* Write a balanced equation for the synthesis of a triacylglycerol, starting from glycerol and fatty acids.

4. *Making a phospholipid.* Write a balanced equation for the synthesis of phosphatidylethanolamine by the de novo pathway, starting from ethanolamine, glycerol, and fatty acids.

5. *ATP needs.* How many high-phosphoryl-transfer-potential molecules are required to synthesize phosphatidylethanolamine from ethanolamine and diacylglycerol? Assume that the ethanolamine is the activated component.

6. *Identifying differences*. Differentiate among sphingomyelin, a cerebroside, and a ganglioside.

7. *Let's count the ways.* There may be 50 ways to leave your lover, but, in principle, there are only three ways to make a glycerol-based phospholipid. Describe the three pathways.

8. *Activated donors.* What is the activated reactant in each of the following biosyntheses?

(a) Phosphatidylinositol from inositol
(b) Phosphatidylethanolamine from ethanolamine
(c) Ceramide from sphingosine
(d) Sphingomyelin from ceramide
(e) Cerebroside from ceramide
(f) Ganglioside G_{M1} from ganglioside G_{M2}
(g) Farnesyl pyrophosphate from geranyl pyrophosphate

9. *No DAG, no TAG*. What would be the effect of a mutation that decreased the activity of phosphatidic acid phosphatase?

10. *The Law of Three Stages*. What are the three stages required for the synthesis of cholesterol?

11. *Many regulations to follow.* Outline the mechanisms of the regulation of cholesterol biosynthesis.

12. *Telltale labels.* What is the distribution of isotopic labeling in cholesterol synthesized from each of the following precursors?

(a) Mevalonate labeled with ^{14}C in its carboxyl carbon atom
(b) Malonyl CoA labeled with ^{14}C in its carboxyl carbon atom

13. *Too much, too soon*. What is familial hypercholesterolemia and what are its causes?

14. *Familial hypercholesterolemia.* Several classes of LDL-receptor mutations have been identified as causes of this disease. Suppose that you have been given cells from patients with different mutations, an antibody specific for the LDL receptor that can be seen with an electron microscope, and access to an electron microscope. What differences in antibody distribution might you expect to find in the cells from different patients?

15. *Breakfast conversation*. You and a friend are eating breakfast together. While eating, your friend is reading the back of her cereal box and comes across the following statement: "Cholesterol plays beneficial roles in your body, making cells, hormones, and tissues." Knowing that you are taking biochemistry, she asks if the statement makes sense. What do you reply?

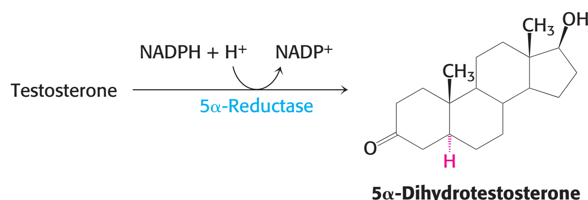
16. *A good thing*. What are statins? What is their pharmacological function?

17. *Too much of a good thing*. Would the development of a "super statin" that inhibited all HMG CoA reductase activity be a useful drug? Explain.

18. *RNA editing.* A shortened version (apo B-48) of apolipoprotein B is formed by the intestine, whereas the full-length protein (apo B-100) is synthesized by the liver. A glutamine codon (CAA) is changed into a stop codon. Propose a simple mechanism for this change.

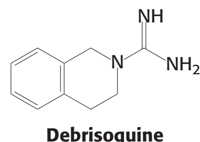
19. *A means of entry*. Describe the process of receptor-mediated endocytosis by using LDL as an example.

20. *Inspiration for drug design.* Some actions of androgens are mediated by dihydrotestosterone, which is formed by the reduction of testosterone. This finishing touch is catalyzed by an NADPH-dependent 5α-reductase.



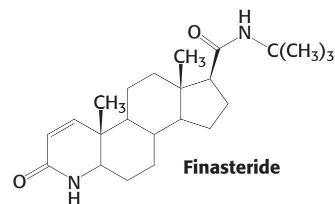
Chromosomal XY males with a genetic deficiency of this reductase are born with a male internal urogenital tract but predominantly female external genitalia. These people are usually reared as girls. At puberty, they masculinize because the testosterone level rises. The testes of these reductase-deficient men are normal, whereas their prostate glands remain small. How might this information be used to design a drug to treat *benign prostatic hypertrophy,* a common consequence of the normal aging process in men? A majority of men older than age 55 have some degree of prostatic enlargement, which often leads to urinary obstruction.

21. *Drug idiosyncrasies.* Debrisoquine, a β-adrenergic blocking agent, has been used to treat hypertension. The optimal dose varies greatly (20–400 mg daily) in a population of patients. The urine of most patients taking the drug contains a high level of 4-hydroxydebrisoquine. However, those most sensitive to the drug (about 8% of the group studied) excrete debrisoquine and very little of the 4-hydroxy derivative. Propose a molecular basis for this drug idiosyncrasy. Why should caution be exercised in giving other drugs to patients who are very sensitive to debrisoquine?



22. *Removal of odorants.* Many odorant molecules are highly hydrophobic and concentrate within the olfactory epithelium. They would give a persistent signal independent of their concentration in the environment if they were not rapidly modified. Propose a mechanism for converting hydrophobic odorants into water-soluble derivatives that can be rapidly eliminated.

23. *Development difficulties.* Propecia (finasteride) is a synthetic steroid that functions as a competitive and specific inhibitor of 5α-reductase, the enzyme responsible for the synthesis of dihydrotestosterone from testosterone.



It is now widely used to retard the development of male pattern hair loss. Pregnant women are advised to avoid handling this drug. Why is it vitally important that pregnant women avoid contact with Propecia?

24. *Life-style consequences.* Human beings and the plant *Arabidopsis* evolved from the same distant ancestor possessing a small number of cytochrome P450 genes. Human beings have approximately 50 such genes, whereas *Arabidopsis* has more than 250 of them. Propose a role for the large number of P450 isozymes in plants.

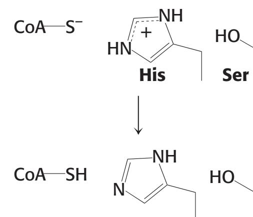
25. *Personalized medicine.* The cytochrome P450 system metabolizes many medicinally useful drugs. Although all human beings have the same number of P450 genes, individual polymorphisms exist that alter the specificity and efficiency of the proteins encoded by the genes. How could knowledge of individual polymorphisms be useful clinically?

26. *Honey-bee crisis*. In 2006, there was a sudden, unexplained die-off of honey-bee colonies throughout the United States. The die-off was economically significant because one-third of the human diet comes from insect-pollinated plants, and honey bees are responsible for 80% of the pollination. In October of 2006, the sequence of the honey bee genome was reported. Interestingly, the genome was found to contain far fewer cytochrome P450 genes than do the genomes of other insects. Suggest how the die-off and the paucity of P450 genes may be related.

27. *Let the sun shine in.* At a biochemical level, vitamin D functions like a steroid hormone (see Chapter 31). Therefore, it is sometimes referred to as an honorary steroid. Why is vitamin D not an actual steroid?

Mechanism Problems

28. *An interfering phosphate.* In the course of the overall reaction catalyzed by HMG-CoA reductase, a histidine residue protonates a coenzyme A thiolate, CoA–S^-, generated in an earlier step.



A nearby serine residue can be phosphorylated by AMP-dependent kinase, which results in a loss of activity. Propose an explanation for why phosphorylation of the serine residue inhibits enzyme activity.

29. *Demethylation.* Methyl amines are often demethylated by cytochrome P450 enzymes. Propose a mechanism for the formation of methylamine from dimethylamine catalyzed by cytochrome P450. What is the other product?

Chapter Integration Problems

30. *Similarities*. Compare the role of CTP in phosphoglyceride synthesis with the role of UTP in glycogen synthesis.

31. *Hold on tight or you might be thrown to the cytoplasm.* Many proteins are modified by the covalent attachment of a farnesyl (C_{15}) or a geranylgeranyl (C_{20}) unit to the carboxyl-terminal cysteine residue of the protein. Suggest why this modification might occur.

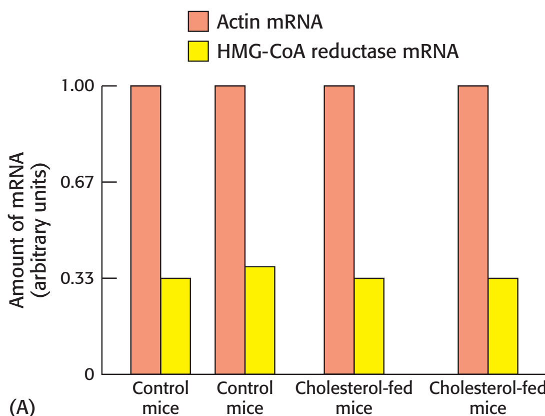
32. *Fork in the road.* 3-Hydroxy-3-methylglutaryl CoA is on the pathway for cholesterol biosynthesis. It is also a component of another pathway. Name the pathway. What determines which pathway 3-hydroxy-3-methylglutaryl CoA follows?

33. *Requires a club membership*. How is methionine metabolism related to the synthesis of phosphatidylcholine?

34. *ATP requirements*. Explain how cholesterol synthesis depends on the activity of ATP-citrate lyase.

Data Interpretation and Chapter Integration Problem

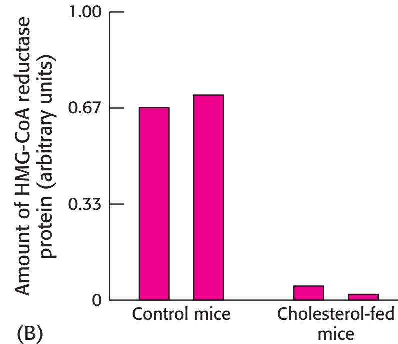
35. *Cholesterol feeding.* Mice were divided into four groups, two of which were fed a normal diet and two of which were fed a cholesterol-rich diet. HMG-CoA reductase mRNA and protein from liver were then isolated and quantified. Graph A shows the results of the mRNA isolation.



(a) What is the effect of cholesterol feeding on the amount of HMG-CoA reductase mRNA?

(b) What is the purpose of also isolating the mRNA for the protein actin, which is not under the control of the sterol regulatory element?

HMG-CoA reductase protein was isolated by precipitation with a monoclonal antibody to HMG-CoA reductase. The amount of HMG-CoA protein in each group is shown in graph B.



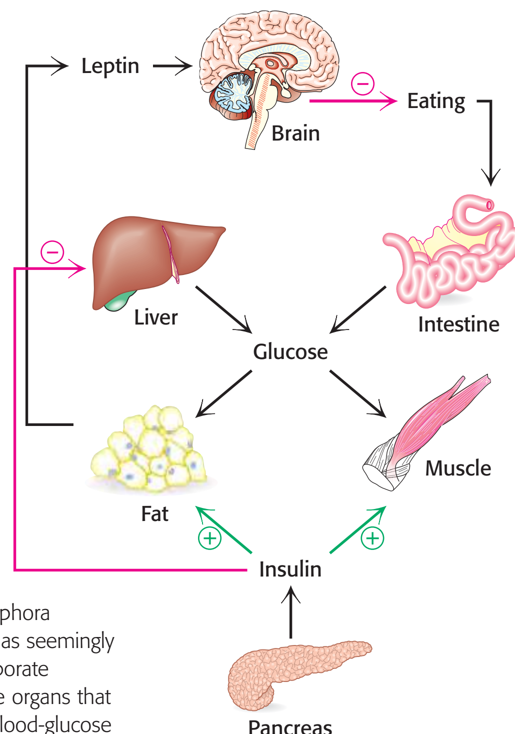
(c) What is the effect of the cholesterol diet on the amount of HMG-CoA reductase protein?

(d) Why is this result surprising in light of the results in graph A?

(e) Suggest possible explanations for the results in graph B.

CHAPTER 27

The Integration of Metabolism



The image at the left shows a detail of runners on a Greek amphora painted in the sixth century B.C. Athletic feats, as well as others as seemingly simple as the maintenance of blood-glucose levels, require elaborate metabolic integration. The representation at the right shows the organs that have essential roles in the metabolic integration that regulates blood-glucose levels during exercise and at rest. Insulin and leptin (secreted by adipocytes) are two of the hormones that modulate the metabolic pathways of organs throughout the body such that adequate energy is available to meet the demands of living. [(Left) Copyright © The Metropolitan Museum of Art/Art Resource, NY.]

OUTLINE

27.1 Caloric Homeostasis Is a Means of Regulating Body Weight

27.2 The Brain Plays a Key Role in Caloric Homeostasis

27.3 Diabetes Is a Common Metabolic Disease Often Resulting from Obesity

27.4 Exercise Beneficially Alters the Biochemistry of Cells

27.5 Food Intake and Starvation Induce Metabolic Changes

27.6 Ethanol Alters Energy Metabolism in the Liver

We have been examining the biochemistry of metabolism one pathway at a time. We have seen how useful energy is extracted from fuels and used to power biosynthetic reactions and signal-transduction pathways. In the Chapters 28 through 30, we will extend our study of biosynthetic reactions to the synthesis of proteins and nucleic acids. Before we do that, however, in this chapter we will take a step back to examine large-scale biochemical interactions that constitute the physiology of the organisms. In keeping with a central theme of life—energy manipulations—we will look at the regulation of energy at the organismal level, which boils down to an apparently simple but actually quite complex question: At the biochemical level, how does an organism know when to eat and when to refrain from eating? The ability to maintain adequate but not excessive energy stores is called *caloric homeostasis* or *energy homeostasis*.

Next, we will examine a significant perturbation of caloric homeostasis—obesity—and how this physiological condition affects insulin action, frequently resulting in diabetes. We then turn our attention to a biochemical examination of one of the most beneficial activities that humans can engage in—exercise—and see how exercise mitigates the effects of diabetes as well as how different forms of exercise use different sources of fuel.

At the opposite end of the physiological spectrum from obesity and overnutrition are fasting and starvation, and we will examine the biochemical responses to these challenges. The chapter ends with a consideration of another biochemical energy perturbation—excess alcohol consumption.

We have already encountered instances of organismal energy regulation when we considered the actions of insulin and glucagon. Recall that insulin, secreted by the β cells of the pancreas, causes glucose to be removed from the blood and stimulates the synthesis of glycogen and lipids. Glucagon, secreted by the α cells of the pancreas, has effects opposite those of insulin. Glucagon increases the level of blood glucose by stimulating glycogen breakdown and gluconeogenesis. This chapter introduces two hormones that play key roles in caloric homeostasis. Leptin and adiponectin, secreted by the adipose tissue, work in concert with insulin to regulate caloric homeostasis.

27.1 Caloric Homeostasis Is a Means of Regulating Body Weight

By now in our study of biochemistry, we are well aware of the fact that many biochemicals, most notably carbohydrates and lipids, are potential sources of energy. We consume these energy sources as foods, convert the energy into ATP, and use the ATP to power our lives. Like all energy transformations, our energy consumption and expenditure are governed by the laws of thermodynamics. Recall that the Second Law of Thermodynamics states that energy can neither be created nor destroyed. Translated into the practical terms of our diets,

$$\text{Energy consumed} = \text{energy expended} + \text{energy stored}$$

This simple equation has severe physiological and health implications: according to the Second Law of Thermodynamics, if we consume more energy than we expend, we will become overweight or obese. Obesity is generally defined as a body mass index (BMI) of more than 30 kg m^{-2}, whereas overweight is defined as a BMI of more than 25 kg m^{-2} (Figure 27.1). Recall that excess fat is stored in adipocytes as triacylglycerols. The number of adipocytes becomes fixed in adults, and so obesity results in engorged adipocytes. Indeed, the cell may increase as much as 1000-fold in size.

We are all aware that many of us, especially in the developed world, are becoming obese or have already attained that state. In the United States, obesity has become an epidemic, with nearly 30% of adults classified as such. Obesity is identified as a risk factor in a host of pathological conditions including diabetes mellitus, hypertension, and cardiovascular disease (Table 27.1). The cause of obesity is quite simple in most cases: more food is consumed than is needed, and the excess calories are stored as fat. We will consider the biochemical basis of pathologies caused by obesity later in the chapter.

Before we undertake a biochemical analysis of the results of overconsumption, let us consider why the obesity epidemic is occurring in the first place. There are two complementary explanations. The first is a commonly held view that our bodies are programmed to rapidly store excess calories in times of plenty, an evolutionary adaptation from times past when humans

Height in feet and inches (in cm)

Weight in pounds (in kg)	4′8″ (142)	4′10″ (147)	5′0″ (152)	5′2″ (157)	5′4″ (163)	5′6″ (168)	5′8″ (173)	5′10″ (178)	6′0″ (183)	6′2″ (188)	6′4″ (193)
260 (117.9)	58	54	51	48	45	42	40	37	35	33	32
250 (113.4)	56	52	49	46	43	40	38	36	34	32	30
240 (108.9)	54	50	47	44	41	39	36	34	33	31	29
230 (104.3)	52	48	45	42	39	37	35	33	31	30	28
220 (99.8)	49	46	43	40	38	36	33	32	30	28	27
210 (95.3)	47	44	41	38	36	34	32	30	28	27	26
200 (90.7)	45	42	39	37	34	32	30	29	27	26	24
190 (86.2)	43	40	37	35	33	31	29	27	26	24	23
180 (81.6)	40	38	35	33	31	29	27	26	24	23	22
170 (77.1)	38	36	33	31	29	27	26	24	23	22	21
160 (72.6)	36	33	31	29	27	26	24	23	22	21	19
150 (68.0)	34	31	29	27	26	24	23	22	20	19	18
140 (63.5)	31	29	27	26	24	23	21	20	19	18	17
130 (59.0)	29	27	25	24	22	21	20	19	18	17	16
120 (54.4)	27	25	23	22	21	19	18	17	16	15	15
110 (49.9)	25	23	21	20	19	18	17	16	15	14	13
100 (45.4)	22	21	20	18	17	16	15	14	14	13	12
90 (40.8)	20	19	18	16	15	15	14	13	12	12	11
80 (36.3)	18	17	16	15	14	13	12	11	11	10	10

BMI	Category
>30	Obese
25–30	Overweight
18.5–25	Normal
<18.5	Underweight

$$\text{BMI} = \frac{\text{weight}}{\text{height}^2}$$

Figure 27.1 Body mass index (BMI). The BMI value for an individual person is a reliable indicator of obesity for most people. [Data from the Centers for Disease Control.]

were not assured of having ample food, as many of us are today. Consequently, we store calories as if a fast might begin tomorrow, but no such fast arrives. The second possible explanation is that we no longer face the risks of predation. Evidence indicates that predation was a common cause of death for our ancestors. An obese individual would more likely have been culled from a group of our ancestors than would a more nimble, lean individual. As the risk of predation declined, leanness became less beneficial. Regardless of why we may have a propensity to gain weight, this propensity can be negated behaviorally—by eating less and exercising more. However, genetic studies indicate that the tendency toward obesity may be highly heritable.

As disturbing as the obesity epidemic is, an equally intriguing, almost amazing observation is that many people are able to maintain an approximately constant weight throughout adult life. A few simple calculations of a simplified situation illustrates how remarkable this feat is. Consider a 120-pound woman whose weight does not change significantly between

Table 27.1 Health consequences of obesity or being overweight

Coronary heart disease
Type 2 diabetes
Cancers (endometrial, breast, and colon)
Hypertension (high blood pressure)
Dyslipidemia (disruption of lipid metabolism, e.g., high cholesterol and triglycerides)
Stroke
Liver and gallbladder disease
Sleep apnea and respiratory problems
Osteoarthritis (degeneration of cartilage and underlying bone at a joint)
Gynecological problems (abnormal menses, infertility)

Source: Centers for Disease Control and Prevention Web site (www.cdc.gov).

the ages of 25 and 65. Let us say that the woman requires 2000 kcal day^{-1}. Over the 40 years under consideration, she will have consumed

$$40 \text{ years} \times 365 \text{ days year}^{-1} \times 2000 \text{ kcal day}^{-1} = 2.9 \times 10^7 \text{ kcals in 40 years}$$

For simplicity's sake, let us assume that the woman's diet consists predominantly of fatty acids derived from lipids. The energy density of fatty acids is 9 kcal g^{-1}. Thus, over the 40-year span, our subject has ingested

$$2.9 \times 10^7 \text{ kcal}/9 \text{ kcal g}^{-1} = 3.2 \times 10^6 \text{ g} = 3200 \text{ kg of food}$$

which is equivalent to more than 6 tons of food! Yet, remarkably, her weight has remained constant without her having to accurately and constantly calibrate and equalize her energy intake and energy output. Although will power, exercise, and a bathroom scale often play a role in this homeostasis, some biochemical signaling must be taking place to help with her energy regulation. Indeed, it is the case; but, before we consider this regulation, let us be cruel, but not too cruel, to our hypothetical subject and say that her weight increased 10% over the 40-year span, a percentage weight gain that most 65-year-olds would delightfully accept. Thus, at 65, she weighs 132 pounds. On a daily basis, what increase in energy intake—food consumption—would have resulted in a 12-pound weight gain over 40 years? Again, let us consider only fats.

$$12 \text{ pounds} = 5.4 \text{ kg} = 5.4 \times 10^3 \text{ g} = \text{total weight gain}$$

$$5.4 \times 10^3 \text{ g } (40 \text{ years } 365 \text{ days year}^{-1})^{-1} = 0.37 \text{ g day}^{-1}$$

Thus, to gain 12 pounds in 40 years, our subject needed to eat only 0.37 g of food per day more than she needed to meet her biochemical needs. How much food is it? Consider butter, which is essentially pure fat; 0.38 g of butter is approximately one-quarter of a pat. So, the simple excess of the equivalent of one-quarter pat of butter per day will lead to a (modest) weight gain of 12 pounds over 40 years—a startling if depressing fact. However, even just a small sampling of your friends and family shows us that this hypothetical weight gain does not apply to everyone. People vary significantly in the way in which their bodies are able to regulate energy intake; an extra pat of butter a day may not make a difference in the long run to one person, whereas another person may end up with much more than a 10% weight gain over 40 years. All things being equal, Problem 33 illustrates how little excess consumption is required to become obese over the 40-year time period.

27.2 The Brain Plays a Key Role in Caloric Homeostasis

What makes this remarkable balance of energy input and output possible? As you might imagine, the answer is complicated, entailing many biochemical signals as well as a host of behavioral factors. We will focus on a few key biochemical signals, and divide our discussion into two parts: short-term signals that are active during a meal and long-term signals that report on the overall energy status of the body. These signals originate in the gastrointestinal tract, the β cells of the pancreas, and fat cells. The primary target of these signals is the brain, in particular a groups of neurons in a region of the hypothalamus called the arcuate nucleus.

Signals from the gastrointestinal tract induce feelings of satiety

Short-term signals relay feelings of satiety from the gut to various regions of the brain and thus reduce the urge to eat (Figure 27.2). The best-studied short-term signal is cholecystokinin (CCK). *Cholecystokinin* is actually a

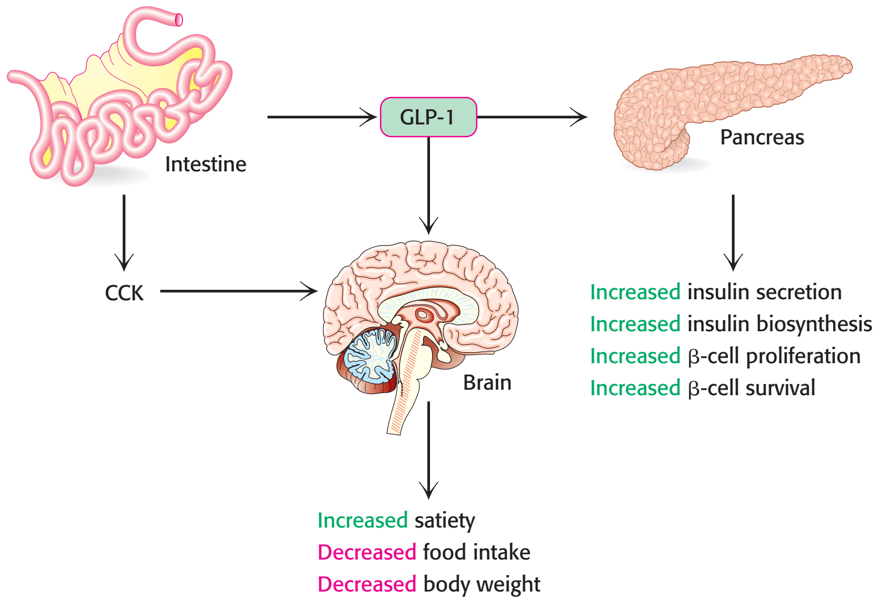


Figure 27.2 Satiation signals. Cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1) are signal molecules that induce feelings of satiety in the brain. CCK is secreted by specialized cells of the small intestine in response to a meal and activates satiation pathways in the brain. GLP-1, secreted by L cells in the intestine, also activates satiation pathways in the brain and potentiates insulin action in the pancreas. [After S. C. Wood. *Cell Metab.* 9:489–498, 2009, Fig. 1.]

family of peptide hormones of various lengths (from 8 to 58 amino acids in length, depending on posttranslational processing) secreted into the blood by cells in the duodenum and jejunum regions of the small intestine as a postprandial satiation signal. The CCK binds to the CCK receptor, a G-protein-coupled receptor (p. 406) located in various peripheral neurons that relay signals to the brain. This binding initiates a signal-transduction pathway in the brain that generates a feeling of satiety. CCK also plays an important role in digestion, stimulating the secretion of pancreatic enzymes and bile salts from the gallbladder.

Another important gut signal is *glucagon-like peptide 1* (GLP-1), a hormone of approximately 30 amino acids in length. GLP-1 is secreted by intestinal L cells, hormone-secreting cells located throughout the lining of the gastrointestinal tract, and has a variety of effects, all apparently facilitated by binding to a GLP-1 receptor, another G-protein-coupled receptor. Like CCK, GLP-1 induces feelings of satiety that inhibit further eating. GLP-1 also potentiates glucose-induced insulin secretion by the β cells of the pancreas while inhibiting glucagon secretion. Although we have examined only two short-term signals, many others are believed to exist (Table 27.2). Most of the short-term signals thus far identified are appetite suppressants. Ghrelin, a peptide that is 28 amino acids in length and secreted by the stomach, acts on regions of the hypothalamus to stimulate appetite through its receptor, a G-protein-coupled receptor. Ghrelin secretion increases before a meal and decreases afterward.

Table 27.2 Gastrointestinal peptides that regulate food intake

Appetite-suppressing signals
Cholecystokinin
Glucagon-like peptide 1
Glucagon-like peptide 2
Amylin
Somatostatin
Bombesin
Enterostatin
Apolipoprotein A-IV
Gastric inhibitory peptide
Appetite-enhancing peptides
Ghrelin

Source: After M. H. Stipanuk, Ed., *Biochemical, Physiological, Molecular Aspects of Human Nutrition,* 2d ed. (Saunders/Elsevier, 2006), p. 627, Box 22-1.

Leptin and insulin regulate long-term control over caloric homeostasis

Two key signal molecules regulate energy homeostasis over the time scale of hours or days: *leptin,* which is secreted by the adipocytes, and *insulin,* which is secreted by the β cells of the pancreas. Leptin reports on the status of the triacylglycerol stores, whereas insulin reports on the status of glucose in the blood—in other words, of carbohydrate availability. We will consider leptin first.

Adipose tissue was formerly considered an inert depot of triacylglycerols. However, recent work has shown that adipose tissue is an active endocrine tissue, secreting signal molecules called *adipokines*, such as leptin, that regulate a host of physiological processes. Leptin is secreted by the adipocytes in direct proportion to the amount of fat present. The more fat

in a body, the more leptin is secreted. Leptin binding to its receptor throughout the body increases the sensitivity of muscle and the liver to insulin, stimulates β oxidation of fatty acids, and decreases triacylglycerol synthesis.

Let us consider the effects of leptin in the brain. Leptin exerts its effect by binding to membrane receptors in various regions of the brain, particularly in the arcuate nucleus of the hypothalamus. There, one population of neurons expresses appetite-stimulating (orexigenic) peptides, called neuropeptide Y (NPY) and agouti-related peptide (AgRP). Leptin inhibits the NPY/AgRP neurons, preventing the release of NPY and AgRP and thus repressing the desire to eat. Fasting, on the other hand, stimulates the production of NPY and AgRP (Figure 27.3) owing to the decrease in leptin levels that results from diminishing adipose tissue.

The second population of neurons containing leptin receptors expresses a large precursor polypeptide, proopiomelanocortin (POMC). In response to leptin binding to its receptor on POMC neurons, POMC is proteolytically processed to yield a variety of signal molecules, one of which, *melanocyte-stimulating hormone* (MSH), is especially important in this context. MSH, originally discovered as a stimulator of melanocytes (cells that synthesize the pigment melanin), activates appetite-suppressing (anorexigenic) neurons and thus inhibits food consumption. Fasting inhibits MSH activity and thus stimulates eating. AgRP inhibits MSH activity by acting as an antagonist, binding to the MSH receptor but failing to activate the receptor (see Figure 27.3). Thus, the net effect of leptin binding to its receptor is the initiation of a complex signal-transduction pathway that ultimately curtails food intake.

Insulin receptors are also present in the hypothalamus, although the mechanism of insulin action in the brain is less clear than that of leptin. Insulin appears to inhibit NPY/AgRP-producing neurons, thus inhibiting food consumption.

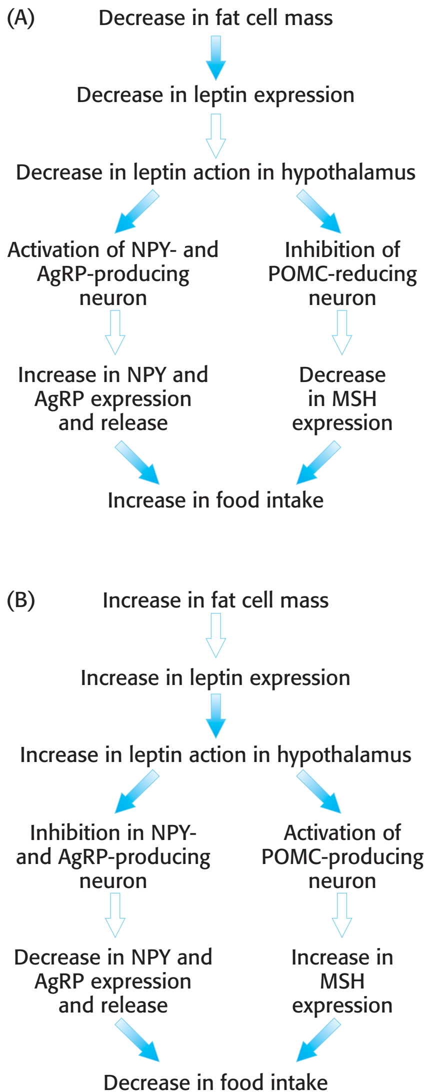


Figure 27.3 The effects of leptin in the brain. Leptin is an adipokine secreted by adipose tissue in direct relation to fat mass. (A) When leptin levels fall, as in fasting, appetite-enhancing neuropeptides NPY and AgRP are secreted, whereas the secretion of appetite-suppressing signals such as MSH is inhibited. (B) When fat mass increases, leptin inhibits NPY and AgRP secretion while stimulating the release of appetite-suppressing hormone MSH. [After M. H. Stipanuk, *Biochemical, Physiological, & Molecular Aspects of Human Nutrition,* 2d ed. (Saunders-Elsevier, 2006), Fig. 22-2.]

Leptin is one of several hormones secreted by adipose tissue

Leptin was the first adipokine discovered because of the dramatic effects of its absence. Researchers discovered a strain of mice called ob/ob mice, which lack leptin and, as a result, are extremely obese. These mice display hyperphagia (overeating), hyperlipidemia (accumulation of triacylglycerides in muscle and liver), and an insensitivity to insulin. Since the discovery of leptin, other adipokines have been detected. For instance, *adiponectin* is another signal molecule produced by the adipocytes that acts in a similar fashion to leptin. Both leptin and adiponectin exert their effects through the key regulatory enzyme, AMP-dependent protein kinase (AMPK). Recall that this enzyme is active when AMP levels are elevated and ATP levels are diminished, and this activation leads to a decrease in anabolism and an increase in catabolism, most notably an increase in fatty acid oxidation. In insulin-resistant obese animals such as the ob/ob mice, leptin levels increase while those of adiponectin decrease.

Adipocytes also produce two hormones, *RBP4* and *resistin*, that promote insulin resistance. Although it is unclear why adipocytes secrete hormones that facilitate insulin resistance, a pathological condition, we can speculate on the answer. These signal molecules may help to fine-tune the actions of leptin and adiponectin or perhaps to act as "brakes" on the action of leptin and adiponectin to prevent hypoglycemia in the fasted state. Some evidence indicates that enlarged adipocytes that result from obesity may secrete higher levels of insulin-antagonizing hormones and thus contribute to insulin resistance.

Leptin resistance may be a contributing factor to obesity

If leptin is produced in proportion to body-fat mass and leptin inhibits eating, why do people become obese? Obese people, in most cases, have both functioning leptin and a high blood concentration of leptin. The failure to respond to the anorexigenic effects of leptin is called *leptin resistance*. What is the basis of leptin resistance?

As for most questions in the exciting area of energy homeostasis, the answer is not well worked out, but recent evidence suggests that a group of proteins called *suppressors of cytokine signaling* (SOCS) may take part. These proteins fine-tune some hormonal systems by inhibiting receptor action. SOCS proteins inhibit receptor signaling by a number of means (Figure 27.4). Consider, for example, the effect of SOCS proteins on the insulin receptor. Recall that insulin stimulates the autophosphorylation of tyrosine residues on the insulin receptor, initiating the insulin-signaling pathway (see Figure 27.5). SOCS proteins bind to phosphorylated tyrosine residues on receptors or other members of the signal-transduction pathway, thereby disrupting signal flow and thus altering the cell's biochemical activity. In other cases, the binding of SOCS proteins to components of the signal-transduction pathway may also enhance proteolytic degradation of these components by the proteasome. Evidence in support of a role for SOCS in leptin resistance comes from mice that have had SOCS selectively deleted from POMC-expressing neurons. These mice display an enhanced sensitivity to leptin and are resistant to weight gain even when fed a high-fat diet. The reason why the activity of SOCS proteins increases, leading to leptin resistance, remains to be determined.

Dieting is used to combat obesity

Given the obesity epidemic that we currently face and its associated disorders, much attention has been focused on determining the most effective

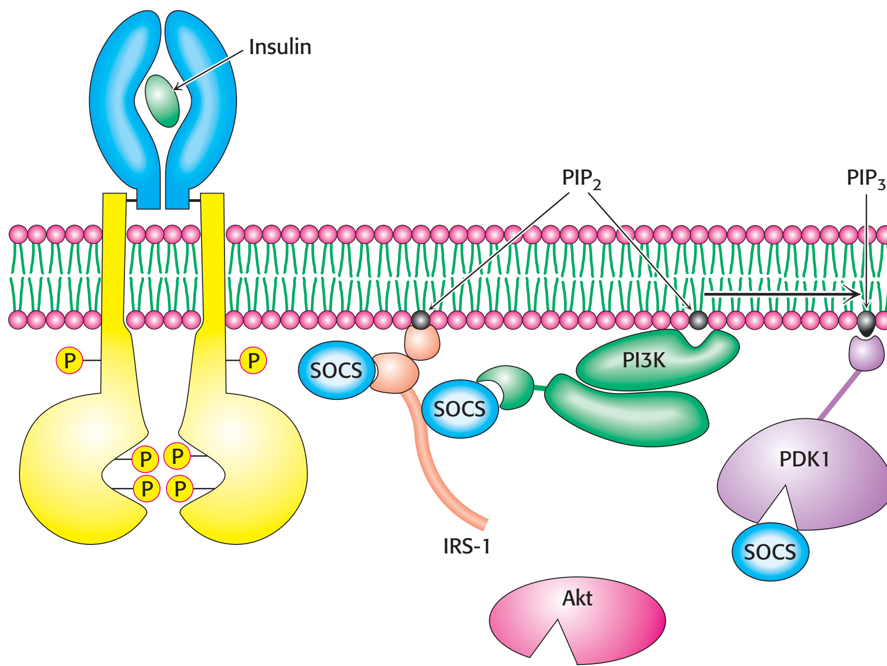


Figure 27.4 Suppressors of cytokine signaling (SOCS) regulate receptor function. SOCS proteins disrupt interactions of components of the insulin-signaling pathway and thereby inhibit the pathway. The binding of a signal component by SOCS results in proteasomal degradation in some cases. (Akt, a protein kinase; IRS-1, insulin-receptor substrate 1; PDK1, PIP_3-dependent protein kinase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; SOCS, suppressor of cytokine signaling.)

weight-loss diet. In general, two categories of diet try to help us control our caloric intake—low-carbohydrate diets and low-fat diets. Low-carbohydrate diets usually emphasize protein consumption. Although studies of the effects of diets on humans are immensely complex, data are beginning to accumulate suggesting that low-carbohydrate–high-protein diets may be the most effective for losing weight. The exact reasons are not clear, but there are two common hypotheses. First, proteins seem to induce a feeling of satiation more effectively than do fats or carbohydrates. Second, proteins require more energy to digest than do fats or carbohydrates, and the increased energy expenditure contributes to weight loss. For instance, recent studies show that a diet that is 30% protein requires almost 30% more energy to digest than that required by a diet that is 10% protein. The mechanisms by which protein-rich diets enhance energy expenditure and feelings of satiation remain to be determined. Regardless of the type of diet, the adage "Eat less, exercise more" always applies.

27.3 Diabetes Is a Common Metabolic Disease Often Resulting from Obesity

Having taken an overview of the regulation of body weight, we now examine the biochemical results when regulation fails because of behavior, genetics, or a combination of the two. The most common result of such a failure is obesity, a condition in which excess energy is stored as triacylglycerides. Recall that all excess food consumption is ultimately converted into triacylglycerides. Humans maintain about a day's worth of glycogen and, after these stores have been replenished, excess carbohydrates are converted into fats and then into triacylglycerols. Amino acids are not stored at all, and so excess amino acids are ultimately converted into fats also. Thus, regardless of the type of food consumed, excess consumption results in increased fat stores.

We begin our consideration of the effects of disruptions in caloric homeostasis with *diabetes mellitus,* a complex disease characterized by grossly abnormal fuel usage: *glucose is overproduced by the liver and underutilized by other organs*. The incidence of diabetes mellitus (usually referred to simply as *diabetes*) is about 5% of the population. Indeed, diabetes is the most common serious metabolic disease in the world; it affects hundreds of millions. *Type 1 diabetes* is caused by the autoimmune destruction of the insulin-secreting β cells in the pancreas and usually begins before age 20. Type 1 diabetes is also referred to as insulin-dependent diabetes, meaning that the affected person requires the administration of insulin to live. Most diabetics, in contrast, have a normal or even higher level of insulin in their blood, but they are quite unresponsive to the hormone, a characteristic called *insulin resistance*. This form of the disease, known as *type 2 diabetes*, typically arises later in life than does the insulin-dependent form. Type 2 diabetes accounts for approximately 90% of the diabetes cases throughout the world and is the most common metabolic disease in the world. In the United States, it is the leading cause of blindness, kidney failure, and amputation. *Obesity is a significant predisposing factor for the development of type 2 diabetes.*

Diabetes

Named for the excessive urination in the disease. Aretaeus, a Cappadocian physician of the second century A.D., wrote: "The epithet diabetes has been assigned to the disorder, being something like passing of water by a siphon." He perceptively characterized diabetes as "being a melting-down of the flesh and limbs into urine."

Mellitus

From Latin, meaning "sweetened with honey." Refers to the presence of sugar in the urine of patients having the disease. *Mellitus* distinguishes this disease from diabetes *insipidus,* which is caused by impaired renal reabsorption of water.

Insulin initiates a complex signal-transduction pathway in muscle

What is the biochemical basis of insulin resistance? How does insulin resistance lead to failure of the β cells of the pancreas that results in type 2 diabetes? How does obesity contribute to this progression? To answer these questions and begin to unravel the mysteries of metabolic disorders, let us

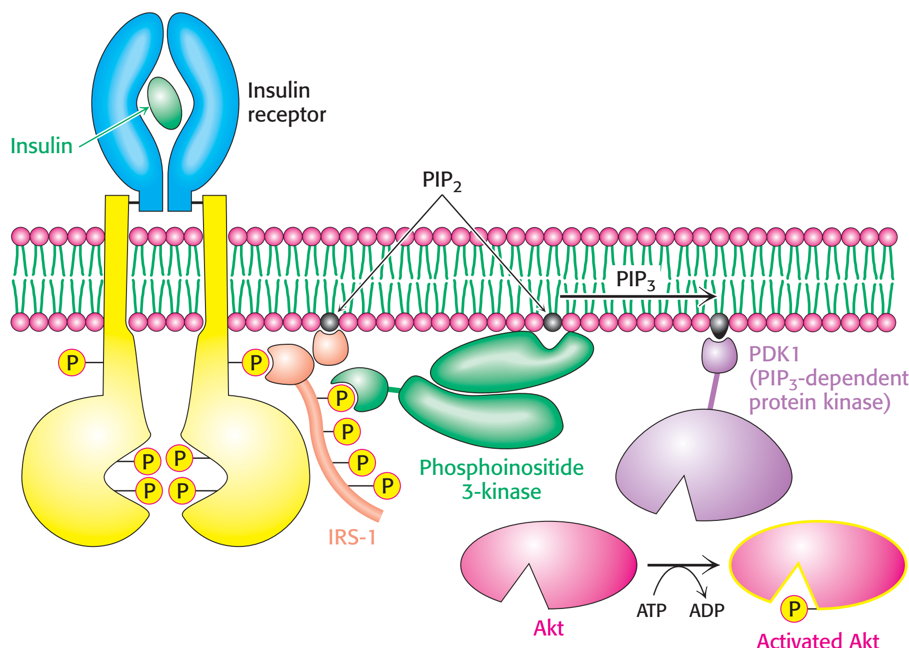
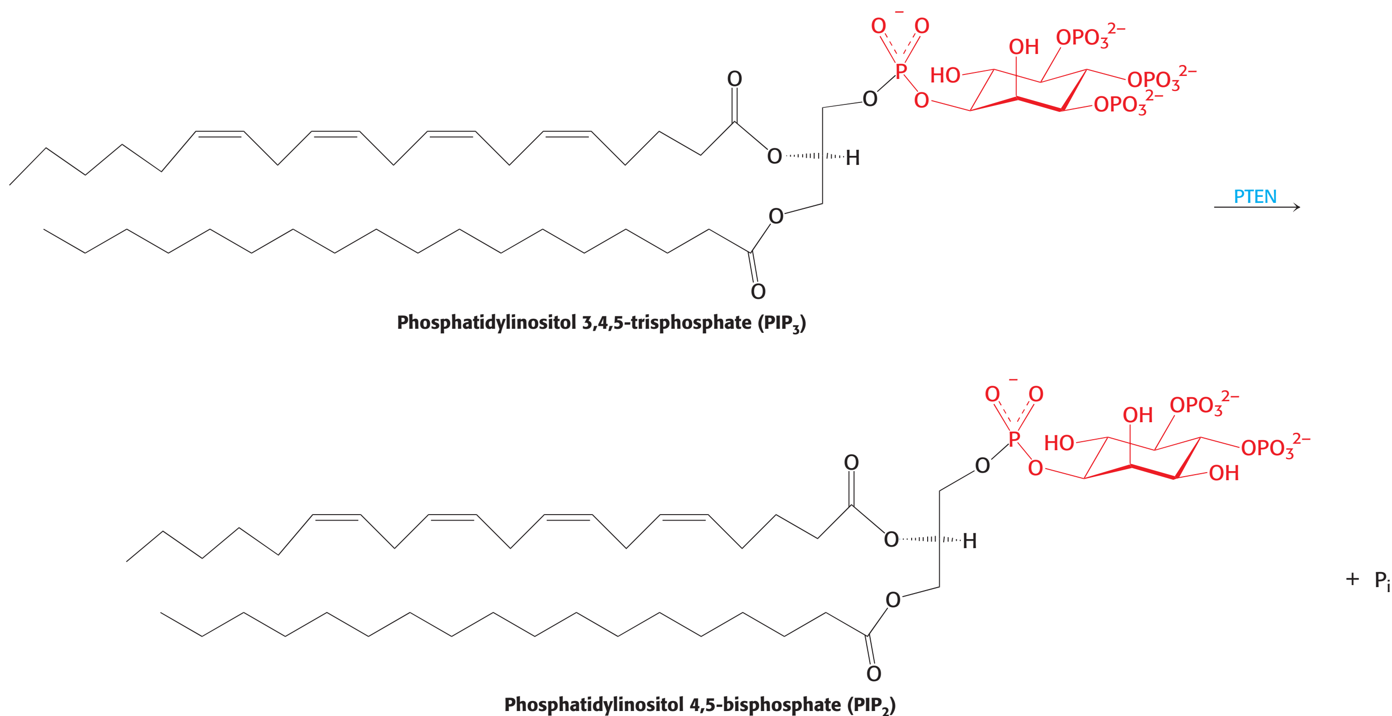


Figure 27.5 Insulin signaling. The binding of insulin results in the cross-phosphorylation and activation of the insulin receptor. Phosphorylated sites on the receptor act as binding sites for insulin-receptor substrates such as IRS-1. The lipid kinase phosphoinositide 3-kinase binds to phosphorylated sites on IRS-1 through its regulatory domain and then converts PIP_2 into PIP_3. Binding to PIP_3 activates PIP_3-dependent protein kinase, which phosphorylates and activates kinases such as Akt1. Activated Akt1 can then diffuse throughout the cell and continue the signal-transduction pathway.

examine the mechanism of action of insulin in muscle, the largest tissue regulated by insulin.

In a normal cell, insulin binds to a receptor, which dimerizes and autophosphorylates on tyrosine residues, with each subunit of the dimer phosphorylating its partner. Phosphorylation of the receptor generates binding sites for insulin-receptor substrates (IRSs), such as IRS-1 (Figure 27.5). Subsequent phosphorylation of IRS-1 by the tyrosine kinase activity of the insulin receptor engages the insulin-signaling pathway. Phosphorylated IRS-1 binds to phosphatidylinositol 3-kinase (PI3K) and activates it. PI3K catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) into phosphatidylinositol 3,4,5-trisphosphate (PIP_3), a second messenger. PIP_3 activates the phosphatidylinositol-dependent protein kinase (PDK), which in turn activates several other kinases, most notably protein kinase B (PKB), also known as Akt. Protein kinase Akt facilitates the translocation of GLUT4-containing vesicles to the cell membrane, which leads to a more robust absorption of glucose from the blood. Moreover, Akt phosphorylates and inhibits glycogen synthase kinase (GSK3). Recall that GSK3 inhibits glycogen synthase (p. 629). Thus, insulin also leads to the activation of glycogen synthase and enhances glycogen synthesis.

Like all signal pathways, the insulin-signaling cascade must be capable of being turned off. Three different processes contribute to the down-regulation of insulin signaling. First, phosphatases deactivate the insulin receptor and destroy a key second messenger. *Tyrosine phosphatase IB* removes phosphoryl groups from the receptor, thus inactivating it. The second messenger PIP_3 is inactivated by the phosphatase *PTEN* (phosphatase and tensin homolog), which dephosphorylates it, forming PIP_2, which itself has no second-messenger properties.



Second, the IRS protein can be inactivated by phosphorylation on serine residues by specific Ser/Thr kinases. These kinases are activated by overnutrition and other stress signals and may play a role in the development of insulin resistance. Finally, SOCS proteins, the regulatory proteins discussed earlier, interact with the insulin receptor and IRS-1 and apparently facilitate their proteolytic degradation by the proteasome complex.

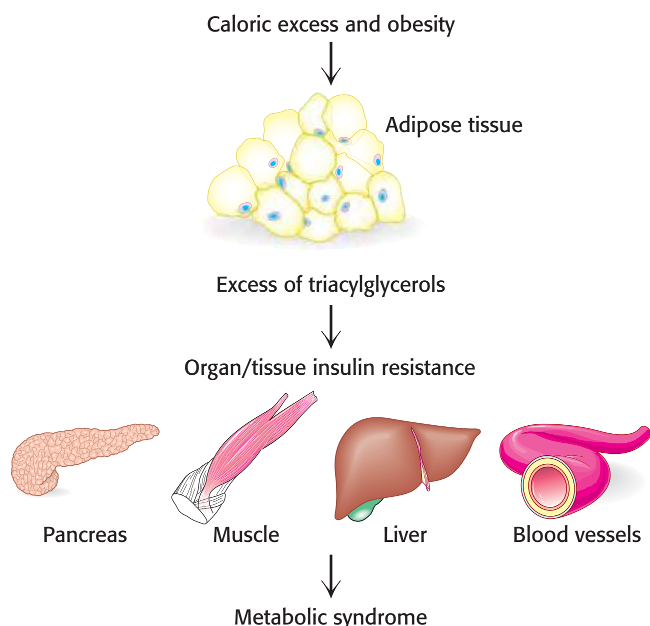


Figure 27.6 The storage capacity of fat tissue can be exceeded in obesity. In caloric excess, the storage capacity of adipocytes can be exceeded with deleterious results. The excess fat accumulates in other tissues, resulting in biochemical malfunction of the tissues. When the pancreas, muscle, liver, and cells lining the blood vessels are affected, metabolic syndrome, a condition that often precedes type 2 diabetes may result. [After S. Fröjdö, H. Vidal, and L. Pirola. *Biochim. Biophys. Acta* 1792:83–92, 2009, Fig. 1.]

Metabolic syndrome often precedes type 2 diabetes

With our knowledge of the key components of energy homeostasis, let us begin our investigation of the biochemical basis of insulin resistance and type 2 diabetes. Obesity is a contributing factor to the development of insulin resistance, which is an early development on the path to type 2 diabetes. Indeed, a cluster of pathologies—including insulin resistance, hyperglycemia, dyslipidemia (high blood levels of triacylglycerols, cholesterol, and low-density lipoproteins)—often develop together. This clustering, called *metabolic syndrome,* is thought to be a predecessor of type 2 diabetes.

A consequence of obesity is that the amount of triacylglycerides consumed exceeds the adipose tissue's storage capacity. As a result, other tissues begin to accumulate fat, most notably liver and muscle (Figure 27.6). For reasons to be presented later in the chapter, this accumulation results in insulin resistance and ultimately in pancreatic failure. We will focus on muscle and the β cells of the pancreas.

Excess fatty acids in muscle modify metabolism

We have seen many times the importance of fats as fuels for cells. In regard to obesity, more fats are present than can be processed by muscle. Although the

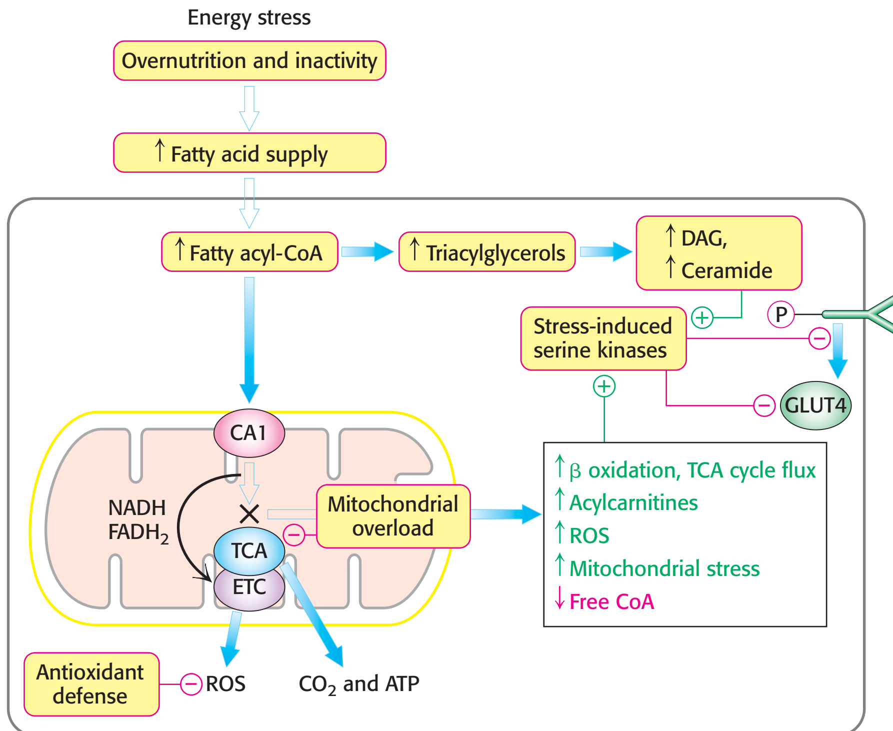


Figure 27.7 Excess fat in peripheral tissues can result in insulin insensitivity. Excess fat accumulation in peripheral tissues, most notably muscle, can disrupt some signal-transduction pathways and inappropriately activate others. In particular, diacylglycerides and ceramide activate stress-induced pathways that interfere with insulin signaling, resulting in insulin resistance. (Abbreviations: DAG, diacylglycerol; TGs, triacylglycerides; ROS, reactive oxygen species; CT1, carnitine acyltransferase 1; GLUT4, glucose transporter; ETC, electron-transport chain.)

rate of β oxidation increases in response to the high concentration of fats, mitochondria are not capable of processing all of the fatty acids by β oxidation; fatty acids accumulate in the mitochondria and eventually spill over into the cytoplasm. Indeed, the inability to process all of the fatty acids results in their reincorporation into triacylglycerols and the accumulation of fat in the cytoplasm. In the cytoplasm, levels of diacylglycerol and ceramide (a component of sphingolipids) also increase. Diacylglycerol is a second messenger that activates protein kinase C (PKC) (p. 409). When active, PKC and other Ser/Thr protein kinases are capable of phosphorylating IRS and reducing the ability of IRS to propagate the insulin signal. Ceramide or its metabolites inhibit glucose uptake and glycogen synthesis, apparently by inhibiting PDK and PKB (p. 799). The result is a diet-induced insulin resistance (Figure 27.7).

Insulin resistance in muscle facilitates pancreatic failure

What is the effect of overnutrition on the pancreas? This question is important because a primary function of the pancreas is to respond to the presence of glucose in the blood by secreting insulin, a process referred to as *glucose-stimulated insulin secretion* (GSIS). Indeed, the β cell is a virtual insulin factory. Proinsulin mRNA constitutes 20% of the total mRNA in the pancreas, whereas 50% of the total protein synthesizes in the pancreas as proinsulin, a precursor of insulin.

Glucose enters the β cells of the pancreas through the glucose transporter GLUT2. Recall that GLUT2 will allow glucose transport only when blood glucose is plentiful, ensuring that insulin is secreted only when glucose is abundant, such as after a meal. The β cell metabolizes glucose to

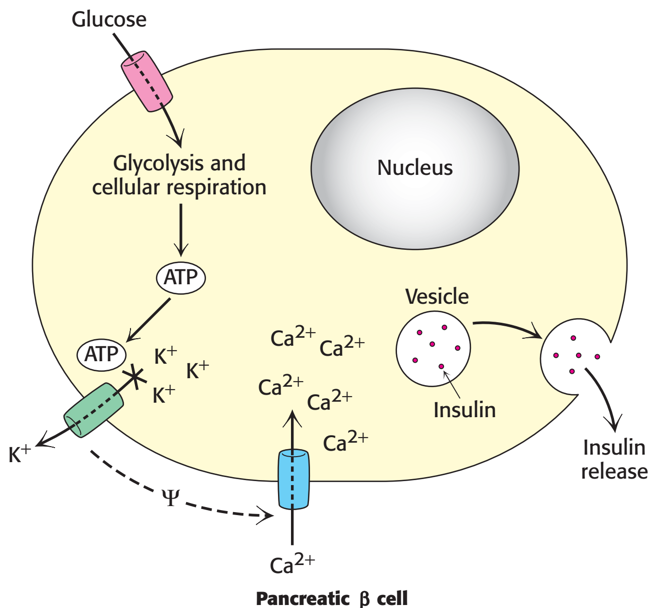


Figure 27.8 Insulin release is regulated by ATP. The metabolism of glucose by glycolysis and cellular respiration increases the concentration of ATP, which causes an ATP-sensitive potassium channel to close. The closure of this channel alters the charge across the membrane (Ψ) and causes a calcium channel to open. The influx of calcium causes insulin-containing granules to fuse with the plasma membrane, releasing insulin into the blood.

CO_2 and H_2O in the process of cellular respiration, generating ATP (Chapters 16, 17, and 18). The resulting increase in the ATP/ADP ratio closes an ATP-sensitive K^+ channel that, when open, allows potassium to flow out of the cell (Figure 27.8). The resulting alteration in the cellular ionic environment opens a Ca^{2+} channel. The influx of Ca^{2+} causes insulin-containing secretory vesicles to fuse with the cell membrane and release insulin into the blood. Thus, the increase in energy charge resulting from the metabolism of glucose has been translated by the membrane proteins into a physiological response—the secretion of insulin and the subsequent removal of glucose from the blood.

What aspect of β-cell function ultimately fails as a result of overnutrition, causing the transition from insulin resistance to full-fledged type 2 diabetes? Recall that, under normal circumstances, the β cells of the pancreas synthesize large amounts of proinsulin. The proinsulin folds in the endoplasmic reticulum, is processed to insulin, and is subsequently packaged into vesicles for secretion. As insulin resistance develops in the muscle, the β cells respond by synthesizing yet more insulin in a futile attempt do drive insulin action. The ability of the endoplasmic reticulum to process all of the proinsulin and insulin becomes compromised, a condition known as *endoplasmic reticulum* (ER) *stress*, and unfolded or misfolded proteins accumulate. ER stress initiates a signal pathway called the *unfolded protein response* (UPR), a pathway intended to save the cell. UPR consists of several steps. First, general protein synthesis is inhibited so as to prevent more proteins from entering the ER. Second, chaperone synthesis is stimulated. Recall that chaperones are proteins that assist the folding of other proteins. Third, misfolded proteins are removed from the ER and are subsequently delivered to the proteasome for destruction. Finally, if the described response fails to alleviate the ER stress, apoptosis is triggered, which ultimately leads to cell death and full-fledged type 2 diabetes.

What is the treatment for type 2 diabetes? Most are behavioral in nature. Diabetics are advised to count calories, making sure that energy intake does not exceed energy output; to consume a diet rich in vegetables, fruits, and grains; and to get plenty of aerobic exercise. Note that these guidelines are the same as those for healthy living, even for those not suffering from type 2 diabetes. Treatments specific for type 2 diabetes include the monitoring of blood-glucose levels so that these levels are within the target range (normal is 3.6 to 6.1 mM). For those who are not able to maintain proper glucose levels with the behaviors described herein, drug treatments are required. The administration of insulin may be necessary on pancreatic failure, and treatment with the use of metformin (Glucophage), which activates AMPK, may be effective.

Metabolic derangements in type 1 diabetes result from insulin insufficiency and glucagon excess

We now turn to the more-straightforward type 1 diabetes. In type 1 diabetes, insulin production is insufficient because of autoimmune destruction of the β cells of the pancreas. Consequently, the glucagon/insulin ratio is at higher-than-normal levels. In essence, the diabetic person is in biochemical fasting mode despite a high concentration of blood glucose. Because insulin is deficient, *the entry of glucose into adipose and muscle cells is impaired*. The

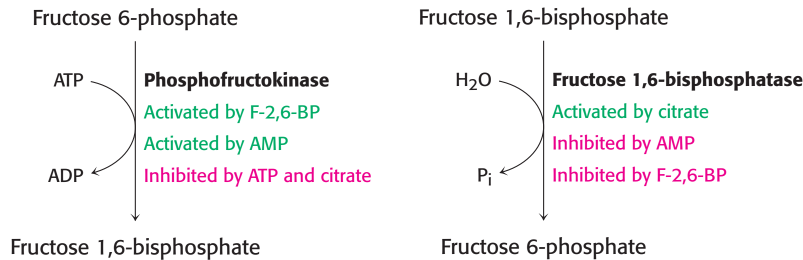


Figure 27.9 Regulation of glycolysis and gluconeogenesis. Phosphofructokinase is the key enzyme in the regulation of glycolysis, whereas fructose 1,6-bisphosphatase is the principal enzyme controlling the rate of gluconeogenesis. Note the reciprocal relation between the pathways and the signal molecules.

liver becomes stuck in a gluconeogenic and ketogenic state. The gluconeogenic state is characterized by excessive production of glucose. The excessive level of glucagon relative to that of insulin leads to a decrease in the amount of fructose 2,6-bisphosphate (F-2,6-BP), which stimulates glycolysis and inhibits gluconeogenesis in the liver. Hence, glycolysis is inhibited and gluconeogenesis is stimulated because of the opposite effects of F-2, 6-BP on phosphofructokinase and fructose-1,6-bisphosphatase (Section 16.4; Figure 27.9). Essentially, the cells' response to a lack of insulin amplifies the amount of glucose in the blood. The high glucagon/insulin ratio in diabetes also promotes glycogen breakdown. Hence, *an excessive amount of glucose is produced by the liver and released into the blood*. Glucose is excreted in the urine (hence the name *mellitus*) when its concentration in the blood exceeds the reabsorptive capacity of the renal tubules. Water accompanies the excreted glucose, and so an untreated diabetic in the acute phase of the disease is hungry and thirsty.

Because carbohydrate utilization is impaired, a lack of insulin leads to the uncontrolled breakdown of lipids and proteins, resulting in the ketogenic state. Large amounts of acetyl CoA are then produced by β oxidation. However, much of the acetyl CoA cannot enter the citric acid cycle, because there is insufficient oxaloacetate for the condensation step. Recall that mammals can synthesize oxaloacetate from pyruvate, a product of glycolysis, but not from acetyl CoA; instead, they generate ketone bodies. *A striking feature of diabetes is the shift in fuel usage from carbohydrates to fats; glucose, more abundant than ever, is spurned*. In high concentrations, ketone bodies overwhelm the kidney's capacity to maintain acid–base balance. The untreated diabetic can go into a coma because of a lowered blood-pH level and dehydration. Interestingly, diabetic ketosis is rarely a problem in type 2 diabetes because insulin is active enough to prevent excessive lipolysis in liver and adipose tissue.

What is the treatment for type 1 diabetes? Many of the behaviors applied to type 2 diabetes apply to type 1: watching calories, exercising, and eating a healthy diet. Likewise, blood-glucose levels must be monitored. Insulin treatments are required for survival.

27.4 Exercise Beneficially Alters the Biochemistry of Cells

Exercise, coupled with a healthy diet, is one of the most effective treatments for diabetes as well as a host of other pathological conditions including coronary disease, hypertension, depression, and a variety of cancers. With regard to diabetes, exercise increases the insulin sensitivity

of people who are insulin resistant or type 2 diabetics. What is the basis of this beneficial effect?

Mitochondrial biogenesis is stimulated by muscular activity

When muscle is stimulated to contract during exercise by receiving nerve impulses from motor neurons, calcium is released from the sarcoplasmic reticulum. Calcium induces muscle contraction, as will be discussed Chapter 35. Recall that calcium is also a potent second messenger and frequently works in association with the calcium-binding protein calmodulin (p. 410). In its capacity as a second messenger, calcium stimulates various calcium-dependent enzymes, such as calmodulin-dependent protein kinase. The calcium-dependent enzymes, as well as AMPK, subsequently activate particular transcription-factor complexes. As we will see in Chapters 29 and 31, transcription factors are proteins that control gene expression. Two patterns of gene expression, in particular, change in response to regular exercise (Figure 27.10). Regular exercise enhances the production of proteins required for fatty acid metabolism, such as the enzymes of β oxidation. Interestingly, fatty acids themselves function as signal molecules to activate the transcription of enzymes of fatty acid metabolism. Additionally, another set of transcription factors activated by the calcium signal cascade institutes metabolic reprogramming that leads to increased mitochondrial biogenesis. In concert, *the increase in fatty acid oxidizing capability and additional mitochondria allow for the efficient metabolism of fatty acids*. Because an excess of fatty acids results in insulin resistance, as already discussed, efficient metabolism of fatty acids results in *an increase in insulin sensitivity*. Indeed, muscles of well-trained athletes may contain high concentrations of triacylglycerides and still maintain exquisite sensitivity to insulin.

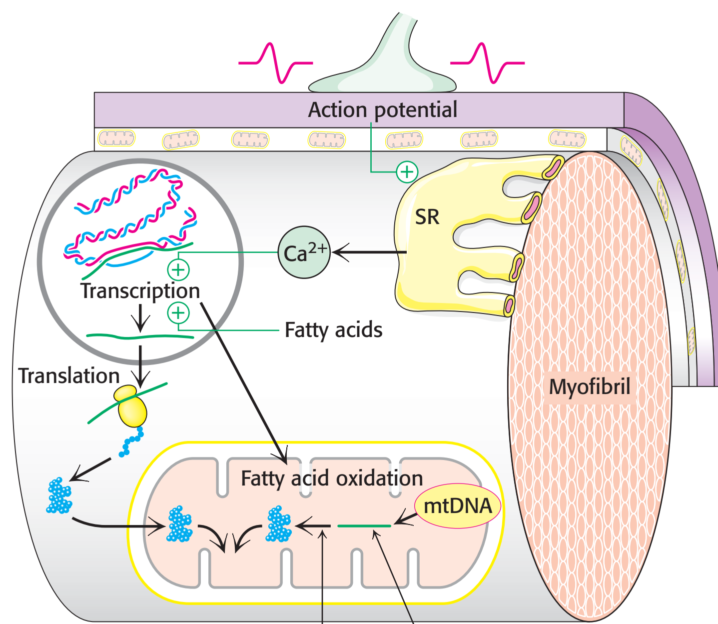


Figure 27.10 Exercise results in mitochondrial biogenesis and enhanced fat metabolism. An action potential causes Ca^{2+} release from the sarcoplasmic reticulum (SR), the muscle-cell equivalent of the endoplasmic reticulum. The Ca^{2+}, in addition to instigating muscle contraction, activates nuclear transcription factors that activate genes that, in conjunction with mitochondrial genes, are responsible for mitochondrial biogenesis. Fatty acids activate a different set of genes that increase the fatty acid oxidation capability of mitochondria. [After D. A. Hood. *J Appl. Physiol.* 90:1137–1157, 2001, Fig. 2.]

Fuel choice during exercise is determined by the intensity and duration of activity

In keeping with our theme of energy use under different physiological conditions, we now examine how fuels are used in different types of exercise. The fuels used in anaerobic exercises—sprinting, for example—differ from those used in aerobic exercises—such as distance running. The selection of fuels during these different forms of exercise illustrates many important facets of energy transduction and metabolic integration. ATP directly powers myosin, the protein immediately responsible for converting chemical energy into movement (Chapter 35). However, the amount of ATP in muscle is small. Hence, the power output and, in turn, the velocity of running depend on the rate of ATP production from other fuels. As shown in Table 27.3, *creatine phosphate* (phosphocreatine) can swiftly transfer its high-potential phosphoryl group to ADP to generate ATP. However, the amount of creatine phosphate, like that of ATP itself, is limited. Creatine phosphate and ATP can power intense muscle contraction for 5 to 6 s. Maximum speed in a sprint can thus be maintained for only 5 to 6 s (see Figure 15.7). Thus, the winner in a 100-meter sprint is the runner who both achieves the highest initial velocity and then slows down the least.

During a ~10-second sprint, the ATP level in muscle drops from 5.2 to 3.7 mM, and that of creatine phosphate decreases from 9.1 to 2.6 mM. Anaerobic glycolysis provides fuel to make up for the loss of ATP and creatine phosphate. *A 100-meter sprint is powered by stored ATP, creatine phosphate, and the anaerobic glycolysis of muscle glycogen*. The conversion of muscle glycogen into lactate can generate a good deal more ATP, but the rate is slower than that of phosphoryl-group transfer from creatine phosphate. Because of anaerobic glycolysis, the blood-lactate level is elevated from 1.6 to 8.3 mM. The release of H^+ from the intensely active muscle concomitantly lowers the blood pH from 7.42 to 7.24. This pace cannot be sustained in a 1000-meter run (~132 s) for two reasons. First, creatine phosphate is consumed within a few seconds. Second, the lactate produced would cause acidosis. Thus, alternative fuel sources are needed.

The complete oxidation of muscle glycogen to CO_2 by aerobic respiration substantially increases the energy yield, but this aerobic process is a good deal slower than anaerobic glycolysis. However, as the distance of a run increases, aerobic respiration, or oxidative phosphorylation, becomes increasingly important. For instance, *part of the ATP consumed in a 1000-meter run must come from oxidative phosphorylation*. Because ATP is produced more slowly by oxidative phosphorylation than by glycolysis (see Table 27.3), the runner's pace is necessarily slower than in

Table 27.3 Fuel sources for muscle contraction

Fuel source	Maximal rate of ATP production (mmol s^{-1})	Total ~P available (mmol)
Muscle ATP		223
Creatine phosphate	73.3	446
Conversion of muscle glycogen into lactate	39.1	6,700
Conversion of muscle glycogen into CO_2	16.7	84,000
Conversion of liver glycogen into CO_2	6.2	19,000
Conversion of adipose-tissue fatty acids into CO_2	6.7	4,000,000

Note: Fuels stored are estimated for a 70-kg person having a muscle mass of 28 kg.
Source: After E. Hultman and R. C. Harris. In *Principles of Exercise Biochemistry,* edited by J. R. Poortmans (Karger, 2004), pp. 78–119.

a 100-meter sprint. The championship velocity for the 1000-meter run is about 7.6 m s^{-1}, compared with approximately 10.4 m s^{-1} for the 100-meter event (Figure 27.11).

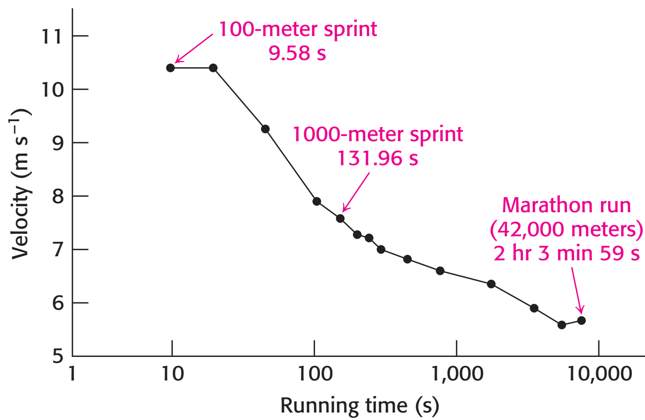


Figure 27.11 Dependency of the velocity of running on the duration of the race. The values shown are world track records. [Data from trackandfieldnews.com.]

The running of a marathon (26 miles 385 yards, or 42,200 meters) requires a different selection of fuels and is characterized by cooperation between muscle, liver, and adipose tissue. Liver glycogen complements muscle glycogen as an energy store that can be tapped. However, the total body glycogen stores (103 mol of ATP at best) are insufficient to provide the 150 mol of ATP needed for this grueling ~2-hour event. Much larger quantities of ATP can be obtained by the oxidation of fatty acids derived from the breakdown of *fat in adipose tissue,* but the maximal rate of ATP generation is slower yet than that of glycogen oxidation and is more than 10-fold slower than that with creatine phosphate. Thus, *ATP is generated much more slowly from high-capacity stores than from limited ones*, accounting for the different velocities of anaerobic and aerobic events. *ATP generation from fatty acids is essential for distance running*. Fats are rapidly consumed in activities such as distance running, explaining why extended aerobic exercise is beneficial for people who are insulin resistant. However, for an elite marathoner, fats cannot be the sole source of fuel. Studies have shown that, when muscle glycogen has been depleted, the power output of the muscle falls to approximately 50% of maximum. Power output decreases despite the fact that ample supplies of fat are available, suggesting that fats can supply only about 50% of maximal aerobic effort. A marathon would take about 6 hours to run if all the ATP came from fatty acid oxidation, because it is much slower than glycogen oxidation. Elite runners consume about equal amounts of glycogen and fatty acids during a marathon to achieve a mean velocity of 5.5 m s^{-1}, about half the velocity of a 100-meter sprint. How is an optimal mix of these fuels achieved? *A low blood-sugar level leads to a high glucagon/insulin ratio, which in turn mobilizes fatty acids from adipose tissue*. Fatty acids readily enter muscle, where they are degraded by β oxidation to acetyl CoA and then to CO_2. The elevated acetyl CoA level decreases the activity of the pyruvate dehydrogenase complex to block the conversion of pyruvate into acetyl CoA. Hence, fatty acid oxidation decreases the funneling of glucose into the citric acid cycle and oxidative phosphorylation. Glucose is spared so that just enough remains available at the end of the marathon. The simultaneous use of both fuels gives a higher mean velocity than would be attained if glycogen were totally consumed before the start of fatty acid oxidation.

If carbohydrate-rich meals are consumed after glycogen depletion, glycogen stores are rapidly restored. In addition, glycogen synthesis continues during the consumption of carbohydrate-rich meals, increasing glycogen stores far above normal. This phenomenon is called "super compensation" or, more commonly, carbo-loading.

27.5 Food Intake and Starvation Induce Metabolic Changes

Thus far, we have been considering metabolism in the context of excess consumption of calories, as in obesity, or extreme caloric needs, as in exercise. We now look at the opposite physiological condition—lack of calories.

The starved–fed cycle is the physiological response to a fast

We begin with a physiological condition called the *starved–fed cycle*, which we all experience in the hours after an evening meal and through the night's fast. This nightly starved–fed cycle has three stages: the well-fed state after a meal, the early fasting during the night, and the refed state after breakfast. A major goal of the many biochemical alterations in this period is to maintain *glucose homeostasis*—that is, a constant blood-glucose level. Maintaining glucose homeostasis is especially important because glucose is normally the only fuel source for the brain. As discussed earlier, the major defect in diabetes is the inability to perform this vital task. The two primary signals regulating the starved–fed cycle are insulin and glucagon.

1. *The Well-Fed, or Postprandial, State.* After we consume and digest an evening meal, glucose and amino acids are transported from the intestine to the blood. The dietary lipids are packaged into chylomicrons and transported to the blood by the lymphatic system. This fed condition leads to the secretion of insulin, which in cooperation with glucagon, maintains glucose homeostasis. In essence, insulin signals the fed state; it stimulates the storage of fuels and the synthesis of proteins in a variety of ways. Insulin stimulates glycogen synthesis in both muscle and the liver and suppresses gluconeogenesis by the liver. Insulin also accelerates glycolysis in the liver, which in turn increases the synthesis of fatty acids.

The liver helps to limit the amount of glucose in the blood during times of plenty by storing it as glycogen so as to be able to release glucose in times of scarcity. How is the excess blood glucose present after a meal removed? The liver is able to trap large quantities of glucose because it possesses an isozyme of hexokinase called *glucokinase*, which converts glucose into glucose 6-phosphate, which cannot be transported out of the cell. Recall that glucokinase has a high K_M value and is thus active only when blood-glucose levels are high. Furthermore, glucokinase is not inhibited by glucose 6-phosphate as hexokinase is. Consequently, *the liver forms glucose 6-phosphate more rapidly as the blood-glucose level rises. The increase in glucose 6-phosphate coupled with insulin action leads to a buildup of glycogen stores*. The hormonal effects on glycogen synthesis and storage are reinforced by a direct action of glucose itself. *Phosphorylase* a *is a glucose sensor in addition to being the enzyme that cleaves glycogen*. When the glucose level is high, the binding of glucose to phosphorylase *a* renders the enzyme susceptible to the action of a phosphatase that converts it into phosphorylase *b,* which does not readily degrade glycogen (Section 21.2). Thus, *glucose allosterically shifts the glycogen system from a degradative to a synthetic mode*.

The high insulin level in the fed state also promotes *the entry of glucose into muscle and adipose tissue*. Insulin stimulates the synthesis of glycogen by muscle as well as by the liver. The entry of glucose into adipose tissue provides glycerol 3-phosphate for the synthesis of triacylglycerols. The action of insulin also extends to amino acid and protein metabolism. Insulin promotes the uptake of branched-chain amino acids (valine, leucine, and isoleucine) by muscle. Indeed, insulin has a general stimulating effect on protein synthesis, which favors a building up of muscle protein. In addition, it inhibits the intracellular degradation of proteins.

2. *The Early Fasting, or Postabsortive, State.* The blood-glucose level begins to drop several hours after a meal, leading to a decrease in insulin secretion and a rise in glucagon secretion; glucagon is secreted by the α cells of the pancreas in response to a *low blood-sugar level in the fasting state.* Just as insulin signals the fed state, glucagon signals the starved state. It serves to mobilize glycogen stores when there is no dietary intake of

glucose. *The main target organ of glucagon is the liver*. Glucagon stimulates glycogen breakdown and inhibits glycogen synthesis by triggering the cyclic AMP cascade leading to the phosphorylation and activation of phosphorylase and the inhibition of glycogen synthase (Section 21.5). Glucagon also inhibits fatty acid synthesis by diminishing the production of pyruvate and by lowering the activity of acetyl CoA carboxylase by maintaining it in a phosphorylated state. In addition, glucagon stimulates gluconeogenesis in the liver and blocks glycolysis by lowering the level of F-2,6-BP (see Figure 27.9).

All known actions of glucagon are mediated by protein kinases that are activated by cyclic AMP. The activation of the cyclic AMP cascade results in a higher level of phosphorylase *a* activity and a lower level of glycogen synthase *a* activity. Glucagon's effect on this cascade is reinforced by the low concentration of glucose in the blood. The diminished binding of glucose to phosphorylase *a* makes the enzyme less susceptible to the hydrolytic action of the phosphatase. Instead, the phosphatase remains bound to phosphorylase *a*, and so the synthase stays in the inactive phosphorylated form. Consequently, there is a rapid mobilization of glycogen.

The large amount of glucose formed by the hydrolysis of glucose 6-phosphate derived from glycogen is then released from the liver into the blood. The entry of glucose into muscle and adipose tissue decreases in response to a low insulin level. The diminished utilization of glucose by muscle and adipose tissue also contributes to the maintenance of the blood-glucose level. The net result of these actions of glucagon is to *markedly increase the release of glucose by the liver*. Both muscle and the liver use fatty acids as fuel when the blood-glucose level drops, saving the glucose for use by the brain and red blood cells. Thus, *the blood-glucose level is kept at or above 4.4 mM (80 mg* dl^{-1}*) by three major factors*: (1) *the mobilization of glycogen and the release of glucose by the liver,* (2) *the release of fatty acids by adipose tissue, and* (3) *the shift in the fuel used from glucose to fatty acids by muscle and the liver*.

What is the result of the depletion of the liver's glycogen stores? Gluconeogenesis from lactate and alanine continues, but this process merely replaces glucose that had already been converted into lactate and alanine by tissues such as muscle and red blood cells. Moreover, the brain oxidizes glucose completely to CO_2 and H_2O. Thus, for the net synthesis of glucose to take place, another source of carbon is required. Glycerol released from adipose tissue on lipolysis provides some of the carbon atoms, with the remaining carbon atoms coming from the hydrolysis of muscle proteins.

3. *The Refed State.* What are the biochemical responses to a hearty breakfast? Fat is processed exactly as it is processed in the normal fed state. However, it is not the case for glucose. The liver does not initially absorb glucose from the blood, but, instead, leaves it for the other tissues. Moreover, the liver remains in a gluconeogenic mode. Now, however, the newly synthesized glucose is used to replenish the liver's glycogen stores. As the blood-glucose levels continue to rise, the liver completes the replenishment of its glycogen stores and begins to process the remaining excess glucose for fatty acid synthesis.

Metabolic adaptations in prolonged starvation minimize protein degradation

Earlier, we considered the metabolic results of overnutrition, a condition becoming all too common in prosperous nations. Let us now examine the opposite extreme. What are the adaptations if fasting is prolonged to the point of starvation, a circumstance affecting nearly a billion people world-

Table 27.4 Fuel reserves in a typical 70-kg man

	Available energy in kilojoules (kcal)					
Organ	Glucose or glycogen		Triacylglycerols		Mobilizable proteins	
Blood	250	(60)	20	(45)	0	(0)
Liver	1700	(400)	2000	(450)	1700	(400)
Brain	30	(8)	0	(0)	0	(0)
Muscle	5000	(1200)	2000	(450)	100,000	(24,000)
Adipose tissue	330	(80)	560,000	(135,000)	170	(40)

Source: After G. F. Cahill, Jr. *Clin. Endocrinol. Metab.* 5(1976):398.

wide? A typical well-nourished 70-kg man has fuel reserves totaling about 670,000 kJ (161,000 kcal; see Table 27.4). The energy need for a 24-hour period ranges from about 6700 kJ (1600 kcal) to 25,000 kJ (6000 kcal), depending on the extent of activity. Thus, stored fuels suffice to meet caloric needs in starvation for 1 to 3 months. However, the carbohydrate reserves are exhausted in only a day.

Even under starvation conditions, the blood-glucose level must be maintained above 2.2 mM (40 mg dl^{-1}). *The first priority of metabolism in starvation is to provide sufficient glucose to the brain and other tissues (such as red blood cells) that are absolutely dependent on this fuel*. However, precursors of glucose are not abundant. Most energy is stored in the fatty acyl moieties of triacylglycerols. However, recall that fatty acids cannot be converted into glucose, because acetyl CoA resulting from fatty acid breakdown cannot be transformed into pyruvate (p. 656). The glycerol moiety of triacylglycerol can be converted into glucose, but only a limited amount is available. The only other potential source of glucose is the carbon skeletons of amino acids derived from the breakdown of proteins. However, proteins are not stored, and so any breakdown will necessitate a loss of function. Thus, *the second priority of metabolism in starvation is to preserve protein, which is accomplished by shifting the fuel being used from glucose to fatty acids and ketone bodies* (Figure 27.12).

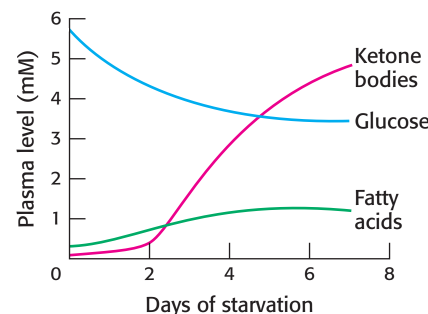


Figure 27.12 Fuel choice during starvation. The plasma levels of fatty acids and ketone bodies increase in starvation, whereas that of glucose decreases.

The metabolic changes on the first day of starvation are like those after an overnight fast. The low blood-sugar level leads to decreased secretion of insulin and increased secretion of glucagon. *The dominant metabolic processes are the mobilization of triacylglycerols in adipose tissue and gluconeogenesis by the liver. The liver obtains energy for its own needs by oxidizing fatty acids released from adipose tissue*. The concentrations of acetyl CoA and citrate consequently increase, which switches off glycolysis. The uptake of glucose by muscle is markedly diminished because of the low insulin level, whereas fatty acids enter freely. Consequently, *muscle uses no glucose and relies exclusively on fatty acids for fuel*. The β oxidation of fatty acids by muscle halts the conversion of pyruvate into acetyl CoA, because acetyl CoA stimulates the phosphorylation of the pyruvate dehydrogenase complex, which renders it inactive (Section 17.3). Hence, pyruvate, lactate, and alanine are exported to the liver for conversion into glucose. Glycerol derived from the cleavage of triacylglycerols is another raw material for the synthesis of glucose by the liver.

Proteolysis also provides carbon skeletons for gluconeogenesis. During starvation, degraded proteins are not replenished and serve as carbon sources for glucose synthesis. Initial sources of protein are those that turn over rapidly, such as proteins of the intestinal epithelium and the secretions of the pancreas. Proteolysis of muscle protein provides some of the three-carbon precursors of glucose. However, survival for most animals depends on being able to move rapidly, which requires a large muscle mass, and so muscle loss must be minimized.

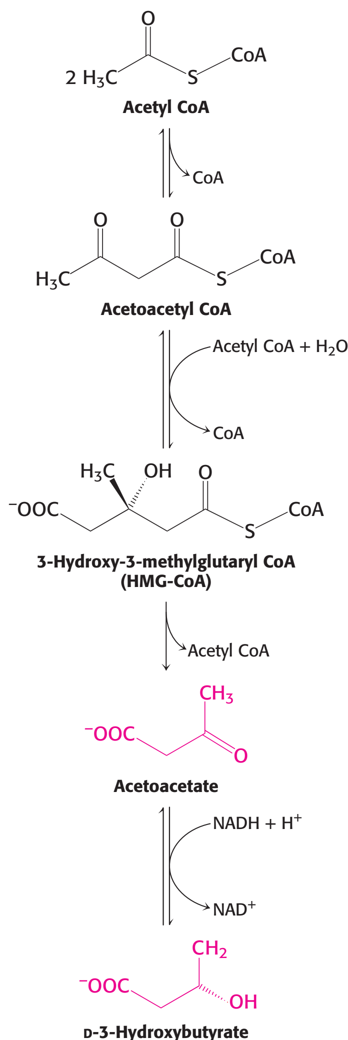


Figure 27.13 Synthesis of ketone bodies by the liver.

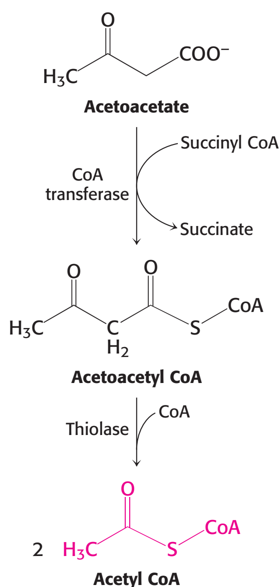


Figure 27.14 Entry of ketone bodies into the citric acid cycle.

Table 27.5 Fuel metabolism in starvation

Fuel exchanges and consumption	Amount formed or consumed in 24 hours (grams)	
	3d day	40th day
Fuel use by the brain		
Glucose	100	40
Ketone bodies	50	100
All other use of glucose	50	40
Fuel mobilization		
Adipose-tissue lipolysis	180	180
Muscle-protein degradation	75	20
Fuel output of the liver		
Glucose	150	80
Ketone bodies	150	150

How is the loss of muscle curtailed? After about 3 days of starvation, the liver forms large amounts of acetoacetate and D-3-hydroxybutyrate (ketone bodies; Figure 27.13). Their synthesis from acetyl CoA increases markedly because the citric acid cycle is unable to oxidize all the acetyl units generated by the degradation of fatty acids. Gluconeogenesis depletes the supply of oxaloacetate, which is essential for the entry of acetyl CoA into the citric acid cycle. Consequently, the liver produces large quantities of ketone bodies, which are released into the blood. At this time, *the brain begins to consume significant amounts of acetoacetate in place of glucose*. After 3 days of starvation, about a quarter of the energy needs of the brain are met by ketone bodies (Table 27.5). The heart also uses ketone bodies as fuel.

After several weeks of starvation, ketone bodies become the major fuel of the brain. Acetoacetate is activated by the transfer of CoA from succinyl CoA to give acetoacetyl CoA (Figure 27.14). Cleavage by thiolase then yields two molecules of acetyl CoA, which enter the citric acid cycle. In essence, *ketone bodies are equivalents of fatty acids that are an accessible fuel source for the brain*. Only 40 g of glucose is then needed per day for the brain, compared with about 120 g in the first day of starvation. *The effective conversion of fatty acids into ketone bodies by the liver and their use by the brain markedly diminishes the need for glucose. Hence, less muscle is degraded than in the first days of starvation*. The breakdown of 20 g of muscle daily compared with 75 g early in starvation is most important for survival. A person's survival time is mainly determined by the size of the triacylglycerol depot.

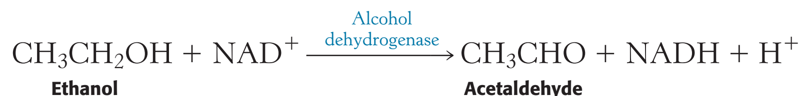
What happens after depletion of the triacylglycerol stores? The only source of fuel that remains is protein. Protein degradation accelerates, and death inevitably results from a loss of heart, liver, or kidney function.

27.6 Ethanol Alters Energy Metabolism in the Liver

Ethanol has been a part of the human diet for centuries. However, its consumption in excess can result in a number of health problems, most notably liver damage. What is the biochemical basis of these health problems?

Ethanol metabolism leads to an excess of NADH

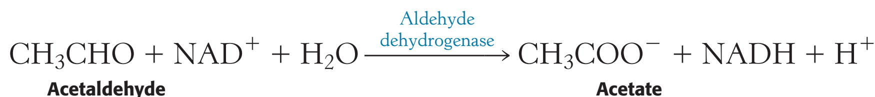
Ethanol cannot be excreted and must be metabolized, primarily by the liver. This metabolism is accomplished by two pathways. The first pathway comprises two steps. The first step, catalyzed by the enzyme *alcohol dehydrogenase*, takes place in the cytoplasm:



$$CH_3CH_2OH + NAD^+ \xrightarrow{\text{Alcohol dehydrogenase}} CH_3CHO + NADH + H^+$$

Ethanol → **Acetaldehyde**

The second step, catalyzed by *aldehyde dehydrogenase,* takes place in mitochondria:



$$CH_3CHO + NAD^+ + H_2O \xrightarrow{\text{Aldehyde dehydrogenase}} CH_3COO^- + NADH + H^+$$

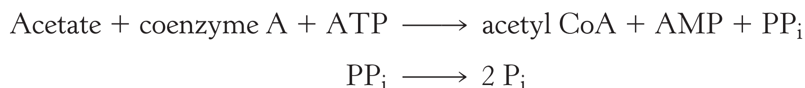
Acetaldehyde → **Acetate**

Note that *ethanol consumption leads to an accumulation of NADH*. This high concentration of NADH inhibits gluconeogenesis by preventing the oxidation of lactate to pyruvate. In fact, the high concentration of NADH will cause the reverse reaction to predominate, and lactate will accumulate. The consequences may be hypoglycemia and lactic acidosis.

The overabundance of NADH also inhibits fatty acid oxidation. The metabolic purpose of fatty acid oxidation is to generate NADH for ATP generation by oxidative phosphorylation, but an alcohol consumer's NADH needs are met by ethanol metabolism. In fact, the excess NADH signals that conditions are right for fatty acid synthesis. Hence, triacylglycerols accumulate in the liver, leading to a condition known as "fatty liver" that is exacerbated in obese persons. The biochemical effects of ethanol consumption can be quite rapid. For instance, fat accumulates in the liver within a few days of moderate alcohol consumption. This accumulation is reversible with a decrease in alcohol intake.

The second pathway for ethanol metabolism is called the ethanol-inducible *microsomal ethanol-oxidizing system* (MEOS). This cytochrome P450-dependent pathway (Section 26.4) generates acetaldehyde and subsequently acetate while oxidizing biosynthetic reducing power, NADPH, to $NADP^+$. Because it uses oxygen, this pathway generates free radicals that damage tissues. Moreover, because the system consumes NADPH, the antioxidant glutathione cannot be regenerated (Section 20.5), exacerbating the oxidative stress.

What are the effects of the other metabolites of ethanol? Liver mitochondria can convert acetate into acetyl CoA in a reaction requiring ATP. The enzyme is the thiokinase that normally activates short-chain fatty acids.



$$\text{Acetate} + \text{coenzyme A} + \text{ATP} \longrightarrow \text{acetyl CoA} + \text{AMP} + PP_i$$

$$PP_i \longrightarrow 2\ P_i$$

However, further processing of the acetyl CoA by the citric acid cycle is blocked, because NADH inhibits two important citric acid cycle regulatory enzymes—isocitrate dehydrogenase and α-ketoglutarate dehydrogenase. The accumulation of acetyl CoA has several consequences. First, ketone bodies will form and be released into the blood, aggravating the acidic condition already resulting from the high lactate concentration. The processing of the acetate in the liver becomes inefficient, leading to a buildup of acetaldehyde. This very reactive compound forms covalent bonds with many important functional groups in proteins, impairing protein function. If ethanol is consistently consumed at high levels, the acetaldehyde can significantly damage the liver, eventually leading to cell death.

Liver damage from excessive ethanol consumption occurs in three stages. The first stage is the aforementioned development of fatty liver. In the second stage—alcoholic hepatitis—groups of cells die and inflammation results. This stage can itself be fatal. In stage three—cirrhosis—fibrous

structure and scar tissue are produced around the dead cells. Cirrhosis impairs many of the liver's biochemical functions. The cirrhotic liver is unable to convert ammonia into urea, and blood levels of ammonia rise. Ammonia is toxic to the nervous system and can cause coma and death. Cirrhosis of the liver arises in about 25% of alcoholics, and about 75% of all cases of liver cirrhosis are the result of alcoholism. Viral hepatitis is a non-alcoholic cause of liver cirrhosis.

Excess ethanol consumption disrupts vitamin metabolism

The adverse effects of ethanol are not limited to the metabolism of ethanol itself. Vitamin A (retinol) is converted into retinoic acid, an important signal molecule for growth and development in vertebrates, by the same dehydrogenases that metabolize ethanol. Consequently, this activation does not take place in the presence of ethanol, which acts as a competitive inhibitor. Moreover, the MEOS system induced by ethanol inactivates retinoic acid. These disruptions in the retinoic acid signaling pathway are believed to be responsible, at least in part, for fetal alcohol syndrome as well as the development of a variety of cancers.

The disruption of vitamin A metabolism is a direct result of the biochemical changes induced by excess ethanol consumption. Other disruptions in metabolism result from another common characteristic of alcoholics—malnutrition. Alcoholics will frequently drink instead of eating. A dramatic neurological disorder, referred to as *Wernicke–Korsakoff syndrome,* results from insufficient intake of the vitamin thiamine. Symptoms include mental confusion, unsteady gait, and lack of fine motor skills. The symptoms of Wernicke–Korsakoff syndrome are similar to those of beriberi (Section 17.4) because both conditions result from a lack of thiamine. Thiamine is converted into the coenzyme thiamine pyrophosphate, a key constituent of the pyruvate dehydrogenase complex. Recall that this complex links glycolysis with the citric acid cycle. Disruptions in the pyruvate dehydrogenase complex are most evident as neurological disorders because the brain is normally dependent on glucose for energy generation.

Alcoholic scurvy is occasionally observed because of an insufficient ingestion of vitamin C. Vitamin C is required for the formation of stable collagen fibers. The symptoms of scurvy include skin lesions and blood-vessel fragility. Most notable are bleeding gums, the loss of teeth, and periodontal infections. Gums are especially sensitive to a lack of vitamin C because the collagen in gums turns over rapidly. What is the biochemical basis for scurvy? Vitamin C is required for the continued activity of prolyl hydroxylase. This enzyme synthesizes

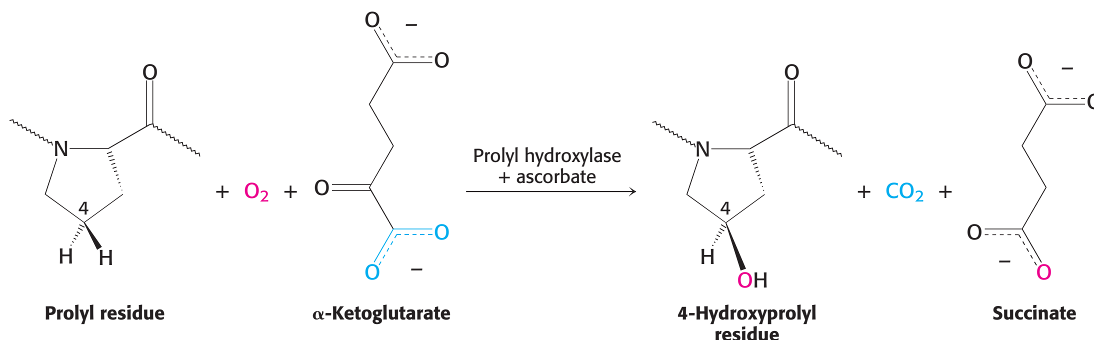


Figure 27.15 Formation of 4-hydroxyproline. Proline is hydroxylated at C-4 by the action of prolyl hydroxylase, an enzyme that activates molecular oxygen.

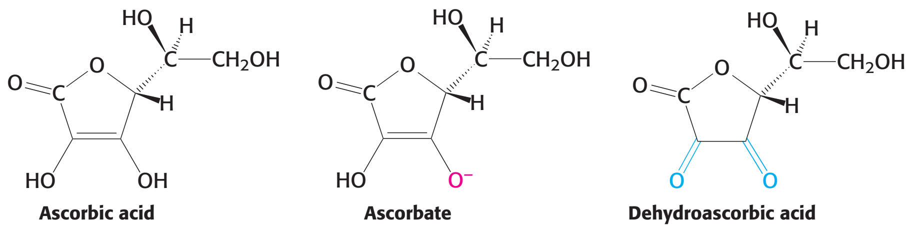


Figure 27.16 Forms of ascorbic acid (vitamin C). Ascorbate is the ionized form of vitamin C, and dehydroascorbic acid is the oxidized form of ascorbate.

4-hydroxyproline, an amino acid that is required in collagen. To form this unusual amino acid, proline residues on the amino side of glycine residues in nascent collagen chains become hydroxylated. One oxygen atom from O_2 becomes attached to C-4 of proline while the other oxygen atom is taken up by α-ketoglutarate, which is converted into succinate (Figure 27.15). This reaction is catalyzed by *prolyl hydroxylase,* a *dioxygenase,* which requires an Fe^{2+} ion to activate O_2. The enzyme also converts α-ketoglutarate into succinate without hydroxylating proline. In this partial reaction, an oxidized iron complex is formed, which inactivates the enzyme. How is the active enzyme regenerated? *Ascorbate (vitamin C)* comes to the rescue by reducing the ferric ion of the inactivated enzyme. In the recovery process, ascorbate is oxidized to dehydroascorbic acid (Figure 27.16). Thus, ascorbate serves here as a specific *antioxidant*. Why does impaired hydroxylation have such devastating consequences? *Collagen synthesized in the absence of ascorbate is less stable than the normal protein*. Hydroxyproline stabilizes the collagen triple helix by forming interstrand hydrogen bonds. The abnormal fibers formed by insufficiently hydroxylated collagen account for the symptoms of scurvy.

Summary

27.1 Caloric Homeostasis Is a Means of Regulating Body Weight

Many people are able to maintain a near-constant body weight throughout adult life. This ability is a demonstration of caloric homeostasis, a physiological condition in which energy needs match energy intake. When energy intake is greater than energy needs, weight gain results. In the developed world, obesity is at epidemic proportions and is implicated as a contributing factor in a host of pathological conditions.

27.2 The Brain Plays a Key Role in Caloric Homeostasis

Various signal molecules act on the brain to control appetite. Short-term signals such as CCK and GLP-1 relay satiety signals to the brain while eating is in progress. Long-term signals include leptin and insulin. Leptin, secreted by adipose tissue in direct proportion to adipose-tissue mass, is an indication of fat stores. Leptin inhibits eating. Insulin also works in the brain, signaling carbohydrate availability.

Leptin acts by binding to a receptor in brain neurons, which initiates signal-transduction pathways that reduce appetite. Obesity can develop in individuals with normal amounts of leptin and the leptin receptor, suggesting that such individuals are leptin resistant. Suppressors of cytokine signaling may inhibit leptin signaling, leading to leptin resistance and obesity.

27.3 Diabetes Is a Common Metabolic Disease Often Resulting from Obesity

Diabetes is the most common metabolic disease in the world. Type 1 diabetes results when insulin is absent due to autoimmune destruction of the β cells of the pancreas. Type 2 diabetes is characterized by normal or higher levels of insulin, but the target tissues of insulin, notably muscle, do not respond to the hormone, a condition called insulin resistance. Obesity is a significant predisposing factor for type 2 diabetes.

In muscle, excess fats accumulate in an obese individual. These fats are processed to second messengers that activate signal-transduction pathways that inhibit insulin signaling, leading to insulin resistance. Insulin resistance in target tissues ultimately leads to pancreatic β-cell failure. The pancreas tries to compensate for a lack of insulin action by synthesizing more insulin, resulting in ER stress and subsequent activation of apoptotic pathways that lead to β-cell death.

Type 1 diabetes is due to metabolic derangements resulting in an insufficiency of insulin and an excess of glucagon relative to a person's needs. The result is an elevated blood-glucose level, the mobilization of triacylglycerols, and excessive ketone-body formation. Accelerated ketone-body formation can lead to acidosis, coma, and death in untreated insulin-dependent diabetics.

27.4 Exercise Beneficially Alters the Biochemistry of Cells

Exercise is a useful prescription for insulin resistance and type 2 diabetes. Muscle activity stimulates mitochondrial biogenesis in a calcium-dependent manner. The increase in the number of mitochondria facilitates fatty acid oxidation in the muscle, resulting in increased insulin sensitivity.

Fuel choice in exercise is determined by the intensity and duration of the bout of exercise. Sprinting and marathon running are powered by different fuels to maximize power output. The 100-meter sprint is powered by stored ATP, creatine phosphate, and anaerobic glycolysis. In contrast, the oxidation of both muscle glycogen and fatty acids derived from adipose tissue is essential in the running of a marathon, a highly aerobic process.

27.5 Food Intake and Starvation Induce Metabolic Changes

Insulin signals the fed state; it stimulates the formation of glycogen and triacylglycerols and the synthesis of proteins. In contrast, glucagon signals a low blood-glucose level; it stimulates glycogen breakdown and gluconeogenesis by the liver and triacylglycerol hydrolysis by adipose tissue. After a meal, the rise in the blood-glucose level leads to an increased secretion of insulin and a decreased secretion of glucagon. Consequently, glycogen is synthesized in muscle and the liver. When the blood-glucose level drops several hours later, glucose is then formed by the degradation of glycogen and by the gluconeogenic pathway, and fatty acids are released by the hydrolysis of triacylglycerols. The liver and muscle then increasingly use fatty acids instead of glucose to meet their own energy needs so that glucose is conserved for use by the brain and the red blood cells.

The metabolic adaptations in starvation serve to minimize protein degradation. Large amounts of ketone bodies are formed by the liver from fatty acids and released into the blood within a few days after the onset of starvation. After several weeks of starvation, ketone bodies become the major fuel of the brain. The diminished need for glucose

decreases the rate of muscle breakdown, and so the likelihood of survival is enhanced.

27.6 Ethanol Alters Energy Metabolism in the Liver

The oxidation of ethanol results in an unregulated overproduction of NADH, which has several consequences. A rise in the blood levels of lactic acid and ketone bodies causes a fall in blood pH, or acidosis. The liver is damaged because the excess NADH causes excessive fat formation as well as the generation of acetaldehyde, a reactive molecule. Severe liver damage can result.

Key Terms

caloric homeostasis (energy homeostasis) (p. 791)
cholecystokinin (CCK) (p. 794)
glucagon-like peptide 1 (GLP-1) (p. 795)
leptin (p. 795)
insulin (p. 795)
leptin resistance (p. 797)
type 1 diabetes (p. 798)
insulin resistance (p. 798)
type 2 diabetes (p. 798)
metabolic syndrome (p. 800)
endoplasmic reticulum (ER) stress (p. 802)
unfolded protein response (UPR) (p. 802)
starved–fed cycle (p. 807)
glucose homeostasis (p. 807)

Problems

1. *Depot fat*. Adipose tissue was once only considered a storage site for fat. Why is this view no longer considered correct?

2. *Balancing act*. What is meant by caloric homeostasis?

3. *Dynamic duo*. What are the key hormones responsible for maintaining caloric homeostasis?

4. *Dual roles*. What two biochemical roles does CCK play? GLP-1?

5. *Failure to communicate*. Leptin inhibits eating and is secreted in amounts in direct proportion to body fat. Moreover, obese people have normal amounts of leptin and leptin receptor. Why, then, do people become obese?

6. *Many signals*. Match the characteristic (1–9) with the appropriate hormone (a–f).

1. Secreted by adipose tissue	(a) leptin
2. Stimulates liver gluconeogenesis	(b) adiponectin
3. GPCR pathway	(c) GLP-1
4. Satiety signal	(d) CCK
5. Enhances insulin secretion	(e) insulin
6. Secreted by the pancreas during a fast	(f) glucagon
7. Secreted after a meal	
8. Stimulates glycogen synthesis	
9. Missing in type 1 diabetes	

7. *A key chemical*. What are the sources of glucose 6-phosphate in liver cells?

8. *Neither option is good*. Differentiate between type 1 and type 2 diabetes.

9. *Fighting diabetes*. Leptin is considered an "antidiabetogenic" hormone. Explain.

10. *Metabolic energy and power*. The rate of energy expenditure of a typical 70-kg person at rest is about 70 watts (W), like that of a light bulb.

(a) Express this rate in kilojoules per second and in kilocalories per second.

(b) How many electrons flow through the mitochondrial electron-transport chain per second under these conditions?

(c) Estimate the corresponding rate of ATP production.

(d) The total ATP content of the body is about 50 g. Estimate how often an ATP molecule turns over in a person at rest.

11. *Respiratory quotient (RQ)*. This classic metabolic index is defined as the volume of CO_2 released divided by the volume of O_2 consumed.

(a) Calculate the RQ values for the complete oxidation of glucose and of tripalmitoylglycerol.

(b) What do RQ measurements reveal about the contributions of different energy sources during intense exercise? (Assume that protein degradation is negligible.)

12. *Camel's hump*. Compare the H_2O yield from the complete oxidation of 1 g of glucose with that of 1 g of tripalmitoylglycerol. Relate these values to the evolutionary selection of the contents of a camel's hump.

13. *Hungry–nourished*. What is meant by the starved–fed cycle?

14. *Of course, too much is bad for you*. What are the primary means of processing ethanol?

15. *Started out with burgundy, but soon hit the harder stuff*. Describe the three stages of ethanol consumption that lead to liver damage and possibly death.

16. *The wages of sin*. How long does a person have to jog to offset the calories obtained from eating 10 macadamia nuts (75 kJ, or 18 kcal, per nut)? (Assume an incremental power consumption of 400 W.)

17. *Sweet hazard*. Ingesting large amounts of glucose before a marathon might seem to be a good way of increasing the fuel stores. However, experienced runners do not ingest glucose before a race. What is the biochemical reason for their avoidance of this potential fuel? (Hint: Consider the effect of glucose ingestion on the level of insulin.)

18. *Lipodystrophy*. Lipodystrophy is a condition in which an individual lacks adipose tissue. The muscles and liver from such individuals are insulin resistant, and both tissues accumulate large amounts of triacylglycerides (hyperlipidemia). The administration of leptin partly ameliorates this condition. What does it indicate about the relation of adipose tissue to insulin action?

19. *Therapeutic target*. What would be the effect of a mutation in the gene for PTP1B (protein tyrosine phosphatase 1B) that inactivated the enzyme in a person who has type 2 diabetes?

20. *An effect of diabetes*. Insulin-dependent diabetes is often accompanied by hypertriglyceridemia, which is an excess blood level of triacylglycerols in the form of very low density lipoproteins. Suggest a biochemical explanation.

21. *Sharing the wealth*. The hormone glucagon signifies the starved state, yet it inhibits glycolysis in the liver. How does this inhibition of an energy-production pathway benefit the organism?

22. *Compartmentation*. Glycolysis takes place in the cytoplasm, whereas fatty acid degradation takes place in mitochondria. What metabolic pathways depend on the interplay of reactions that take place in both compartments?

23. *Kwashiorkor*. The most common form of malnutrition in children in the world, kwashiorkor, is caused by a diet having ample calories but little protein. The high levels of carbohydrate result in high levels of insulin. What is the effect of high levels of insulin on

(a) lipid utilization?

(b) protein metabolism?

(c) Children suffering from kwashiorkor often have large distended bellies caused by water from the blood leaking into extracellular spaces. Suggest a biochemical basis for this condition.

24. *One for all, all for one*. How is the metabolism of the liver coordinated with that of skeletal muscle during strenuous exercise?

25. *A little help, please?* What is the advantage of converting pyruvate into lactate in skeletal muscle?

26. *Fuel choice*. What is the major fuel for resting muscle? What is the major fuel for muscle under strenuous work conditions?

27. *Hefty reimbursement*. Endurance athletes sometimes follow the exercise-and-diet plan described here: 7 days before an event, do exhaustive exercises so as to all but deplete glycogen stores. For the next 2 to 3 days, consume few carbohydrates and do moderate- to low-intensity exercises. Finally, 3 to 4 days before the event, consume a diet rich in carbohydrates. Explain the benefits of this regime.

28. *Oxygen deficit*. After light exercise, the oxygen consumed in recovery is approximately equal to the oxygen deficit, which is the amount of additional oxygen that would have been consumed had oxygen consumption reached steady state immediately. How is the oxygen consumed in recovery used?

29. *Excess postexercise oxygen consumption*. The oxygen consumed after strenuous exercise stops is significantly greater than the oxygen deficit and is termed *excess postexercise oxygen consumption* (EPOC). Why is so much more oxygen required after intense exercise?

30. *Psychotropic effects*. Ethanol is unusual in that it is freely soluble in both water and lipids. Thus, it has access to all regions of the highly vascularized brain. Although the molecular basis of ethanol action in the brain is not clear, ethanol evidently influences a number of neurotransmitter receptors and ion channels. Suggest a biochemical explanation for the diverse effects of ethanol.

31. *Fiber type*. Skeletal muscle has several distinct fiber types. Type I is used primarily for aerobic activity, whereas type II is specialized for short, intense bursts of activity. How could you distinguish between these types of muscle fiber if you viewed them with an electron microscope?

32. *Tour de France*. Cyclists in the Tour de France (more than 2000 miles in 3 weeks) require about 836,000 kJ (200,000 kcal) of energy, or 41,840 kJ (10,000 kcal) day^{-1} (a resting male requires ~8368 kJ, or 2000 kcal, day^{-1}).

(a) With the assumptions that the energy yield of ATP is about 50.2 kJ (12 kcal) mol^{-1} and that ATP has a molecular weight of 503 g mol^{-1}, how much ATP would be expended by a Tour de France cyclist?

(b) Pure ATP can be purchased at a cost of approximately $150 per gram. How much would it cost to power a cyclist through the Tour de France if the ATP had to be purchased?

33. *Spare tire*. Suppose that our test subject from the beginning of the chapter gained 55 pounds between the ages of 25 and 65, and that her weight at 65 years of age is 175 pounds. Calculate how many excess calories she consumed per day to gain the 55 pounds over 40 years. Assume that our test subject is 5 feet 6 inches tall. What is her BMI? Would she be considered obese at 175 lbs?

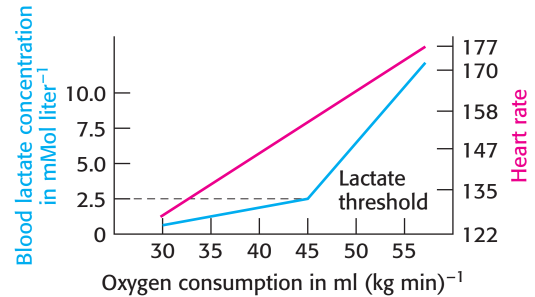
34. *Responding to stress.* Why does it make good physiological sense that regular bouts of prolonged exercise will result in mitochondrial biogenesis?

35. *Too much of a good thing*. What is the relation between fatty acid oxidation and insulin resistance in the muscle?

36. *Aneurin? Really?* Why are the symptoms of beriberi similar to those of Wernicke–Korsakoff syndrome?

Data Interpretation Problem

37. *Lactate threshold*. The graph shows the relation between blood-lactate levels, oxygen consumption, and heart rate during exercise of increasing intensity. The values for oxygen consumption and heart rate are indicators of the degree of exertion.



(a) Why is some lactate produced even when exercise is moderate?

(b) Biochemically, what is taking place when the lactate concentration begins to rise rapidly, a point called the lactate threshold?

(c) Endurance athletes will sometimes measure blood-lactate levels during training so that they know their lactate threshold. Then, during events, they will race just at or below their lactate threshold until the late stages of the race. Biochemically, why is this practice wise?

(d) Training can increase the lactate threshold. Explain.

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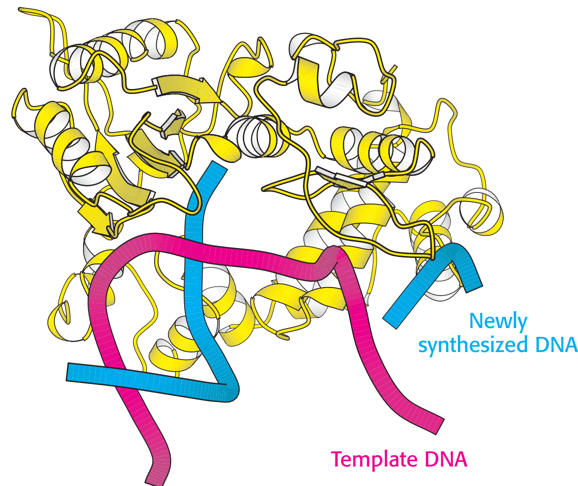
CHAPTER 28

DNA Replication, Repair, and Recombination



Faithful copying is essential to the storage of genetic information. With the precision of a diligent monk copying an illuminated manuscript, a DNA polymerase (above) copies DNA strands, preserving the precise sequence of bases with very few errors. [(Left)The Pierpont Morgan Library/Art Resource.]

Perhaps the most exciting aspect of the structure of DNA deduced by Watson and Crick was, as expressed in their words, that the "specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material." A double helix separated into two single strands can be replicated because each strand serves as a template on which its complementary strand can be assembled (Figure 28.1). To preserve the information encoded in DNA through many cell divisions, copying of the genetic information must be extremely faithful. To replicate the human genome without mistakes, an error rate of less than 1 bp per 3×10^9 bp must be achieved. Such remarkable accuracy is achieved through a multilayered system of accurate DNA synthesis (which has an error rate of 1 per 10^3–10^4 bases inserted), proofreading during DNA synthesis (which reduces that error rate to approximately 1 per 10^6–10^7 bp), and postreplication mismatch repair (which reduces the error rate to approximately 1 per 10^9–10^{10} bp).

Even after DNA has been initially replicated, the genome is still not safe. Although DNA is remarkably robust, ultraviolet light as well as a range of chemical species can damage DNA, introducing changes in the DNA

OUTLINE

28.1 DNA Replication Proceeds by the Polymerization of Deoxyribonucleoside Triphosphates Along a Template

28.2 DNA Unwinding and Supercoiling Are Controlled by Topoisomerases

28.3 DNA Replication Is Highly Coordinated

28.4 Many Types of DNA Damage Can Be Repaired

28.5 DNA Recombination Plays Important Roles in Replication, Repair, and Other Processes

sequence (mutations) or lesions that can block further DNA replication (Figure 28.2). All organisms contain DNA-repair systems that detect DNA damage and act to preserve the original sequence. Mutations in genes that encode components of DNA-repair systems are key factors in the development of cancer. Among the most potentially devastating types of DNA damage are double-stranded breaks in DNA. With both strands of the double helix broken in a local region, neither strand is intact to act as a template for future DNA synthesis. A mechanism used to repair such lesions relies on DNA recombination—that is, the reassortment of DNA sequences present on two different double helices. In addition to its role in DNA repair, recombination is crucial for the generation of genetic diversity in meiosis. Recombination is also the key to generating a highly diverse repertoire of genes for key molecules in the immune system (Chapter 34).

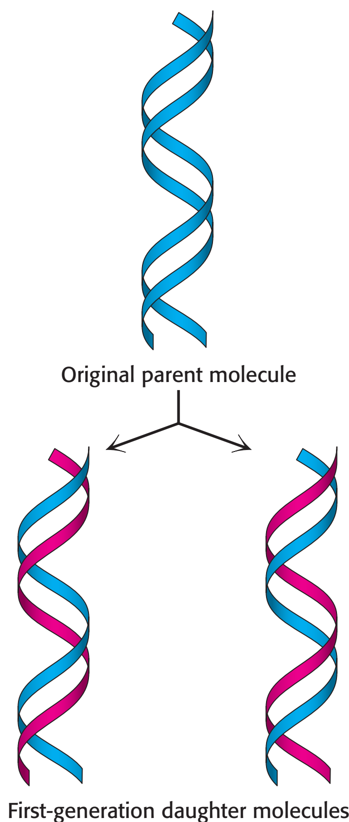


Figure 28.1 DNA replication. Each strand of one double helix (shown in blue) acts as a template for the synthesis of a new complementary strand (shown in red).

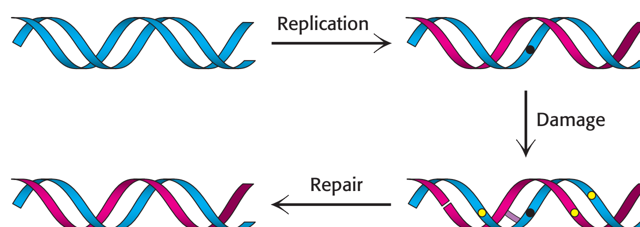


Figure 28.2 DNA Replication, damage, and repair. Some errors (shown as a black dot) may arise in the replication processes. Additional defects (shown in yellow) including modified bases, cross-links, and single- and double-strand breaks are introduced into DNA by subsequent DNA-damaging reactions. Many of the errors are detected and subsequently repaired.

28.1 DNA Replication Proceeds by the Polymerization of Deoxyribonucleoside Triphosphates Along a Template

The base sequences of newly synthesized DNA must faithfully match the sequences of parent DNA. To achieve faithful replication, each strand within the parent double helix acts as a *template* for the synthesis of a new DNA strand with a complementary sequence. The building blocks for the synthesis of the new strands are deoxyribonucleoside triphosphates. They are added, one at a time, to the 3′ end of an existing strand of DNA.

Although this reaction is in principle quite simple, it is significantly complicated by specific features of the DNA double helix. First, the two strands of the double helix run in opposite directions. Because DNA strand synthesis always proceeds in the 5′-to-3′ direction, the DNA replication process must have special mechanisms to accommodate the oppositely directed strands. Second, the two strands of the double helix interact with one another in such a way that the bases, key templates for replication, are on the inside of the helix. Thus, the two strands must be separated from each other so as to generate appropriate templates. Finally, the two strands of the double helix wrap around each other. Thus, strand separation also entails the unwinding of the double helix. This unwinding creates supercoils that must themselves be resolved as replication continues. We begin with a consideration of the chemistry that underlies the formation of the phosphodiester backbone of newly synthesized DNA.

DNA polymerases require a template and a primer

DNA polymerases catalyze the formation of polynucleotide chains. Each incoming nucleoside triphosphate first forms an appropriate base pair with a base in the template. Only then does the DNA polymerase link the incom-

ing base with the predecessor in the chain. Thus, *DNA polymerases are template-directed enzymes.*

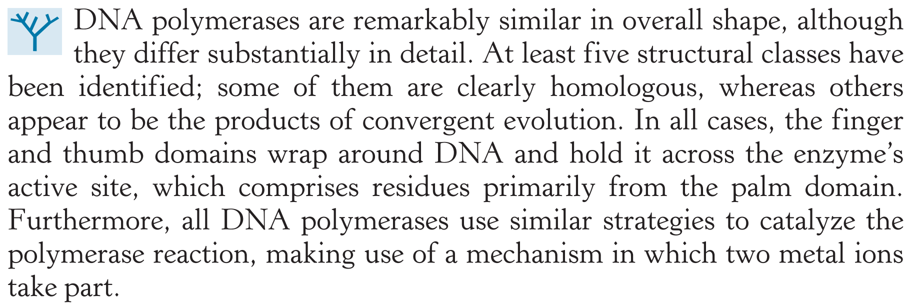
DNA polymerases add nucleotides to the 3′ end of a polynucleotide chain. The polymerase catalyzes the nucleophilic attack by the 3′-hydroxyl-group terminus of the polynucleotide chain on the α phosphoryl group of the nucleoside triphosphate to be added (see Figure 4.25). To initiate this reaction, DNA polymerases require a *primer* with a free 3′-hydroxyl group already base-paired to the template. They cannot start from scratch by adding nucleotides to a free single-stranded DNA template. RNA polymerase, in contrast, can initiate RNA synthesis without a primer, as we shall see in Chapter 29.

Primer
The initial segment of a polymer that is to be extended on which elongation depends.

Template
A sequence of DNA or RNA that directs the synthesis of a complementary sequence.

All DNA polymerases have structural features in common

The three-dimensional structures of a number of DNA polymerase enzymes are known. The first such structure was elucidated by Tom Steitz and coworkers, who determined the structure of the so-called *Klenow fragment* of DNA polymerase I from *E. coli* (Figure 28.3). This fragment comprises two main parts of the full enzyme, including the polymerase unit. This unit approximates the shape of a right hand with domains that are referred to as the fingers, the thumb, and the palm. In addition to the polymerase, the Klenow fragment includes a domain with 3′ ⟶ 5′ *exonuclease* activity that participates in proofreading and correcting the polynucleotide product.


DNA polymerases are remarkably similar in overall shape, although they differ substantially in detail. At least five structural classes have been identified; some of them are clearly homologous, whereas others appear to be the products of convergent evolution. In all cases, the finger and thumb domains wrap around DNA and hold it across the enzyme's active site, which comprises residues primarily from the palm domain. Furthermore, all DNA polymerases use similar strategies to catalyze the polymerase reaction, making use of a mechanism in which two metal ions take part.

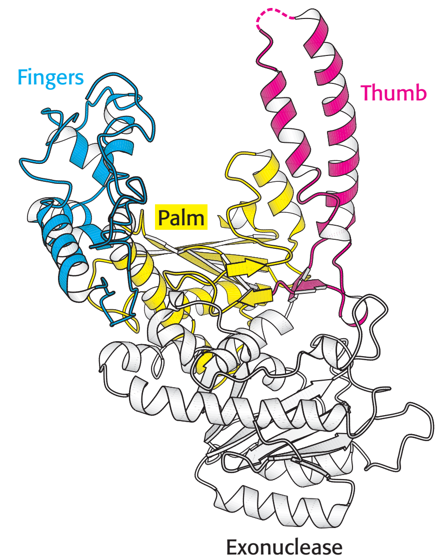


Figure 28.3 DNA polymerase structure. The first DNA polymerase structure determined was that of a fragment of *E. coli* DNA polymerase I called the Klenow fragment. *Notice* that, like other DNA polymerases, the polymerase unit resembles a right hand with fingers (blue), palm (yellow), and thumb (red). The Klenow fragment also includes an exonuclease domain that removes incorrect nucleotide bases. [Drawn from 1DPI.pdb.]

Two bound metal ions participate in the polymerase reaction

Like all enzymes with nucleoside triphosphate substrates, DNA polymerases require metal ions for activity. Examination of the structures of DNA polymerases with bound substrates and substrate analogs reveals the presence of two metal ions in the active site. One metal ion binds both the deoxynucleoside triphosphate (dNTP) and the 3′-hydroxyl group of the primer, whereas the other interacts only with the dNTP (Figure 28.4). The two metal ions

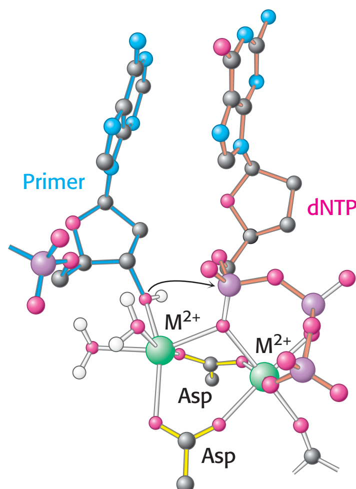


Figure 28.4 DNA polymerase mechanism. Two metal ions (typically, Mg^{2+}) participate in the DNA polymerase reaction. One metal ion coordinates the 3′-hydroxyl group of the primer, whereas the other metal ion interacts only with the dNTP. The phosphoryl group of the nucleoside triphosphate bridges between the two metal ions. The hydroxyl group of the primer attacks the phosphoryl group to form a new O–P bond.

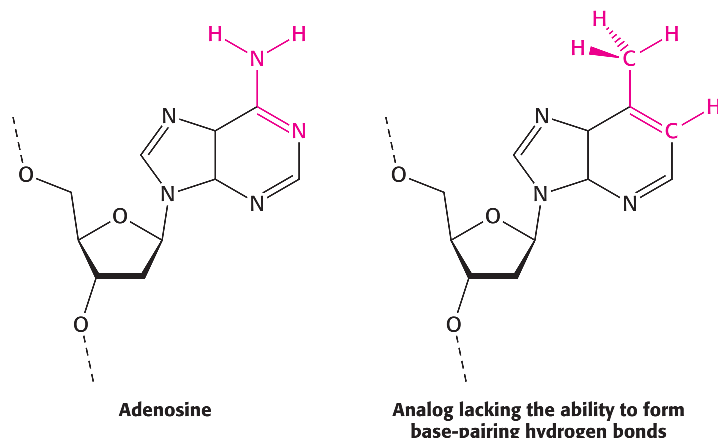


Figure 28.5 Shape complementarity. The base analog on the right has the same shape as adenosine, but groups that form hydrogen bonds between base pairs have been replaced by groups (shown in red) not capable of hydrogen bonding. Nonetheless, studies reveal that, when incorporated into the template strand, this analog directs the insertion of thymidine in DNA replication.

are bridged by the carboxylate groups of two aspartate residues in the palm domain of the polymerase. These side chains hold the metal ions in the proper positions and orientations. The metal ion bound to the primer activates the 3′-hydroxyl group of the primer, facilitating its attack on the α phosphoryl group of the dNTP substrate in the active site. The two metal ions together help stabilize the negative charge that accumulates on the pentacoordinate transition state. The metal ion initially bound to dNTP stabilizes the negative charge on the pyrophosphate product.

The specificity of replication is dictated by complementarity of shape between bases

DNA must be replicated with high fidelity. Each base added to the growing chain should, with high probability, be the Watson–Crick complement of the base in the corresponding position in the template strand. The binding of the dNTP containing the proper base is favored by the formation of a base pair with its partner on the template strand. Although hydrogen bonding contributes to the formation of this base pair, overall shape complementarity is crucial. Studies show that a nucleotide with a base that is very similar in shape to adenine but lacks the ability to form base-pairing hydrogen bonds can still direct the incorporation of thymidine, both in vitro and in vivo (Figure 28.5).

An examination of the crystal structures of various DNA polymerases reveals why shape complementarity is so important. First, residues of the enzyme form hydrogen bonds with *the minor-groove side of the base pair in the active site* (Figure 28.6). In the minor groove, hydrogen-bond acceptors are present in the same positions for all Watson–Crick base pairs. These interactions act as a "ruler" that measures whether a properly spaced base pair has formed in the active site.

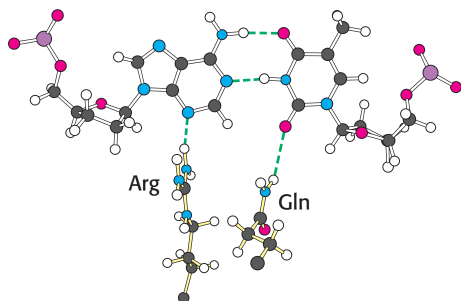


Figure 28.6 Minor-groove interactions. DNA polymerases donate two hydrogen bonds to base pairs in the minor groove. Hydrogen-bond acceptors are present in these two positions for all Watson–Crick base pairs, including the A–T base pair shown.

Second, DNA polymerases close down around the incoming dNTP (Figure 28.7). The binding of a deoxyribonucleoside triphosphate into the active site of a DNA polymerase triggers a conformational change: the finger domain rotates to form a tight pocket into which only a properly shaped base pair will readily fit. Many of the residues lining this pocket are important to ensure the efficiency and fidelity of DNA synthesis. For example, mutation of a conserved tyrosine residue that forms part of the pocket results in a polymerase that is approximately 40 times as error prone as the parent polymerase.

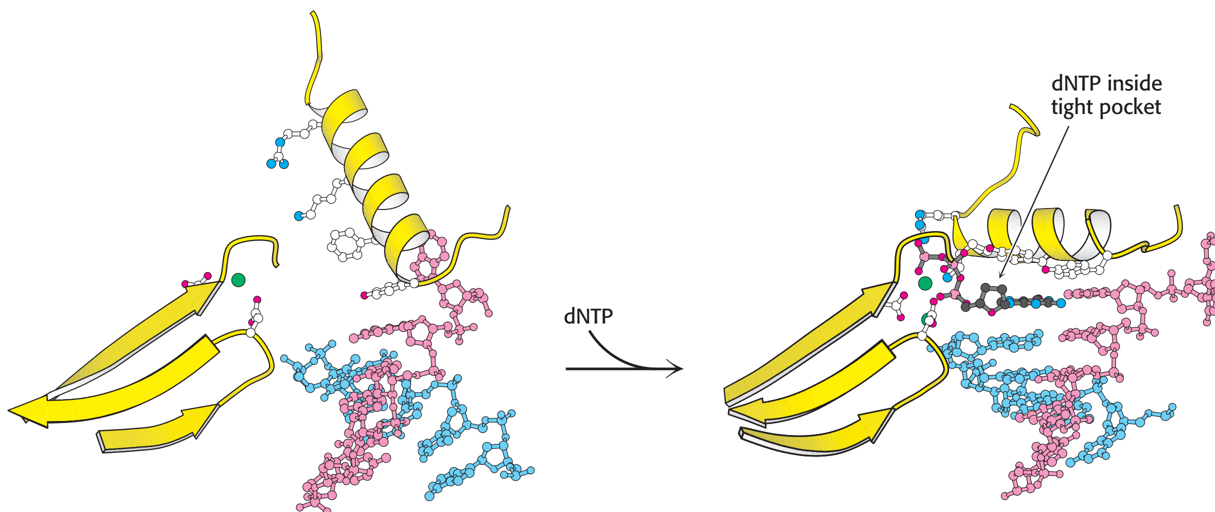


Figure 28.7 Shape selectivity. The binding of a deoxyribonucleoside triphosphate (dNTP) to DNA polymerase induces a conformational change, generating a tight pocket for the base pair consisting of the dNTP and its partner on the template strand. Such a conformational change is possible only when the dNTP corresponds to the Watson–Crick partner of the template base. [Drawn from 2BDP.pdb and 1T7P.pdb.]

An RNA primer synthesized by primase enables DNA synthesis to begin

DNA polymerases cannot initiate DNA synthesis without a primer, a section of nucleic acid having a free 3′ end that forms a double helix with the template. How is this primer formed? An important clue came from the observation that RNA synthesis is essential for the initiation of DNA synthesis. In fact, *RNA primes the synthesis of DNA*. An RNA polymerase called *primase* synthesizes a short stretch of RNA (about five nucleotides) that is complementary to one of the template DNA strands (Figure 28.8). Primase, like other RNA polymerases, can initiate synthesis without a primer. After DNA synthesis has been initiated, the short stretch of RNA is removed by hydrolysis and replaced by DNA.

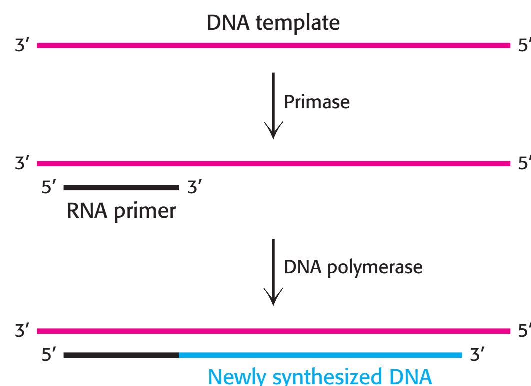


Figure 28.8 Priming. DNA replication is primed by a short stretch of RNA that is synthesized by primase, an RNA polymerase. The RNA primer is removed at a later stage of replication.

One strand of DNA is made continuously, whereas the other strand is synthesized in fragments

Both strands of parental DNA serve as templates for the synthesis of new DNA. The site of DNA synthesis is called the *replication fork* because the complex formed by the newly synthesized daughter helices arising from the parental duplex resembles a two-pronged fork. Recall that the two strands are antiparallel; that is, they run in opposite directions. During DNA replication, both daughter strands appear on cursory examination to grow in the same direction. However, all known DNA polymerases synthesize DNA in the 5′ → 3′ direction but not in the 3′ → 5′ direction. How then does one of the daughter DNA strands appear to grow in the 3′ → 5′ direction?

This dilemma was resolved by Reiji Okazaki, who found that *a significant proportion of newly synthesized DNA exists as small fragments*. These units of about a thousand nucleotides (called *Okazaki fragments*) are present briefly in the vicinity of the replication fork (Figure 28.9).

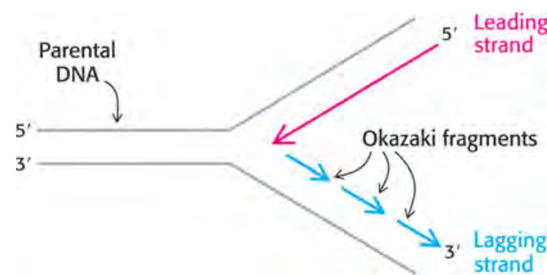


Figure 28.9 Okazaki fragments. At a replication fork, both strands are synthesized in the 5′ → 3′ direction. The leading strand is synthesized continuously, whereas the lagging strand is synthesized in short pieces termed Okazaki fragments.

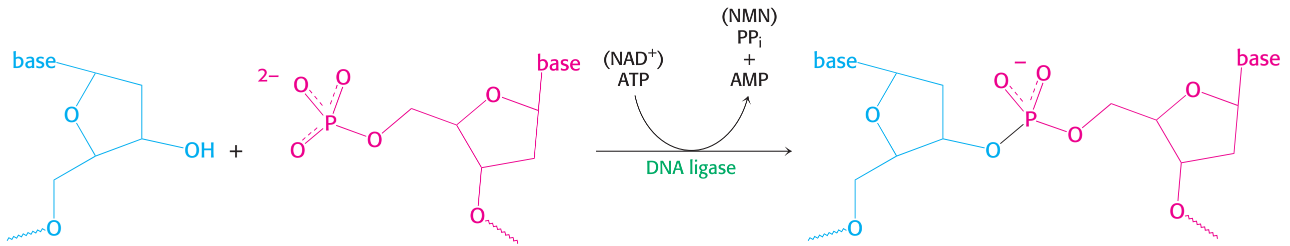


Figure 28.10 DNA ligase reaction. DNA ligase catalyzes the joining of one DNA strand with a free 3′-hydroxyl group to another with a free 5′-phosphoryl group. In eukaryotes and archaea, ATP is cleaved to AMP and PP_i to drive this reaction. In bacteria, NAD^+ is cleaved to AMP and nicotinamide mononucleotide (NMN).

As replication proceeds, these fragments become covalently joined through the action of the enzyme DNA ligase to form a continuous daughter strand. The other new strand is synthesized continuously. The strand formed from Okazaki fragments is termed the *lagging strand,* whereas the one synthesized without interruption is the *leading strand.* The discontinuous assembly of the lagging strand enables $5' \rightarrow 3'$ polymerization at the nucleotide level to give rise to overall growth in the $3' \rightarrow 5'$ direction.

DNA ligase joins ends of DNA in duplex regions

The joining of Okazaki fragments requires an enzyme that catalyzes the joining of the ends of two DNA chains. The existence of circular DNA molecules also points to the existence of such an enzyme. In 1967, scientists in several laboratories simultaneously discovered *DNA ligase. This enzyme catalyzes the formation of a phosphodiester bond between the 3′-hydroxyl group at the end of one DNA chain and the 5′-phosphoryl group at the end of the other* (Figure 28.10). An energy source is required to drive this thermodynamically uphill reaction. In eukaryotes and archaea, ATP is the energy source. In bacteria, NAD^+ typically plays this role.

DNA ligase cannot link two molecules of single-stranded DNA or circularize single-stranded DNA. Rather, *ligase seals breaks in double-stranded DNA molecules.* The enzyme from *E. coli* ordinarily forms a phosphodiester bridge only if there are at least a few bases of single-stranded DNA on the end of a double-stranded fragment that can come together with those on another fragment to form base pairs. Ligase encoded by T4 bacteriophage can link two blunt-ended double-helical fragments, a capability that is exploited in recombinant DNA technology.

The separation of DNA strands requires specific helicases and ATP hydrolysis

For a double-stranded DNA molecule to replicate, the two strands of the double helix must be separated from each other, at least locally. This separation allows each strand to act as a template on which a new polynucleotide chain can be assembled. Specific enzymes, termed *helicases,* utilize the energy of ATP hydrolysis to power strand separation.

Helicases are a large and diverse family of enzymes taking part in many biological processes. The helicases in DNA replication are typically oligomers containing six subunits that form a ring structure. The structure of one such helicase, that from bacteriophage T7, has been determined and has been a source of considerable insight into the helicase mechanism (Figure 28.11). Each of the subunits within this hexameric structure has a core structure that includes a P-loop NTPase domain (see Figure 9.51). In addition to the P-loop, each subunit has two loops that extend toward the center of the ring structure and interact with DNA. Each subunit interacts closely with its two neighbors within the ring structure. Closer examination of this structure reveals that the ring deviates significantly from six-fold symmetry. This deviation is even more apparent when the nonhydrolyzable

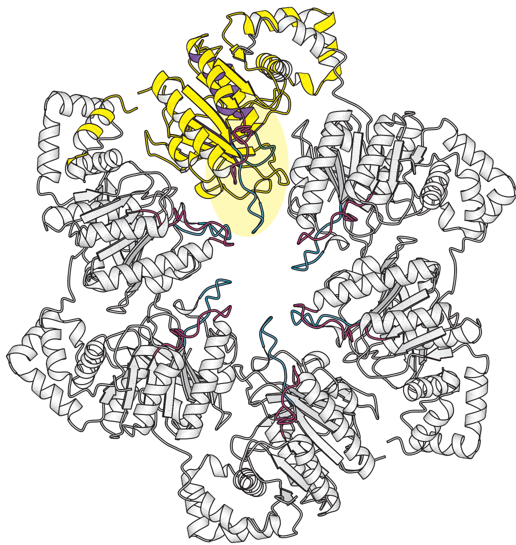
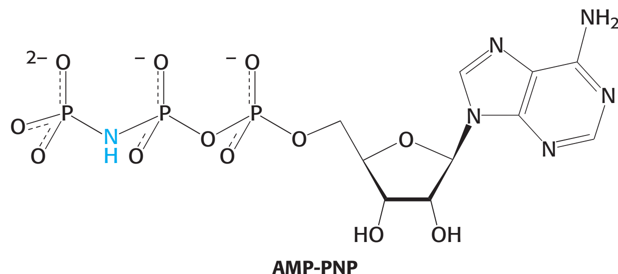


Figure 28.11 Helicase structure. The structure of the hexameric helicase from bacteriophage T7. One of the six subunits is shown in yellow with the P-loop NTPase shown in purple. The loops that participate in DNA binding are highlighted by a yellow oval. *Notice* that each subunit interacts closely with its neighbors and that the DNA-binding loops line the hole in the center of the structure. [Drawn from 1E0K.pdb.]

ATP analog AMP-PNP has been added in laboratory studies of helicase's structure.



The AMP-PNP binds to only four of the six subunits within the ring (Figure 28.12). Furthermore, the four nucleotide-binding sites are not identical but fall into two classes. One class appears to be well positioned to bind ATP but not catalyze its hydrolysis, whereas the other class is more well suited to catalyze the hydrolysis but not release the hydrolysis products. The classes are analogous to myosin's two different conformations—one for binding ATP and one for hydrolyzing it (Section 9.4). Finally, the six subunits fall into three classes with regard to their orientation with respect to the overall ring structure, with differences in rotation around an axis in the plane of the ring of approximately 30°. These differences in orientation affect the position of the two DNA-binding loops in each subunit.

These observations are consistent with the following mechanism for the helicase (Figure 28.13). Only a single strand of DNA can fit through the center on the ring. This single strand binds to loops on two adjacent subunits, one of which has bound ATP and the other of which has bound ADP + P_i. The binding of ATP to the domains that initially had no bound nucleotides leads to a conformational change within the entire hexamer, leading to the release of ADP + P_i from two subunits and the binding of the single-stranded DNA by one of the domains that just bound ATP. This conformational change pulls the DNA through the center of the hexamer. The protein acts as a wedge, forcing the two strands of the double helix apart. This cycle then repeats itself, moving two bases along the DNA strand with each cycle.

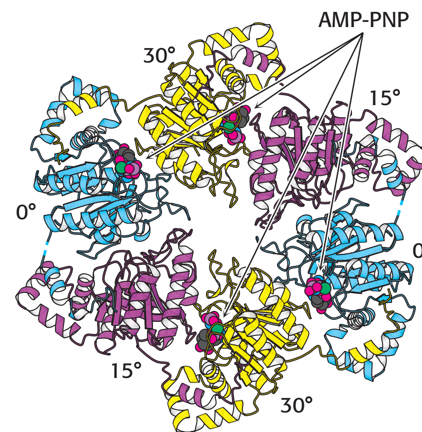


Figure 28.12 Helicase asymmetry. The structure of the T7 helicase complexes with the ATP analog AMP-PNP is shown. The three classes of helicase subunits are shown in blue, red, and yellow. The rotation relative to the plane of the hexamer is shown for each subunit. *Notice* that only four of the subunits, those shown in blue and yellow, bind AMP-PNP. [Drawn from 1E0K.pdb.]

28.2 DNA Unwinding and Supercoiling Are Controlled by Topoisomerases

As a helicase moves along unwinding DNA, the DNA in front of the helicase will become overwound in the absence of other changes. As discussed in Chapter 4, DNA double helices that are torsionally stressed tend to fold

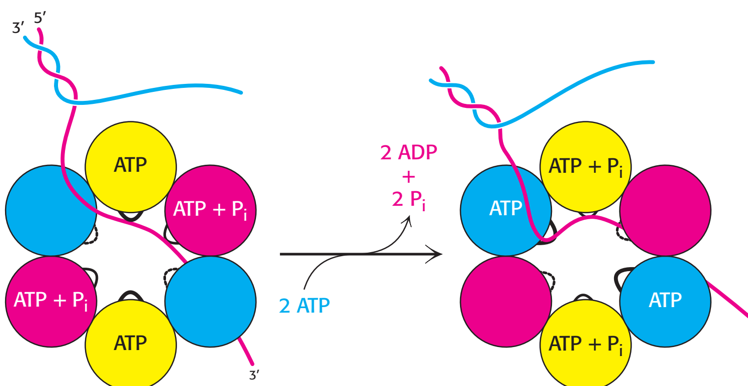


Figure 28.13 Helicase mechanism. One of the strands of the double helix passes through the hole in the center of the helicase, bound to the loops of two adjacent subunits. Two of the subunits do not contain bound nucleotides. On the binding of ATP to these two subunits and the release of ADP + P_i from two other subunits, the helicase hexamer undergoes a conformational change, pulling the DNA through the helicase. The helicase acts as a wedge to force separation of the two strands of DNA.

up on themselves to form tertiary structures created by *supercoiling*. We will first consider the supercoiling of DNA in quantitative terms and then turn to topoisomerases, enzymes that can directly modulate DNA winding and supercoiling. Supercoiling is most readily understood by considering circular DNA molecules, but it also applies to linear DNA molecules constrained to be in loops by other means. Most DNA molecules inside cells are subject to supercoiling.

Consider a linear 260-bp DNA duplex in the B-DNA form (Figure 28.14A). Because the number of base pairs per turn in an unstressed DNA molecule averages 10.4, this linear DNA molecule has 25 (260/10.4) turns. The ends of this helix can be joined to produce a *relaxed* circular DNA (Figure 28.14B). A different circular DNA can be formed by unwinding the linear duplex by two turns before joining its ends (Figure 28.14C). What is the structural consequence of unwinding before ligation? Two limiting conformations are possible. The DNA can fold into a structure containing 23 turns of B helix and an unwound loop (Figure 28.14D). Alternatively, the double helix can fold up to cross itself. Such crossings are called *supercoils*. In particular, a supercoiled structure with 25 turns of B helix and 2 turns of *right-handed* (termed *negative*) superhelix can be formed (Figure 28.14E).

Supercoiling markedly alters the overall form of DNA. *A supercoiled DNA molecule is more compact than a relaxed DNA molecule of the same length.* Hence, supercoiled DNA moves faster than relaxed DNA when analyzed by centrifugation or electrophoresis. Unwinding will cause supercoiling in circular DNA molecules, whether covalently closed or constrained in closed configurations by other means.

The linking number of DNA, a topological property, determines the degree of supercoiling

Our understanding of the conformation of DNA is enriched by concepts drawn from topology, a branch of mathematics dealing with structural properties that are unchanged by deformations such as stretching and bending. A key topological property of a circular DNA molecule is its *linking number (Lk)*, which is equal to the number of times that a strand of DNA winds in the right-handed direction around the helix axis when the axis lies in a plane, as in Figure 28.14A. For the relaxed DNA shown in Figure 28.14B, $Lk = 25$. For the partly unwound molecule shown in part D and the supercoiled one shown in part E, $Lk = 23$ because the linear duplex was unwound two complete turns before closure. Molecules differing only in linking number are *topological isomers,* or *topoisomers,* of one another. *Topoisomers of DNA can be interconverted only by cutting one or both DNA strands and then rejoining them.*

The unwound DNA and supercoiled DNA shown in Figure 28.14D and E are topologically identical but geometrically different. They have the same value of Lk but differ in *twist (Tw)* and *writhe (Wr)*. Although the rigorous definitions of twist and writhe are complex, twist is a measure of the helical winding of the DNA strands around each other, whereas writhe is a measure of the coiling of the axis of the double helix—that is, supercoiling. A right-handed coil is assigned a negative number (negative supercoiling) and a left-handed coil is assigned a positive number (positive supercoiling).

Is there a relation between Tw and Wr? Indeed, there is. Topology tells us that the sum of Tw and Wr is equal to Lk.

$$Lk = Tw + Wr$$

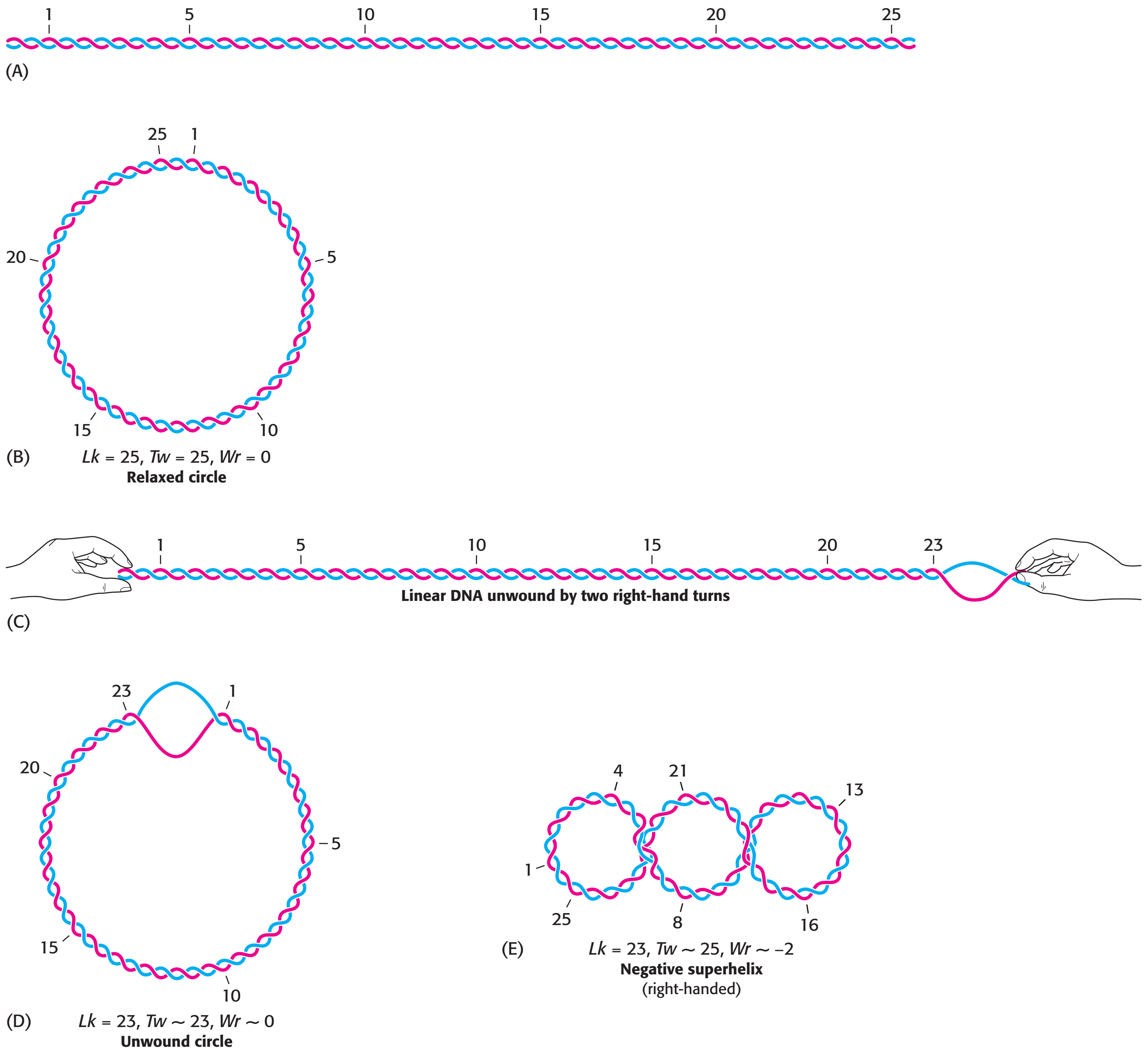


Figure 28.14 Linking number. The relations between the linking number *(Lk),* twisting number *(Tw),* and writhing number *(Wr)* of a circular DNA molecule revealed schematically. [After W. Saenger, *Principles of Nucleic Acid Structure* (Springer Verlag, 1984), p. 452.]

In Figure 28.14, the partly unwound circular DNA has $Tw \sim 23$, meaning the helix has 23 turns and $Wr \sim 0$, meaning the helix has not crossed itself to create a supercoil. The supercoiled DNA, however has $Tw \sim 25$ and $Wr \sim -2$. These forms can be interconverted without cleaving the DNA chain because they have the same value of *Lk*—namely, 23. The partitioning of *Lk* (which must be an integer) between *Tw* and *Wr* (which need not be integers) is determined by energetics. The free energy is minimized when about 70% of the change in *Lk* is expressed in *Wr* and 30% is expressed in *Tw.* Hence, the most stable form would be one with $Tw = 24.4$ and $Wr = -1.4$. Thus, *a lowering of* Lk *causes both right-handed (negative) supercoiling of the DNA axis and unwinding of the duplex*. Topoisomers differing by just 1 in *Lk,* and

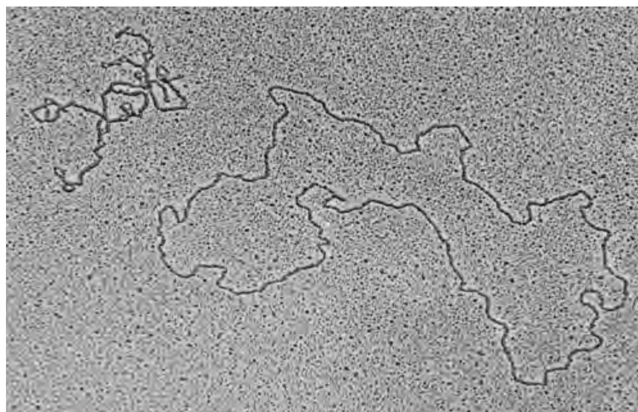


Figure 28.15 Topoisomers. An electron micrograph showing negatively supercoiled and relaxed DNA. [Courtesy of Dr. Jack Griffith.]

consequently by 0.7 in *Wr,* can be readily separated by agarose gel electrophoresis because their hydrodynamic volumes are quite different; *supercoiling condenses DNA* (Figure 28.15).

Topoisomerases prepare the double helix for unwinding

Most naturally occurring DNA molecules are negatively supercoiled. What is the basis for this prevalence? As already stated, negative supercoiling arises from the unwinding or underwinding of the DNA. In essence, negative supercoiling prepares DNA for processes requiring separation of the DNA strands, such as replication. Positive supercoiling condenses DNA as effectively, but it makes strand separation more difficult.

The presence of supercoils in the immediate area of unwinding would, however, make unwinding difficult. Therefore, negative supercoils must be continuously removed, and the DNA relaxed, as the double helix unwinds. Specific enzymes called *topoisomerases* that introduce or eliminate supercoils were discovered by James Wang and Martin Gellert. *Type I topoisomerases* catalyze the relaxation of supercoiled DNA, a thermodynamically favorable process. *Type II topoisomerases* utilize free energy from ATP hydrolysis to add negative supercoils to DNA. Both type I and type II topoisomerases play important roles in DNA replication as well as in transcription and recombination.

These enzymes alter the linking number of DNA by catalyzing a three-step process: (1) the *cleavage* of one or both strands of DNA, (2) the *passage* of a segment of DNA through this break, and (3) the *resealing* of the DNA break. Type I topoisomerases cleave just one strand of DNA, whereas type II enzymes cleave both strands. The two types of enzymes have several common features, including the use of key tyrosine residues to form covalent links to the polynucleotide backbone that is transiently broken.

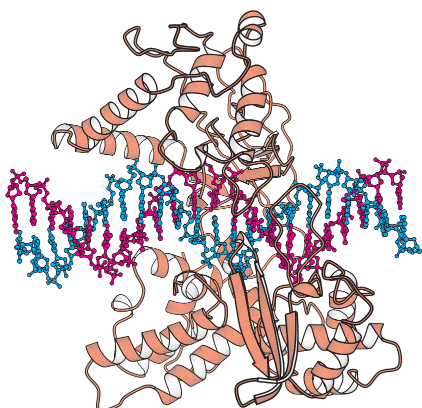


Figure 28.16 Structure of topoisomerase I. The structure of a complex between a fragment of human topoisomerase I and DNA is shown. *Notice* that DNA lies in a central cavity within the enzyme. [Drawn from 1EJ9.pdb.]

Type I topoisomerases relax supercoiled structures

The three-dimensional structures of several type I topoisomerases have been determined (Figure 28.16). These structures reveal many features of the reaction mechanism. Human type I topoisomerase comprises four domains, which are arranged around a central cavity having a diameter of 20 Å, just the correct size to accommodate a double-stranded DNA molecule. This cavity also includes a tyrosine residue (Tyr 723), which acts as a nucleophile to cleave the DNA backbone in the course of catalysis.

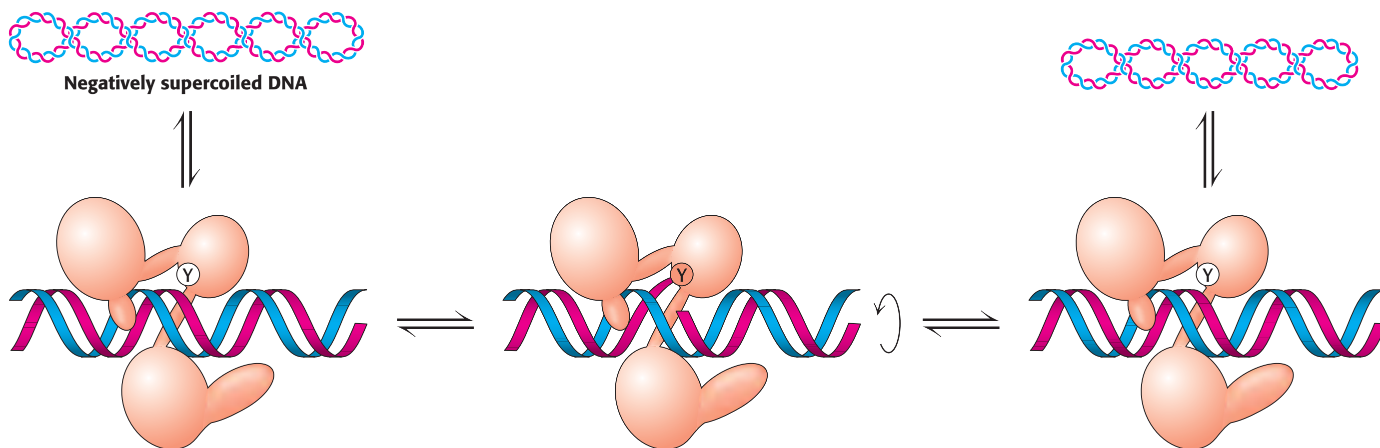
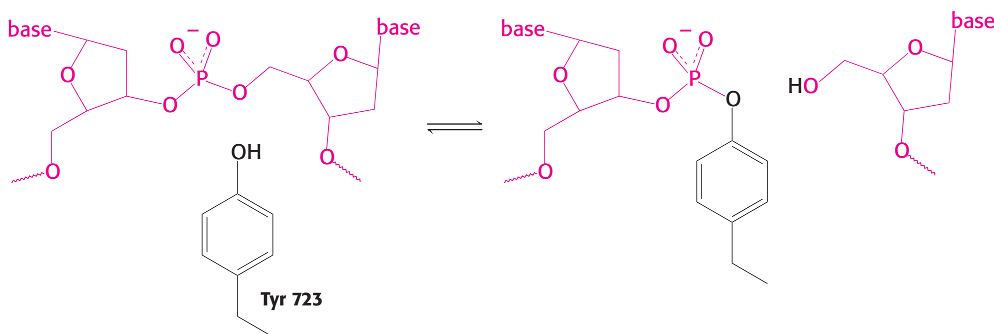


Figure 28.17 Topoisomerase I mechanism. On binding to DNA, topoisomerase I cleaves one strand of the DNA by means of a tyrosine (Y) residue attacking a phosphoryl group. When the strand has been cleaved, it rotates in a controlled manner around the other strand. The reaction is completed by religation of the cleaved strand. This process results in partial or complete relaxation of a supercoiled plasmid.

From analyses of these structures and the results of other studies, the relaxation of negatively supercoiled DNA molecules is known to proceed in the following manner (Figure 28.17). First, the DNA molecule binds inside the cavity of the topoisomerase. The hydroxyl group of tyrosine 723 attacks a phosphoryl group on one strand of the DNA backbone to form a phosphodiester linkage between the enzyme and the DNA, cleaving the DNA and releasing a free 5′-hydroxyl group.



With the backbone of one strand cleaved, the DNA can now rotate around the remaining strand, its movement driven by the release of energy stored because of the supercoiling. The rotation of the DNA unwinds the supercoils. The enzyme controls the rotation so that the unwinding is not rapid. The free hydroxyl group of the DNA attacks the phosphotyrosine residue to reseal the backbone and release tyrosine. The DNA is then free to dissociate from the enzyme. Thus, reversible cleavage of one strand of supercoiled DNA allows controlled rotation to partly relax the supercoils.

Type II topoisomerases can introduce negative supercoils through coupling to ATP hydrolysis

Supercoiling requires an input of energy because a supercoiled molecule, in contrast with its relaxed counterpart, is torsionally stressed. The introduction of an additional supercoil into a 3000-bp plasmid typically requires about 30 kJ mol^{-1} (7 kcal mol^{-1}).

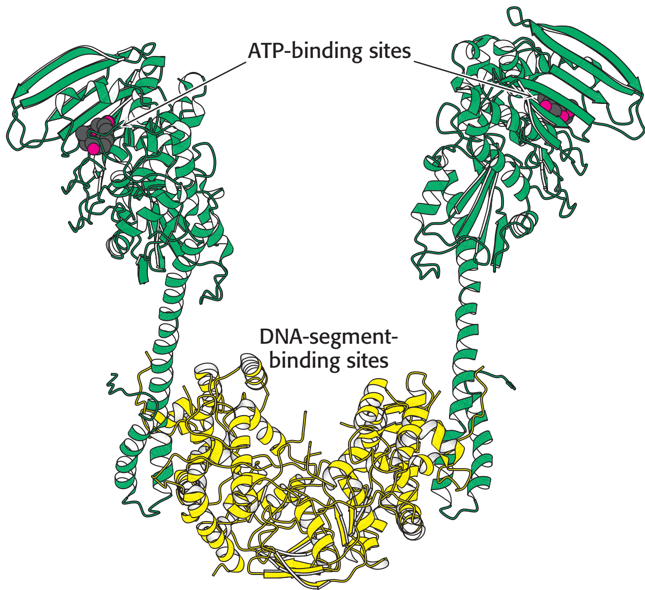


Figure 28.18 Structure of topoisomerase II. The dimeric structure of a typical topoisomerase II, that from the archaeon *Sulfolobus shibatae. Notice* that each half of the enzyme has one domain (shown in yellow) that contains a region for binding a DNA double helix and another domain (shown in green) that contains ATP binding sites. [Drawn from 2ZBK.pdb.]

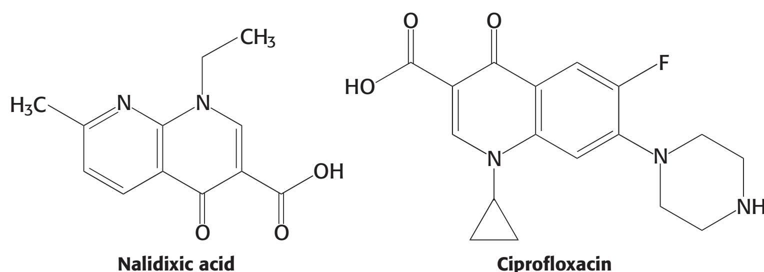
Supercoiling can be catalyzed by type II topoisomerases. These elegant molecular machines couple the binding and hydrolysis of ATP to the directed passage of one DNA double helix through another, temporarily cleaved DNA double helix. These enzymes have several mechanistic features in common with the type I topoisomerases.

Topoisomerase II molecules are dimeric with a large internal cavity (Figure 28.18). The large cavity has gates at both the top and the bottom that are crucial to topoisomerase action. The reaction begins with the binding of one double helix (hereafter referred to as the G, for gate, segment) to the enzyme (Figure 28.19). Each strand is positioned next to a tyrosine residue, one from each monomer, capable of forming a covalent linkage with the DNA backbone. This complex then loosely binds a second DNA double helix (hereafter referred to as the T, for transported, segment). Each monomer of the enzyme has a domain that binds ATP; this ATP binding leads to a conformational change that strongly favors the coming together of the two domains. As these domains come closer together, they trap the bound T segment. This conformational change also forces the separation and cleavage of the two strands of the G segment. Each strand is linked to the enzyme by a tyrosine–phosphodiester bond. Unlike the type I enzymes,

Figure 28.19 Topoisomerase II mechanism. Topoisomerase II first binds one DNA duplex termed the G (for gate) segment. The binding of ATP to the two N-terminal domains brings these two domains together. This conformational change leads to the cleavage of both strands of the G segment and the binding of an additional DNA duplex, the T segment. This T segment then moves through the break in the G segment and out the bottom of the enzyme. The hydrolysis of ATP resets the enzyme with the G segment still bound.

the type II topoisomerases hold the DNA tightly so that it cannot rotate. The T segment then passes through the cleaved G segment and into the large central cavity. The ligation of the G segment leads to the release of the T segment through the gate at the bottom of the enzyme. The hydrolysis of ATP and the release of ADP and orthophosphate allow the ATP-binding domains to separate, preparing the enzyme to bind another T segment. The overall process leads to a decrease in the linking number by two.

The bacterial topoisomerase II (often called DNA gyrase) is the target of several antibiotics that inhibit the prokaryotic enzyme much more than the eukaryotic one. *Novobiocin* blocks the binding of ATP to gyrase. *Nalidixic acid* and *ciprofloxacin,* in contrast, interfere with the breakage and rejoining of DNA chains. These two gyrase inhibitors are widely used to treat urinary-tract and other infections including those due to *Bacillus anthracis* (anthrax). *Camptothecin,* an antitumor agent, inhibits human topoisomerase I by stabilizing the form of the enzyme covalently linked to DNA.



28.3 DNA Replication Is Highly Coordinated

DNA replication must be very rapid, given the sizes of the genomes and the rates of cell division. The *E. coli* genome contains 4.6 million base pairs and is copied in less than 40 minutes. Thus, 2000 bases are incorporated per second. Enzyme activities must be highly coordinated to replicate entire genomes precisely and rapidly.

We begin our consideration of the coordination of DNA replication by looking at *E. coli,* which has been extensively studied. For this organism with a relatively small genome, replication begins at a single site and continues around the circular chromosome. The coordination of eukaryotic DNA replication is more complex because there are many initiation sites throughout the genome and an additional enzyme is needed to replicate the ends of linear chromosomes.

DNA replication requires highly processive polymerases

Replicative polymerases are characterized by their *very high catalytic potency, fidelity, and processivity. Processivity* refers to the ability of an enzyme to catalyze many consecutive reactions without releasing its substrate. These polymerases are assemblies of many subunits that have evolved to grasp their templates and not let go until many nucleotides have been added. The source of the processivity was revealed by the determination of the three-dimensional structure of the β_2 subunit of the *E. coli* replicative polymerase called DNA polymerase III (Figure 28.20). This unit keeps the polymerase associated with the DNA double helix. It has the form of a star-shaped ring. A 35-Å-diameter hole in its center can readily accommodate a duplex DNA molecule, yet leaves enough space between the DNA and the protein to allow rapid sliding during replication. To achieve a catalytic rate of 1000

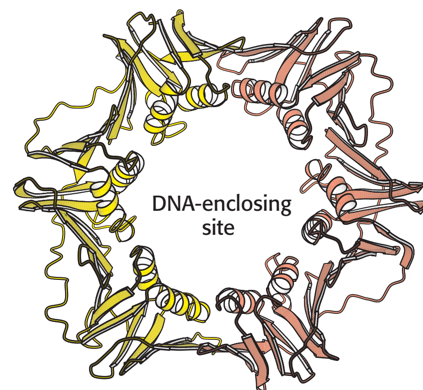


Figure 28.20 Structure of a sliding DNA clamp. The dimeric β subunit of DNA polymerase III forms a ring that surrounds the DNA duplex. *Notice* the central cavity through which the DNA template slides. Clasping the DNA molecule in the ring, the polymerase enzyme is able to move without falling off the DNA substrate. [Drawn from 2POL.pdb.]

Processive enzyme

From the Latin *procedere,* "to go forward."

An enzyme that catalyzes multiple rounds of the elongation or digestion of a polymer while the polymer stays bound. A *distributive enzyme,* in contrast, releases its polymeric substrate between successive catalytic steps.

nucleotides polymerized per second requires that 100 turns of duplex DNA (a length of 3400 Å, or 0.34 mm) slide through the central hole of β_2 per second. Thus, *β_2 plays a key role in replication by serving as a sliding DNA clamp*.

How does DNA become entrapped inside the sliding clamp? Replicative polymerases also include assemblies of subunits that function as *clamp loaders*. These enzymes grasp the sliding clamp and, utilizing the energy of ATP binding, pull apart one of the interfaces between the two subunits of the sliding clamp. DNA can move through the gap, inserting itself through the central hole. ATP hydrolysis then releases the clamp, which closes around the DNA.

The leading and lagging strands are synthesized in a coordinated fashion

Replicative polymerases such as DNA polymerase III synthesize the leading and lagging strands simultaneously at the replication fork (Figure 28.21). DNA polymerase III begins the synthesis of the leading strand starting from the RNA primer formed by primase. The duplex DNA ahead of the polymerase is unwound by a hexameric helicase called DnaB. Copies of single-stranded-binding protein (SSB) bind to the unwound strands, keeping the strands separated so that both strands can serve as templates. The leading strand is synthesized continuously by polymerase III. Topoisomerase II concurrently introduces right-handed (negative) supercoils to avert a topological crisis.

The mode of synthesis of the lagging strand is necessarily more complex. As mentioned earlier, the lagging strand is synthesized in fragments so that $5' \rightarrow 3'$ polymerization leads to overall growth in the $3' \rightarrow 5'$ direction. Yet the synthesis of the lagging strand is coordinated with the synthesis of the leading strand. How is this coordination accomplished? Examination of the subunit composition of the DNA polymerase III holoenzyme reveals an elegant solution (Figure 28.22). The holoenzyme includes two copies of the polymerase core enzyme, which consists of the DNA polymerase itself (the α subunit); the ε subunit, a 3′-to-5′ proofreading exonuclease; another subunit called θ; and two copies of the dimeric β-subunit sliding clamp. The core enzymes are linked to a central structure having the subunit composition $\gamma\tau_2\delta\delta'\chi\phi$. The $\gamma\tau_2\delta\delta'$ complex is the clamp loader, and the χ and ϕ

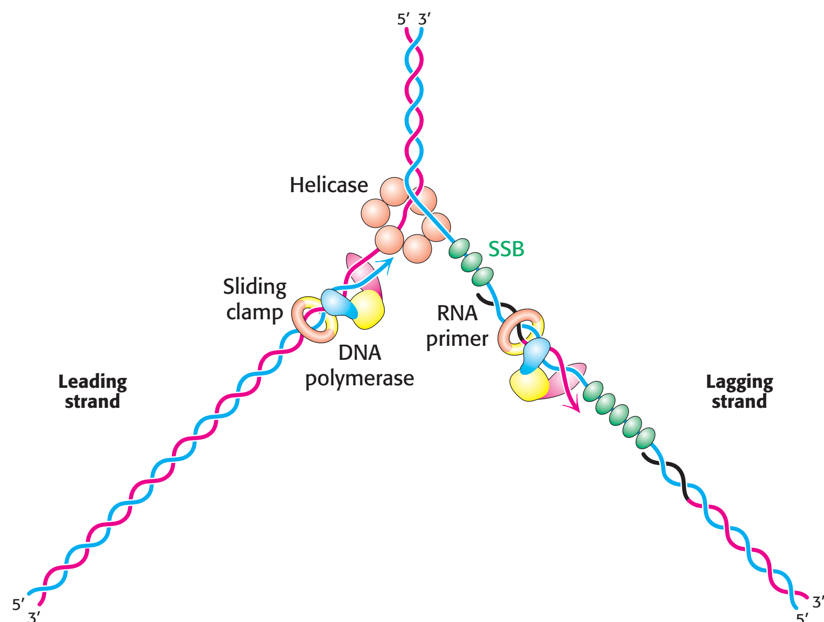


Figure 28.21 Replication fork. A schematic view of the arrangement of DNA polymerase III and associated enzymes and proteins present in the replication of DNA. The helicase separates the two strands of the parent double helix, allowing DNA polymerases to use each strand as a template for DNA synthesis. Abbreviation: SSB, single-stranded-binding protein.

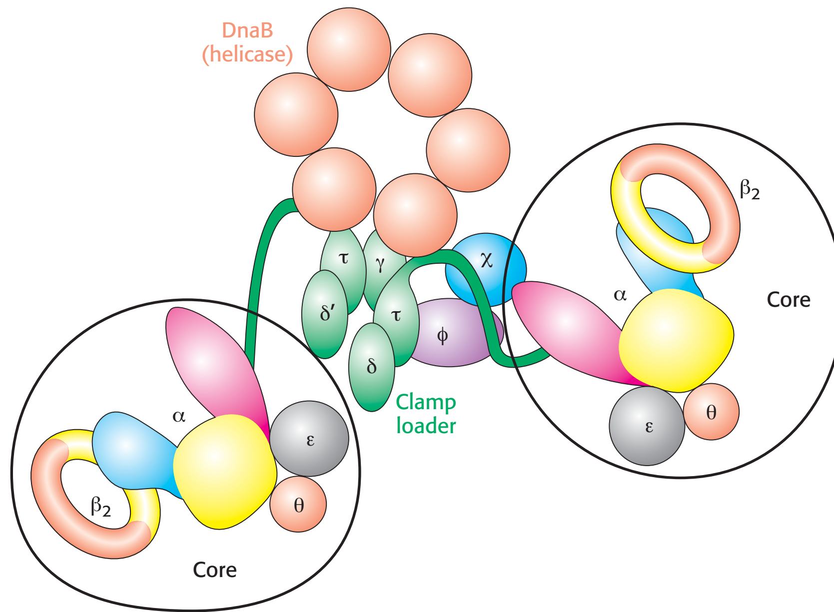


Figure 28.22 DNA polymerase holoenzyme. Each holoenzyme consists of two copies of the polymerase core enzyme, which comprises the α, ε, and θ subunits and two copies of the β subunit, linked to a central structure. The central structure includes the clamp-loader complex and the hexameric helicase DnaB.

subunits interact with the single-stranded-DNA–binding protein. The entire apparatus interacts with the hexameric helicase DnaB. Eukaryotic replicative polymerases have similar, albeit slightly more complicated, subunit compositions and structures.

The lagging-strand template is looped out so that it passes through the polymerase site in one subunit of a dimeric polymerase III in the same direction as that of the leading-strand template in the other subunit, $5' \rightarrow 3'$. DNA polymerase III lets go of the lagging-strand template after adding about 1000 nucleotides by releasing the sliding clamp. A new loop is then formed, a sliding clamp is added, and primase again synthesizes a short stretch of RNA primer to initiate the formation of another Okazaki fragment. This mode of replication has been termed the *trombone model* because the size of the loop lengthens and shortens like the slide on a trombone (Figure 28.23).

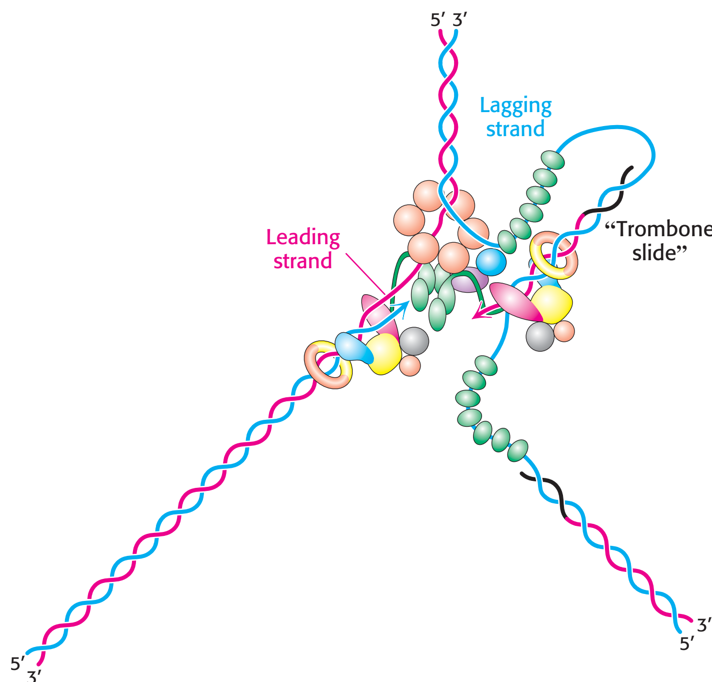


Figure 28.23 Trombone model. The replication of the leading and lagging strands is coordinated by the looping out of the lagging strand to form a structure that acts somewhat as a trombone slide, growing as the replication fork moves forward. When the polymerase on the lagging strand reaches a region that has been replicated, the sliding clamp is released and a new loop is formed.

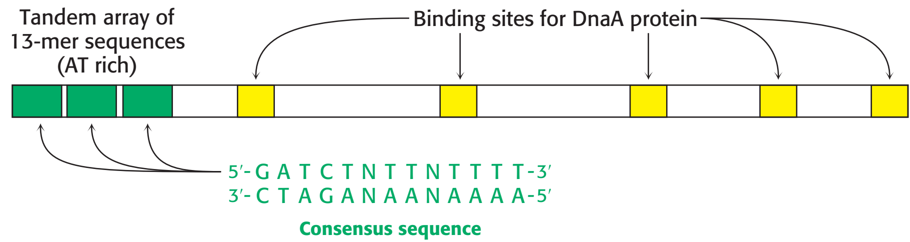


Figure 28.24 Origin of replication in *E. coli.* The *oriC* locus has a length of 245 bp. It contains a tandem array of three nearly identical 13-nucleotide sequences (green) and five binding sites (yellow) for the DnaA protein.

The gaps between fragments of the nascent lagging strand are filled by DNA polymerase I. This essential enzyme also uses its $5' \rightarrow 3'$ exonuclease activity to remove the RNA primer lying ahead of the polymerase site. The primer cannot be erased by DNA polymerase III, because the enzyme lacks $5' \rightarrow 3'$ editing capability. Finally, DNA ligase connects the fragments.

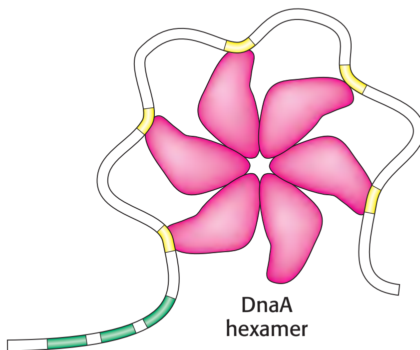


Figure 28.25 Assembly of DnaA. Monomers of DnaA bind to their binding sites (shown in yellow) in *oriC* and come together to form a complex structure, possibly the cyclic hexamer shown here. This structure marks the origin of replication and favors DNA strand separation in the AT-rich sites (green).

DNA replication in *Escherichia coli* begins at a unique site

In *E. coli,* DNA replication starts at a unique site within the entire 4.6×10^6 bp genome. This *origin of replication,* called the oriC *locus,* is a 245-bp region that has several unusual features (Figure 28.24). The *oriC* locus contains five copies of a sequence that are preferred binding sites for the origin-recognition protein DnaA. In addition, the locus contains a tandem array of 13-bp sequences that are rich in AT base pairs. Several steps are required to prepare for the start of replication:

1. *The binding of DnaA proteins to DNA is the first step in the preparation for replication.* DnaA is a member of the P-loop NTPase family related to the hexameric helicases. Each DnaA monomer comprises an ATPase domain linked to a DNA-binding domain at its C-terminus. DnaA molecules are able to bind to each other through their ATPase domains; a group of bound DnaA molecules will break apart on the binding and hydrolysis of ATP. The binding of DnaA molecules to one another signals the start of the preparatory phase, and their breaking apart signals the end of that phase. The DnaA proteins bind to the five high-affinity sites in *oriC* and then come together with DnaA molecules bound to lower-affinity sites to form an oligomer, possibly a cyclic hexamer. The DNA is wrapped around the outside of the DnaA hexamer (Figure 28.25).

2. *Single DNA strands are exposed in the prepriming complex*. With DNA wrapped around a DnaA hexamer, additional proteins are brought into play. The hexameric helicase DnaB is loaded around the DNA with the help of the helicase loader protein DnaC. Local regions of *oriC,* including the AT regions, are unwound and trapped by the single-stranded-DNA–binding protein. The result of this process is the generation of a structure called the *prepriming complex,* which makes single-stranded DNA accessible to other proteins (Figure 28.26). Significantly, the primase, DnaG, is now able to insert the RNA primer.

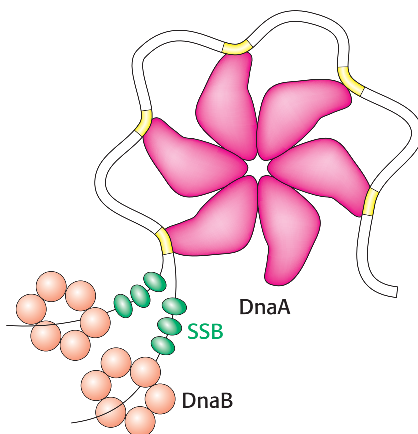


Figure 28.26 Prepriming complex. The AT-rich regions are unwound and trapped by the single-stranded-binding protein (SSB). The hexameric DNA helicase DnaB is loaded on each strand. At this stage, the complex is ready for the synthesis of the RNA primers and and assembly of the DNA polymerase III holoenzyme.

3. *The polymerase holoenzyme assembles*. The DNA polymerase III holoenzyme assembles on the prepriming complex, initiated by interactions between DnaB and the sliding-clamp subunit of DNA polymerase III. These interactions also trigger ATP hydrolysis within the DnaA subunits, signaling the initiation of DNA replication. The breakup of the DnaA assembly prevents additional rounds of replication from beginning at the replication origin.

DNA synthesis in eukaryotes is initiated at multiple sites

Replication in eukaryotes is mechanistically similar to replication in prokaryotes but is more challenging for a number of reasons. One of them is sheer size: *E. coli* must replicate 4.6 million base pairs, whereas a human diploid cell must replicate more than 6 billion base pairs. Second, the genetic information for *E. coli* is contained on 1 chromosome, whereas, in human beings, 23 pairs of chromosomes must be replicated. Finally, whereas the *E. coli* chromosome is circular, human chromosomes are linear. Unless countermeasures are taken, linear chromosomes are subject to shortening with each round of replication.

The first two challenges are met by the use of multiple origins of replication. In human beings, replication requires about 30,000 origins of replication, with each chromosome containing several hundred. Each origin of replication is the starting site for a replication unit, or *replicon.* In contrast with *E. coli,* the origins of replication in human beings do not contain regions of sharply defined sequence. Instead, more broadly defined AT-rich sequences are the sites around which the *origin of replication complexes* (ORCs) are assembled.

1. *The assembly of the ORC is the first step in the preparation for replication*. In human beings, the ORC is composed of six different proteins, each homologous to DnaA. These proteins likely come together to form a hexameric structure analogous to the assembly formed by DnaA.

2. *Licensing factors recruit a helicase that exposes single strands of DNA*. After the ORC has been assembled, additional proteins are recruited, including Cdc6, a homolog of the ORC subunits, and Cdt1. These proteins, in turn, recruit a hexameric helicase with six distinct subunits called Mcm2-7. These proteins, including the helicase, are sometimes called *licensing factors* because they permit the formation of the initiation complex. After the initiation complex has formed, Mcm2-7 separates the parental DNA strands, and the single strands are stabilized by the binding of *replication protein A,* a single-stranded-DNA–binding protein.

3. *Two distinct polymerases are needed to copy a eukaryotic replicon*. An initiator polymerase called *polymerase* α begins replication but is soon replaced by a more processive enzyme. This process is called *polymerase switching* because one polymerase has replaced another. This second enzyme, called *DNA polymerase* δ*,* is the principal replicative polymerase in eukaryotes (Table 28.1).

Table 28.1 Some types of DNA polymerases

Name	Function
Prokaryotic Polymerases	
DNA polymerase I	Erases primer and fills in gaps on lagging strand
DNA polymerase II (error-prone polymerase)	DNA repair
DNA polymerase III	Primary enzyme of DNA synthesis
Eukaryotic Polymerases	
DNA polymerase α	Initiator polymerase
Primase subunit	Synthesizes the RNA primer
DNA polymerase unit	Adds stretch of about 20 nucleotides to the primer
DNA polymerase β (error-prone polymerase)	DNA repair
DNA polymerase δ	Primary enzyme of DNA synthesis

Replication begins with the binding of DNA polymerase α. This enzyme includes a primase subunit, used to synthesize the RNA primer, as well as an active DNA polymerase. After this polymerase has added a stretch of about 20 deoxynucleotides to the primer, another replication protein, called *replication factor C* (RFC), displaces DNA polymerase α. Replication factor C attracts a sliding clamp called *proliferating cell nuclear antigen* (PCNA), which is homologous to the β_2 subunit of *E. coli* polymerase III. The binding of PCNA to DNA polymerase δ renders the enzyme highly processive and suitable for long stretches of replication. Replication continues in both directions from the origin of replication until adjacent replicons meet and fuse. RNA primers are removed and the DNA fragments are ligated by DNA ligase.

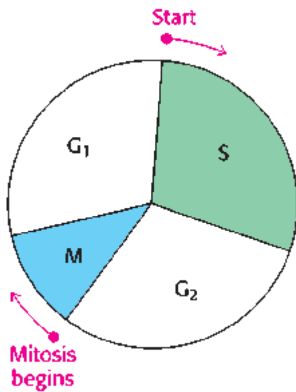


Figure 28.27 Eukaryotic cell cycle. DNA replication and cell division must take place in a highly coordinated fashion in eukaryotes. Mitosis (M) takes place only after DNA synthesis (S). Two gaps (G_1 and G_2) in time separate the two processes.

The use of multiple origins of replication requires mechanisms for ensuring that each sequence is replicated once and only once. The events of eukaryotic DNA replication are linked to the eukaryotic *cell cycle* (Figure 28.27). The processes of DNA synthesis and cell division are coordinated in the cell cycle so that the replication of all DNA sequences is complete before the cell progresses into the next phase of the cycle. This coordination requires several *checkpoints* that control the progression along the cycle. A family of small proteins termed *cyclins* are synthesized and degraded by proteasomal digestion in the course of the cell cycle. Cyclins act by binding to specific *cyclic-dependent protein kinases* and activating them. One such kinase, cyclin-dependent kinase 2 (cdk2) binds to assemblies at origins of replication and regulates replication through a number of interlocking mechanisms.

Telomeres are unique structures at the ends of linear chromosomes

Whereas the genomes of essentially all prokaryotes are circular, the chromosomes of human beings and other eukaryotes are linear. The free ends of linear DNA molecules introduce several complications that must be resolved by special enzymes. In particular, complete replication of DNA ends is difficult because polymerases act only in the $5' \rightarrow 3'$ direction. The lagging strand would have an incomplete $5'$ end after the removal of the RNA primer. Each round of replication would further shorten the chromosome.

The first clue to how this problem is resolved came from sequence analyses of the ends of chromosomes, which are called *telomeres* (from the Greek *telos,* "an end"). Telomeric DNA contains hundreds of tandem repeats of a six-nucleotide sequence. One of the strands is G rich at the $3'$ end, and it is slightly longer than the other strand. In human beings, the repeating G-rich sequence is AGGGTT.

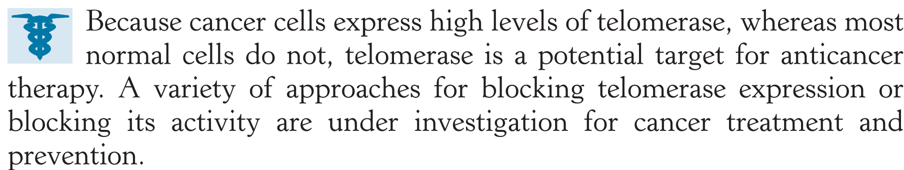
The structure adopted by telomeres has been extensively investigated. Recent evidence suggests that they may form large duplex loops (Figure 28.28). The single-stranded region at the very end of the structure has been proposed to loop back to form a DNA duplex with another part of the repeated sequence, displacing a part of the original telomeric duplex. This looplike structure is formed and stabilized by specific telomere-binding proteins. Such structures would nicely mask and protect the end of the chromosome.

Figure 28.28 Proposed model for telomeres. A single-stranded segment of the G-rich strand extends from the end of the telomere. In one model for telomeres, this single-stranded region invades the duplex to form a large duplex loop.

Telomeres are replicated by telomerase, a specialized polymerase that carries its own RNA template

How are the repeated sequences generated? An enzyme, termed *telomerase,* that executes this function has been purified and characterized. When a primer ending in GGTT is added to human telomerase in the presence of deoxynucleoside triphosphates, the sequences GGTTAGGGTT and GGTTAGGGTTAGGGTT, as well as longer products, are generated. Elizabeth Blackburn and Carol Greider discovered that the enzyme adding the repeats contains an RNA molecule that serves as the template for the elongation of the G-rich strand (Figure 28.29). Thus, telomerase carries the information necessary to generate the telomere sequences. The exact number of repeated sequences is not crucial.

Subsequently, a protein component of telomerases also was identified. This component is related to reverse transcriptases, enzymes first discovered in retroviruses that copy RNA into DNA. Thus, *telomerase is a specialized reverse transcriptase that carries its own template.* Telomerase is generally expressed at high levels only in rapidly growing cells. Thus, telomeres and telomerase can play important roles in cancer-cell biology and in cell aging.



Because cancer cells express high levels of telomerase, whereas most normal cells do not, telomerase is a potential target for anticancer therapy. A variety of approaches for blocking telomerase expression or blocking its activity are under investigation for cancer treatment and prevention.

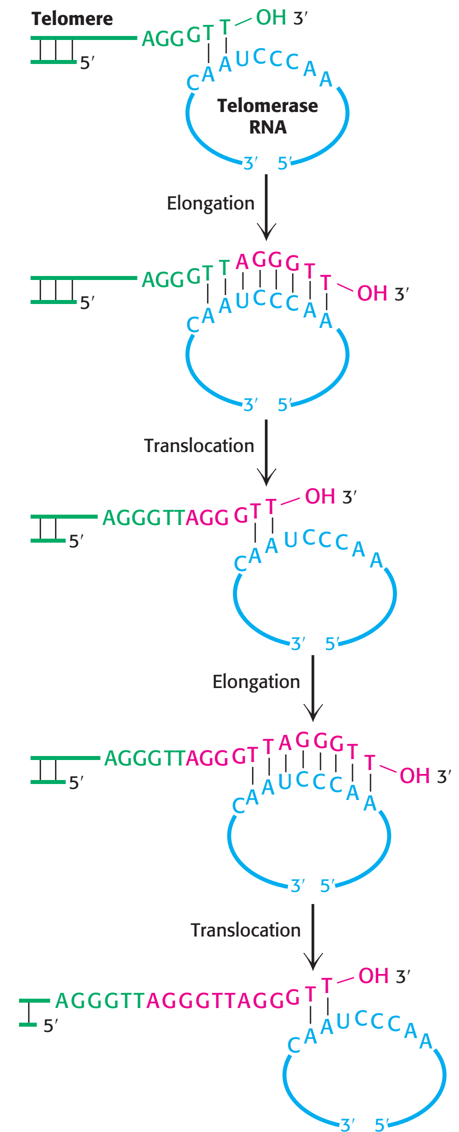


Figure 28.29 Telomere formation. Mechanism of synthesis of the G-rich strand of telomeric DNA. The RNA template of telomerase is shown in blue and the nucleotides added to the G-rich strand of the primer are shown in red. [After E. H. Blackburn. *Nature* 350:569–573, 1991.]

28.4 Many Types of DNA Damage Can Be Repaired

We have examined how even very large and complex genomes can, in principle, be replicated with considerable fidelity. However, DNA does become damaged, both in the course of replication and through other processes. Damage to DNA can be as simple as the misincorporation of a single base or it can take more-complex forms such as the chemical modification of bases, chemical cross-links between the two strands of the double helix, or breaks in one or both of the phosphodiester backbones. The results may be cell death or cell transformation, changes in the DNA sequence that can be inherited by future generations, or blockage of the DNA replication process itself. A variety of DNA-repair systems have evolved that can recognize these defects and, in many cases, restore the DNA molecule to its undamaged form. We begin with some of the sources of DNA damage.

Errors can arise in DNA replication

Errors introduced in the replication process are the simplest source of damage in the double helix. With the addition of each base, there is the possibility that an incorrect base might be incorporated, forming a non-Watson–Crick base pair. These non-Watson–Crick base pairs can locally distort the DNA double helix. Furthermore, such mismatches can be *mutagenic;* that is, they can result in permanent changes in the DNA sequence. When a double helix containing a non-Watson–Crick base pair is replicated, the two daughter double helices will have different sequences because the mismatched base is very likely to pair with its Watson–Crick partner. Errors other than mismatches include insertions, deletions, and breaks in one or both strands. Furthermore, replicative polymerases can stall or even fall off a damaged template entirely. As a consequence, replication of the genome may halt before it is complete.

A variety of mechanisms have evolved to deal with such interruptions, including specialized DNA polymerases that can replicate DNA across many lesions. A drawback is that such polymerases are substantially more error prone than are normal replicative polymerases. Nonetheless, these *error-prone polymerases* allow the completion of a draft sequence of the genome that can be at least partly repaired by DNA-repair processes. DNA recombination (Section 28.5) provides an additional mechanism for salvaging interruptions in DNA replication.

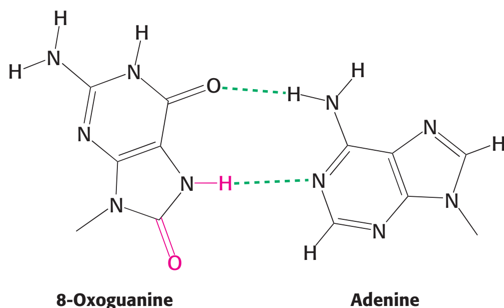


Figure 28.30 Oxoguanine–adenine base pair. When guanine is oxidized to 8-oxoguanine, the damaged base can form a base pair with adenine through an edge of the base that does not normally participate in base-pair formation.

Bases can be damaged by oxidizing agents, alkylating agents, and light

A variety of chemical agents can alter specific bases within DNA after replication is complete. Such *mutagens* include reactive oxygen species such as hydroxyl radical. For example, hydroxyl radical reacts with guanine to form 8-oxoguanine. 8-Oxoguanine is mutagenetic because it often pairs with adenine rather than cytosine in DNA replication. Its choice of pairing partner differs from that of guanine because it uses a different edge of the base to form base pairs (Figure 28.30). Deamination is another potentially deleterious process. For example, adenine can be deaminated to form hypoxanthine (Figure 28.31). This process is mutagenic because hypoxanthine pairs with cytosine rather than thymine. Guanine and cytosine also can be deaminated to yield bases that pair differently from the parent base.

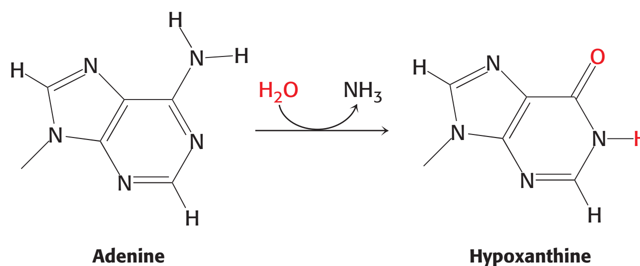


Figure 28.31 Adenine deamination. The base adenine can be deaminated to form hypoxanthine. Hypoxanthine forms base pairs with cytosine in a manner similar to that of guanine, and so the deamination reaction can result in mutation.

In addition to oxidation and deamination, nucleotide bases are subject to alkylation. Electrophilic centers can be attacked by nucleophiles such as N-7 of guanine and adenine to form alkylated adducts. Some compounds are converted into highly active electrophiles through the action of enzymes that normally play a role in detoxification. A striking example is aflatoxin B_1, a compound produced by molds that grow on peanuts and other foods. A cytochrome P450 enzyme (p. 781) converts this compound into a highly reactive epoxide (Figure 28.32). This agent reacts with the N-7 atom of guanosine to form a mutagenic adduct that frequently leads to a G–C-to-T–A transversion.

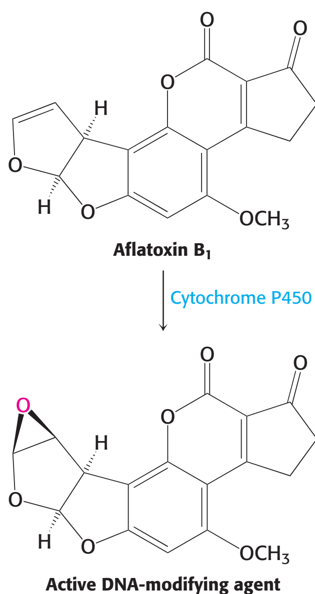


Figure 28.32 Aflatoxin activation. The compound, produced by molds that grow on peanuts, is activated by cytochrome P450 to form a highly reactive species that modifies bases such as guanine in DNA, leading to mutations.

The ultraviolet component of sunlight is a ubiquitous DNA-damaging agent. Its major effect is to covalently link adjacent pyrimidine residues along a DNA strand (Figure 28.33). Such a pyrimidine dimer cannot fit into a double helix, and so replication and gene expression are blocked until the lesion is removed.

A thymine dimer is an example of an *intra*strand cross-link because both participating bases are in the same strand of the double helix. Cross-links between bases on opposite strands also can be introduced by various agents. Psoralens are compounds produced by a Chinese herb that form such *inter*strand cross-links (Figure 28.34). Interstrand cross-links disrupt replication because they prevent strand separation.

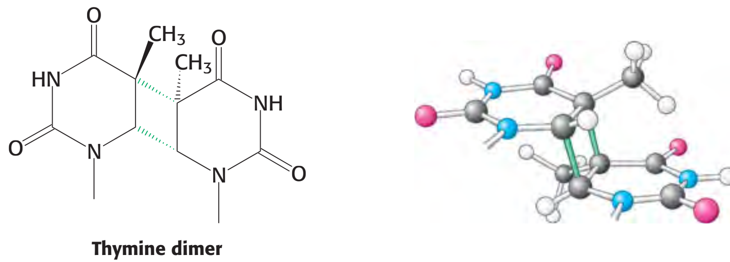


Figure 28.33 Cross-linked dimer of two thymine bases. Ultraviolet light induces cross-links between adjacent pyrimidines along one strand of DNA.

High-energy electromagnetic radiation such as x-rays can damage DNA by producing high concentrations of reactive species in solution. X-ray exposure can induce several types of DNA damage including single- and double-stranded breaks in DNA. This ability to induce such DNA damage led Hermann Muller to discover the mutagenic effects of x-rays in *Drosophila* in 1927. This discovery contributed to the development of *Drosophila* as one of the premier organisms for genetic studies.

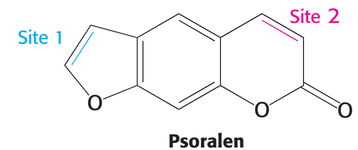


Figure 28.34 A cross-linking agent. The compound psoralen and its derivatives can form interstrand cross-links through two reactive sites that can form adducts with nucleotide bases.

DNA damage can be detected and repaired by a variety of systems

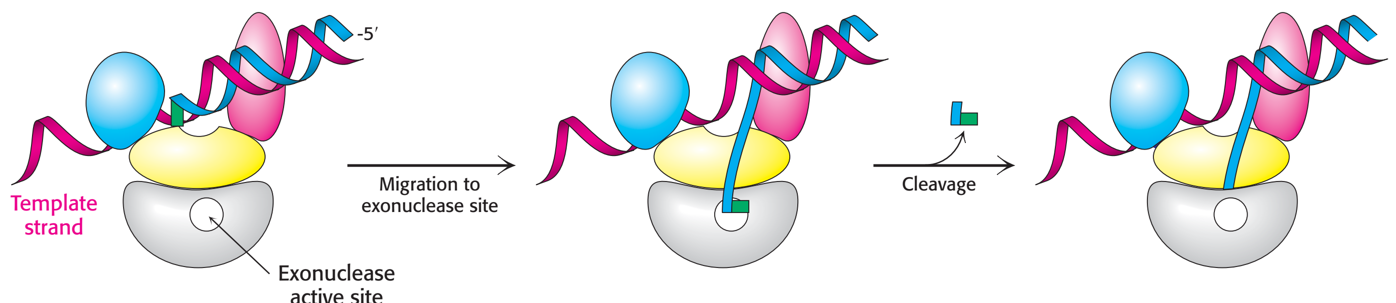
To protect the genetic message, a wide range of DNA-repair systems are present in most organisms. Many systems repair DNA by using sequence information from the uncompromised strand. Such single-strand replication systems follow a similar mechanistic outline:

1. Recognize the offending base(s).
2. Remove the offending base(s).
3. Repair the resulting gap with a DNA polymerase and DNA ligase.

We will briefly consider examples of several repair pathways. Although many of these examples are taken from *E. coli*, corresponding repair systems are present in most other organisms, including humans.

The replicative DNA polymerases themselves are able to correct many DNA mismatches produced in the course of replication. For example, the ε subunit of *E. coli* DNA polymerase III functions as a 3′-to-5′ exonuclease. This domain removes mismatched nucleotides from the 3′ end of DNA by hydrolysis. How does the enzyme sense whether a newly added base is correct? As a new strand of DNA is synthesized, it is *proofread*. If an incorrect base is inserted, then DNA synthesis slows down owing to the difficulty of threading a non-Watson–Crick base pair into the polymerase. In addition, the mismatched base is weakly bound and therefore able to fluctuate in position. The delay from the slowdown allows time for these fluctuations to take the newly synthesized strand out of the polymerase active site and into the exonuclease active site (Figure 28.35). There, the DNA is degraded, one

Figure 28.35 Proofreading. The growing polynucleotide chain occasionally leaves the polymerase site and migrates to the active site of exonuclease. There, one or more nucleotides are excised from the newly synthesized chain, removing potentially incorrect bases.



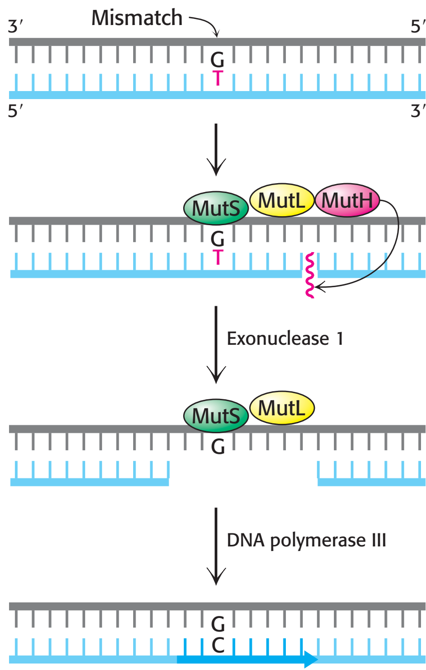


Figure 28.36 Mismatch repair. DNA mismatch repair in *E. coli* is initiated by the interplay of MutS, MutL, and MutH proteins. A G–T mismatch is recognized by MutS. MutH cleaves the backbone in the vicinity of the mismatch. A segment of the DNA strand containing the erroneous T is removed by exonuclease I and synthesized anew by DNA polymerase III. [After R. F. Service. *Science* 263:1559–1560, 1994.]

nucleotide at a time, until it moves back into the polymerase active site and synthesis continues.

A second mechanism is present in essentially all cells to correct errors made in replication that are not corrected by proofreading (Figure 28.36). *Mismatch-repair* systems consist of at least two proteins, one for detecting the mismatch and the other for recruiting an endonuclease that cleaves the newly synthesized DNA strand close to the lesion to facilitate repair. In *E. coli,* these proteins are called MutS and MutL and the endonuclease is called MutH.

Another mechanism of DNA repair is *direct repair,* one example of which is the photochemical cleavage of pyrimidine dimers. Nearly all cells contain a *photoreactivating enzyme* called *DNA photolyase.* The *E. coli* enzyme, a 35-kd protein that contains bound N^5,N^{10}-methenyltetrahydrofolate and flavin adenine dinucleotide (FAD) cofactors, binds to the distorted region of DNA. The enzyme uses light energy—specifically, the absorption of a photon by the N^5,N^{10}-methenyltetrahydrofolate coenzyme—to form an excited state that cleaves the dimer into its component bases.

The excision of modified bases such as 3-methyladenine by the *E. coli* enzyme *AlkA* is an example of *base-excision repair.* The binding of this enzyme to damaged DNA flips the affected base out of the DNA double helix and into the active site of the enzyme (Figure 28.37). The enzyme then acts as *a glycosylase,* cleaving the glycosidic bond to release the damaged base. At this stage, the DNA backbone is intact, but a base is missing. This hole is called an *AP site* because it is apurinic (devoid of A or G) or apyrimidinic (devoid of C or T). An *AP endonuclease* recognizes this defect and nicks the backbone adjacent to the missing base. *Deoxyribose phosphodiesterase* excises the residual deoxyribose phosphate unit, and DNA polymerase I inserts an undamaged nucleotide, as dictated by the base on the undamaged complementary strand. Finally, the repaired strand is sealed by DNA ligase.

One of the best-understood examples of *nucleotide-excision repair* is utilized for the excision of a pyrimidine dimer. Three enzymatic activities are essential for this repair process in *E. coli* (Figure 28.38). First, an enzyme complex consisting of the proteins encoded by the *uvrABC* genes detects the distortion produced by the DNA damage. The UvrABC enzyme then cuts

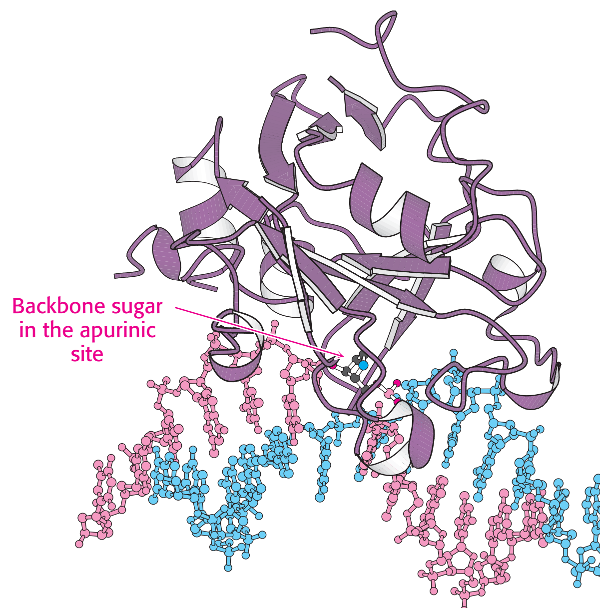


Figure 28.37 Structure of DNA-repair enzyme. A complex between the DNA-repair enzyme AlkA and an analog of a DNA molecule missing a purine base (an apurinic site) is shown. *Notice* that the backbone sugar in the apurinic site is flipped out of the double helix into the active site of the enzyme. [Drawn from 1BNK.pdb.]

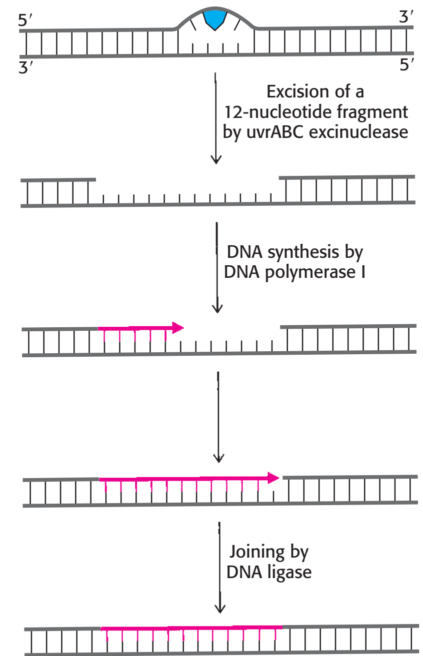
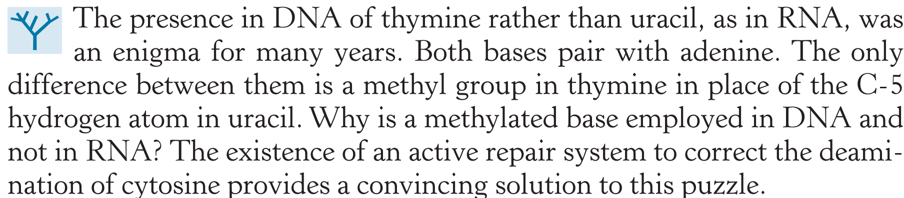


Figure 28.38 Nucleotide-excision repair. Repair of a region of DNA containing a thymine dimer by the sequential action of a specific exinuclease, a DNA polymerase, and a DNA ligase. The thymine dimer is shown in blue and the new region of DNA is in red. [After P. C. Hanawalt. *Endevour* 31:83, 1982.]

the damaged DNA strand at two sites, 8 nucleotides away from the damaged site on the 5′ side and 4 nucleotides away on the 3′ side. The 12-residue oligonucleotide excised by this highly specific *excinuclease* (from the Latin *exci,* "to cut out") then diffuses away. DNA polymerase I enters the gap to carry out repair synthesis. The 3′ end of the nicked strand is the primer, and the intact complementary strand is the template. Finally, the 3′ end of the newly synthesized stretch of DNA and the original part of the DNA chain are joined by DNA ligase.

DNA ligase is able to seal simple breaks in one strand of the DNA backbone. However, alternative mechanisms are required to repair breaks on both strands that are close enough together to separate the DNA into two double helices. Several distinct mechanisms are able to repair such damage. One mechanism, *nonhomologous end joining* (NHEJ), does not depend on other DNA molecules in the cell. In NHEJ, the free double-stranded ends are bound by a heterodimer of two proteins, Ku70 and Ku80. These proteins stabilize the ends and mark them for subsequent manipulations. Through mechanisms that are not yet well understood, the Ku70/80 heterodimers act as handles used by other proteins to draw the two double-stranded ends close together so that enzymes can seal the break.

Alternative mechanisms of double-stranded-break repair can operate if an intact stretch of double-stranded DNA with an identical or very similar sequence is present in the cell. These repair processes use homologous recombination, presented in Section 28.5.



The presence of thymine instead of uracil in DNA permits the repair of deaminated cytosine

The presence in DNA of thymine rather than uracil, as in RNA, was an enigma for many years. Both bases pair with adenine. The only difference between them is a methyl group in thymine in place of the C-5 hydrogen atom in uracil. Why is a methylated base employed in DNA and not in RNA? The existence of an active repair system to correct the deamination of cytosine provides a convincing solution to this puzzle.

Cytosine in DNA spontaneously deaminates at a perceptible rate to form uracil. The deamination of cytosine is potentially mutagenic because uracil pairs with adenine, and so one of the daughter strands will contain a U–A base pair rather than the original C–G base pair. This mutation is prevented by a repair system that recognizes uracil to be foreign to DNA (Figure 28.39). The repair enzyme, *uracil DNA glycosylase,* is homologous to AlkA. The enzyme hydrolyzes the glycosidic bond between the uracil and deoxyribose moieties but does not attack thymine-containing nucleotides. The AP site generated is repaired to reinsert cytosine. Thus, *the methyl group on thymine is a tag that distinguishes thymine from deaminated cytosine.* If thymine were not used in DNA, uracil correctly in place would be indistinguishable from uracil formed by deamination. The defect would persist unnoticed, and so a C–G base pair would necessarily be mutated to U–A in one of the daughter DNA molecules. This mutation is prevented by a repair system that searches for uracil and leaves thymine alone. *Thymine is used instead of uracil in DNA to enhance the fidelity of the genetic message.*

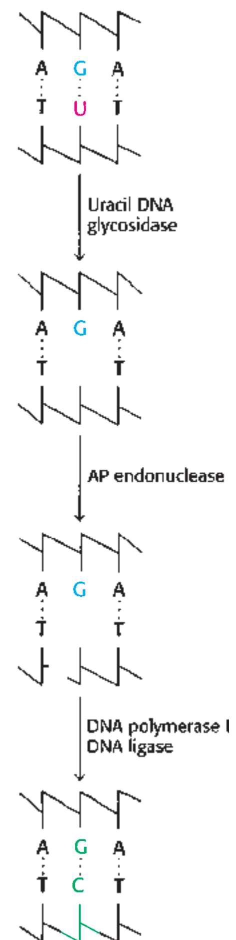


Figure 28.39 Uracil repair. Uridine bases in DNA, formed by the deamination of cytidine, are excised and replaced by cytidine.

Some genetic diseases are caused by the expansion of repeats of three nucleotides

Some genetic diseases are caused by the presence of DNA sequences that are inherently prone to errors in the course of repair and replication. A particularly important class of such diseases is characterized by the presence of long tandem arrays of repeats of three nucleotides. An example is *Huntington disease,* an autosomal dominant neurological disorder with a variable age of onset. The mutated gene in this disease expresses a protein in the brain called huntingtin, which contains a stretch of consecutive glutamine residues. These glutamine residues are encoded by a tandem array of CAG sequences within the gene. In unaffected persons, this array is between 6 and 31 repeats, whereas, in those with the disease, the array is between 36 and 82 repeats or longer. Moreover, the array tends to become longer from one generation to the next. The consequence is a phenomenon called *anticipation:* the children of an affected parent tend to show symptoms of the disease at an earlier age than did the parent.

The tendency of these *trinucleotide repeats* to expand is explained by the formation of alternative structures in the course of DNA repair. On cleavage of the DNA backbone, part of the array can loop out without disrupting base-pairing outside this region. Then, in replication, DNA polymerase extends this strand through the remainder of the array, leading to an increase in the number of copies of the trinucleotide sequence.

A number of other neurological diseases are characterized by expanding arrays of trinucleotide repeats. How do these long stretches of repeated amino acids cause disease? For huntingtin, it appears that the polyglutamine stretches become increasingly prone to aggregate as their length increases; the additional consequences of such aggregation are still under investigation.

Many cancers are caused by the defective repair of DNA

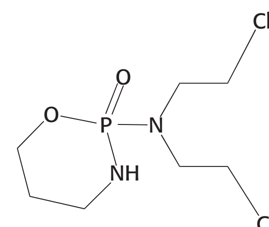
As described in Chapter 14, cancers are caused by mutations in genes associated with growth control. Defects in DNA-repair systems increase the overall frequency of mutations and, hence, the likelihood of cancer-causing mutations. Indeed, the synergy between studies of mutations that predispose people to cancer and studies of DNA repair in model organisms has been tremendous in revealing the biochemistry of DNA-repair pathways. Genes for DNA-repair proteins are often *tumor-suppressor genes;* that is, they suppress tumor development when at least one copy of the gene is free of a deleterious mutation. When both copies of a gene are mutated, however, tumors develop at rates greater than those for the population at large. People who inherit defects in a single tumor-suppressor allele do not necessarily develop cancer but are susceptible to developing the disease because only the one remaining normal copy of the gene must develop a new defect to further the development of cancer.

Consider, for example, *xeroderma pigmentosum,* a rare human skin disease. The skin of an affected person is extremely sensitive to sunlight or ultraviolet light. In infancy, severe changes in the skin become evident and worsen with time. The skin becomes dry, and there is a marked atrophy of the dermis. Keratoses appear, the eyelids become scarred, and the cornea ulcerates. Skin cancer usually develops at several sites. Many patients die before age 30 from metastases of these malignant skin tumors. Studies of xeroderma pigmentosum patients have revealed that mutations occur in genes for a number of different proteins. These proteins are components of the human nucleotide-excision-repair pathway, including homologs of the UvrABC subunits.

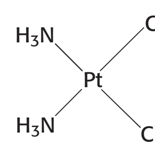
Defects in other repair systems can increase the frequency of other tumors. For example, *hereditary nonpolyposis colorectal cancer* (HNPCC, or *Lynch syndrome*) results from defective DNA mismatch repair. HNPCC is

not rare—as many as 1 in 200 people will develop this form of cancer. Mutations in two genes, called *hMSH2* and *hMLH1*, account for most cases of this hereditary predisposition to cancer. The striking finding is that these genes encode the human counterparts of MutS and MutL of *E. coli*. Mutations in *hMSH2* and *hMLH1* seem likely to allow mutations to accumulate throughout the genome. In time, genes important in controlling cell proliferation become altered, resulting in the onset of cancer.

Not all tumor-suppressor genes are specific to particular types of cancer. *The gene for a protein called p53 is mutated in more than half of all tumors*. The p53 protein helps control the fate of damaged cells. First, it plays a central role in sensing DNA damage, especially double-stranded breaks. Then, after sensing damage, the protein either promotes a DNA-repair pathway or activates the apoptosis pathway, leading to cell death. Most mutations in the p53 gene are sporatic; that is, they occur in somatic cells rather than being inherited. People who inherit a deleterious mutation in one copy of the p53 gene suffer from *Li-Fraumeni syndrome* and have a high probability of developing several types of cancer.



Cyclophosphamide



Cisplatin

Cancer cells often have two characteristics that make them especially vulnerable to agents that damage DNA molecules. First, they divide frequently, and so their DNA replication pathways are more active than they are in most cells. Second, as already noted, cancer cells often have defects in DNA-repair pathways. Several agents widely used in cancer chemotherapy, including cyclophosphamide and cisplatin, act by damaging DNA. Cancer cells are less able to avoid the effect of the induced damage than are normal cells, providing a therapeutic window for specifically killing cancer cells.

Many potential carcinogens can be detected by their mutagenic action on bacteria

Many human cancers are caused by exposure to chemicals that cause mutations. It is important to identify such compounds and to ascertain their potency so that human exposure to them can be minimized. Bruce Ames devised a simple and sensitive test for detecting chemical mutagens. In the *Ames test,* a thin layer of agar containing about 10^9 bacteria of a specially constructed tester strain of *Salmonella* is placed on a petri plate. These bacteria are unable to grow in the absence of histidine, because a mutation is present in one of the genes for the biosynthesis of this amino acid. The addition of a chemical mutagen to the center of the plate results in many new mutations. A small proportion of them reverse the original mutation, and histidine can be synthesized. These *revertants* multiply in the absence of an external source of histidine and appear as discrete colonies after the plate has been incubated at 37°C for 2 days (Figure 28.40). For example, 0.5 μg of 2-aminoanthracene gives 11,000 revertant colonies, compared with only 30 spontaneous revertants in its absence. A series of concentrations of a chemical can be readily tested to generate a dose–response curve. These curves are usually linear, which suggests that there is no threshold concentration for mutagenesis.

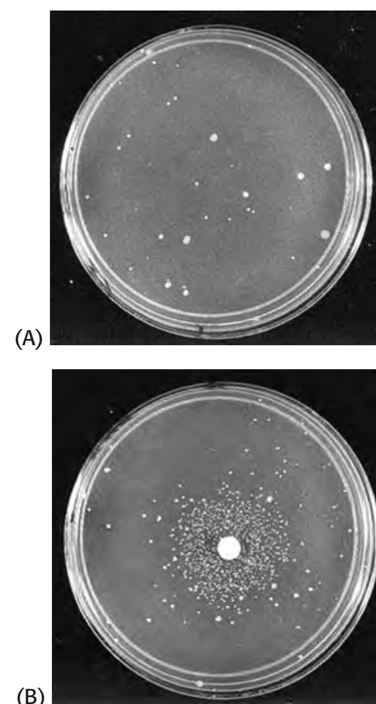


Figure 28.40 Ames test. (A) A petri plate containing about 10^9 *Salmonella* bacteria that cannot synthesize histidine and (B) a petri plate containing a filter-paper disc with a mutagen, which produces a large number of revertants that can synthesize histidine. After 2 days, the revertants appear as rings of colonies around the disc. The small number of visible colonies in plate A are spontaneous revertants. [From B. N. Ames, J. McCann, and E. Yamasake. *Mutat. Res.* 31:347–364, 1975.]

Some of the tester strains are responsive to *base-pair substitutions,* whereas others detect *deletions or additions of base pairs (frameshifts)*. The sensitivity of these specially designed strains has been enhanced by the genetic deletion of their excision-repair systems. Potential mutagens enter the tester strains easily because the lipopolysaccharide barrier that normally coats the surface of *Salmonella* is incomplete in these strains. A key feature of this detection system is the inclusion of a *mammalian liver homogenate*. Recall that some potential carcinogens such as aflatoxin are converted into their active forms by enzyme systems in the liver or other mammalian tissues. Bacteria lack these enzymes, and so the test plate requires a few milligrams of a liver homogenate to activate this group of mutagens.

The *Salmonella* test is extensively used to help evaluate the mutagenic and carcinogenic risks of a large number of chemicals. This rapid and inexpensive bacterial assay for mutagenicity complements epidemiological surveys and animal tests that are necessarily slower, more laborious, and far more expensive. The *Salmonella* test for mutagenicity is an outgrowth of studies of gene–protein relations in bacteria. It is a striking example of how fundamental research in molecular biology can lead directly to important advances in public health.

28.5 DNA Recombination Plays Important Roles in Replication, Repair, and Other Processes

Most processes associated with DNA replication function to copy the genetic message as faithfully as possible. However, several biochemical processes require the *recombination* of genetic material between two DNA molecules. In genetic recombination, two daughter molecules are formed by the exchange of genetic material between two parent molecules (Figure 28.41). Recombination is essential in the following processes.

1. When replication stalls, recombination processes can reset the replication machinery so that replication can continue.
2. Some double-stranded breaks in DNA are repaired by recombination.
3. In meiosis, the limited exchange of genetic material between paired chromosomes provides a simple mechanism for generating genetic diversity in a population.
4. As we shall see in Chapter 34, recombination plays a crucial role in generating molecular diversity for antibodies and some other molecules in the immune system.
5. Some viruses employ recombination pathways to integrate their genetic material into the DNA of a host cell.
6. Recombination is used to manipulate genes in, for example, the generation of "gene knockout" mice (Section 5.4).

Recombination is most efficient between DNA sequences that are similar in sequence. In homologous recombination, parent DNA duplexes align at regions of sequence similarity, and new DNA molecules are formed by the breakage and joining of homologous segments.

Figure 28.41 Recombination. Two DNA molecules can recombine with each other to form new DNA molecules that have segments from both parent molecules.

RecA can initiate recombination by promoting strand invasion

In many recombination pathways, a DNA molecule with a free end recombines with a DNA molecule having no free ends available for interaction. DNA molecules with free ends are the common result of double-stranded DNA breaks, but they may also be generated in DNA replication if the replication complex stalls. This type of recombination has been studied extensively in *E. coli,* but it also takes place in other organisms through the

Figure 28.42 Strand invasion. This process, promoted by proteins such as RecA, can initiate recombination.

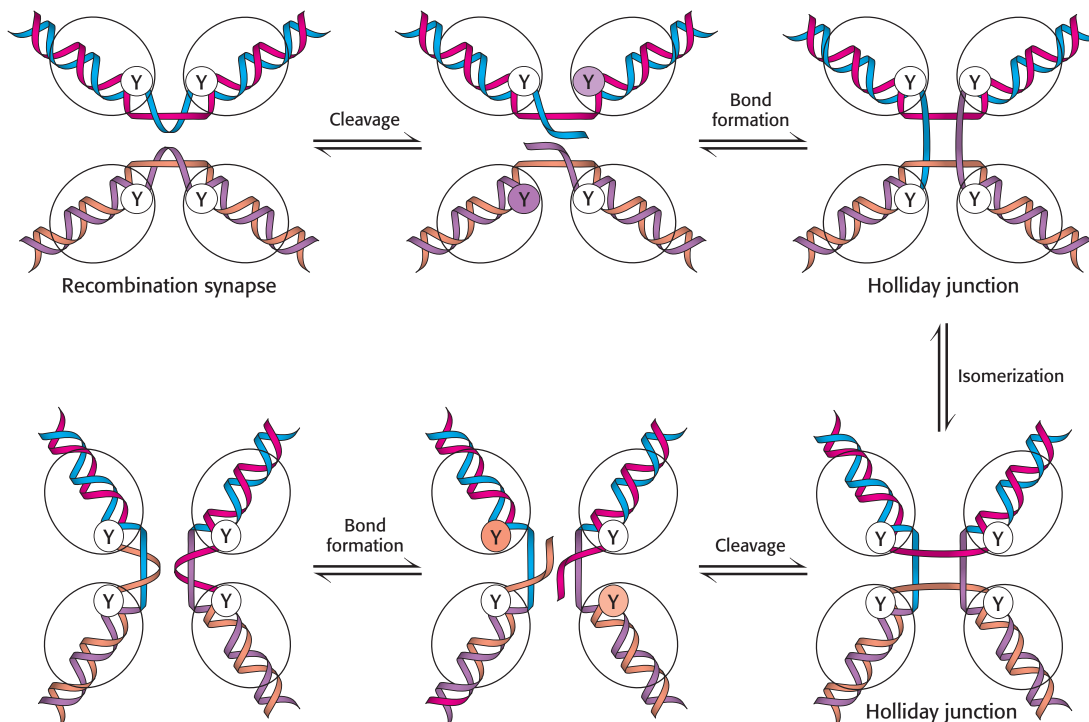
action of proteins homologous to those of *E. coli*. Often dozens of proteins participate in the complete recombination process. However, the key protein is *RecA*. To accomplish the exchange, the single-stranded DNA displaces one of the strands of the double helix (Figure 28.42). The resulting three-stranded structure is called a *displacement loop* or *D-loop*. This process is often referred to as *strand invasion*. Because a free 3′ end is now base-paired to a contiguous strand of DNA, the 3′ end can act as a primer to initiate new DNA synthesis. Strand invasion can initiate many processes, including the repair of double-stranded breaks and the reinitiation of replication after the replication apparatus has come off its template. In the repair of a break, the recombination partner is an intact DNA molecule with an overlapping sequence.

Some recombination reactions proceed through Holliday-junction intermediates

In recombination pathways for meiosis and some other processes, intermediates form that are composed of four polynucleotide chains in a crosslike structure. Intermediates with these crosslike structures are often referred to as *Holliday junctions,* after Robin Holliday, who proposed their role in recombination in 1964. Such intermediates have been characterized by a wide range of techniques, including x-ray crystallography.

Specific enzymes, termed *recombinases,* bind to these structures and resolve them into separated DNA duplexes. The Cre recombinase from bacteriophage P1 has been extensively studied. The mechanism begins with the recombinase binding to the DNA substrates (Figure 28.43).

Figure 28.43 Recombination mechanism. Recombination begins as two DNA molecules come together to form a recombination synapse. One strand from each duplex is cleaved by the recombinase enzyme; the 3′ end of each of the cleaved strands is linked to a tyrosine (Y) residue on the recombinase enzyme. New phosphodiester bonds are formed when a 5′ end of the other cleaved strand in the complex attacks these tyrosine–DNA adducts. After isomerization, these steps are repeated to form the recombined products.



Four molecules of the enzyme and two DNA molecules come together to form a *recombination synapse*. The reaction begins with the cleavage of one strand from each duplex. The 5′-hydroxyl group of each cleaved strand remains free, whereas the 3′-phosphoryl group becomes linked to a specific tyrosine residue in the recombinase. The free 5′ ends invade the other duplex in the synapse and attack the DNA–tyrosine units to form new phosphodiester bonds and free the tyrosine residues. These reactions result in the formation of a Holliday junction. This junction can then isomerize to form a structure in which the polynucleotide chains in the center of the structure are reoriented. From this junction, the processes of strand cleavage and phosphodiester-bond formation repeat. The result is a synapse containing the two recombined duplexes. Dissociation of this complex generates the final recombined products.

Cre catalyzes the formation of Holliday junctions as well as their resolution. In contrast, other proteins bind to Holliday junctions that have already been formed by other processes and resolve them into separate duplexes. In many cases, these proteins also promote the process of branch migration whereby a Holliday junction is moved along the two component double helices. Branch migration can affect which segments of DNA are exchanged in a recombination process.

Summary

28.1 DNA Replication Proceeds by the Polymerization of Deoxyribonucleoside Triphosphates Along a Template

DNA polymerases are template-directed enzymes that catalyze the formation of phosphodiester bonds by nucleophilic attack by the 3′-hydroxyl group on the innermost phosphorus atom of a deoxyribonucleoside 5′-triphosphate. The complementarity of shape between correctly matched nucleotide bases is crucial to ensuring the fidelity of base incorporation. DNA polymerases cannot start chains de novo; a primer with a free 3′-hydroxyl group is required. Thus DNA synthesis is initiated by the synthesis of an RNA primer, the task of a specialized primase enzyme. After serving as a primer, the RNA is degraded and replaced by DNA. DNA polymerases always synthesize a DNA strand in the 5′-to-3′ direction. So that both strands of the double helix can be synthesized in the same direction simultaneously, one strand is synthesized continuously while the other is synthesized in fragments called Okazaki fragments. Gaps between the fragments are sealed by DNA ligases. ATP-driven helicases prepare the way for DNA replication by separating the strands of the double helix.

28.2 DNA Unwinding and Supercoiling Are Controlled by Topoisomerases

A key topological property of DNA is its linking number *(Lk)*, which is defined as the number of times one strand of DNA winds around the other in the right-hand direction when the DNA axis is constrained to lie in a plane. Molecules differing in linking number are topoisomers of one another and can be interconverted only by cutting one or both DNA strands; these reactions are catalyzed by topoisomerases. Changes in linking number generally lead to changes in both the number of turns of double helix and the number of turns of superhelix. Topoisomerase II catalyzes the ATP-driven introduction of negative supercoils, which leads to the compaction of DNA and renders it more susceptible to unwinding. Supercoiled DNA can be relaxed by topoisomerase I or topoisomerase II. Topoisomerase I acts by transiently

cleaving one strand of DNA in a double helix, whereas topoisomerase II transiently cleaves both strands simultaneously.

28.3 DNA Replication Is Highly Coordinated

Replicative DNA polymerases are processive; that is, they catalyze the addition of many nucleotides without dissociating from the template. A major contributor to processivity is the DNA sliding clamp, such as the dimeric β subunit of the *E. coli* replicative polymerase. The sliding clamp has a ring structure that encircles the DNA double helix and keeps the enzyme and DNA associated. The DNA polymerase holoenzyme is a large DNA-copying machine formed by two DNA polymerase enzymes, one to act on each template strand, associated with other subunits including a sliding clamp and a clamp loader.

The synthesis of the leading and lagging strands of a double-stranded DNA template is coordinated. As a replicative polymerase moves along a DNA template, the leading strand is copied smoothly while the lagging strand forms loops that change length in the course of the synthesis of each Okazaki fragment. The mode of action is referred to as the trombone model.

DNA replication is initiated at a single site within the *E. coli* genome. A set of specific proteins recognize this origin of replication and assemble the enzymes needed for DNA synthesis, including a helicase that promotes strand separation. The initiation of replication in eukaryotes is more complex. DNA synthesis is initiated at thousands of sites throughout the genome. Assemblies homologous to those in *E. coli,* but more complicated, are assembled at each eukaryotic origin of replication. A special polymerase called telomerase that relies on an RNA template synthesizes specialized structures called telomeres at the ends of linear chromosomes.

28.4 Many Types of DNA Damage Can Be Repaired

A wide variety of DNA damage can occur. For example, mismatched bases may be incorporated in the course of DNA replication or individual bases may be damaged by oxidation or alkylation after DNA replication. Other forms of damage are the formation of cross-links and the introduction of single- or double-stranded breaks in the DNA backbone. Several different repair systems detect and repair DNA damage. Repair begins with the process of proofreading in DNA replication: mismatched bases that were incorporated in the course of synthesis are excised by exonuclease activity present in replicative polymerases. Some DNA lesions such as thymine dimers can be directly reversed through the action of specific enzymes. Other DNA-repair pathways act through the excision of single damaged bases (base-excision repair) or short segments of nucleotides (nucleotide-excision repair). Double-stranded breaks in DNA can be repaired by homologous or nonhomologous end-joining processes. Defects in DNA-repair components are associated with susceptibility to many different sorts of cancer. Such defects are a common target of cancer treatments. Many potential carcinogens can be detected by their mutagenic action on bacteria (the Ames test).

28.5 DNA Recombination Plays Important Roles in Replication, Repair, and Other Processes

Recombination is the exchange of segments between two DNA molecules. Recombination is important in some types of DNA repair as well as other processes such as meiosis, the generation of antibody diversity, and the life cycles of some viruses. Some recombination

pathways are initiated by strand invasion, in which a single strand at the end of a DNA double helix forms base pairs with one strand of DNA in another double helix and displaces the other strand. A common intermediate formed in other recombination pathways is the Holliday junction, which consists of four strands of DNA that come together to form a crosslike structure. Recombinases promote recombination reactions through the introduction of specific DNA breaks and the formation and resolution of Holliday-junction intermediates.

Key Terms

template (p. 820)
DNA polymerase (p. 820)
primer (p. 821)
exonuclease (p. 821)
primase (p. 823)
replication fork (p. 823)
Okazaki fragment (p. 823)
lagging strand (p. 824)
leading stand (p. 824)
DNA ligase (p. 824)
helicase (p. 824)
supercoil (p. 826)
linking number (p. 826)
topoisomer (p. 826)
twist (p. 826)
writhe (p. 826)
topoisomerase (p. 828)
processivity (p. 831)
sliding clamp (p. 832)
trombone model (p. 833)
origin of replication (p. 834)
origin of replication complex (ORC) (p. 835)
cell cycle (p. 836)
telomere (p. 836)
telomerase (p. 837)
mutagen (p. 837)
mismatch repair (p. 840)
direct repair (p. 840)
base-excision repair (p. 840)
nucleotide-excision repair (p. 840)
nonhomologous end joining (NHEJ) (p. 841)
trinucleotide repeat (p. 842)
tumor-suppressor gene (p. 842)
Ames test (p. 843)
RecA (p. 845)
Holliday junction (p. 845)
recombinase (p. 845)
recombination synapse (p. 846)

Problems

1. *Activated intermediates.* DNA polymerase I, DNA ligase, and topoisomerase I catalyze the formation of phosphodiester bonds. What is the activated intermediate in the linkage reaction catalyzed by each of these enzymes? What is the leaving group?

2. *Life in a hot tub.* An archaeon *(Sulfolobus acidocaldarius)* found in acidic hot springs contains a topoisomerase that catalyzes the ATP-driven introduction of positive supercoils into DNA. How might this enzyme be advantageous to this unusual organism?

3. *Which way?* Provide a chemical explanation of why DNA synthesis proceeds in a 5′-to-3′ direction.

4. *Nucleotide requirement*. DNA replication does not take place in the absence of the ribonucleotides ATP, CTP, GTP, and UTP. Propose an explanation.

5. *Close contact.* Examination of the structure of DNA polymerases bound to nucleotide analogs reveals that conserved residues come within van der Waals contact of C-2′ of the bound nucleotide. What is the potential significance of this interaction?

6. *Molecular motors in replication.* (a) How fast does template DNA spin (expressed in revolutions per second) at an *E. coli* replication fork? (b) What is the velocity of movement (in micrometers per second) of DNA polymerase III holoenzyme relative to the template?

7. *Wound tighter than a drum.* Why would replication come to a halt in the absence of topoisomerase II?

8. *The missing link*. One form of a plasmid shows a twist of $Tw = 48$ and a writhe of $Wr = 3$. What is the linking number? What would the value of writhe be for a form with twist $Tw = 50$ if the linking number is the same as that for the preceding form.

9. *Telomeres and cancer.* Telomerase is not active in most human cells. Some cancer biologists have suggested that activation of the telomerase gene would be a requirement for a cell to become cancerous. Explain why it might be the case.

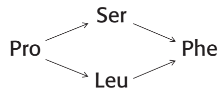
10. *Backward?* Bacteriophage T7 helicase moves along DNA in the 5′-to-3′ direction. Other helicases have been reported to move in the 3′-to-5′ direction. Is there any fun-

damental reason why you would expected helicases to move in one direction or the other?

11. *Nick translation.* Suppose that you wish to make a sample of DNA duplex highly radioactive to use as a DNA probe. You have a DNA endonuclease that cleaves the DNA internally to generate 3′-OH and 5′-phosphoryl groups, intact DNA polymerase I, and radioactive dNTPs. Suggest a means for making the DNA radioactive.

12. *Revealing tracks.* Suppose that replication is initiated in a medium containing *moderately* radioactive tritiated thymine. After a few minutes of incubation, the bacteria are transferred to a medium containing *highly* radioactive tritiated thymidine. Sketch the autoradiographic pattern that would be seen for (a) undirectional replication and (b) bidirectional replication, each from a single origin.

13. *Mutagenic trail.* Suppose that the single-stranded RNA from tobacco mosaic virus was treated with a chemical mutagen, that mutants were obtained having serine or leucine instead of proline at a specific position, and that further treatment of these mutants with the same mutagen yielded phenylalanine at this position.



(a) What are the plausible codon assignments for these four amino acids?

(b) Was the mutagen 5-bromouracil, nitrous acid, or an acridine dye?

14. *Induced spectrum.* DNA photolyases convert the energy of light in the near-ultraviolet or visible region (300–500 nm) into chemical energy to break the cyclobutane ring of pyrimidine dimers. In the absence of substrate, these photoreactivating enzymes do not absorb light of wavelengths longer than 300 nm. Why is the substrate-induced absorption band advantageous?

15. *Missing telomerase.* Cells lacking telomerase can grow for several cell divisions without obvious defects. However, after more cell divisions, such cells tend to show chromosomes that have fused together. Propose an explanation for the formation of the chromosomes.

16. *I need to unwind.* With the assumption that the energy required to break an average base pair in DNA is 10 kJ mol^{-1} (2.4 kcal mol^{-1}), estimate the maximum number of base pairs that could be broken per ATP hydrolyzed by a helicase operating under standard conditions.

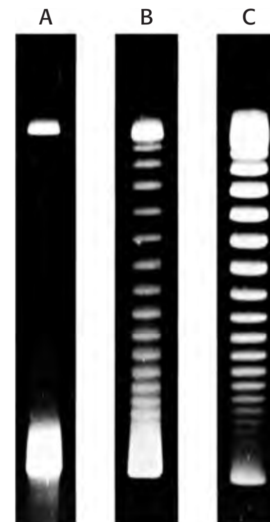
17. *Triplet oxidation*. The oxidation of guanine bases in the context of triplet repeats such as CAGCAGCAG can lead to the expansion of the repeat. Explain.

Mechanism Problem

18. *A revealing analog.* AMP-PNP, the β,γ-imido analog of ATP (p. 825), is hydrolyzed very slowly by most ATPases. The addition of AMP-PNP to topoisomerase II and circular DNA leads to the negative supercoiling of a single molecule of DNA per enzyme. DNA remains bound to the enzyme in the presence of this analog. What does this finding reveal about the catalytic mechanism?

Data Interpretation and Chapter Integration Problems

19. *Like a ladder.* Circular DNA from SV40 virus was isolated and subjected to gel electrophoresis. The results are shown in lane A (the control) of the adjoining gel patterns.



[From W. Keller. *PNAS* 72: 2553, 1975.]

(a) Why does the DNA separate in agarose gel electrophoresis? How does the DNA in each band differ?

The DNA was then incubated with topoisomerase I for 5 minutes and again analyzed by gel electrophoresis with the results shown in lane B.

(b) What types of DNA do the various bands represent?

Another sample of DNA was incubated with topoisomerase I for 30 minutes and again analyzed as shown in lane C.

(c) What is the significance of the fact that more of the DNA is in slower-moving forms?

20. *Ames test.* The illustration on the next page shows four petri plates used for the Ames test. A piece of filter paper (white circle in the center of each plate) was soaked in one of

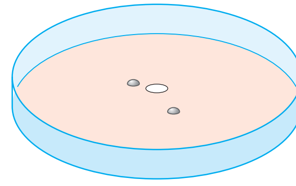
four preparations and then placed on a petri plate. The four preparations contained (A) purified water (control), (B) a known mutagen, (C) a chemical whose mutagenicity is under investigation, and (D) the same chemical after treatment with liver homogenate. The number of revertants, visible as colonies on the petri plates, was determined in each case.

(a) What was the purpose of the control plate, which was exposed only to water?

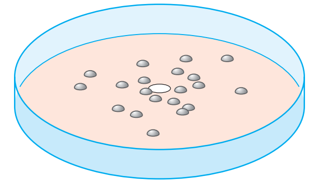
(b) Why was it wise to use a known mutagen in the experimental system?

(c) How would you interpret the results obtained with the experimental compound?

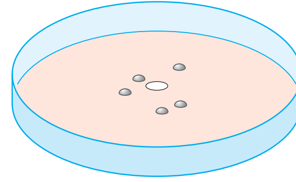
(d) What liver components would you think are responsible for the effects observed in preparation D?



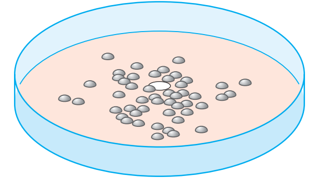
(A) Control: No mutagen



(B) + Known mutagen



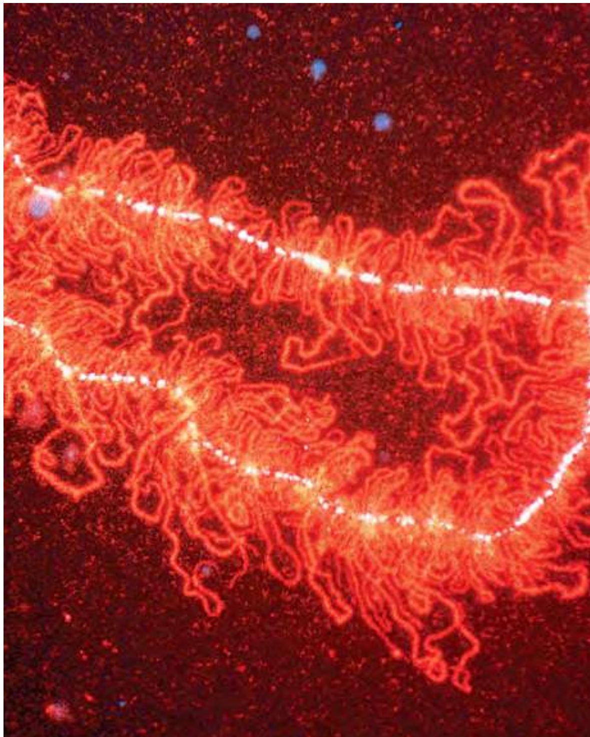
(C) + Experimental sample

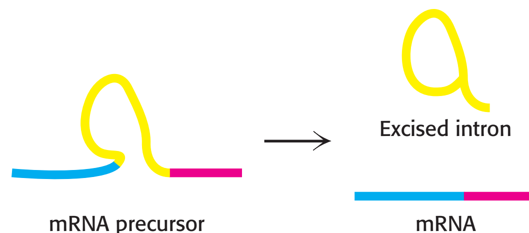


(D) + Experimental sample after treatment with liver homogenate

CHAPTER 29

RNA Synthesis and Processing





RNA synthesis is a key step in the expression of genetic information. For eukaryotic cells, the initial RNA transcript (the mRNA precursor) is often spliced, removing introns that do not encode protein sequences. Often, the same pre-mRNA is spliced differently in different cell types or at different developmental stages. In the image at the left, proteins associated with RNA splicing (stained with a fluorescent antibody) highlight regions of the newt genome that are being actively transcribed.[(Left) Courtesy of Dr. Mark B. Roth and Dr. Joseph G. Gall.]

DNA stores genetic information in a stable form that can be readily replicated. The expression of this genetic information requires its flow from DNA to RNA and, usually, to protein, as was introduced in Chapter 4. This chapter examines RNA synthesis, or *transcription,* which is the process of synthesizing an RNA transcript with the transfer of sequence information from a DNA template. We begin with a discussion of RNA polymerases, the large and complex enzymes that carry out the synthetic process. We then turn to transcription in bacteria and focus on the three stages of transcription: promoter binding and initiation, elongation of the nascent RNA transcript, and termination. We then examine transcription in eukaryotes, focusing on the distinctions between bacterial and eukaryotic transcription.

RNA transcripts in eukaryotes are extensively modified, as exemplified by the capping of the 5′ end of an mRNA precursor and the addition of a long poly(A) tail to its 3′ end. One of the most striking examples of RNA modification is the splicing of mRNA precursors, which is catalyzed by

OUTLINE

29.1 RNA Polymerases Catalyze Transcription

29.2 Transcription in Eukaryotes Is Highly Regulated

29.3 The Transcription Products of Eukaryotic Polymerases Are Processed

29.4 The Discovery of Catalytic RNA Was Revealing in Regard to Both Mechanism and Evolution

Chapter 29 includes material from the sixth edition by Susan J. Baserga and Erica A. Champion.

spliceosomes, protein complexes consisting of small nuclear ribonucleoprotein particles (snRNPs). Remarkably, some RNA molecules can splice themselves in the absence of protein. This landmark discovery by Thomas Cech and Sidney Altman revealed that RNA molecules can serve as catalysts and greatly influenced our view of molecular evolution.

RNA splicing is not merely a curiosity. At least 15% of all genetic diseases have been associated with mutations that affect RNA splicing. Moreover, the same pre-mRNA can be spliced differently in various cell types, at different stages of development, or in response to other biological signals. In addition, individual bases in some pre-mRNA molecules are changed in a process called *RNA editing*. One of the biggest surprises of the sequencing of the human genome was that only about 23,000 genes were identified compared with previous estimates of 100,000 or more. The ability of one gene to encode more than one distinct mRNA by alternative splicing and, hence, more than one protein may play a key role in expanding the repertoire of our genomes.

RNA synthesis comprises three stages: Initiation, elongation, and termination

RNA synthesis is catalyzed by large enzymes called *RNA polymerases*. The basic biochemistry of RNA synthesis is common to all organisms, commonality that has been beautifully illustrated by the three-dimensional structures of representative RNA polymerases from bacteria and eukaryotes (Figure 29.1). Despite substantial differences in size and number of polypeptide subunits, the overall structures of these enzymes are quite similar, revealing a common evolutionary origin.

RNA synthesis, like all biological polymerization reactions, takes place in three stages: *initiation, elongation,* and *termination*. RNA polymerases perform multiple functions in this process:

1. They search DNA for initiation sites, also called *promoter sites* or simply *promoters*. For instance, *E. coli* DNA has about 2000 promoter sites in its 4.8×10^6 bp genome.

2. They unwind a short stretch of double-helical DNA to produce single-stranded DNA templates from which the sequence of bases can be easily read out.

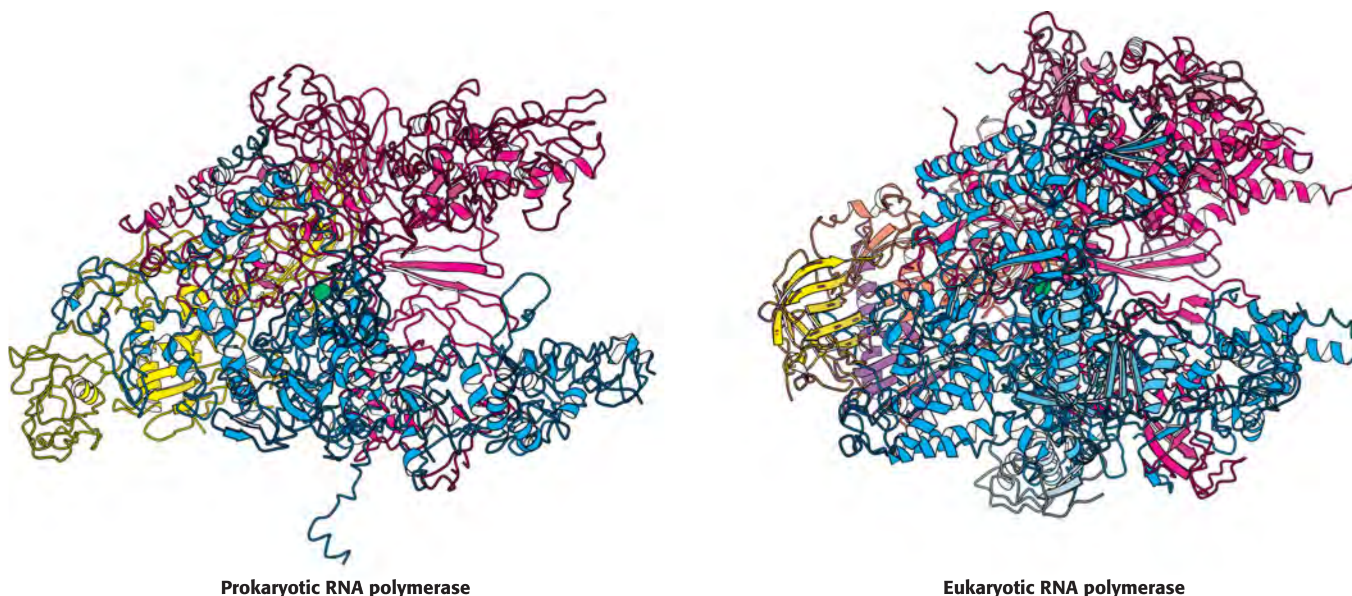


Figure 29.1 RNA polymerase structures. The three-dimensional structures of RNA polymerases from a bacterium *(Thermus aquaticus)* and a eukaryote *(Saccharomyces cerevisiae).* The two largest subunits for each structure are shown in dark red and dark blue. *Notice* that both structures contain a central metal ion (green) in their active sites, near a large cleft on the right. The similarity of these structures reveals that these enzymes have the same evolutionary origin and have many mechanistic features in common. [Drawn from 1I6V.pdb and 1I6H.pdb.]

3. They select the correct ribonucleoside triphosphate and catalyze the formation of a phosphodiester bond. This process is repeated many times as the enzyme moves along the DNA template. RNA polymerase is completely processive—a transcript is synthesized from start to end by a single RNA polymerase molecule.

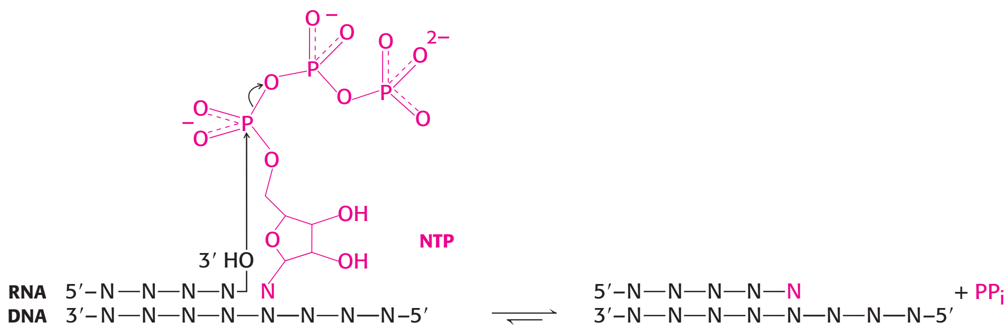
4. They detect termination signals that specify where a transcript ends.

5. They interact with activator and repressor proteins that modulate the rate of transcription initiation over a wide range. Gene expression is controlled substantially at the level of transcription, as will be discussed in detail in Chapters 31 and 32.

The chemistry of RNA synthesis is identical for all forms of RNA, including messenger RNAs, transfer RNAs, ribosomal RNAs, and small regulatory RNAs. The basic steps just outlined apply to all forms. Their synthetic processes differ mainly in regulation, posttranscriptional processing, and the specific RNA polymerase that participates.

29.1 RNA Polymerases Catalyze Transcription

The fundamental reaction of RNA synthesis is the formation of a phosphodiester bond. The 3′-hydroxyl group of the last nucleotide in the chain nucleophilically attacks the α phosphoryl group of the incoming nucleoside triphosphate with the concomitant release of a pyrophosphate.



This reaction is thermodynamically favorable, and the subsequent degradation of the pyrophosphate to orthophosphate locks the reaction in the direction of RNA synthesis. The catalytic sites of RNA polymerases include two metal ions, normally magnesium ions (Figure 29.2). One ion remains tightly bound to the enzyme, whereas the other ion comes in with the nucleoside triphosphate and leaves with the pyrophosphate. Three conserved aspartate residues participate in binding these metal ions.

RNA polymerases are very large, complex enzymes. For example, the RNA polymerase of *E. coli* consists of 5 kinds of subunits with the composition $\alpha_2\beta\beta'\omega$ (Table 29.1). A typical eukaryotic RNA polymerase is larger and more complex, having 12 subunits and a total molecular mass of more than 0.5 million daltons. Despite this complexity, the structures of RNA polymerases have been determined in detail by x-ray crystallography in work pioneered by Roger Kornberg and Seth Darst.

The polymerization reactions catalyzed by RNA polymerases take place within a complex in DNA termed a *transcription bubble* (Figure 29.3). This complex consists of

Table 29.1 Subunits of RNA polymerase from *E. coli*

Subunit	Gene	Number	Mass (kd)
α	*rpoA*	2	37
β	*rpoB*	1	151
β′	*rpoC*	1	155
ω	*rpoZ*	1	10
σ^{70}	*rpoD*	1	70

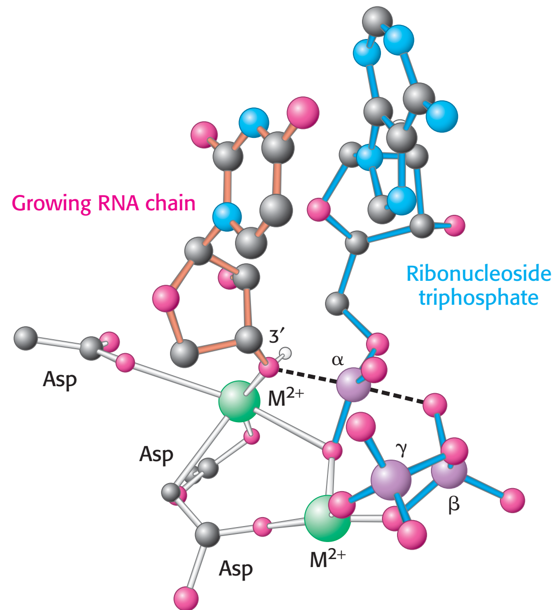


Figure 29.2 RNA polymerase active site. A model of the transition state for phosphodiester-bond formation in the active site of RNA polymerase. The 3′-hydroxyl group of the growing RNA chain attacks the α-phosphoryl group of the incoming nucleoside triphosphate, resulting in the release of pyrophosphate. This transition state is structurally similar to that in the active site of DNA polymerase (see Figure 28.4).

double-stranded DNA that has been locally unwound in a region of approximately 17 base pairs. The edges of the bases that normally take part in Watson–Crick base pairs are exposed in the unwound region. We will begin with a detailed examination of the elongation process, including the role of the DNA template read by RNA polymerase and the reactions catalyzed by the polymerase, before returning to the more-complex processes of initiation and termination.

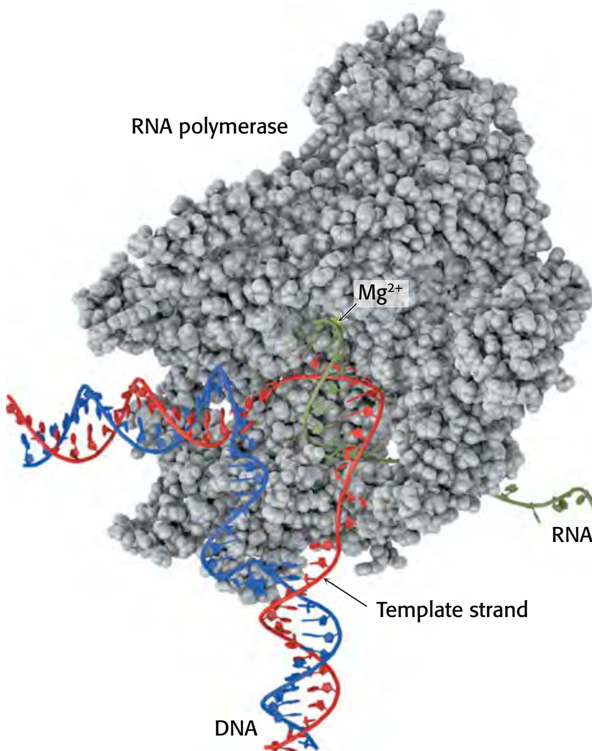


Figure 29.3 Transcription bubble. RNA polymerase separates a region of the double helix to form a structure called the "transcription bubble." The red (template strand) and blue (nontemplate) strands of DNA are shown along with RNA molecule being synthesized (shown in green). The position of the active site magnesium is indicated.

RNA chains are formed de novo and grow in the 5′-to-3′ direction

Let us begin our examination of transcription by considering the DNA template. The first nucleotide (the start site) of a DNA sequence to be transcribed is denoted as +1 and the second one as +2; the nucleotide preceding the start site is denoted as −1. These designations refer to the coding strand of DNA. Recall that the sequence of the *template strand of DNA* is the *complement* of that of the RNA transcript (Figure 29.4). In contrast, the *coding strand of DNA* has the *same* sequence as that of the RNA transcript except for thymine (T) in place of uracil (U). The coding strand is also known as the *sense* (+) *strand,* and the template strand as the *antisense* (−) *strand*.

In contrast with DNA synthesis, *RNA synthesis can start de novo, without the requirement for a primer*. Most newly synthesized RNA chains carry a highly distinctive tag on the 5′ end: the first base at that end is either *pppG* or *pppA*.

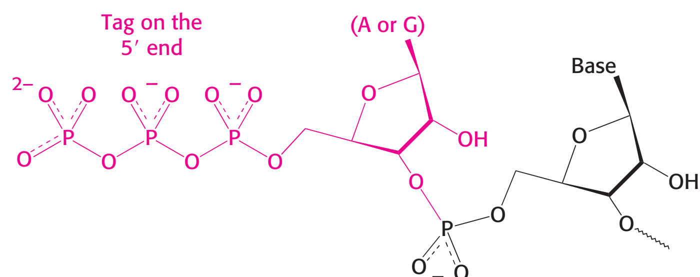


Figure 29.4 Template and coding strands. The template, or antisense (−), strand is complementary in sequence to the RNA transcript.

The presence of the triphosphate moiety confirms that RNA synthesis starts at the 5′ end.

The dinucleotide shown on page 854 is synthesized by RNA polymerase as part of the complex process of initiation, which will be discussed later in the chapter. After initiation takes place, RNA polymerase elongates the nucleic acid chain in the following manner (Figure 29.5). A ribonucleoside triphosphate binds in the active site of the RNA polymerase, directly adjacent to the growing RNA chain. The incoming ribonucleoside triphosphate forms a Watson–Crick base pair with the template strand. The 3′-hydroxyl group of the growing RNA chain, oriented and activated by the tightly bound metal ion, attacks the α-phosphoryl group to form a new phosphodiester bond, displacing pyrophosphate.

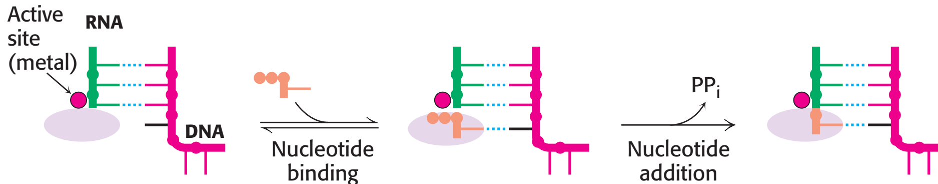


Figure 29.5 Elongation mechanism. A ribonucleoside triphosphate binds adjacent to the growing RNA chain and forms a Watson–Crick base pair with a base on the DNA template strand. The 3′-hydroxyl group at the end of the RNA chain attacks the newly bound nucleotide and forms a new phosphodiester bond, releasing pyrophosphate.

To proceed to the next step, the RNA–DNA hybrid must move relative to the polymerase to bring the 3′ end of the newly added nucleotide into proper position for the next nucleotide to be added (Figure 29.6). This translocation step does not include breaking any bonds between base pairs and is reversible but, once it has taken place, the addition of the next nucleotide, favored by the triphosphate cleavage and pyrophosphate release and cleavage, drives the polymerization reaction forward.

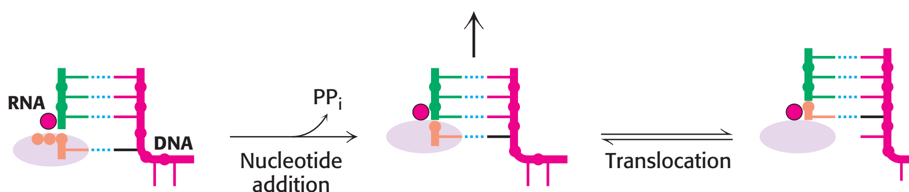


Figure 29.6 Translocation. After nucleotide addition, the RNA–DNA hybrid can translocate through the RNA polymerase, bringing a new DNA base into position to base-pair with an incoming nucleoside triphosphate.

The lengths of the RNA–DNA hybrid and of the unwound region of DNA stay rather constant as RNA polymerase moves along the DNA template. The length of the RNA–DNA hybrid is determined by a structure within the enzyme that forces the RNA–DNA hybrid to separate, allowing the RNA chain to exit from the enzyme and the DNA chain to rejoin its DNA partner (Figure 29.7).

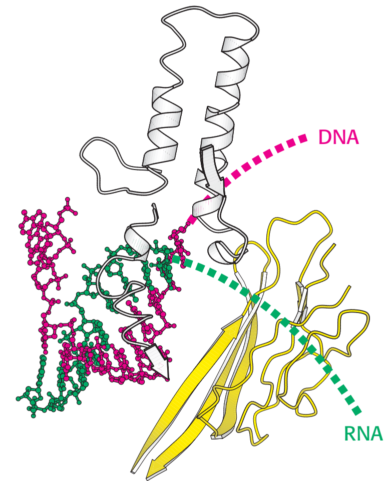


Figure 29.7 RNA–DNA hybrid separation. A structure within RNA polymerase forces the separation of the RNA–DNA hybrid. *Notice* that the DNA strand exits in one direction and the RNA product exits in another. [Drawn from 1I6H.pdb.]

RNA polymerases backtrack and correct errors

The RNA–DNA hybrid can also move in the direction opposite that of elongation (Figure 29.8). This backtracking is less favorable energetically than moving forward because it breaks the bonds between a base pair. However, backtracking is very important for *proofreading*. The incorporation of an incorrect nucleotide introduces a non-Watson–Crick base pair. In this case, breaking the bonds between this base pair and backtracking is less costly energetically. After the polymerase has backtracked, the phosphodiester bond one base pair before the one that has just formed is adjacent to the metal ion in the active site. In this position, a hydrolysis reaction in which a water molecule attacks the phosphate can result in the cleavage of the phosphodiester bond and the release of a dinucleotide that includes the incorrect nucleotide.

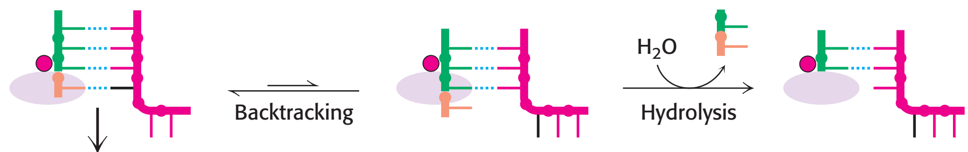


Figure 29.8 Backtracking. The RNA–DNA hybrid can occasionally backtrack within the RNA polymerase. In the backtracked position, hydrolysis can take place, producing a configuration equivalent to that after translocation. Backtracking is more likely if a mismatched base is added, facilitating proofreading.

Studies of single molecules of RNA polymerase have confirmed that the enzymes hesitate and backtrack to correct errors. Furthermore, these proofreading activities are often enhanced by accessory proteins. The final error rate of the order of one mistake per 10^4 or 10^5 nucleotides is higher than that for DNA replication, including all error-correcting mechanisms. The lower fidelity of RNA synthesis can be tolerated because mistakes are not transmitted to progeny. For most genes, many RNA transcripts are synthesized; a few defective transcripts are unlikely to be harmful.

RNA polymerase binds to promoter sites on the DNA template to initiate transcription

The elongation process is common to all organisms. In contrast, the processes of initiation and termination differ substantially in bacteria and eukaryotes. We begin with a discussion of these processes in bacteria, starting with initiation of transcription. The bacterial RNA polymerase discussed earlier with the composition $\alpha_2\beta\beta'\omega$ is referred to as the *core enzyme*. The inclusion of an additional subunit produces the *holoenzyme* with composition $\sigma_2\beta\beta'\omega\sigma$. The σ subunit helps find sites on DNA where transcrip-

tion begins, referred to as *promoter sites* or, simply, *promoters*. At these sites, the σ subunit participates in the initiation of RNA synthesis and then dissociates from the rest of the enzyme.

Sequences upstream of the promoter site are important in determining where transcription begins. A striking pattern was evident when the sequences of bacterial promoters were compared. *Two common motifs are present on the upstream side of the transcription start site*. They are known as the *−10 sequence* and the *−35 sequence* because they are centered at about 10 and 35 nucleotides upstream of the start site. The region containing these sequences is called the *core promoter*. The −10 and −35 sequences are each 6 bp long. Their *consensus sequences*, deduced from analyses of many promoters (Figure 29.9), are

```
       −35                 −10         +1
5′~~TTGACA~~~~~~~~~~~~~~TATAAT~~~~Start
                                     site
```

Promoters differ markedly in their efficacy. Some genes are transcribed frequently—as often as every 2 seconds in *E. coli*. The promoters for these genes are referred to as *strong promoters*. In contrast, other genes are transcribed much less frequently, about once in 10 minutes; the promoters for these genes are *weak promoters*. The −10 and −35 regions of most strong promoters have sequences that correspond closely to the consensus sequences, whereas weak promoters tend to have multiple substitutions at these sites. Indeed, mutation of a single base in either the −10 sequence or the −35 sequence can diminish promoter activity. The distance between these conserved sequences also is important; a separation of 17 nucleotides is optimal. Thus, *the efficiency or strength of a promoter sequence serves to regulate transcription*. Regulatory proteins that bind to specific sequences near promoter sites and interact with RNA polymerase (Chapter 31) also markedly influence the frequency of transcription of many genes.

Outside the core promoter in a subset of highly expressed genes is the *upstream element* (also called the UP element for *up*stream element). This sequence is present from 40 to 60 nucleotides upstream of the transcription start site. The UP element is bound by the α subunit of RNA polymerase and serves to increase the efficiency of transcription by creating an additional interaction site for the polymerase.

Sigma subunits of RNA polymerase recognize promoter sites

To initiate transcription, the $\alpha_2\beta\beta'\omega$ core of RNA polymerase must bind the promoter. However, it is the σ *subunit* that makes this binding possible by enabling *RNA polymerase to recognize promoter sites*. In the presence of the σ subunit, the RNA polymerase binds weakly to the DNA and slides along the double helix until it dissociates or encounters a promoter. The σ subunit recognizes the promoter through several interactions with the nucleotide bases of the promoter DNA. The structure of a bacterial RNA polymerase holoenzyme bound to a promoter site shows the σ subunit interacting with DNA at the −10 and −35 regions essential to promoter recognition (Figure 29.10).

```
                              Transcription
                                 starts here
                                      ↓
      5′      −10                     3′
(A)   G A T A T A A T G A G C A A A
(B)   C G T A T A A T T T G A C C A
(C)   G T T A A C T A G T A C G C A
(D)   G T G A T A C T G A G C A C A
(E)   G T T T T C A T G C C T C C A
          T A T A A T
```

Figure 29.9 Bacterial promoter sequences. A comparison of five sequences from prokaryotic promoters reveals a recurring sequence of TATAAT centered on position −10. The −10 consensus sequence (in red) was deduced from a large number of promoter sequences. The sequences are from the (A) *uvrD*, (B) *uncI*, and (C) *trp* operons of *E. coli;* from (D) λ phage; and from (E) ϕX174 phage.

σ subunit

−35 element

−10 element

Figure 29.10 RNA polymerase holoenzyme complex. *Notice* that the σ subunit (blue) of the bacterial RNA polymerase holoenzyme makes sequence-specific contacts with the −10 (green) and −35 promoter elements (yellow). The active site of the polymerase is revealed by bound metal ion (red). [Drawn from 1L9Z.pdb].

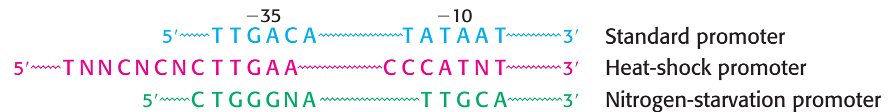


Figure 29.11 Alternative promoter sequences. A comparison of the consensus sequences of standard, heat-shock, and nitrogen-starvation promoters of *E. coli.* These promoters are recognized by σ^{70}, σ^{32}, and σ^{54}, respectively.

Therefore, *the σ subunit is responsible for the specific binding of the RNA polymerase to a promoter site on the template DNA*. The σ subunit is generally released when the nascent RNA chain reaches 9 or 10 nucleotides in length. After its release, it can assist initiation by another core enzyme. Thus, the *σ subunit acts catalytically*.

E. coli has seven distinct σ factors for recognizing several types of promoter sequences in *E. coli* DNA. The type that recognizes the consensus sequences described earlier is called σ^{70} because it has a mass of 70 kd. A different σ factor comes into play when the temperature is raised abruptly. *E. coli* responds by synthesizing σ^{32}, which recognizes the promoters of *heat-shock genes*. These promoters exhibit −10 sequences that are somewhat different from the −10 sequence for standard promoters (Figure 29.11). The increased transcription of heat-shock genes leads to the coordinated synthesis of a series of protective proteins. Other σ factors respond to environmental conditions, such as nitrogen starvation. These findings demonstrate that *σ plays a key role in determining where RNA polymerase initiates transcription*.

Some other bacteria contain a much larger number of σ factors. For example, the genome of the soil bacterium *Streptomyces coelicolor* encodes more than 60 σ factors recognized on the basis of their amino acid sequences. This repertoire allows these cells to adjust their gene-expression programs to the wide range of conditions, in regard to nutrients and competing organisms, that they may experience.

RNA polymerases must unwind the template double helix for transcription to take place

Although RNA polymerases can search for promoter sites when bound to double-helical DNA, a segment of the DNA double helix must be unwound before synthesis can begin. The transition from the *closed promoter complex* (in which DNA is double helical) to the *open promoter complex* (in which a DNA segment is unwound) is an essential event in transcription (Figure 29.12). The free energy necessary to break the bonds between approximately 17 base pairs in the double helix is derived from additional interactions that are possible when the DNA distorts to wrap around the RNA polymerase and from interactions between the single-stranded DNA regions and other parts of the enzyme. These interactions stabilize the open promoter complex and help pull the template strand into the active site. The −35 element remains in a double-helical state, whereas the −10 element is unwound. The stage is now set for the formation of the first phosphodiester bond of the new RNA chain.

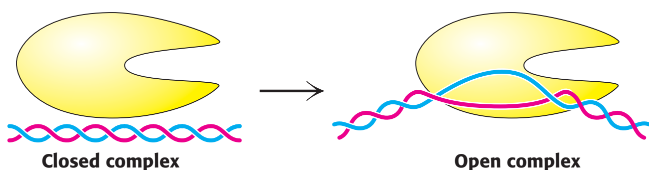


Figure 29.12 DNA unwinding. The transition from the closed promoter complex to the open promoter complex requires the unwinding of approximately 17 base pairs of DNA.

Elongation takes place at transcription bubbles that move along the DNA template

The elongation phase of RNA synthesis begins with the formation of the first phosphodiester bond. Repeated cycles of nucleotide addition can take

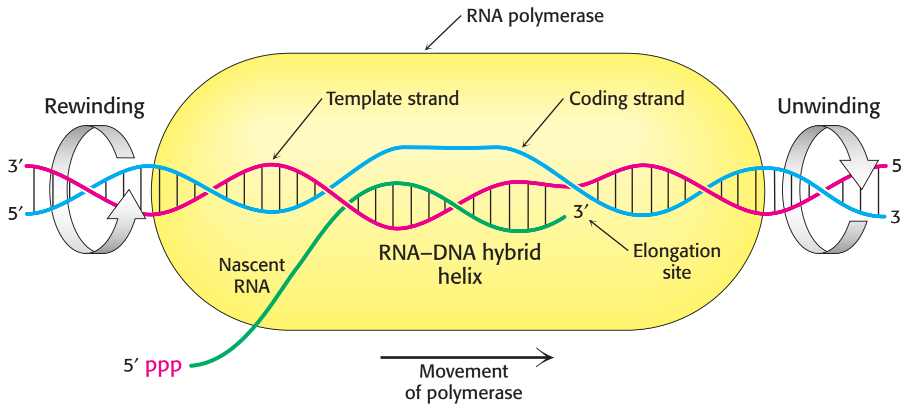


Figure 29.13 Transcription bubble. (A) A schematic representation of a transcription bubble in the elongation of an RNA transcript. Duplex DNA is unwound at the forward end of RNA polymerase and rewound at its rear end. The RNA–DNA hybrid rotates during elongation.

place at this point. However, until about 10 nucleotides have been added, RNA polymerase sometimes releases the short RNA, which dissociates from the DNA. Once RNA polymerase passes this point, the enzyme stays bound to its template until a termination signal is reached. The region containing RNA polymerase, DNA, and nascent RNA corresponds to the transcription bubble (Figure 29.13). The newly synthesized RNA forms a hybrid helix with the template DNA strand. This RNA–DNA helix is about 8 bp long, which corresponds to nearly one turn of a double helix. The 3′-hydroxyl group of the RNA in this hybrid helix is positioned so that it can attack the α-phosphorus atom of an incoming ribonucleoside triphosphate. The core enzyme also contains a binding site for the coding strand of DNA. About 17 bp of DNA are unwound throughout the elongation phase, as in the initiation phase. The transcription bubble moves a distance of 170 Å (17 nm) in a second, which corresponds to a rate of elongation of about 50 nucleotides per second. Although rapid, it is much slower than the rate of DNA synthesis, which is 800 nucleotides per second.

Sequences within the newly transcribed RNA signal termination

In bacteria, the termination of transcription is as precisely controlled as its initiation. In the termination phase of transcription, the formation of phosphodiester bonds ceases, the RNA–DNA hybrid dissociates, the unwound region of DNA rewinds, and RNA polymerase releases the DNA. What determines where transcription is terminated? *The transcribed regions of DNA templates contain stop signals*. The simplest one is a *palindromic GC-rich region followed by an AT-rich region*. The RNA transcript of this DNA palindrome is self-complementary (Figure 29.14). Hence, its bases can pair to form a hairpin structure with a stem and loop, a structure favored by its high content of G and C residues. Guanine–cytosine base pairs are more stable than adenine–thymine pairs because of the extra hydrogen bond in the base pair. This stable hairpin is followed by a sequence of four or more uracil residues, which also are crucial for termination. The RNA transcript ends within or just after them.

How does this combination hairpin–oligo(U) structure terminate transcription? First, RNA polymerase likely pauses immediately after it has synthesized a stretch of RNA that folds into a hairpin. Furthermore, the RNA–DNA hybrid helix produced after the hairpin is unstable because its rU–dA base pairs are the weakest of the four kinds. Hence, the pause in transcription caused by the hairpin permits the weakly bound *nascent RNA to dissociate from the DNA template and then*

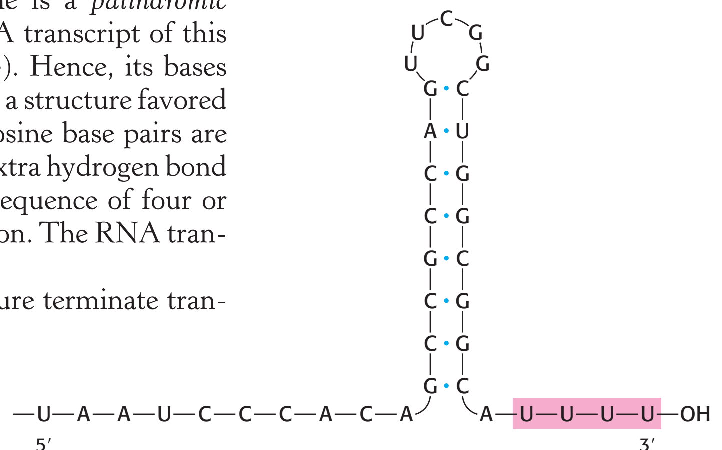


Figure 29.14 Termination signal. A termination signal found at the 3′ end of an mRNA transcript consists of a series of bases that form a stable stem-loop structure and a series of U residues.

from the enzyme. The solitary DNA template strand rejoins its partner to re-form the DNA duplex, and the transcription bubble closes.

Some messenger RNAs directly sense metabolite concentrations

As we shall explore in Chapters 31 and 32, the expression of many genes is controlled in response to the concentrations of metabolites and signaling molecules within cells. One set of control mechanisms depends on the remarkable ability of some mRNA molecules to form special secondary structures, some of which are capable of directly binding small molecules. These structures are termed *riboswitches*. Consider a riboswitch that controls the synthesis of genes that participate in the biosynthesis of riboflavin (p. 441) in *Bacillus subtilis* (Figure 29.15). When flavin mononucleotide (FMN), a key intermediate in riboflavin biosynthesis, is present at high concentration, it binds to a conformation of the RNA transcript with an FMN-binding pocket that also includes a hairpin structure that favors premature termination. By trapping the RNA transcript in this termination-favoring conformation, FMN prevents the production of functional mRNA. However, when FMN is present at low concentration, it does not readily bind to RNA, and an alternative conformation forms without the terminator, allowing the production of the full-length mRNA. The occurrence of riboswitches serves as a vivid illustration of how RNAs are capable of forming elaborate, functional structures, though we tend to depict them as simple lines in the absence of specific information.

The *rho* protein helps to terminate the transcription of some genes

RNA polymerase needs no help to terminate transcription at a hairpin followed by several U residues. At other sites, however, termination requires the participation of an additional factor. This discovery was prompted by the observation that some RNA molecules synthesized in vitro by RNA polymerase acting alone are *longer* than those made in vivo. The missing factor, a protein that caused the correct termination, was isolated and named *rho* (ρ). Additional information about the action of ρ was obtained by adding this termination factor to an incubation mixture at various times after the initiation of RNA synthesis (Figure 29.16). RNAs with sedimentation coefficients of 10S, 13S, and 17S were obtained when ρ was added at initiation, a few seconds after initiation, and 2 minutes after initiation, respectively. If no ρ was added, transcription yielded a 23S RNA product. It is evident that the template contains at least three termination sites that respond to ρ (yielding 10S, 13S, and 17S RNA) and one termination site that

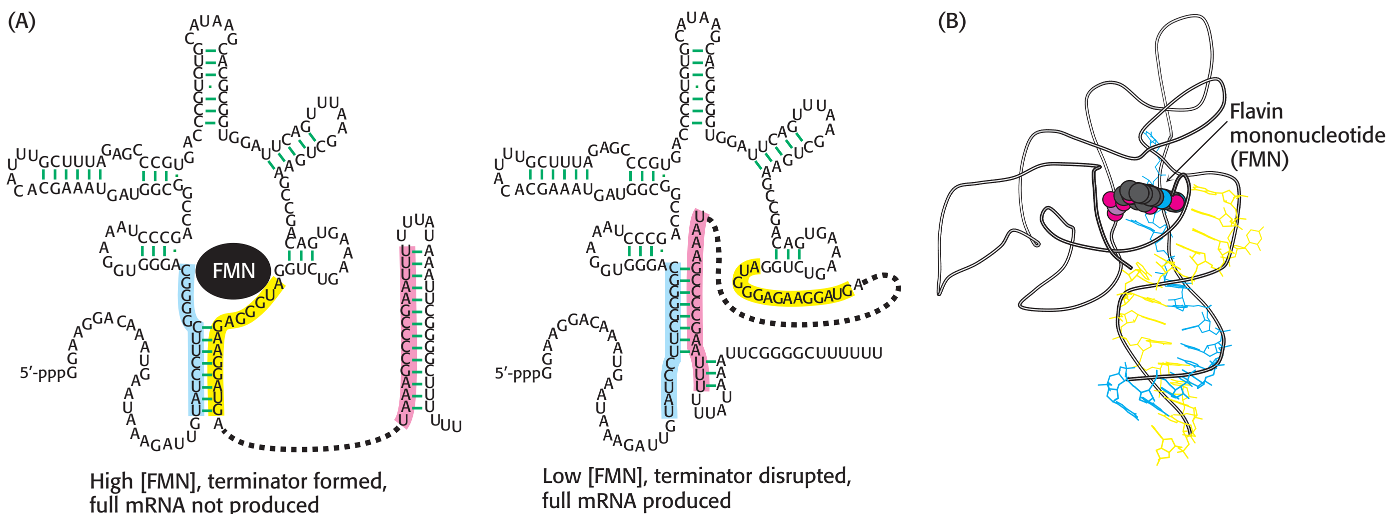


Figure 29.15 Riboswitch. (A) The 5′-end of an mRNA that encodes proteins engaged in the production of flavin mononucleotide (FMN) folds to form a structure that is stabilized by binding FMN. This structure includes a terminator that leads to premature termination of the mRNA. At lower concentrations of FMN, an alternative structure that lacks the terminator is formed, leading to the production of full-length mRNA. (B) The three-dimensional structure of a related FMN-binding riboswitch bound to FMN. The blue and yellow stretches correspond to regions highlighted in the same colors in part A. *Notice* how the yellow strand contacts the bound FMN, stabilizing the structure. [Drawn from 3F2Q.pdb].

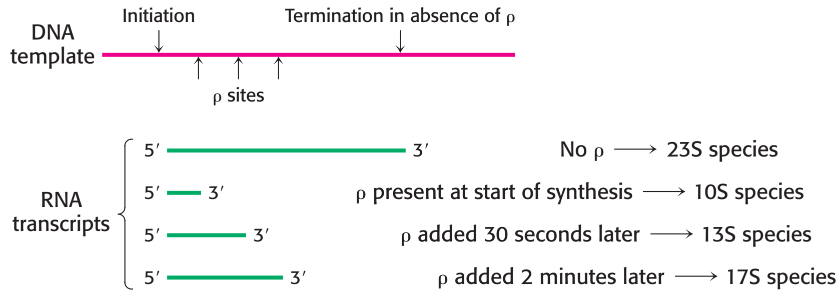


Figure 29.16 Effect of ρ protein on the size of RNA transcripts.

does not (yielding 23S RNA). Thus, specific termination at a site producing 23S RNA can take place in the absence of ρ. However, ρ detects additional termination signals that are not recognized by RNA polymerase alone.

How does ρ provoke the termination of RNA synthesis? *A key clue is the finding that ρ hydrolyzes ATP in the presence of single-stranded RNA but not in the presence of DNA or duplex RNA.* Hexameric ρ is a helicase, homologous to the helicases that we encountered in our consideration of DNA replication (Section 28.1). A stretch of nucleotides is bound in such a way that the RNA passes through the center of the structure (Figure 29.17). The ρ protein is brought into action by sequences located in the nascent RNA that are rich in cytosine and poor in guanine. The helicase activity of ρ enables the protein to pull the nascent RNA while pursuing RNA polymerase. When ρ catches RNA polymerase at the transcription bubble, it breaks the RNA–DNA hybrid helix by functioning as an RNA–DNA helicase.

Proteins in addition to ρ may provoke termination. For example, the *nusA protein* enables RNA polymerase in *E. coli* to recognize a characteristic class of termination sites. *A common feature of protein-independent and protein-dependent termination is that the functioning signals lie in newly synthesized RNA rather than in the DNA template.*

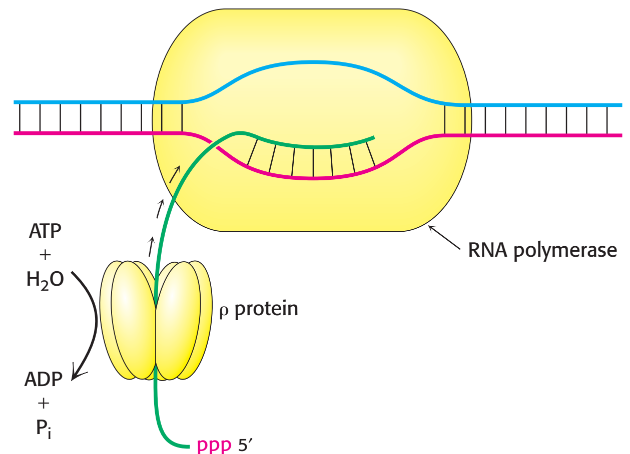
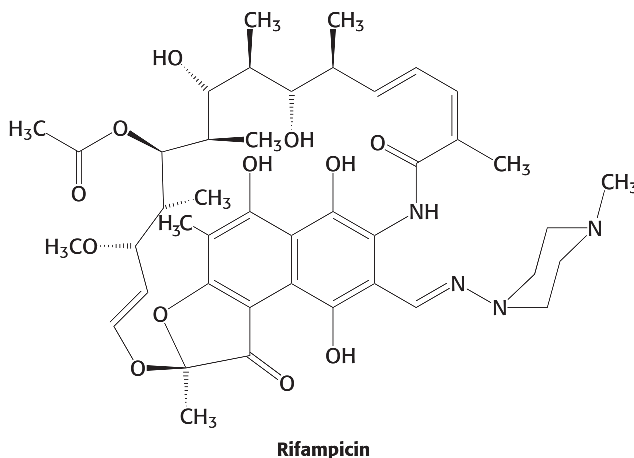


Figure 29.17 Mechanism for the termination of transcription by ρ protein. This protein is an ATP-dependent helicase that binds the nascent RNA chain and pulls it away from RNA polymerase and the DNA template.

Some antibiotics inhibit transcription

Many antibiotics are highly specific inhibitors of biological processes in bacteria. Rifampicin and actinomycin are two antibiotics that inhibit bacterial transcription, although in quite different ways. *Rifampicin* is a semisynthetic derivative of *rifamycins,* which are compounds derived from a strain of *Streptomyces.*



Rifampicin

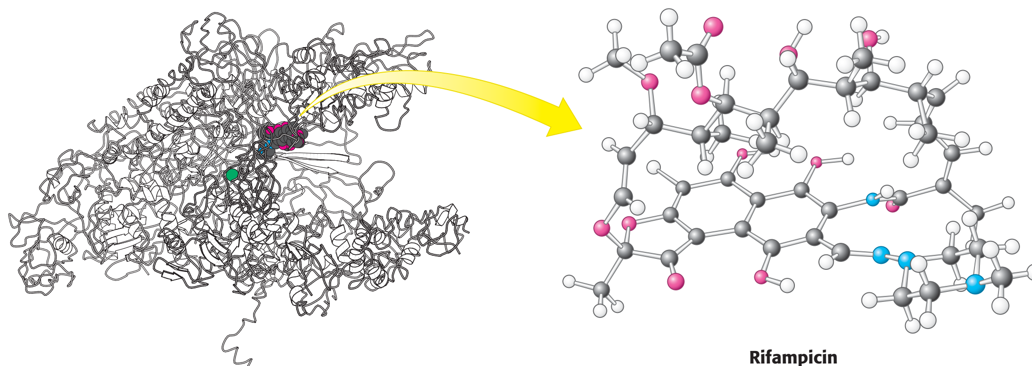


Figure 29.18 Antibiotic action. Rifampicin binds to a pocket in the channel that is normally occupied by the newly formed RNA–DNA hybrid. Thus, the antibiotic blocks elongation after only two or three nucleotides have been added.

This antibiotic *specifically inhibits the initiation of RNA synthesis*. Rifampicin interferes with the formation of the first few phosphodiester bonds in the RNA chain. The structure of a complex between a prokaryotic RNA polymerase and rifampicin reveals that the antibiotic blocks the channel into which the RNA–DNA hybrid generated by the enzyme must pass (Figure 29.18). The binding site is 12 Å from the active site itself. Rifampicin can inhibit only the initiation of transcription, not elongation, because the RNA–DNA hybrid present in the enzyme during elongation prevents the antibiotic from binding. The pocket in which rifampicin binds is conserved among bacterial RNA polymerases, but not eukaryotic polymerases, and so rifampicin can be used as an antibiotic in antituberculosis therapy.

Actinomycin D, a peptide-containing antibiotic from a different strain of *Streptomyces,* inhibits transcription by an entirely different mechanism. *Actinomycin D binds tightly and specifically to double-helical DNA and thereby prevents it from being an effective template for RNA synthesis.* The results of spectroscopic, hydrodynamic, and structural studies of complexes of actinomycin D and DNA reveal that the phenoxazone ring of actinomycin slips in between base pairs in DNA (Figure 29.19). This mode of binding is called *intercalation*. At low concentrations, actinomycin D inhibits transcription without significantly affecting DNA replication or protein synthesis. Hence, *actinomycin D is extensively used as a highly specific inhibitor of the formation of new RNA in both prokaryotic and eukaryotic cells.* Its ability to inhibit the growth of rapidly dividing cells makes it an effective therapeutic agent in the treatment of some cancers.

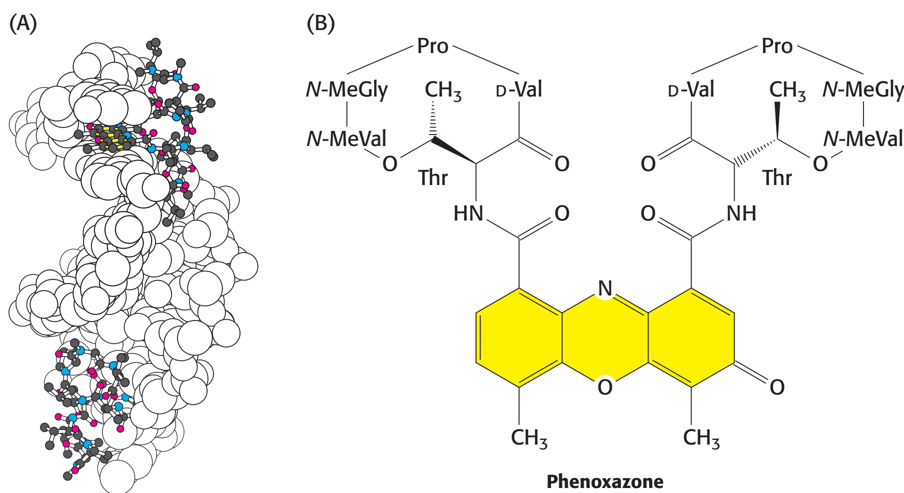


Figure 29.19 Actinomycin–DNA complex structure. (A) The structure of a complex between a DNA duplex (shown as a space-filling model) and actinomycin B (shown as a ball-and-stick model). Two actinomycin B molecules are bound in the complex. (B) The structure of actinomycin B showing the phenoxazone ring. *Notice* how the phenoxazone (yellow) slides between base pairs of the DNA. Abbreviation: Me, methyl. [Drawn from 1I3W.pdb]

Precursors of transfer and ribosomal RNA are cleaved and chemically modified after transcription in prokaryotes

In prokaryotes, messenger RNA molecules undergo little or no modification after synthesis by RNA polymerase. Indeed, many mRNA molecules are translated while they are being transcribed. In contrast, *transfer RNA and ribosomal RNA molecules are generated by cleavage and other modifications of nascent RNA chains*. For example, in *E. coli,* the three rRNAs and a tRNA are excised from a single primary RNA transcript that also contains spacer regions (Figure 29.20). Other transcripts contain arrays of several kinds of tRNA or of several copies of the same tRNA. The nucleases that cleave and trim these precursors of rRNA and tRNA are highly precise. *Ribonuclease P* (RNase P), for example, generates the correct 5′ terminus of all tRNA molecules in *E. coli.* Sidney Altman and his coworkers showed that this interesting enzyme contains a catalytically active RNA molecule. *Ribonuclease III* (RNase III) excises 5S, 16S, and 23S rRNA precursors from the primary transcript by cleaving double-helical hairpin regions at specific sites.

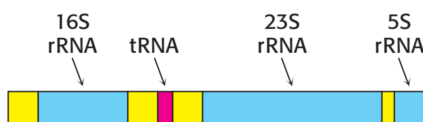
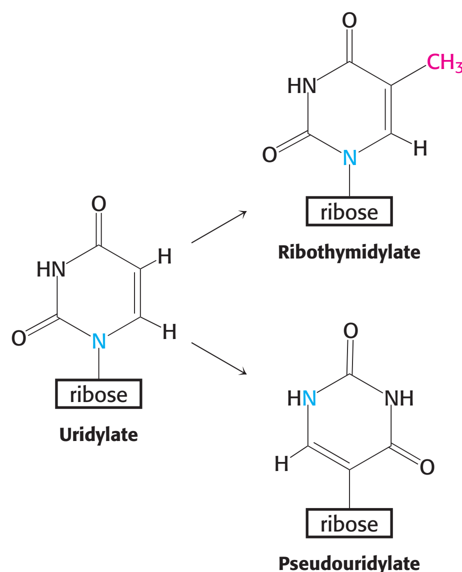


Figure 29.20 Primary transcript. Cleavage of this transcript produces 5S, 16S, and 23S rRNA molecules and a tRNA molecule. Spacer regions are shown in yellow.

A second type of processing is the *addition of nucleotides to the termini of some RNA chains*. For example, CCA, a terminal sequence required for the function of all tRNAs, is added to the 3′ ends of tRNA molecules for which this terminal sequence is not encoded in the DNA. The enzyme that catalyzes the addition of CCA is atypical for an RNA polymerase in that it does not use a DNA template. A third type of processing is the *modification of bases and ribose units* of ribosomal RNAs. In prokaryotes, some bases of rRNA are methylated. Unusual bases are found in all tRNA molecules. They are formed by the enzymatic modification of a standard ribonucleotide in a tRNA precursor. For example, uridylate residues are modified after transcription to form *ribothymidylate* and *pseudouridylate*. These modifications generate diversity, allowing greater structural and functional versatility.



29.2 Transcription in Eukaryotes Is Highly Regulated

We turn now to transcription in eukaryotes, a much more complex process than in bacteria. Eukaryotic cells have a remarkable ability to regulate precisely the time at which each gene is transcribed and how much RNA is produced. This ability has allowed some eukaryotes to evolve into multicellular organisms, with distinct tissues. *That is, multicellular eukaryotes use differential transcriptional regulation to create different cell types*. Gene expression is influenced by three important characteristics unique to eukaryotes: the nuclear membrane, complex transcriptional regulation, and RNA processing.

1. *The Nuclear Membrane. In eukaryotes, transcription and translation take place in different cellular compartments:* transcription takes place in the membrane-bounded nucleus, whereas translation takes place outside the nucleus in the cytoplasm. In bacteria, the two processes are closely coupled (Figure 29.21). Indeed, the translation of bacterial mRNA begins while the transcript is still being synthesized. *The spatial and temporal separation of transcription and translation enables eukaryotes to regulate gene expression in much more intricate ways, contributing to the richness of eukaryotic form and function.*

2. *Complex Transcriptional Regulation*. Like bacteria, eukaryotes rely on conserved sequences in DNA to regulate the initiation of transcription. But bacteria have only three promoter elements (the −10, −35, and UP elements), whereas eukaryotes use a variety of types of promoter elements, each identified by its own conserved sequence. Not all possible types will be present together in the same promoter. *In eukaryotes, elements that regulate transcription can be found at a variety of locations in DNA,* upstream or downstream of the start site and sometimes at distances much farther from the start site than in prokaryotes. For example, enhancer elements located on DNA far from the start site increase the promoter activity of specific genes.

3. *RNA Processing*. Although both bacteria and eukaryotes modify RNA, *eukaryotes very extensively process nascent RNA destined to become mRNA.* This processing includes modifications to both ends and, most significantly, splicing out segments of the primary transcript. RNA processing is described in Section 29.3.

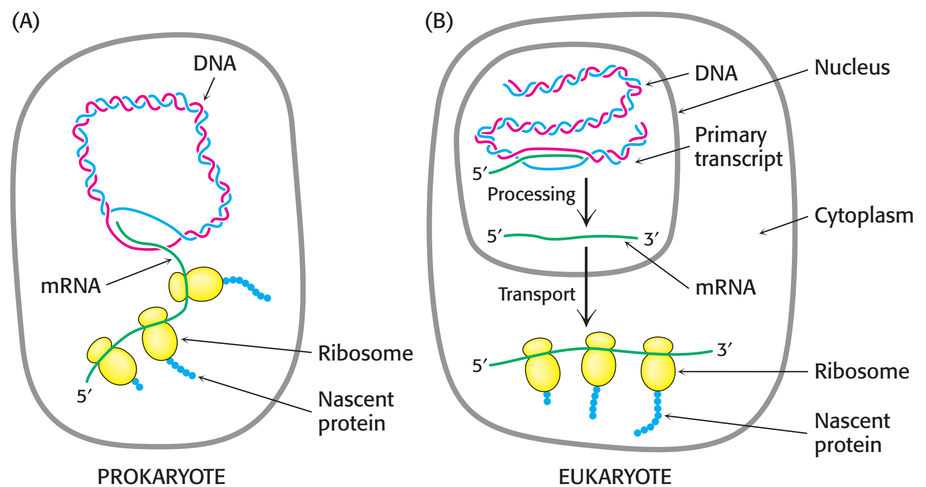


Figure 29.21 Transcription and translation. These two processes are closely coupled in prokaryotes whereas they are spatially and temporally separate in eukaryotes. (A) In prokaryotes the primary transcript serves as mRNA and is used immediately as the template for protein synthesis. (B) In eukaryotes, mRNA precursors are processed and spliced in the nucleus before being transported to the cytoplasm for translation into protein. [After J. Darnell, H. Lodish, and D. Baltimore. *Molecular Cell Biology,* 2d ed. (Scientific American Books, 1990), p. 230.]

Table 29.2 Eukaryotic RNA polymerases

Type	Location	Cellular transcripts	Effects of α-amanitin
I	Nucleolus	18S, 5.8S, and 28S rRNA	Insensitive
II	Nucleoplasm	mRNA precursors and snRNA	Strongly inhibited
III	Nucleoplasm	tRNA and 5S rRNA	Inhibited by high concentrations

Three types of RNA polymerase synthesize RNA in eukaryotic cells

In bacteria, RNA is synthesized by a single kind of polymerase. In contrast, the nucleus of a typical eukaryotic cell contains three types of RNA polymerase differing in template specificity and location in the nucleus (Table 29.2). All these polymerases are large proteins, containing from 8 to 14 subunits and having total molecular masses greater than 500 kd. *RNA polymerase I* is located in specialized structures within the nucleus called nucleoli, where it transcribes the tandem array of genes for 18S, 5.8S, and 28S ribosomal RNA. The other ribosomal RNA molecule (5S rRNA) and all the transfer RNA molecules are synthesized by *RNA polymerase III,* which is located in the nucleoplasm rather than in nucleoli. *RNA polymerase II,* which also is located in the nucleoplasm, synthesizes the precursors of messenger RNA as well as several small RNA molecules, such as those of the splicing apparatus and many of the precursors to small regulatory RNAs.

Although all eukaryotic RNA polymerases are homologous to one another and to prokaryotic RNA polymerases, RNA polymerase II contains a unique *carboxyl-terminal domain* on the 220-kd subunit called the CTD; this domain is unusual because it contains multiple repeats of a YSPTSPS consensus sequence. The activity of RNA polymerase II is regulated by phosphorylation mainly on the serine residues of the CTD.

Another major distinction among the polymerases lies in their responses to the toxin *α-amanitin,* a cyclic octapeptide that contains several modified amino acids.

α-Amanitin

α-Amanitin is produced by the poisonous mushroom *Amanita phalloides,* which is also called the *death cap* or the *destroying angel*. More than a hundred deaths result worldwide each year from the ingestion of poisonous mushrooms. α-Amanitin binds very tightly (K_d = 10 nM) to RNA polymerase II and thereby blocks the elongation phase of RNA synthesis. Higher concentrations of α-amanitin (1 μM) inhibit polymerase III, whereas

Amanita phalloides, also called the *death cap.* [Archenzo/WikimediaCommons.]

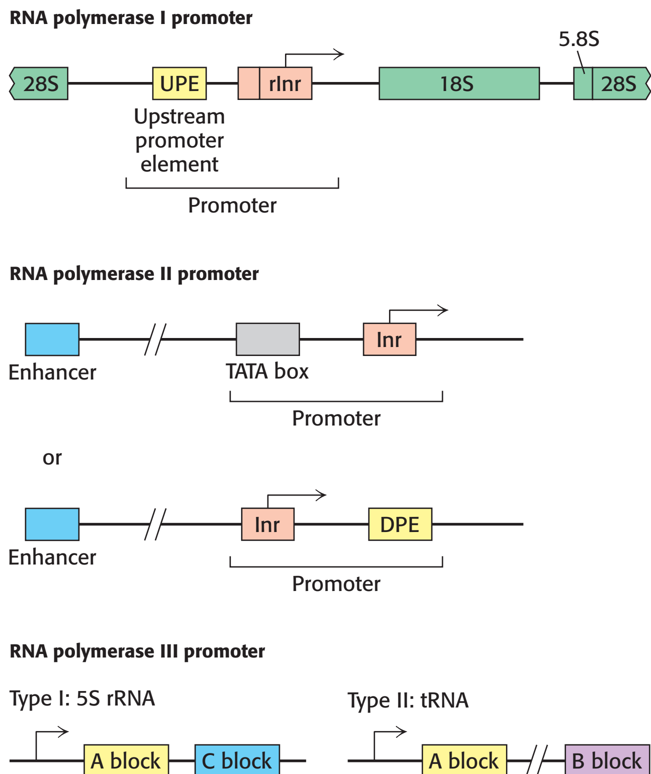


Figure 29.22 Common eukaryotic promoter elements. Each eukaryotic RNA polymerase recognizes a set of promoter elements—sequences in DNA that promote transcription. The RNA polymerase I promoter consists of a ribosomal initator (rInr) and an upstream promoter element (UPE). The RNA polymerase II promoter likewise includes an initator element (Inr) and may also include either a TATA box or a downstream promoter element (DPE). Separate from the promoter region, enhancer elements bind specific transcription factors. RNA polymerase III promoters consist of conserved sequences that lie within the transcribed genes.

polymerase I is insensitive to this toxin. This pattern of sensitivity is highly conserved throughout the animal and plant kingdoms.

Eukaryotic polymerases also differ from each other in the promoters to which they bind. Eukaryotic genes, like prokaryotic genes, require promoters for transcription initiation. Like prokaryotic promoters, eukaryotic promoters consist of conserved sequences that serve to attract the polymerase to the start site. However, eukaryotic promoters differ distinctly in sequence and position, depending on the type of RNA polymerase to which they bind (Figure 29.22).

1. *RNA Polymerase I.* The ribosomal DNA (rDNA) transcribed by polymerase I is arranged in several hundred tandem repeats, each containing a copy of each of three rRNA genes. The promoter sequences are located in stretches of DNA separating the genes. At the transcriptional start site lies a TATA-like sequence called the *ribosomal initiator element* (rInr). Farther upstream, 150 to 200 bp from the start site, is the *upstream promoter element* (UPE). Both elements aid transcription by binding proteins that recruit RNA polymerase I.

2. *RNA Polymerase II.* Promoters for RNA polymerase II, like prokaryotic promoters, include a set of consensus sequences that define the start site and recruit the polymerase. However, the promoter can contain any combination of a number of possible consensus sequences. Unique to eukaryotes, they also include enhancer elements that can be very distant (more than 1 kb) from the start site.

3. *RNA Polymerase III.* Promoters for RNA polymerase III are *within* the transcribed sequence, downstream of the start site. There are two types of intergenic promoters for RNA polymerase III. Type I promoters, found in the 5S rRNA gene, contain two short conserved sequences known as the A block and the C block. Type II promoters, found in tRNA genes, consist of two 11-bp sequences, the A block and the B block, situated about 15 bp from either end of the gene.

Three common elements can be found in the RNA polymerase II promoter region

RNA polymerase II transcribes all of the protein-coding genes in eukaryotic cells. Promoters for RNA polymerase II, like those for bacterial polymerases, are generally located on the 5′ side of the start site for transcription. Because these sequences are on the *same* molecule of DNA as the genes being transcribed, they are called *cis-acting elements.* The most commonly recognized cis-acting element for genes transcribed by RNA polymerase II is called the *TATA box* on the basis of its consensus sequence (Figure 29.23). The TATA box is usually found between positions −30 and −100. Note that the eukaryotic TATA box closely resembles the prokaryotic −10 sequence (TATAAT) but is farther from the start site. The mutation of a single base in the TATA box markedly impairs promoter activity. Thus, the precise sequence, not just a high content of AT pairs, is essential.

The TATA box is often paired with an *initiator element* (Inr), a sequence found at the transcriptional start site, between positions –3 and +5. This sequence defines the start site because the other promoter elements are

5′ T_{82} A_{97} T_{93} A_{85} A_{63} A_{88} A_{50} 3′
TATA box

Figure 29.23 TATA box. Comparisons of the sequences of more than 100 eukaryotic promoters led to the consensus sequence shown. The subscripts denote the frequency (%) of the base at that position.

5′ GGNCAATCT 3′
CAAT box

5′ GGGCGG 3′
GC box

Figure 29.24 CAAT box and GC box. Consensus sequences for the CAAT and GC boxes of eukaryotic promoters for mRNA precursors.

at variable distances from that site. Its presence increases transcriptional activity.

A third element, the *downstream core promoter element* (DPE), is commonly found in conjunction with the Inr in transcripts that lack the TATA box. In contrast with the TATA box, the DPE is found downstream of the start site, between positions +28 and +32.

Additional regulatory sequences are located between –40 and –150. Many promoters contain a *CAAT box,* and some contain a *GC box* (Figure 29.24). Constitutive genes (genes that are continuously expressed rather than regulated) tend to have GC boxes in their promoters. The positions of these upstream sequences vary from one promoter to another, in contrast with the quite constant location of the −35 region in prokaryotes. Another difference is that the CAAT box and the GC box can be effective when present on the template (antisense) strand, unlike the −35 region, which must be present on the coding (sense) strand. These differences between prokaryotes and eukaryotes correspond to fundamentally different mechanisms for the recognition of cis-acting elements. The −10 and −35 sequences in prokaryotic promoters are binding sites for RNA polymerase and its associated σ factor. In contrast, the TATA, CAAT, and GC boxes and other cis-acting elements in eukaryotic promoters are recognized by proteins other than by RNA polymerase itself.

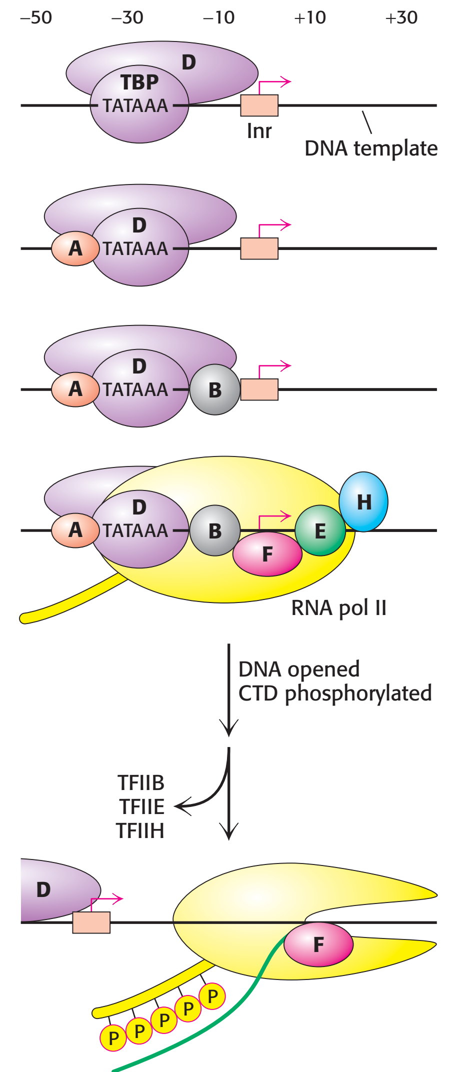


Figure 29.25 Transcription initation. Transcription factors TFIIA, B, D, E, F, and H are essential in initiating transcription by RNA polymerase II. The step-by-step assembly of these general transcription factors begins with the binding of TFIID (purple) to the TATA box. [The TATA-box-binding protein (TBP), a component of TFIID, recognizes the TATA box.] After assembly, TFIIH opens the DNA double helix and phosphorylates the carboxyl-terminal domain (CTD), allowing the polymerase to leave the promoter and begin transcription. The red arrow marks the transcription start site.

The TFIID protein complex initiates the assembly of the active transcription complex

Cis-acting elements constitute only part of the puzzle of eukaryotic gene expression. *Transcription factors* that bind to these elements also are required. For example, RNA polymerase II is guided to the start site by a set of transcription factors known collectively as *TFII* (*TF* stands for transcription factor, and *II* refers to RNA polymerase II). Individual TFII factors are called TFIIA, TFIIB, and so on.

In TATA-box promoters, the key initial event is the recognition of the TATA box by the TATA-box-binding protein (TBP), a 30-kd component of the 700-kd TFIID complex (Figure 29.25). In TATA-less promoters, other proteins in the TFIID complex bind the core promoter elements but, because less is known about these interactions, we will consider only the TATA-box–TBP binding interaction. TBP binds 10^5 times as tightly to the TATA box as to nonconsensus sequences; the dissociation constant of the TBP–TATA-box complex is approximately 1 nM. TBP is a saddle-shaped protein consisting of two similar domains (Figure 29.26). The TATA box of DNA binds to the concave surface of TBP. This binding induces large conformational changes in the bound DNA. The double helix is substantially unwound to widen its *minor groove,* enabling it to make extensive contact with the antiparallel β strands on the concave side of TBP. Hydrophobic interactions are prominent at this interface. Four phenylalanine residues,

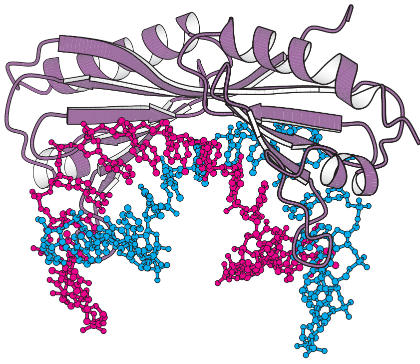


Figure 29.26 Complex formed by TATA-box-binding protein and DNA. The saddlelike structure of the protein sits atop a DNA fragment. *Notice* that the DNA is significantly unwound and bent. [Drawn from 1CDW.pdb.]

for example, are intercalated between base pairs of the TATA box. The flexibility of AT-rich sequences is generally exploited here in bending the DNA. Immediately outside the TATA box, classical B-DNA resumes. The TBP–TATA-box complex is distinctly asymmetric. The asymmetry is crucial for specifying a unique start site and ensuring that transcription proceeds unidirectionally.

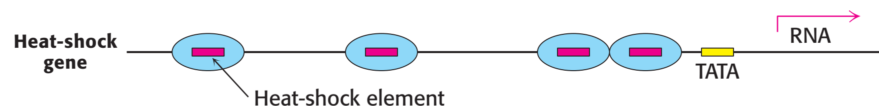
TBP bound to the TATA box is the heart of the initiation complex (see Figure 29.25). The surface of the TBP saddle provides docking sites for the binding of other components. Additional transcription factors assemble on this nucleus in a defined sequence. TFIIA is recruited, followed by TFIIB; then TFIIF, RNA polymerase II, TFIIE, and TFIIH join the other factors to form a complex called the *basal transcription apparatus*. During the formation of the basal transcription apparatus, the carboxyl-terminal domain (CTD) is unphosphorylated and plays a role in transcription regulation through its binding to an enhancer-associated complex called mediator (see Section 32.2). The phosphorylated CTD stabilizes transcription elongation by RNA polymerase II and recruits RNA-processing enzymes that act in the course of elongation. *Phosphorylation of the CTD by TFIIH marks the transition from initiation to elongation.* The importance of the carboxyl-terminal domain is highlighted by the finding that yeast containing mutant polymerase II with fewer than 10 repeats in the CTD is not viable. Most of the factors are released before the polymerase leaves the promoter and can then participate in another round of initiation.

Multiple transcription factors interact with eukaryotic promoters

The basal transcription complex described in the preceding section initiates transcription at a low frequency. Additional transcription factors that bind to other sites are required to achieve a high rate of mRNA synthesis. Their role is to selectively stimulate specific genes. Upstream stimulatory sites in eukaryotic genes are diverse in sequence and variable in position. Their variety suggests that they are recognized by many different specific proteins. Indeed, many transcription factors have been isolated, and their binding sites have been identified by footprinting experiments. For example, *heat-shock transcription factor* (HSTF) is expressed in *Drosophila* after an abrupt increase in temperature. This 93-kd DNA-binding protein binds to the following consensus sequence:

5′–CNNGAANNTCCNNG–3′

Several copies of this sequence, known as the *heat-shock response element*, are present starting at a site 15 bp upstream of the TATA box.



HSTF differs from σ^{32}, a heat-shock protein of *E. coli* (p. 858), in binding directly to response elements in heat-shock promoters rather than first becoming associated with RNA polymerase.

Enhancer sequences can stimulate transcription at start sites thousands of bases away

The activities of many promoters in higher eukaryotes are greatly increased by another type of cis-acting element called an *enhancer*. Enhancer sequences have no promoter activity of their own *yet can exert their stimulatory actions*

over distances of several thousand base pairs. They can be upstream, downstream, or even in the midst of a transcribed gene. Moreover, enhancers are effective when present on *either DNA strand* (equivalently, in either orientation).

A particular enhancer is effective only in certain cells. For example, the immunoglobulin enhancer functions in B lymphocytes but not elsewhere. Cancer can result if the relation between genes and enhancers is disrupted. In Burkitt lymphoma and B-cell leukemia, a chromosomal translocation brings the proto-oncogene *myc* (a transcription factor itself) under the control of a powerful immunoglobin enhancer. The consequent dysregulation of the *myc* gene is believed to play a role in the progression of the cancer.

Transcription factors and other proteins that bind to regulatory sites on DNA can be regarded as passwords that cooperatively open multiple locks, giving RNA polymerase access to specific genes. The discovery of promoters and enhancers has allowed us to gain a better understanding of how genes are selectively expressed in eukaryotic cells. The regulation of gene transcription, discussed in Chapter 32, is the fundamental means of controlling gene expression.

Although bacteria lack TBP, archaea utilize a TBP molecule that is structurally quite similar to the eukaryotic protein. In fact, transcriptional control processes in archaea are, in general, much more similar to those in eukaryotes than are the processes in bacteria. Many components of the eukaryotic transcriptional machinery evolved from an ancestor of archaea.

29.3 The Transcription Products of Eukaryotic Polymerases Are Processed

Virtually all the initial products of transcription are further processed in eukaryotes. For example, primary transcripts (pre-mRNA molecules), the products of RNA polymerase II action, acquire a cap at their 5′ ends and a poly(A) tail at their 3′ends. Most importantly, *nearly all mRNA precursors in higher eukaryotes are spliced*. Introns are precisely excised from primary transcripts, and exons are joined to form mature mRNAs with continuous messages. Some mature mRNAs are only a tenth the size of their precursors, which can be as large as 30 kb or more. The pattern of splicing can be regulated in the course of development to generate variations on a theme, such as membrane-bound or secreted forms of antibody molecules. Alternative splicing enlarges the repertoire of proteins in eukaryotes and is one clear illustration of why the proteome is more complex than the genome. The particular processing steps and the factors taking part vary according to the type of RNA polymerase.

RNA polymerase I produces three ribosomal RNAs

Several RNA molecules are key components of ribosomes. RNA polymerase I transcription produces a single precursor (45S in mammals) that encodes three RNA components of the ribosome: the 18S rRNA, the 28S rRNA, and the 5.8S rRNA (Figure 29.27). The 18S rRNA is the RNA component of the small ribosomal subunit (40S), and the 28S and 5.8S rRNAs are two RNA components of the large ribosomal subunit (60S). The other RNA component of the large ribosomal subunit, the 5S rRNA, is transcribed by RNA polymerase III as a separate transcript.

The cleavage of the precursor into three separate rRNAs is actually the final step in its processing. First, the nucleotides of the pre-rRNA sequences

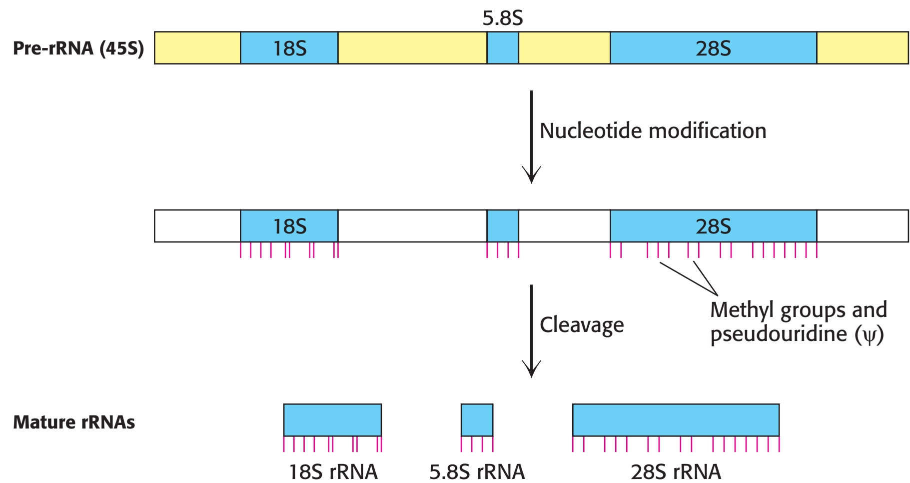


Figure 29.27 Processing of eukaryotic pre-rRNA. The mammalian pre-rRNA transcript contains the RNA sequences destined to become the 18S, 5.8S, and 28S rRNAs of the small and large ribosomal subunits. First, nucleotides are modified: small nucleolar ribonucleoproteins methylate specific ribose groups and convert selected uridines into pseudouridines (indicated by red lines). Next, the pre-rRNA is cleaved and packaged to form mature ribosomes, in a highly regulated process in which more than 200 proteins take part.

destined for the ribosome undergo extensive modification, on both ribose and base components, directed by many *small nucleolar ribonucleoproteins* (snoRNPs), each of which consists of one snoRNA and several proteins. The pre-rRNA is assembled with ribosomal proteins, as guided by processing factors, in a large ribonucleoprotein. For instance, the small-subunit (SSU) processome is required for 18S rRNA synthesis and can be visualized in electron micrographs as a terminal knob at the 5′ ends of the nascent rRNAs (Fig. 29.28). Finally, rRNA cleavage (sometimes coupled with additional processing steps) releases the mature rRNAs assembled with ribosomal proteins as ribosomes. Like those of RNA polymerase I transcription itself, most of these processing steps take place in the cell nucleolus, a nuclear subcompartment.

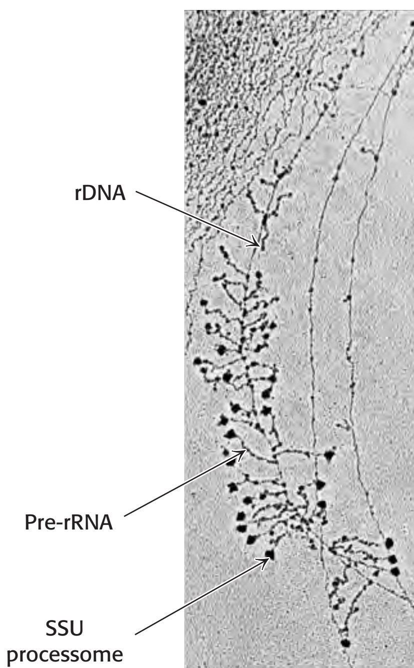


Figure 29.28 Visualization of rRNA transcription and processing in eukaryotes. Transcription of rRNA and its assembly into precursor ribosomes can be visualized by electron microscopy. The structures resemble Christmas trees: the trunk is the rDNA and each branch is a pre-rRNA transcript. Transcription starts at the top of the tree, where the shortest transcripts can be seen, and progresses down the rDNA to the end of the gene. The terminal knobs visible at the end of some pre-rRNA transcripts likely correspond to the SSU processome, a large ribonucleoprotein required for processing the pre-rRNA. [From F. Dragon et al. *Nature* 417:967–970, 2002.]

RNA polymerase III produces transfer RNA

Eukaryotic tRNA transcripts are among the most processed of all RNA polymerase III transcripts. Like those of prokaryotic tRNAs, the 5′ leader is cleaved by RNase P, the 3′ trailer is removed, and CCA is added by the CCA-adding enzyme (Figure 29.29). Eukaryotic tRNAs are also heavily modified on base and ribose moieties; these modifications are important for function. In contrast with prokaryotic tRNAs, many eukaryotic pre-tRNAs are also spliced by an endonuclease and a ligase to remove an intron.

The product of RNA polymerase II, the pre-mRNA transcript, acquires a 5′ cap and a 3′ poly(A) tail

Perhaps the most extensively studied transcription product is the product of RNA polymerase II: most of this RNA will be processed to mRNA. The immediate product of RNA polymerase II is sometimes referred to as precursor-to-messenger RNA, or *pre-mRNA*. Most pre-mRNA molecules are spliced to remove the introns. Moreover, both the 5′ and the 3′ ends are modified, and both modifications are retained as the pre-mRNA is converted into mRNA.

As in prokaryotes, eukaryotic transcription usually begins with A or G. However, the 5′ triphosphate end of the nascent RNA chain is immediately modified. First, a phosphoryl group is released by hydrolysis. The diphosphate 5′ end then attacks the α-phosphorus atom of GTP to form a very unusual 5′–5′ triphosphate linkage. This distinctive terminus is called a *cap* (Figure 29.30). The N-7 nitrogen of the terminal guanine is then methylated by *S*-adenosylmethionine to form *cap 0*. The adjacent riboses may be

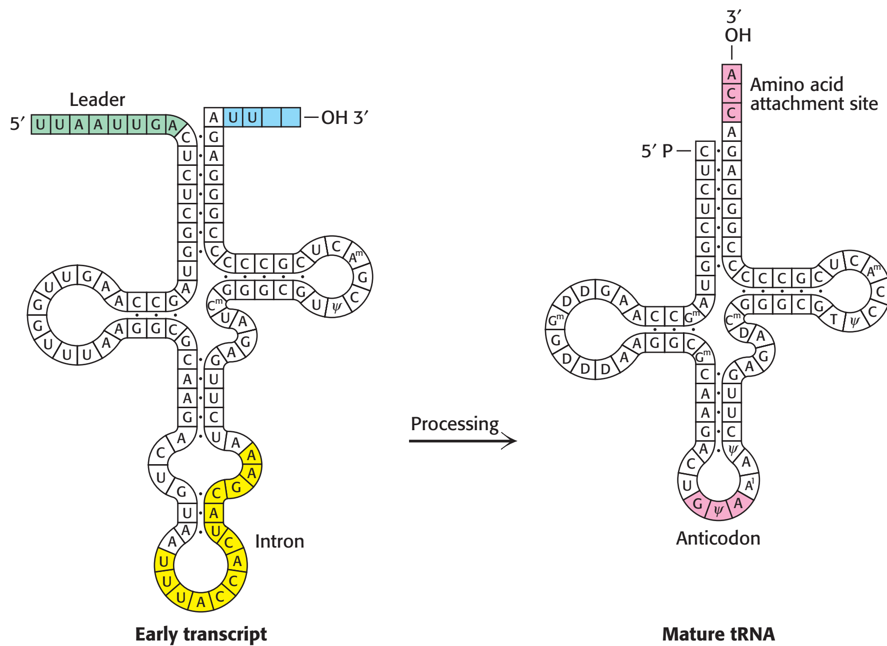


Figure 29.29 Transfer RNA precursor processing. The conversion of a yeast tRNA precursor into a mature tRNA requires the removal of a 14-nucleotide intron (yellow), the cleavage of a 5′ leader (green), and the removal of UU and the attachment of CCA at the 3′ end (red). In addition, several bases are modified.

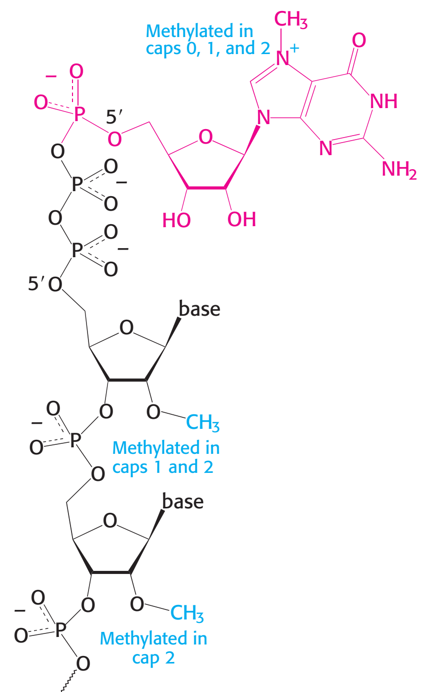


Figure 29.30 Capping the 5′ end. Caps at the 5′ end of eukaryotic mRNA include 7-methylguanylate (red) attached by a triphosphate linkage to the ribose at the 5′ end. None of the riboses are methylated in cap 0, one is methylated in cap 1, and both are methylated in cap 2.

methylated to form *cap 1* or *cap 2*. Transfer RNA and ribosomal RNA molecules, in contrast with messenger RNAs and with small RNAs that participate in splicing, do not have caps. Caps contribute to the stability of mRNAs by protecting their 5′ ends from phosphatases and nucleases. In addition, caps enhance the translation of mRNA by eukaryotic protein-synthesizing systems.

As mentioned earlier, pre-mRNA is also modified at the 3′ end. *Most eukaryotic mRNAs contain a polyadenylate, poly(A), tail at that end,* added after transcription has ended. The DNA template does not encode this poly(A) tail. Indeed, the nucleotide preceding poly(A) is not the last nucleotide to be transcribed. Some primary transcripts contain hundreds of nucleotides beyond the 3′ end of the mature mRNA.

How is the 3′ end of the pre-mRNA given its final form? *Eukaryotic primary transcripts are cleaved by a specific endonuclease that recognizes the sequence AAUAAA* (Figure 29.31). Cleavage does not take place if this sequence or a segment of some 20 nucleotides on its 3′ side is deleted. The

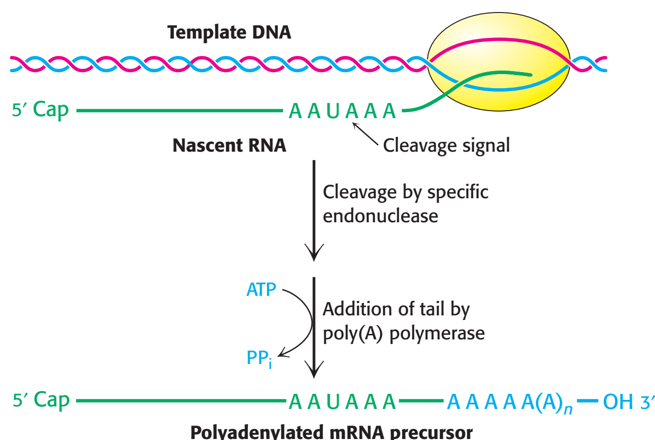


Figure 29.31 Polyadenylation of a primary transcript. A specific endonuclease cleaves the RNA downstream of AAUAAA. Poly(A) polymerase then adds about 250 adenylate residues.

presence of internal AAUAAA sequences in some mature mRNAs indicates that AAUAAA is only part of the cleavage signal; its context also is important. After cleavage of the pre-RNA by the endonuclease, a *poly(A) polymerase* adds about 250 adenylate residues to the 3′ end of the transcript; ATP is the donor in this reaction.

The role of the poly(A) tail is still not firmly established despite much effort. However, evidence is accumulating that it enhances translation efficiency and the stability of mRNA. Blocking the synthesis of the poly(A) tail by exposure to *3′-deoxyadenosine (cordycepin)* does not interfere with the synthesis of the primary transcript. Messenger RNA devoid of a poly(A) tail can be transported out of the nucleus. However, an mRNA molecule devoid of a poly(A) tail is usually a much less effective template for protein synthesis than is one with a poly(A) tail. Indeed, some mRNAs are stored in an unadenylated form and receive the poly(A) tail only when translation is imminent. The half-life of an mRNA molecule may be determined in part by the rate of degradation of its poly(A) tail.

Small regulatory RNAs are cleaved from larger precursors

Cleavage plays a role in the processing of small single-stranded RNAs (approximately 20–23 nucleotides) called *microRNAs*. MicroRNAs play key roles in gene regulation in eukaryotes, as we shall see in Chapter 32. They are generated from initial transcripts produced by RNA polymerase II and, in some cases, RNA polymerase III. These transcripts fold into hairpin structures that are cleaved by specific nucleases at various stages (Figure 29.32). The final single-stranded RNAs are bound by members of the Argonaute family of proteins to exert their roles on controlling gene expression.

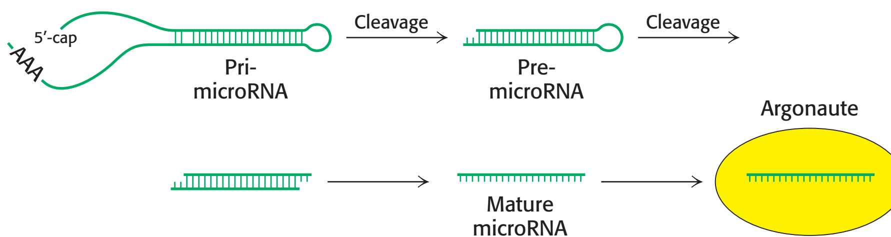


Figure 29.32 Small regulatory RNA production. A pathway from a transcription product including a microRNA to the mature microRNA bound to an Argonaute protein. The initial transcription product, a pri-microRNA, is first cleaved to a small double-stranded RNA called a pre-microRNA. One of the strands of the pre-microRNA, the mature microRNA, is then bound by an Argonaute protein.

RNA editing changes the proteins encoded by mRNA

Remarkably, the amino acid sequence information encoded by some mRNAs is altered after transcription. *RNA editing* is the term for a change in the nucleotide sequence of RNA after transcription by processes other than RNA splicing. RNA editing is prominent in some systems already discussed. *Apolipoprotein B* (apo B) plays an important role in the transport of triacylglycerols and cholesterol by forming an amphipathic spherical shell around the lipids carried in lipoprotein particles (Section 26.3). Apo B exists in two forms, a 512-kd *apo B-100* and a 240-kd *apo B-48*. The larger form, synthesized by the liver, participates in the transport of lipids synthesized in the cell. The smaller form, synthesized by the small intestine, carries dietary fat in the form of chylomicrons. Apo B-48 contains the 2152 N-terminal residues of the 4536-residue apo B-100. This truncated molecule can form lipoprotein particles but cannot bind to the low-density-lipoprotein receptor on cell surfaces. What is the relationship between these two forms of apo B? Experiments revealed that a totally unexpected mechanism for generating diversity is at work: *the changing of the nucleotide sequence of mRNA after its synthesis* (Figure 29.33). *A specific cytidine residue of mRNA*

is deaminated to uridine, which changes the codon at residue 2153 from CAA (Gln) to UAA (stop). The deaminase that catalyzes this reaction is present in the small intestine, but not in the liver, and is expressed only at certain developmental stages.

RNA editing is not confined to apolipoprotein B. Glutamate opens cation-specific channels in the vertebrate central nervous system by binding to receptors in postsynaptic membranes. RNA editing changes a single glutamine codon (CAG) in the mRNA for the glutamate receptor to the codon for arginine (read as CGG). The substitution of Arg for Gln in the receptor prevents Ca^{2+}, but not Na^{+}, from flowing through the channel.

RNA editing is likely much more common than was formerly thought. The chemical reactivity of nucleotide bases, including the susceptibility to deamination that necessitates complex DNA-repair mechanisms, has been harnessed as an engine for generating molecular diversity at the RNA and, hence, protein levels.

In trypanosomes (parasitic protozoans), a different kind of RNA editing markedly changes several mitochondrial mRNAs. Nearly half the uridine residues in these mRNAs are inserted by RNA editing. A *guide RNA molecule* identifies the sequences to be modified, and a *poly(U) tail* on the guide donates uridine residues to the mRNAs undergoing editing. DNA sequences evidently do not always faithfully disclose the sequence of encoded proteins: functionally crucial changes to mRNA can take place.

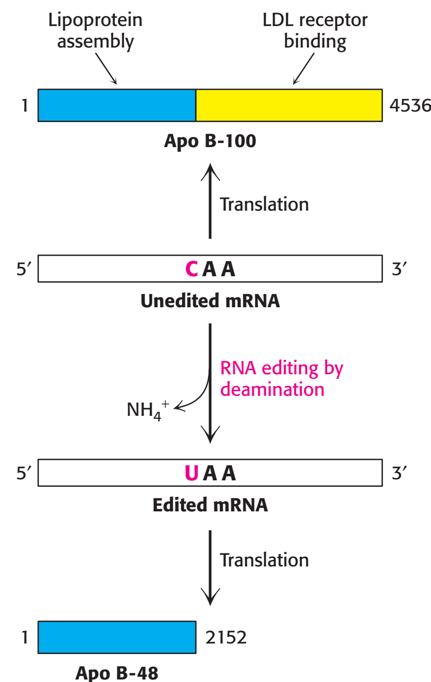


Figure 29.33 RNA editing. Enzyme-catalyzed deamination of a specific cytidine residue in the mRNA for apolipoprotein B-100 changes a codon for glutamine (CAA) to a stop codon (UAA). Apolipoprotein B-48, a truncated version of the protein lacking the LDL receptor-binding domain, is generated by this posttranscriptional change in the mRNA sequence. [After P. Hodges and J. Scott. *Trends Biochem. Sci.* 17:77, 1992.]

Sequences at the ends of introns specify splice sites in mRNA precursors

Most genes in higher eukaryotes are composed of exons and introns (Section 4.7). The introns must be excised and the exons must be linked to form the final mRNA in a process called *RNA splicing*. This splicing must be exquisitely sensitive: splicing just one nucleotide upstream or downstream of the intended site would create a one-nucleotide shift, which would alter the reading frame on the 3′ side of the splice to give an entirely different amino acid sequence, likely including a premature stop codon. Thus, the correct splice site must be clearly marked. Does a particular sequence denote the splice site? The sequences of thousands of intron–exon junctions within RNA transcripts are known. In eukaryotes from yeast to mammals, these sequences have a common structural motif: *the intron begins with GU and ends with AG*. The consensus sequence at the 5′ splice in vertebrates is AGGUAAGU, where the GU is invariant (Figure 29.34). At the 3′ end of an intron, the consensus sequence is a stretch of *10 pyrimidines* (U or C; termed the *polypyrimidine tract*), followed by any base and then by C, and ending with the invariant AG. Introns also have an important internal site located between 20 and 50 nucleotides upstream of the 3′ splice site; it is called the *branch site* for reasons that will be evident shortly. In yeast, the branch-site sequence is nearly always UACUAAC, whereas, in mammals, a variety of sequences are found.

The 5′ and 3′ splice sites and the branch site are essential for determining where splicing takes place. Mutations in each of these three critical

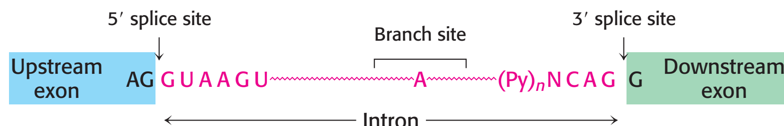


Figure 29.34 Splice sites. Consensus sequences for the 5′ splice site and the 3′ splice site are shown. Py stands for pyrimidine.

regions lead to aberrant splicing. Introns vary in length from 50 to 10,000 nucleotides, and so the splicing machinery may have to find the 3′ site several thousand nucleotides away. Specific sequences near the splice sites (in both the introns and the exons) play an important role in splicing regulation, particularly in designating splice sites when there are many alternatives (p. 878). Researchers are currently attempting to determine the factors that contribute to splice-site selection for individual mRNAs. Despite our knowledge of splice-site sequences, predicting pre-mRNAs and their protein products from genomic DNA sequence information remains a challenge.

Splicing consists of two sequential transesterification reactions

The splicing of nascent mRNA molecules is a complicated process. It requires the cooperation of several small RNAs and proteins that form a large complex called a *spliceosome*. However, the chemistry of the splicing process is simple. Splicing begins with the cleavage of the phosphodiester bond between the upstream exon (exon 1) and the 5′ end of the intron (Figure 29.35). The attacking group in this reaction is the 2′-OH group of an adenylate residue in the branch site. A 2′–5′ phosphodiester bond is formed between this A residue and the 5′ terminal phosphate of the intron. This reaction is a transesterification.

Note that this adenylate residue is also joined to two other nucleotides by normal 3’–5’ phosphodiester bonds (Figure 29.36). Hence a *branch* is generated at this site, and a *lariat intermediate* is formed.

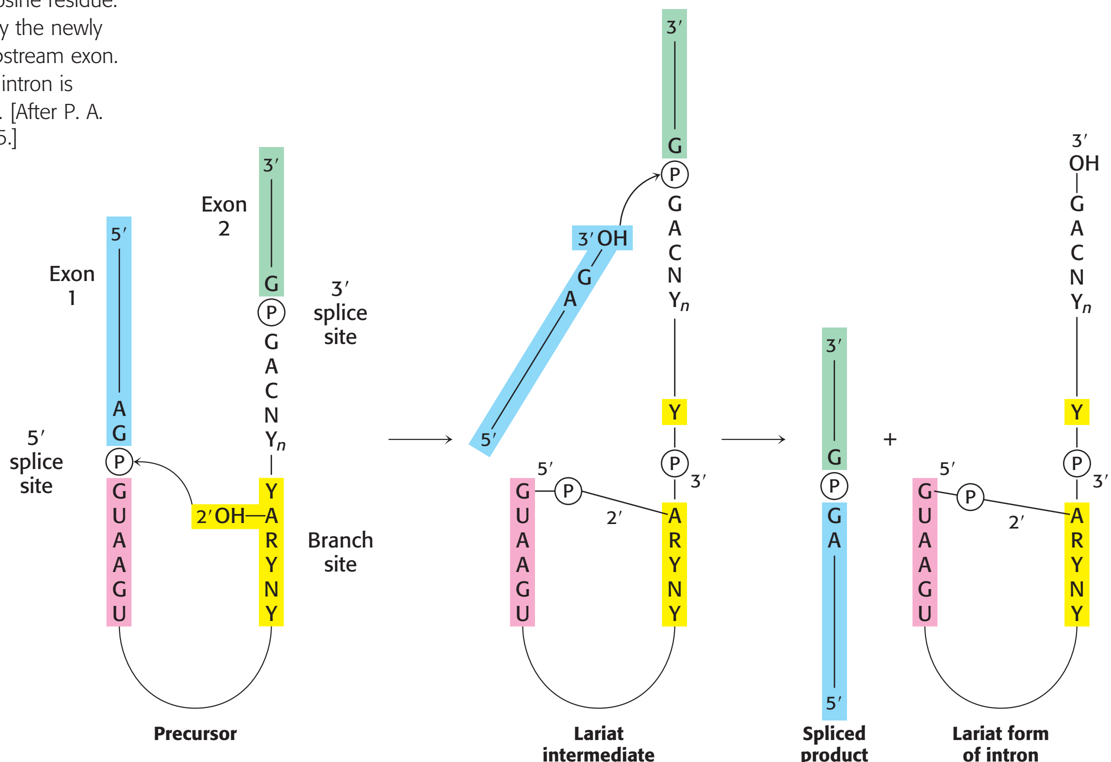


Figure 29.35 Splicing mechanism used for mRNA precursors. The upstream (5′) exon is shown in blue, the downstream (3′) exon in green, and the branch site in yellow. Y stands for a pyrimidine nucleotide, R for a purine nucleotide, and N for any nucleotide. The 5′ splice site is attacked by the 2′-OH group of the branch-site adenosine residue. The 3′ splice site is attacked by the newly formed 3′-OH group of the upstream exon. The exons are joined, and the intron is released in the form of a lariat. [After P. A. Sharp. *Cell* 42:397–408, 1985.]

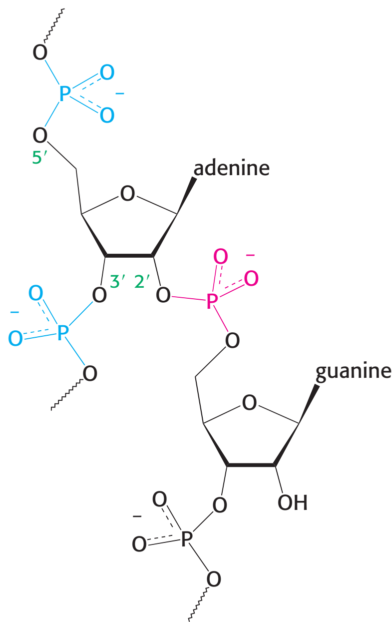


Figure 29.36 Splicing branch point. The structure of the branch point in the lariat intermediate in which the adenylate residue is joined to three nucleotides by phosphodiester bonds. The new 2′-to-5′ linkage is shown in red, and the usual 3′-to-5′ linkages are shown in blue.

The 3′-OH terminus of exon 1 then attacks the phosphodiester bond between the intron and exon 2. Exons 1 and 2 become joined, and the intron is released in lariat form. Again, this reaction is a transesterification. Splicing is thus accomplished by two *transesterification reactions* rather than by hydrolysis followed by ligation. The first reaction generates a free 3′-OH group at the 3′ end of exon 1, and the second reaction links this group to the 5′-phosphate of exon 2. *The number of phosphodiester bonds stays the same during these steps,* which is crucial because it allows the splicing reaction itself to proceed without an energy source such as ATP or GTP.

Small nuclear RNAs in spliceosomes catalyze the splicing of mRNA precursors

The nucleus contains many types of small RNA molecules with fewer than 300 nucleotides, referred to as *snRNAs* (small nuclear RNAs). A few of them—designated U1, U2, U4, U5, and U6—are essential for splicing mRNA precursors. The secondary structures of these RNAs are highly conserved in organisms ranging from yeast to human beings. These RNA molecules are associated with specific proteins to form complexes termed *snRNPs* (*s*mall *n*uclear *r*ibo*n*ucleo*p*rotein particles); investigators often speak of them as "snurps." Spliceosomes are large (60S) dynamic assemblies composed of snRNPs, hundreds of other proteins called *splicing factors,* and the mRNA precursors being processed (Table 29.3).

In mammalian cells, splicing begins with the recognition of the 5′ splice site by the U1 snRNP (Figure 29.37). U1 snRNA contains a highly conserved six-nucleotide sequence, not covered by protein in the snRNP, that

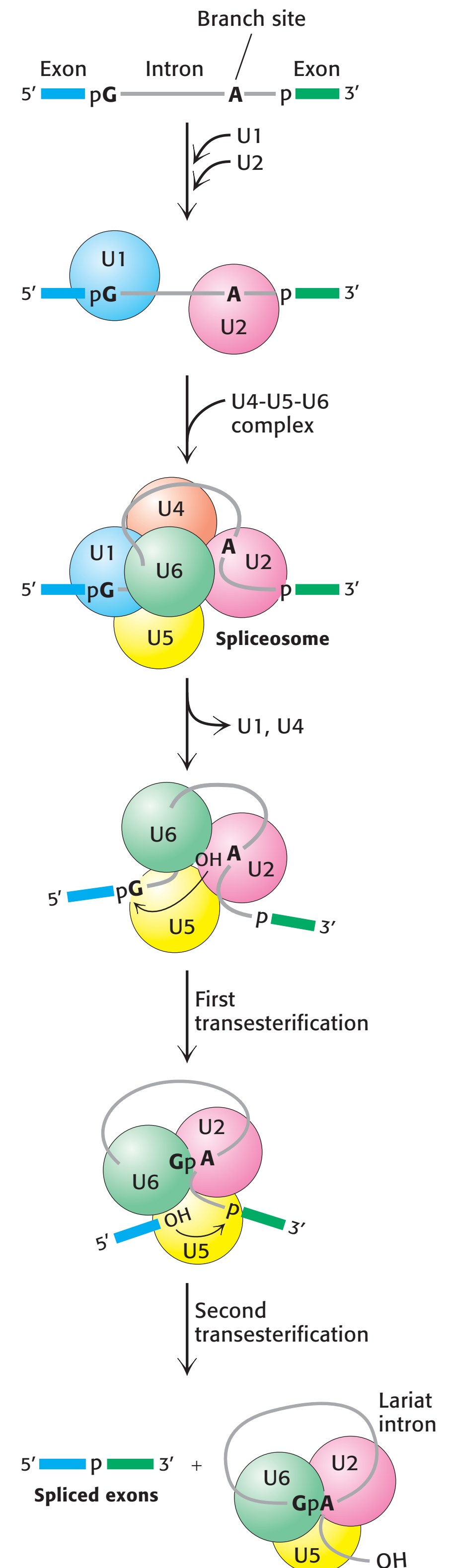
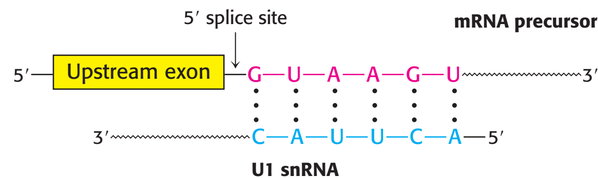


Figure 29.37 Spliceosome assembly and action. U1 binds the 5′ splice site and U2 binds to the branch point. A preformed U4-U5-U6 complex then joins the assembly to form the complete spliceosome. The U6 snRNA re-folds and binds the 5′ splice site, displacing U1. Extensive interactions between U6 and U2 displace U4. Then, in the first transesterification step, the branch-site adenosine attacks the 5′ splice site, making a lariat intermediate. U5 holds the two exons in close proximity, and the second transesterification takes place, with the 5′ splice-site hydroxyl group attacking the 3′ splice site. These reactions result in the mature spliced mRNA and a lariat form of the intron bound by U2, U5, and U6. [After T. Villa, J. A. Pleiss, and C. Guthrie, *Cell* 109:149–152, 2002.]

Table 29.3 Small nuclear ribonucleoprotein particles (snRNPs) in the splicing of mRNA precursors

snRNP	Size of snRNA (nucleotides)	Role
U1	165	Binds the 5′ splice site
U2	185	Binds the branch site and forms part of the catalytic center
U5	116	Binds the 5′ splice site and then the 3′ splice site
U4	145	Masks the catalytic activity of U6
U6	106	Catalyzes splicing

base-pairs to the 5′ splice site of the pre-mRNA. This binding initiates spliceosome assembly on the pre-mRNA molecule.



U2 snRNP then binds the branch site in the intron by base-pairing between a highly conserved sequence in U2 snRNA and the pre-mRNA. U2 snRNP binding requires ATP hydrolysis. A preassembled U4-U5-U6 tri-snRNP joins this complex of U1, U2, and the mRNA precursor to form the spliceosome. This association also requires ATP hydrolysis.

A revealing view of the interplay of RNA molecules in this assembly came from examining the pattern of cross-links formed by *psoralen,* a reagent that joins neighboring pyrimidines in base-paired regions on treatment with light. These cross-links suggest that splicing takes place in the following way. First, U5 interacts with exon sequences in the 5′ splice site and subsequently with the 3′ exon. Next, U6 disengages from U4 and undergoes an intramolecular rearrangement that permits base-pairing with U2 as well as interaction with the 5′ end of the intron, displacing U1 from the spliceosome. The U2-U6 helix is indispensable for splicing, suggesting that *U2 and U6 snRNAs probably form the catalytic center of the spliceosome* (Figure 29.38). U4 serves as an inhibitor that masks U6 until the specific splice sites are aligned. These rearrangements result in the first transesterification reaction, cleaving the 5′ exon and generating the lariat intermediate.

Further rearrangements of RNA in the spliceosome facilitate the second transesterification. In these rearrangements, U5 aligns the free 5′ exon with the 3′ exon such that the 3′-hydroxyl group of the 5′ exon is positioned to nucleophilically attack the 3′ splice site to generate the spliced product. U2, U5, and U6 bound to the excised lariat intron are released to complete the splicing reaction.

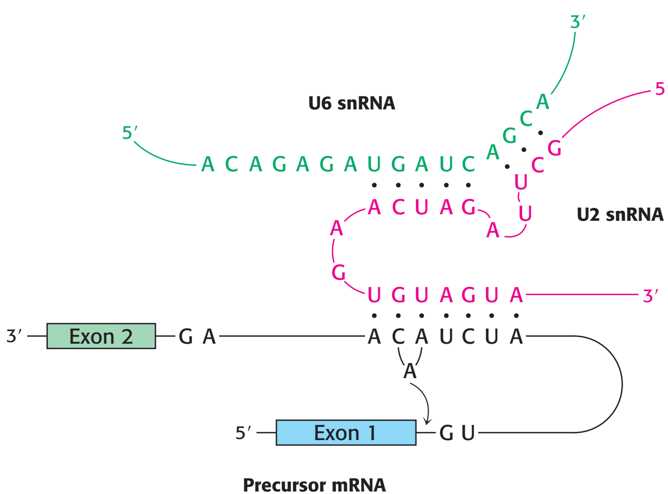


Figure 29.38 Splicing catalytic center. The catalytic center of the spliceosome is formed by U2 snRNA (red) and U6 snRNA (green), which are base paired. U2 is also base paired to the branch site of the mRNA precursor. [After H. D. Madhani and C. Guthrie. *Cell* 71:803–817, 1992.]

Many of the steps in the splicing process require ATP hydrolysis. How is the free energy associated with ATP hydrolysis used to power splicing? To achieve the well-ordered rearrangements necessary for splicing, ATP-powered RNA helicases must unwind RNA helices and allow alternative base-pairing arrangements to form. Thus, two features of the splicing process

are noteworthy. First, *RNA molecules play key roles in directing the alignment of splice sites and in carrying out catalysis*. Second, *ATP-powered helicases unwind RNA duplex intermediates that facilitate catalysis and induce the release of snRNPs from the mRNA*.

Transcription and processing of mRNA are coupled

Although the transcription and processing of mRNAs have been described herein as separate events in gene expression, experimental evidence suggests that the two steps are coordinated by the carboxyl-terminal domain of RNA polymerase II. We have seen that the CTD consists of a unique repeated seven-amino-acid sequence, YSPTSPS. Either S_2 or S_5 or both may be phosphorylated in the various repeats. The phosphorylation state of the CTD is controlled by a number of kinases and phosphatases and leads the CTD to bind many of the proteins having roles in RNA transcription and processing. The CTD contributes to efficient transcription by recruiting these proteins to the pre-mRNA (Figure 29.39), including:

1. capping enzymes, which methylate the 5′ guanine on the pre-mRNA immediately after transcription begins;

2. components of the splicing machinery, which initiate the excision of each intron as it is synthesized; and

3. an endonuclease that cleaves the transcript at the poly(A) addition site, creating a free 3′-OH group that is the target for 3′ adenylation.

These events take place sequentially, directed by the phosphorylation state of the CTD.

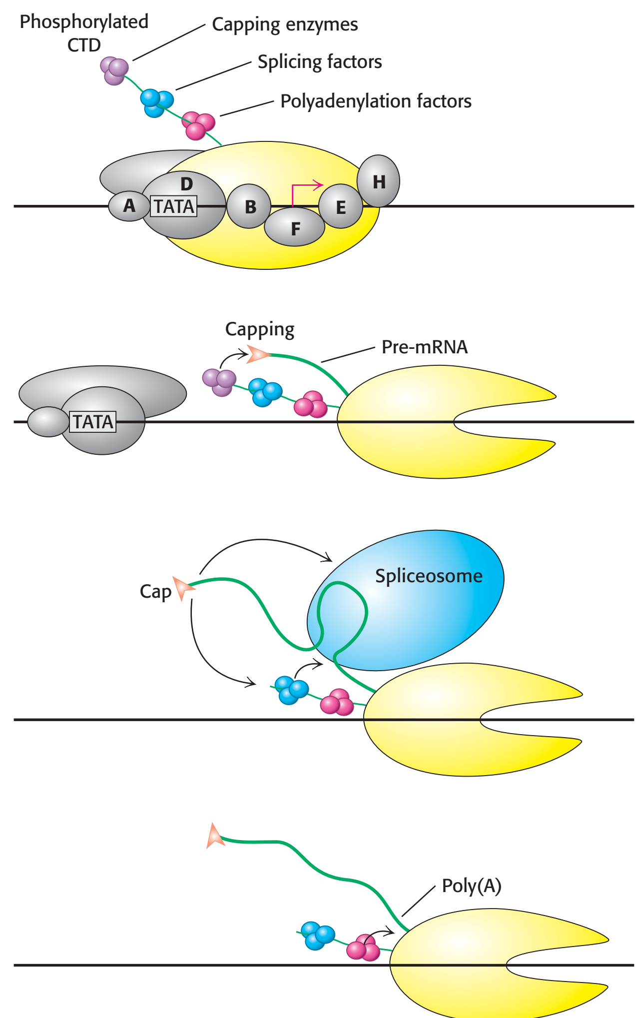
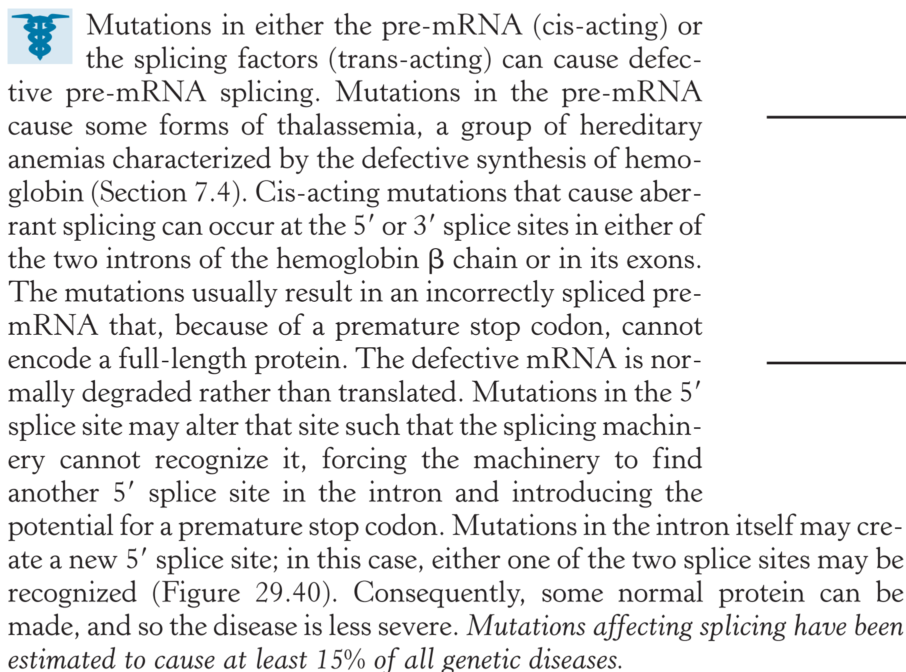


Figure 29.39 The CTD: Coupling transcription to pre-mRNA processing. The transcription factor TFIIH phosphorylates the carboxyl-terminal domain (CTD) of RNA polymerase II, signaling the transition from transcription initiation to elongation. The phosphorylated CTD binds factors required for pre-mRNA capping, splicing, and polyadenylation. These proteins are brought in close proximity to their sites of action on the nascent pre-mRNA as it is transcribed during elongation. [After P. A. Sharp. *TIBS* 30:279–281, 2005.]

Mutations that affect pre-mRNA splicing cause disease



Mutations in either the pre-mRNA (cis-acting) or the splicing factors (trans-acting) can cause defective pre-mRNA splicing. Mutations in the pre-mRNA cause some forms of thalassemia, a group of hereditary anemias characterized by the defective synthesis of hemoglobin (Section 7.4). Cis-acting mutations that cause aberrant splicing can occur at the 5′ or 3′ splice sites in either of the two introns of the hemoglobin β chain or in its exons. The mutations usually result in an incorrectly spliced pre-mRNA that, because of a premature stop codon, cannot encode a full-length protein. The defective mRNA is normally degraded rather than translated. Mutations in the 5′ splice site may alter that site such that the splicing machinery cannot recognize it, forcing the machinery to find another 5′ splice site in the intron and introducing the potential for a premature stop codon. Mutations in the intron itself may create a new 5′ splice site; in this case, either one of the two splice sites may be recognized (Figure 29.40). Consequently, some normal protein can be made, and so the disease is less severe. *Mutations affecting splicing have been estimated to cause at least 15% of all genetic diseases.*

Disease-causing mutations may also appear in splicing factors. Retinitis pigmentosa is a disease of acquired blindness, first described in 1857, with an incidence of 1/3500. About 5% of the autosomal dominant form of retinitis pigmentosa is likely due to mutations in the hPrp8 protein, a pre-mRNA

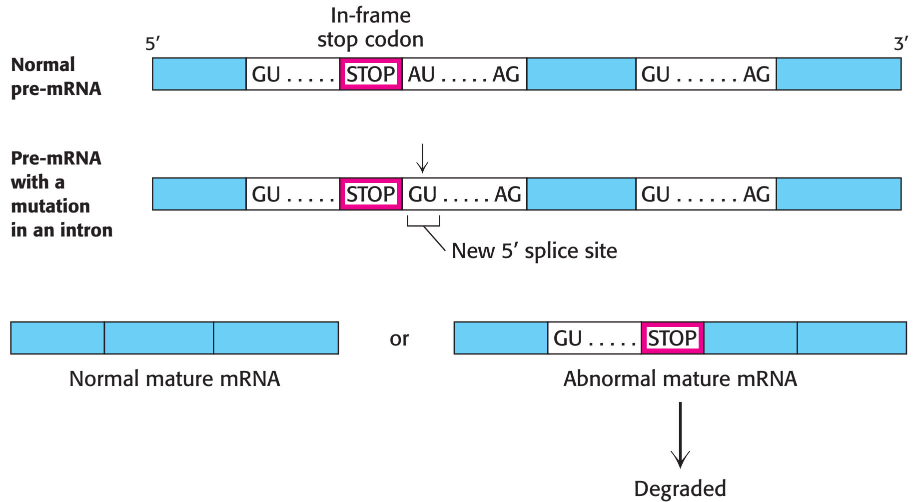


Figure 29.40 A splicing mutation that causes thalassemia. An A-to-G mutation within the first intron of the gene for the human hemoglobin β chain creates a new 5′ splice site (GU). Both 5′ splice sites are recognized by the U1 snRNP; so splicing may sometimes create a normal mature mRNA and an abnormal mature mRNA that contains intron sequences. The normal mature mRNA is translated into a hemoglobin β chain. Because it includes intron sequences, the abnormal mature mRNA now has a premature stop codon and is degraded.

splicing factor that is a component of the U4-U5-U6 tri-snRNP. How a mutation in a splicing factor that is present in all cells causes disease only in the retina is not clear; nevertheless, retinitis pigmentosa is a good example of how mutations that disrupt spliceosome function can cause disease.

Most human pre-mRNAs can be spliced in alternative ways to yield different proteins

Alternative splicing is a widespread mechanism for generating protein diversity. Different combinations of exons from the same gene may be spliced into a mature RNA, producing distinct forms of a protein for specific tissues, developmental stages, or signaling pathways. What controls which splicing sites are selected? The selection is determined by the binding of trans-acting splicing factors to cis-acting sequences in the pre-mRNA. Most alternative splicing leads to changes in the coding sequence, resulting in proteins with different functions. *Alternative splicing provides a powerful mechanism for expanding the versatility of genomic sequences through combinatorial control.* Consider a gene with five positions at which splicing can take place. With the assumption that these alternative splicing pathways can be regulated independently, a total of $2^5 = 32$ different mRNAs can be generated.

Sequencing of the human genome has revealed that most pre-mRNAs are alternatively spliced, leading to a much greater number of proteins than would be predicted from the number of genes. An example of alternative splicing leading to the expression of two different proteins, each in a different tissue, is provided by the gene encoding both calcitonin and calcitonin-gene-related peptide (CGRP; Figure 29.41). In the thyroid gland, the inclusion of exon 4 in one splicing pathway produces calcitonin, a peptide hormone that regulates calcium and phosphorus metabolism. In neuronal

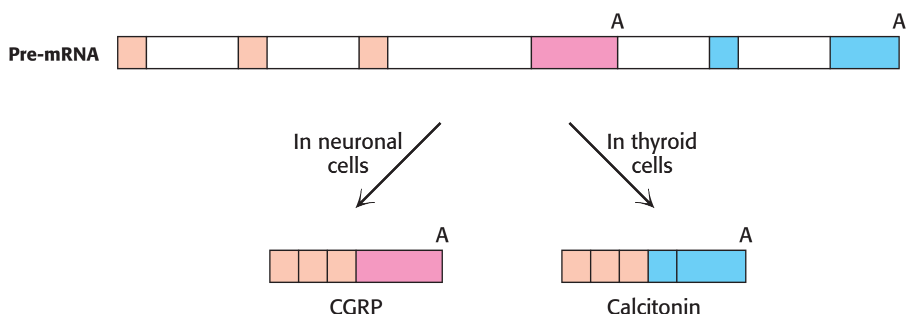


Figure 29.41 An example of alternative splicing. In human beings, two very different hormones are produced from a single calcitonin/CGRP pre-mRNA. Alternative splicing produces the mature mRNA for either calcitonin or CGRP (calcitonin-gene-related protein), depending on the cell type in which the gene is expressed. Each alternative transcript incorporates one of two alternative polyadenylation signals (A) present in the pre-mRNA.

Table 29.4 Selected human diseases attributed to defects in alternative splicing

Disorder	Gene or its product
Acute intermittent porphyria	Porphobilinogen deaminase
Breast and ovarian cancer	*BRCA*1
Cystic fibrosis	CFTR
Frontotemporal dementia	τ protein
Hemophilia A	Factor VIII
HGPRT deficiency (Lesch–Nyhan syndrome)	Hypoxanthine-guanine phosphoribosyltransferase
Leigh encephalomyelopathy	Pyruvate dehydrogenase E1α
Severe combined immunodeficiency	Adenosine deaminase
Spinal muscle atrophy	*SMN*1 or *SMN*2

cells, the exclusion of exon 4 in another splicing pathway produces CGRP, a peptide hormone that acts as a vasodilator. A single pre-mRNA thus yields two very different peptide hormones, depending on cell type. In this case, only two proteins result from alternative splicing; however, in other cases, many more can be produced. An extreme example is the *Drosophila* pre-mRNA that encodes DSCAM, a neuronal protein affecting axon connectivity. Alternative splicing of this pre-mRNA has the potential to produce 38,016 different combinations of exons, a greater number than the total number of genes in the *Drosophila* genome. However, only a fraction of these potential mRNAs appear to be produced owing to regulatory mechanisms that are not yet well understood. Several human diseases that can be attributed to defects in alternative splicing are listed in Table 29.4. Further understanding of alternative splicing and the mechanisms of splice-site selection will be crucial to understanding how the proteome is represented by the human genome.

29.4 The Discovery of Catalytic RNA Was Revealing in Regard to Both Mechanism and Evolution

RNAs form a surprisingly versatile class of molecules. As we have seen, splicing is catalyzed largely by RNA molecules, with proteins playing a secondary role. Another enzyme that contains a key RNA component is ribonuclease P, which catalyzes the maturation of tRNA by endonucleolytic cleavage of nucleotides from the 5′ end of the precursor molecule. Finally, as we shall see in Chapter 30, the RNA component of ribosomes is the catalyst that carries out protein synthesis.

The versatility of RNA first became clear from observations of the processing of ribosomal RNA in a single-cell eukaryote. In *Tetrahymena* (a ciliated protozoan), a 414-nucleotide intron is removed from a 6.4-kb precursor to yield the mature 26S rRNA molecule (Figure 29.42). In an elegant series of studies of this splicing reaction, Thomas Cech and his coworkers established that the RNA spliced itself to precisely excise the intron. These remarkable experiments demonstrated that an RNA molecule can *splice itself* in the absence of protein. Indeed, the RNA alone is catalytic and, under certain conditions, is thus a *ribozyme*. More than 1500 similar introns have since been found in species as widely dispersed as bacteria and eukaryotes, though not in vertebrates. Collectively, they are referred to as *group I introns*.

The *self-splicing* reaction in the group I intron requires an added guanosine nucleotide. Nucleotides were originally included in the reaction mixture because it was thought that ATP or GTP might be needed as an energy

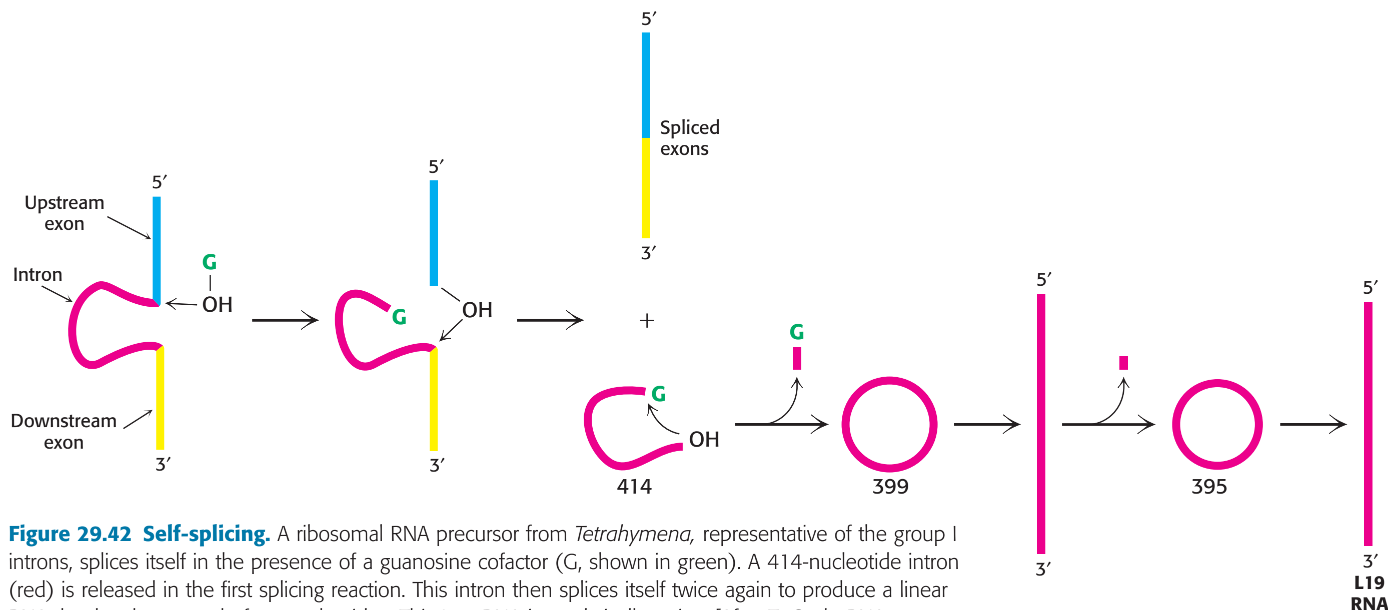


Figure 29.42 Self-splicing. A ribosomal RNA precursor from *Tetrahymena,* representative of the group I introns, splices itself in the presence of a guanosine cofactor (G, shown in green). A 414-nucleotide intron (red) is released in the first splicing reaction. This intron then splices itself twice again to produce a linear RNA that has lost a total of 19 nucleotides. This L19 RNA is catalytically active. [After T. Cech. RNA as an enzyme. Copyright © 1986 by Scientific American, Inc. All rights reserved.]

source. Instead, the nucleotides were found to be necessary as cofactors. The required cofactor proved to be a guanosine unit, in the form of guanosine, GMP, GDP, or GTP. G (denoting any one of these species) serves not as an energy source but as an attacking group that becomes transiently incorporated into the RNA (see Figure 29.42). G binds to the RNA and then attacks the 5′ splice site to form a phosphodiester bond with the 5′ end of the intron. This transesterification reaction generates a 3′-OH group at the end of the upstream exon. This newly attached 3′-OH group then attacks the 3′ splice site. This second transesterification reaction joins the two exons and leads to the release of the 414-nucleotide intron.

Self-splicing depends on the structural integrity of the RNA precursor. Much of the group I intron is needed for self-splicing. This molecule, like many RNAs, has a folded structure formed by many double-helical stems and loops (Figure 29.43), with a well-defined pocket for binding the guanosine. Examination of the three-dimensional structure of a catalytically active group I intron determined by x-ray crystallography reveals the

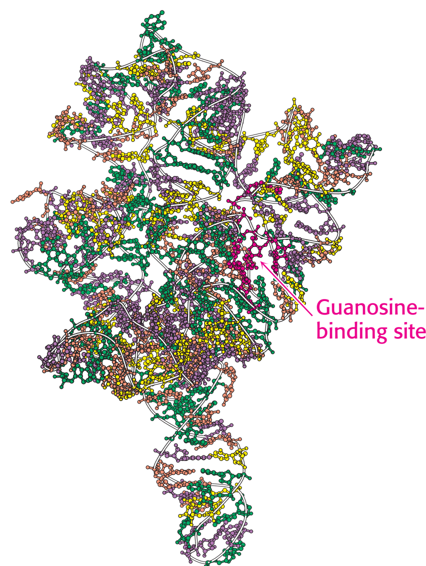


Figure 29.43 Structure of a self-splicing intron. The structure of a large fragment of the self-splicing intron from *Tetrahymena* reveals a complex folding pattern of helices and loops. Bases are shown in green, A; yellow, C; purple, G; and orange, U. [Drawn from 1GRZ.pdb].

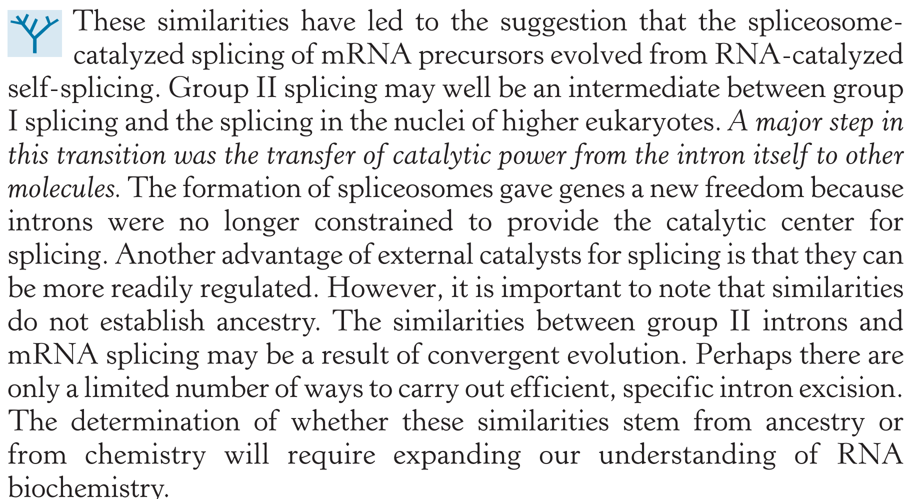
coordination of magnesium ions in the active site analogous to that observed in protein enzymes such as DNA polymerase.

Analysis of the base sequence of the rRNA precursor suggested that the splice sites are aligned with the catalytic residues by base-pairing between the *internal guide sequence* (IGS) in the intron and the 5′ and 3′ exons (Figure 29.44). The IGS first brings together the guanosine cofactor and the 5′ splice site so that the 3′-OH group of G can nucleophilically attack the phosphorus atom at this splice site. The IGS then holds the downstream exon in position for attack by the newly formed 3′-OH group of the upstream exon. A phosphodiester bond is formed between the two exons, and the intron is released as a linear molecule. Like catalysis by protein enzymes, self-catalysis of bond formation and breakage in this rRNA precursor is highly specific.

The finding of enzymatic activity in the self-splicing intron and in the RNA component of RNase P has opened new areas of inquiry and changed the way in which we think about molecular evolution. As mentioned in an earlier chapter, the discovery that RNA can be a catalyst as well as an information carrier suggests that an RNA world may have existed early in the evolution of life, before the appearance of DNA and protein.

Messenger RNA precursors in the mitochondria of yeast and fungi also undergo self-splicing, as do some RNA precursors in the chloroplasts of unicellular organisms such as *Chlamydomonas*. Self-splicing reactions can be classified according to the nature of the unit that attacks the upstream splice site. Group I self-splicing is mediated by a guanosine cofactor, as in *Tetrahymena*. The attacking moiety in group II splicing is the 2′-OH group of a specific adenylate of the intron (Figure 29.45).

Group I and group II self-splicing resembles spliceosome-catalyzed splicing in two respects. First, in the initial step, a ribose hydroxyl group attacks the 5′ splice site. The newly formed 3′-OH terminus of the upstream exon then attacks the 3′ splice site to form a phosphodiester bond with the downstream exon. Second, both reactions are transesterifications in which the phosphate moieties at each splice site are retained in the products. The number of phosphodiester bonds stays constant. Group II splicing is like the spliceosome-catalyzed splicing of mRNA precursors in several additional ways. The attack at the 5′ splice site is carried out by a part of the intron itself (the 2′-OH group of adenosine) rather than by an external cofactor (G). In both cases, the intron is released in the form of a lariat. Moreover, in some instances, the group II intron is transcribed in pieces that assemble through hydrogen bonding to the catalytic intron, in a manner analogous to the assembly of the snRNAs in the spliceosome.



These similarities have led to the suggestion that the spliceosome-catalyzed splicing of mRNA precursors evolved from RNA-catalyzed self-splicing. Group II splicing may well be an intermediate between group I splicing and the splicing in the nuclei of higher eukaryotes. *A major step in this transition was the transfer of catalytic power from the intron itself to other molecules.* The formation of spliceosomes gave genes a new freedom because introns were no longer constrained to provide the catalytic center for splicing. Another advantage of external catalysts for splicing is that they can be more readily regulated. However, it is important to note that similarities do not establish ancestry. The similarities between group II introns and mRNA splicing may be a result of convergent evolution. Perhaps there are only a limited number of ways to carry out efficient, specific intron excision. The determination of whether these similarities stem from ancestry or from chemistry will require expanding our understanding of RNA biochemistry.

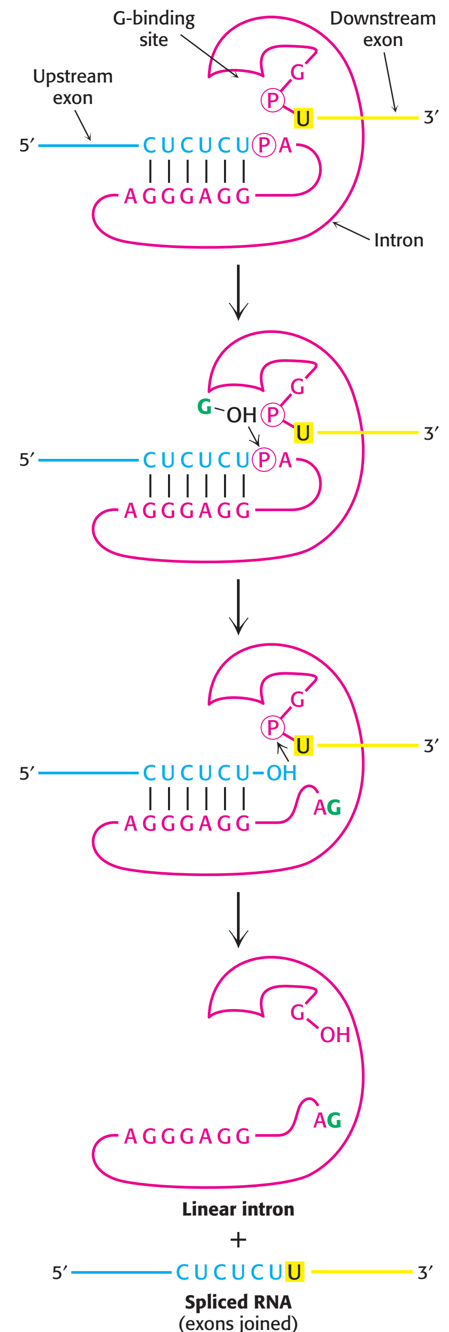


Figure 29.44 Self-splicing mechanism. The catalytic mechanism of the group I intron includes a series of transesterification reactions. [After T. Cech. RNA as an enzyme. Copyright © 1986 by Scientific American, Inc. All rights reserved.]

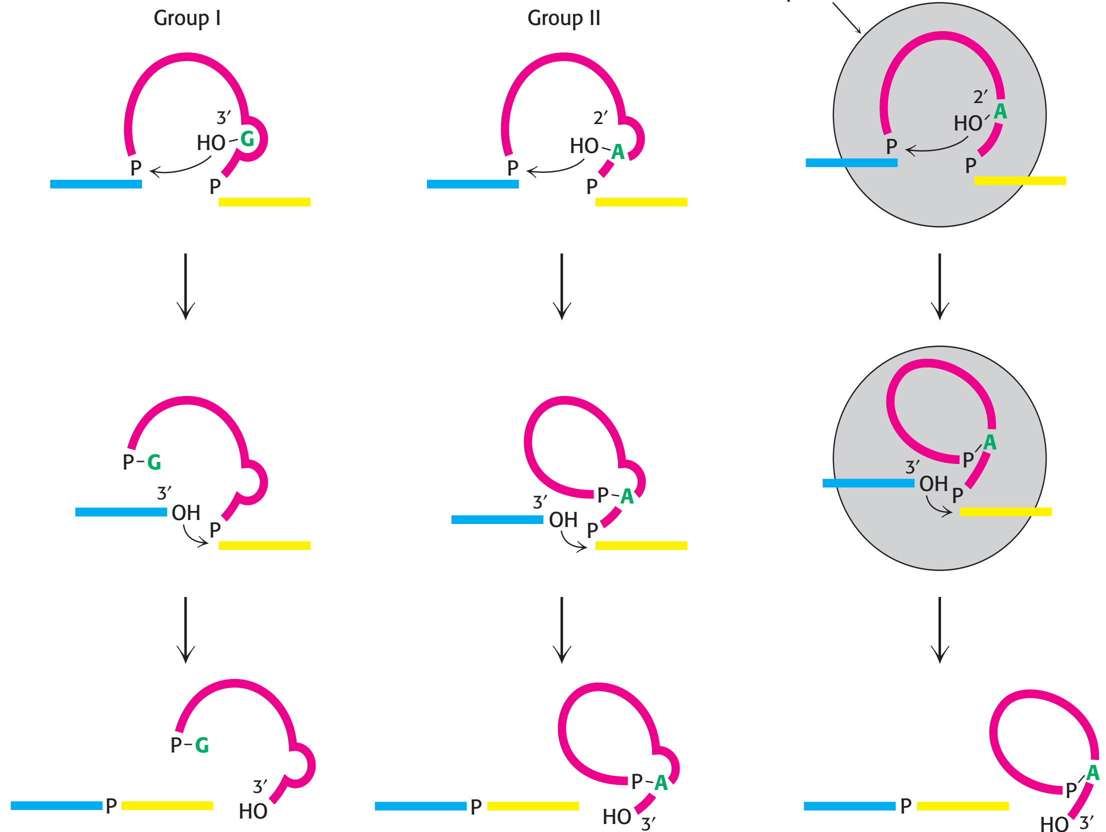


Figure 29.45 Comparison of splicing pathways. The exons being joined are shown in blue and yellow and the attacking unit is shown in green. The catalytic site is formed by the intron itself (red) in group I and group II splicing. In contrast, the splicing of nuclear mRNA precursors is catalyzed by snRNAs and their associated proteins in the spliceosome. [After P. A. Sharp. *Science* 235:766–771, 1987.]

Summary

29.1 RNA Polymerases Catalyze Transcription

All cellular RNA molecules are synthesized by RNA polymerases according to instructions given by DNA templates. The activated monomer substrates are ribonucleoside triphosphates. The direction of RNA synthesis is $5' \longrightarrow 3'$, as in DNA synthesis. RNA polymerases, unlike DNA polymerases, do not need a primer.

RNA polymerase in *E. coli* is a multisubunit enzyme. The subunit composition of the ~500-kd holoenzyme is $\alpha_2\beta\beta'\omega\sigma$ and that of the core enzyme is $\alpha_2\beta\beta'\omega$. Transcription is initiated at promoter sites consisting of two sequences, one centered near −10 and the other near −35; that is, 10 and 35 nucleotides away from the start site in the 5′ (upstream) direction. The consensus sequence of the −10 region is TATAAT. The σ subunit enables the holoenzyme to recognize promoter sites. When the growth temperature is raised, *E. coli* expresses a special σ subunit that selectively binds the distinctive promoter of heat-shock genes. RNA polymerase must unwind the template double helix for transcription to take place. Unwinding exposes some 17 bases on the template strand and sets the stage for the formation of the first

phosphodiester bond. Newly synthesized RNA chains usually start with pppG or pppA. The σ subunit usually dissociates from the holoenzyme after the initiation of the new chain. Elongation takes place at transcription bubbles that move along the DNA template at a rate of about 50 nucleotides per second. RNA polymerase occasionally backtracks, a process that can facilitate proofreading of the RNA transcript. The nascent RNA chain contains stop signals that end transcription. One stop signal is an RNA hairpin, which is followed by several U residues. A different stop signal is read by the *rho* protein, an ATPase. Some genes are regulated by riboswitches, structures that form in RNA transcripts and bind specific metabolites. In *E. coli,* precursors of transfer RNA and ribosomal RNA are cleaved and chemically modified after transcription, whereas messenger RNA is used unchanged as a template for protein synthesis.

29.2 Transcription in Eukaryotes Is Highly Regulated

RNA synthesis in eukaryotes takes place in the nucleus, whereas protein synthesis takes place in the cytoplasm. There are three types of RNA polymerase in the nucleus: RNA polymerase I makes ribosomal RNA precursors, II makes messenger RNA precursors, and III makes transfer RNA precursors. Eukaryotic promoters are complex, being composed of several different elements. Promoters for RNA polymerase II may be located on the 5′ side or the 3′ side of the start site for transcription. A common type of eukaryotic promoter consists of a TATA box centered between −30 and −100 and paired with an initiator element. Eukaryotic promoter elements are recognized by proteins called transcription factors rather than by RNA polymerase II. The saddle-shaped TATA-box-binding protein unwinds and sharply bends DNA at TATA-box sequences and serves as a focal point for the assembly of transcription complexes. The TATA-box-binding protein initiates the assembly of the active transcription complex. The activity of many promoters is greatly increased by enhancer sequences that have no promoter activity of their own. Enhancer sequences can act over distances of several kilobases, and they can be located either upstream or downstream of a gene.

29.3 The Transcription Products of Eukaryotic Polymerases Are Processed

The 5′ ends of mRNA precursors become capped and methylated in the course of transcription. A 3′ poly(A) tail is added to most mRNA precursors after the nascent chain has been cleaved by an endonuclease. RNA editing processes alter the nucleotide sequence of some mRNAs, such as the one for apolipoprotein B.

The splicing of mRNA precursors is carried out by spliceosomes, which consist of small nuclear ribonucleoprotein particles. Splice sites in mRNA precursors are specified by sequences at ends of introns and by branch sites near their 3′ ends. The 2′-OH group of an adenosine residue in the branch site attacks the 5′ splice site to form a lariat intermediate. The newly generated 3′-OH terminus of the upstream exon then attacks the 3′ splice site to become joined to the downstream exon. Splicing thus consists of two transesterification reactions, with the number of phosphodiester bonds remaining constant during reactions. Small nuclear RNAs in spliceosomes catalyze the splicing of mRNA precursors. In particular, U2 and U6 snRNAs form the active centers of spliceosomes.

The events in posttranscriptional processing of mRNA are controlled by the phosphorylation state of the carboxy-terminal domain, part of RNA polymerase II.

29.4 The Discovery of Catalytic RNA Was Revealing in Regard to Both Mechanism and Evolution

Some RNA molecules, such as those containing the group I intron, undergo self-splicing in the absence of protein. A self-modified version of this rRNA intron displays true catalytic activity and is thus a ribozyme. Spliceosome-catalyzed splicing may have evolved from self-splicing. The discovery of catalytic RNA has opened new vistas in our exploration of early stages of molecular evolution and the origins of life.

Key Terms

transcription (p. 851)
RNA polymerase (p. 852)
promoter site (p. 852)
transcription bubble (p. 853)
consensus sequence (p. 857)
sigma (σ) subunit (p. 857)
riboswitch (p. 860)
rho (ρ) protein (p. 860)
carboxy-terminal domain (CTD) (p. 865)
TATA box (p. 866)
transcription factor (p. 867)
enhancer (p. 868)
small nucleolar ribonucleoprotein (snoRNP) (p. 870)
pre-mRNA (p. 870)
5′ cap (p. 870)
poly(A) tail (p. 871)
microRNA (p. 872)
RNA editing (p. 872)
RNA splicing (p. 873)
spliceosome (p. 874)
small nuclear RNA (snRNA) (p. 875)
small nuclear ribonucleoprotein particle (snRNP) (p. 875)
alternative splicing (p. 878)
catalytic RNA (p. 879)
self-splicing (p. 879)

Problems

1. *Complements.* The sequence of part of an mRNA is

5′–AUGGGGAACAGCAAGAGUGGGGCCCUGUCCAAGGAG–3′

What is the sequence of the DNA coding strand? Of the DNA template strand?

2. *Checking for errors.* Why is RNA synthesis not as carefully monitored for errors as is DNA synthesis?

3. *Speed is not of the essence.* Why is it advantageous for DNA synthesis to be more rapid than RNA synthesis?

4. *Active sites.* The overall structures of RNA polymerase and DNA polymerase are very different, yet their active sites show considerable similarities. What do the similarities suggest about the evolutionary relationship between these two important enzymes?

5. *Potent inhibitor.* Heparin inhibits transcription by binding to RNA polymerase. What properties of heparin allow it to bind so effectively to RNA polymerase?

6. *A loose cannon.* Sigma protein by itself does not bind to promoter sites. Predict the effect of a mutation enabling σ to bind to the –10 region in the absence of other subunits of RNA polymerase.

7. *Stuck sigma.* What would be the likely effect of a mutation that prevents σ from dissociating from the RNA polymerase core?

8. *Transcription time.* What is the minimum length of time required for the synthesis by *E. coli* polymerase of an mRNA encoding a 100-kd protein?

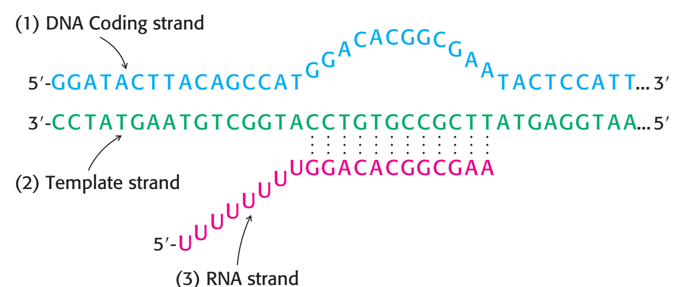
9. *Rapid search.* RNA polymerase finds promoter sites very rapidly. The observed rate constant for the binding of the RNA polymerase holoenzyme to promoter sequences is $10^{10}\ M^{-1}s^{-1}$. The rate constant for two macromolecules encountering each other is typically $10^{8}\ M^{-1}s^{-1}$. Propose an explanation for the 100-fold larger rate for a protein finding a particular site along a DNA molecule.

10. *Where to begin?* Identify the likely transcription start site in the following DNA sequence:

5′–GCCGTTGACACCGTTCGGCGATCGATCCGCTATAATGTGTGGATCCGCTT–3′
5′–CGGCAACTGTGGCAAGCCGCTAGCTAGGCGATATTACACACCTAGGCGAA–3′

11. *Between bubbles.* How far apart are transcription bubbles on *E. coli* genes that are being transcribed at a maximal rate?

12. *A revealing bubble.* Consider the synthetic RNA–DNA transcription bubble illustrated here. Refer to the coding DNA strand, the template strand, and the RNA strand as strands 1, 2, and 3, respectively.



(a) Suppose that strand 3 is labeled with ^{32}P at its 5′ end and that polyacrylamide gel electrophoresis is carried out under nondenaturing conditions. Predict the autoradiographic pattern for (i) strand 3 alone, (ii) strands 1 and 3, (iii) strands 2 and 3, (iv) strands 1, 2, and 3, and (v) strands 1, 2, and 3 and core RNA polymerase.

(b) What is the likely effect of rifampicin on RNA synthesis in this system?

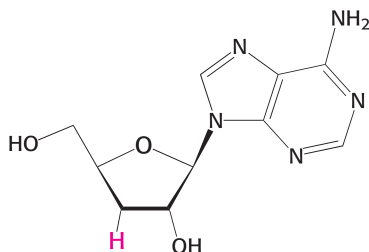
(c) Heparin blocks elongation of the RNA primer if it is added to core RNA polymerase before the onset of transcription but not if added after transcription starts. Account for this difference.

(d) Suppose that synthesis is carried out in the presence of ATP, CTP, and UTP. Compare the length of the longest product obtained with that expected when all four ribonucleoside triphosphates are present.

13. *Proofreading marks*. The major products of proofreading by RNA polymerase are dinucleotides rather than mononucleotides. Why?

14. *Abortive cycling.* Di- and trinucleotides are occasionally released from RNA polymerase at the very start of transcription, a process called abortive cycling. This process requires the restart of transcription. Suggest a plausible explanation for abortive cycling.

15. *Polymerase inhibition.* Cordycepin inhibits poly(A) synthesis at low concentrations and RNA synthesis at higher concentrations.



Cordycepin (3′-deoxyadenosine)

(a) What is the basis of inhibition by cordycepin?

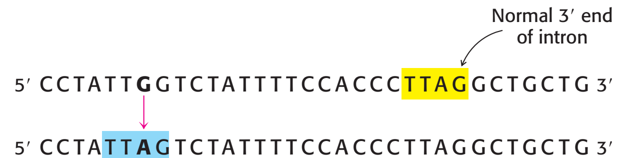
(b) Why is poly(A) synthesis more sensitive to the presence of cordycepin?

(c) Does cordycepin need to be modified to exert its effect?

16. *Alternative splicing.* A gene contains eight sites where alternative splicing is possible. Assuming that the splicing pattern at each site is independent of that at all other sites, how many splicing products are possible?

17. *Supercoiling*. Negative supercoiling of DNA favors the transcription of genes because it facilitates unwinding. However, not all promoter sites are stimulated by negative supercoiling. The promoter site for topoisomerase II itself is a noteworthy exception. Negative supercoiling decreases the rate of transcription of this gene. Propose a possible mechanism for this effect and suggest a reason why it may occur.

18. *An extra piece.* In one type of mutation leading to a form of thalassemia, the mutation of a single base (G to A) generates a new 3′ splice site (blue in the illustration below) akin to the normal one (yellow) but farther upstream.



What is the amino acid sequence of the extra segment of protein synthesized in a thalassemic patient having a mutation leading to aberrant splicing? The reading frame after the splice site begins with TCT.

19. *A long-tailed messenger*. Another thalassemic patient had a mutation leading to the production of an mRNA for the β chain of hemoglobin that was 900 nucleotides longer than the normal one. The poly(A) tail of this mutant mRNA was located a few nucleotides after the only AAUAAA sequence in the additional sequence. Propose a mutation that would lead to the production of this altered mRNA.

Mechanism Problem

20. *RNA editing*. Many uridine molecules are inserted into some mitochondrial mRNAs in trypanosomes. The uridine residues come from the poly(U) tail of a donor strand. Nucleoside triphosphates do not participate in this reaction. Propose a reaction mechanism that accounts for these findings. (Hint: Relate RNA editing to RNA splicing.)

Chapter Integration Problems

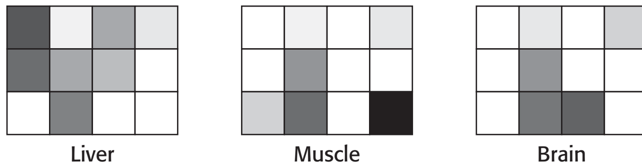
21. *Proteome complexity*. What processes considered in this chapter make the proteome more complex than the genome? What processes might further enhance this complexity?

22. *Separation technique*. Suggest a means by which you could separate mRNA from the other types of RNA in a eukaryotic cell.

Data Interpretation Problems

23. *Run-off experiment*. Nuclei were isolated from brain, liver, and muscle. The nuclei were then incubated with α-[^{32}P]UTP under conditions that allow RNA synthesis, except that an inhibitor of RNA initiation was present. The radioactive RNA was isolated and annealed to various DNA sequences that had been attached to a gene chip. In

the adjoining graphs, the intensity of the shading indicates roughly how much mRNA was attached to each DNA sequence.



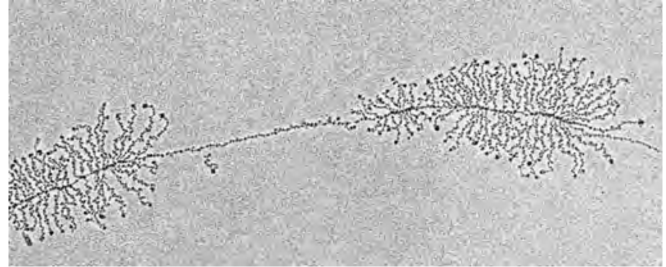
(a) Why does the intensity of hybridization differ between genes?

(b) What is the significance of the fact that some of the RNA molecules display different hybridization patterns in different tissues?

(c) Some genes are expressed in all three tissues. What would you guess is the nature of these genes?

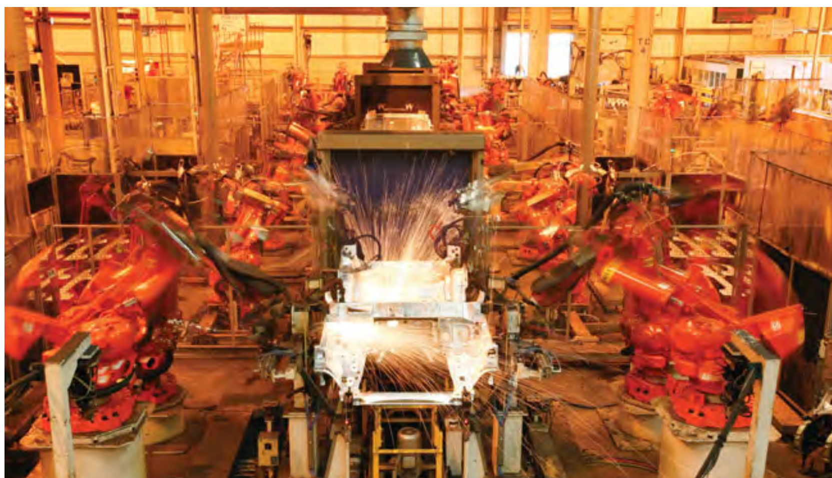
(d) Suggest a reason why an initiation inhibitor was included in the reaction mixture.

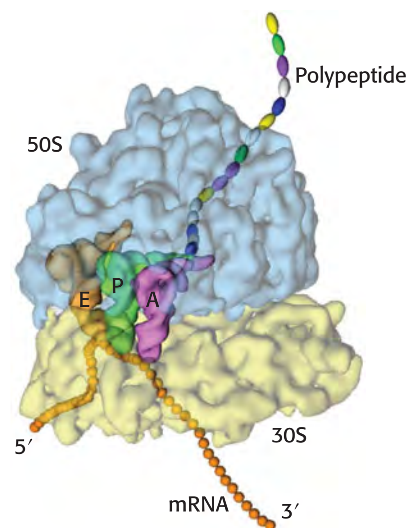
24. *Christmas trees*. The adjoining autoradiograph depicts several bacterial genes undergoing transcription. Identify the DNA. What are the strands of increasing length? Where is the beginning of transcription? The end of transcription? On the page, what is the direction of RNA synthesis? What can you conclude about the number of enzymes participating in RNA synthesis on a given gene?



CHAPTER 30

Protein Synthesis





The ribosome, shown at the right, is a factory for the manufacture of polypeptides. Amino acids are carried into the ribosome, one at a time, connected to transfer RNA molecules. Each amino acid is joined to the growing polypeptide chain, which detaches from the ribosome only after the polypeptide has been completed. This assembly-line approach allows even very long polypeptide chains to be assembled rapidly and with impressive accuracy. [(Left) Images of Birmingham Premium/Alamy.]

Genetic information is most important because of the proteins that it encodes, in that proteins play most of the functional roles in cells. In Chapters 28 and 29, we examined how DNA is replicated and transcribed into RNA. We now turn to the mechanism of protein synthesis, a process called *translation* because the four-letter alphabet of nucleic acids is translated into the entirely different twenty-letter alphabet of proteins. Translation is a conceptually more complex process than either replication or transcription, both of which take place within the framework of a common base-pairing language. As befits its position linking the nucleic acid and protein languages, the process of protein synthesis critically depends on both nucleic acid and protein factors. Protein synthesis takes place on *ribosomes*—enormous complexes containing three large RNA molecules and more than 50 proteins. Among the great triumphs in biochemistry in recent years has been the determination of the structure of the ribosome and its components so that its function can be examined in atomic detail. Perhaps the most significant conclusion from these studies is that *the ribosome is a ribozyme;* that is, the RNA components play the most fundamental roles. These observations strongly support the notion that life evolved through an RNA world, and the ribosome is a surviving inhabitant of that world.

Transfer RNA molecules (tRNAs) and messenger RNA (mRNA) also are key participants in protein synthesis. The link between amino acids and nucleic acids is first made by enzymes called aminoacyl-tRNA synthetases.

OUTLINE

30.1 Protein Synthesis Requires the Translation of Nucleotide Sequences into Amino Acid Sequences

30.2 Aminoacyl Transfer RNA Synthetases Read the Genetic Code

30.3 The Ribosome Is the Site of Protein Synthesis

30.4 Eukaryotic Protein Synthesis Differs from Prokaryotic Protein Synthesis Primarily in Translation Initiation

30.5 A Variety of Antibiotics and Toxins Can Inhibit Protein Synthesis

30.6 Ribosomes Bound to the Endoplasmic Reticulum Manufacture Secretory and Membrane Proteins

By specifically linking a particular amino acid to each tRNA, these enzymes translate the genetic code.

Although RNA is paramount in the process of translation, protein factors also are required for the efficient synthesis of a protein. Protein factors participate in the initiation, elongation, and termination of protein synthesis. P-loop NTPases of the G-protein family play particularly important roles. Recall that these proteins serve as molecular switches as they cycle between a GTP-bound form and a GDP-bound form (Section 14.1). This chapter focuses primarily on protein synthesis in prokaryotes because it illustrates many general principles and is well understood. Some distinctive features of protein synthesis in eukaryotes also are presented.

30.1 Protein Synthesis Requires the Translation of Nucleotide Sequences into Amino Acid Sequences

The basics of protein synthesis are the same across all kingdoms of life—evidence that the protein-synthesis system arose very early in evolution. An mRNA is decoded, or read, in the 5′-to-3′ direction, one codon at a time, and the corresponding protein is synthesized in the amino-to-carboxyl direction by the sequential addition of amino acids to the carboxyl end of the growing peptide chain (Figure 30.1). The amino acids arrive at the growing chain in activated form as aminoacyl-tRNAs, created by joining the carboxyl group of an amino acid to the 3′ end of a tRNA molecule. The linking of an amino acid to its corresponding tRNA is catalyzed by an *aminoacyl-tRNA synthetase.* ATP cleavage drives this activation reaction. For each amino acid, there is usually one activating enzyme and at least one kind of tRNA.

The synthesis of long proteins requires a low error frequency

The process of transcription is analogous to copying, word for word, a page of a book. There is no change of alphabet or vocabulary; so the likelihood of a change in meaning is small. Translating the base sequence of an mRNA molecule into a sequence of amino acids is analogous to translating the page of a book into another language. Translation is a complex process, entailing many steps and dozens of molecules. The potential for error exists at each step. The complexity of translation creates a conflict between two requirements: the process must be both accurate and fast enough to meet a cell's needs. In *E. coli,* translation can take place at a rate of 50 amino acids per second, a truly impressive speed, considering the complexity of the process.

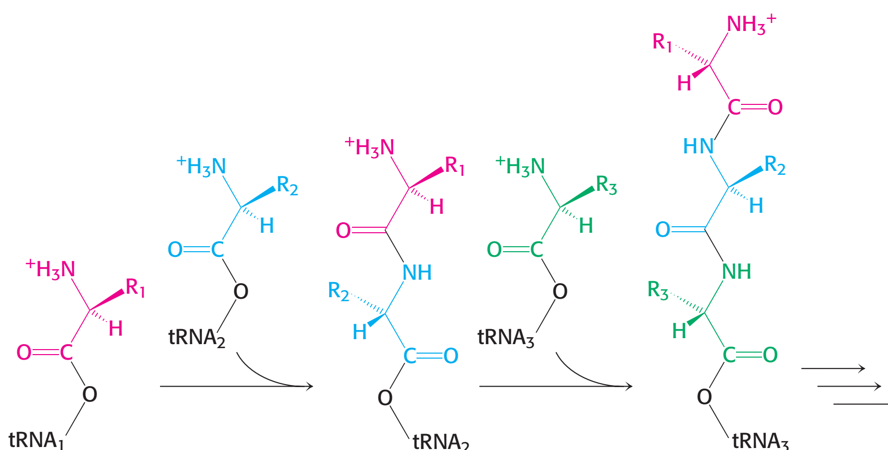


Figure 30.1 Polypeptide-chain growth. Proteins are synthesized by the successive addition of amino acids to the carboxyl terminus.

How accurate must protein synthesis be? Let us consider error rates. The probability of forming a protein with no errors depends on the number of amino acid residues and on the frequency (ε) of insertion of a wrong amino acid. As Table 30.1 shows, an error frequency of 10^{-2} is intolerable, even for quite small proteins. An ε value of 10^{-3} usually leads to the error-free synthesis of a 300-residue protein (~33 kd) but not of a 1000-residue protein (~110 kd). Thus, the error frequency must not exceed approximately 10^{-4} to produce the larger proteins effectively. Lower error frequencies are conceivable; however, except for the largest proteins, they will not dramatically increase the percentage of proteins with accurate sequences. In addition, such lower error rates are likely to be possible only by a reduction in the rate of protein synthesis because additional time for proofreading is required. *In fact, the observed values of ε are close to 10^{-4}*. An error frequency of about 10^{-4} per amino acid residue was selected in the course of evolution to accurately produce proteins consisting of as many as 1000 amino acids while maintaining a remarkably rapid rate for protein synthesis.

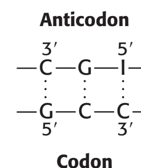
Table 30.1 Accuracy of protein synthesis

Frequency of inserting an incorrect amino acid	Probability of synthesizing an error-free protein		
	Number of amino acid residues		
	100	300	1000
10^{-2}	0.366	0.049	0.000
10^{-3}	0.905	0.741	0.368
10^{-4}	0.990	0.970	0.905
10^{-5}	0.999	0.997	0.990

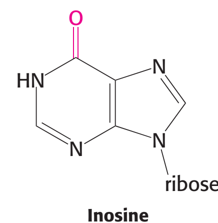
Note: The probability p of forming a protein with no errors depends on n, the number of amino acids, and ε, the frequency of insertion of a wrong amino acid: $p = (1 - \varepsilon)^n$.

Transfer RNA molecules have a common design

The fidelity of protein synthesis requires accurate recognition of three-base *codons* on messenger RNA. Recall that the genetic code relates each amino acid to a three-letter codon (Section 4.6). An amino acid cannot itself recognize a codon. Consequently, an amino acid is attached to a specific tRNA molecule that can recognize the codon by Watson–Crick base-pairing. *Transfer RNA serves as the adapter molecule that binds to a specific codon and brings with it an amino acid for incorporation into the polypeptide chain.*



Consider yeast alanyl-tRNA, so called because it will carry the amino acid alanine. Yeast alanyl-tRNA was the first nucleic acid sequenced. This adapter molecule is a single chain of 76 ribonucleotides (Figure 30.2). The 5′ terminus is phosphorylated (pG), whereas the 3′ terminus has a free hydroxyl group. The *amino acid-attachment site* is the 3′-hydroxyl group of the adenosine residue at the 3′ terminus of the molecule. The sequence 5′-IGC-3′ in the middle of the molecule is the *anticodon,* where I is the purine base inosine. It is complementary to 5′-GCC-3′, one of the codons for alanine.



Thousands of tRNA sequences are now known. The striking finding is that all of them can be arranged in a cloverleaf pattern in which about half the residues are base-paired (Figure 30.3). Hence, *tRNA molecules have many common structural features.* This finding is not unexpected, because all tRNA molecules must be able to interact in nearly the same way with the ribosomes, mRNAs, and protein factors that participate in translation.

All known transfer RNA molecules have the following features:

1. Each is a single chain containing between *73 and 93 ribonucleotides* (~25 kd).

2. They contain *many unusual bases,* typically between 7 and 15 per molecule. Some of these bases are methylated or dimethylated derivatives of A, U, C, and G formed by enzymatic modification of a precursor tRNA. Some methylations prevent the formation of certain base pairs, thereby

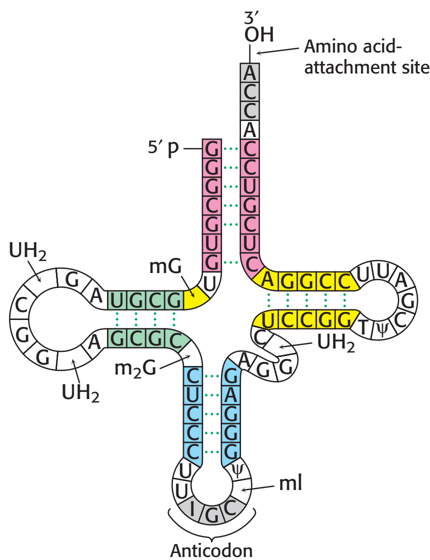


Figure 30.2 Alanyl-tRNA sequence. The base sequence of yeast alanyl-tRNA and the deduced cloverleaf secondary structure are shown. Modified nucleosides are abbreviated as follows: methylinosine (mI), dihydrouridine (UH$_2$), ribothymidine (T), pseudouridine (ψ), methylguanosine (mG), and dimethylguanosine (m$_2$G). Inosine (I), another modified nucleoside, is part of the anticodon.

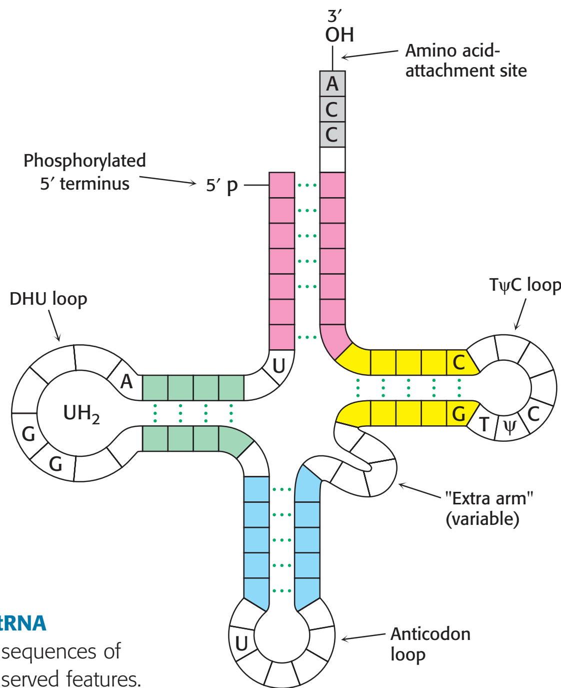
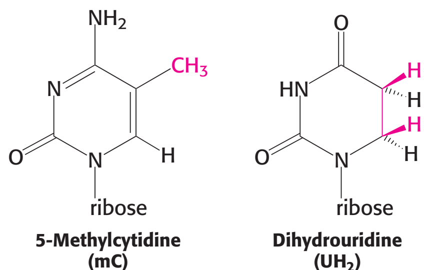


Figure 30.3 General structure of tRNA molecules. Comparison of the base sequences of many tRNAs reveals a number of conserved features.



rendering such bases accessible for interactions with other bases. In addition, methylation imparts a hydrophobic character to some regions of tRNAs, which may be important for their interaction with synthetases and ribosomal proteins. Other modifications alter codon recognition, as will be described shortly.

3. The molecule is L-shaped (Figure 30.4).

4. About half the nucleotides in tRNAs are base-paired to form double helices. The four helical regions are arranged to form two apparently continuous segments of double helix. These segments are like A-form DNA, as expected for an RNA helix (p. 119). One helix, containing the 5′ and 3′ ends, runs horizontally in the model shown in Figure 30.5. The other helix, which

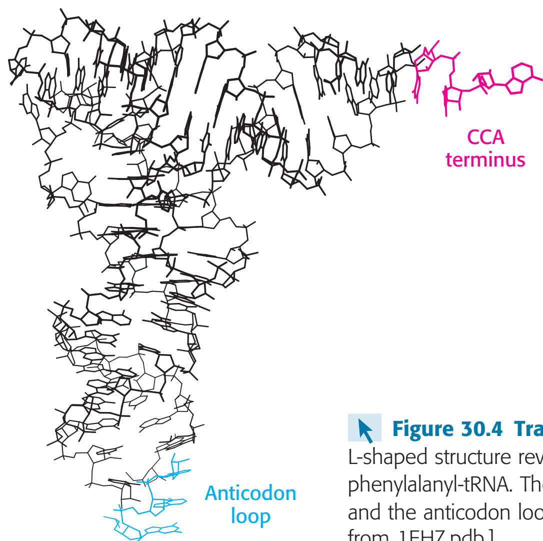


Figure 30.4 Transfer RNA structure. *Notice* the L-shaped structure revealed by this skeletal model of yeast phenylalanyl-tRNA. The CCA region is at the end of one arm, and the anticodon loop is at the end of the other. [Drawn from 1EHZ.pdb.]

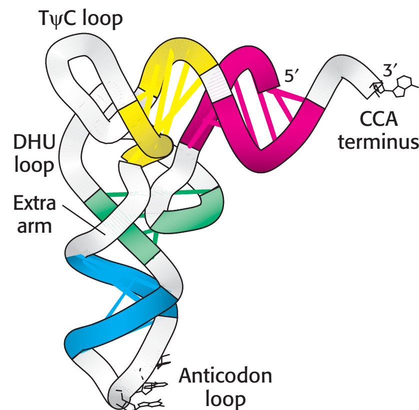


Figure 30.5 Helix stacking in tRNA. The four double-stranded regions of the tRNA (see Figure 30.3) stack to form an L-shaped structure. [Drawn from 1EHZ.pdb.]

contains the anticodon and runs vertically in Figure 30.5, forms the other arm of the L.

Five groups of bases are not base-paired in this way: the 3′ *CCA terminal region,* which is part of a region called the *acceptor stem;* the *TψC loop,* which acquired its name from the sequence ribothymine-pseudouracil-cytosine; the *"extra arm,"* which contains a variable number of residues; *the DHU loop,* which contains several dihydrouracil residues; and the *anticodon loop.* Most of the bases in the nonhelical regions participate in hydrogen-bonding interactions, even if the interactions are not like those in Watson–Crick base pairs. The structural diversity generated by this combination of helices and loops containing modified bases ensures that the tRNAs can be uniquely distinguished, though structurally similar overall.

5. The 5′ end of a tRNA is phosphorylated. The 5′ terminal residue is usually pG.

6. An activated amino acid is attached to a hydroxyl group of the adenosine residue in the amino acid-attachment site, located at the end of the 3′ CCA component of the acceptor stem (Figure 30.6). This single-stranded region can change conformation in the course of amino acid activation and protein synthesis.

7. The anticodon loop, which is present in a loop near the center of the sequence, is at the other end of the L, making accessible the three bases that make up the anticodon.

Thus, the architecture of the tRNA molecule is well suited to its role as adaptor: the anticodon is available to interact with an appropriate codon on mRNA while the end that is linked to an activated amino acid is well positioned to participate in peptide-bond formation.

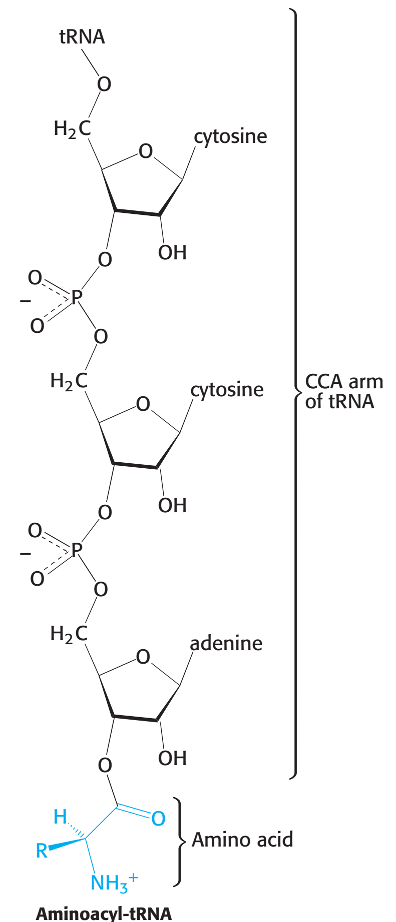
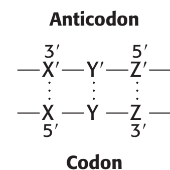


Figure 30.6 Aminoacyl-tRNA. Amino acids are coupled to tRNAs through ester linkages to either the 2′- or the 3′-hydroxyl group of the 3′-adenosine residue. A linkage to the 3′-hydroxyl group is shown.

Some transfer RNA molecules recognize more than one codon because of wobble in base-pairing

What are the rules that govern the recognition of a codon by the anticodon of a tRNA? A simple hypothesis is that each of the bases of the codon forms a Watson–Crick type of base pair with a complementary base on the anticodon of the tRNA. The codon and anticodon would then be lined up in an antiparallel fashion. In the diagram in the margin, the prime denotes the complementary base. Thus X and X′ would be either A and U (or U and A) or G and C (or C and G). According to this model, a particular anticodon can recognize only one codon.



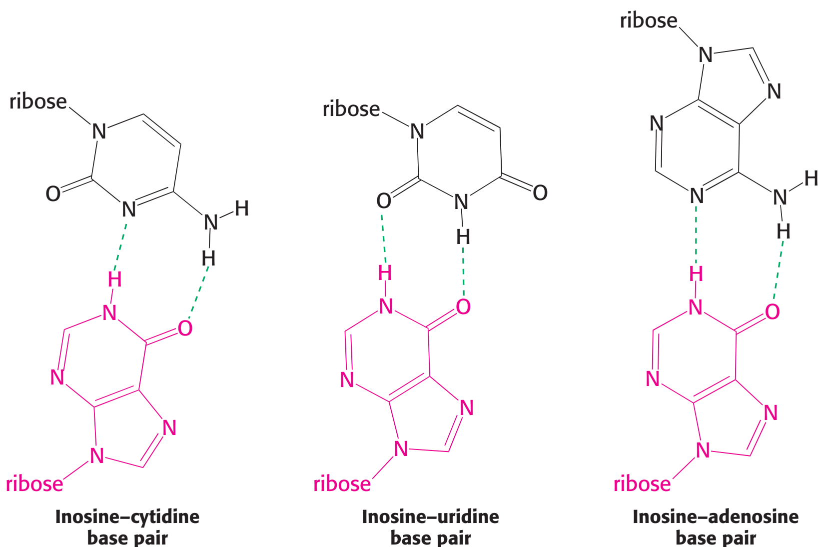
The facts are otherwise. As found experimentally, *some tRNA molecules can recognize more than one codon*. For example, the yeast alanyl-tRNA binds to *three* codons: GCU, GCC, and GCA. The first two bases of these codons are the same, whereas the third is different. Could it be that recognition of the third base of a codon is sometimes less discriminating than recognition of the other two? The pattern of degeneracy of the genetic code indicates that it might be so. XYU and XYC always encode the same amino acid; XYA and XYG usually do. Francis Crick surmised from these data that the steric criteria might be less stringent for pairing of the third base than for the other two. Models of various base pairs were built to determine which ones are similar to the standard A · U and G · C base pairs with regard to the distance and angle between the glycosidic bonds. Inosine was

Table 30.2 Allowed pairings at the third base of the codon according to the wobble hypothesis

First base of anticodon	Third base of codon
C	G
A	U
U	A or G
G	U or C
I	U, C, or A

included in this study because it appeared in several anticodons. With the assumption of some steric freedom ("wobble") in the pairing of the third base of the codon, the combinations shown in Table 30.2 seemed plausible.

The *wobble hypothesis* is now firmly established. The anticodons of tRNAs of known sequence bind to the codons predicted by this hypothesis. For example, the anticodon of yeast alanyl-tRNA is IGC. This tRNA recognizes the codons GCU, GCC, and GCA. Recall that, by convention, nucleotide sequences are written in the $5' \longrightarrow 3'$ direction unless otherwise noted. Hence, I (the $5'$ base of this anticodon) pairs with U, C, or A (the $3'$ base of the codon), as predicted.



Two generalizations concerning the codon–anticodon interaction can be made:

1. The first two bases of a codon pair in the standard way. Recognition is precise. Hence, *codons that differ in either of their first two bases must be recognized by different tRNAs.* For example, both UUA and CUA encode leucine but are read by different tRNAs.

2. The first base of an anticodon determines whether a particular tRNA molecule reads one, two, or three kinds of codons: C or A (one codon), U or G (two codons), or I (three codons). Thus, *part of the degeneracy of the genetic code arises from imprecision (wobble) in the pairing of the third base of the codon with the first base of the anticodon.* We see here a strong reason for the frequent appearance of inosine, one of the unusual nucleosides, in anticodons. *Inosine maximizes the number of codons that can be read by a particular tRNA molecule.* The inosine bases in tRNA are formed by the deamination of adenosine after the synthesis of the primary transcript.

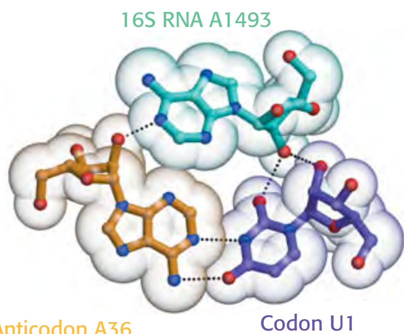


Figure 30.7 16S rRNA monitors base-pairing between the codon and the anticodon. Adenine 1493, one of three universally conserved bases in 16S rRNA, forms hydrogen bonds with the bases in both the codon and the anticodon only if the codon and anticodon are correctly paired. [From J. M. Ogle and V. Ramakrishnan. *Annu. Rev. Biochem.* 74:129–177, 2005, Fig. 2a.]

Why is wobble tolerated in the third position of the codon but not in the first two? This question is answered by considering the interaction of the tRNA with the ribosome. As we will see, ribosomes are huge RNA–protein complexes consisting of two subunits, the 30S and 50S subunits. The 30S subunit has an RNA molecule, the16S rRNA, that has three universally conserved bases—adenine 1492, adenine 1493, and guanine 530—that form hydrogen bonds on the minor-groove side but only with correctly formed base pairs of the codon–anticodon duplex (Figure 30.7). These

interactions serve to check whether Watson–Crick base pairs are present in the first two positions of the codon–anticodon duplex. No such inspection device is present for the third position; so more-varied base pairs are tolerated. This mechanism for ensuring fidelity is analogous to the minor-groove interactions utilized by DNA polymerase for a similar purpose (Section 28.1). *Thus, the ribosome plays an active role in decoding the codon–anticodon interactions.*

30.2 Aminoacyl Transfer RNA Synthetases Read the Genetic Code

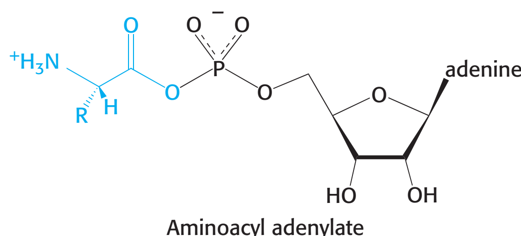
Before codon and anticodon meet, the amino acids required for protein synthesis must first be attached to specific tRNA molecules. The linkage of an amino acid to a tRNA is crucial for two reasons. *First, the attachment of a given amino acid to a particular tRNA establishes the genetic code.* When an amino acid has been linked to a tRNA, it will be incorporated into a growing polypeptide chain at a position dictated by the anticodon of the tRNA. *Second, because the formation of a peptide bond between free amino acids is not thermodynamically favorable, the amino acid must first be activated in order for protein synthesis to proceed. The activated intermediates in protein synthesis are amino acid esters,* in which the carboxyl group of an amino acid is linked to either the 2′- or the 3′-hydroxyl group of the ribose unit at the 3′ end of tRNA. An amino acid ester of tRNA is called an *aminoacyl-tRNA* or sometimes a *charged tRNA* (see Figure 30.6). For a specific amino acid attached to its cognate tRNA—for instance, threonine—the charged tRNA is designated Thr-$tRNA^{Thr}$.

Amino acids are first activated by adenylation

The activation reaction is catalyzed by specific *aminoacyl-tRNA synthetases,* which are also called *activating enzymes*. The first step is the formation of an *aminoacyl adenylate* from an amino acid and ATP.

$$\text{Amino acid} + \text{ATP} \rightleftharpoons \text{aminoacyl-AMP} + \text{PP}_i$$

This activated species is a mixed anhydride in which the carboxyl group of the amino acid is linked to the phosphoryl group of AMP; hence, it is also known as *aminoacyl-AMP*.



Aminoacyl adenylate

The next step is the transfer of the aminoacyl group of aminoacyl-AMP to a particular tRNA molecule to form *aminoacyl-tRNA*.

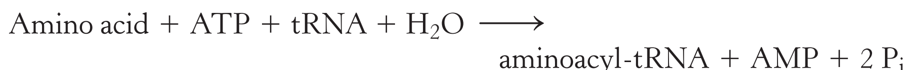
$$\text{Aminoacyl-AMP} + \text{tRNA} \rightleftharpoons \text{aminoacyl-tRNA} + \text{AMP}$$

The sum of these activation and transfer steps is

$$\text{Amino acid} + \text{ATP} + \text{tRNA} \rightleftharpoons \text{aminoacyl-tRNA} + \text{AMP} + \text{PP}_i$$

The $\Delta G°'$ of this reaction is close to 0, because the free energy of hydrolysis of the ester bond of aminoacyl-tRNA is similar to that for the hydrolysis

of ATP to AMP and PP_i. As we have seen many times, the reaction is driven by the hydrolysis of pyrophosphate. The sum of these three reactions is highly exergonic:

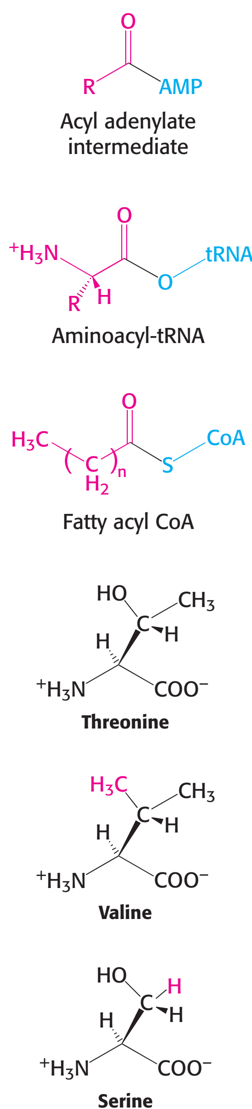


$$\text{Amino acid} + \text{ATP} + \text{tRNA} + H_2O \longrightarrow \text{aminoacyl-tRNA} + \text{AMP} + 2\,P_i$$

Thus, *the equivalent of two molecules of ATP is consumed in the synthesis of each aminoacyl-tRNA*. One of them is consumed in forming the ester linkage of aminoacyl-tRNA, whereas the other is consumed in driving the reaction forward.

The activation and transfer steps for a particular amino acid are catalyzed by the same aminoacyl-tRNA synthetase. Indeed, *the aminoacyl-AMP intermediate does not dissociate from the synthetase*. Rather, it is tightly bound to the active site of the enzyme by noncovalent interactions. Aminoacyl-AMP is normally a transient intermediate in the synthesis of aminoacyl-tRNA, but it is relatively stable and readily isolated if tRNA is absent from the reaction mixture.

We have already encountered an acyl adenylate intermediate in fatty acid activation (Section 22.2). The major difference between these reactions is that the acceptor of the acyl group is CoA in fatty acid activation and tRNA in amino acid activation. The energetics of these biosyntheses are very similar: both are made irreversible by the hydrolysis of pyrophosphate.



Aminoacyl-tRNA synthetases have highly discriminating amino acid activation sites

Each aminoacyl-tRNA synthetase is highly specific for a given amino acid. Indeed, a synthetase will incorporate the incorrect amino acid only once in 10^4 or 10^5 catalytic reactions. How is this level of specificity achieved? Each aminoacyl-tRNA synthetase takes advantage of the properties of its amino acid substrate. Let us consider the challenge faced by threonyl-tRNA synthetase. Threonine is particularly similar to two other amino acids—namely, valine and serine. Valine has almost exactly the same shape as that of threonine, except that valine has a methyl group in place of a hydroxyl group. Serine has a hydroxyl group, as does threonine, but lacks the methyl group. How can the threonyl-tRNA synthetase avoid coupling these incorrect amino acids to threonyl-tRNA?

The structure of the amino acid-binding site of threonyl-tRNA synthetase reveals how valine is avoided (Figure 30.8). The synthetase contains a zinc ion, bound to the enzyme by two histidine residues and one

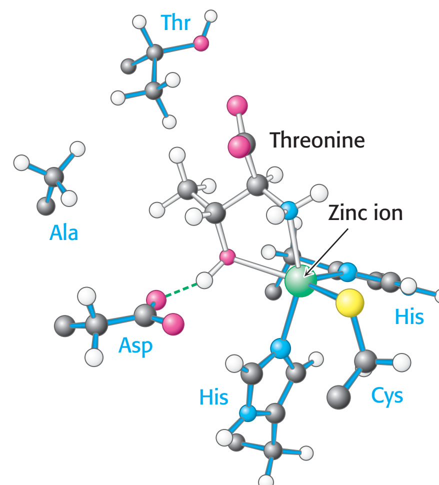


Figure 30.8 Active site of threonyl-tRNA synthetase. *Notice* that the amino acid-binding site includes a zinc ion (green ball) that coordinates threonine through its amino and hydroxyl groups.

cysteine residue. The remaining coordination sites are available for substrate binding. Threonine coordinates to the zinc ion through its amino group and its side-chain hydroxyl group. The side-chain hydroxyl group is further recognized by an aspartate residue that hydrogen bonds to it. The methyl group present in valine in place of this hydroxyl group cannot participate in these interactions; it is excluded from this active site and, hence, does not become adenylated and transferred to threonyl-tRNA (abbreviated $tRNA^{Thr}$). The use of a zinc ion appears to be unique to threonyl-tRNA synthetase; other aminoacyl-tRNA synthetases have different strategies for recognizing their cognate amino acids. The carboxylate group of the correctly positioned threonine is available to attack the α phosphoryl group of ATP to form the aminoacyl adenylate.

The zinc site is less able to discriminate against serine because this amino acid does have a hydroxyl group that can bind to the zinc ion. Indeed, with only this mechanism available, threonyl-tRNA synthetase does mistakenly couple serine to threonyl-tRNA at a rate 10^{-2} to 10^{-3} times that for threonine. As noted on page 888, this error rate is likely to lead to many translation errors. How is a higher level of specificity achieved?

Proofreading by aminoacyl-tRNA synthetases increases the fidelity of protein synthesis

Threonyl-tRNA synthetase can be incubated with $tRNA^{Thr}$ that has been covalently linked with serine (Ser-$tRNA^{Thr}$); the tRNA has been "mischarged." The reaction is immediate: a rapid hydrolysis of the aminoacyl-tRNA forms serine and free tRNA. In contrast, incubation with correctly charged Thr-$tRNA^{Thr}$ results in no reaction. Thus, threonyl-tRNA synthetase contains an additional functional site that hydrolyzes Ser-$tRNA^{Thr}$ but not Thr-$tRNA^{Thr}$. This editing site provides an opportunity for the synthetase to correct its mistakes and improve its fidelity to less than one mistake in 10^4. The results of structural and mutagenesis studies revealed that the editing site is more than 20 Å from the activation site (Figure 30.9). This editing site readily accepts and cleaves Ser-$tRNA^{Thr}$ but does not cleave Thr-$tRNA^{Thr}$. The discrimination of serine from threonine is easy because threonine contains an *extra* methyl group; a site that conforms to the structure of serine will sterically exclude threonine.

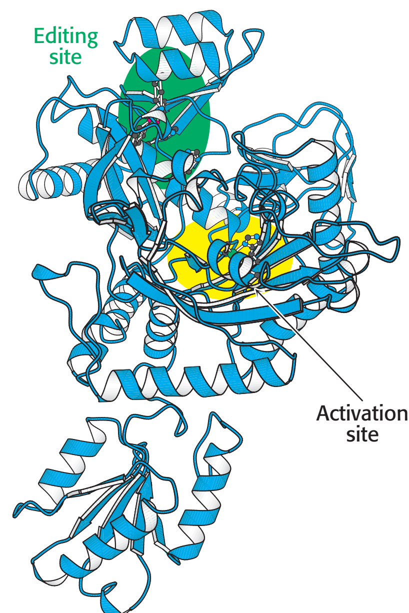


Figure 30.9 Editing site. Mutagenesis studies revealed the position of the editing site (shown in green) in threonyl-tRNA synthetase. Only one subunit of the dimeric enzyme is shown here and in subsequent illustrations. [Drawn from 1QF6.pdb.]

Most aminoacyl-tRNA synthetases contain editing sites in addition to activation sites. These complementary pairs of sites function as a *double sieve* to ensure very high fidelity. In general, the acylation site rejects amino acids that are *larger* than the correct one because there is insufficient room for them, whereas the hydrolytic site cleaves activated species that are *smaller* than the correct one.

The structure of the complex between threonyl-tRNA synthetase and its substrate reveals that the aminoacylated CCA can swing out of the activation site and into the editing site (Figure 30.10). Thus, the aminoacyl-tRNA can be edited without dissociating from the synthetase. This proofreading, which depends on the conformational flexibility of a short stretch of polynucleotide sequence, is entirely analogous to that of DNA polymerase (Section 28.1). In both cases, editing without dissociation significantly improves fidelity with only modest costs in time and energy.

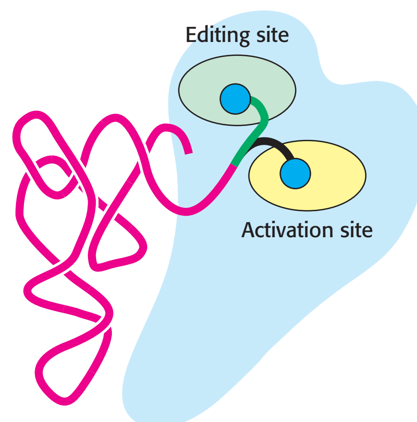


Figure 30.10 Editing of aminoacyl-tRNA. The flexible CCA arm of an aminoacyl-tRNA can move the amino acid between the activation site and the editing site. If the amino acid fits well into the editing site, the amino acid is removed by hydrolysis.

A few synthetases achieve high accuracy without editing. For example, tyrosyl-tRNA synthetase has no difficulty discriminating between tyrosine and phenylalanine; the hydroxyl group on the tyrosine ring enables tyrosine to bind to the enzyme 10^4 times as strongly as phenylalanine. *Proof-reading has been selected in evolution only when fidelity must be enhanced beyond what can be obtained through an initial binding interaction.*

Synthetases recognize various features of transfer RNA molecules

How do synthetases choose their tRNA partners? This enormously important step is the point at which "translation" takes place—at which the correlation between the amino acid and the nucleic acid worlds is made. In a sense, aminoacyl-tRNA synthetases are the only molecules in biology that "know" the genetic code. Their precise recognition of tRNAs is as important for high-fidelity protein synthesis as is the accurate selection of amino acids. In general, tRNA recognition by the synthetase is different for each synthetase and tRNA pair. Consequently, generalities are difficult to make. We will examine the interaction of threonyl-tRNA synthase with its tRNA partner.

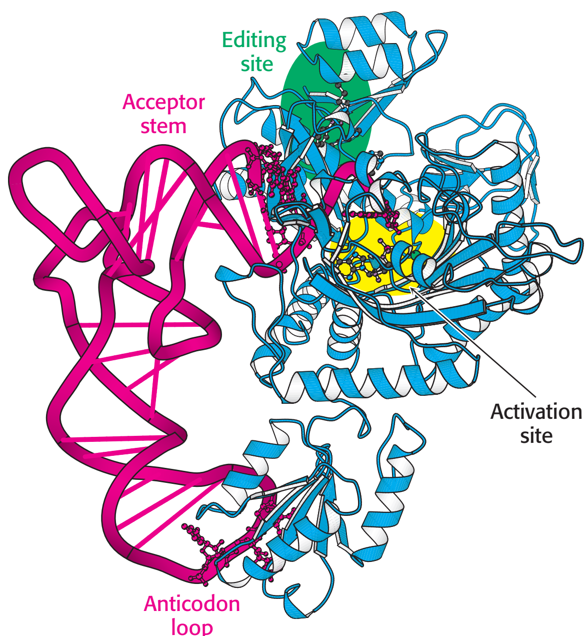


Figure 30.11 Threonyl-tRNA synthetase complex. The structure shows the complex between threonyl-tRNA synthetase and $tRNA^{Thr}$. *Notice* that the synthetase binds to both the acceptor stem and the anticodon loop. [Drawn from 1QF6.pdb.]

Some synthetases recognize their tRNA partners primarily on the basis of their anticodons, although they may also recognize other aspects of tRNA structure that vary among different tRNAs. The most direct evidence comes from crystallographic studies of complexes formed between synthetases and their cognate tRNAs. Consider, for example, the structure of the complex between threonyl-tRNA synthetase and $tRNA^{Thr}$ (Figure 30.11). As expected, the CCA arm extends into the zinc-containing activation site, where it is well positioned to accept threonine from threonyl adenylate. The enzyme interacts extensively not only with the acceptor stem of the tRNA, but also with the anticodon loop. The interactions with the anticodon loop are particularly revealing. Each base within the sequence 5′-CGU-3′ of the anticodon participates in hydrogen bonds with the enzyme; those with the second two bases (G and U) appear to be more important because the synthetase interacts just as efficiently with the anticodons GGU and UGU. Although interactions between the enzyme and the anticodon are often crucial for correct recognition, Figure 30.12 shows that many aspects of tRNA molecules are recognized by synthetases. Note that many of the recognition sites are loops rich in unusual bases that can provide structural identifiers.

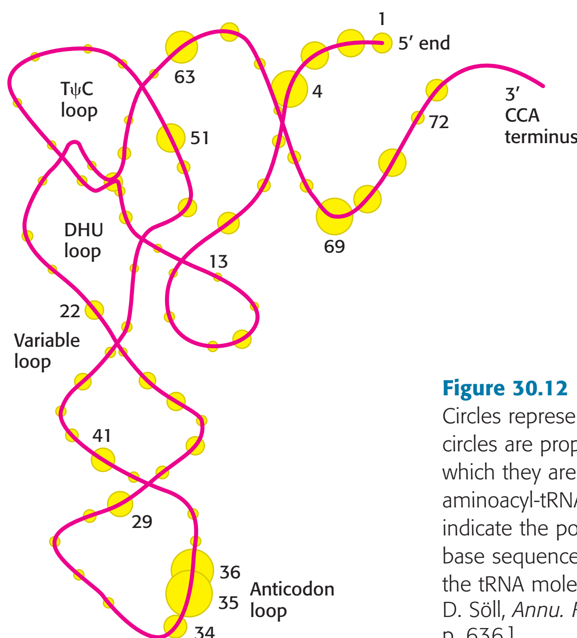


Figure 30.12 Recognition sites on tRNA. Circles represent nucleotides, and the sizes of the circles are proportional to the frequency with which they are used as recognition sites by aminoacyl-tRNA synthetases. The numbers indicate the positions of the nucleotides in the base sequence, beginning from the 5′ end of the tRNA molecule. [After M. Ibba, and D. Söll, *Annu. Rev. Biochem.* 69:617–650, 1981, p. 636.]

Aminoacyl-tRNA synthetases can be divided into two classes

At least one aminoacyl-tRNA synthetase exists for each amino acid. The diverse sizes, subunit composition, and sequences of these enzymes were bewildering for many years. Could it be that essentially all synthetases evolved independently? The determination of the three-dimensional structures of several synthetases followed by more-refined sequence comparisons revealed that different synthetases are, in fact, related. Specifically, synthetases fall into two classes, termed *class I* and *class II,* each of which includes enzymes specific for 10 of the 20 amino acids (Table 30.3). Intriguingly, synthetases from the two classes bind to different faces of the tRNA molecule (Figure 30.13). The CCA arm of tRNA adopts different conformations to accommodate these interactions; the arm is in the helical conformation observed for free tRNA (see Figures 30.4 and 30.5) for class II enzymes and in a hairpin conformation for class I enzymes. These two classes also differ in other ways.

Table 30.3 Classification and subunit structure of aminoacyl-tRNA synthetases in *E. coli*

Class I	Class II
Arg (α)	Ala (α4)
Cys (α)	Asn (α2)
Gln (α)	Asp (α2)
Glu (α)	Gly (α2β2)
Ile (α)	His (α2)
Leu (α)	Lys (α2)
Met (α)	Phe (α2β2)
Trp (α2)	Ser (α2)
Tyr (α2)	Pro (α2)
Val (α)	Thr (α2)

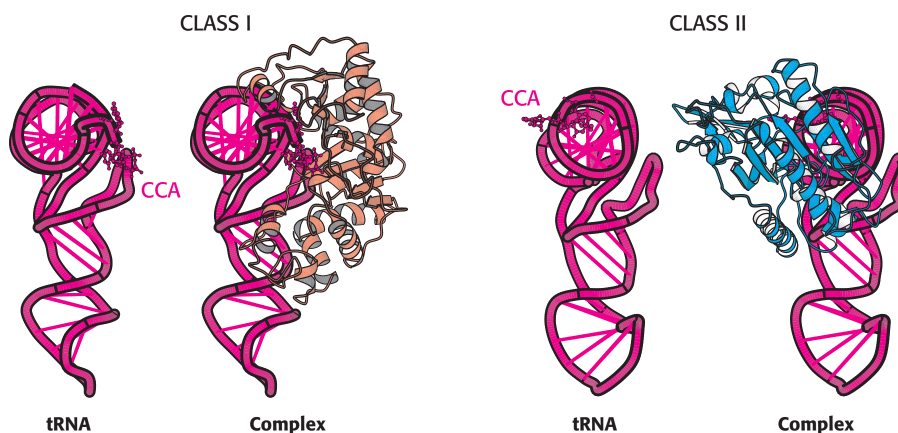


Figure 30.13 Classes of aminoacyl-tRNA synthetases. *Notice* that class I and class II synthetases recognize different faces of the tRNA molecule. The CCA arm of tRNA adopts different conformations in complexes with the two classes of synthetase. Note that the CCA arm of the tRNA is turned toward the viewer (see Figures 30.4 and 30.5). [Drawn from 1EUY.pdb and 1QF6.pdb.]

1. Class I enzymes acylate the 2′-hydroxyl group of the terminal adenosine of tRNA, whereas class II enzymes (except the enzyme for Phe-tRNA) acylate the 3′-hydroxyl group.
2. The two classes bind ATP in different conformations.
3. Most class I enzymes are monomeric, whereas most class II enzymes are dimeric.

Why did two distinct classes of aminoacyl-tRNA synthetases evolve? The observation that the two classes bind to distinct faces of tRNA suggests a possibility. Recognition sites on both faces of tRNA may have been required to allow the recognition of 20 different tRNAs.

30.3 The Ribosome Is the Site of Protein Synthesis

We turn now to ribosomes, the molecular machines that coordinate the interplay of charged tRNAs, mRNA, and proteins that leads to protein synthesis. An *E. coli* ribosome is a ribonucleoprotein assembly with a mass of about 2500 kd, a diameter of approximately 250 Å, and a sedimentation coefficient of 70S. The 20,000 ribosomes in a bacterial cell constitute nearly a fourth of its mass.

A ribosome can be dissociated into a *large subunit (50S)* and a *small subunit (30S)*. These subunits can be further split into their constituent proteins

and RNAs. The 30S subunit contains 21 different proteins (referred to as S1 through S21) and a 16S RNA molecule. The 50S subunit contains 34 different proteins (L1 through L34) and two RNA molecules, a 23S and a 5S species. A ribosome contains one copy of each RNA molecule, two copies each of the L7 and L12 proteins, and one copy of each of the other proteins. The L7 protein is identical with L12 except that its amino terminus is acetylated. Both the 30S and the 50S subunits can be reconstituted in vitro from their constituent proteins and RNA, as was first achieved by Masayasu Nomura in 1968. *This reconstitution is an outstanding example of the principle that supramolecular complexes can form spontaneously from their macromolecular constituents*.

Astounding progress on the structure of the ribosome has been made by x-ray crystallographic methods, after the pioneering work by Ada Yonath. The structures of both the 30S and the 50S subunits as well as the complete 70S ribosome have been determined at or close to atomic resolution (Figure 30.14). The determination of the structure of the 70S ribosome requires the positioning of more than 100,000 atoms. The features of these structures are in remarkable agreement with interpretations of less-direct experimental probes. These structures provide an invaluable framework for examining the mechanism of protein synthesis.

Ribosomal RNAs (5S, 16S, and 23S rRNA) play a central role in protein synthesis

The prefix *ribo* in the name *ribosome* is apt because RNA constitutes nearly two-thirds of the mass of these large molecular assemblies. The three RNAs present—5S, 16S, and 23S—are critical for ribosomal architecture and function. They are formed by the cleavage of primary 30S transcripts and further processing. These molecules fold into structures that allow them to form internal base pairs. Their base-pairing patterns were deduced by comparing the nucleotide sequences of many species to detect conserved sequences as well as conserved base pairings. For instance, the 16S RNA of one species may have a G–C base pair, whereas another may have an A–U base pair, but the location of the base pair is the same in both molecules. Chemical modification and digestion experiments supported the structures deduced from sequence comparisons (Figure 30.15). The striking finding is that across all species *ribosomal RNAs* (rRNAs) *are folded into defined structures that have many short duplex regions*. This conclusion and essentially all features of the secondary structure have been confirmed by the x-ray crystallographically determined structures.

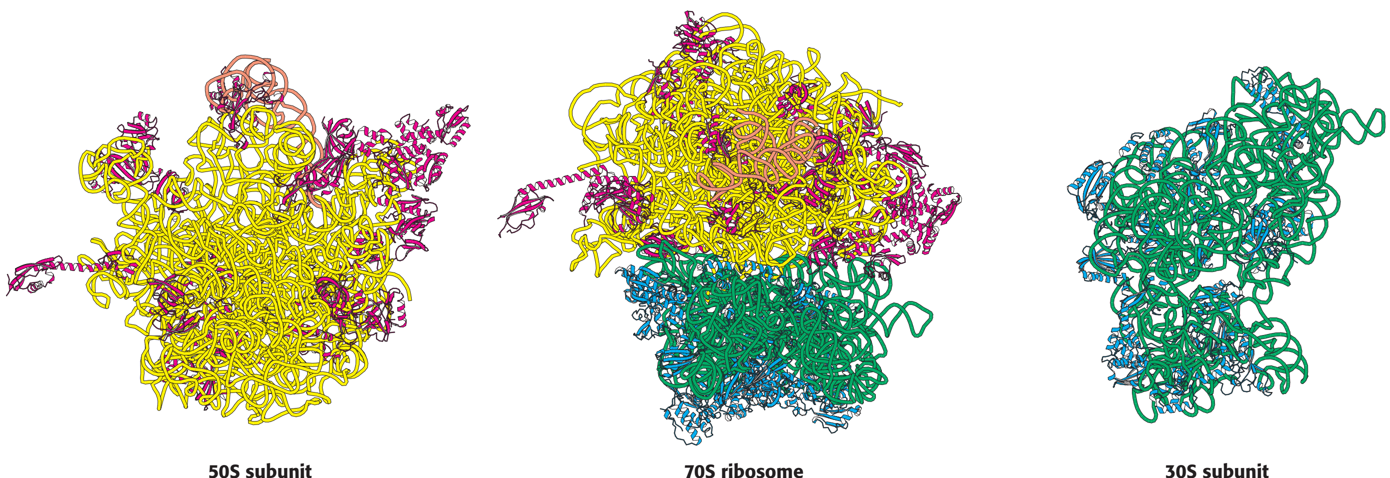


Figure 30.14 The ribosome at high resolution. Detailed models of the ribosome based on the results of x-ray crystallographic studies of the 70S ribosome and the 30S and 50S subunits: (left) view of the part of the 50S subunit that interacts with the 30S subunit; (center) side view of the 70S ribosome; (right) view of the part of the 30S subunit that interacts with the 50S subunit. 23S RNA is shown in yellow, 5S RNA in orange, 16S RNA in green, proteins of the 50S subunit in red, and proteins of the 30S subunit in blue. *Notice* that the interface between the 50S and the 30S subunits consists entirely of RNA. [Drawn from 1GIX.pdb and 1GIY.pdb.]

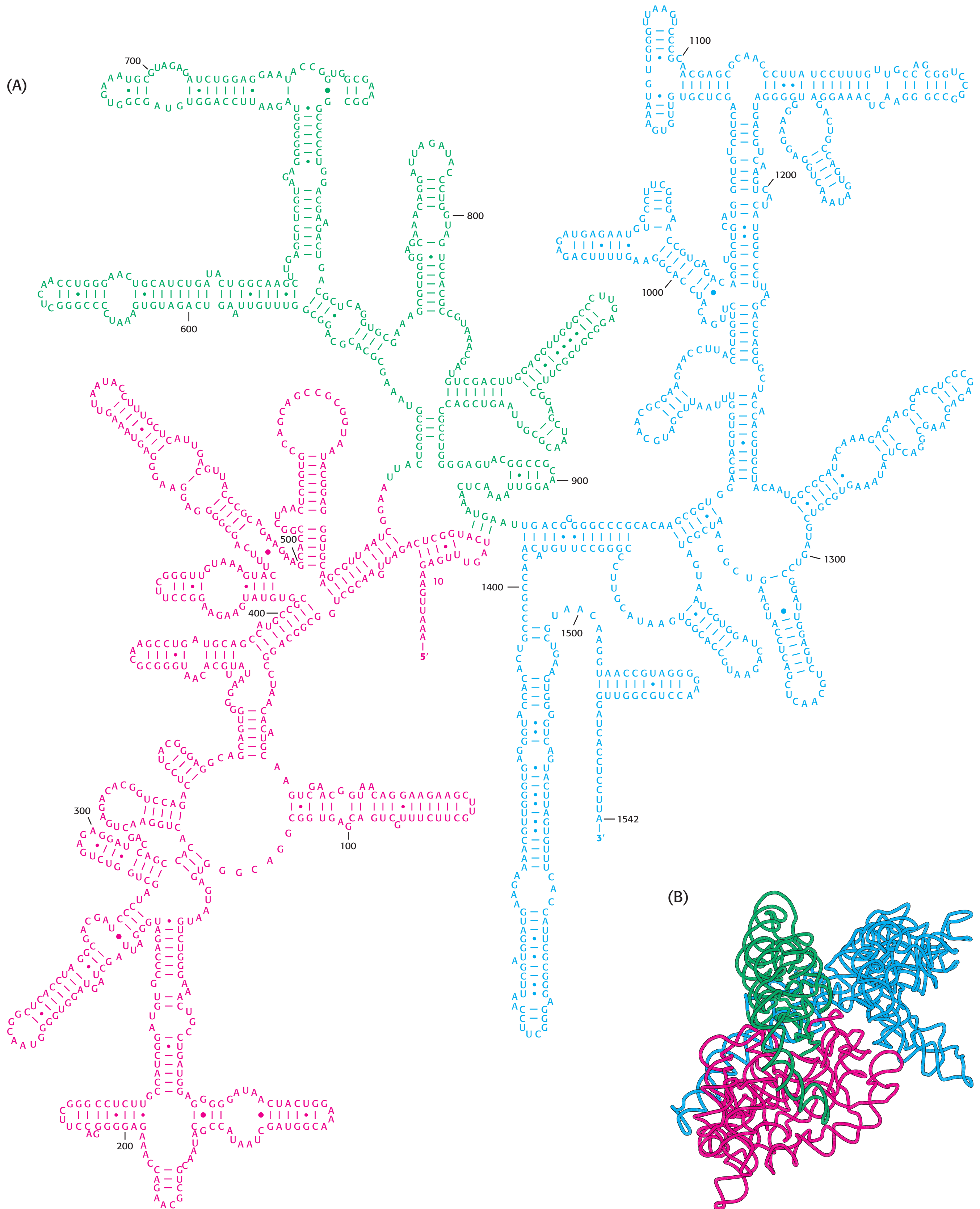


Figure 30.15 Ribosomal RNA folding pattern. (A) The secondary structure of 16S ribosomal RNA deduced from sequence comparison and the results of chemical studies. (B) The tertiary structure of 16S RNA determined by x-ray crystallography. [(A) Courtesy of Dr. Bryn Weiser and Dr. Harry Noller; (B) drawn from 1FJG.pdb.]

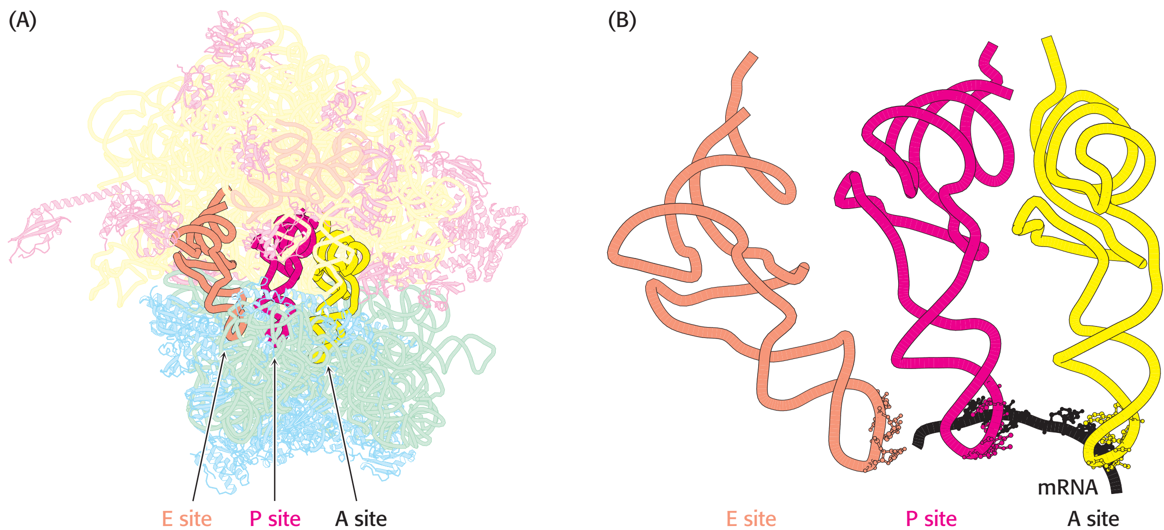


Figure 30.16 Transfer RNA-binding sites. (A) Three tRNA-binding sites are present on the 70S ribosome. They are called the A (for aminoacyl), P (for peptidyl), and E (for exit) sites. Each tRNA molecule contacts both the 30S and the 50S subunit. (B) The tRNA molecules in sites A and P are base-paired with mRNA. [(B) Drawn from 1JGP. pdb.]

For many years, ribosomal proteins were presumed to orchestrate protein synthesis and ribosomal RNAs were presumed to serve primarily as structural scaffolding. The current view is almost the reverse. The discovery of catalytic RNA made biochemists receptive to the possibility that RNA plays a much more active role in ribosomal function. The detailed structures make it clear that the key sites in the ribosome, such as those that catalyze the formation of the peptide bond and interact with mRNA and tRNA, are composed almost entirely of RNA. Contributions from the proteins are minor. Many of the proteins have elongated structures that "snake" their way into the RNA matrix. The almost inescapable conclusion is that the ribosome initially consisted only of RNA and that the proteins were added later to fine-tune its functional properties. This conclusion has the pleasing consequence of dodging a "chicken and egg" question: How can complex proteins be synthesized if complex proteins are required for protein synthesis?

Ribosomes have three tRNA-binding sites that bridge the 30S and 50S subunits

Three tRNA-binding sites in ribosomes are arranged to allow the formation of peptide bonds between amino acids encoded by the codons on mRNA (Figure 30.16). The mRNA fragment being translated at a given moment is bound within the 30S subunit. Each of the tRNA molecules is in contact with both the *30S subunit* and the *50S subunit*. At the 30S end, two of the three tRNA molecules are bound to the mRNA through anticodon–codon base pairs. These binding sites are called the A site (for *a*minoacyl) and the P site (for *p*eptidyl). The third tRNA molecule is bound to an adjacent site called the E site (for *e*xit).

The other end of each tRNA molecule, the end without the anticodon, interacts with the 50S subunit. The acceptor stems of the tRNA molecules occupying the A site and the P site converge at a site where a peptide bond is formed. A tunnel connects this site to the back of the ribosome, through which the polypeptide chain passes during synthesis (Figure 30.17).

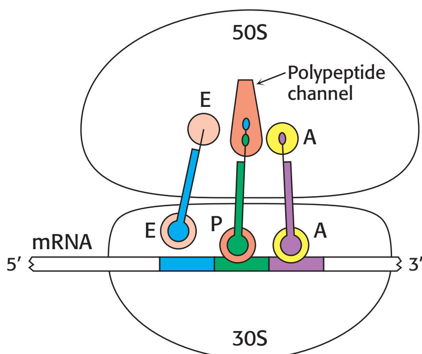


Figure 30.17 An active ribosome. This schematic representation shows the relations among the key components of the translation machinery.

The start signal is usually AUG preceded by several bases that pair with 16S rRNA

How does protein synthesis start? The simplest possibility would be for the first 3 nucleotides of each mRNA to serve as the first codon; no special start

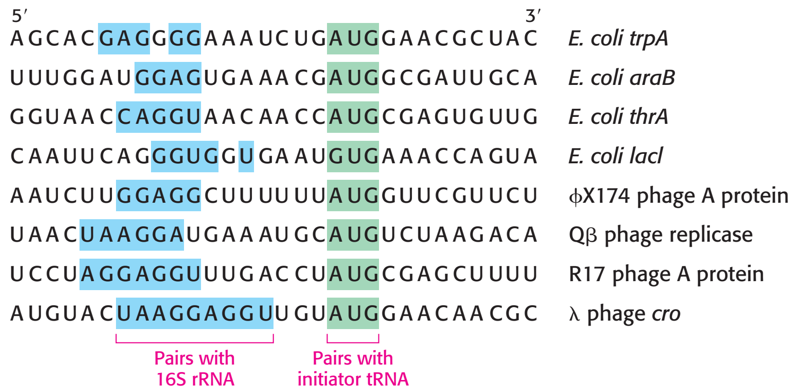


Figure 30.18 Initiation sites. Sequences of mRNA initiation sites for protein synthesis in some bacterial and viral mRNA molecules. Comparison of these sequences reveals some recurring features.

signal would then be needed. However, experiments show that translation does not begin immediately at the 5′ terminus of mRNA. Indeed, the first translated codon is nearly always more than 25 nucleotides away from the 5′ end. Furthermore, in prokaryotes, many mRNA molecules are *polycistronic*, or polygenic—that is, they encode two or more polypeptide chains. For example, a single mRNA molecule about 7000 nucleotides long specifies five enzymes in the biosynthetic pathway for tryptophan in *E. coli*. Each of these five proteins has its own start and stop signals on the mRNA. In fact, *all known mRNA molecules contain signals that define the beginning and end of each encoded polypeptide chain*.

A clue to the mechanism of initiation was the finding that nearly half the amino-terminal residues of proteins in *E. coli* are methionine. In fact, the initiating codon in mRNA is AUG (methionine) or, less frequently, GUG (valine) or, rarely UUG (leucine). What additional signals are necessary to specify a translation start site? The first step toward answering this question was the isolation of initiator regions from a number of mRNAs. This isolation was accomplished by using pancreatic ribonuclease to digest mRNA–ribosome complexes (formed under conditions in which protein synthesis could begin but elongation could not take place). As expected, each initiator region displays an AUG (or GUG or UUG) codon (Figure 30.18). In addition, each initiator region contains a purine-rich sequence centered about 10 nucleotides on the 5′ side of the initiator codon.

The role of this purine-rich region, called the *Shine–Dalgarno sequence*, became evident when the sequence of 16S rRNA was elucidated. The 3′ end of this rRNA component of the 30S subunit contains a sequence of several bases that is complementary to the purine-rich region in the initiator sites of mRNA. Mutagenesis of the CCUCC sequence near the 3′ end of 16S rRNA to ACACA markedly interferes with the recognition of start sites in mRNA. This result and other evidence show that the initiator region of mRNA binds very near the 3′ end of the 16S rRNA. The number of base pairs linking mRNA and 16S rRNA ranges from three to nine. Thus, *two kinds of interactions determine where protein synthesis starts: (1) the pairing of mRNA bases with the 3′ end of 16S rRNA and (2) the pairing of the initiator codon on mRNA with the anticodon of an initiator tRNA molecule*.

Bacterial protein synthesis is initiated by formylmethionyl transfer RNA

As stated earlier, methionine is the first amino acid in many *E. coli* proteins. However, the methionine residue found at the amino-terminal end of *E. coli* proteins is usually modified. In fact, *protein synthesis in bacteria starts with the modified amino acid N-formylmethionine* (fMet). A special tRNA brings formylmethionine to the ribosome to initiate protein synthesis. This *initiator tRNA* (abbreviated as $tRNA_f$) differs from the tRNA that inserts

methionine in internal positions (abbreviated as $tRNA_m$). The subscript "f" indicates that methionine attached to the initiator tRNA can be formylated, whereas it cannot be formylated when attached to $tRNA_m$. Although virtually all proteins synthesized in *E. coli* begin with formylmethionine, in approximately one-half of the proteins, *N*-formylmethionine is removed when the nascent chain is 10 amino acids long.

Methionine is linked to these two kinds of tRNAs by the same aminoacyl-tRNA synthetase. A specific enzyme then formylates the amino group of the methionine molecule that is attached to $tRNA_f$ (Figure 30.19). The activated formyl donor in this reaction is N^{10}-formyltetrahydrofolate, a folate derivative that carries activated one-carbon units (Section 24.2). Free methionine and methionyl-$tRNA_m$ are not substrates for this transformylase.

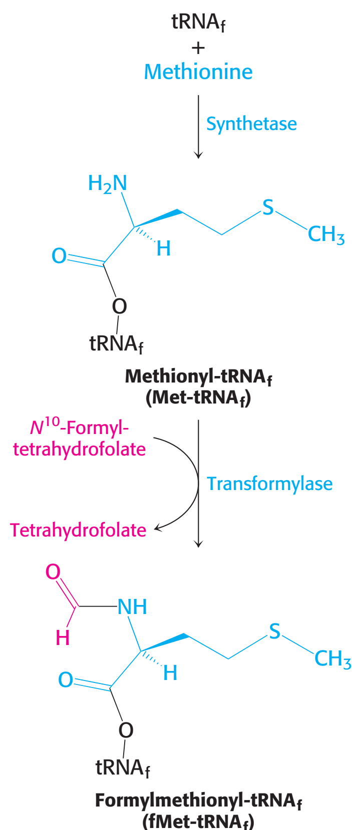


Figure 30.19 Formylation of methionyl-tRNA. Initiator tRNA ($tRNA_f$) is first charged with methionine, and then a formyl group is transferred to the methionyl-$tRNA_f$ from N^{10}-formyltetrahydrofolate.

Formylmethionyl-$tRNA_f$ is placed in the P site of the ribosome in the formation of the 70S initiation complex

Messenger RNA and formylmethionyl-$tRNA_f$ must be brought to the ribosome for protein synthesis to begin. How is this task accomplished? Three protein *initiation factors* (IF1, IF2, and IF3) are essential. The 30S ribosomal subunit first forms a complex with IF1 and IF3 (Figure 30.20). The binding of these factors to the 30S subunit prevents it from prematurely joining the 50S subunit to form a dead-end 70S complex, devoid of mRNA and fMet-$tRNA_f$. IF1 binds near the A site and directs the fMet-$tRNA_f$ to the P site. Initiation factor 2, a member of the G-protein family, binds GTP, and the concomitant conformational change enables IF2 to associate with fMet-$tRNA_f$. The IF2–GTP–initiator-tRNA complex binds with mRNA (correctly positioned by the interaction of the Shine–Dalgarno sequence with the 16S rRNA) and the 30S subunit to form the *30S initiation complex*. Structural changes then lead to the ejection of IF1 and IF3. IF2 stimulates the association of the 50S subunit to the complex. The GTP bound to IF2 is hydrolyzed, leading to the release of IF2. The result is a *70S initiation complex*. The formation of the 70S initiation complex is the rate-limiting step in protein synthesis.

When the 70S initiation complex has been formed, the ribosome is ready for the elongation phase of protein synthesis. The fMet-$tRNA_f$ molecule occupies the P site on the ribosome, positioned so that its anticodon pairs with the initiating codon on mRNA. The other two sites for tRNA molecules, the A site and the E site, are empty. This interaction establishes the *reading frame* for the translation of the entire mRNA. After the initiator codon has been located, groups of three nonoverlapping nucleotides are defined.

Elongation factors deliver aminoacyl-tRNA to the ribosome

At this point, fMet-$tRNA_f$ occupies the P site, and the A site is vacant. The particular species inserted into the empty A site depends on the mRNA codon in the A site. However, the appropriate aminoacyl-tRNA does not simply leave the synthetase and diffuse to the A site. Rather, it is delivered to the A site in association with a 43-kd protein called *elongation factor Tu* (EF-Tu), another member of the G-protein family, which requires GTP for activity. EF-Tu binds aminoacyl-tRNA only in its GTP form (Figure 30.21) and releases it to the ribosome in its GDP form. The binding of EF-Tu to aminoacyl-tRNA serves two functions. First, EF-Tu protects the delicate ester linkage in aminoacyl-tRNA from hydrolysis. Second, the GTP in EF-Tu is hydrolyzed to GDP only when an appropriate complex between the EF-Tu–aminoacyl-tRNA complex and the ribosome has

formed. If the anticodon is not properly paired with the codon, hydrolysis does not take place and the aminoacyl-tRNA is not transferred to the ribosome. This mechanism allows the free energy of GTP hydrolysis to contribute to the accuracy of protein synthesis.

EF-Tu is then reset to its GTP form by a second elongation factor, *elongation factor Ts*. EF-Ts induces the dissociation of GDP. GTP binds to EF-Tu, and EF-Ts is concomitantly released. It is noteworthy that *EF-Tu does not interact with fMet-tRNA$_f$*. Hence, this initiator tRNA is not delivered to the A site. In contrast, Met-tRNA$_m$, like all other aminoacyl-tRNAs, does bind to EF-Tu. These findings account for the fact that *internal AUG codons are not read by the initiator tRNA*. Conversely, IF2 recognizes fMet-tRNA$_f$ but no other tRNA. The cycle of elongation continues until a termination codon is met.

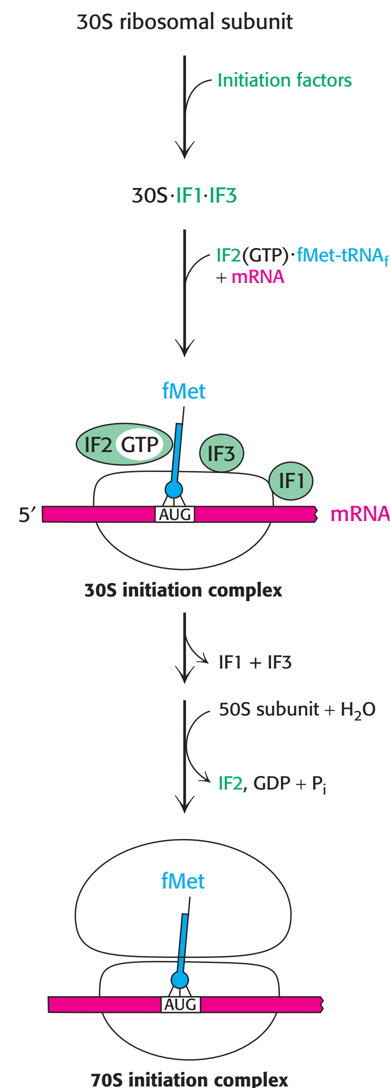
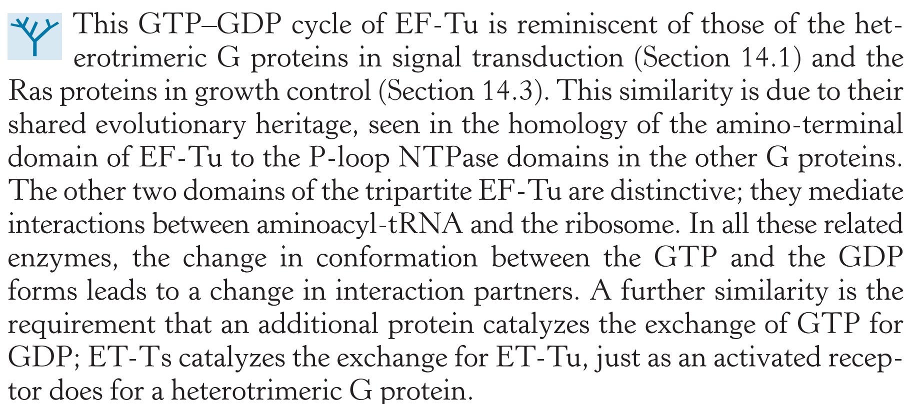


Figure 30.20 Translation initiation in prokaryotes. Initiation factors aid the assembly first of the 30S initiation complex and then of the 70S initiation complex.


This GTP–GDP cycle of EF-Tu is reminiscent of those of the heterotrimeric G proteins in signal transduction (Section 14.1) and the Ras proteins in growth control (Section 14.3). This similarity is due to their shared evolutionary heritage, seen in the homology of the amino-terminal domain of EF-Tu to the P-loop NTPase domains in the other G proteins. The other two domains of the tripartite EF-Tu are distinctive; they mediate interactions between aminoacyl-tRNA and the ribosome. In all these related enzymes, the change in conformation between the GTP and the GDP forms leads to a change in interaction partners. A further similarity is the requirement that an additional protein catalyzes the exchange of GTP for GDP; ET-Ts catalyzes the exchange for ET-Tu, just as an activated receptor does for a heterotrimeric G protein.

Peptidyl transferase catalyzes peptide-bond synthesis

With both the P site and the A site occupied by aminoacyl-tRNA, the stage is set for the formation of a peptide bond: the formylmethionine molecule linked to the initiator tRNA will be transferred to the amino group of the amino acid in the A site. The formation of the peptide bond, one of the most important reactions in life, is a thermodynamically spontaneous reaction catalyzed by a site on the 23S rRNA of the 50S subunit called the *peptidyl transferase center*. This catalytic center is located deep in the 50S subunit near the tunnel that allows the nascent peptide to leave the ribosome.

The ribosome derives much of its catalytic power from what is called *catalysis by proximity and orientation*. The ribosome positions and orients

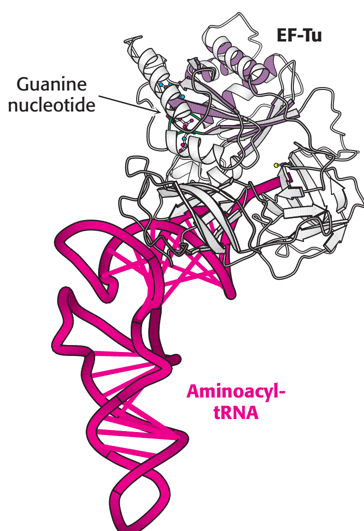


Figure 30.21 Structure of elongation factor Tu. The structure of a complex between elongation factor Tu (EF-Tu) and an aminoacyl-tRNA. *Notice* the P-loop NTPase domain (purple shading) at the amino-terminal end of EF-Tu. This NTPase domain is similar to those in other G proteins. [Drawn from 1B23.pdb.]

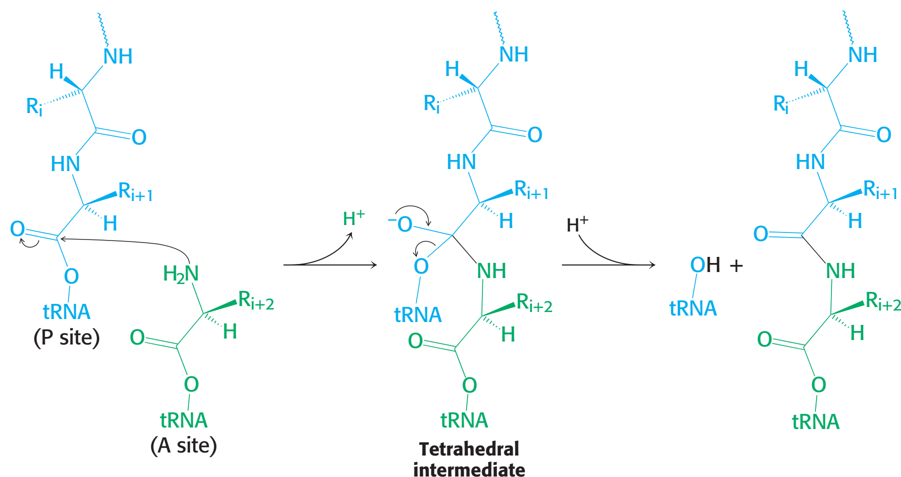


Figure 30.22 Peptide-bond formation. The amino group of the aminoacyl-tRNA attacks the carbonyl group of the ester linkage of the peptidyl-tRNA to form a tetrahedral intermediate. This intermediate collapses to form the peptide bond and release the deacylated tRNA.

the two substrates so that they are situated to take advantage of the inherent reactivity of an amine group (on the aminoacyl-tRNA in the A site) with an ester (on the initiator tRNA in the P site). The amino group of the aminoacyl-tRNA in the A site is positioned to attack the ester linkage between the initiator tRNA and the formylmethionine molecule in the P site (Figure 30.22). The peptidyl transferase center includes bases that promote this reaction by helping to form an —NH_2 group on the A-site aminoacyl-tRNA and by helping to stabilize the tetrahedral intermediate that forms. This reaction is, in many ways, analogous to the reverse of the reaction catalyzed by serine proteases such as chymotrypsin (Section 9.1). The peptidyl-tRNA is analogous to the acyl-enzyme form of a serine protease. In a serine protease, the acyl-enzyme is generated with the use of the free energy associated with cleaving an amide bond. In the ribosome, the free energy necessary to form the analogous species, an aminoacyl-tRNA, comes from the ATP that is cleaved by the aminoacyl-tRNA synthetase before the arrival of the tRNA at the ribosome.

The formation of a peptide bond is followed by the GTP-driven translocation of tRNAs and mRNA

With the formation of the peptide bond, the peptide chain is now attached to the tRNA whose anticodon is in the A site on the 30S subunit. The two subunits rotate with respect to one another, and this structural change places the CCA end of the same tRNA and its peptide in the P site of the large subunit (Figure 30.23). However, protein synthesis cannot continue without the translocation of the mRNA and the tRNAs within the ribosome. The mRNA must move by a distance of three nucleotides so that the next codon is positioned in the A site for interaction with the incoming aminoacyl-tRNA. At the same time, the deacylated tRNA moves out of the P site into the E site on the 30S subunit and the peptidyl-tRNA moves out of the A site into the P site on the 30S subunit. The movement of the peptidyl-tRNA into the P site shifts the mRNA by one codon, exposing the next codon to be translated in the A site.

The three-dimensional structure of the ribosome undergoes significant change during translocation, and evidence suggests that translocation may result from properties of the ribosome itself. However, protein factors accelerate the process. Translocation is enhanced by *elongation factor G* (EF-G, also called *translocase*). A possible mechanism for accelerating the translocation

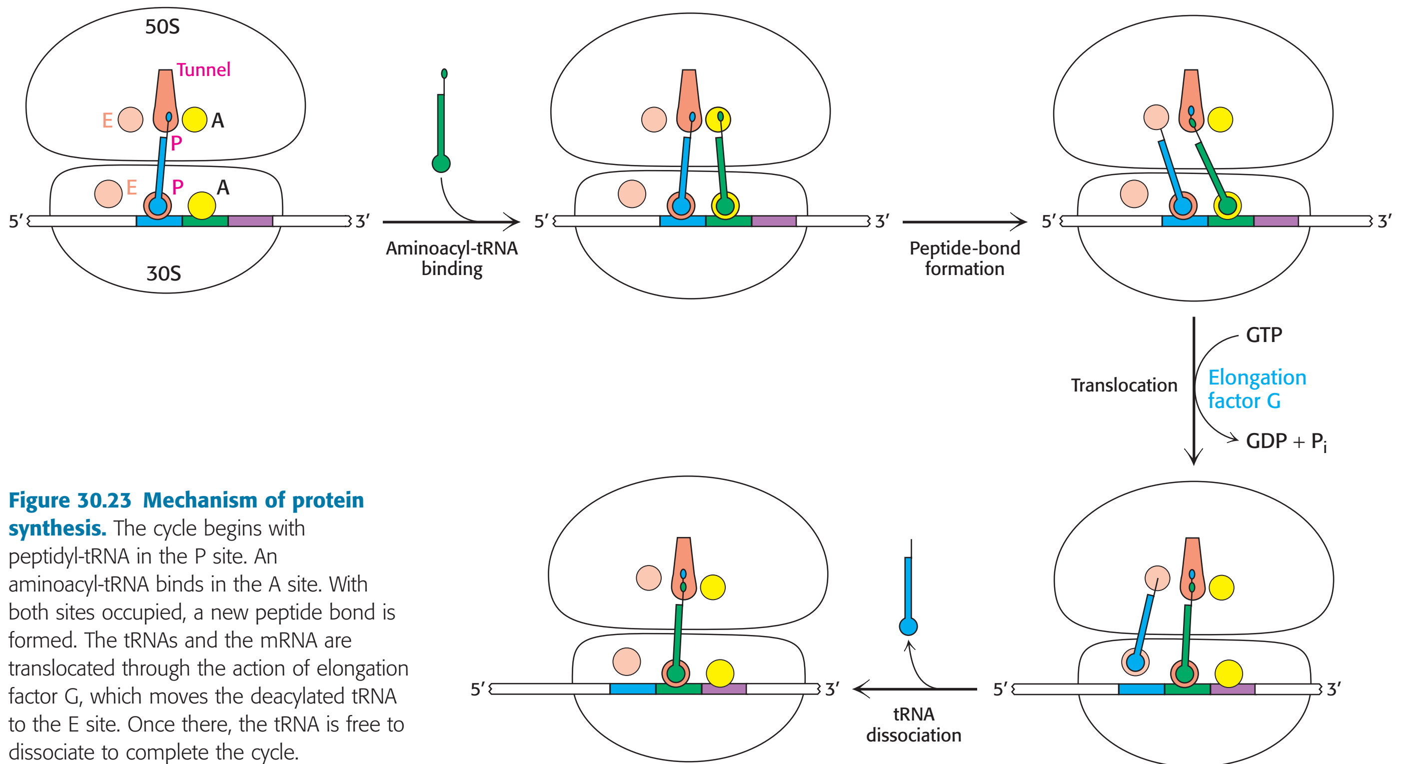


Figure 30.23 Mechanism of protein synthesis. The cycle begins with peptidyl-tRNA in the P site. An aminoacyl-tRNA binds in the A site. With both sites occupied, a new peptide bond is formed. The tRNAs and the mRNA are translocated through the action of elongation factor G, which moves the deacylated tRNA to the E site. Once there, the tRNA is free to dissociate to complete the cycle.

process in shown in Figure 30.24. First, EF-G in the GTP form binds to the ribosome near the A site, interacting with the 23S rRNA of the 50S subunit. The binding of EF-G to the ribosome stimulates the GTPase activity of EF-G. On GTP hydrolysis, EF-G undergoes a conformational change that displaces the peptidyl-tRNA in the A site to the P site, which carries the mRNA and the deacylated tRNA with it. The dissociation of EF-G leaves the ribosome ready to accept the next aminoacyl-tRNA into the A site.

Note that *the peptide chain remains in the P site on the 50S subunit throughout this cycle,* growing into the exit tunnel. This cycle is repeated, with mRNA translation taking place in the 5′ → 3′ direction, as new aminoacyl-tRNAs move into the A site, allowing the polypeptide to be elongated until a stop signal is found.

The direction of translation has important consequences. Recall that transcription also is in the 5′ → 3′ direction (Section 29.1). If the direction of translation were opposite that of transcription, only fully synthesized mRNA could be translated. In contrast, because the directions are the same,

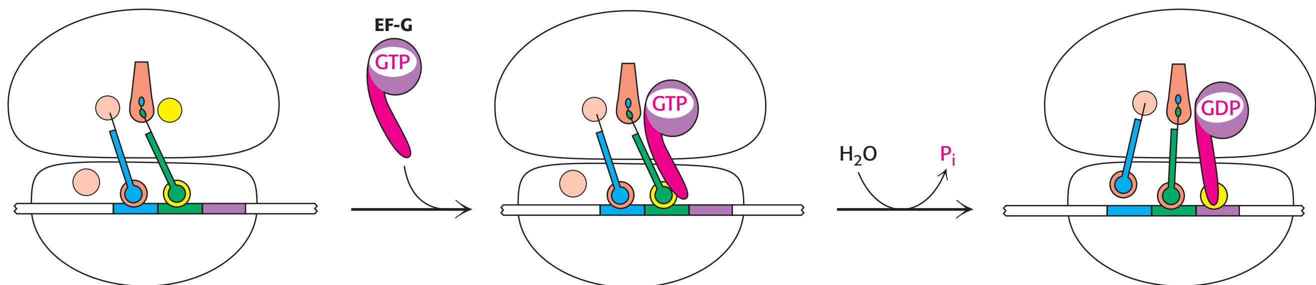


Figure 30.24 Translocation mechanism. In the GTP form, EF-G binds to the EF-Tu-binding site on the 50S subunit. This binding stimulates GTP hydrolysis, inducing a conformational change in EF-G that forces the tRNAs and mRNA to move through the ribosome by a distance corresponding to one codon.

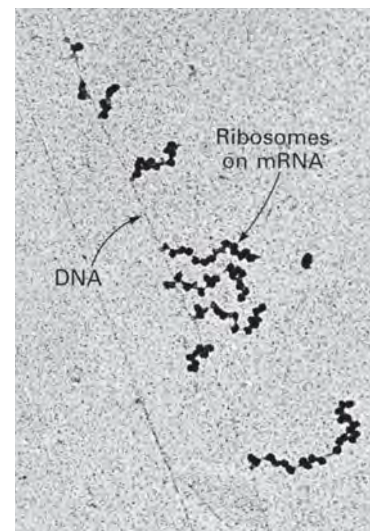
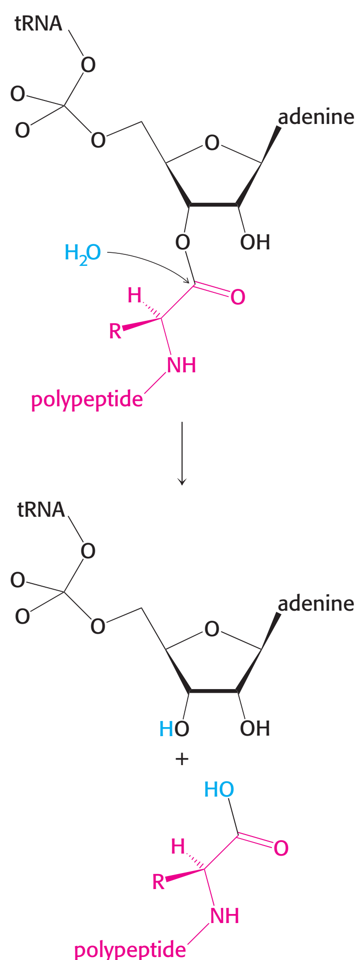


Figure 30.25 Polysomes. Transcription of a segment of DNA from *E. coli* generates mRNA molecules that are immediately translated by multiple ribosomes. [From O. L. Miller, Jr., B. A. Hamkalo, and C. A. Thomas, Jr. *Science* 169:392–395, 1970.]

mRNA can be translated while it is being synthesized. In prokaryotes, almost no time is lost between transcription and translation. The 5′ end of mRNA interacts with ribosomes very soon after it is made, much before the 3′ end of the mRNA molecule is finished. *An important feature of prokaryotic gene expression is that translation and transcription are closely coupled in space and time*.

Many ribosomes can be translating an mRNA molecule simultaneously. This parallel synthesis markedly increases the efficiency of mRNA translation. The group of ribosomes bound to an mRNA molecule is called a *polyribosome* or a *polysome* (Figure 30.25). Recent work shows that the ribosomes are arranged so as to protect the mRNA and to facilitate easy exchange of the substrates and products with the cytoplasm. The ribosomes in the polysome are in a helical array around the mRNA with the tRNA binding sites and peptide exit tunnel exposed to the cytoplasm.



Protein synthesis is terminated by release factors that read stop codons

The final phase of translation is termination. How does the synthesis of a polypeptide chain come to an end when a stop codon is encountered? No tRNAs with anticodons complementary to the stop codons—UAA, UGA, or UAG—exist in normal cells. Instead, these *stop codons are recognized by proteins called release factors* (RFs). One of these release factors, RF1, recognizes UAA or UAG. A second factor, RF2, recognizes UAA or UGA. A third factor, RF3, another GTPase, mediates interactions between RF1 or RF2 and the ribosome.

RF1 and RF2 are compact proteins that, in eukaryotes, resemble a tRNA molecule. When bound to the ribosome, the proteins unfold to bridge the gap between the stop codon on the mRNA and the peptidyl transferase center on the 50S subunit (Figure 30.26). The RF interacts with the peptidyl transferase center with a loop containing a highly conserved glycine-glycine-glutamine (GGQ) sequence, with the glutamine methylated on the amide nitrogen atom of the R group. This modified glutamine is crucial in promoting, assisted by the peptidyl transferase, a water molecule's attack on the ester linkage between the tRNA and the polypeptide chain, freeing the polypeptide chain. The detached polypeptide leaves the ribosome. Transfer RNA and messenger RNA remain briefly attached to the 70S ribosome until the entire complex is dissociated through the hydrolysis of GTP in response to the binding of EF-G and another factor, called the *ribosome release factor* (RRF).

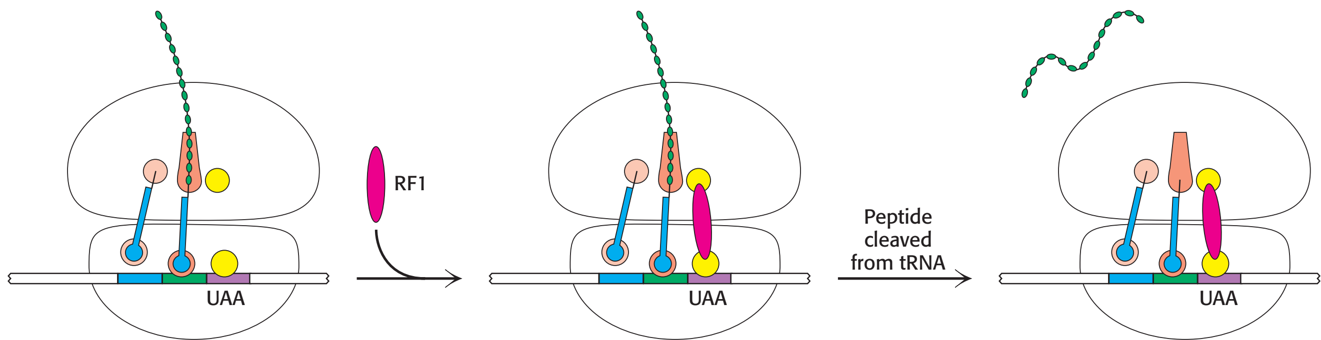


Figure 30.26 Termination of protein synthesis. A release factor recognizes a stop codon in the A site and stimulates the release of the completed protein from the tRNA in the P site.

30.4 Eukaryotic Protein Synthesis Differs from Prokaryotic Protein Synthesis Primarily in Translation Initiation

The basic plan of protein synthesis in eukaryotes and archaea is similar to that in bacteria. The major structural and mechanistic themes recur in all domains of life. However, eukaryotic protein synthesis entails more protein components than does prokaryotic protein synthesis, and some steps are more intricate. Some noteworthy similarities and differences are as follows:

1. *Ribosomes.* Eukaryotic ribosomes are larger. They consist of a 60S large subunit and a 40S small subunit, which come together to form an 80S particle having a mass of 4200 kd, compared with 2700 kd for the prokaryotic 70S ribosome. The 40S subunit contains an 18S RNA that is homologous to the prokaryotic 16S RNA. The 60S subunit contains three RNAs: the 5S RNA, which is homologous to the prokaryotic 5S rRNA; the 28S RNA, which is homologous to the prokaryotic 23S molecules; and the 5.8S RNA, which is homologous to the 5′ end of the 23S RNA of prokaryotes.

2. *Initiator tRNA.* In eukaryotes, the initiating amino acid is methionine rather than *N*-formylmethionine. However, as in prokaryotes, a special tRNA participates in initiation. This aminoacyl-tRNA is called Met-$tRNA_i$ or Met-$tRNA_f$ (the subscript "i" stands for initiation, and "f" indicates that it can be formylated in vitro).

3. *Initiation.* The initiating codon in eukaryotes is always AUG. Eukaryotes, in contrast with prokaryotes, do not have a specific purine-rich sequence on the 5′ side to distinguish initiator AUGs from internal ones. Instead, the AUG nearest the 5′ end of mRNA is usually selected as the start site. A 40S ribosome, with a bound Met-$tRNA_i$, attaches to the cap at the 5′ end of eukaryotic mRNA (Section 29.3) and searches for an AUG codon by moving step-by-step in the 3′ direction (Figure 30.27). This scanning process is catalyzed by helicases that move along the mRNA powered by ATP hydrolysis. Pairing of the anticodon of Met-$tRNA_i$ with the AUG codon of mRNA signals that the target has been found. In almost all cases, eukaryotic mRNA has only one start site and hence is the template for only a single protein. In contrast, a prokaryotic mRNA can have multiple Shine–Dalgarno sequences and, hence, start sites, and it can serve as a template for the synthesis of several proteins.

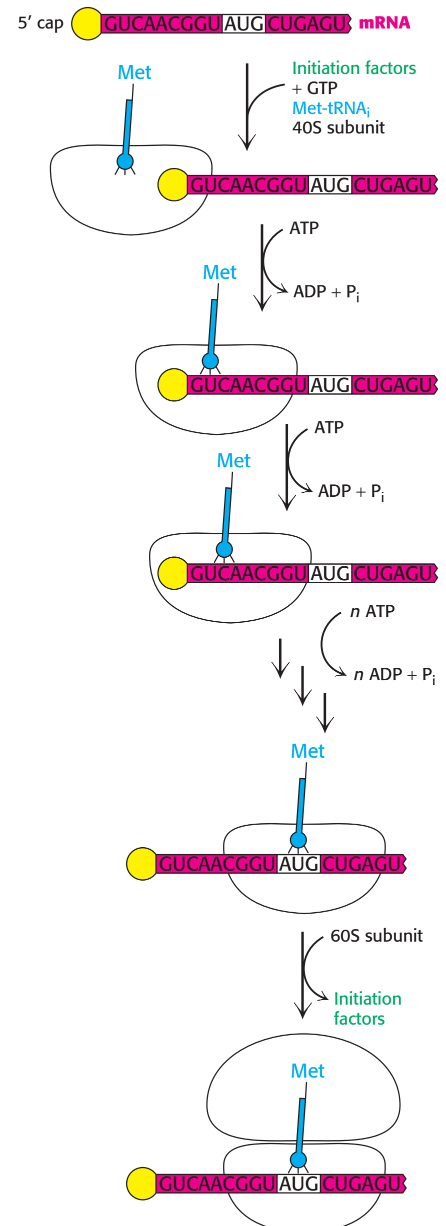


Figure 30.27 Eukaryotic translation initiation. In eukaryotes, translation initiation starts with the assembly of a complex on the 5′ cap that includes the 40S subunit and Met-$tRNA_i$. Driven by ATP hydrolysis, this complex scans the mRNA until the first AUG is reached. The 60S subunit is then added to form the 80S initiation complex.

Eukaryotes utilize many more initiation factors than do prokaryotes, and their interplay is much more intricate. The prefix *eIF* denotes a eukaryotic initiation factor. For example, eIF-4E is a protein that binds directly to the 7-methylguanosine cap (Section 29.3), whereas eIF-2, in association with GTP, delivers the met-$tRNA_i$ to the ribosome. The difference in initiation mechanism between prokaryotes and eukaryotes is, in part, a consequence of the difference in RNA processing. The 5′ end of mRNA is readily available to ribosomes immediately after transcription in prokaryotes. In contrast, in eukaryotes pre-mRNA must be processed and transported to the cytoplasm before translation is initiated. The 5′ cap provides an easily recognizable starting point. In addition, the complexity of eukaryotic translation initiation provides another mechanism for regulation of gene expression that we shall explore further in Chapter 31.

Although most eukaryotic mRNA molecules rely on the 5′ cap to initiate protein synthesis, recent work has established that some mRNA molecules can recruit ribosomes for initiation without the use of a 5′-cap and cap-binding proteins. In these mRNAs, highly structured RNA sequences called *i*nternal *r*ibosome *e*ntry *s*ites (IRES) facilitate 40S ribosome binding to the mRNA. IRES were first discovered in the genomes of RNA viruses and have since been found in other viruses, as well as in a subset of cellular mRNA that appears to take part in development and cell stress. The molecular mechanism by which IRES function to initiate protein synthesis remains to be determined.

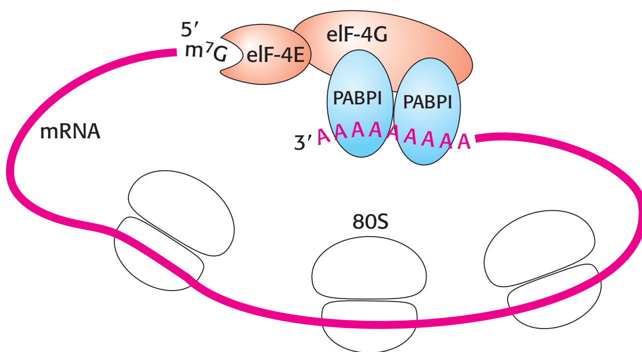


Figure 30.28 Protein interactions circularize eukaryotic mRNA. [After H. Lodish et al., *Molecular Cell Biology,* 5th ed. (W. H. Freeman and Company, 2004), Fig. 4.31.]

4. *The Structure of mRNA.* The 5′-cap bearing eukaryotic mRNA is circular. The eIF-4E protein that binds to the mRNA cap structure also binds to the poly(A) tail through two protein intermediaries. The eIF-4E that is already bound to the cap then binds to the eIF-4G protein, which in turn binds to a protein associated with the poly(A) tail, the poly(A)-binding protein (PABPI; Figure 30.28). Cap and tail are thus brought together to form a circle of mRNA. The circular structure may facilitate the rebinding of the ribosomes following protein-synthesis termination. Regulation of the activity of eIF-4G is a key control point under normal and pathological conditions. Altered function of eIF-4G has been implicated in fragile-X syndrome, the most common form of inherited mental impairment, as well as prostate and other cancers.

5. *Elongation and Termination.* Eukaryotic elongation factors EF1α and EF1βγ are the counterparts of prokaryotic EF-Tu and EF-Ts. The GTP form of EF1α delivers aminoacyl-tRNA to the A site of the ribosome, and EF1βγ catalyzes the exchange of GTP for bound GDP. Eukaryotic EF2 mediates GTP-driven translocation in much the same way as does prokaryotic EF-G. Termination in eukaryotes is carried out by a single release factor, eRF1, compared with two in prokaryotes. Finally, eIF-3, like its prokaryotic counterpart IF3, prevents the reassociation of ribosomal subunits in the absence of an initiation complex.

6. *Organization.* The components of the translation machinery in higher eukaryotes are organized into large complexes associated with the cytoskeleton. This association is believed to facilitate the efficiency of protein synthesis. Recall that organization of elaborate biochemical processes into physical complexes is a reoccurring theme in biochemistry (Section 18.5 and Section 25.2).

Mutations in initiation factor 2 cause a curious pathological condition

Defective eIF4-G is not the only initiation factor that results in pathological conditions. Mutations in eukaryotic initiation factor 2

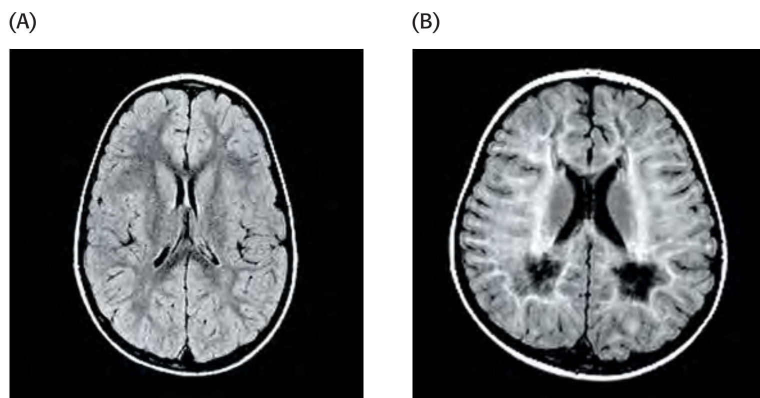


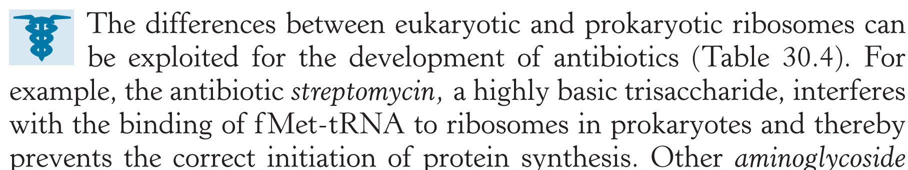
Figure 30.29 The effects of vanishing white matter disease. (A) In the normal brain, magnetic resonance imaging (MRI) visualizes the white matter as dark gray. (B) In the diseased brain, this MRI reveals that white matter is replaced by cerebrospinal fluid, seen as white. [(A) Courtesy of Marjo S. van der Knaap, M.D., Ph.D., VU University Medical Center, The Netherlands. (B) M. S. van der knapp et al., *Lancet Neurology* 5: 413–423.]

result in a mysterious disease, called *vanishing white matter* (VWM) *disease,* in which nerve cells in the brain disappear and are replaced by cerebrospinal fluid (Figure 30.29). The white matter of the brain consists predominately of nerve axons that connect the gray matter of the brain to the rest of the body. Death, resulting from fever or extended coma, can be anywhere from a few years to decades after the onset of the disease, which is usually in young children but can be shortly after birth or even in adulthood. An especially puzzling aspect of the disease is its tissue specificity. A mutation in a biochemical process as fundamental to life as protein-synthesis initiation would be predicted to be lethal or at least to affect all tissues of the body. Diseases such as VWM graphically show that, although much progress has been made in biochemistry, much more research will be required to understand the complexities of health and disease.

39.5 A Variety of Antibiotics and Toxins Can Inhibit Protein Synthesis

Many chemicals that inhibit various aspects of protein synthesis have been identified. These chemicals are powerful experimental tools and clinically useful drugs.

Some antibiotics inhibit protein synthesis



The differences between eukaryotic and prokaryotic ribosomes can be exploited for the development of antibiotics (Table 30.4). For example, the antibiotic *streptomycin,* a highly basic trisaccharide, interferes with the binding of fMet-tRNA to ribosomes in prokaryotes and thereby prevents the correct initiation of protein synthesis. Other *aminoglycoside*

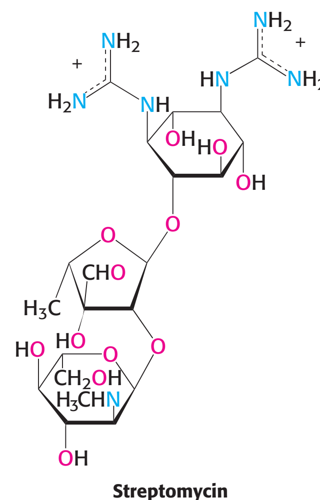


Table 30.4 Antibiotic inhibitors of protein synthesis

Antibiotic	Action
Streptomycin and other aminoglycosides	Inhibit initiation and cause the misreading of mRNA (prokaryotes)
Tetracycline	Binds to the 30S subunit and inhibits the binding of aminoacyl-tRNAs (prokaryotes)
Chloramphenicol	Inhibits the peptidyl transferase activity of the 50S ribosomal subunit (prokaryotes)
Cycloheximide	Inhibits translocation (eukaryotes)
Erythromycin	Binds to the 50S subunit and inhibits translocation (prokaryotes)
Puromycin	Causes premature chain termination by acting as an analog of aminoacyl-tRNA (prokaryotes and eukaryotes)

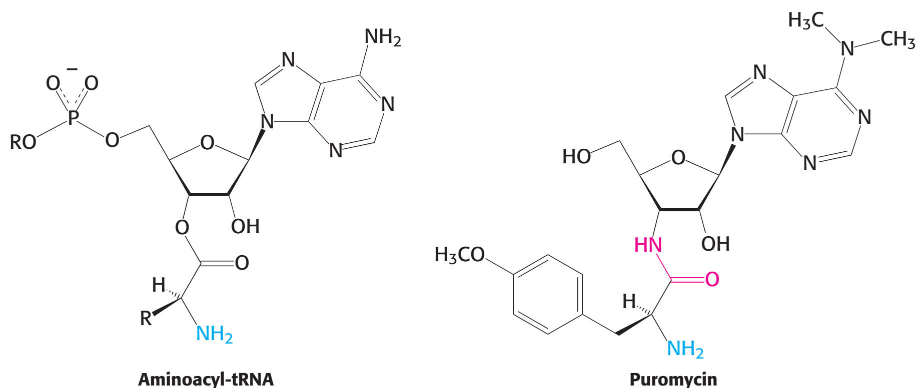


Figure 30.30 Antibiotic action of puromycin. Puromycin resembles the aminoacyl terminus of an aminoacyl-tRNA. Its amino group joins the carboxyl group of the growing polypeptide chain to form an adduct that dissociates from the ribosome. This adduct is stable because puromycin has an amide (shown in red) rather than an ester linkage.

antibiotics such as neomycin, kanamycin, and gentamycin interfere with the interaction between tRNA and the 16S rRNA of the 30S subunit (p. 892) of prokaryotic ribosomes. *Chloramphenicol* acts by inhibiting peptidyl transferase activity. *Erythromycin* binds to the 50S subunit and blocks translocation.

The antibiotic *puromycin* inhibits protein synthesis in both prokaryotes and eukaryotes by causing nascent polypeptide chains to be released before their synthesis is completed. Puromycin is an analog of the terminal part of aminoacyl-tRNA (Figure 30.30). It binds to the A site on the ribosome and inhibits the entry of aminoacyl-tRNA. Furthermore, puromycin contains an α-amino group. This amino group, like the one on aminoacyl-tRNA, forms a peptide bond with the carboxyl group of the growing peptide chain. The product, a peptide having a covalently attached puromycin residue at its carboxyl end, dissociates from the ribosome. No longer used medicinally, puromycin is utilized mainly as an experimental tool for the investigation of protein synthesis. *Cycloheximide,* another antibiotic, blocks translocation in eukaryotic ribosomes, making a useful laboratory tool for blocking protein synthesis in eukaryotic cells.

Diphtheria toxin blocks protein synthesis in eukaryotes by inhibiting translocation

Many antibiotics, harvested from prokaryotes for medicinal purposes, are inhibitors of prokaryotic protein synthesis. However, some prokaryotes produce protein-synthesis inhibitors that inhibit eukaryotic protein synthesis, leading to diseases such as diphtheria, which was a major cause of death in childhood before the advent of effective immunization. Symptoms include painful sore throat, hoarseness, fever, and difficulty breathing. The lethal effects of this disease are due mainly to a protein toxin produced by *Corynebacterium diphtheriae,* a bacterium that grows in the upper respiratory tract of an infected person. A few micrograms of diphtheria toxin is usually lethal in an unimmunized person because it inhibits protein synthesis. Shortly after entering a target cell, the toxin is cleaved into a 21-kd A fragment and a 40-kd B fragment. The A fragment of the toxin catalyzes the covalent modification of elongation factor 2, the elongation factor catalyzing translocation in eukaryotic protein synthesis, whereas the B fragment enables the A fragment to enter the cytoplasm of its target cell.

A single A fragment of the toxin in the cytoplasm can kill a cell. Why is it so lethal? EF2 contains *diphthamide,* an unusual amino acid residue of unknown function that is formed by the posttranslational modification of histidine. The A fragment of the diphtheria toxin catalyzes the transfer of the ADP ribose unit of NAD^+ to the diphthamide ring (Figure 30.31). *This ADP*

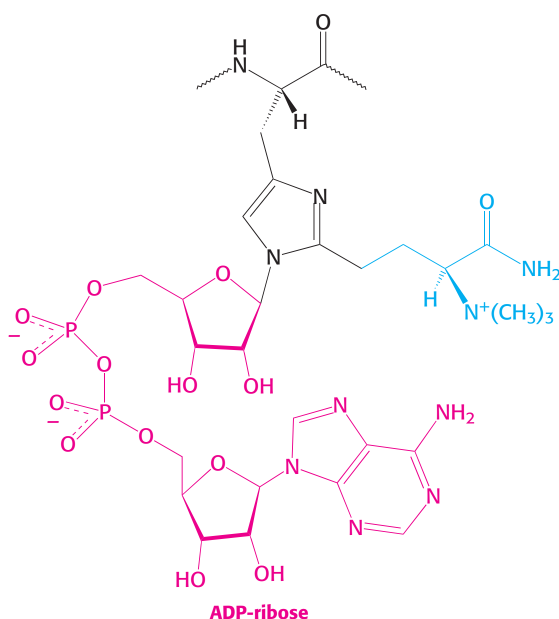


Figure 30.31 Blocking of translocation by diphtheria toxin. Diphtheria toxin blocks protein synthesis in eukaryotes by catalyzing the transfer of an ADP-ribose unit from NAD^+ to diphthamide, a modified amino acid residue in elongation factor 2 (translocase). Diphthamide is formed by the posttranslational modification (blue) of a histidine residue.

ribosylation of a single side chain of EF2 blocks EF2's capacity to carry out the translocation of the growing polypeptide chain. Protein synthesis ceases, accounting for the remarkable toxicity of diphtheria toxin.

Ricin fatally modifies 28S ribosomal RNA

Ricin is a biomolecule frequently in the news because of its potential use as a bioterrorism agent. Ricin is a small protein (65 kd) found in the seeds of the castor oil plant, *Ricinus communis* (Figure 30.32). It is indeed a deadly molecule because as little as 500 μg is lethal for an adult human being, and a single molecule can inhibit all protein synthesis in a cell, resulting in cell death.

Ricin is a heterodimeric protein composed of a catalytic A chain joined by a single disulfide bond to a B chain. The B chain allows the toxin to bind to the target cell, and this binding leads to an endocytotic uptake of the dimer and the eventual release of the A chain into the cytoplasm. The A chain cleaves adenine from a particular adenosine nucleotide on the 28S rRNA. Removal of the adenine base completely inactivates the ribosome by preventing the binding of elongation factors. Thus, ricin and diphtheria toxin both act by inhibiting protein-synthesis elongation; ricin does so by covalently modifying rRNA, and diphtheria toxin does so by covalently modifying the elongation factor.

Figure 30.32 Castor beans. The seeds of castor beans from *Ricinus communis* are a rich source of oils with a wide variety of uses, including the production of biodiesel fuels. The seeds are also rich in the toxin ricin. [Ted Kinsman/Photo Researchers.]

30.6 Ribosomes Bound to the Endoplasmic Reticulum Manufacture Secretory and Membrane Proteins

A newly synthesized protein in *E. coli* can stay in the cytoplasm or it can be sent to the plasma membrane, the outer membrane, the space between them, or the extracellular medium. Eukaryotic cells can direct proteins to internal sites such as lysosomes, mitochondria, chloroplasts, and the nucleus. How is sorting accomplished? In eukaryotes, a key choice is made soon after the synthesis of a protein begins. The ultimate destination of a protein depends broadly on the location of the ribosome on which it is being synthesized.

In eukaryotic cells, a ribosome remains free in the cytoplasm unless it is directed to the *endoplasmic reticulum* (ER), the extensive membrane system that comprises about half the total membrane of a cell. The region that binds ribosomes is called the *rough ER* because of its studded appearance, in contrast with the *smooth ER,* which is devoid of ribosomes (Figure 30.33). Free ribosomes synthesize proteins that remain within the cell, either within the cytoplasm or directed to organelles bounded by a double membrane, such as the nucleus, mitochondria, and chloroplasts. Ribosomes bound to the ER usually synthesize proteins destined to leave the cell or to at least contact the cell exterior from a position in the cell membrane. These proteins fall into three major classes: *secretory proteins* (proteins exported by the cell), *lysosomal proteins,* and *proteins spanning the plasma membrane.* Virtually all integral membrane proteins of the cell, except those located in the membranes of mitochondria and chloroplasts, are formed by ribosomes bound to the ER.

A variety of strategies are used to send proteins synthesized by free ribosomes to the nucleus, peroxisomes, mitochondria, and chloroplasts of eukaryotic cells. However, in this section, we will focus on the targeting of proteins produced by ribosomes bound to the endoplasmic reticulum.

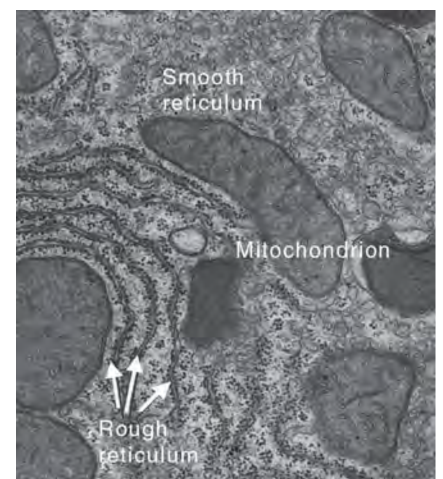


Figure 30.33 Ribosomes are bound to the endoplasmic reticulum. In this electron micrograph, ribosomes appear as small black dots binding to the cytoplasmic side of the endoplasmic reticulum to give a rough appearance. In contrast, the smooth endoplasmic reticulum is devoid of ribosomes. [From G. K. Voletz, M. M. Rolls, and T. A. Rapoport, *EMBO Rep.* 3:944–950, 2002.]

Signal sequences mark proteins for translocation across the endoplasmic reticulum membrane

The synthesis of proteins destined to leave the cell or become embedded in the plasma membrane begins on a free ribosome but, shortly after synthesis

Protein	Signal sequence	Cleavage site
Human growth hormone	MATGSRTSLLLAFGLLCLPWLQEGSA	FPT
Human proinsulin	MALWMRLLPLLALLALWGPDPAAA	FVN
Bovine proalbumin	MKWVTFISLLLLFSSAYS	RGV
Mouse antibody H chain	MKVLSLLYLLTAIPHIMS	DVQ
Chicken lysozyme	MRSLLILVLCFLPKLAALG	KVF
Bee promellitin	MKFLVNVALVFMVVYISYIYA	APE
Drosophila glue protein	MKLLVVAVIACMLIGFADPASG	CKD
Zea maize protein 19	MAAKIFCLIMLLGLSASAATA	SIF
Yeast invertase	MLLOAFLFLLAGFAAKISA	SMT
Human influenza virus A	MKAKLLVLLYAFVAG	DQI

Figure 30.34 Amino-terminal signal sequences of some eukaryotic secretory and plasma-membrane proteins. The hydrophobic core (yellow) is preceded by basic residues (blue) and followed by a cleavage site (red) for signal peptidase.

begins, it is halted until the ribosome is directed to the cytoplasmic side of the endoplasmic reticulum. When the ribosome docks with the ER membrane, protein synthesis begins again. As the newly forming peptide chain exits the ribosome, it is transported, cotranslationally, through the membrane into the lumen of the endoplasmic reticulum.

Free ribosomes that are synthesizing proteins for use in the cell are identical with those attached to the ER. What is the process that directs the ribosome synthesizing a protein destined to enter the ER to bind to the ER? The translocation consists of four components.

1. *The Signal Sequence.* The signal sequence is *a sequence of 9 to 12 hydrophobic amino acid residues, sometimes containing positively charged amino acids* (Figure 30.34). This sequence is usually near the amino terminus of the nascent polypeptide chain. The presence of the signal sequence identifies the nascent peptide as one that must cross the ER membrane. Some signal sequences are maintained in the mature protein, whereas others are cleaved by a *signal peptidase* on the lumenal side of the ER membrane (see Figure 30.36).

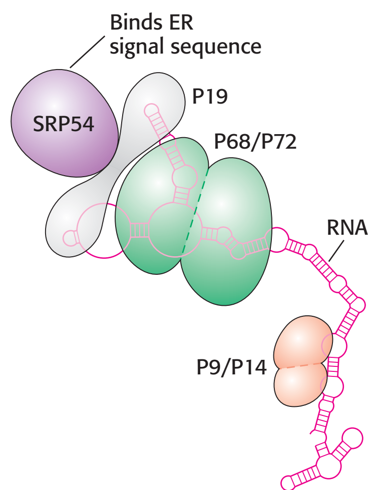


Figure 30.35 The signal-recognition particle. The signal-recognition particle (SRP) consists of six proteins (one of which is SRP54) and one 300-nucleotide RNA molecule. The RNA has a complex structure with many double-helical stretches punctuated by single-stranded regions, shown as circles. [After H. Lodish et al., *Molecular Cell Biology,* 5th ed. (W. H. Freeman and Company, 2004). See K. Strub et al., *Mol. Cell Biol.* 11:3949–3959, 1991, and S. High and B. Dobberstein, *J. Cell Biol.* 113:229–233, 1991.]

2. *The Signal-Recognition Particle (SRP).* The signal-recognition particle recognizes the signal sequence and binds the sequence and the ribosome as soon as the signal sequence exits the ribosome. SRP then shepherds the ribosome and its nascent polypeptide chain to the ER membrane. SRP is a ribonucleoprotein consisting of a 7S RNA and six different proteins (Figure 30.35). One protein, SRP54, is a GTPase that is crucial for SRP function. SRP binds all ribosomes but binds tightly only to ribosomes that display the signal sequence. SRP samples ribosomes until it locates one exhibiting a signal sequence. After SRP is bound to the signal sequence, interactions between the ribosome and the SRP occlude the elongation-factor-binding site, thereby halting protein synthesis.

3. *The SRP Receptor (SR).* The SRP–ribosome complex diffuses to the endoplasmic reticulum, where SRP binds the SRP receptor, an integral membrane protein consisting of two subunits, SRα and SRβ. SRα is, like SRP54, a GTPase.

4. *The Translocon.* The SRP–SR complex delivers the ribosome to the ER membrane. There it docks with the translocation machinery, called the *translocon,* a multisubunit assembly of integral and peripheral membrane proteins. The translocon is a protein-conducting channel. This channel

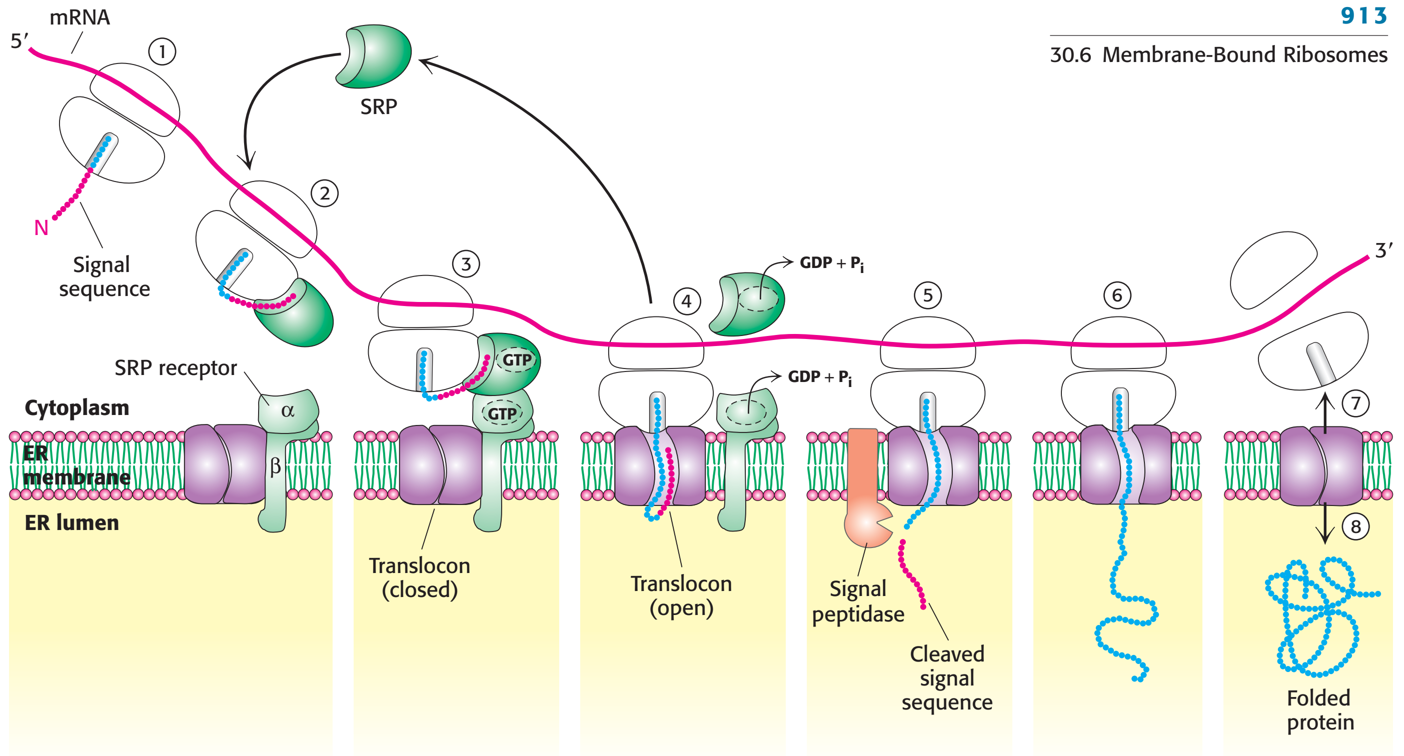


Figure 30.36 The SRP targeting cycle. (1) Protein synthesis begins on free ribosomes. (2) After the signal sequence has exited the ribosome, it is bound by the SRP, and protein synthesis halts. (3) The SRP–ribosome complex docks with the SRP receptor in the ER membrane. (4) The SRP and the SRP receptor simultaneously hydrolyze bound GTPs. Protein synthesis resumes and the SRP is free to bind another signal sequence. (5) The signal peptidase may remove the signal sequence as it enters the lumen of the ER. (6) Protein synthesis continues as the protein is synthesized directly into the ER. (7) On completion of protein synthesis, the ribosome is released. (8) The protein tunnel in the translocon closes. [After H. Lodish et al., *Molecular Cell Biology,* 5th ed. (W. H. Freeman and Company, 2004), Fig. 16.6.]

opens when the translocon and ribosome bind to each other. Protein synthesis resumes with the growing polypeptide chain passing through the translocon channel into the lumen of the ER.

The interactions of the components of the translocation machinery are shown in Figure 30.36. For the SRP–SR complex to form, both the SRP54 and the SRα subunits of SR must bind GTP. For the SRP–SR complex to then deliver the ribosome to the translocon, the two GTP molecules—one in SRP and the other in SR—are aligned in what is essentially an active site shared by the two proteins. After the ribosome has been passed along to the translocon, the GTPs are hydrolyzed, SRP and SR dissociate, and SRP is free to search for another signal sequence to begin the cycle anew. Thus, SRP acts catalytically. The signal peptidase, which is associated with the translocon in the lumen of the ER, removes the signal sequence from most proteins.

Transport vesicles carry cargo proteins to their final destination

As the proteins are synthesized, they fold to form their three-dimensional structures in the lumen of the ER. Some proteins are modified by the attachment of N-linked carbohydrates (Section 11.3). Finally, the proteins must be sorted and transported to their final destinations. Regardless of the destination, the principles of transport are the same. Transport is mediated by *transport vesicles* that bud off the endoplasmic reticulum (Figure 30.37). Transport vesicles from the ER carry their cargo (the proteins) to the Golgi complex, where the vesicles fuse and deposit the cargo inside the complex.

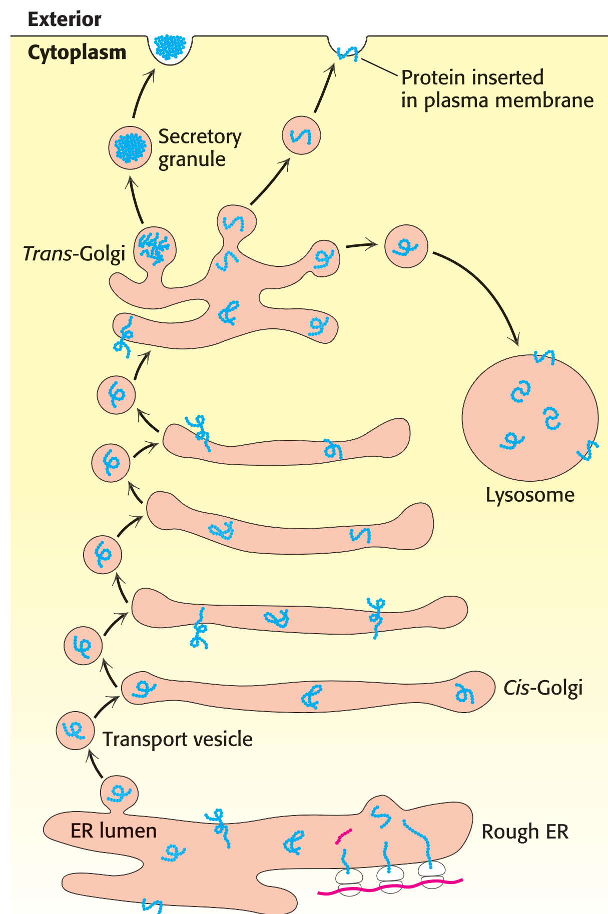


Figure 30.37 Protein-sorting pathways. Newly synthesized proteins in the lumen of the ER are collected into membrane buds. These buds pinch off to form transport vesicles. The transport vesicles carry the cargo proteins to the Golgi complex where the cargo proteins are modified. Transport vesicles then carry the cargo to the final destination as directed by the v-SNARE and t-SNARE proteins.

There the cargo proteins are modified—for instance, by the attachment of carbohydrates. From the Golgi complex, transport vesicles carry the cargo proteins to their final destinations, as shown in Figure 30.37.

How does a protein end up at the correct destination? A newly synthesized protein will float inside the ER lumen until it binds to an integral membrane protein called a *cargo receptor*. This binding sequesters the cargo protein into a small region of the membrane that can subsequently form a membrane bud. The bud will carry the protein to a specific destination—plasma membrane, lysosome, or cell exterior. The key to ensuring that the protein reaches the proper destination is that the protein must bind to a receptor in the ER region associated with the protein's destination. To ensure the proper match of protein with ER region, cargo receptors recognize various characteristics of the cargo protein, such as a particular amino acid sequence or an added carbohydrate.

The formation of buds is facilitated by the binding of *coat proteins* (COPs) to the cytoplasmic side of the bud. The coat proteins associate with one another to pinch off the vesicle. After the transport vesicle has formed and is released, the coat proteins are shed to reveal another integral protein called *v-SNARE* ("v" for *v*esicle). v-SNARE will bind to a particular *t-SNARE* ("t" for *t*arget) in the target membrane. This binding leads to the fusion of the transport vesicle to the target membrane, and the cargo is delivered. Thus, the assignment of identical v-SNARE proteins to the same region of the ER membrane causes an ER region to be associated with a particular destination.

Summary

30.1 Protein Synthesis Requires the Translation of Nucleotide Sequences into Amino Acid Sequences

Protein synthesis is called translation because information present as a nucleic acid sequence is translated into a different language, the sequence of amino acids in a protein. This complex process is mediated by the coordinated interplay of more than a hundred macromolecules, including mRNA, rRNAs, tRNAs, aminoacyl-tRNA synthetases, and protein factors. Given that a protein typically comprises from 100 to 1000 amino acids, the frequency at which an incorrect amino acid is incorporated in the course of protein synthesis must be less than 10^{-4}. Transfer RNAs are the adaptors that make the link between a nucleic acid and an amino acid. These molecules, single chains of about 80 nucleotides, have an L-shaped structure.

30.2 Aminoacyl Transfer RNA Synthetases Read the Genetic Code

Each amino acid is activated and linked to a specific transfer RNA by an enzyme called an aminoacyl-tRNA synthetase. Such an enzyme links the carboxyl group of an amino acid to the 2′- or 3′-hydroxyl group of the adenosine unit of a CCA sequence at the 3′ end of the tRNA by an ester linkage. There is at least one specific aminoacyl-tRNA synthetase and at least one specific tRNA for each amino acid. A synthetase utilizes both the functional groups and the shape of its cognate amino acid to prevent the attachment of an incorrect amino acid to a tRNA. Some synthetases have a separate active site at which incorrectly linked amino acids are removed by hydrolysis. A synthetase recognizes the anticodon, the acceptor stem, and sometimes other parts of its tRNA substrate. By specifically recognizing both amino acids and tRNAs, aminoacyl-tRNA synthetases implement the instruction of the genetic code.

The codons of messenger RNA recognize the anticodons of transfer RNAs rather than the amino acids attached to the tRNAs. A codon on mRNA forms base pairs with the anticodon of the tRNA. Some tRNAs are recognized by more than one codon because pairing of the third base of a codon is less crucial than that of the other two (the wobble mechanism). There exist two evolutionary distinct classes of synthetases, each recognizing 10 amino acids. The two classes recognize opposite faces of tRNA molecules.

30.3 The Ribosome Is the Site of Protein Synthesis

Protein synthesis takes place on ribosomes—ribonucleoprotein particles (about two-thirds RNA and one-third protein) consisting of large and small subunits. In *E. coli,* the 70S ribosome (2500 kd) is made up of 30S and 50S subunits. The 30S subunit consists of 16S ribosomal RNA and 21 different proteins; the 50S subunit consists of 23S and 5S rRNA and 34 different proteins. The ribosome includes three sites for tRNA binding called the A (aminoacyl) site, the P (peptidyl) site, and the E (exit) site.

Protein synthesis takes place in three phases: initiation, elongation, and termination. In prokaryotes, mRNA, formylmethionyl-$tRNA_f$ (the special initiator tRNA that recognizes AUG), and a 30S ribosomal subunit come together with the assistance of initiation factors to form a 30S initiation complex. A 50S ribosomal subunit then joins this complex to form a 70S initiation complex, in which fMet-$tRNA_f$ occupies the P site of the ribosome.

Elongation factor Tu delivers the appropriate aminoacyl-tRNA to the ribosome's A (aminoacyl) site as an EF-Tu–aminoacyl-tRNA–GTP ternary complex. EF-Tu serves both to protect the aminoacyl-tRNA from premature cleavage and to increase the fidelity of protein synthesis by ensuring that the correct anticodon–codon pairing has taken place before hydrolyzing GTP and releasing aminoacyl-tRNA into the A site. A peptide bond is formed when the amino group of the aminoacyl-tRNA nucleophilically attacks the ester linkage of the peptidyl-tRNA. On peptide-bond formation, the tRNAs and mRNA must be translocated for the next cycle to begin. The deacylated tRNA moves to the E site and then leaves the ribosome, and the peptidyl-tRNA moves from the A site into the P site. Elongation factor G uses the free energy of GTP hydrolysis to drive translocation. Protein synthesis is terminated by release factors, which recognize the termination codons UAA, UGA, and UAG and cause the hydrolysis of the ester bond between the polypeptide and tRNA.

30.4 Eukaryotic Protein Synthesis Differs from Prokaryotic Protein Synthesis Primarily in Translation Initiation

The basic plan of protein synthesis in eukaryotes is similar to that in prokaryotes, but there are some significant differences between them. Eukaryotic ribosomes (80S) consist of a 40S small subunit and a 60S large subunit. The initiating amino acid is again methionine, but it is not formylated. The initiation of protein synthesis is more complex in eukaryotes than in prokaryotes. In eukaryotes, the AUG closest to the 5′ end of mRNA is nearly always the start site. The 40S ribosome finds this site by binding to the 5′ cap and then scanning the RNA until AUG is reached. The regulation of translation in eukaryotes provides a means for regulating gene expression.

30.5 A Variety of Antibiotics and Toxins Can Inhibit Protein Synthesis

Many clinically important antibiotics function by inhibiting protein synthesis. All steps of protein synthesis are susceptible to inhibition by one antibiotic or another. Diphtheria toxin inhibits protein synthesis by covalently modifying an elongation factor, thereby preventing elongation. Ricin, a toxin from castor beans, inhibits elongation by removing a crucial adenine residue from rRNA.

30.6 Ribosomes Bound to the Endoplasmic Reticulum Manufacture Secretory and Membrane Proteins

Proteins contain signals that determine their ultimate destination. The synthesis of all proteins begins on free ribosomes in the cytoplasm. In eukaryotes, protein synthesis continues in the cytoplasm unless the nascent chain contains a signal sequence that directs the ribosome to the endoplasmic reticulum. Amino-terminal signal sequences consist of a hydrophobic segment of 9 to 12 residues preceded by a positively charged amino acid. Signal-recognition particle, a ribonucleoprotein assembly, recognizes signal sequences and brings ribosomes bearing them to the ER. A GTP–GDP cycle releases the signal sequence from SRP and then detaches SRP from its receptor. The nascent chain is then translocated across the ER membrane. Proteins are transported throughout the cell in transport vesicles.

Key Terms

translation (p. 887)
ribosome (p. 887)
aminoacyl-tRNA synthetase (p. 888)
codon (p. 889)
transfer RNA (tRNA) (p. 889)
anticodon (p. 889)

wobble hypothesis (p. 892)
30S subunit (p. 897)
50S subunit (p. 897)
Shine–Dalgarno sequence (p. 901)
initiation factor (p. 902)
elongation factor Tu (EF-Tu) (p. 902)
elongation factor Ts (EF-Ts) (p. 903)
peptidyl transferase center (p. 903)
elongation factor G (EF-G) (translocase) (p. 904)
polysome (p. 906)
release factor (RF) (p. 906)
signal sequence (p. 912)
signal peptidase (p. 912)
signal-recognition particle (SRP) (p. 912)
SRP receptor (SR) (p. 912)
translocon (p. 912)
transport vesicle (p. 913)
coat proteins (p. 914)
v-SNARE (p. 914)
t-SNARE (p. 914)

Problems

1. *Babel fish*. Why is protein synthesis also called translation?

2. *Careful, but not too careful*. Why is it crucial that protein synthesis has an error frequency of 10^{-4}?

3. *Commonalities*. What features are common to all tRNA molecules?

4. *The ol' two step*. What two reaction steps are required for the formation of an aminoacyl-tRNA?

5. *The same but different*. Why must tRNA molecules have both unique structural features and common structural features?

6. *Charge it*. In the context of protein synthesis, what is meant by an activated amino acid?

7. *Synthetase mechanism*. The formation of isoleucyl-tRNA proceeds through the reversible formation of an enzyme-bound Ile-AMP intermediate. Predict whether ^{32}P-labeled ATP is formed from $^{32}PP_i$ when each of the following sets of components is incubated with the specific activating enzyme:

(a) ATP and $^{32}PP_i$

(b) tRNA, ATP, and $^{32}PP_i$

(c) Isoleucine, ATP, and $^{32}PP_i$

8. *1 = 2, for sufficiently large values of 1*. The energetic equivalent of two molecules of ATP is used to activate an amino acid, yet only one molecule of ATP is used. Explain.

9. *Sieves*. Using threonyl-tRNA synthetase as an example, account for the specificity of threonyl-tRNA formation.

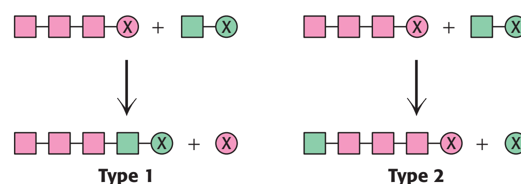
10. *Use all available information*. Suggest a reason why there are two classes of aminoacyl-tRNA synthetases, with each class recognizing a different face of the tRNA.

11. *Going wobbly*. Explain how it is possible that some tRNA molecules recognize more than one codon.

12. *Light and heavy ribosomes*. Ribosomes were isolated from bacteria grown in a "heavy" medium (^{13}C and ^{15}N) and from bacteria grown in a "light" medium (^{12}C and ^{14}N). These 60S ribosomes were added to an in vitro system engaged in protein synthesis. An aliquot removed several hours later was analyzed by density-gradient centrifugation. How many bands of 70S ribosomes would you expect to see in the density gradient?

13. *The price of protein synthesis*. What is the smallest number of molecules of ATP and GTP consumed in the synthesis of a 200-residue protein, starting from amino acids? Assume that the hydrolysis of PP_i is equivalent to the hydrolysis of ATP for this calculation.

14. *Contrasting modes of elongation*. The two basic mechanisms for the elongation of biomolecules are represented in the adjoining illustration. In type 1, the activating group (X) is released from the growing chain. In type 2, the activating group is released from the incoming unit as it is added to the growing chain. Indicate whether each of the following biosyntheses is by means of a type 1 or a type 2 mechanism:



(a) Glycogen synthesis

(b) Fatty acid synthesis

(c) $C_5 \longrightarrow C_{10} \longrightarrow C_{15}$ in cholesterol synthesis

(d) DNA synthesis

(e) RNA synthesis

(f) Protein synthesis

15. *Correct phasing*. What is meant by the phrase *reading frame?*

16. *Suppressing frameshifts*. The insertion of a base in a coding sequence leads to a shift in the reading frame, which in most cases produces a nonfunctional protein. Propose a mutation in a tRNA that might suppress frameshifting.

17. *Tagging a ribosomal site*. Design an affinity-labeling reagent for one of the tRNA-binding sites in *E. coli* ribosomes.

18. *Viral mutation.* An mRNA transcript of a T7 phage gene contains the base sequence

↓
5′–AACUGCACGAGGUAACACAAGAUGGCU–3′

Predict the effect of a mutation that changes the G marked by an arrow to A.

19. *A new translation.* A transfer RNA with a UGU anticodon is enzymatically conjugated to ^{14}C-labeled cysteine. The cysteine unit is then chemically modified to alanine. The altered aminoacyl-tRNA is added to a protein-synthesizing system containing normal components except for this tRNA. The mRNA added to this mixture contains the following sequence:

5′–UUUUGCCAUGUUUGUGCU–3′

What is the sequence of the corresponding radiolabeled peptide?

20. *Two synthetic modes.* Compare and contrast protein synthesis by ribosomes with protein synthesis by the solid-phase method (see Section 3.5).

21. *Enhancing fidelity.* Compare the accuracy of DNA replication, RNA synthesis, and protein synthesis. Which mechanisms are used to ensure the fidelity of each of these processes?

22. *Triggered GTP hydrolysis*. Ribosomes markedly accelerate the hydrolysis of GTP bound to the complex of EF-Tu and aminoacyl-tRNA. What is the biological significance of this enhancement of GTPase activity by ribosomes?

23. *Blocking translation*. Devise an experimental strategy for switching off the expression of a specific mRNA without changing the gene encoding the protein or the gene's control elements.

24. *Directional problem*. Suppose that you have a protein-synthesis system that is synthesizing a protein designated A. Furthermore, you know that protein A has four trypsin-sensitive sites, equally spaced in the protein, that, on digestion with trypsin, yield the peptides A_1, A_2, A_3, A_4, and A_5. Peptide A_1 is the amino-terminal peptide, and A_5 is the carboxyl-terminal peptide. Finally, you know that your system requires 4 minutes to synthesize a complete protein A. At $t = 0$, you add all 20 amino acids, each carrying a ^{14}C label.

(a) At $t = 1$ minute, you isolate intact protein A from the system, cleave it with trypsin, and isolate the five peptides. Which peptide is most heavily labeled?

(b) At $t = 3$ minutes, what will be the order of the labeling of peptides from greatest to least?

(c) What does this experiment tell you about the direction of protein synthesis?

25. *Translator*. Aminoacyl-tRNA synthetases are the only component of gene expression that decodes the genetic code. Explain.

26. *A timing device*. EF-Tu, a member of the G-protein family, plays a crucial role in the elongation process of translation. Suppose that a slowly hydrolyzable analog of GTP were added to an elongating system. What would be the effect on the rate of protein synthesis?

27. *Not just RNA*. What are the roles of the protein factors required for protein synthesis?

28. *Membrane transport*. What four components are required for the translocation of proteins across the endoplasmic reticulum membrane?

29. *Push. Don't pull*. What is the energy source that powers the cotranslational movement of proteins across the endoplasmic reticulum?

30. *You have to know where to look*. Prokaryotic messenger RNAs usually contain many AUG codons. How does the ribosome identify the AUG specifying initiation?

31. *Fundamentally the same, yet . . .* List the differences between prokaryotic and eukaryotic protein synthesis.

32. *Like a border collie*. What is the role of the signal-recognition particle in protein translocation?

33. *An assembly line*. Why is the fact that protein synthesis takes place on polysomes advantageous?

34. *Match 'em*

(a) Initiation	1. GTP
(b) Elongation	2. AUG
(c) Termination	3. fMet
	4. RRF
	5. IF2
	6. Shine–Dalgarno
	7. EF-Tu
	8. Peptidyl transferase
	9. UGA
	10. Transformylase

35. *Wasted effort?* Transfer RNA molecules are quite large, given that the anticodon consists of only three nucleotides. What is the purpose of the rest of the tRNA molecule?

Mechanism Problems

36. *Molecular attack*. What is the nucleophile in the reaction catalyzed by peptidyl transferase? Suggest a plausible mechanism for this reaction.

37. *Evolutionary amino acid choice*. Ornithine is structurally similar to lysine except ornithine's side chain is one methylene group shorter than that of lysine. Attempts to chemically synthesize and isolate ornithinyl-tRNA proved unsuccessful. Propose a mechanistic explanation. (Hint: Six-membered rings are more stable than seven-membered rings.)

Chapter Integration Problems

38. *Déjà vu*. Which protein in G-protein cascades plays a role similar to that of elongation factor Ts?

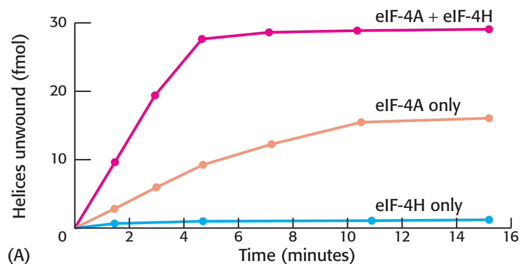
39. *Family resemblance*. Eukaryotic elongation factor 2 is inhibited by ADP ribosylation catalyzed by diphtheria toxin. What other G proteins are sensitive to this mode of inhibition?

40. *The exceptional E. coli*. In contrast with *E. coli*, most bacteria do not have a full complement of aminoacyl-tRNA synthetases. For instance, *Helicobacter pylori*, the cause of stomach ulcers, has $tRNA^{Gln}$, but no Gln-tRNA synthetase. However, glutamine is a common amino acid in *H. pylori* proteins. Suggest a means by which glutamine can be incorporated into proteins in *H. pylori*. (Hint: Glu-tRNA synthetase can misacylate $tRNA^{Gln}$.)

41. *The final step*. What aspect of primary structure allows the transfer of linear nucleic acid information into the functional three-dimensional structure of proteins?

Data Interpretation Problems

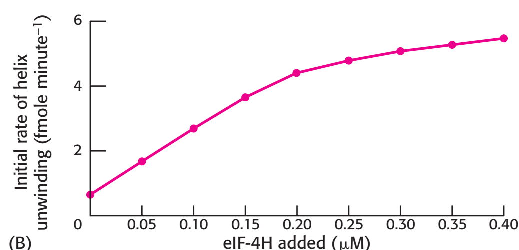
42. *Helicase helper*. The initiation factor eIF-4 displays ATP-dependent RNA helicase activity. Another initiation factor, eIF-4H, has been proposed to assist the action of eIF-4. Graph A shows some of the experimental results from an assay that can measure the activity of eIF-4 helicase in the presence of eIF-4H.



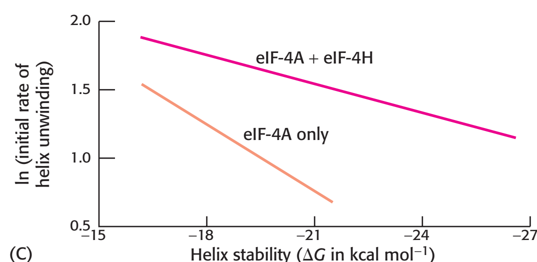
(a) What are the effects on eIF-4 helicase activity in the presence of eIF-4H?

(b) Why did measuring the helicase activity of eIF-4H alone serve as an important control?

(c) The initial rate of helicase activity of 0.2 μM of eIF-4 was then measured with varying amounts of eIF-4H (graph B). What ratio of eIF-4H to eIF-4 yielded optimal activity?



(d) Next, the effect of RNA–RNA helix stability on the initial rate of unwinding in the presence and absence of eIF-4H was tested (graph C). How does the effect of eIF-4H vary with helix stability?

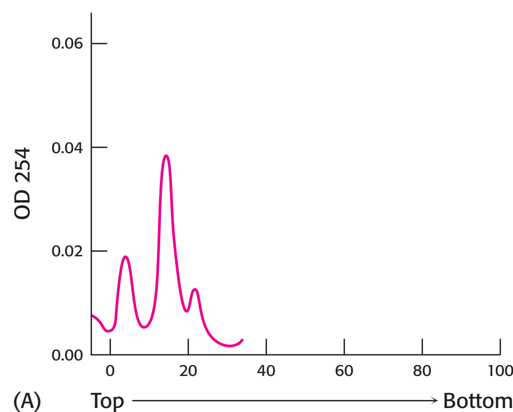


(e) How might eIF-4H affect the helicase activity of eIF-4A?

[Data after N. J. Richter, G. W. Rodgers, Jr., J. O. Hensold, and W. C. Merrick. Further biochemical and kinetic characterization of human eukaryotic initiation factor 4H. *J. Biol. Chem.* 274:35415–35424, 1999.]

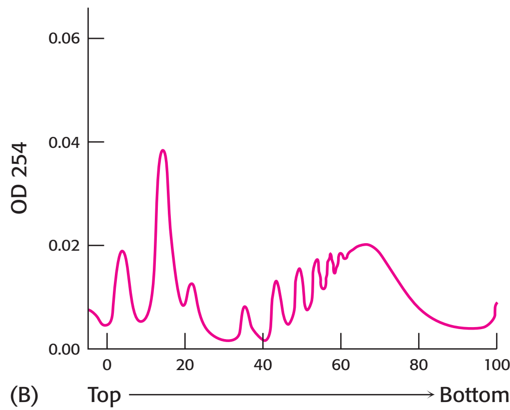
43. *Size separation*. The protein-synthesizing machinery was isolated from eukaryotic cells and briefly treated with a low concentration of RNase. The sample was then subjected to sucrose gradient centrifugation. The gradient was fractionated and the absorbance, or optical density (OD), at 254 nm was recorded for each fraction. The following plot was obtained.

(a) What do the three peaks of absorbance in graph A represent?

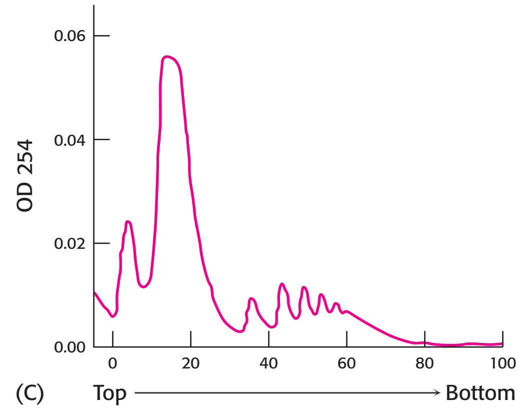


The experiment was repeated except that, this time, the RNase treatment was omitted.

(b) Why is the centrifugation pattern in graph B more complex? What do the series of peaks near the bottom of the centrifuge tube represent?



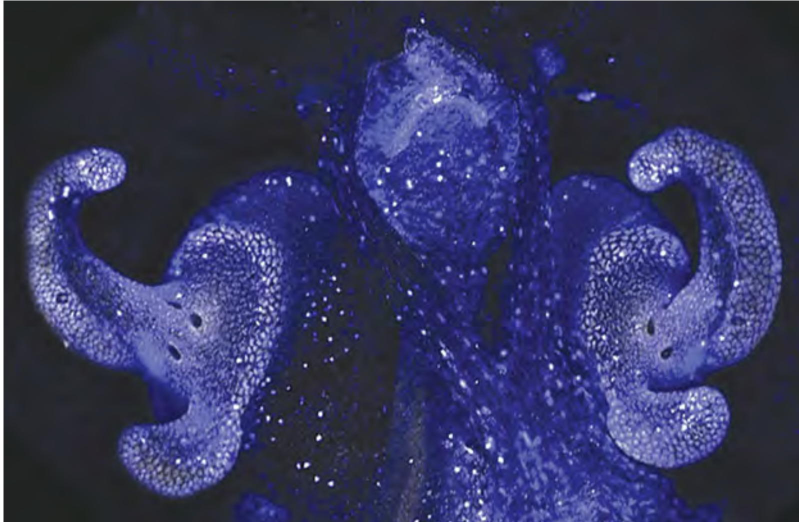
Before the isolation of the protein-synthesizing machinery, the cells were grown in low concentrations of oxygen (hypoxic conditions). Again the experiment was repeated without RNase treatment (graph C).

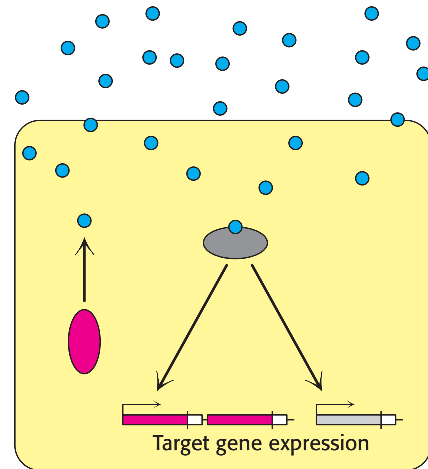


(c) What is the effect of growing cells under hypoxic conditions?

[Data after M. Koritzinsky et al. *EMBO J.* 25:1114–1125, 2006.]

The Control of Gene Expression in Prokaryotes





Bacteria respond to changes in their environments. A micrograph of the light organ of a newly hatched squid (*Euprymna scolopes*) is shown on the left. The light spots are due to colonies of the bacteria *Vibrio fischerili* that live symbiotically within these organs. These bacteria become luminescent when they reach an appropriately high density. The density is sensed by the circuit shown on the right in which each bacterium releases a small molecule into the environment. The molecule is subsequently taken up by other bacterial cells, which start a signaling cascade that stimulates the expression of specific genes. [S. V. Nyholm et al. *PNAS* 97(2000): 10231–10235. Copyright 2000 National Academy of Science.]

OUTLINE

31.1 Many DNA-Binding Proteins Recognize Specific DNA Sequences

31.2 Prokaryotic DNA-Binding Proteins Bind Specifically to Regulatory Sites in Operons

31.3 Regulatory Circuits Can Result in Switching Between Patterns of Gene Expression

31.4 Gene Expression Can Be Controlled at Posttranscriptional Levels

Even simple prokaryotic cells must respond to changes in their metabolism or in their environments. Much of this response takes place through changes in gene expression. A gene is *expressed* when it is transcribed into RNA and, for most genes, translated into proteins. Genomes comprise thousands of genes. Some of these genes are expressed all the time. These genes are subject to *constitutive expression*. Many other genes are expressed only under some circumstances—that is, under a particular set of physiological conditions. These genes are subject to *regulated expression*. For example, the level of expression of some genes in bacteria may vary more than a 1000-fold in response to the supply of nutrients or to environmental challenges.

In this chapter, we will examine gene-regulation mechanisms in prokaryotes, particularly *E. coli,* because many of these processes were first discovered in this organism. In Chapter 32, we will turn to gene-regulation mechanisms in eukaryotes. We shall see both substantial similarities and fundamental differences in comparing gene-regulatory mechanisms of the two types of organisms.

How is gene expression controlled? *Gene activity is controlled first and foremost at the level of transcription.* Whether a gene is transcribed is determined

largely by the interplay between specific DNA sequences and certain proteins that bind to these sequences. Most often, these proteins repress the expression of specific genes by blocking the access of RNA polymerase to their promotors. In some cases, however, the proteins can activate the expression of specific genes. We shall learn about several different strategies that allow the coordinated regulation of sets of genes. Some genes are also controlled at stages beyond the level of transcription and we shall examine several mechanisms at these stages. Finally, we will examine several important examples of how gene expression is regulated in response to changes in the concentrations of specific molecules in the environment of prokaryotic cells.

31.1 Many DNA-Binding Proteins Recognize Specific DNA Sequences

How do regulatory systems distinguish the genes that need to be activated or repressed from genes that are constitutive? After all, the DNA sequences of genes themselves do not have any distinguishing features that would allow regulatory systems to recognize them. Instead, gene regulation depends on other sequences in the genome. In prokaryotes, these regulatory sites are close to the region of the DNA that is transcribed. Regulatory sites are usually binding sites for specific DNA-binding proteins, which can stimulate or repress gene expression. These regulatory sites were first identified in *E. coli* in studies of changes in gene expression. In the presence of the sugar lactose, the bacterium starts to express a gene encoding β-galactosidase, an enzyme that can process lactose for use as a carbon and energy source. The sequence of the regulatory site for this gene is shown in Figure 31.1. The nucleotide sequence of this site shows a nearly perfect inverted repeat, indicating that the DNA in this region has an approximate twofold axis of symmetry. Recall that cleavage sites for restriction enzymes such as *Eco*RV have similar symmetry properties (Section 9.3). Symmetry in such regulatory sites usually corresponds to symmetry in the protein that binds the site. *Symmetry matching is a recurring theme in protein–DNA interactions.*

Figure 31.1 Sequence of the *lac* regulatory site. The nucleotide sequence of this regulatory site shows a nearly perfect inverted repeat, corresponding to twofold rotational symmetry in the DNA. Parts of the sequences that are related by this symmetry are shown in the same color.

To understand these protein–DNA interactions in detail, scientists examined the structure of the complex between an oligonucleotide that includes this site and the DNA-binding unit that recognizes it (Figure 31.2).

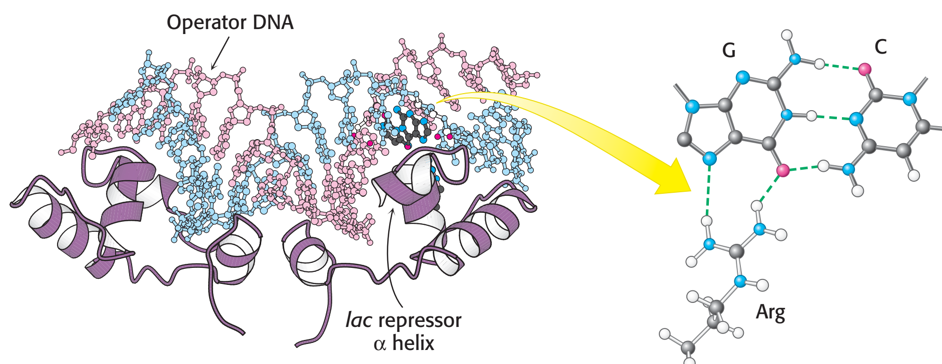


Figure 31.2 The *lac* repressor–DNA complex. The DNA-binding domain from a gene-regulatory protein, the *lac* repressor, binds to α DNA fragment containing its preferred binding site (referred to as operator DNA) by inserting an α helix into the major groove of operator DNA. *Notice* that a specific contact forms between an arginine residue of the repressor and a G–C base pair in the binding site. [Drawn from 1EFA.pdb.]

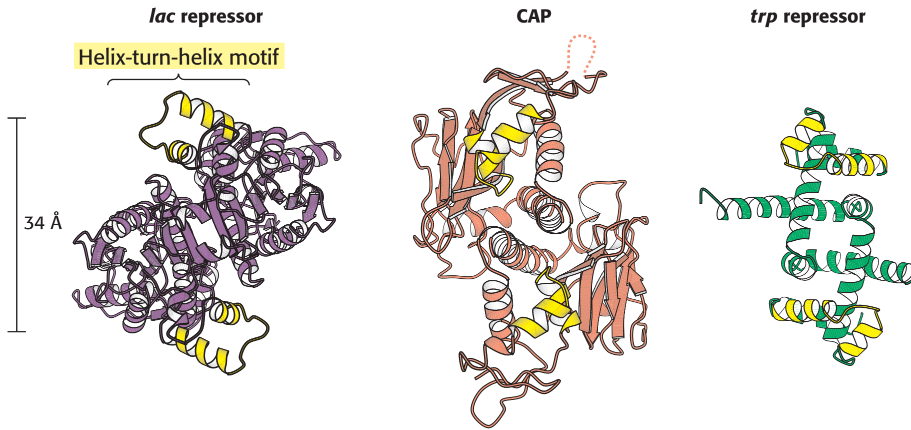


Figure 31.3 Helix-turn-helix motif. These structures show three sequence-specific DNA-binding proteins that interact with DNA through a helix-turn-helix motif (highlighted in yellow). *Notice* that, in each case, the helix-turn-helix units within a protein dimer are approximately 34 Å apart, corresponding to one full turn of DNA. [Drawn from 1EFA, 1RUN, and 1TRO.pdb.]

The DNA-binding unit comes from a protein called the *lac repressor,* which represses the expression of the lactose-processing gene. As expected, this DNA-binding unit binds as a dimer, and the twofold axis of symmetry of the dimer matches the symmetry of the DNA. An α helix from each monomer of the protein is inserted into the major groove of the DNA, where amino acid side chains make specific contacts with exposed edges of the base pairs. For example, the side chain of an arginine residue of the protein forms a pair of hydrogen bonds with a guanine residue of the DNA, which would not be possible with any other base. This interaction and similar ones allow the *lac* repressor to bind more tightly to this site than to the wide range of other sites present in the *E. coli* genome.

The helix-turn-helix motif is common to many prokaryotic DNA-binding proteins

Are similar strategies utilized by other prokaryotic DNA-binding proteins? The structures of many such proteins have now been determined, and amino acid sequences are known for many more. Strikingly, the DNA-binding surfaces of many, but not all, of these proteins consist of a pair of α helices separated by a tight turn (Figure 31.3). In complexes with DNA, the second of these two helices (often called the *recognition helix*) lies in the major groove, where amino acid side chains make contact with the edges of base pairs. In contrast, residues of the first helix participate primarily in contacts with the DNA backbone. *Helix-turn-helix motifs* are present on many proteins that bind DNA as dimers, and thus two of the units will be present, one on each monomer.

Although the helix-turn-helix motif is the most commonly observed DNA-binding unit in prokaryotes, not all regulatory proteins bind DNA through such units. A striking example is provided by the *E. coli* methionine repressor (Figure 31.4). This protein binds DNA through the insertion of a pair of β strands into the major groove.

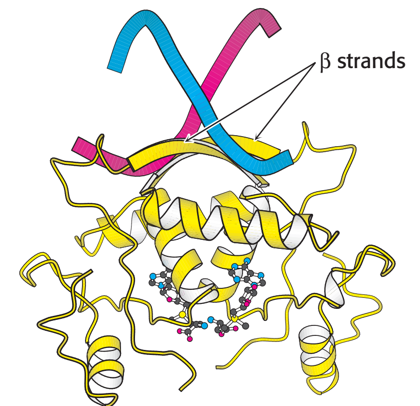


Figure 31.4 DNA recognition through β strands. A methionine repressor is shown bound to DNA. *Notice* that residues in β strands, rather than in α helices, participate in the crucial interactions between the protein and the DNA. [Drawn from 1CMA.pdb.]

31.2 Prokaryotic DNA-Binding Proteins Bind Specifically to Regulatory Sites in Operons

A historically important example reveals many common principles of gene regulation by DNA-binding proteins. Bacteria such as *E. coli* usually rely on glucose as their source of carbon and energy, even when other sugars are available. However, when glucose is scarce, *E. coli* can use lactose as their carbon source, even though this disaccharide does not lie on any major metabolic pathways. An essential enzyme in the metabolism of lactose is

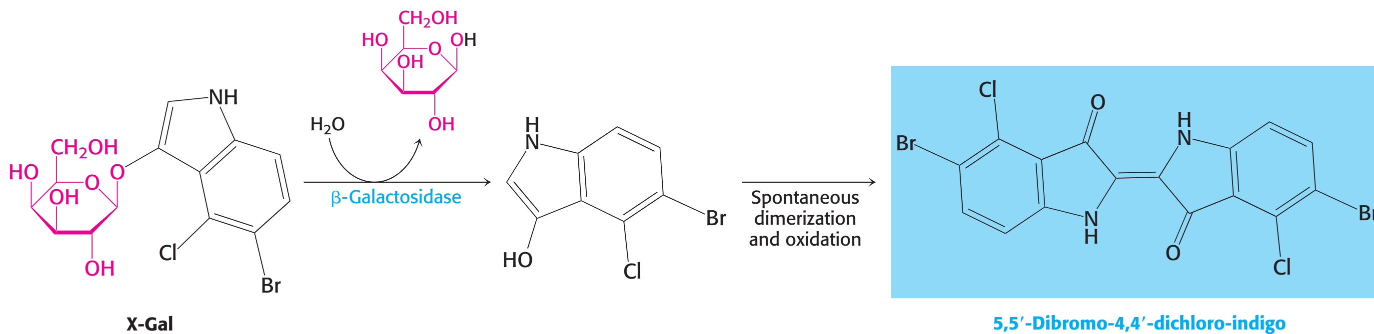
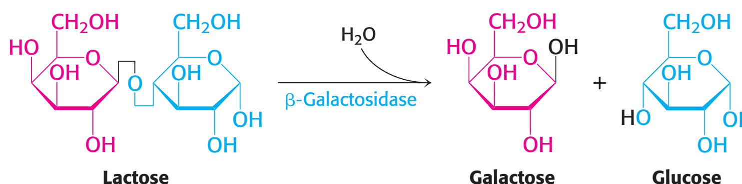


Figure 31.5 Monitoring the β-galactosidase reaction. The galactoside substrate X-Gal produces a colored product on cleavage by β-galactosidase. The appearance of this colored product provides a convenient means for monitoring the amount of the enzyme both in vitro and in vivo.

β-galactosidase, which hydrolyzes lactose into galactose and glucose. These products are then metabolized by pathways discussed in Chapter 16.



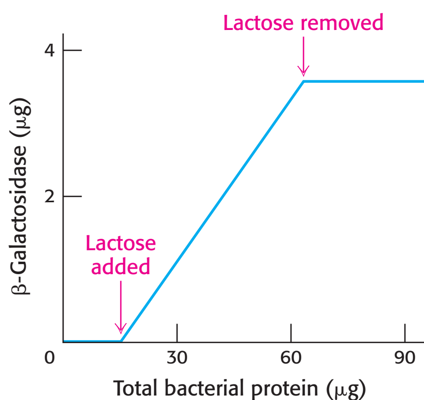


Figure 31.6 β-Galactosidase induction. The addition of lactose to an *E. coli* culture causes the production of β-galactosidase to increase from very low amounts to much larger amounts. The increase in the amount of enzyme parallels the increase in the number of cells in the growing culture. β-Galactosidase constitutes 6.6% of the total protein synthesized in the presence of lactose.

This reaction can be conveniently followed in the laboratory through the use of alternative galactoside substrates that form colored products such as X-Gal (Figure 31.5). An *E. coli* cell growing on a carbon source such as glucose or glycerol contains fewer than 10 molecules of β-galactosidase. In contrast, the same cell will contain several thousand molecules of the enzyme when grown on lactose (Figure 31.6). The presence of lactose in the culture medium induces a large increase in the amount of β-galactosidase by eliciting the synthesis of new enzyme molecules rather than by activating a preexisting but inactive precursor.

A crucial clue to the mechanism of gene regulation was the observation that two other proteins are synthesized in concert with β-galactosidase—namely, *galactoside permease* and *thiogalactoside transacetylase.* The permease is required for the transport of lactose across the bacterial cell membrane (Section 13.3). The transacetylase is not essential for lactose metabolism but appears to play a role in the detoxification of compounds that also may be transported by the permease. Thus, *the expression levels of a set of enzymes that all contribute to the adaptation to a given change in the environment change together.* Such a coordinated unit of gene expression is called an *operon.*

An operon consists of regulatory elements and protein-encoding genes

The parallel regulation of β-galactosidase, the permease, and the transacetylase suggested that the expression of genes encoding these enzymes is controlled by a common mechanism. François Jacob and Jacques Monod proposed the *operon model* to account for this parallel regulation as well as the results of other genetic experiments. The genetic elements of the model are a *regulator gene* that encodes a regulatory protein, a regulatory DNA sequence called *an operator site,* and a *set of structural genes* (Figure 31.7).

The regulator gene encodes a *repressor* protein that binds to the operator site. The binding of the repressor to the operator prevents transcription of the structural genes. The operator and its associated structural genes constitute

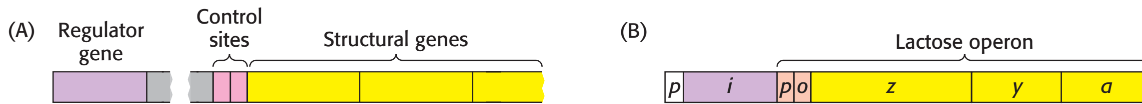


Figure 31.7 Operons. (A) The general structure of an operon as conceived by Jacob and Monod. (B) The structure of the lactose operon. In addition to the promoter, *p,* in the operon, a second promoter is present in front of the regulator gene, *i,* to drive the synthesis of the regulator.

the operon. For the *lactose (lac) operon,* the *i* gene encodes the repressor, *o* is the operator site, and the *z, y,* and *a* genes are the structural genes for β-galactosidase, the permease, and the transacetylase, respectively. The operon also contains a promoter site (denoted by *p*), which directs the RNA polymerase to the correct transcription initiation site. The *z, y,* and *a* genes are transcribed to give a single mRNA molecule that encodes all three proteins. An mRNA molecule encoding more than one protein is known as a *polygenic* or *polycistronic* transcript.

The *lac* repressor protein in the absence of lactose binds to the operator and blocks transcription

In the absence of lactose, the lactose operon is repressed. How does the *lac* repressor mediate this repression? The *lac repressor* exists as a tetramer of 37-kd subunits with two pairs of subunits coming together to form the DNA-binding unit previously discussed. In the absence of lactose, the repressor binds very tightly and rapidly to the operator. When the *lac* repressor is bound to DNA, the repressor prevents RNA polymerase from transcribing the protein-coding genes inasmuch as the operator site is directly adjacent to and downstream of the promoter site where the repressor would block the progress of RNA polymerase.

How does the *lac* repressor locate the operator site in the *E. coli* chromosome? The *lac* repressor binds 4×10^6 times as strongly to operator DNA as it does to random sites in the genome. This high degree of selectivity allows the repressor to find the operator efficiently even with a large excess (4.6×10^6) of other sites within the *E. coli* genome. The dissociation constant for the repressor–operator complex is approximately 0.1 pM (10^{-13} M). The rate constant for association ($\approx 10^{10}\ M^{-1}\ s^{-1}$) is strikingly high, indicating that the repressor finds the operator primarily by diffusing along a DNA molecule (a one-dimensional search) rather than encountering it from the aqueous medium (a three-dimensional search). This diffusion has been confirmed by studies that monitored the behavior of fluorescently labeled single molecules of *lac* repressor inside living *E. coli* cells.

Inspection of the complete *E. coli* genome sequence reveals two sites within 500 bp of the primary operator site that approximate the sequence of the operator. When one dimeric DNA-binding unit binds to the operator site, the other DNA-binding unit of the *lac* repressor tetramer can bind to one of these sites with similar sequences. The DNA between the two bound sites forms a loop. No other sites that closely match the sequence of the *lac operator* site are present in the rest of the *E. coli* genome sequence. Thus, *the DNA-binding specificity of the* lac *repressor is sufficient to specify a nearly unique site within the* E. coli *genome.*

The three-dimensional structure of the *lac* repressor has been determined in various forms. Each monomer consists of a small amino-terminal domain that binds DNA and a larger domain that mediates the formation of the dimeric DNA-binding unit and the tetramer (Figure 31.8). A pair of the amino-terminal domains come together to form the functional DNA-binding unit. Each monomer has a helix-turn-helix unit that interacts with the major groove of the bound DNA.

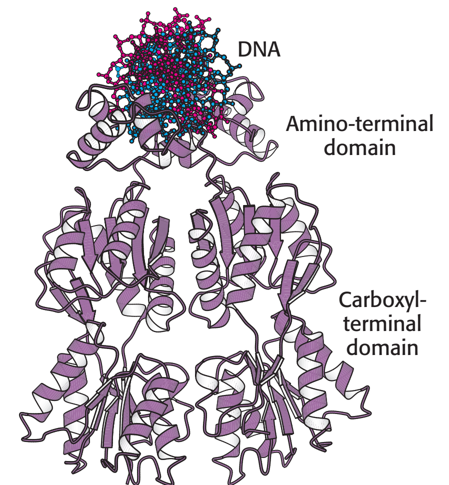
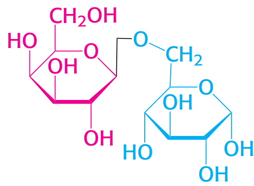
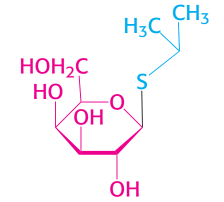


Figure 31.8 Structure of the *lac* repressor. A *lac* repressor dimer is shown bound to DNA. *Notice* that the amino-terminal domain binds to DNA, whereas the carboxyl-terminal domain forms a separate structure. A part of the structure that mediates the formation of lac repressor tetramers is not shown. [Drawn from 1EFA.pdb.]



1,6-Allolactose



Isopropylthiogalactoside (IPTG)

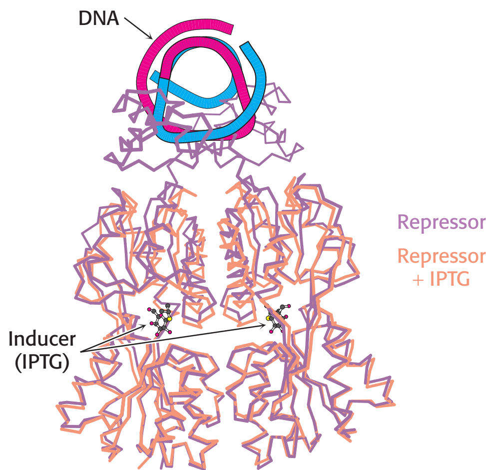


Figure 31.9 Effects of IPTG on *lac* repressor structure. The structure of the *lac* repressor bound to the inducer isopropylthiogalactoside (IPTG), shown in orange, is superimposed on the structure of the *lac* repressor bound to DNA, shown in purple. *Notice* that the binding of IPTG induces structural changes that alter the relation between the two DNA-binding domains so that they cannot interact effectively with DNA. The DNA-binding domains of the *lac* repressor bound to IPTG are not shown, because these regions are not well ordered in the crystals studied.

Ligand binding can induce structural changes in regulatory proteins

In the situation just described, glucose is present and lactose is absent, and the *lac* operon is repressed. How does the presence of lactose trigger the relief of this repression and, hence, the expression of the *lac* operon? Interestingly, lactose itself does not have this effect; rather, *allolactose,* a combination of galactose and glucose with an α-1,6 rather than an α-1,4 linkage, does. Allolactose is thus referred to as the *inducer* of the *lac* operon. Allolactose is a side product of the β-galactosidase reaction and is produced at low levels by the few molecules of β-galactosidase that are present before induction. Some other β-galactosides such as *isopropylthiogalactoside* (IPTG) are potent inducers of β-galactosidase expression, although they are not substrates of the enzyme. IPTG is useful in the laboratory as a tool for inducing gene expression in engineered bacterial strains.

The inducer triggers gene expression by preventing the *lac* repressor from binding the operator. *The inducer binds to the* lac *repressor and thereby greatly reduces the repressor's affinity for operator DNA.* An inducer molecule binds in the center of the large domain within each monomer. This binding leads to conformational changes that modify the relation between the two small DNA-binding domains (Figure 31.9). These domains can no longer easily contact DNA simultaneously, leading to a dramatic reduction in DNA-binding affinity.

Let us recapitulate the processes that regulate gene expression in the lactose operon (Figure 31.10). In the absence of inducer, the *lac* repressor is bound to DNA in a manner that blocks RNA polymerase from transcribing the *z, y,* and *a* genes. Thus, very little β-galactosidase, permease, or transacetylase are produced. The addition of lactose to the environment leads to the formation of allolactose. This inducer binds to the *lac* repressor, leading to conformational changes and the release of DNA by the *lac* repressor. With the operator site unoccupied, RNA polymerase can then transcribe the other *lac* genes and the bacterium will produce the proteins necessary for the efficient use of lactose.

The structure of the large domain of the *lac* repressor is similar to those of a large class of proteins that are present in *E. coli* and other bacteria. This family of homologous proteins binds ligands such as sugars and amino acids at their centers. Remarkably, domains of this family are utilized by eukaryotes in taste proteins and in neurotransmitter receptors, as will be discussed in Chapter 33.

The operon is a common regulatory unit in prokaryotes

Many other gene-regulatory networks function in ways analogous to those of the *lac* operon. For example, genes taking part in purine and, to

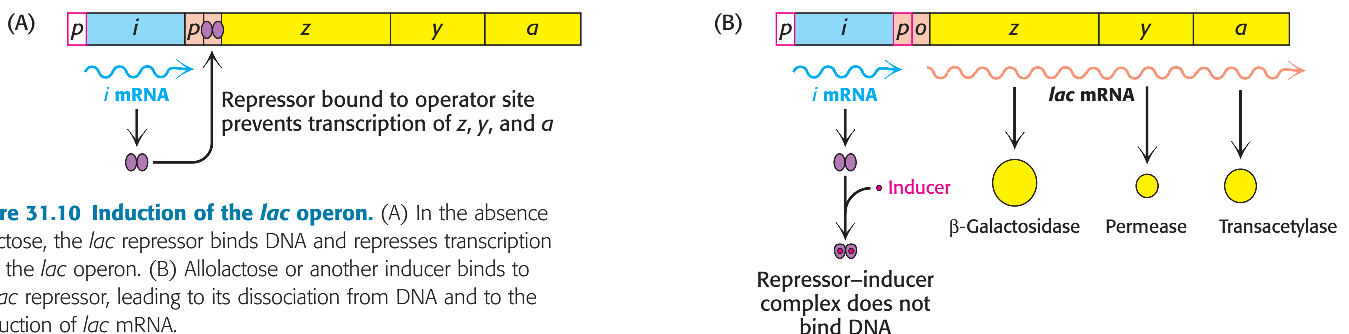


Figure 31.10 Induction of the *lac* operon. (A) In the absence of lactose, the *lac* repressor binds DNA and represses transcription from the *lac* operon. (B) Allolactose or another inducer binds to the *lac* repressor, leading to its dissociation from DNA and to the production of *lac* mRNA.

a lesser degree, pyrimidine biosynthesis are repressed by the *pur repressor*. This dimeric protein is 31% identical in sequence with the *lac* repressor and has a similar three-dimensional structure. However, the behavior of the *pur* repressor is opposite that of the *lac* repressor: whereas the *lac* repressor is *released* from DNA by binding to a small molecule, *the* pur *repressor binds DNA specifically, blocking transcription, only when bound to a small molecule*. Such a small molecule is called a *corepressor*. For the *pur* repressor, the corepressor can be either guanine or hypoxanthine. The dimeric *pur* repressor binds to inverted-repeat DNA sites of the form 5′-AN**G**C**AA**N**CG**N**TT**N**C**NT-3′, in which the bases shown in boldface type are particularly important. Examination of the *E. coli* genome sequence reveals the presence of more than 20 such sites, regulating 19 operons and including more than 25 genes (Figure 31.11).

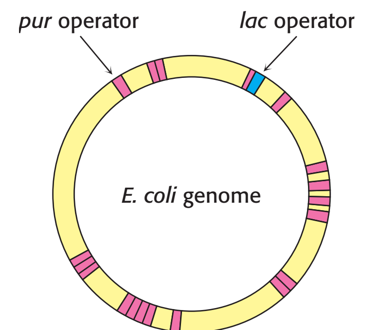


Figure 31.11 Binding-site distributions. The *E. coli* genome contains only a single region that closely matches the sequence of the *lac* operator (shown in blue). In contrast, 20 sites match the sequence of the *pur* operator (shown in red). Thus, the *pur* repressor regulates the expression of many more genes than does the *lac* repressor.

Because the DNA binding sites for these regulatory proteins are short, it is likely that they evolved independently and are not related by divergence from an ancestral regulatory site. Once a ligand-regulated DNA-binding protein is present in a cell, binding sites for the protein may arise by mutation adjacent to additional genes. Binding sites for the *pur* repressor have evolved in the regulatory regions of a wide range of genes taking part in nucleotide biosynthesis. All such genes can then be regulated in a concerted manner.

The organization of prokaryotic genes into operons is useful for the analysis of completed genome sequences. Sometimes a gene of unknown function is discovered to be part of an operon containing well-characterized genes. Such associations can provide powerful clues to the biochemical and physiological functions of the uncharacterized gene.

Transcription can be stimulated by proteins that contact RNA polymerase

All the DNA-binding proteins discussed thus far function by inhibiting transcription until some environmental condition, such as the presence of lactose, is met. There are also DNA-binding proteins that stimulate transcription. One particularly well studied example is a protein in *E. coli* that stimulates the expression of catabolic enzymes.

E. coli grown on glucose, a preferred energy source, have very low levels of catabolic enzymes for metabolizing other sugars. Clearly, the synthesis of these enzymes when glucose is abundant would be wasteful. Glucose has an inhibitory effect on the genes encoding these enzymes, an effect called *catabolite repression*. It is due to the fact that glucose lowers the concentration of cyclic AMP in *E. coli*. When its concentration is high, cAMP stimulates the concerted transcription of many catabolic enzymes by acting through a protein called the *catabolite activator protein* (CAP), which is also known as the cAMP receptor protein (CRP).

When bound to cAMP, CAP stimulates the transcription of lactose- and arabinose-catabolizing genes. CAP is a sequence-specific DNA-binding protein. Within the *lac* operon, CAP binds to an inverted repeat that is centered near position −61 relative to the start site for transcription (Figure 31.12). This site is approximately 70 base pairs from the operator site. As expected from the symmetry of the binding site, CAP functions as a dimer of identical subunits.

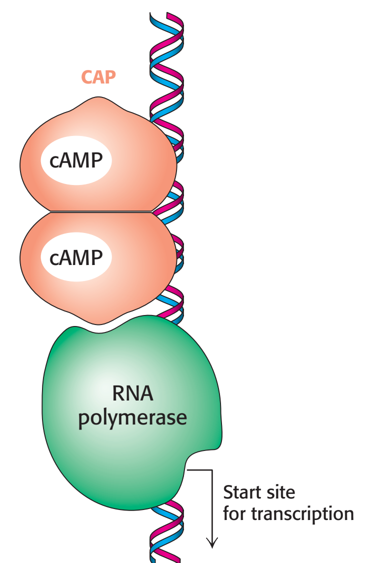


Figure 31.12 Binding site for catabolite activator protein (CAP). This protein binds as a dimer to an inverted repeat that is at the position −61 relative to the start site of transcription. The CAP-binding site on DNA is adjacent to the position at which RNA polymerase binds.

The CAP–cAMP complex stimulates the initiation of transcription by approximately a factor of 50. Energetically favorable contacts between CAP and RNA polymerase increase the likelihood that transcription will be initiated at sites to which the CAP–cAMP complex is bound (Figure 31.13).

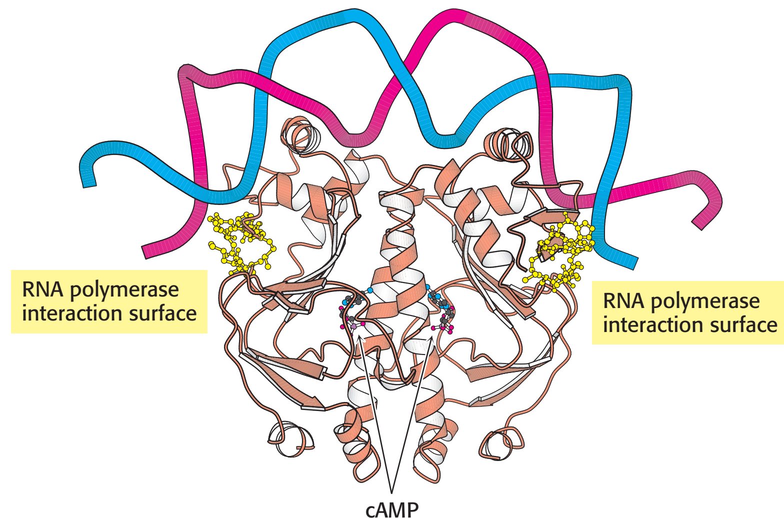


Figure 31.13 Structure of a dimer of CAP bound to DNA. The residues shown in yellow in each CAP monomer have been implicated in direct interactions with RNA polymerase. [Drawn from 1RUN.pdb.]

Thus, in regard to the *lac* operon, gene expression is maximal when the binding of allolactose relieves the inhibition by the *lac* repressor and the CAP–cAMP complex stimulates the binding of RNA polymerase.

The *E. coli* genome contains many CAP-binding sites in positions appropriate for interactions with RNA polymerase. Thus, an increase in the cAMP level inside an *E. coli* bacterium results in the formation of CAP–cAMP complexes that bind to many promoters and stimulate the transcription of genes encoding a variety of catabolic enzymes.

31.3 Regulatory Circuits Can Result in Switching Between Patterns of Gene Expression

The study of viruses that infect bacteria has led to significant advances in our understanding of the processes that control gene expression. Again, sequence-specific DNA-binding proteins play key roles in these processes. Investigations of bacteriophage λ have been particularly revealing. We examined the alternative infection modes of λ phage in Chapter 5. In the lytic pathway, most of the genes in the viral genome are transcribed, initiating the production of many virus particles and leading to the eventual lysis of the bacterial cell with the concomitant release of approximately 100 virus particles. In the lysogenic pathway, the viral genome is incorporated into the bacterial DNA where most of the viral genes remain unexpressed, allowing the viral genome to be carried along as the bacteria replicate. Two key proteins and a set of regulatory sequences in the viral genome are responsible for the switch that determines which of these two pathways is followed.

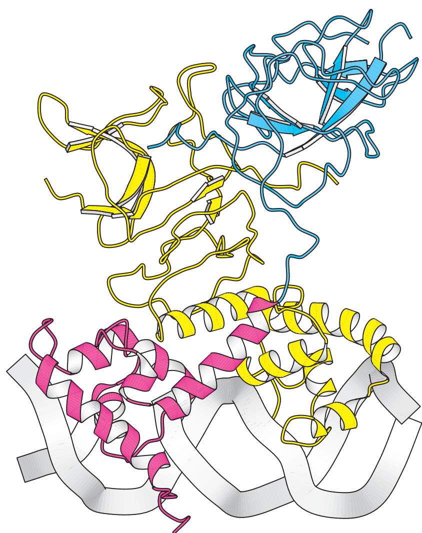


Figure 31.14 Structure of the λ repressor bound to DNA. The λ repressor binds to DNA as a dimer. The amino-terminal domain of one subunit is shown in red and the carboxyl-terminal domain is shown in blue. In the other subunit, both domains are shown in yellow. *Notice* how α helices on the amino-terminal domains fit into the major groove of the DNA. [Drawn from 3DBN.pdb.]

Lambda repressor regulates its own expression

The first protein that we shall consider is the λ repressor, sometimes known as the λ cI protein. This protein is key because it blocks, either directly or indirectly, the transcription of almost all genes encoded by the virus. The one exception is the gene that encodes the λ repressor itself. The λ repressor consists of an amino-terminal DNA-binding domain and a carboxyl-terminal domain that participates in protein oligomerization (Figure 31.14). This protein binds to a number of key sites in the λ phage genome. The sites of greatest interest for our present discussion are in the so-called right operator (Figure 31.15). This region includes three binding sites for the λ repressor dimer as well as two promoters within a region of approximately 80 base pairs. One promoter drives the expression of the gene for the λ repressor

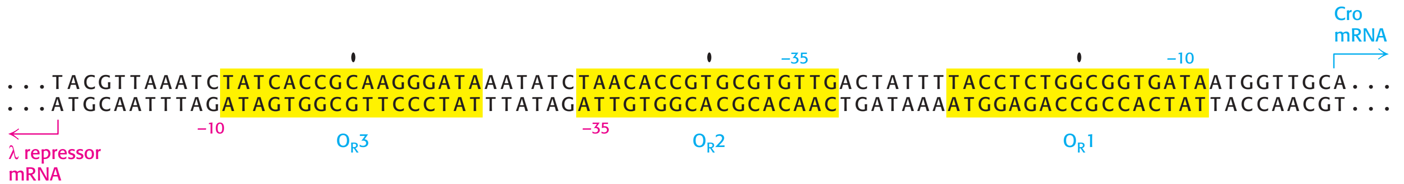


Figure 31.15 Sequence of the λ right operator. The three operator sites (O_R1, O_R2, and O_R3) are shaded yellow with their centers indicated. The start sites for the λ repressor mRNA and the Cro mRNA are indicated, as are their −10 and −35 sequences.

itself, whereas the other drives the expression of a number of other viral genes. The λ repressor does not have the same affinity for the three sites; it binds the site O_R1 with the highest affinity. In addition, the binding to adjacent sites is cooperative so that, after a λ repressor dimer has bound at O_R1, the likelihood that a protein will bind to the adjacent site O_R2 increases by approximately 25-fold. Thus, when λ repressor is present in the cell at moderate concentrations, the most likely configuration has λ repressor bound at O_R1 and O_R2, but not at O_R3. In this configuration, the λ repressor dimer bound at O_R1 blocks access to the promoter on the right side of the operator sites, repressing transcription of the adjacent gene, which encodes a protein termed Cro (*c*ontroller of *r*epressor and *o*thers), while the repressor dimer at O_R2 can be in contact with RNA polymerase and stimulate transcription of the promoter that controls the transcription of the gene that encodes the λ repressor itself. Thus, the λ repressor stimulates its own production. As the concentration of the λ repressor increases further, an additional repressor dimer can bind to the O_R3 site, blocking the other promoter and repressing the production of additional repressor. Thus, the right operator serves to maintain the λ repressor in a narrow, stable concentration range (Figure 31.16). The λ repressor also blocks other promoters in the λ phage genome so that the repressor is the only phage protein produced, which corresponds to the lysogenic state.

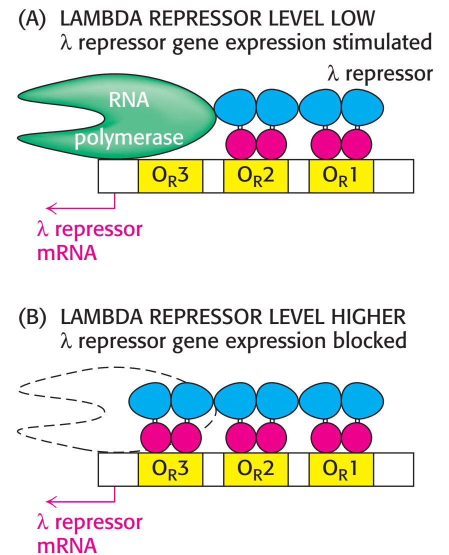


Figure 31.16 The λ repressor controls its own synthesis. When λ repressor levels are relatively low, the repressor binds to sites O_R1 and O_R2 and stimulates the transcription of the gene than encodes the λ repressor itself. When λ repressor levels are higher, the repressor also binds to site O_R3, blocking access to its promoter and repressing transcription from this gene.

A circuit based on lambda repressor and Cro form a genetic switch

What stimulates the switch to the lytic pathway? Changes such as DNA damage initiate the cleavage of the λ repressor at a specific bond between the DNA-binding and oligomerization domains. This process is mediated by the *E. coli* RecA protein (Section 28.5). After this cleavage has taken place, the affinity of the λ repressor for DNA is reduced. After the λ repressor is no longer bound to the O_R1 site, the Cro gene can be transcribed. Cro is a small protein that binds to the same sites as the λ repressor does, but with a different order of affinity for the three sites in the right operator. In particular, Cro has the highest affinity for O_R3. Cro bound in this site blocks the production of new repressor. The absence of repressor leads to the production of other phage genes, leading to the production of virus particles and the eventually lysis of the host cells. Thus, this genetic circuit acts a switch with two stable states: (1) repressor high, Cro low, corresponding to the lysogenic state, and (2) Cro high, repressor low, corresponding to the lytic state (Figure 31.17). Regulatory circuits with different DNA-binding proteins controlling the expression of each other's genes constitute a common motif for controlling gene expression.

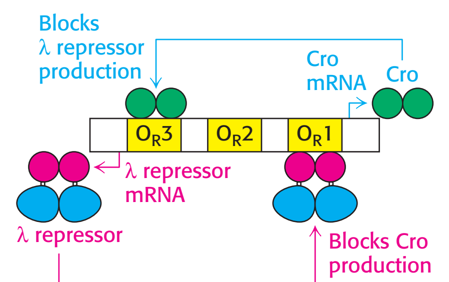


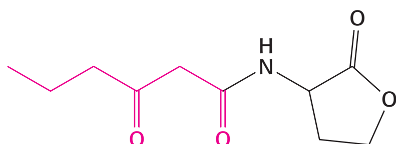
Figure 31.17 The λ repressor and Cro form a genetic circuit. The λ repressor blocks the production of Cro by binding most favorably to site O_R1 whereas Cro blocks the production of the λ repressor by binding most favorably to site O_R3. This circuit forms a switch that determines whether the lysogenic or the lytic pathway is followed.

Many prokaryotic cells release chemical signals that regulate gene expression in other cells

Prokaryotic cells have been traditionally viewed as solitary single cells. However, it is becoming increasingly clear that, in many circumstances,

prokaryotic cells live in complex communities, interacting with other cells of their own and different species. *These social interactions change the patterns of gene expression within the cells.*

An important type of interaction is called *quorum sensing*. This phenomenon was discovered in the bacteria *Vibrio fischeri,* a species of bacterium that can live inside a specialized light organ in the bobtail squid. In this symbiotic relation, the bacteria produce luciferase and fluoresce, providing protection for the squid (by preventing being backlit by moonlight) in exchange for a protected place to live and reproduce. When these bacteria are grown in culture at low density, they are not fluorescent. However, when the cell density reaches a critical level, the gene for luciferase is expressed and the cells fluoresce. A key observation was that, when *V. fischeri* cells were transferred to a sterile medium in which other *V. fischeri* cells had been grown to high density, the cells became fluorescent even at low cell density. This experiment revealed that a chemical, subsequently shown to be *N*-3-oxo-hexanoyl homoserine lactone (hereafter AHL for acyl-homoserine lactone), had been released into the medium that triggers the development of the fluorescence (Figure 31.18). This compound and other compounds that play similar roles are termed *autoinducers*.



Acyl-homoserine lactone (AHL)

Figure 31.18 Autoinducer structure. The structure of the acyl-homeserine lactone *N*-3-oxo-hexanoyl homoserine lactone, the autoinducer from *V. fischeri*. The autoinducers from other bacterial species can have different acyl groups (shown in red).

Cells of *V. fischeri* release the autoinducer into their environment and other *V. fischeri* cells take up the chemical. *V. fischeri* cells express a DNA-binding protein LuxR that serves as the receptor for the autoinducer. LuxR comprises two domains, one of which binds AHL and the other of which binds DNA through a helix-turn-helix motif (Figure 31.19). After the AHL concentration inside the cell has increased to an appropriate level, a substantial fraction of the LuxR molecules bind AHL. When bound to AHL, LuxR dimers bind to specific sites on DNA and increase the rate of transcription initiation at specific genes. These target genes include an operon that includes LuxA and LuxB, which together encode the luciferase enzyme, and LuxI, which produces an enzyme that catalyzes the formation of more AHL.

Because each cell produces only a small amount of the autoinducer, this regulatory system allows each *V. fischeri* cell to determine the density of the *V. fischeri* population in its environment—hence the term *quorum sensing* for this process. Studies of other prokaryotic cells are revealing an elaborate chemical language of different autoinducers (as well as autorepressors that serve to repress specific genes). The "words" in this language include other acyl-homoserine lactone molecules with different acyl chain lengths and functionalities as well as other distinct classes of molecules.

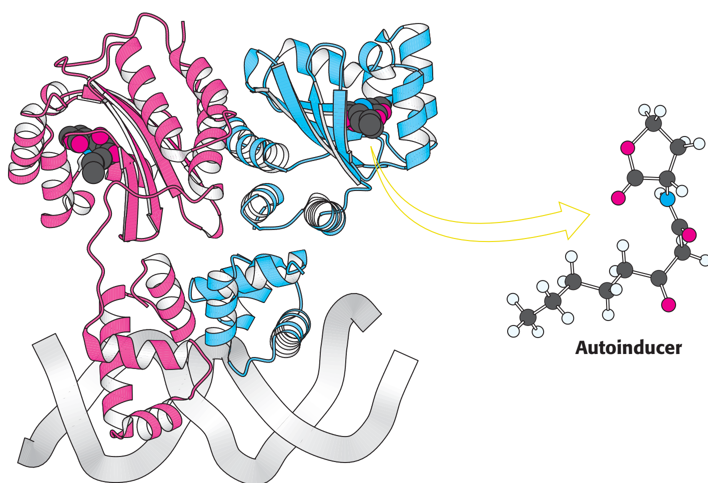


Figure 31.19 Quorum-sensing gene regulator. The structure of a homolog of LuxR (TraR from the bacterium *Agrobacterium tumefaciens*) is shown. *Notice* that the dimeric protein binds to DNA through an α-helical domain, whereas the autoinducer binds to a separate domain.

Biofilms are complex communities of prokaryotes

Many species of prokaryotes can be found in specialized structures termed *biofilms* that can form on surfaces. Biofilms are of considerable medical importance because organisms within them are often quite resistant to the immune response of the host as well as to antibiotics. Quorum sensing appears to play a major role in the formation of biofilms in that cells are able to sense other cells in their environments and to promote the formation of communities with particular compositions. Some genes controlled by quorum-sensing mechanisms promote the formation of specific molecules that serve as scaffolds for the biofilm. An intrigu-

ing recent discovery is that many of the organisms that are present in biofilms on or in our bodies (perhaps 95% or more) have not been grown in culture. Through DNA-sequencing methods, we are developing a better census of our microbiome and are working toward understanding the gene-regulatory mechanisms that support these complex communities.

31.4 Gene Expression Can Be Controlled at Posttranscriptional Levels

The modulation of the rate of transcription initiation is the most common mechanism of gene regulation. However, other stages of transcription also can be targets for regulation. In addition, the process of translation provides other points of intervention for regulating the level of a protein produced in a cell. In Chapter 29, we considered riboswitches that control transcription termination (Section 29.1). Other riboswitches control gene expression by other mechanisms such as the formation of structures that inhibit translation. Additional mechanisms for posttranscriptional gene regulation have been discovered.

Attenuation is a prokaryotic mechanism for regulating transcription through the modulation of nascent RNA secondary structure

A means for regulating transcription in bacteria was discovered by Charles Yanofsky and his colleagues as a result of their studies of the tryptophan operon. This operon encodes five enzymes that convert chorismate into tryptophan. Analysis of the 5′ end of *trp* mRNA revealed the presence of a *leader sequence* of 162 nucleotides before the initiation codon of the first enzyme. The next striking observation was that bacteria produced a transcript consisting of only the first 130 nucleotides when the tryptophan level was high, but they produced a 7000-nucleotide *trp* mRNA, including the entire leader sequence, when tryptophan was scarce. Thus, when trytophan is plentiful and the biosynthetic enzymes are not needed, transcription is abruptly broken off before any mRNA coding for the enzymes is produced. The site of termination is called the attenuator, and this mode of regulation is called *attenuation*.

Attenuation depends on features at the 5′ end of the mRNA product (Figure 31.20). The first part of the leader sequence encodes a 14-amino-acid leader peptide. Following the open reading frame for the peptide is the attenuator, a region of RNA that is capable of forming several alternative structures. Recall that transcription and translation are tightly coupled in bacteria. Thus, the translation of the *trp* mRNA begins soon after the ribosome-binding site has been synthesized.

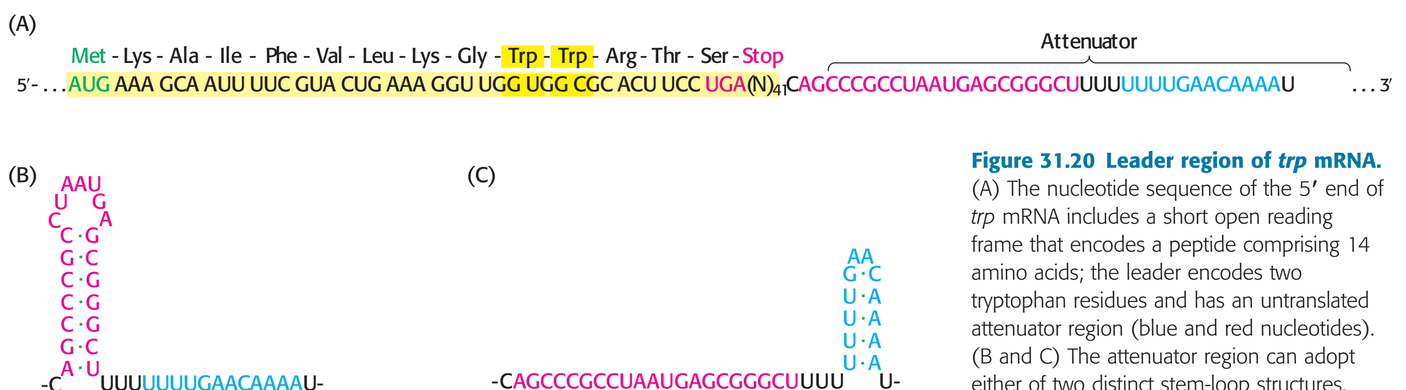


Figure 31.20 Leader region of *trp* mRNA. (A) The nucleotide sequence of the 5′ end of *trp* mRNA includes a short open reading frame that encodes a peptide comprising 14 amino acids; the leader encodes two tryptophan residues and has an untranslated attenuator region (blue and red nucleotides). (B and C) The attenuator region can adopt either of two distinct stem-loop structures.

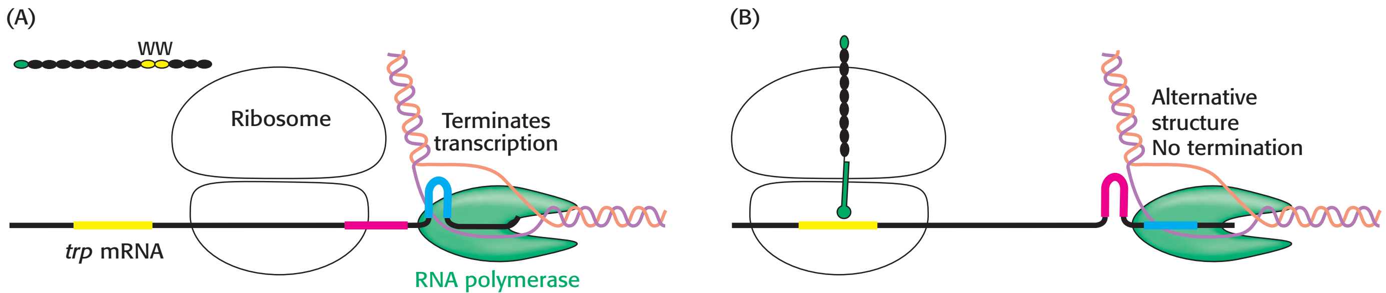


Figure 31.21 Attenuation. (A) In the presence of adequate concentrations of tryptophan (and, hence, Trp-tRNA), translation proceeds rapidly and an RNA structure forms that terminates transcription. (B) At low concentrations of tryptophan, translation stalls while awaiting Trp-tRNA, giving time for an alternative RNA structure to form that does not terminate transcription efficiently.

How does the level of tryptophan alter transcription of the *trp* operon? An important clue was the finding that the 14-amino-acid leader peptide includes two adjacent tryptophan residues. A ribosome is able to translate the leader region of the mRNA product only in the presence of adequate concentrations of tryptophan. When enough tryptophan is present, a stem-loop structure forms in the attenuator region, which leads to the release of RNA polymerase from the DNA (Figure 31.21). However, when tryptophan is scarce, transcription is terminated less frequently. Little tryptophanyl-tRNA is present, and so the ribosome stalls at the tandem UGG codons encoding tryptophan. This delay leaves the adjacent region of the mRNA exposed as transcription continues. An alternative RNA structure that does not function as a terminator is formed, and transcription continues into and through the coding regions for the enzymes. Thus, attenuation provides an elegant means of sensing the supply of tryptophan required for protein synthesis.

Several other operons for the biosynthesis of amino acids in *E. coli* also are regulated by attenuator sites. The leader peptide of each contains an abundance of the amino acid residues of the type synthesized by the operon (Figure 31.22). For example, the leader peptide for the phenylalanine operon includes 7 phenylalanine residues among 15 residues. The threonine operon encodes enzymes required for the synthesis of both threonine and isoleucine; the leader peptide contains 8 threonine and 4 isoleucine residues in a 16-residue sequence. The leader peptide for the histidine operon includes 7 histidine residues in a row. In each case, low levels of the corresponding charged tRNA cause the ribosome to stall, trapping the

(A)
Met - Lys - Arg - Ile - Ser - Thr - Thr - Ile - Thr - Thr - Thr - Ile - Thr - Ile - Thr - Thr -
5′ AUG AAA CGC AUU AGC ACC ACC AUU ACC ACC ACC AUC ACC AUU ACC ACA 3′

(B)
Met - Lys - His - Ile - Pro - Phe - Phe - Phe - Ala - Phe - Phe - Phe - Thr - Phe - Pro - Stop
5′ AUG AAA CAC AUA CCG UUU UUC UUC GCA UUC UUU UUU ACC UUC CCC UGA 3′

(C)
Met - Thr - Arg - Val - Gln - Phe - Lys - His - His - His - His - His - His - His - Pro - Asp -
5′ AUG ACA CGC GUU CAA UUU AAA CAC CAC CAU CAU CAC CAU CAU CCU GAC 3′

Figure 31.22 Leader peptide sequences. Amino acid sequences and the corresponding mRNA nucleotide sequences of the (A) threonine operon, (B) phenylalanine operon, and (C) histidine operon. In each case, an abundance of one amino acid in the leader peptide sequence leads to attenuation.

nascent mRNA in a state that can form a structure that allows RNA polymerase to read through the attenuator site. Evolution has apparently converged on this strategy repeatedly as a mechanism for controlling amino acid biosynthesis.

Summary

31.1 Many DNA-Binding Proteins Recognize Specific DNA Sequences

The regulation of gene expression depends on the interplay between specific sequences within the genome and proteins that bind specifically to these sites. Specific DNA-binding proteins recognize regulatory sites that usually lie adjacent to the genes whose transcription is regulated by these proteins. The proteins of the largest family contain a helix-turn-helix motif. The first helix of this motif inserts into the major groove of DNA and makes specific hydrogen-bonding and other contacts with the edges of the base pairs.

31.2 Prokaryotic DNA-Binding Proteins Bind Specifically to Regulatory Sites in Operons

In prokaryotes, many genes are clustered into operons, which are units of coordinated genetic expression. An operon consists of control sites (an operator and a promoter) and a set of structural genes. In addition, regulator genes encode proteins that interact with the operator and promoter sites to stimulate or inhibit transcription. The treatment of *E. coli* with lactose induces an increase in the production of β-galactosidase and two additional proteins that are encoded in the lactose operon. In the absence of lactose or a similar galactoside inducer, the *lac* repressor protein binds to an operator site on the DNA and blocks transcription. The binding of allolactose, a derivative of lactose, to the *lac* repressor induces a conformational change that leads to dissociation from DNA. RNA polymerase can then move through the operator to transcribe the *lac* operon.

Some proteins activate transcription by directly contacting RNA polymerase. For example, cyclic AMP stimulates the transcription of many catabolic operons by binding to the catabolite activator protein. The binding of the cAMP–CAP complex to a specific site in the promoter region of an inducible catabolic operon enhances the binding of RNA polymerase and the initiation of transcription.

31.3 Regulatory Circuits Can Result in Switching Between Patterns of Gene Expression

The study of bacterial viruses, particularly bacteriophage λ, has revealed key aspects of gene-regulatory networks. Bacteriophage λ can develop by either a lytic or a lysogenic pathway. A key regulatory protein, the λ repressor, regulates its own expression, promoting transcription of the gene that encodes the repressor when repressor levels are low and blocking transcription of the gene when levels are high. This behavior depends on the λ right operator, which includes three sites to which λ repressor dimers can bind. Cooperative binding of the λ repressor to two of the sites stabilizes the state in which two λ repressor dimers are bound. The Cro protein binds to the same sites as does the λ repressor, but with reversed affinities. When Cro is present at sufficient concentrations, it blocks the transcription of the gene for the λ repressor while allowing the transcription of its own gene. Thus, these two proteins and the operator form a genetic switch that can exist in either of two states.

Some prokaryotic species participate in quorum sensing. This process includes the release of chemicals called autoinducers into the medium surrounding the cells. These autoinducers are often, but not always, acyl homoserine lactones. Autoinducers are taken up by surrounding cells. When the autoinducer concentration reaches an appropriate level, it is bound by receptor proteins that activate the expression of genes, including those that promote the synthesis of more autoinducer. These chemically mediated social interactions allow these prokaryotes to change their gene-expression patterns in response to the number of other cells in their environments. Biofilms are complex communities of prokaryotes that are promoted by quorum-sensing mechanisms.

31.4 Gene Expression Can Be Controlled at Posttranscriptional Levels

Gene expression can also be regulated at the level of translation. In prokaryotes, many operons important in amino acid biosynthesis are regulated by attenuation, a process that depends on the formation of alternative structures in mRNA, one of which favors the termination of transcription. Attenuation is mediated by the translation of a leader region of mRNA. A ribosome stalled by the absence of an aminoacyl-tRNA needed to translate the leader mRNA alters the structure of mRNA, allowing RNA polymerase to transcribe the operon beyond the attenuator site.

Key Terms

helix-turn-helix motif (p. 923)
β-galactosidase (p. 924)
operon model (p. 924)
repressor (p. 924)
lac repressor (p. 925)
lac operator (p. 925)
inducer (p. 926)
isopropylthiogalactoside (IPTG) (p. 926)
pur repressor (p. 927)
corepressor (p. 927)
catabolite repression (p. 927)
catabolite activator protein (CAP) (p. 927)
quorum sensing (p. 930)
autoinducer (p. 930)
biofilm (p. 930)
attenuation (p. 931)

Problems

1. *Missing genes.* Predict the effects of deleting the following regions of DNA:

(a) The gene encoding *lac* repressor

(b) The *lac* operator

(c) The gene encoding CAP

2. *Minimal concentration.* Calculate the concentration of *lac* repressor, assuming that one molecule is present per cell. Assume that each *E. coli* cell has a volume of 10^{-12} cm^3. Would you expect the single molecule to be free or bound to DNA?

3. *Counting sites.* Calculate the expected number of times that a given 8-base-pair DNA site should be present in the *E. coli* genome. Assume that all four bases are equally probable. Repeat for a 10-base-pair site and a 12-base-pair site.

4. *The same but not the same.* The *lac* repressor and the *pur* repressor are homologous proteins with very similar three-dimensional structures, yet they have different effects on gene expression. Describe two important ways in which the gene-regulatory properties of these proteins differ.

5. *The opposite direction.* Some compounds called anti-inducers bind to repressors such as the *lac* repressor and inhibit the action of inducers; that is, transcription is repressed and higher concentrations of inducer are required to induce transcription. Propose a mechanism of action for anti-inducers.

6. *Inverted repeats.* Suppose that a nearly perfect 20-base-pair inverted repeat is observed in a DNA sequence. Provide two possible explanations.

7. *Broken operators.* Consider a hypothetical mutation in O_R2 that blocks both λ repressor and Cro binding. How would this mutation affect the likelihood of bacteriophage λ entering the lytic phase?

8. *Promoters.* Compare the −10 and −35 sequences for the λ repressor and Cro genes in the right operator. How many differences are there between these sequences?

9. *Positive and negative feedback*. What is the effect of an increased Cro concentration on the expression of the gene for the λ repressor? Of an increased concentration of λ repressor on the expression of the Cro gene? Of an increased concentration of λ repressor on the expression of the λ repressor gene?

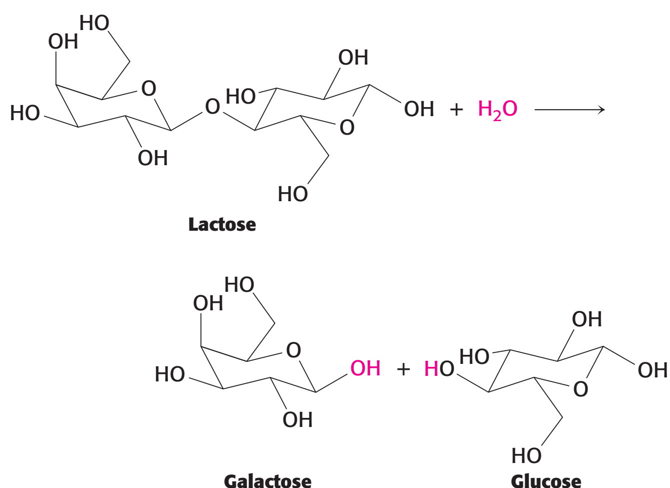
10. *Leaderless*. The mRNA for the λ repressor begins with 5′-AUG-3′, which encodes the methionine residue that begins the protein. What is unusual about this beginning? Would it cause the mRNA to translate efficiently or not?

11. *Quorum count*. Suppose you have a series of compounds that you wish to test for the autoinducer activity in *Vibrio fischeri*. Propose a simple assay, assuming that you can grow *V. fischeri* cultures at low cell densities.

12. *Codon utilization*. There are four codons that encode threonine. Consider the leader sequence in Figure 31.22A. What codons are used and with what frequency?

Mechanism Problem

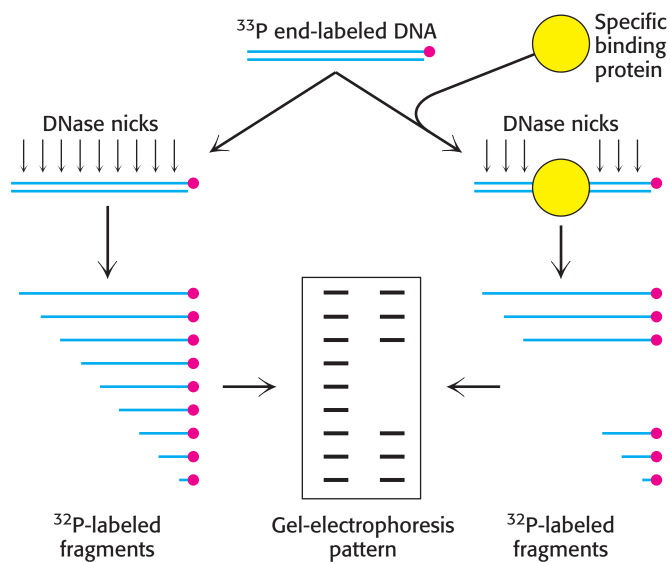
13. *Follow the stereochemistry*. The hydrolysis of lactose is catalyzed by β-galactosidase.



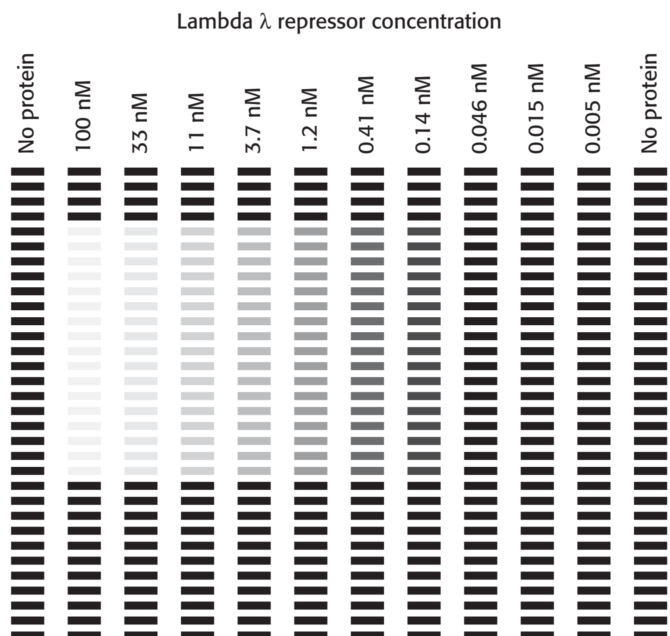
Does the overall reaction proceed with retention or inversion of configuration? Given that each step likely proceeds with inversion of configuration, what does the overall change in stereochemistry suggest about the mechanism? A key residue in the reaction has been identified to be Glu 537. Propose an overall mechanism for the hydrolysis of lactose.

Data Interpretation Problem

14. *Leaving tracks*. A powerful method for examining protein–DNA interactions is called DNA footprinting. In this method, a DNA fragment containing a potential binding site is radiolabeled on one end. The labeled DNA is then treated with a DNA-cleaving agent such as DNase I such that each DNA molecule within the population is cut only once. The same cleavage process is carried out in the presence of the DNA-binding protein. The bound protein protects some sites within the DNA from cleavage. The patterns of DNA fragments in the cleaved pool of DNA molecules are then examined by electrophoresis followed by autoradiography.



This method is applied to a DNA fragment containing a single binding site for the λ repressor in the presence of difference concentrations of the λ repressor. The results are shown below:



Estimate the dissociation constant for the λ repressor–DNA complex and the standard free energy of binding.

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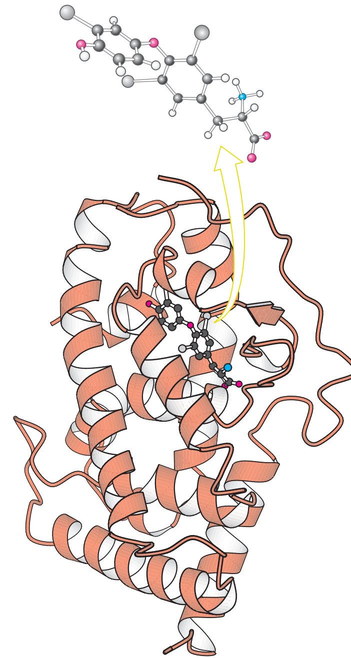
The Control of Gene Expression in Eukaryotes



Complex biological processes often require coordinated control of the expression of many genes. The maturation of a tadpole into a frog is largely controlled by thyroid hormone. This hormone regulates gene expression by binding to a protein, the thyroid-hormone receptor, as shown at the right. In response to the hormone's binding, this protein binds to specific DNA sites in the genome and modulates the expression of nearby genes. [(Right) Sharon Cummings/Dembinsky Photo Associates.]

OUTLINE

32.1 Eukaryotic DNA Is Organized into Chromatin

32.2 Transcription Factors Bind DNA and Regulate Transcription Initiation

32.3 The Control of Gene Expression Can Require Chromatin Remodeling

32.4 Eukaryotic Gene Expression Can Be Controlled at Posttranscriptional Levels

Many of the most important and intriguing features in modern biology and medicine, such as the pathways crucial for the development of multicellular organisms, the changes that distinguish normal cells and cancer cells, and the evolutionary changes leading to new species, entail networks of gene-regulatory pathways. Gene regulation in eukaryotes is significantly more complex than in prokaryotes in several ways. First, the genomes being regulated are significantly larger. The *E. coli* genome consists of a single, circular chromosome containing 4.6 Mb. This genome encodes approximately 2000 proteins. In comparison, one of the simplest eukaryotes, *Saccharomyces cerevisiae* (baker's yeast), contains 16 chromosomes ranging in size from 0.2 to 2.2 Mb (Figure 32.1). The yeast genome totals 12 Mb and encodes approximately 6000 proteins. The genome within a human cell contains 23 pairs of chromosomes ranging in size from 50 to 250 Mb. Approximately 23,000 genes are present within the 3000 Mb of human DNA.

Second, whereas prokaryotic genomic DNA is relatively accessible, eukaryotic DNA is packaged into chromatin, a complex between the DNA and a special set of proteins (Figure 32.2). Although the principles for the construction of chromatin are relatively simple, the chromatin structure for a complete genome is quite complex. Importantly, in a given eukaryotic cell,

Megabases

A length of DNA consisting of 10^6 base pairs (if double stranded) or 10^6 bases (if single stranded).

1 Mb = 10^3 kb = 10^6 bases

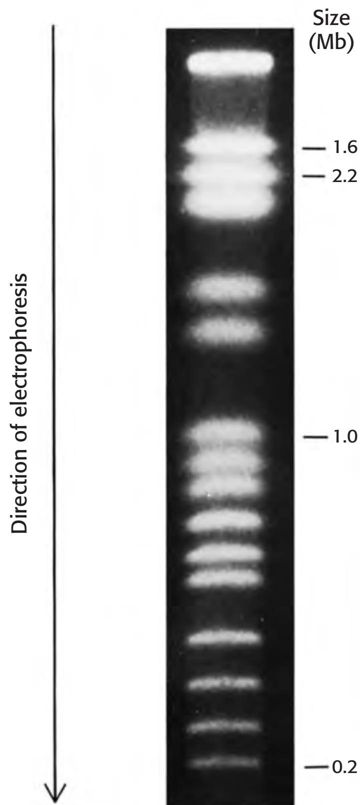


Figure 32.1 Yeast chromosomes. Pulsed-field electrophoresis allows the separation of 16 yeast chromosomes. [From G. Chu, D. Vollrath, and R. W. Davis. *Science* 234:1582–1585, 1986.]

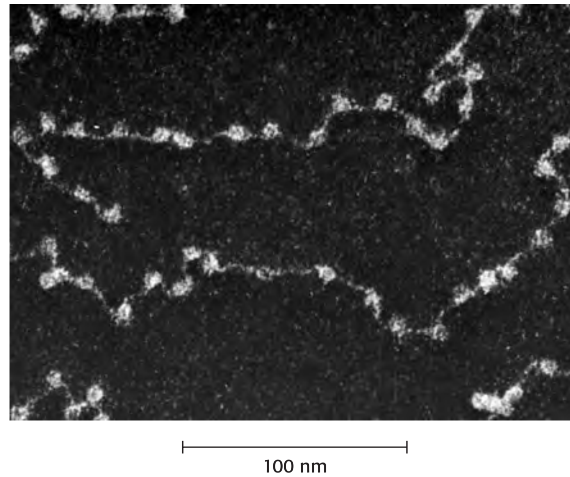


Figure 32.2 Chromatin structure. An electron micrograph of chromatin showing its "beads on a string" character. The beads correspond to DNA complexes with specific proteins. [Courtesy of Dr. Ada Olins and Dr. Donald Olins.]

some genes and their associated regulatory regions are relatively accessible for transcription and regulation, whereas other genes are tightly packaged and are thus rendered inactive. Eukaryotic gene regulation frequently requires the manipulation of chromatin structure.

A manifestation of this complexity is the presence of many different *cell types* in most eukaryotes. A liver cell, a pancreatic cell, and an embryonic stem cell contain the same DNA sequences, yet the subset of genes highly expressed in cells from the pancreas, which secretes digestive enzymes, differs markedly from the subset highly expressed in the liver, the site of lipid transport and energy transduction. Embryonic stem cells do not express any subset of genes at high levels; the most highly expressed genes are "housekeeping" genes involved in the cytoskeleton and processes such as translation (Table 32.1). The existence of stable cell types is due to differences in the *epigenome,* differences in chromatin structure, and covalent modifications of the DNA, not in the DNA sequence itself.

In addition, eukaryotic genes are not generally organized into operons. Instead, genes that encode proteins for steps within a given pathway are often spread widely across the genome. This characteristic requires that other mechanisms function to regulate genes in a coordinated way.

Despite these differences, some aspects of gene regulation in eukaryotes are quite similar to those in prokaryotes. In particular, activator and repressor proteins that recognize specific DNA sequences are central to many gene-regulatory processes. In this chapter, we will focus first on chromatin structure. We will then turn to transcription factors—DNA-binding proteins similar in many ways to the prokaryotic proteins that we encountered in the preceding chapter. Eukaryotic transcription factors can act directly by interacting with the transcriptional machinery or indirectly by influencing chromatin structure. Finally, we examine selected posttranscriptional gene-regulatory mechanisms, including those based on microRNAs, an important class of gene-regulatory molecules discovered in recent years.

32.1 Eukaryotic DNA Is Organized into Chromatin

Eukaryotic DNA is tightly bound to a group of small basic proteins called *histones.* In fact, histones constitute half the mass of a eukaryotic chromosome. The entire complex of a cell's DNA and associated protein is called

Table 32.1 Highly expressed protein-encoding genes of the pancreas, liver, and embryonic stem cells (as percentage of total mRNA pool)

Rank	Proteins expressed in pancreas	%	Proteins expressed in liver	%	Proteins expressed in stem cells	%
1	Procarboxypeptidase A1	7.6	Albumin	3.5	Glyceraldehyde-3-phosphate dehydrogenase	0.7
2	Pancreatic trypsinogen 2	5.5	Apolipoprotein A-I	2.8	Translation elongation factor 1 α1	0.6
3	Chymotrypsinogen	4.4	Apolipoprotein C-I	2.5	Tubulin α	0.5
4	Pancreatic trypsin 1	3.7	Apolipoprotein C-III	2.1	Translationally controlled tumor protein	0.5
5	Elastase IIIB	2.4	ATPase 6/8	1.5	Cyclophilin A	0.4
6	Protease E	1.9	Cytochrome oxidase 3	1.1	Cofilin	0.4
7	Pancreatic lipase	1.9	Cytochrome oxidase 2	1.1	Nucleophosmin	0.3
8	Procarboxypeptidase B	1.7	α_1-Antitrypsin	1.0	Connexin 43	0.3
9	Pancreatic amylase	1.7	Cytochrome oxidase 1	0.9	Phosphoglycerate mutase	0.2
10	Bile-salt-stimulated lipase	1.4	Apolipoprotein E	0.9	Translation elongation factor 1 β2	0.2

Sources: Data for pancreas from V. E. Velculescu, L. Zhang, B. Vogelstein, and K. W. Kinzler. *Science* 270:484–487, 1995. Data for liver from T. Yamashita, S. Hashimoto, S. Kaneko, S. Nagai, N. Toyoda, T. Suzuki, K. Kobayashi, and K. Matsushima. *Biochem. Biophys. Res. Commun.* 269:110–116, 2000. Data for stem cells from M. Richards, S. P. Tan, J. H. Tan, W. K. Chan, and A. Bongso. *Stem Cells* 22:51–64, 2004.

chromatin. Chromatin serves to compact and organize eukaryotic DNA and its presence has dramatic consequences for gene regulation.

Nucleosomes are complexes of DNA and histones

Chromatin is made up of repeating units, each containing 200 bp of DNA and two copies each of four histone proteins H2A, H2B, H3, and H4. Histones have strikingly basic properties because a quarter of the residues in each histone are either arginine or lysine, positively charged amino acids that strongly interact with the negatively charged DNA. The protein complex is called the *histone octamer*. The repeating units of the histone octamer and the associated DNA are known as *nucleosomes*. Chromatin viewed with the electron microscope has the appearance of beads on a string (see Figure 32.2); each bead has a diameter of approximately 100 Å. Partial digestion of chromatin with DNase yields the isolated beads. These particles consist of fragments of DNA about 200 bp in length bound to the histone octamer. More-extensive digestion yields a shorter DNA fragment of 145 bp bound to the octamer. The smaller complex formed by the histone octamer and the 145-bp DNA fragment is the *nucleosome core particle*. The DNA connecting core particles in undigested chromatin is called linker DNA. Histone H1 binds, in part, to the linker DNA.

DNA wraps around histone octamers to form nucleosomes

The overall structure of the nucleosome was revealed through electron microscopic and x-ray crystallographic studies pioneered by Aaron Klug and his colleagues. More recently, the three-dimensional structure of a reconstituted nucleosome core particle (Figure 32.3) was determined to higher resolution by x-ray diffraction methods. The four types of histone

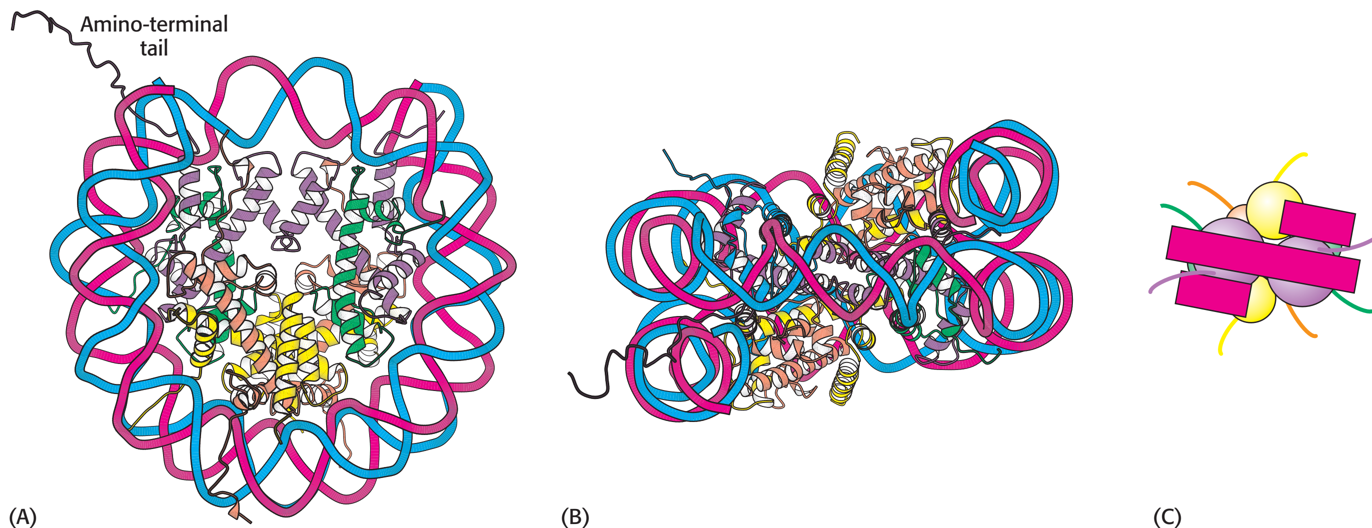


Figure 32.3 Nucleosome core particle. The structure consists of a core of eight histone proteins surrounded by DNA. (A) A view showing the DNA wrapping around the histone core. (B) A view related to that in part A by a 90-degree rotation. *Notice* that the DNA forms a left-handed superhelix as it wraps around the core. (C) A schematic view. [Drawn from 1AOI.pdb.]

that make up the protein core are homologous and similar in structure (Figure 32.4). The eight histones in the core are arranged into a $(H3)_2(H4)_2$ tetramer and a pair of H2A–H2B dimers. The tetramer and dimers come together to form a left-handed superhelical ramp around which the DNA wraps. In addition, each histone has an amino-terminal tail that extends out from the core structure. These tails are flexible and contain a number of lysine and arginine residues. As we shall see, *covalent modifications of these tails play an essential role in regulating gene expression.*

The DNA forms a left-handed superhelix as it wraps around the outside of the histone octamer. The protein core forms contacts with the inner surface of the DNA superhelix at many points, particularly along the phosphodiester backbone and the minor groove. Nucleosomes will form on almost all DNA sites, although some sequences are preferred because the dinucleotide steps are properly spaced to favor bending around the histone core. A histone with a different structure from that of the others, called

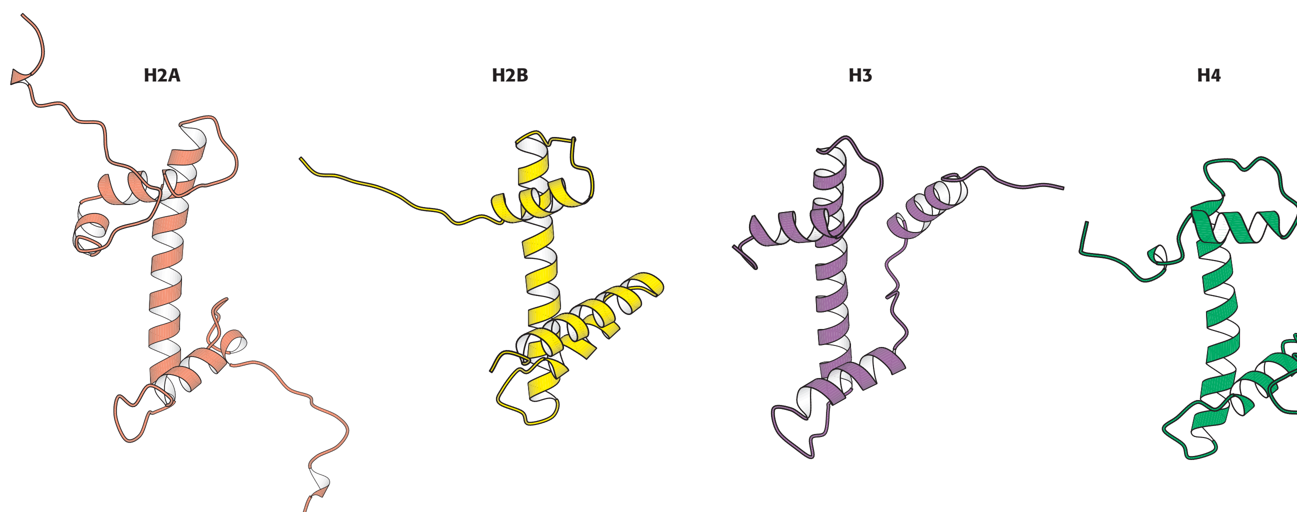


Figure 32.4 Homologous histones. Histones H2A, H2B, H3, and H4 adopt a similar three-dimensional structure as a consequence of common ancestry. Some parts of the tails at the termini of the proteins are not shown. [Drawn from 1AOI.pdb.]

histone H1, seals off the nucleosome at the location at which the linker DNA enters and leaves. The amino acid sequences of histones, including their amino-terminal tails, are remarkably conserved from yeast to human beings.

The winding of DNA around the nucleosome core contributes to the packing of DNA by decreasing its linear extent. An extended 200-bp stretch of DNA would have a length of about 680 Å. Wrapping this DNA around the histone octamer reduces the length to approximately 100 Å along the long dimension of the nucleosome. Thus the DNA is compacted by a factor of 7. However, human chromosomes in metaphase, which are highly condensed, are compacted by a factor of 10^4. Clearly, the nucleosome is just the first step in DNA compaction. What is the next step? The nucleosomes themselves are arranged in a helical array approximately 360 Å across, forming a series of stacked layers approximately 110 Å apart (Figure 32.5). The folding of these fibers of nucleosomes into loops further compacts DNA.

The wrapping of DNA around the histone octamer as a left-handed helix also stores negative supercoils; if the DNA in a nucleosome is straightened out, the DNA will be underwound. This underwinding is exactly what is needed to separate the two DNA strands during replication and transcription.

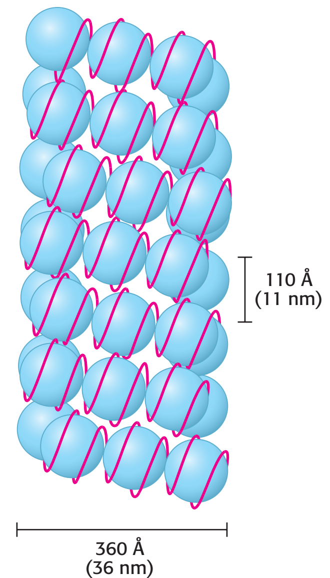


Figure 32.5 Higher-order chromatin structure. A proposed model for chromatin arranged in a helical array consisting of six nucleosomes per turn of helix. The DNA double helix (shown in red) is wound around each histone octamer (shown in blue). [After J. T. Finch and A. Klug. *Proc. Natl. Acad. Sci. U. S. A.* 73:1897–1901, 1976.]

32.2 Transcription Factors Bind DNA and Regulate Transcription Initiation

DNA-binding *transcription factors* are key to gene regulation in eukaryotes, just as they are in prokaryotes. However, the roles of eukaryotic transcription factors are different in several ways. First, whereas the DNA-binding sites crucial for the control of gene expression in prokaryotes are usually quite close to promoters, those in eukaryotes can be farther away from promoters and can exert their action at a distance. Second, most prokaryotic genes are regulated by single transcription factors, and multiple genes in a pathway are expressed in a coordinated fashion because such genes are often transcribed as part of a polycistronic mRNA. In eukaryotes, the expression of each gene is typically controlled by multiple transcription factors, and the coordinated expression of different genes depends on having similar transcription-factor-binding sites in each gene in the set. Third, in prokaryotes, transcription factors usually interact directly with RNA polymerase. In eukaryotes, some transcription factors interact directly with RNA polymerase, whereas others interact with other proteins associated with RNA polymerase and still others act by modifying the chromatin structure. Let us now examine eukaryotic transcription factors in more detail.

Eukaryotic transcription factors usually consist of several domains. The *DNA-binding domain* binds to regulatory sequences that can either be adjacent to the promoter or at some distance from it. Most commonly, transcription factors include additional domains that help activate transcription. When a transcription factor is bound to the DNA, its *activation domain* promotes transcription by interacting with RNA polymerase II, by interacting with other associated proteins, or by modifying the local structure of chromatin.

A range of DNA-binding structures are employed by eukaryotic DNA-binding proteins

The structures of many eukaryotic DNA-binding proteins have been determined and a range of structural motifs have been observed, but we will focus on three that reveal the common features and the diversity of these

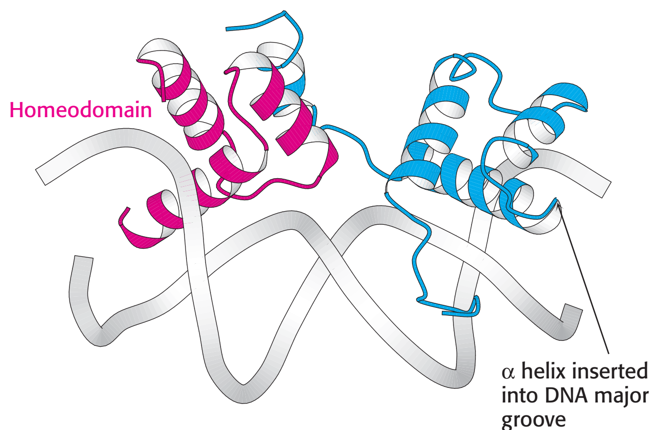


Figure 32.6 Homeodomain structure. The structure of a heterodimer formed from two different DNA-binding domains, each based on a homeodomain. *Notice* that each homeodomain has a helix-turn-helix motif with one helix inserted into the major groove of DNA. [Drawn from 1AKH.pdb.]

motifs. The first class of eukaryotic DNA-binding unit that we will consider is the *homeodomain* (Figure 32.6). The structure of this domain and its mode of recognition of DNA are very similar to those of the prokaryotic helix-turn-helix proteins. In eukaryotes, homeodomain proteins often form heterodimeric structures, sometimes with other homeodomain proteins, that recognize asymmetric DNA sequences.

The second class of eukaryotic DNA-binding unit comprises the *basic-leucine zipper* (*b*Zip) *proteins* (Figure 32.7). This DNA-binding unit consists of a pair of long α helices. The first part of each α helix is a basic region that lies in the major groove of the DNA and makes contacts responsible for DNA-site recognition. The second part of each α helix forms a coiled-coil structure with its partner. Because these units are often stabilized by appropriately spaced leucine residues, these structures are often referred to as *leucine zippers*.

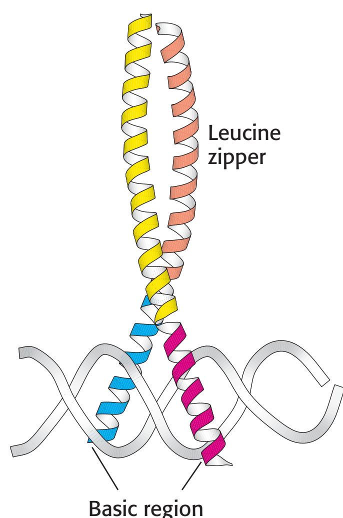


Figure 32.7 Basic-leucine zipper. This heterodimer comprises two basic-leucine zipper proteins. *Notice* that the basic region lies in the major groove of DNA. The leucine-zipper part stabilizes the protein dimer. [Drawn from 1FOS.pdb.]

The final class of eukaryotic DNA-binding units that we will consider here are the *Cys_2His_2 zinc-finger domains* (Figure 32.8). A DNA-binding unit of this class comprises tandem sets of small domains, each of which binds a zinc ion through conserved sets of two cysteine and two histidine residues. These domains, often called *zinc-finger domains,* form a string that follows the major groove of DNA. An α helix from each domain makes specific contact with the edges of base pairs within the groove. Some proteins contain arrays of 10 or more zinc-finger domains, potentially enabling them to contact long stretches of DNA. The human genome encodes several hundred proteins that contain zinc-finger domains of this class. We will encounter another class of zinc-based DNA-binding domain when we consider nuclear hormone receptors in Section 32.3.

Activation domains interact with other proteins

The activation domains of transcription factors generally recruit other proteins that promote transcription. In some cases, these activation domains interact directly with RNA polymerase II or closely associated proteins. The activation domains act through intermediary proteins that bridge between the transcription factors and the polymerase. An important target of activators is *mediator,* a complex of 25 to 30 subunits conserved from

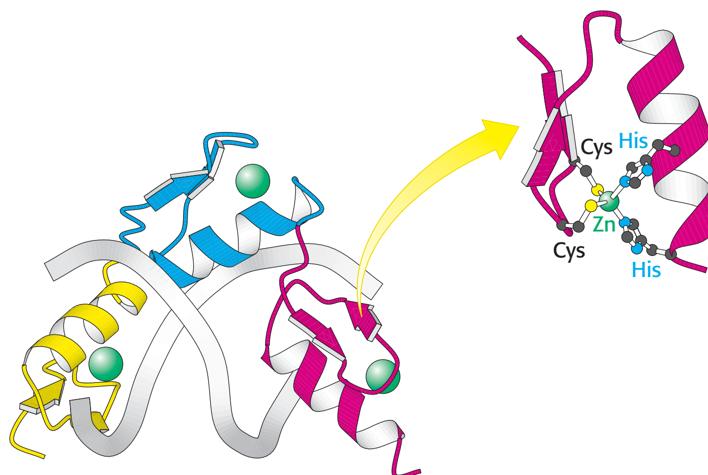


Figure 32.8 Zinc-finger domains. A DNA-binding domain comprising three Cys_2His_2 zinc-finger domains (shown in yellow, blue, and red) is shown in a complex with DNA. Each zinc-finger domain is stabilized by a bound zinc ion (shown in green) through interactions with two cysteine residues and two histidine residues. *Notice* how the protein wraps around the DNA in the major groove. [Drawn from 1AAY.pdb.]

yeast to human beings, that acts as a bridge between transcription factors and promoter-bound RNA polymerase II (Figure 32.9).

Activation domains are less conserved than DNA-binding domains. In fact, very little sequence similarity has been found. For example, they may be acidic, hydrophobic, glutamine rich, or proline rich. However, certain features are common to activation domains. First, they are often *redundant;* that is, a part of the activation domain can be deleted without loss of function. Second, they are *modular* and can activate transcription when paired with a variety of DNA-binding domains. Third, activation domains can act *synergistically:* two activation domains acting together create a stronger effect than either acting separately.

We have been considering the case in which gene control increases the expression level of a gene. In many cases, the expression of a gene must be decreased by blocking transcription. The agents in such cases are *transcriptional repressors*. Like activators, transcriptional repressors act in many cases by altering chromatin structure.

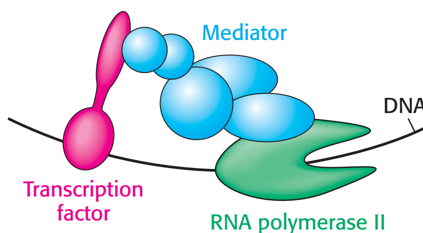


Figure 32.9 Mediator. Mediator, a large complex of protein subunits, acts as a bridge between transcription factors bearing activation domains and RNA polymerase II. These interactions help recruit and stabilize RNA polymerase II near specific genes that are then transcribed.

Multiple transcription factors interact with eukaryotic regulatory regions

The basal transcription complex described in Chapter 29 initiates transcription at a low frequency. Recall that several general transcription factors join with RNA polymerase II to form the basal transcription complex. Additional transcription factors must bind to other sites that can be near the promoter or quite distant for a gene to achieve a higher rate of mRNA synthesis. In contrast with the regulators of prokaryotic transcription, few eukaryotic transcription factors have any effect on transcription on their own. Instead, each factor recruits other proteins to build up large complexes that interact with the transcriptional machinery to activate transcription.

A major advantage of this mode of regulation is that a given regulatory protein can have different effects, depending on what other proteins are present in the same cell. This phenomenon, called *combinatorial control,* is crucial to multicellular organisms that have many different cell types. Even in unicellular eukaryotes such as yeast, combinatorial control allows the generation of distinct cell types.

Enhancers can stimulate transcription in specific cell types

Transcription factors can often act even if their binding sites lie at a considerable distance from the promoter. These distant regulatory sites are called *enhancers* (Chapter 29). Enhancers function by serving as binding sites for specific transcription factors. An enhancer is effective only in the specific cell types in which appropriate regulatory proteins are expressed. In many cases, these DNA-binding proteins influence transcription initiation by perturbing the local chromatin structure to expose a gene or its regulatory sites rather than by direct interactions with RNA polymerase. This mechanism accounts for the ability of enhancers to act at a distance.

The properties of enhancers are illustrated by studies of the enhancer controlling the muscle isoform of creatine kinase (Figure 32.10). The

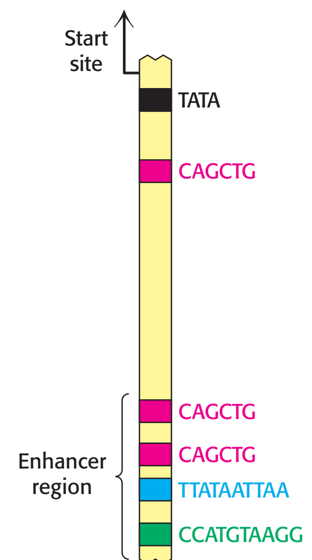


Figure 32.10 Enhancer binding sites. A schematic structure for the region 1 kb upstream of the start site for the muscle creatine kinase gene. One binding site of the form 5′-CAGCTG-3′ is present near the TATA box. The enhancer region farther upstream contains two binding sites for the same protein and two additional binding sites for other proteins.

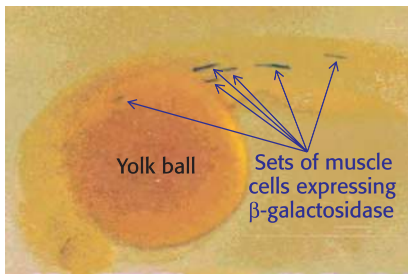


Figure 32.11 An experimental demonstration of enhancer function. A promoter for muscle creatine kinase artificially drives the transcription of β-galactosidase in a zebrafish embryo. Only specific sets of muscle cells produce β-galactosidase, as visualized by the formation of the blue product on treatment of the embryo with X-Gal. [From F. Müller, D. W. Williamson, J. Kobolák, L. Gauvry, G. Goldspink, L. Orbán, and N. MacLean. *Mol. Reprod. Dev.* 47:404–412, 1997.]

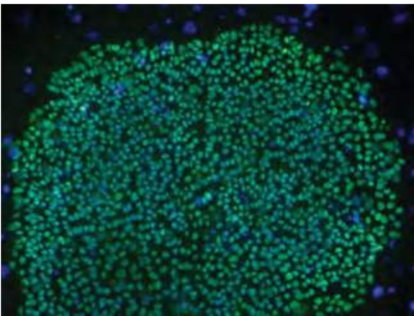


Figure 32.12 Induced pluripotent stem cells. A micrograph of human induced pluripotent stem cells stained green for a transcription factor that is characteristic of pluripotent cells. [From K. Takahashi et al., *Cell* 131:861–872, 2007; with permission from Elsevier, courtesy of Shinya Yamanaka, Kyoto University.]

results of mutagenesis and other studies revealed the presence of an enhancer located between 1350 and 1050 base pairs upstream of the start site of the gene for this enzyme. Experimentally inserting this enhancer near a gene not normally expressed in muscle cells is sufficient to cause the gene to be expressed at high levels in muscle cells but not in other cells (Figure 32.11).

Induced pluripotent stem cells can be generated by introducing four transcription factors into differentiated cells

An important application illustrating the power of transcription factors is the development of *induced pluripotent stem* (iPS) *cells.* Pluripotent stem cells have the ability to differentiate into many different cell types on appropriate treatment. Previously isolated cells derived from embryos show a very high degree of pluripotency. Over time, researchers identified dozens of genes in embryonic stem cells that contributed to this pluripotency when expressed. In a remarkable experiment reported for mouse cells in 2006 and human cells in 2007, Shinya Yamanaka demonstrated that just four genes out of this entire set could induce pluripotency in already-differentiated skin cells. Yamanaka introduced genes encoding four transcription factors into skin cells called fibroblasts. The fibroblasts de-differentiated into cells that appeared to have characteristics very nearly identical with those of embryonic stem cells (Figure 32.12).

These iPS cells represent powerful new research tools and, potentially, a new class of therapeutic agents. The proposed concept is that a sample of a patient's fibroblasts could be readily isolated and converted into iPS cells. These iPS cells could then be treated to differentiate into a desired cell type that could then be transplanted into the patient. For example, such an approach might be used to restore a particular class of nerve cells that had been depleted by a neurodegenerative disease. Although the field of iPS cell research is still in its very early stages, it holds great promise as a possible approach to treatment for many common and difficult-to-treat diseases.

32.3 The Control of Gene Expression Can Require Chromatin Remodeling

Early observations suggested that chromatin structure plays a major role in controlling eukaryotic gene expression. DNA that is densely packaged into chromatin is less susceptible to cleavage by the nonspecific DNA-cleaving enzyme DNase I. Regions adjacent to genes that are being transcribed are more sensitive to cleavage by DNase I than are other sites in the genome, suggesting that the DNA in these regions is less compacted than it is elsewhere and more accessible to proteins. In addition, some sites, usually within 1 kb of the start site of an active gene, are exquisitely sensitive to DNase I and other nucleases. These *hypersensitive sites* correspond to regions that have few nucleosomes or contain nucleosomes in an altered conformational state. *Hypersensitive sites are cell-type specific and developmentally regulated*. For example, globin genes in the precursors of erythroid cells from 20-hour-old chicken embryos are insensitive to DNase I. However, when hemoglobin synthesis begins at 35 hours, regions adjacent to these genes become highly susceptible to digestion. In tissues such as the brain that produce no hemoglobin, the globin genes remain resistant to DNase I throughout development and into adulthood. These studies suggest that a prerequisite for gene expression is a relaxing of the chromatin structure.

Recent experiments even more clearly revealed the role of chromatin structure in regulating access to DNA binding sites. Genes required for galactose utilization in yeast are activated by a transcription factor called GAL4, which recognizes DNA binding sites with two 5′-CGG-3′ sequences on complementary strands separated by 11 base pairs (Figure 32.13). Approximately 4000 potential GAL4 binding sites of the form 5′-CGG$(N)_{11}$CCG-3′ are present in the yeast genome, but only 10 of them regulate genes necessary for galactose metabolism. How is GAL4 targeted to only a small fraction of the potential binding sites? This question is addressed through the use of a technique called *chromatin immunoprecipitation* (ChIP; Figure 32.14). GAL4 is first cross-linked to its DNA binding sites in chromatin. The DNA is then fragmented into small pieces, and antibodies to GAL4 are used to isolate the chromatin fragments containing GAL4. The cross-linking is reversed, and the DNA is isolated and characterized. The results of these studies reveal that only approximately 10 of the 4000 potential GAL4 sites are occupied by GAL4 when the cells are growing on galactose; more than 99% of the sites appear to be blocked, presumably by the local chromatin structure. Thus, whereas in prokaryotes all sites appear to be equally accessible, chromatin structure shields a large number of the potential binding sites in eukaryotic cells. GAL4 is thereby prevented from binding to sites that are unimportant in galactose metabolism. These lines of evidence and others reveal that chromatin structure is altered in active genes compared with inactive ones.

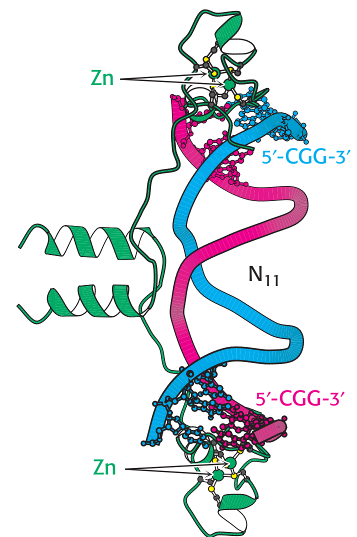
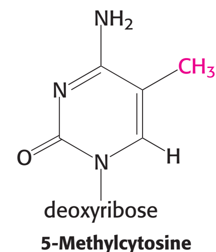


Figure 32.13 GAL4 binding sites. The yeast transcription factor GAL4 binds to DNA sequences of the form 5′-CGG$(N)_{11}$CCG-3′. Two zinc-based domains are present in the DNA-binding region of this protein. *Notice* that these domains contact the 5′-CGG-3′ sequences, leaving the center of the site uncontacted. [Drawn from 1D66.pdb.]

The methylation of DNA can alter patterns of gene expression

The degree of methylation of DNA provides another mechanism, in addition to packaging with histones, for inhibiting gene expression inappropriate to a specific cell type. Carbon 5 of cytosine can be methylated by specific methyltransferases. About 70% of the 5′-CpG-3′ sequences (where "p" represents the phosphate residue in the DNA backbone) in mammalian genomes are methylated. However, the distribution of these methylated cytosines varies, depending on the cell type. Consider the β-globin gene. In cells that are actively expressing hemoglobin, the region from approximately 1 kb upstream of the start site to approximately 100 bp downstream of the start site is less methylated than the corresponding region in cells that do not express this gene. The relative absence of 5-methylcytosines near the start site is referred to as *hypomethylation.* The methyl group of 5-methylcytosine protrudes into the major groove where it could easily interfere with the binding of proteins that stimulate transcription.



5-Methylcytosine

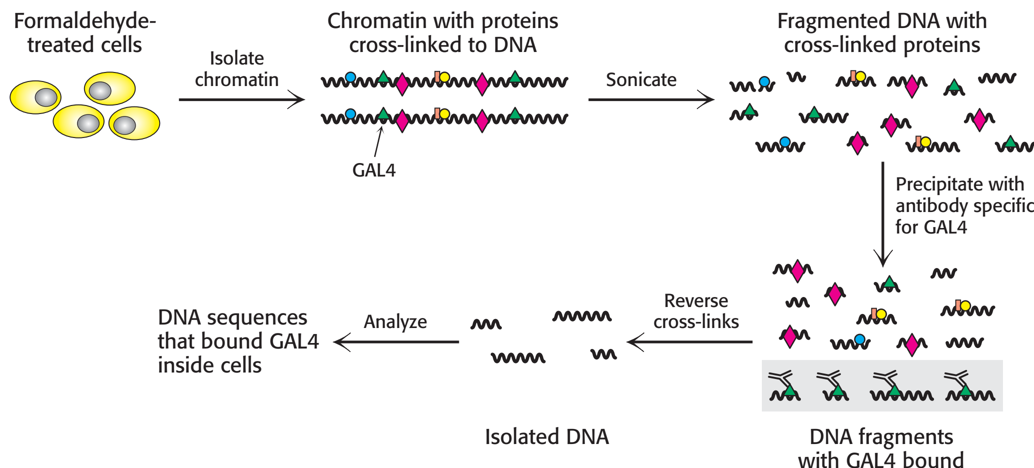
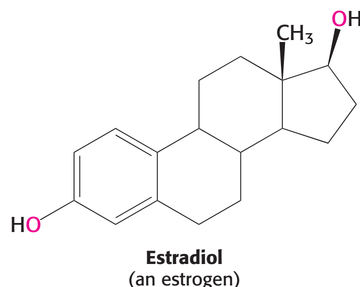


Figure 32.14 Chromatin immunoprecipitation. Cells or isolated nuclei are treated with a formaldehyde to cross-link proteins to DNA. The cells are then lysed and the DNA is fragmented by sonication. DNA fragments bound to a particular protein are isolated through the use of an antibody specific for that protein. The cross-links are then reversed and the DNA fragments are characterized.

The distribution of CpG sequences in mammalian genomes is not uniform. Many CpG sequences have been converted into TpG through mutation by the deamination of 5-methylcytosine to thymine. However, sites near the 5′ ends of genes have been maintained because of their role in gene expression. Thus, most genes are found in *CpG islands,* regions of the genome that contain approximately four times as many CpG sequences as does the remainder of the genome.

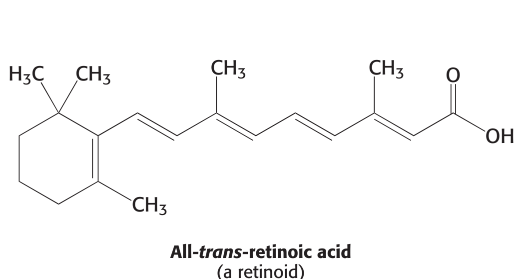


Estradiol
(an estrogen)

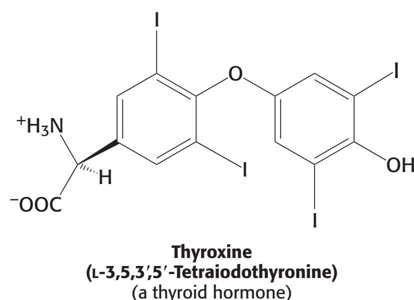
Steroids and related hydrophobic molecules pass through membranes and bind to DNA-binding receptors

We next look at an example that illustrates how transcription factors can stimulate changes in chromatin structure that affect transcription. We will consider in some detail the system that detects and responds to estrogens. Synthesized and released by the ovaries, *estrogens,* such as estradiol, are cholesterol-derived steroid hormones (Section 26.4). They are required for the development of female secondary sex characteristics and, along with progesterone, participate in the ovarian cycle.

Because they are hydrophobic molecules, estrogens easily diffuse across cell membranes. When inside a cell, estrogens bind to highly specific, soluble receptor proteins. Estrogen receptors are members of a large family of proteins that act as receptors for a wide range of hydrophobic molecules, including other steroid hormones, thyroid hormones, and retinoids.



All-*trans*-retinoic acid
(a retinoid)



Thyroxine
(L-3,5,3′,5′-Tetraiodothyronine)
(a thyroid hormone)

The human genome encodes approximately 50 members of this family, often referred to as *nuclear hormone receptors.* The genomes of other multicellular eukaryotes encode similar numbers of nuclear hormone receptors, although they are absent in yeast.

All these receptors have a similar mode of action. On binding of the signal molecule (called, generically, a *ligand*), the ligand–receptor complex modifies the expression of specific genes by binding to control elements in the DNA. Estrogen receptors bind to specific DNA sites (referred to as *estrogen response elements* or EREs) that contain the consensus sequence 5′-**AGGTCA**NNN**TGACCT**-3′. As expected from the symmetry of this sequence, an estrogen receptor binds to such sites as a dimer.

A comparison of the amino acid sequences of members of this family reveals two highly conserved domains: a DNA-binding domain and a ligand-binding domain (Figure 32.15). The DNA-binding domain lies toward the center of the molecule and consists of a set of zinc-based domains different from the Cys_2His_2 zinc-finger proteins introduced in Section 32.2. These zinc-based domains bind to specific DNA sequences by virtue of an α helix that lies in the major groove in the specific DNA complexes formed by estrogen receptors.

Nuclear hormone receptors regulate transcription by recruiting coactivators to the transcription complex

The second highly conserved domain of the nuclear receptor proteins lies near the carboxyl terminus and is the ligand-binding site. This domain folds

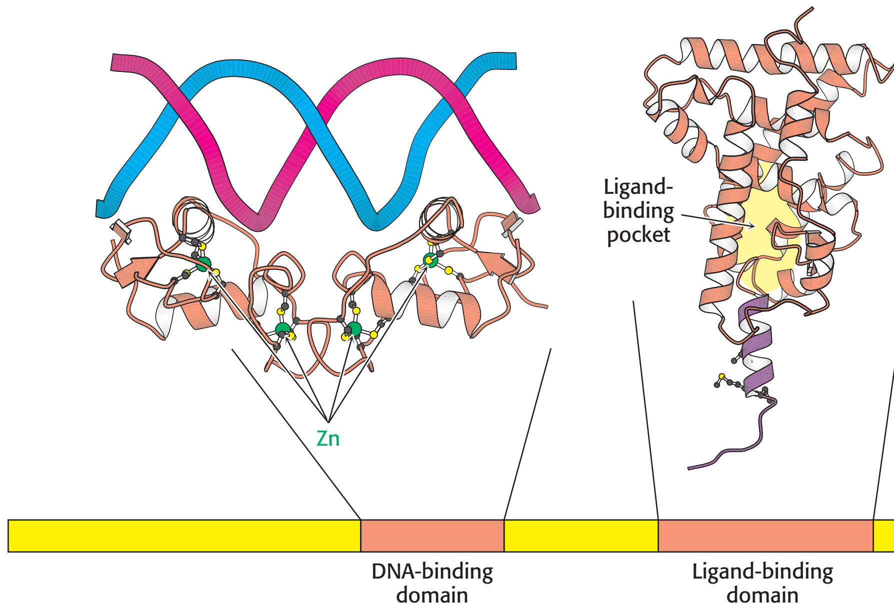


Figure 32.15 Structure of two nuclear hormone-receptor domains. Nuclear hormone receptors contain two crucial conserved domains: (1) a DNA-binding domain toward the center of the sequence and (2) a ligand-binding domain toward the carboxyl terminus. The structure of a dimer of the DNA-binding domain bound to DNA is shown, as is one monomer of the normally dimeric ligand-binding domain. [Drawn from 1HCQ and 1LBD.pdb.]

into a structure that consists almost entirely of α helices, arranged in three layers. The ligand binds in a hydrophobic pocket that lies in the center of this array of helices (Figure 32.16). This domain changes conformation when it binds its ligand, estrogen. How does ligand binding lead to changes in gene expression? The simplest model would have the binding of ligand alter the DNA-binding properties of the receptor, analogously to the *lac* repressor in prokaryotes. However, experiments with purified nuclear hormone receptors revealed that ligand binding does *not* significantly alter DNA-binding affinity and specificity. Another mechanism is operative.

Because ligand binding does not alter the ability of nuclear hormone receptors to bind DNA, investigators sought to determine whether specific proteins might bind to the nuclear hormone receptors only in the presence of ligand. Such searches led to the identification of several related proteins called *coactivators,* such as SRC-1 (*s*teroid *r*eceptor *c*oactivator-1), GRIP-1 (*g*lucocorticoid *r*eceptor *i*nteracting *p*rotein-1), and NcoA-1 (*n*uclear hormone receptor *coa*ctivator-1). These coactivators are referred to as the

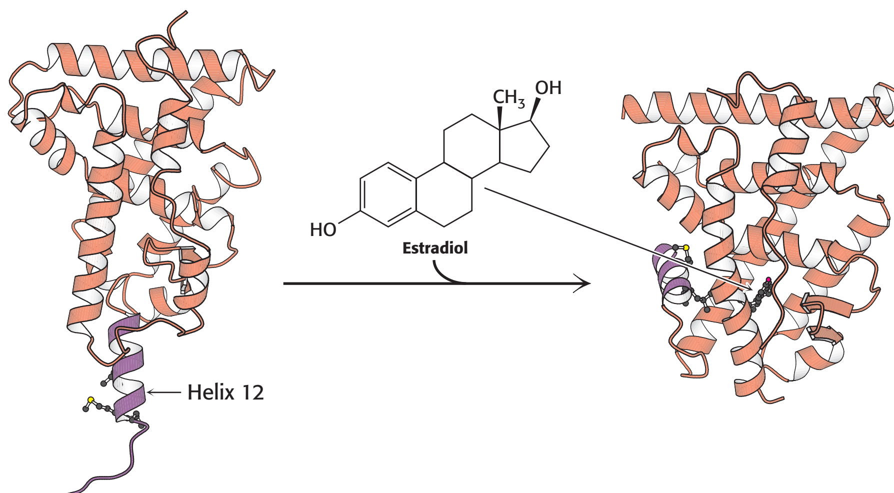


Figure 32.16 Ligand binding to nuclear hormone receptor. The ligand lies completely surrounded within a pocket in the ligand-binding domain. *Notice* that the last α helix, helix 12 (shown in purple), folds into a groove on the side of the structure on ligand binding. [Drawn from 1LDB and 1ERE.pdb.]

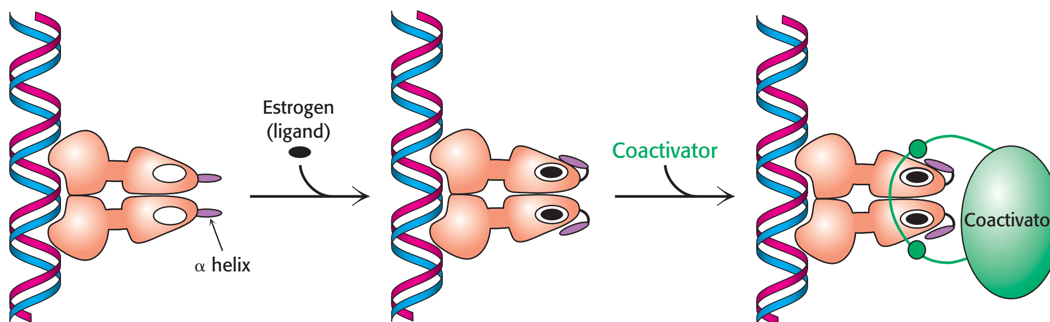
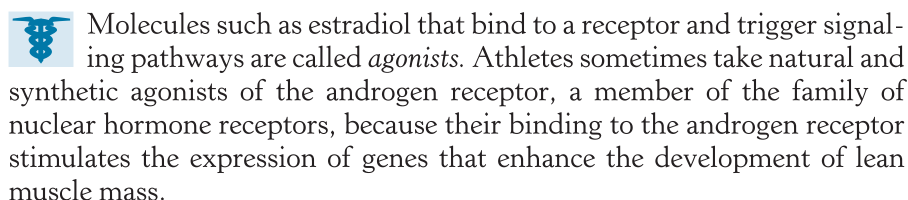


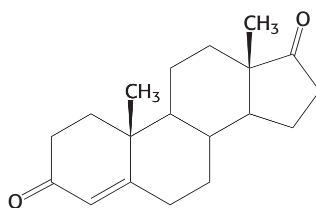
Figure 32.17 Coactivator recruitment. The binding of ligand to a nuclear hormone receptor induces a conformational change in the ligand-binding domain. This change in conformation generates favorable sites for the binding of a coactivator.

p160 family because of their size. The binding of ligand to the receptor induces a conformational change that allows the recruitment of a coactivator (Figure 32.17). In many cases, these coactivators are enzymes that catalyze reactions that lead to the modification of chromatin structure.

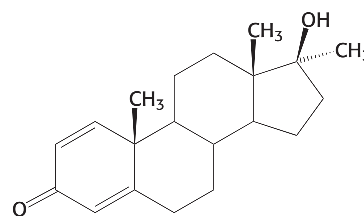
Steroid-hormone receptors are targets for drugs



Molecules such as estradiol that bind to a receptor and trigger signaling pathways are called *agonists.* Athletes sometimes take natural and synthetic agonists of the androgen receptor, a member of the family of nuclear hormone receptors, because their binding to the androgen receptor stimulates the expression of genes that enhance the development of lean muscle mass.



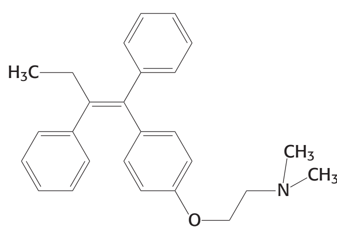
Androstendione
(a natural androgen)



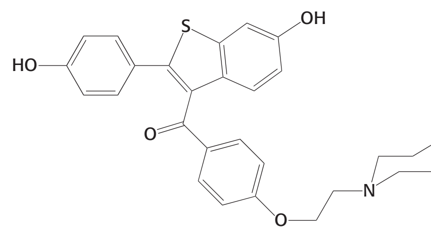
Dianabol
(methandrostenolone)
(a synthetic androgen)

Referred to as *anabolic steroids,* such compounds used in excess are not without side effects. In men, excessive use leads to a decrease in the secretion of testosterone, to testicular atrophy, and sometimes to breast enlargement (gynecomastia) if some of the excess androgen is converted into estrogen. In women, excess testosterone causes a decrease in ovulation and estrogen secretion; it also causes breast regression and growth of facial hair.

Other molecules bind to nuclear hormone receptors but do not effectively trigger signaling pathways. Such compounds are called *antagonists* and are, in many ways, like competitive inhibitors of enzymes. Some important drugs are antagonists that target the estrogen receptor. For example, *tamoxifen* and *raloxifene* are used in the treatment and prevention of breast cancer, because some breast tumors rely on estrogen-mediated pathways for growth. Because some of these compounds have distinct effects on different forms of the estrogen receptor, they are referred to as *selective estrogen receptor modulators* (SERMs).



Tamoxifen



Raloxifene

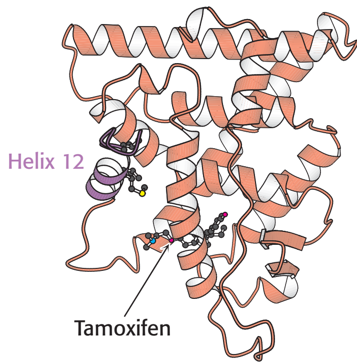


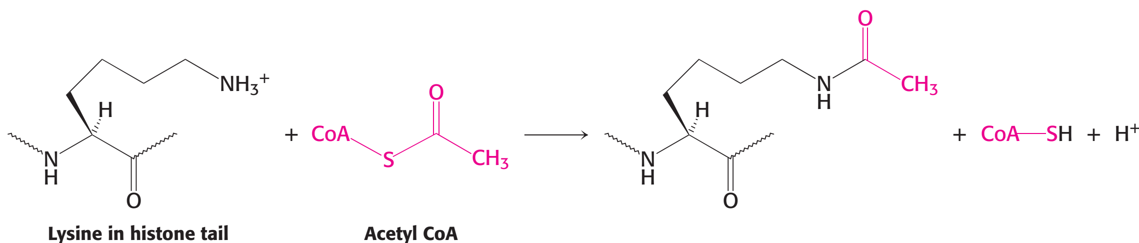
Figure 32.18 Estrogen receptor–tamoxifen complex. Tamoxifen binds in the pocket normally occupied by estrogen. However, *notice* that part of the tamoxifen structure extends from this pocket, and so helix 12 cannot pack in its usual position. Instead, this helix blocks the coactivator-binding site. [Drawn from 3ERT.pdb.]

The determination of the structures of complexes between the estrogen receptor and these drugs revealed the basis for their antagonist effect (Figure 32.18). Tamoxifen binds to the same site as estradiol does. However, tamoxifen has a group that extends out of the normal ligand-binding pocket, as do other antagonists. These groups block the normal conformational changes induced by estrogen. Tamoxifen blocks the binding of coactivators and thus inhibits the activation of gene expression.

Chromatin structure is modulated through covalent modifications of histone tails

We have seen that nuclear receptors respond to signal molecules by recruiting coactivators. Now we can ask, How do coactivators modulate transcriptional activity? *These proteins act to loosen the histone complex from the DNA, exposing additional DNA regions to the transcription machinery.*

Much of the effectiveness of coactivators appears to result from their ability to covalently modify the amino-terminal tails of histones as well as regions on other proteins. Some of the p160 coactivators and the proteins that they recruit catalyze the transfer of acetyl groups from acetyl CoA to specific lysine residues in these amino-terminal tails.



Enzymes that catalyze such reactions are called *histone acetyltransferases* (HATs). The histone tails are readily extended; so they can fit into the HAT active site and become acetylated (Figure 32.19).

What are the consequences of histone acetylation? Lysine bears a positively charged ammonium group at neutral pH. The addition of an acetyl group generates an uncharged amide group. This change dramatically reduces the affinity of the tail for DNA and modestly decreases the affinity of the entire histone complex for DNA, loosening the histone complex from the DNA.

In addition, the acetylated lysine residues interact with a specific *acetyllysine-binding domain* that is present in many proteins that regulate eukaryotic transcription. This domain, termed a *bromodomain*, comprises approximately 110 amino acids that form a four-helix bundle containing a peptide-binding site at one end (Figure 32.20).

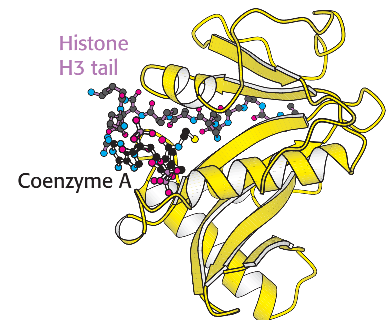


Figure 32.19 Structure of histone acetyltransferase. The amino-terminal tail of histone H3 extends into a pocket in which a lysine side chain can accept an acetyl group from acetyl CoA bound in an adjacent site. [Drawn from 1QSN.pdb.]

Figure 32.20 Structure of a bromodomain. This four-helix-bundle domain binds peptides containing acetyllysine. *Notice* that an acetylated peptide from histone H4 is bound in the structure. [Drawn from 1EGI.pdb.]

Figure 32.21 Chromatin remodeling. Eukaryotic gene regulation begins with an activated transcription factor bound to a specific site on DNA. One scheme for the initiation of transcription by RNA polymerase II requires five steps: (1) recruitment of a coactivator, (2) acetylation of lysine residues in the histone tails, (3) binding of a remodeling-engine complex to the acetylated lysine residues, (4) ATP-dependent remodeling of the chromatin structure to expose a binding site for RNA polymerase or for other factors, and (5) recruitment of RNA polymerase. Only two subunits are shown for each complex, although the actual complexes are much larger. Other schemes are possible.

Bromodomain-containing proteins are components of two large complexes essential for transcription. One is a complex of more than 10 polypeptides that binds to the *TATA-box-binding protein*. Recall that the TATA-box-binding protein is an essential transcription factor for many genes (Section 29.2). Proteins that bind to the TATA-box-binding protein are called *TAFs* (for *TATA-box-binding protein associated factors*). In particular, TAF1 contains a pair of bromodomains near its carboxyl terminus. The two domains are oriented such that each can bind one of two acetyllysine residues at positions 5 and 12 in the histone H4 tail. Thus, *acetylation of the histone tails provides a mechanism for recruiting other components of the transcriptional machinery.*

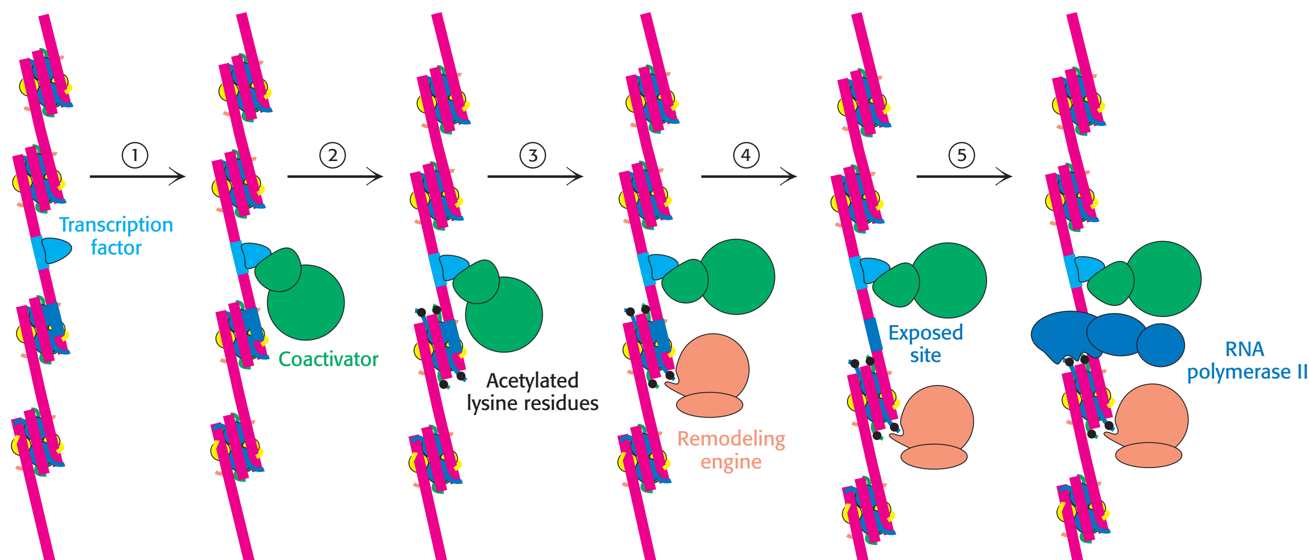
Bromodomains are also present in some components of large complexes known as *chromatin-remodeling complexes* or *chromatin-remodeling engines*. These complexes, which also contain domains homologous to those of helicases, utilize the free energy of ATP hydrolysis to shift the positions of nucleosomes along the DNA and to induce other conformational changes in chromatin (Figure 32.21). Histone acetylation can lead to a reorganization of the chromatin structure, potentially exposing binding sites for other factors. *Thus, histone acetylation can activate transcription through a combination of three mechanisms: by reducing the affinity of the histones for DNA, by recruiting other components of the transcriptional machinery, and by initiating the remodeling of the chromatin structure.*

Nuclear hormone receptors also include regions that interact with components of the mediator complex. Thus, two mechanisms of gene regulation can work in concert. Modification of histones and chromatin remodeling can open up regions of chromatin into which the transcription complex can be recruited through protein–protein interactions.

Histone deacetylases contribute to transcriptional repression

Just as in prokaryotes, some changes in a cell's environment lead to the repression of genes that had been active. The modification of histone tails again plays an important role. However, in repression, a key reaction appears to be the deacetylation of acetylated lysine, catalyzed by specific *histone deacetylase* enzymes.

In many ways, the acetylation and deacetylation of lysine residues in histone tails (and, likely, in other proteins) is analogous to the phosphorylation and dephosphorylation of serine, threonine, and tyrosine residues in other



stages of signaling processes. Like the addition of phosphoryl groups, the addition of acetyl groups can induce conformational changes and generate novel binding sites. Without a means of removal of these groups, however, these signaling switches will become stuck in one position and lose their effectiveness. Like phosphatases, deacetylases help reset the switches.

Acetylation is not the only modification of histones and other proteins in gene-regulation processes. The methylation of specific lysine and arginine residues also can be important. Some of the more common modifications are shown in Table 32.2. The elucidation of the roles of these processes is a very active area of research at present. The relation between various histone modifications and their roles in controlling gene expression is sometimes referred to as "the histone code." Although important generalizations have been discovered, this code clearly is subtle and complicated rather than being a set of hard and fast rules.

Table 32.2 Selected histone modifications

Modification	Associated with
H4 K8 acetylation	Activation
H3 K14 acetylation	Activation
H3 K27 monomethylation	Activation
H3 K27 trimethylation	Repression
H3 R17 methylation	Activation
H2B S14 phosphorylation	DNA repair
H2B K120 ubiquitination	Activatiom

32.4 Eukaryotic Gene Expression Can Be Controlled at Posttranscriptional Levels

Just as in prokaryotes, gene expression in eukaryotes can be regulated subsequent to transcription. We shall consider two examples. The first is the regulation of genes taking part in iron metabolism through key features in RNA secondary structure, similar in many ways to prokaryotic posttranscriptional regulation (Section 31.4). The second entails an entirely new mechanism, first glimpsed with the discovery of RNA interference (p. 165). Certain small regulatory RNA molecules allow the regulation of gene expression through interaction with a range of mRNA molecules. Remarkably, this mechanism, discovered only recently, affects the expression of approximately 60% of all human genes.

Genes associated with iron metabolism are translationally regulated in animals

RNA secondary structure plays a role in the regulation of iron metabolism in eukaryotes. Iron is an essential nutrient, required for the synthesis of hemoglobin, cytochromes, and many other proteins. However, excess iron can be quite harmful because, untamed by a suitable protein environment, iron can initiate a range of free-radical reactions that damage proteins, lipids, and nucleic acids. Animals have evolved sophisticated systems for the accumulation of iron in times of scarcity and for the safe storage of excess iron for later use. Key proteins include *transferrin,* a transport protein that carries iron in the serum, *transferrin receptor,* a membrane protein that binds iron-loaded transferrin and initiates its entry into cells, and *ferritin,* an impressively efficient iron-storage protein found primarily in the liver and kidneys. Twenty-four ferritin polypeptides form a nearly spherical shell that encloses as many as 2400 iron atoms, a ratio of one iron atom per amino acid (Figure 32.22).

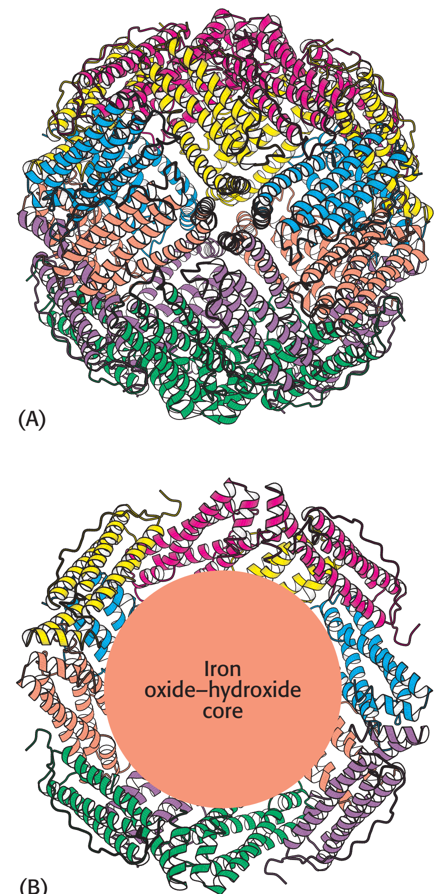


Figure 32.22 Structure of ferritin. (A) Twenty-four ferritin polypeptides form a nearly spherical shell. (B) A cutaway view reveals the core that stores iron as an iron oxide–hydroxide complex [Drawn from 1IES.pdb.]

Ferritin and transferrin-receptor expression levels are reciprocally related in their responses to changes in iron levels. When iron is scarce, the amount of transferrin receptor increases and little or no new ferritin is synthesized. Interestingly, the extent of mRNA synthesis for these proteins does not change correspondingly. Instead, regulation takes place at the level of translation.

Consider ferritin first. Ferritin mRNA includes a stem-loop structure termed an *iron-response element* (IRE) in its 5′ untranslated region (Figure 32.23). This stem-loop binds a 90-kd protein, called an *IRE-binding*

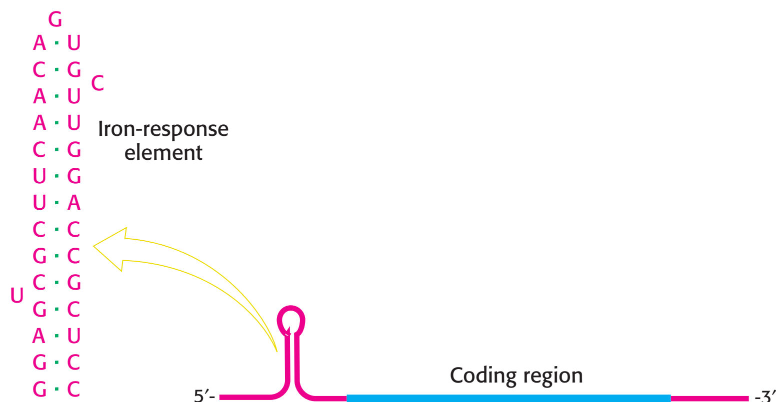


Figure 32.23 Iron-response element. Ferritin mRNA includes a stem-loop structure, termed an iron-response element (IRE), in its 5′ untranslated region. The IRE binds a specific protein that blocks the translation of this mRNA under low iron conditions.

protein (IRP), that blocks the initiation of translation as the IRE binds on the 5′ side of the coding region. When the iron level increases, the IRP binds iron as a 4Fe-4S cluster. The IRP bound to iron cannot bind RNA, because the binding sites for iron and RNA substantially overlap. Thus, in the presence of iron, ferritin mRNA is released from the IRP and translated to produce ferritin, which sequesters the excess iron.

An examination of the nucleotide sequence of transferrin-receptor mRNA reveals the presence of several IRE-like regions. However, these regions are located in the 3′ untranslated region rather than in the 5′ untranslated region (Figure 32.24). Under low-iron conditions, IRP binds to these IREs. However, given the location of these binding sites, the transferrin-receptor mRNA can still be translated. What happens when the iron level increases and the IRP no longer binds transferrin-receptor mRNA? Freed from the IRP, transferrin-receptor mRNA is rapidly degraded. Thus, an increase in the cellular iron level leads to the destruction of transferrin-receptor mRNA and, hence, a reduction in the production of transferrin-receptor protein.

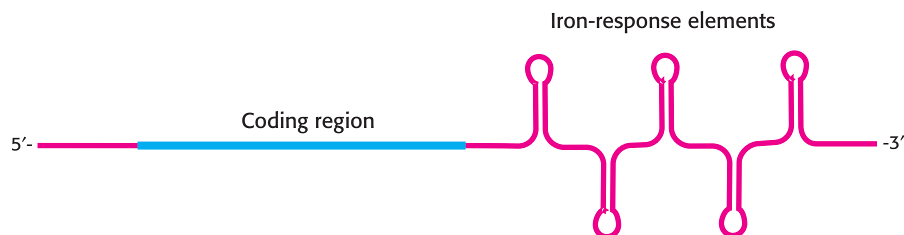
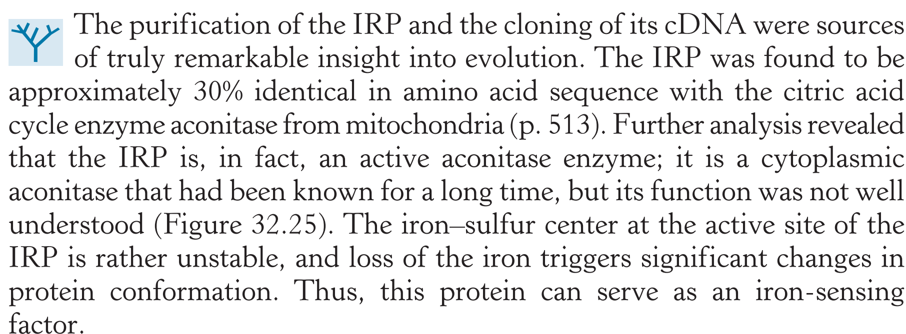


Figure 32.24 Transferrin-receptor mRNA. This mRNA has a set of iron-response elements (IREs) in its 3′ untranslated region. The binding of the IRE-binding protein to these elements stabilizes the mRNA but does not interfere with translation.



The purification of the IRP and the cloning of its cDNA were sources of truly remarkable insight into evolution. The IRP was found to be approximately 30% identical in amino acid sequence with the citric acid cycle enzyme aconitase from mitochondria (p. 513). Further analysis revealed that the IRP is, in fact, an active aconitase enzyme; it is a cytoplasmic aconitase that had been known for a long time, but its function was not well understood (Figure 32.25). The iron–sulfur center at the active site of the IRP is rather unstable, and loss of the iron triggers significant changes in protein conformation. Thus, this protein can serve as an iron-sensing factor.

Other mRNAs, including those taking part in heme synthesis, have been found to contain IREs. Thus, genes encoding proteins required for

iron metabolism acquired sequences that, when transcribed, provided binding sites for the iron-sensing protein. An environmental signal—the concentration of iron—controls the translation of proteins required for the metabolism of this metal. Thus, mutations in the untranslated region of mRNAs have been selected for beneficial regulation by iron levels.

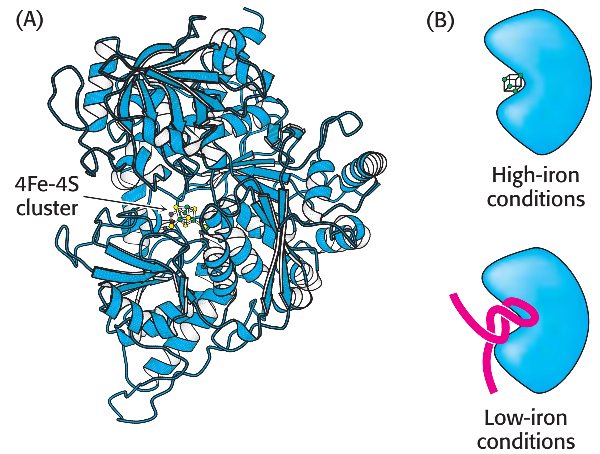


Figure 32.25 The IRP is an aconitase. (A) Aconitase contains an unstable 4Fe-4S cluster at its center. (B) Under conditions of low iron, the 4Fe-4S cluster dissociates and appropriate RNA molecules can bind in its place. [Drawn from 1C96.pdb.]

Small RNAs regulate the expression of many eukaryotic genes

Genetic studies of development in *C. elegans* revealed that a gene called *lin-4* encodes an RNA molecule 61 nucleotides long that can regulate the expression of certain other genes. The 61-nucleotide *lin-4* RNA does not encode a protein, but rather is cleaved into a 22-nucleotide RNA that possesses the regulatory activity. This discovery was the first view of a large class of regulatory RNA molecules that are now called *microRNAs* or *miRNAs*. The key to the activity of microRNAs and their specificity for particular genes is their ability to form Watson–Crick base-pair-stabilized complexes with the mRNAs of those genes.

These miRNAs do not function on their own. Instead, the miRNAs bind to members of a class of proteins called the *Argonaute* family (Figure 32.26). These Argonaute–miRNA complexes can then bind mRNAs that have sequences that are substantially complementary to the miRNAs. Once bound, such an mRNA can be cleaved by the Argonaute–miRNA complex through the action of a magnesium-based active site. Thus, *the miRNAs serve as guide RNAs that determine the specificity of the Argonaute complex* (Figure 32.27). Cleavage of mRNA by the Argonaute–miRNA complex is similar to the mechanism of RNA interference (p. 165). The small RNA molecules participating in RNAi, however, come from a different source. In RNAi, double-stranded RNAs are cleaved into 21-nucleotide fragments, single-stranded components of which are bound by members of the Argonaute family to form a RISC complex that cleaves complementary mRNAs. For miRNAs, the single-stranded RNAs are generated from larger genetically encoded precursors, as described in Chapter 28.

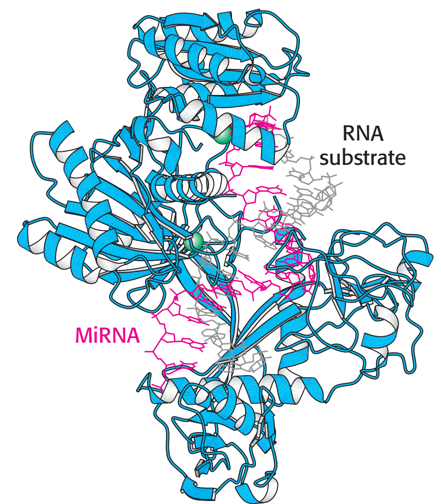


Figure 32.26 MicroRNA–Argonaute complex. The miRNA (shown in red) is bound by the Argonaute protein. *Notice* that the miRNA serves as a guide that binds the RNA substrate (shown in gray) through the formation of a double helix. Two magnesium ions are shown in green. [Drawn from 3HK2.pdb].

The occurrence of gene regulation by miRNAs was originally thought to be limited to a relatively small number of species. However, subsequent studies have revealed that this mode of gene regulation is nearly ubiquitous in eukaryotes. Indeed, more than 700 miRNAs encoded by the human genome have been identified. Each miRNA can regulate many different genes because many different target sequences are present in each mRNA. An estimated 60% of all human genes are regulated by one or more miRNAs. As one example, consider the human miRNA called miR-206. This miRNA down-regulates the expression of one isoform of the estrogen receptor. In addition, this miRNA appears to down-regulate the expression of several different coactivators that interact with the estrogen receptor. Thus, this miRNA can mute the influence of estrogen by blocking the estrogen-initiated signaling pathway at several different steps.

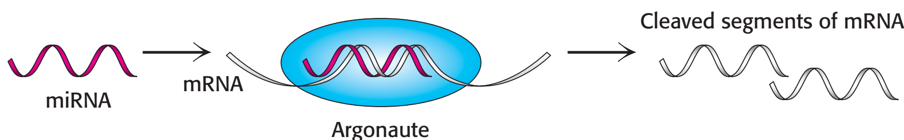


Figure 32.27 MicroRNA action. MicroRNAs bind to members of the Argonaute family where they serve to target specific mRNA molecules for cleavage.

The microRNA pathway has tremendous implications for the evolution of gene-regulatory pathways. Most of the target sites for miRNAs are present in the 3′-untranslated regions of mRNAs. These sequences are quite free to mutate because they do not encode proteins and are not required to fold into any specific structures. Thus, in the context of a set of expressed miRNAs, mutations in this region of any particular gene could, in principle, increase or decrease the affinity for one or more miRNAs and, hence, alter the regulation of the gene.

Summary

32.1 Eukaryotic DNA Is Organized into Chromatin

Eukaryotic DNA is tightly bound to basic proteins called histones; the combination is called chromatin. DNA wraps twice around an octamer of core histones to form a nucleosome. The four core histones are homologous and fold into similar structures. Each core histone has an amino-terminal tail rich in lysine and arginine residues. Nucleosomes are the first stage of compaction of eukaryotic DNA. Chromatin blocks access to many potential DNA binding sites. Changes in chromatin structure play a major role in regulating gene expression.

32.2 Transcription Factors Bind DNA and Regulate Transcription Initiation

Most eukaryotic genes are not expressed unless they are activated by the binding of specific proteins, called transcription factors, to sites on the DNA. These specific DNA-binding proteins interact directly or indirectly with RNA polymerases or their associated proteins. Eukaryotic transcription factors are modular: they consist of separate DNA-binding and activation domains. Important classes of DNA-binding proteins include the homeodomains, the basic-leucine zipper proteins, and Cys_2His_2 zinc-finger proteins. Each of these classes of proteins uses an α helix to make specific contacts with DNA. Activation domains interact with RNA polymerases or their associated factors or with other protein complexes such as mediator. Enhancers are DNA elements that can modulate gene expression from more than 1000 bp away from the start site of transcription. Enhancers are often specific for certain cell types, depending on which DNA-binding proteins are present. The introduction of genes for a specific set of four transcription factors into fibroblasts can cause these cells to dedifferentiate into induced pluripotent stem cells.

32.3 The Control of Gene Expression Can Require Chromatin Remodeling

Chromatin structure is crucial to the control of gene expression; chromatin structure is more open near the transcription start sites of actively transcribed genes. Steroids such as estrogens bind to eukaryotic transcription factors called nuclear hormone receptors. These proteins are capable of binding DNA whether or not ligands are bound. The binding of ligands induces a conformational change that allows the recruitment of additional proteins called coactivators. Among the most important functions of coactivators is to catalyze the addition of acetyl groups to lysine residues in the tails of histone proteins. Histone acetylation decreases the affinity of the histones for DNA, making additional genes accessible for transcription. In addition, acetylated histones are targets for proteins containing specific binding units called bromodomains. Bromodomains are components of two classes of large complexes: (1) chromatin-remodeling engines and (2) factors associated with RNA polymerase II. These complexes open up sites on chromatin and initiate transcription.

32.4 Eukaryotic Gene Expression Can Be Controlled at Posttranscriptional Levels

Genes encoding proteins that transport and store iron are regulated at the translational level. Iron-response elements, structures that are present in certain mRNAs, are bound by an IRE-binding protein when this protein is not binding iron. Whether the expression of a gene is stimulated or inhibited in response to changes in the iron status of a cell depends on the location of the IRE within the mRNA. The IRE-binding protein is a cytoplasmic aconitase that loses its iron–sulfur center under low-iron conditions. MicroRNAs are specialized RNA molecules encoded as parts of larger RNA precursors. Micro RNAs bind to proteins of the Argonaute family; the bound miRNAs function as guides that help bind specific mRNA molecules that are then cleaved.

Key Terms

cell type (p. 938)
histone (p. 938)
chromatin (p. 939)
nucleosome (p. 939)
nucleosome core particle (p. 939)
transcription factor (p. 941)
homeodomain (p. 942)
basic-leucine zipper (bZip) protein (p. 942)
Cys_2His_2 zinc-finger domain (p. 942)
mediator (p. 942)
combinatorial control (p. 943)
enhancer (p. 943)
induced pluripotent stem (iPS) cell (p. 944)
hypersensitive site (p. 944)
chromatin immunoprecipitation (ChIP) (p. 945)
hypomethylation (p. 945)
CpG island (p. 946)
nuclear hormone receptor (p. 946)
estrogen response element (ERE) (p. 946)
coactivator (p. 947)
agonist (p. 948)
anabolic steroid (p. 948)
antagonist (p. 948)
selective estrogen receptor modulator (SERM) (p. 948)
histone acetyltransferase (HAT) (p. 949)
acetyllysine-binding domain (p. 949)
bromodomain (p. 949)
TATA-box-binding protein associated factor (TAF) (p. 950)
chromatin-remodeling complex (p. 950)
histone deacetylase (p. 950)
transferrin (p. 951)
transferrin receptor (p. 951)
ferritin (p. 951)
iron-response element (IRE) (p. 951)
IRE-binding protein (IRP) (p. 951)
microRNA (miRNA) (p. 953)
Argonaute family proteins (p. 953)

Problems

1. *Charge neutralization.* Given the histone amino acid sequences illustrated below, estimate the charge of a histone octamer at pH 7. Assume that histidine residues are uncharged at this pH. How does this charge compare with the charge on 150 base pairs of DNA?

Histone H2A

MSGRGKQGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYSERVGAGAPVYLAAVLEYLTAEILELAGNA
ARDNKKTRIIPRHLQLAIRNDEELNKLLGRVTIAQGGVLPNIQAVLLPKKTESHHKAKGK

Histone H2B

MPEPAKSAPAPKKGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQVHPDTGISSKAMGIMNSFVNDI
FERIAGEASRLAHYNKRSTITSREIQTAVRLLLPGELAKHAVSEGTKAVTKYTSSK

Histone H3

MARTKQTARKSTGGKAPRKQLATKAARKSAPSTGGVKKPHRYRPGTVALREIRRYQKSTELLIRKLPFQR
LVREIAQDFKTDLRFQSAAIGALQEASEAYLVGLFEDTNLCAIHAKRVTIMPKDIQLARRIRGERA

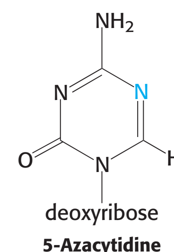
Histone H4

MSGRGKGGKGLGKGGAKRHRKVLRDNIQGITKPAIRRLARRGGVKRISGLIYEETRGVLKVFLENVIRDA
VTYTEHAKRKTVTAMDVVYALKRQGRTLYGFGG

2. *Chromatin immunoprecipitation.* You have used the technique of chromatin immunoprecipitation to isolate DNA fragments containing a DNA-binding protein of interest. Suppose that you wish to know whether a particular known DNA fragment is present in the isolated mixture. How might you detect its presence?

3. *Around we go.* Assuming that 145 base pairs of DNA wrap around the histone octamer 1 3/4 times, estimate the radius of the histone octamer. Assume 3.4 Å per base pair and simplify the calculation by assuming that the wrapping is in two rather than three dimensions and neglecting the thickness of the DNA.

4. *Nitrogen substitution.* Growth of mammalian cells in the presence of 5-azacytidine results in the activation of some normally inactive genes. Propose an explanation.



5-Azacytidine

5. *A new domain.* A protein domain that recognizes 5-methylcytosine in the context of double-stranded DNA has been characterized. What role might proteins containing such a domain play in regulating gene expression? Where on a double-stranded DNA molecule would you expect such a domain to bind?

6. *Hybrid receptor*. Through recombinant DNA methods, a modified steroid hormone receptor was prepared that consists of an estrogen receptor with its ligand-binding domain replaced by the ligand-binding domain from the progesterone receptor. Predict the expected responsiveness of gene expression for cells treated with estrogen or with progesterone.

7. *Different modifications*. What is the effect of acetylation of a lysine residue on the charge of a histone protein? Of lysine methylation?

8. *Transformer*. The following amino acid sequence of one of the four transcription factors is used to generate iPS cells:

HTCDYAGCGKTYTKSSHLKAHLRTHTGEKPYHCDWDGCGWKFARSDELTRHYRKHTGHRPFQCQKCD
RAFSRSDHLALHMKRHF

This transcription factor belongs to one of the three structural classes discussed in Section 32.2. Identify the class.

9. *Coverage*. What percentage of the DNA sites in yeast are accessible, assuming that the fraction of sites observed for GAL4 is typical? To how many base pairs of the 12-Mb yeast genome does this percentage correspond?

10. *Iron regulation*. What effect would you expect from the addition of an IRE to the 5′ end of a gene that is not normally regulated by iron levels? To the 3′ end?

11. *Predicting microRNA regulation*. Suppose that you have identified an miRNA that has the sequence 5′-GCCUAGCCUUAGCAUUGAUUGG-3′. Propose a strategy for identifying mRNA that might be regulated by this miRNA, given the sequences of all mRNAs encoded by the human genome.

Mechanism Problem

12. *Acetyltransferases.* Propose a mechanism for the transfer of an acetyl group from acetyl CoA to the amino group of lysine.

Data Interpretation Problem

13. *Limited restriction.* The restriction enzyme *Hpa*II is a powerful tool for analyzing DNA methylation. This enzyme cleaves sites of the form 5′-CCGG-3′ but will not cleave such sites if the DNA is methylated on any of the cytosine residues. Genomic DNA from different organisms is treated with *Hpa*II and the results are analyzed by gel electrophoresis (see the adjoining patterns). Provide an explanation for the observed patterns.