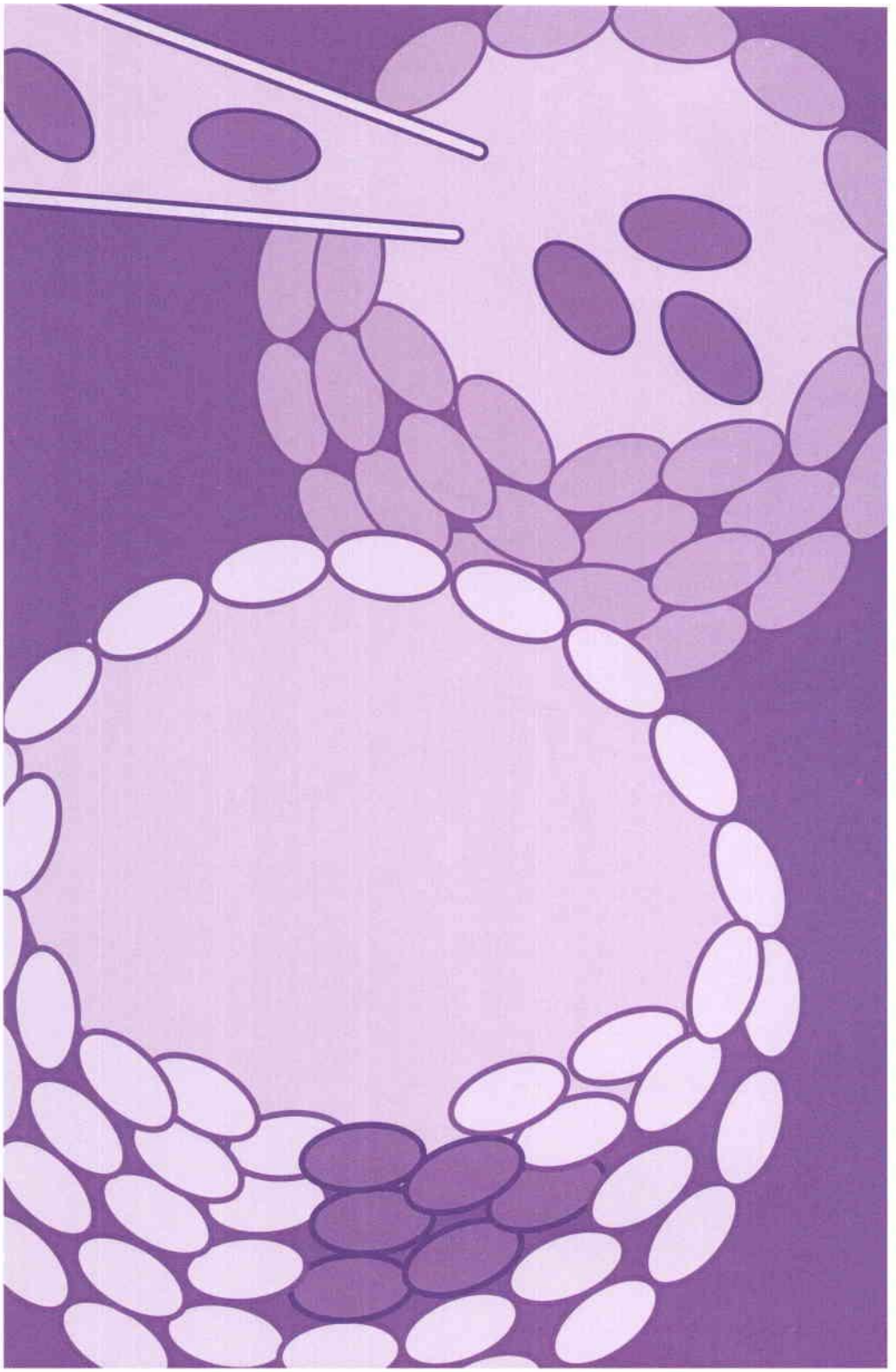
III METHODS

Part III
Chapters



Manipulating Proteins, DNA, and RNA

8

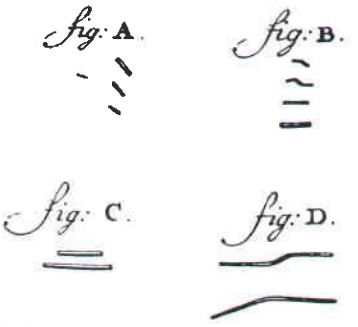
Progress in science is often driven by advances in technology. The entire field of cell biology, for example, came into being when optical craftsmen learned to grind small lenses of sufficiently high quality to observe cells and their substructures. Innovations in lens grinding, rather than any conceptual or philosophical advance, allowed Hooke and van Leeuwenhoek to discover a previously unseen cellular world, where tiny creatures tumble and twirl in a small droplet of water (**Figure 8–1**).

The 21st century promises to be a particularly exciting time for biology. New methods for analyzing proteins, DNA, and RNA are fueling an information explosion and allowing scientists to study cells and their macromolecules in previously unimagined ways. We now have access to the sequences of many billions of nucleotides, providing the complete molecular blueprints for hundreds of organisms—from microbes and mustard weeds to worms, flies, mice, dogs, chimpanzees, and humans. And powerful new techniques are helping us to decipher that information, allowing us not only to compile huge, detailed catalogs of genes and proteins but also to begin to unravel how these components work together to form functional cells and organisms. The long-range goal is nothing short of obtaining a complete understanding of what takes place inside a cell as it responds to its environment and interacts with its neighbors. We want to know which genes are switched on, which mRNA transcripts are present, and which proteins are active—where they are located, with what other proteins and other molecules they associate, and to which pathways or networks they belong. We also want to understand how the cell successfully manages this staggering number of variables and how it chooses among an almost unlimited number of possibilities in performing its diverse biological tasks. Such information will permit us to begin to build a framework for delineating, and eventually predicting, how genes and proteins operate to lay the foundations for life.

In this chapter, we present some of the principal methods used to study the molecular components of cells, particularly proteins, DNA, and RNA. We consider how to separate cells of different types from tissues, how to grow cells outside the body, and how to disrupt cells and isolate their organelles and constituent macromolecules in pure form. We also present the latest techniques used to determine protein structure, function, and interactions, and we discuss the breakthroughs in DNA technology that continue to revolutionize our understanding of cell function.

The techniques and methods described in this chapter have made possible the discoveries that are presented throughout this book, and they are currently being used by tens of thousands of scientists each day.

In This Chapter



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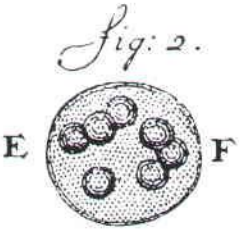


(B)

Figure 8–1 Microscopic life. A sample of "diverse animalcules" seen by van Leeuwenhoek using his simple microscope. (A) Bacteria seen in material he excavated from between his teeth. Those in fig. B he described as "swimming first forward and then backwards" (1692). (B) The eucaryotic green alga *Volvox* (1700). (Courtesy of the John Innes Foundation.)

ISOLATING CELLS AND GROWING THEM IN CULTURE

Although the organelles and large molecules in a cell can be visualized with microscopes, understanding how these components function requires a detailed biochemical analysis. Most biochemical procedures require that large

numbers of cells be physically disrupted to gain access to their components. If the sample is a piece of tissue, composed of different types of cells, heterogeneous cell populations will be mixed together. To obtain as much information as possible about the cells in a tissue, biologists have developed ways of dissociating cells from tissues and separating them according to type. These manipulations result in a relatively homogeneous population of cells that can then be analyzed—either directly or after their number has been greatly increased by allowing the cells to proliferate in culture.

Cells Can Be Isolated from Intact Tissues

Intact tissues provide the most realistic source of material, as they represent the actual cells found within the body. The first step in isolating individual cells is to disrupt the extracellular matrix and cell–cell junctions that hold the cells together. For this purpose, a tissue sample is typically treated with proteolytic enzymes (such as trypsin and collagenase) to digest proteins in the extracellular matrix and with agents (such as ethylenediaminetetraacetic acid, or EDTA) that bind, or chelate, the Ca^{2+} on which cell–cell adhesion depends. The tissue can then be teased apart into single cells by gentle agitation.

For some biochemical preparations, the protein of interest can be obtained in sufficient quantity without having to separate the tissue or organ into cell types. Examples include the preparation of histones from calf thymus, actin from rabbit muscle, or tubulin from cow brain. In other cases, obtaining the desired protein requires enrichment for a specific cell type of interest. Several approaches are used to separate the different cell types from a mixed cell suspension. The most general cell-separation technique uses an antibody coupled to a fluorescent dye to label specific cells. An antibody is chosen that specifically binds to the surface of only one cell type in the tissue. The labeled cells can then be separated from the unlabeled ones in an electronic *fluorescence-activated cell sorter*. In this remarkable machine, individual cells traveling single file in a fine stream pass through a laser beam, and the fluorescence of each cell is rapidly measured. A vibrating nozzle generates tiny droplets, most containing either one cell or no cells. The droplets containing a single cell are automatically given a positive or a negative charge at the moment of formation, depending on whether the cell they contain is fluorescent; they are then deflected by a strong electric field into an appropriate container. Occasional clumps of cells, detected by their increased light scattering, are left uncharged and are discarded into a waste container. Such machines can accurately select 1 fluorescent cell from a pool of 1000 unlabeled cells and sort several thousand cells each second (**Figure 8–2**).

Selected cells can also be obtained by carefully dissecting them from thin tissue slices that have been prepared for microscopic examination (discussed in Chapter 9). In one approach, a tissue section is coated with a thin plastic film and a region containing the cells of interest is irradiated with a focused pulse from an infrared laser. This light pulse melts a small circle of the film, binding the cells underneath. These captured cells are then removed for further analysis. The technique, called *laser capture microdissection*, can be used to separate and analyze cells from different areas of a tumor, allowing their properties or molecular composition to be compared with neighboring normal cells. A related method uses a laser beam to directly cut out a group of cells and catapult them into an appropriate container for future analysis (**Figure 8–3**).

A uniform population of cells obtained by any of these or other separation methods can be used directly for biochemical analysis. After breaking open the cells by mechanical disruption, detergents, and other methods, cytoplasm or individual organelles can be extracted and then specific molecules purified.

Cells Can Be Grown in Culture

Although molecules can be extracted from whole tissues, this is often not the most convenient source of material, requiring, for example, early-morning trips

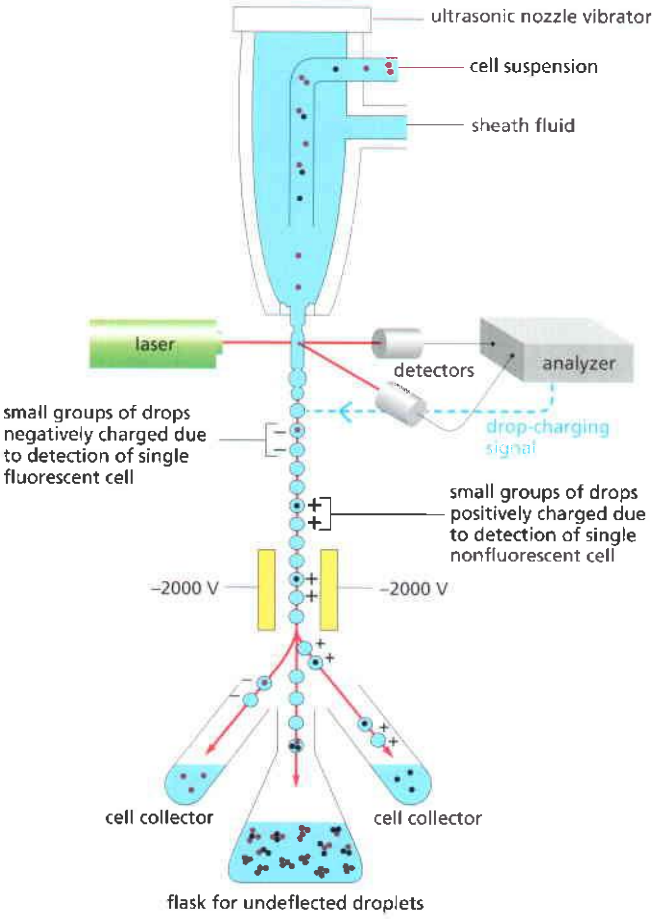


Figure 8–2 A fluorescence-activated cell sorter. A cell passing through the laser beam is monitored for fluorescence. Droplets containing single cells are given a negative or positive charge, depending on whether the cell is fluorescent or not. The droplets are then deflected by an electric field into collection tubes according to their charge. Note that the cell concentration must be adjusted so that most droplets contain no cells and flow to a waste container together with any cell clumps.

to a slaughterhouse. The problem is not only a question of convenience. The livestock commonly used as organ sources are not amenable to genetic manipulation. Moreover, the complexity of intact tissues and organs is an inherent disadvantage when trying to purify particular molecules. Cells grown in culture provide a more homogeneous population of cells from which to extract material, and they are also much more convenient to work with in the laboratory. Given appropriate surroundings, most plant and animal cells can live, multiply, and even express differentiated properties in a tissue-culture dish. The cells can be watched continuously under the microscope or analyzed biochemically, and the effects of adding or removing specific molecules, such as hormones or growth factors, can be systematically explored. In addition, by mixing two cell types, the interactions between one cell type and another can be studied.

Experiments performed on cultured cells are sometimes said to be carried out *in vitro* (literally, "in glass") to contrast them with experiments using intact organisms, which are said to be carried out *in vivo* (literally, "in the living organism"). These terms can be confusing, however, because they are often used in a very different sense by biochemists. In the biochemistry lab, *in vitro* refers to reactions carried out in a test tube in the absence of living cells, whereas *in vivo*

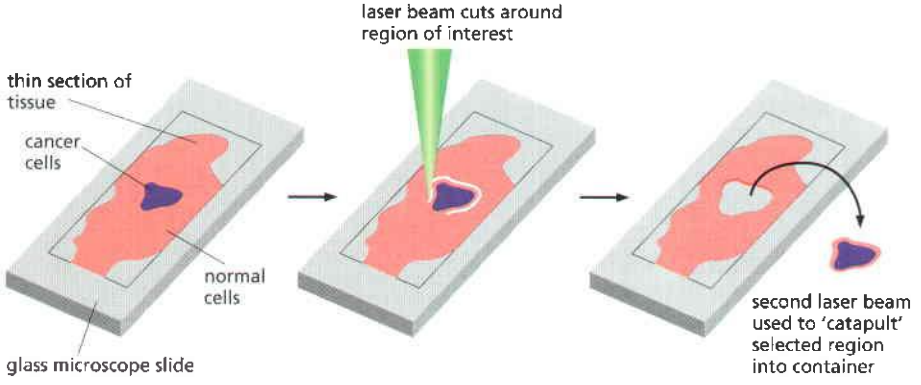


Figure 8–3 Microdissection techniques to select cells from tissue slices. This method uses a laser beam to excise a region of interest and eject it into a container, and it permits the isolation of even a single cell from a tissue sample.

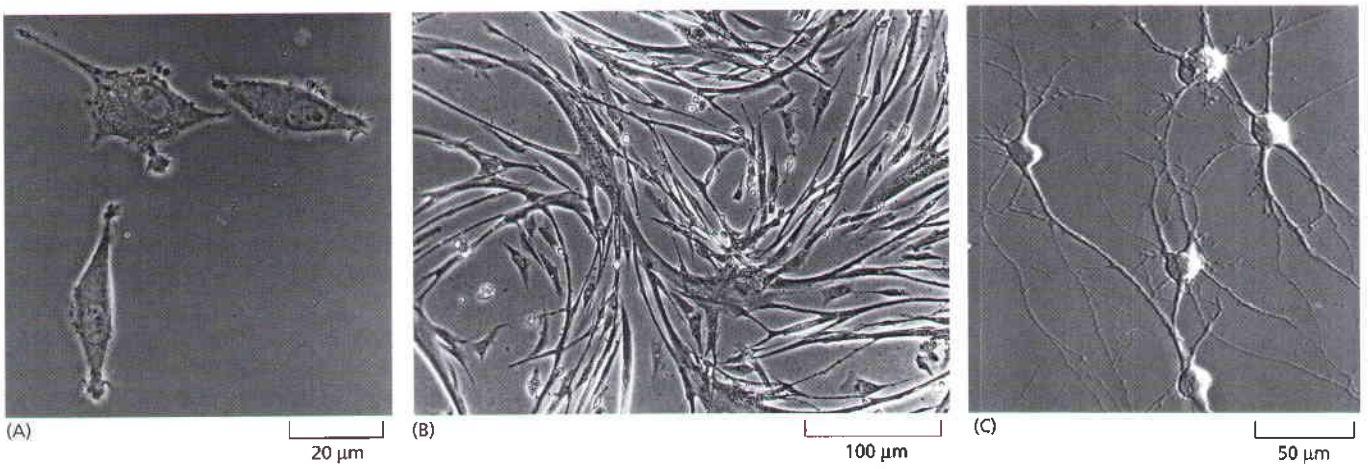


Figure 8–4 Light micrographs of cells in culture. (A) Mouse fibroblasts. (B) Chick myoblasts fusing to form multinucleate muscle cells. (C) Purified rat retinal ganglion nerve cells. (D) Tobacco cells in liquid culture. (A, courtesy of Daniel Zicha; B, courtesy of Rosalind Zalin; C, from A. Meyer-Franke et al., *Neuron* 15:805–819, 1995. With permission from Elsevier; D, courtesy of Gethin Roberts.)

refers to any reaction taking place inside a living cell, even if that cell is growing in culture.

Tissue culture began in 1907 with an experiment designed to settle a controversy in neurobiology. The hypothesis under examination was known as the neuronal doctrine, which states that each nerve fiber is the outgrowth of a single nerve cell and not the product of the fusion of many cells. To test this contention, small pieces of spinal cord were placed on clotted tissue fluid in a warm, moist chamber and observed at regular intervals under the microscope. After a day or so, individual nerve cells could be seen extending long, thin filaments (axons) into the clot. Thus, the neuronal doctrine received strong support, and the foundation was laid for the cell-culture revolution.

These original experiments on nerve fibers used cultures of small tissue fragments called explants. Today, cultures are more commonly made from suspensions of cells dissociated from tissues using the methods described earlier. Unlike bacteria, most tissue cells are not adapted to living suspended in fluid and require a solid surface on which to grow and divide. For cell cultures, this support is usually provided by the surface of a plastic tissue-culture dish. Cells vary in their requirements, however, and many do not proliferate or differentiate unless the culture dish is coated with materials that cells like to adhere to, such as polylysine or extracellular matrix components.

Cultures prepared directly from the tissues of an organism are called *primary cultures*. These can be made with or without an initial fractionation step to separate different cell types. In most cases, cells in primary cultures can be removed from the culture dish and recultured repeatedly in so-called secondary cultures; in this way, they can be repeatedly subcultured *(passaged)* for weeks or months. Such cells often display many of the differentiated properties appropriate to their origin (**Figure 8–4**): fibroblasts continue to secrete collagen; cells derived from embryonic skeletal muscle fuse to form muscle fibers that contract spontaneously in the culture dish; nerve cells extend axons that are electrically excitable and make synapses with other nerve cells; and epithelial cells form extensive sheets with many of the properties of an intact epithelium. Because these properties are maintained in culture, they are accessible to study in ways that are often not possible in intact tissues.

Cell culture is not limited to animal cells. When a piece of plant tissue is cultured in a sterile medium containing nutrients and appropriate growth regulators, many of the cells are stimulated to proliferate indefinitely in a disorganized manner, producing a mass of relatively undifferentiated cells called a *callus*. If the nutrients and growth regulators are carefully manipulated, one can induce the formation of a shoot and then root apical meristems within the callus, and,

in many species, regenerate a whole new plant. Similar to animal cells, callus cultures can be mechanically dissociated into single cells, which will grow and divide as a suspension culture (see Figure 8–4D).

Eucaryotic Cell Lines Are a Widely Used Source of Homogeneous Cells

The cell cultures obtained by disrupting tissues tend to suffer from a problem—eventually the cells die. Most vertebrate cells stop dividing after a finite number of cell divisions in culture, a process called *replicative cell senescence* (discussed in Chapter 17). Normal human fibroblasts, for example, typically divide only 25–40 times in culture before they stop. In these cells, the limited proliferation capacity reflects a progressive shortening and uncapping of the cell's telomeres, the repetitive DNA sequences and associated proteins that cap the ends of each chomosome (discussed in Chapter 5). Human somatic cells in the body have turned off production of the enzyme, called *telomerase*, that normally maintains the telomeres, which is why their telomeres shorten with each cell division. Human fibroblasts can often be coaxed to proliferate indefinitely by providing them with the gene that encodes the catalytic subunit of telomerase; in this case, they can be propagated as an "immortalized" cell line.

Some human cells, however, cannot be immortalized by this trick. Although their telomeres remain long, they still stop dividing after a limited number of divisions because the culture conditions eventually activate cell-cycle *checkpoint mechanisms* (discussed in Chapter 17) that arrest the cell cycle—a process sometimes called "culture shock." In order to immortalize these cells, one has to do more than introduce telomerase. One must also inactivate the checkpoint mechanisms. This can be done by introducing certain cancer-promoting oncogenes, such as those derived from tumor viruses (discussed in Chapter 20). Unlike human cells, most rodent cells do not turn off production of telomerase and therefore their telomeres do not shorten with each cell division. Therefore, if culture shock can be avoided, some rodent cell types will divide indefinitely in culture. In addition, rodent cells often undergo genetic changes in culture that inactivate their checkpoint mechanisms, thereby spontaneously producing immortalized cell lines.

Cell lines can often be most easily generated from cancer cells, but these cultures differ from those prepared from normal cells in several ways, and are referred to as *transformed cell lines*. Transformed cell lines often grow without attaching to a surface, for example, and they can proliferate to a much higher density in a culture dish. Similar properties can be induced experimentally in normal cells by transforming them with a tumor-inducing virus or chemical. The resulting transformed cell lines can usually cause tumors if injected into a susceptible animal (although it is usually only a small subpopulation, called cancer stem cells, that can do so—discussed in Chapter 20).

Both transformed and nontransformed cell lines are extremely useful in cell research as sources of very large numbers of cells of a uniform type, especially since they can be stored in liquid nitrogen at –196°C for an indefinite period and retain their viability when thawed. It is important to keep in mind, however, that the cells in both types of cell lines nearly always differ in important ways from their normal progenitors in the tissues from which they were derived.

Some widely used cell lines are listed in **Table 8–1**. Different lines have different advantages; for example, the PtK epithelial cell lines derived from the rat kangaroo, unlike many other cell lines which round up during mitosis, remain flat during mitosis, allowing the mitotic apparatus to be readily observed in action.

Embryonic Stem Cells Could Revolutionize Medicine

Among the most promising cell lines to be developed—from a medical point of view—are embryonic stem (ES) cells. These remarkable cells, first harvested from the inner cell mass of the early mouse embryo, can proliferate indefinitely

Table 8–1 Some Commonly Used Cell Lines

CELL LINE*	CELL TYPE AND ORIGIN
3T3	fibroblast (mouse)
BHK21	fibroblast (Syrian hamster)
MDCK	epithelial cell (dog)
HeLa	epithelial cell (human)
PtK1	epithelial cell (rat kangaroo)
L6	myoblast (rat)
PC12	chromaffin cell (rat)
SP2	plasma cell (mouse)
COS	kidney (monkey)
293	kidney (human); transformed with adenovirus
CHO	ovary (Chinese hamster)
DT40	lymphoma cell for efficient targeted recombination (chick)
R1	embryonic stem cell (mouse)
E14.1	embryonic stem cell (mouse)
H1, H9	embryonic stem cell (human)
S2	macrophage-like cell *(Drosophila)*
BY2	undifferentiated meristematic cell (tobacco)

*Many of these cell lines were derived from tumors. All of them are capable of indefinite replication in culture and express at least some of the special characteristics of their cell's of origin.

in culture and yet retain an unrestricted developmental potential. If the cells from the culture dish are put back into an early embryonic environment, they can give rise to all the cell types in the body, including germ cells (**Figure 8–5**). Their descendants in the embryo are able to integrate perfectly into whatever site they come to occupy, adopting the character and behavior that normal cells would show at that site.

Cells with properties similar to those of mouse ES cells can now be derived from early human embryos, creating a potentially inexhaustible supply of cells that might be used to replace and repair damaged mature human tissue. Experiments in mice suggest that it may be possible, in the future, to use ES cells to produce specialized cells for therapy—to replace the skeletal muscle fibers that degenerate in victims of muscular dystrophy, the nerve cells that die in patients with Parkinson's disease, the insulin-secreting cells that are destroyed in type I

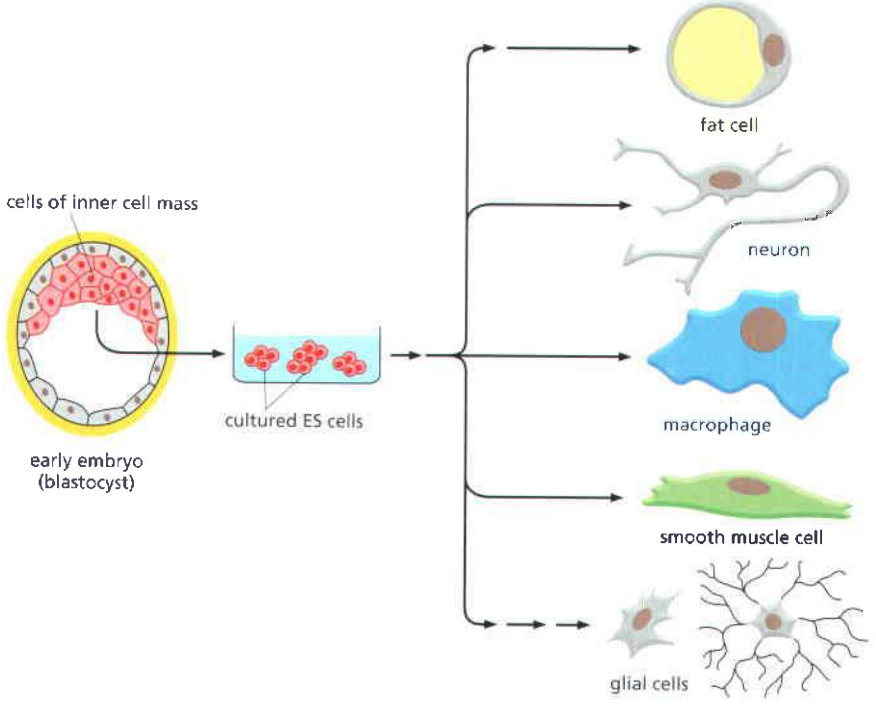


Figure 8–5 Embryonic stem (ES) cells derived from an embryo. These cultured cells can give rise to all of the cell types of the body. ES cells are harvested from the inner cell mass of an early embryo and can be maintained indefinitely as stem cells (discussed in Chapter 23) in culture. If they are put back into an embryo, they will integrate perfectly and differentiate to suit whatever environment they find themselves. The cells can also be kept in culture as an immortal cell line; they can then be supplied with different hormones or growth factors to encourage them to differentiate into specific cell types. (Based on E. Fuchs and J.A. Segré, *Cell* 100:143–155, 2000. With permission from Elsevier.)

diabetics, and the cardiac muscle cells that die during a heart attack. Perhaps one day it may even become possible to grow entire organs from ES cells by a recapitulation of embryonic development. It is important not to transplant ES cells by themselves into adults, as they can produce tumors called teratomas.

There is another major problem associated with the use of ES-cell-derived cells for tissue repair. If the transplanted cells differ genetically from the cells of the patient into whom they are grafted, the patient's immune system will reject and destroy those cells. This problem can be avoided, of course, if the cells used for repair are derived from the patient's own body. As discussed in Chapter 23, many adult tissues contain stem cells dedicated to continuous production of just one or a few specialized cell types, and a great deal of stem-cell research aims to manipulate the behavior of these adult stem cells for use in tissue repair.

ES cell technology, however, in theory at least, also offers another way around the problem of immune rejection, using a strategy known as "therapeutic cloning," as we explain next.

Somatic Cell Nuclear Transplantation May Provide a Way to Generate Personalized Stem Cells

The term "cloning" has been used in confusing ways as a shorthand term for several quite distinct types of procedures. It is important to understand the distinctions, particularly in the context of public debates about the ethics of stem cell research.

As biologists define the term, a *clone* is simply a set of individuals that are genetically identical because they have descended from a single ancestor. The simplest type of cloning is the cloning of cells. Thus, one can take a single epidermal stem cell from the skin and let it grow and divide in culture to obtain a large clone of genetically identical epidermal cells, which can, for example, be used to help reconstruct the skin of a badly burned patient. This kind of cloning is no more than an extension by artificial means of the processes of cell proliferation and repair that occur in a normal human body.

The cloning of entire multicellular animals, called *reproductive cloning*, is a very different enterprise, involving a far more radical departure from the ordinary course of nature. Normally, each individual animal has both a mother and a father, and is not genetically identical to either of them. In reproductive cloning, the need for two parents and sexual union is bypassed. For mammals, this difficult feat has been achieved in sheep and some other domestic animals by *somatic cell nuclear transplantation*. The procedure begins with an unfertilized egg cell. The nucleus of this haploid cell is sucked out and replaced by a nucleus from a regular diploid somatic cell. The diploid donor cell is typically taken from a tissue of an adult individual. The hybrid cell, consisting of a diploid donor nucleus in a host egg cytoplasm, is allowed to develop for a short while in culture. In a small proportion of cases, this procedure can give rise to an early embryo, which is then put into the uterus of a foster mother (**Figure 8–6**). If the experimenter is lucky, development continues like that of a normal embryo,

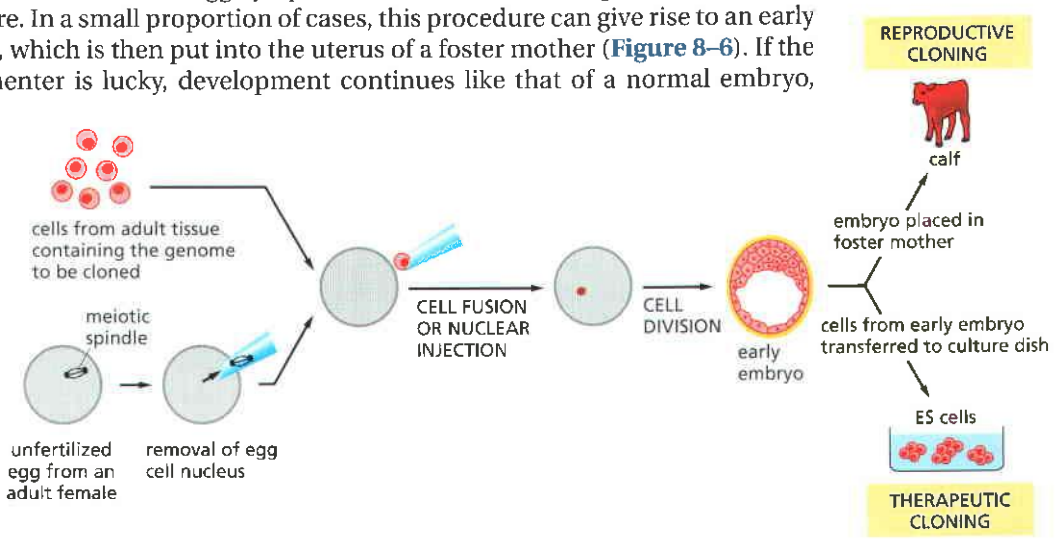


Figure 8–6 Reproductive and therapeutic cloning. Cells from adult tissue can be used for reproductive cloning or for generating personalized ES cells (so-called therapeutic cloning).

giving rise, eventually, to a whole new animal. An individual produced in this way, by reproductive cloning, should be genetically identical to the adult individual that donated the diploid cell (except for the small amount of genetic information in mitochondria, which is inherited solely from the egg cytoplasm).

Therapeutic cloning, which is very different from reproductive cloning, employs the technique of somatic cell nuclear transplantation to produce personalized ES cells (see Figure 8–6). In this case, the very early embryo generated by nuclear transplantation, consisting of about 200 cells, is not transferred to the uterus of a foster mother. Instead, it is used as a source from which ES cells are derived in culture, with the aim of generating various cell types that can be used for tissue repair. Cells obtained by this route are genetically nearly identical to the donor of the original nucleus, so they can be grafted back into the donor, without fear of immunological rejection.

Somatic cell nuclear transfer has an additional potential benefit—for studying inherited human diseases. ES cells that have received a somatic nucleus from an individual with an inherited disorder can be used to directly study the way in which the disease develops as the ES cells are induced to differentiate into distinct cell types. "Disease-specific" ES cells and their differentiated progeny can also be used to study the progression of such diseases and to test and develop new drugs to treat the disorders. These strategies are still in their infancy, and some countries outlaw certain aspects of the research. It remains to be seen whether human ES cells can be produced by nuclear transfer and whether human ES cells will fulfill the great hopes that medical scientists have for them.

Hybridoma Cell Lines Are Factories That Produce Monoclonal Antibodies

As we see throughout this book, antibodies are particularly useful tools for cell biology. Their great specificity allows precise visualization of selected proteins among the many thousands that each cell typically produces. Antibodies are often produced by inoculating animals with the protein of interest and subsequently isolating the antibodies specific to that protein from the serum of the animal. However, only limited quantities of antibodies can be obtained from a single inoculated animal, and the antibodies produced will be a heterogeneous mixture of antibodies that recognize a variety of different antigenic sites on a macromolecule that differs from animal to animal. Moreover, antibodies specific for the antigen will constitute only a fraction of the antibodies found in the serum. An alternative technology, which allows the production of an infinite quantity of identical antibodies and greatly increases the specificity and convenience of antibody-based methods, is the production of monoclonal antibodies by hybridoma cell lines.

This technology, developed in 1975, has revolutionized the production of antibodies for use as tools in cell biology, as well as for the diagnosis and treatment of certain diseases, including rheumatoid arthritis and cancer. The procedure requires hybrid cell technology (**Figure 8–7**), and it involves propagating a clone of cells from a single antibody-secreting B lymphocyte to obtain a homogeneous preparation of antibodies in large quantities. B lymphocytes normally have a limited life-span in culture, but individual antibody-producing B lymphocytes from an immunized mouse or rat, when fused with cells derived from a transformed B lymphocyte cell line, can give rise to hybrids that have both the ability to make a particular antibody and the ability to multiply indefinitely in culture. These **hybridomas** are propagated as individual clones, each of which provides a permanent and stable source of a single type of **monoclonal antibody** (**Figure 8–8**). Each type of monoclonal antibody recognizes a single type of antigenic site—for example, a particular cluster of five or six amino acid side chains on the surface of a protein. Their uniform specificity makes monoclonal antibodies much more useful than conventional antisera for most purposes.

An important advantage of the hybridoma technique is that monoclonal antibodies can be made against molecules that constitute only a minor component of a complex mixture. In an ordinary antiserum made against such a mix-

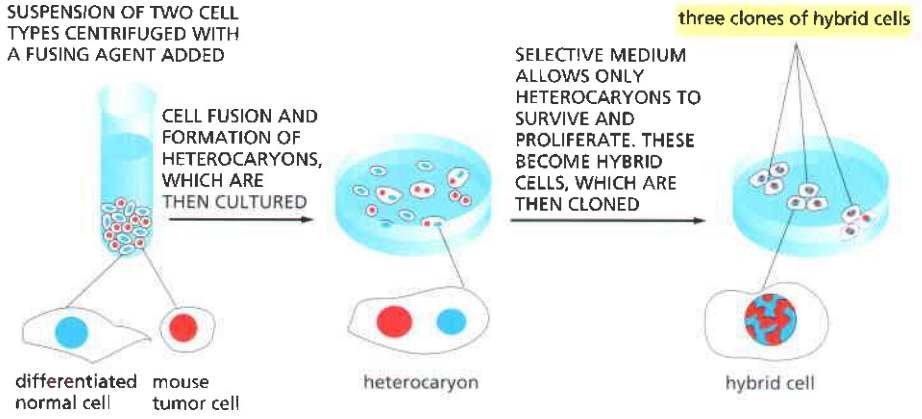


Figure 8–7 The production of hybrid cells. It is possible to fuse one cell with another to form a *heterocaryon*, a combined cell with two separate nuclei. Typically, a suspension of cells is treated with certain inactivated viruses or with polyethylene glycol, each of which alters the plasma membranes of cells in a way that induces them to fuse. Eventually, a heterocaryon proceeds to mitosis and produces a hybrid cell in which the two separate nuclear envelopes have been disassembled, allowing all the chromosomes to be brought together in a single large nucleus. Such hybrid cells can give rise to immortal hybrid cell lines. If one of the parent cells was from a tumor cell line, the hybrid cell is called a hybridoma.

ture, the proportion of antibody molecules that recognize the minor component would be too small to be useful. But if the B lymphocytes that produce the various components of this antiserum are made into hybridomas, it becomes possible to screen individual hybridoma clones from the large mixture to select one that produces the desired type of monoclonal antibody and to propagate the selected hybridoma indefinitely so as to produce that antibody in unlimited quantities. In principle, therefore, a monoclonal antibody can be made against any protein in a biological sample. Once an antibody has been made, it can be used to localize the protein in cells and tissues, to follow its movement, and to purify the protein to study its structure and function.

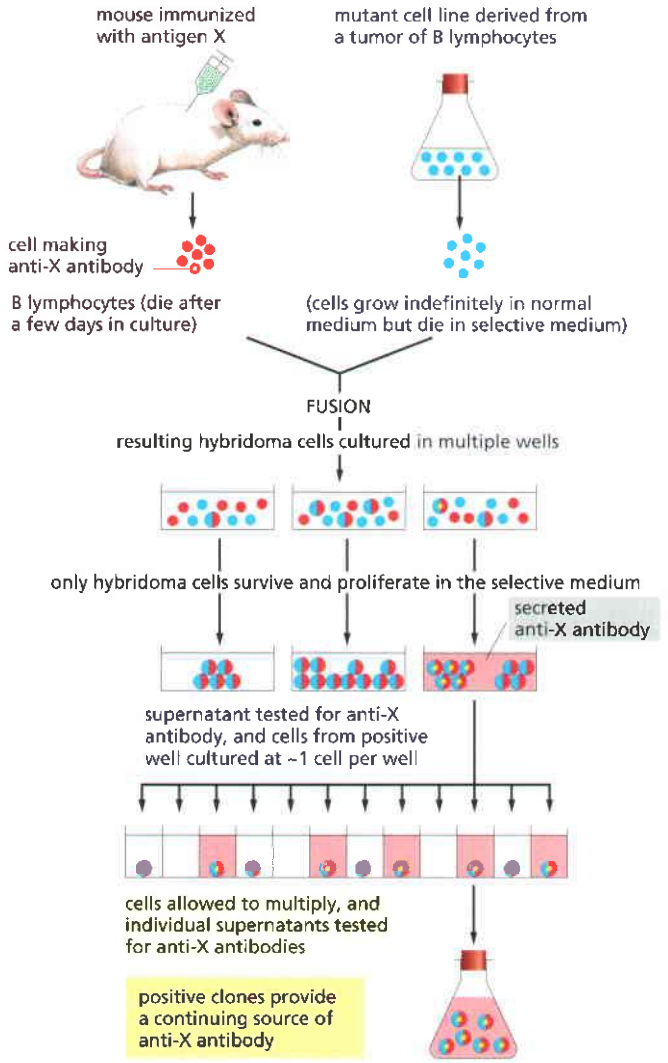


Figure 8–8 Preparation of hybridomas that secrete monoclonal antibodies against a particular antigen. Here, the antigen of interest is designated as "antigen X." The selective growth medium used after the cell fusion step contains an inhibitor (aminopterin) that blocks the normal biosynthetic pathways by which nucleotides are made. The cells must therefore use a bypass pathway to synthesize their nucleic acids. This pathway is defective in the mutant cell line derived from the B cell tumor, but it is intact in the normal cells obtained from the immunized mouse. Because neither cell type used for the initial fusion can survive and proliferate on its own, only the hybridoma cells do so.

Summary

Tissues can be dissociated into their component cells, from which individual cell types can be purified and used for biochemical analysis or for the establishment of cell cultures. Many animal and plant cells survive and proliferate in a culture dish if they are provided with a suitable culture medium containing nutrients and appropriate signal molecules. Although many animal cells stop dividing after a finite number of cell divisions, cells that have been immortalized through spontaneous mutations or genetic manipulation can be maintained indefinitely as cell lines. Embryonic stem cells can proliferate indefinitely in a culture dish, while retaining the ability to differentiate into all the different cell types of the body. They therefore hold great medical promise. Hybridoma cells are widely employed to produce unlimited quantities of uniform monoclonal antibodies, which are used to detect and purify cell proteins, as well as to diagnose and treat diseases.

PURIFYING PROTEINS

The challenge of isolating a single type of protein from the thousands of other proteins present in a cell is a formidable one, but must be overcome in order to study protein function *in vitro*. As we shall see later in this chapter, *recombinant DNA technology* can enormously simplify this task by "tricking" cells into producing large quantities of a given protein, thereby making its purification much easier. Whether the source of the protein is an engineered cell or a natural tissue, a purification procedure usually starts with subcellular fractionation to reduce the complexity of the material, and is then followed by purification steps of increasing specificity.

Cells Can Be Separated into Their Component Fractions

In order to purify a protein, it must first be extracted from inside the cell. Cells can be broken up in various ways: they can be subjected to osmotic shock or ultrasonic vibration, forced through a small orifice, or ground up in a blender. These procedures break many of the membranes of the cell (including the plasma membrane and endoplasmic reticulum) into fragments that immediately reseal to form small closed vesicles. If carefully carried out, however, the disruption procedures leave organelles such as nuclei, mitochondria, the Golgi apparatus, lysosomes, and peroxisomes largely intact. The suspension of cells is thereby reduced to a thick slurry (called a *homogenate* or *extract*) that contains a variety of membrane-enclosed organelles, each with a distinctive size, charge, and density. Provided that the homogenization medium has been carefully chosen (by trial and error for each organelle), the various components—including the vesicles derived from the endoplasmic reticulum, called microsomes—retain most of their original biochemical properties.

The different components of the homogenate must then be separated. Such cell fractionations became possible only after the commercial development in the early 1940s of an instrument known as the *preparative ultracentrifuge*, which rotates extracts of broken cells at high speeds (**Figure 8–9**). This treatment separates cell components by size and density: in general, the largest units experience the largest centrifugal force and move the most rapidly. At relatively low speed, large components such as nuclei sediment to form a pellet at the bottom of the centrifuge tube; at slightly higher speed, a pellet of mitochondria is deposited; and at even higher speeds and with longer periods of centrifugation, first the small closed vesicles and then the ribosomes can be collected (**Figure 8–10**). All of these fractions are impure, but many of the contaminants can be removed by resuspending the pellet and repeating the centrifugation procedure several times.

Centrifugation is the first step in most fractionations, but it separates only components that differ greatly in size. A finer degree of separation can be achieved by layering the homogenate in a thin band on top of a dilute salt solution that fills

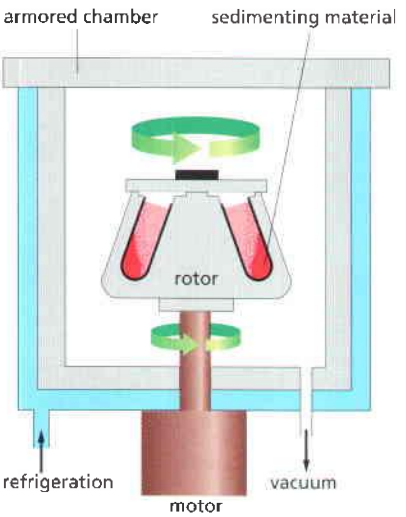


Figure 8–9 The preparative ultracentrifuge. The sample is contained in tubes that are inserted into a ring of cylindrical holes in a metal *rotor*. Rapid rotation of the rotor generates enormous centrifugal forces, which cause particles in the sample to sediment. The vacuum reduces friction, preventing heating of the rotor and allowing the refrigeration system to maintain the sample at 4°C.

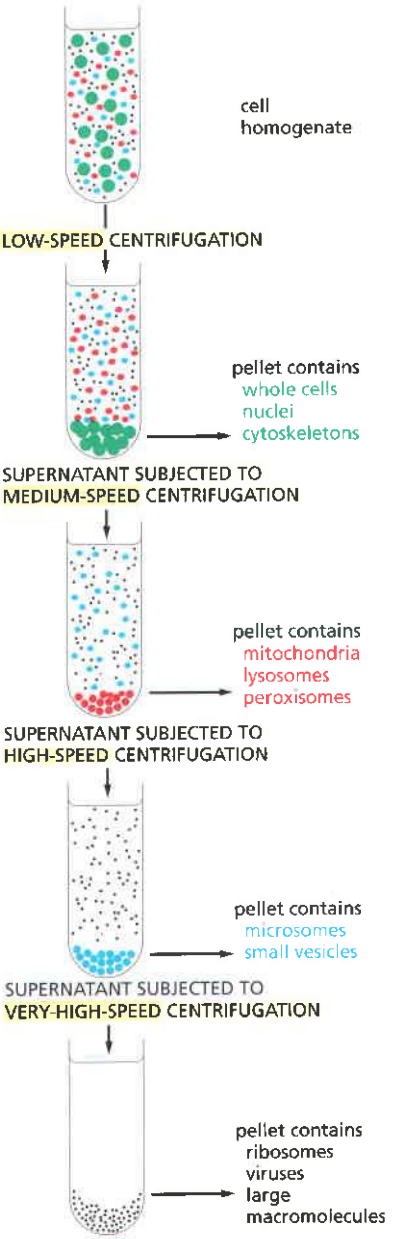


Figure 8–10 Cell fractionation by centrifugation. Repeated centrifugation at progressively higher speeds will fractionate homogenates of cells into their components. In general, the smaller the subcellular component, the greater is the centrifugal force required to sediment it. Typical values for the various centrifugation steps referred to in the figure are:
low speed: 1000 times gravity for 10 minutes
medium speed: 20,000 times gravity for 20 minutes
high speed: 80,000 times gravity for 1 hour
very high speed: 150,000 times gravity for 3 hours

a centrifuge tube. When centrifuged, the various components in the mixture move as a series of distinct bands through the salt solution, each at a different rate, in a process called *velocity sedimentation* (**Figure 8–11**A). For the procedure to work effectively, the bands must be protected from convective mixing, which would normally occur whenever a denser solution (for example, one containing organelles) finds itself on top of a lighter one (the salt solution). This is achieved by augmenting the solution in the tube with a shallow gradient of sucrose prepared by a special mixing device. The resulting density gradient—with the dense end at the bottom of the tube—keeps each region of the salt solution denser than any solution above it, and it thereby prevents convective mixing from distorting the separation.

When sedimented through such dilute sucrose gradients, different cell components separate into distinct bands that can be collected individually. The relative rate at which each component sediments depends primarily on its size and shape—normally being described in terms of its sedimentation coefficient, or S value. Present-day ultracentrifuges rotate at speeds of up to 80,000 rpm and produce forces as high as 500,000 times gravity. These enormous forces drive even small macromolecules, such as tRNA molecules and simple enzymes, to sediment at an appreciable rate and allow them to be separated from one another by size.

The ultracentrifuge is also used to separate cell components on the basis of their buoyant density, independently of their size and shape. In this case the sample is sedimented through a steep density gradient that contains a very high concentration of sucrose or cesium chloride. Each cell component begins to move down the gradient as in Figure 8–11A, but it eventually reaches a position where the density of the solution is equal to its own density. At this point the component floats and can move no farther. A series of distinct bands is thereby produced in the centrifuge tube, with the bands closest to the bottom of the tube containing the components of highest buoyant density (Figure 8–11B). This method, called *equilibrium sedimentation*, is so sensitive that it can separate macromolecules that have incorporated heavy isotopes, such as ^{13}C or ^{15}N, from the same macromolecules that contain the lighter, common isotopes (^{12}C or ^{14}N). In fact, the cesium-chloride method was developed in 1957 to separate the labeled from the unlabeled DNA produced after exposure of a growing population of bacteria to nucleotide precursors containing ^{15}N; this classic experiment provided direct evidence for the semiconservative replication of DNA (see Figure 5–5).

Cell Extracts Provide Accessible Systems to Study Cell Functions

Studies of organelles and other large subcellular components isolated in the ultracentrifuge have contributed enormously to our understanding of the functions of different cell components. Experiments on mitochondria and chloroplasts purified by centrifugation, for example, demonstrated the central function of these organelles in converting energy into forms that the cell can use. Similarly, resealed vesicles formed from fragments of rough and smooth endoplasmic reticulum (microsomes) have been separated from each other and analyzed as functional models of these compartments of the intact cell.

Similarly, highly concentrated cell extracts, especially extracts of *Xenopus laevis* (African clawed frog) oocytes, have played a critical role in the study of

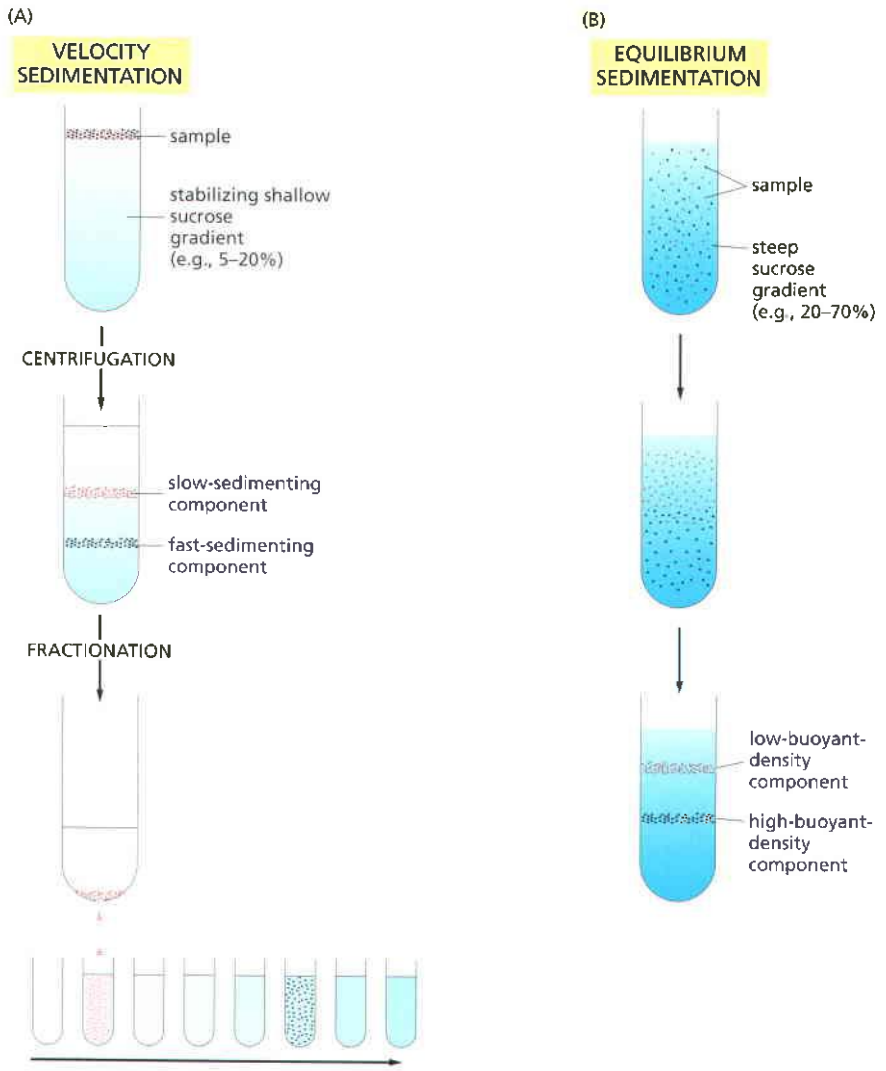


Figure 8–11 Comparison of velocity sedimentation and equilibrium sedimentation. (A) In velocity sedimentation, subcellular components sediment at different speeds according to their size and shape when layered over a dilute solution containing sucrose. To stabilize the sedimenting bands against convective mixing caused by small differences in temperature or solute concentration, the tube contains a continuous shallow gradient of sucrose, which increases in concentration toward the bottom of the tube (typically from 5% to 20% sucrose). After centrifugation, the different components can be collected individually, most simply by puncturing the plastic centrifuge tube and collecting drops from the bottom, as illustrated here. (B) In equilibrium sedimentation, subcellular components move up or down when centrifuged in a gradient until they reach a position where their density matches their surroundings. Although a sucrose gradient is shown here, denser gradients, which are especially useful for protein and nucleic acid separation, can be formed from cesium chloride. The final bands, at equilibrium, can be collected as in (A).

such complex and highly organized processes as the cell-division cycle, the separation of chromosomes on the mitotic spindle, and the vesicular-transport steps involved in the movement of proteins from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane.

Cell extracts also provide, in principle, the starting material for the complete separation of all of the individual macromolecular components of the cell. We now consider how this separation is achieved, focusing on proteins.

Proteins Can Be Separated by Chromatography

Proteins are most often fractionated by **column chromatography**, in which a mixture of proteins in solution is passed through a column containing a porous solid matrix. The different proteins are retarded to different extents by their interaction with the matrix, and they can be collected separately as they flow out of the bottom of the column (**Figure 8–12**). Depending on the choice of matrix, proteins can be separated according to their charge *(ion-exchange chromatography)*, their hydrophobicity *(hydrophobic chromatography)*, their size *(gel-filtration chromatography)*, or their ability to bind to particular small molecules or to other macromolecules *(affinity chromatography)*.

Many types of matrices are commercially available (**Figure 8–13**). Ion-exchange columns are packed with small beads that carry either a positive or a negative charge, so that proteins are fractionated according to the arrangement of charges on their surface. Hydrophobic columns are packed with beads from which hydrophobic side chains protrude, selectively retarding proteins with

exposed hydrophobic regions. Gel-filtration columns, which separate proteins according to their size, are packed with tiny porous beads: molecules that are small enough to enter the pores linger inside successive beads as they pass down the column, while larger molecules remain in the solution flowing between the beads and therefore move more rapidly, emerging from the column first. Besides providing a means of separating molecules, gel-filtration chromatography is a convenient way to determine their size.

Inhomogeneities in the matrices (such as cellulose), which cause an uneven flow of solvent through the column, limit the resolution of conventional column chromatography. Special chromatography resins (usually silica-based) composed of tiny spheres (3–10 μm in diameter) can be packed with a special apparatus to form a uniform column bed. Such **high-performance liquid chromatography (HPLC)** columns attain a high degree of resolution. In HPLC, the solutes equilibrate very rapidly with the interior of the tiny spheres, and so solutes with different affinities for the matrix are efficiently separated from one another even at very fast flow rates. HPLC is therefore the method of choice for separating many proteins and small molecules.

Affinity Chromatography Exploits Specific Binding Sites on Proteins

If one starts with a complex mixture of proteins, the types of column chromatography just discussed do not produce very highly purified fractions: a single passage through the column generally increases the proportion of a given protein in the mixture no more than twentyfold. Because most individual proteins represent less than 1/1000 of the total cell protein, it is usually necessary to use several different types of columns in succession to attain sufficient purity (**Figure 8–14**). A more efficient procedure, known as **affinity chromatography**, takes advantage of the biologically important binding interactions that occur on protein surfaces. If a substrate molecule is covalently coupled to an inert matrix such as a polysaccharide bead, the enzyme that operates on that substrate will often be specifically retained by the matrix and can then be eluted (washed out) in nearly pure form. Likewise, short DNA oligonucleotides of a specifically

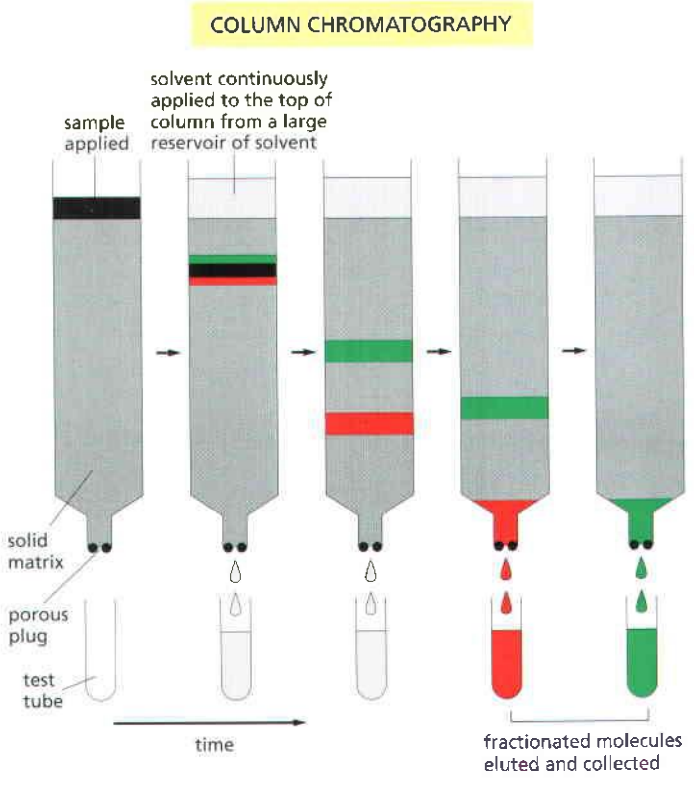


Figure 8–12 The separation of molecules by column chromatography. The sample, a solution containing a mixture of different molecules, is applied to the top of a cylindrical glass or plastic column filled with a permeable solid matrix, such as cellulose. A large amount of solvent is then pumped slowly through the column and collected in separate tubes as it emerges from the bottom. Because various components of the sample travel at different rates through the column, they are fractionated into different tubes.

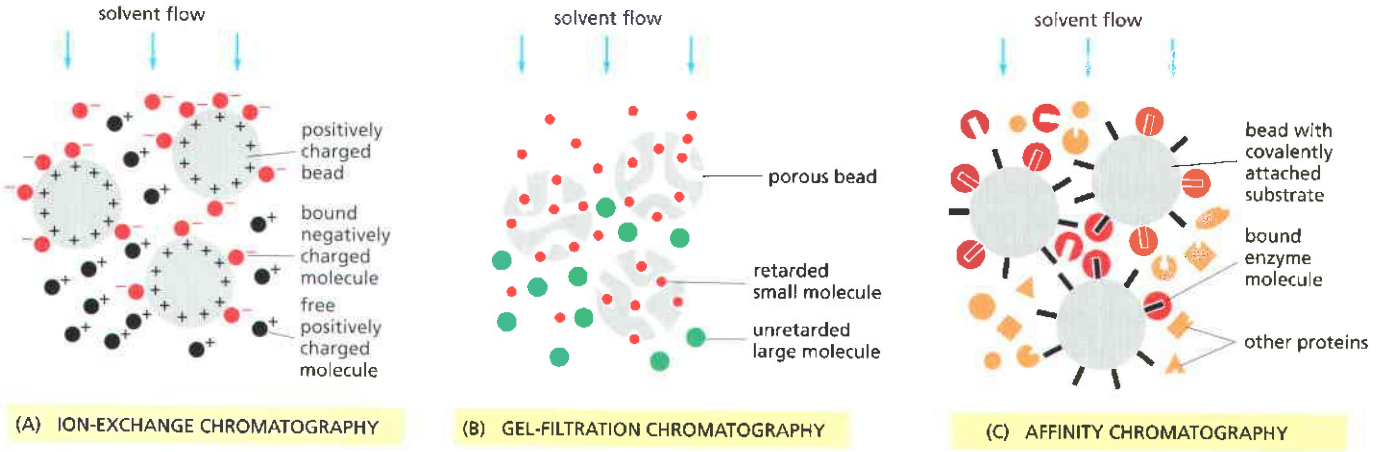


Figure 8–13 Three types of matrices used for chromatography. (A) In ion-exchange chromatography, the insoluble matrix carries ionic charges that retard the movement of molecules of opposite charge. Matrices used for separating proteins include diethylaminoethylcellulose (DEAE-cellulose), which is positively charged, and carboxymethylcellulose (CM-cellulose) and phosphocellulose, which are negatively charged. Analogous matrices based on agarose or other polymers are also frequently used. The strength of the association between the dissolved molecules and the ion-exchange matrix depends on both the ionic strength and the pH of the solution that is passing down the column, which may therefore be varied systematically (as in Figure 8–14) to achieve an effective separation. (B) In gel-filtration chromatography, the matrix is inert but porous. Molecules that are small enough to penetrate into the matrix are thereby delayed and travel more slowly through the column than larger molecules that cannot penetrate. Beads of cross-linked polysaccharide (dextran, agarose, or acrylamide) are available commercially in a wide range of pore sizes, making them suitable for the fractionation of molecules of various molecular weights, from less than 500 daltons to more than 5×10^6 daltons. (C) Affinity chromatography uses an insoluble matrix that is covalently linked to a specific ligand, such as an antibody molecule or an enzyme substrate, that will bind a specific protein. Enzyme molecules that bind to immobilized substrates on such columns can be eluted with a concentrated solution of the free form of the substrate molecule, while molecules that bind to immobilized antibodies can be eluted by dissociating the antibody–antigen complex with concentrated salt solutions or solutions of high or low pH. High degrees of purification can be achieved in a single pass through an affinity column.

designed sequence can be immobilized in this way and used to purify DNA-binding proteins that normally recognize this sequence of nucleotides in chromosomes (see Figure 7–28). Alternatively, specific antibodies can be coupled to a matrix to purify protein molecules recognized by the antibodies. Because of the great specificity of all such affinity columns, 1000- to 10,000-fold purifications can sometimes be achieved in a single pass.

Genetically-Engineered Tags Provide an Easy Way to Purify Proteins

Using the recombinant DNA methods discussed in subsequent sections, any gene can be modified to produce its protein with a special recognition tag attached to it, so as to make subsequent purification of the protein by affinity chromatography simple and rapid. Often the recognition tag is itself an antigenic determinant, or *epitope*, which can be recognized by a highly specific antibody. The antibody, can then be used both to localize the protein in cells and to purify it (**Figure 8–15**). Other types of tags are specifically designed for protein purification. For example, the amino acid histidine binds to certain metal ions, including nickel and copper. If genetic engineering techniques are used to attach a short string of histidines to one end of a protein, the slightly modified protein can be retained selectively on an affinity column containing immobilized nickel ions. Metal affinity chromatography can thereby be used to purify the modified protein from a complex molecular mixture.

In other cases, an entire protein is used as the recognition tag. When cells are engineered to synthesize the small enzyme glutathione S-transferase (GST) attached to a protein of interest, the resulting **fusion protein** can be purified from the other contents of the cell with an affinity column containing glutathione, a

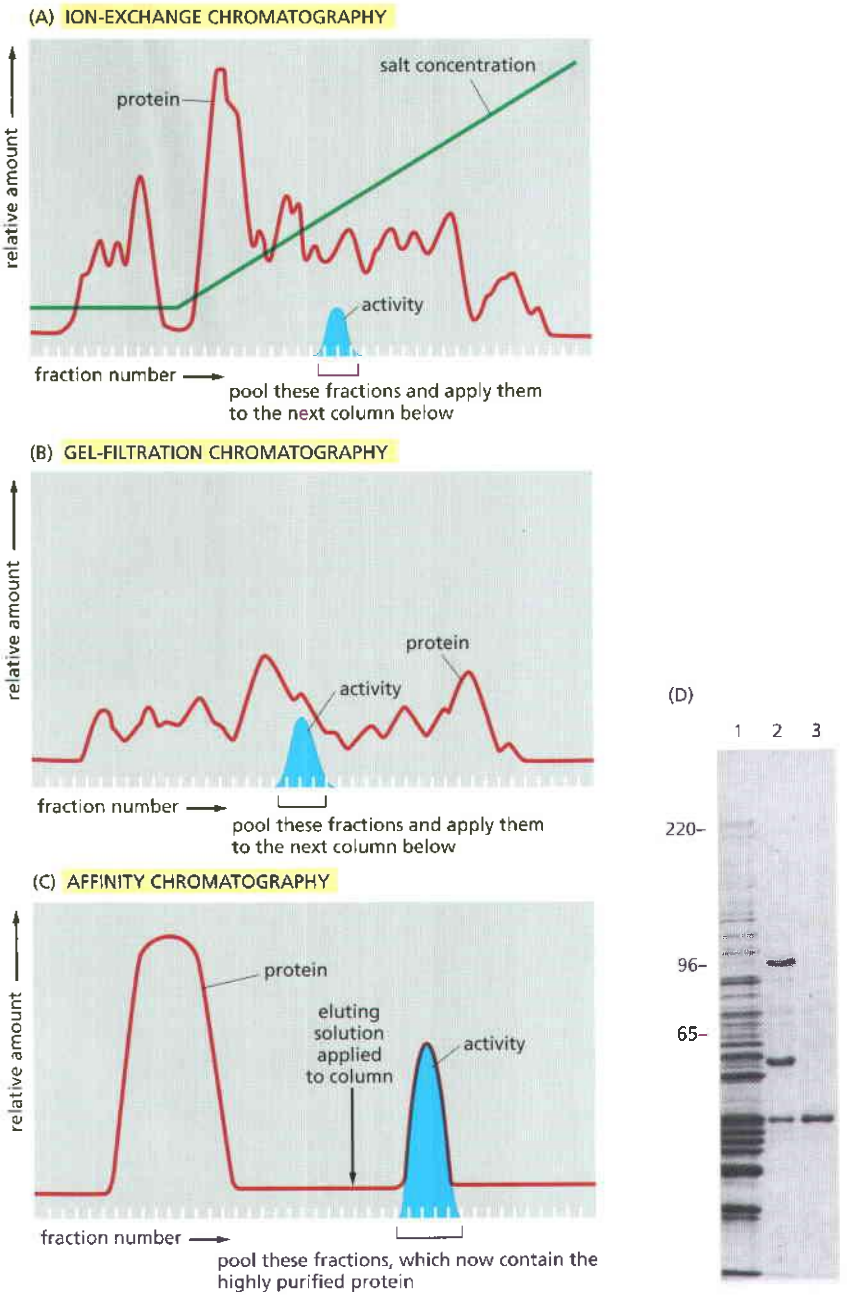


Figure 8–14 Protein purification by chromatography. Typical results obtained when three different chromatographic steps are used in succession to purify a protein. In this example, a homogenate of cells was first fractionated by allowing it to percolate through an ion-exchange resin packed into a column (A). The column was washed to remove all unbound contaminants, and the bound proteins were then eluted by passing a solution containing a gradually increasing concentration of salt onto the top of the column. Proteins with the lowest affinity for the ion-exchange resin passed directly through the column and were collected in the earliest fractions eluted from the bottom of the column. The remaining proteins were eluted in sequence according to their affinity for the resin—those proteins binding most tightly to the resin requiring the highest concentration of salt to remove them. The protein of interest was eluted in several fractions and was detected by its enzymatic activity. The fractions with activity were pooled and then applied to a second, gel-filtration column (B). The elution position of the still-impure protein was again determined by its enzymatic activity, and the active fractions were pooled and purified to homogeneity on an affinity column (C) that contained an immobilized substrate of the enzyme. (D) Affinity purification of cyclin-binding proteins from *S. cerevisiae*, as analyzed by SDS polyacrylamide-gel electrophoresis, which is described below in Figure 8–18. Lane 1 is a total cell extract; lane 2 shows the proteins eluted from an affinity column containing cyclin B2; lane 3 shows one major protein eluted from a cyclin B3 affinity column. Proteins in lanes 2 and 3 were eluted from the affinity columns with salt, and the gel was stained with Coomassie blue. The scale at the left shows the molecular weights of marker proteins, in kilodaltons. (D, from D. Kellogg et al., *J. Cell Biol.* 130:675–685, 1995. With permisison from The Rockefeller University Press.)

substrate molecule that binds specifically and tightly to GST. If the purification is carried out under conditions that do not disrupt protein–protein interactions, the fusion protein can be isolated in association with the proteins it interacts with inside the cell (**Figure 8–16**).

As a further refinement of purification methods using recognition tags, an amino acid sequence that forms a cleavage site for a highly specific proteolytic enzyme can be engineered between the protein of choice and the recognition tag. Because the amino acid sequences at the cleavage site are very rarely found by chance in proteins, the tag can later be cleaved off without destroying the purified protein.

This type of specific cleavage is used in an especially powerful purification methodology known as *tandem affinity purification tagging (tap-tagging)*. Here, one end of a protein is engineered to contain two recognition tags that are separated by a protease cleavage site. The tag on the very end of the construct is chosen to bind irreversibly to an affinity column, allowing the column to be washed extensively to remove all contaminating proteins. Protease cleavage then releases the protein, which is then further purified using the second tag.

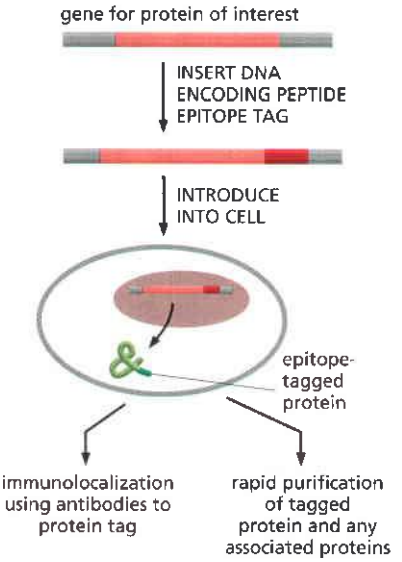


Figure 8–15 Epitope tagging for the localization or purification of proteins. Using standard genetic engineering techniques, a short peptide tag can be added to a protein of interest. If the tag is itself an antigenic determinant, or *epitope*, it can be targeted by an appropriate commercially available antibody. The antibody, suitably labeled, can be used to determine the location of the protein in cells or to purify it by immunoprecipitation or affinity chromatography. In immunoprecipitation, antibodies directed against the epitope tag are added to a solution containing the tagged protein; the antibodies specifically cross-link the tagged protein molecules and precipitate them out of solution as antibody–protein complexes.

Because this two-step strategy provides an especially high degree of protein purification with relatively little effort, it is used extensively in cell biology. Thus, for example, a set of approximately 6000 yeast strains, each with a different gene fused to DNA that encodes a tap-tag, has been constructed to allow any yeast protein to be rapidly purified.

Purified Cell-free Systems Are Required for the Precise Dissection of Molecular Functions

It is important to study biological processes free from all of the complex side reactions that occur in a living cell by using **purified cell-free systems**. To make this possible, cell homogenates are fractionated with the aim of purifying each of the individual macromolecules that are needed to catalyze a biological process of interest. For example, the experiments to decipher the mechanisms of protein synthesis began with a cell homogenate that could translate RNA molecules to produce proteins. Fractionation of this homogenate, step by step, produced in turn the ribosomes, tRNAs, and various enzymes that together constitute the protein-synthetic machinery. Once individual pure components were available, each could be added or withheld separately to define its exact role in the overall process.

A major goal for cell biologists is the reconstitution of every biological process in a purified cell-free system. Only in this way can one define all of the components needed for the process and control their concentrations, as required to work out their precise mechanism of action. Although much remains to be done, a great deal of what we know today about the molecular biology of the cell has been discovered by studies in such cell-free systems. They have been used, for example, to decipher the molecular details of DNA replication and DNA transcription, RNA splicing, protein translation, muscle contraction, and particle transport along microtubules, and many other processes that occur in cells.

Summary

Populations of cells can be analyzed biochemically by disrupting them and fractionating their contents, allowing functional cell-free systems to be developed. Highly purified cell-free systems are needed for determining the molecular details of complex cell processes, requiring extensive purification of all the proteins and other components involved. The proteins in soluble cell extracts can be purified by column

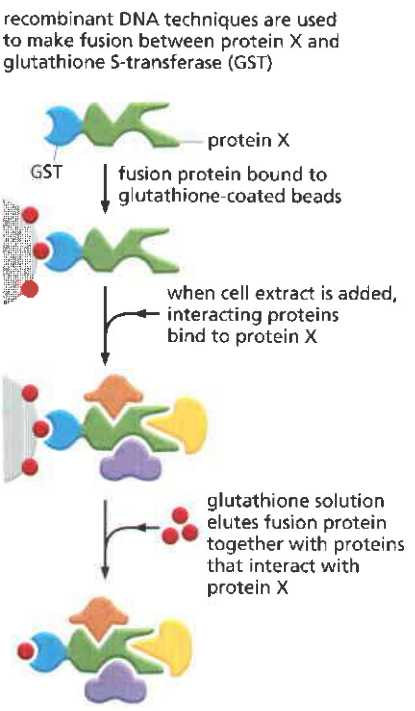


Figure 8–16 Purification of protein complexes by using a GST-tagged fusion protein. GST fusion proteins, produced in cells with recombinant DNA techniques, can be captured on an affinity column containing beads coated with glutathione. Proteins not bound to the beads are washed away. The fusion protein, along with other proteins in the cell that are bound tightly to it, can then be eluted with glutathione. The identities of these additional proteins can be determined by mass spectrometry (see Figure 8–21). Affinity columns can also be made to contain antibodies against GST or another convenient small protein or epitope tag (see Figure 8–15).

chromatography; depending on the type of column matrix, biologically active proteins can be separated on the basis of their molecular weight, hydrophobicity, charge characteristics, or affinity for other molecules. In a typical purification, the sample is passed through several different columns in turn—the enriched fractions obtained from one column are applied to the next. Recombinant DNA techniques, to be described later, allow special recognition tags to be attached to proteins, thereby greatly simplifying their purification.

ANALYZING PROTEINS

Proteins perform most processes in cells: they catalyze metabolic reactions, use nucleotide hydrolysis to do mechanical work, and serve as the major structural elements of the cell. The great variety of protein structures and functions has stimulated the development of a multitude of techniques to study them.

Proteins Can Be Separated by SDS Polyacrylamide-Gel Electrophoresis

Proteins usually possess a net positive or negative charge, depending on the mixture of charged amino acids they contain. An electric field applied to a solution containing a protein molecule causes the protein to migrate at a rate that depends on its net charge and on its size and shape. The most popular application of this property is **SDS polyacrylamide-gel electrophoresis** (**SDS-PAGE**). It uses a highly cross-linked gel of polyacrylamide as the inert matrix through which the proteins migrate. The gel is prepared by polymerization of monomers; the pore size of the gel can be adjusted so that it is small enough to retard the migration of the protein molecules of interest. The proteins themselves are not in a simple aqueous solution but in one that includes a powerful negatively charged detergent, sodium dodecyl sulfate, or SDS (**Figure 8–17**). Because this detergent binds to hydrophobic regions of the protein molecules, causing them to unfold into extended polypeptide chains, the individual protein molecules are released from their associations with other proteins or lipid molecules and rendered freely soluble in the detergent solution. In addition, a reducing agent such as β-mercaptoethanol (see Figure 8–17) is usually added to break any S–S linkages in the proteins, so that all of the constituent polypeptides in multisubunit proteins can be analyzed separately.

What happens when a mixture of SDS-solubilized proteins is run through a slab of polyacrylamide gel? Each protein molecule binds large numbers of the negatively charged detergent molecules, which mask the protein's intrinsic charge and cause it to migrate toward the positive electrode when a voltage is applied. Proteins of the same size tend to move through the gel with similar speeds because (1) their native structure is completely unfolded by the SDS, so that their shapes are the same, and (2) they bind the same amount of SDS and therefore have the same amount of negative charge. Larger proteins, with more charge, are subjected to larger electrical forces and also to a larger drag. In free solution, the two effects would cancel out, but, in the mesh of the polyacrylamide gel, which acts as a molecular sieve, large proteins are retarded much more than small ones. As a result, a complex mixture of proteins is fractionated into a series of discrete protein bands arranged in order of molecular weight (**Figure 8–18**). The major proteins are readily detected by staining the proteins in the gel with a dye such as Coomassie blue. Even minor proteins are seen in gels treated with a silver or gold stain, so that as little as 10 ng of protein can be detected in a band.

SDS-PAGE is widely used because it can separate all types of proteins, including those that are normally insoluble in water—such as the many proteins in membranes. And because the method separates polypeptides by size, it provides information about the molecular weight and the subunit composition of proteins. **Figure 8–19** presents a photograph of a gel that has been used to analyze each of the successive stages in the purification of a protein.

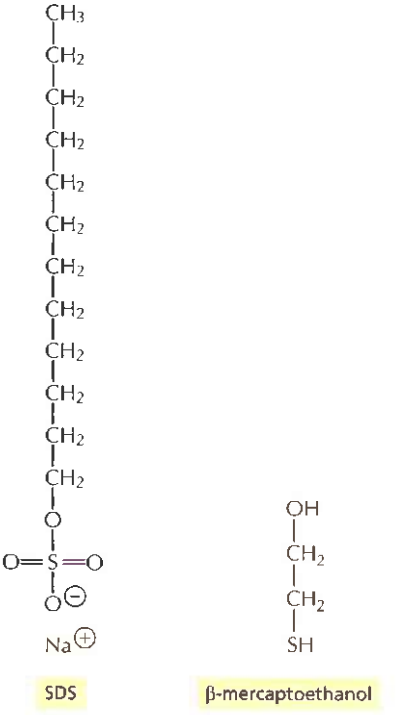


Figure 8–17 The detergent sodium dodecyl sulfate (SDS) and the reducing agent β-mercaptoethanol. These two chemicals are used to solubilize proteins for SDS polyacrylamide-gel electrophoresis. The SDS is shown here in its ionized form.

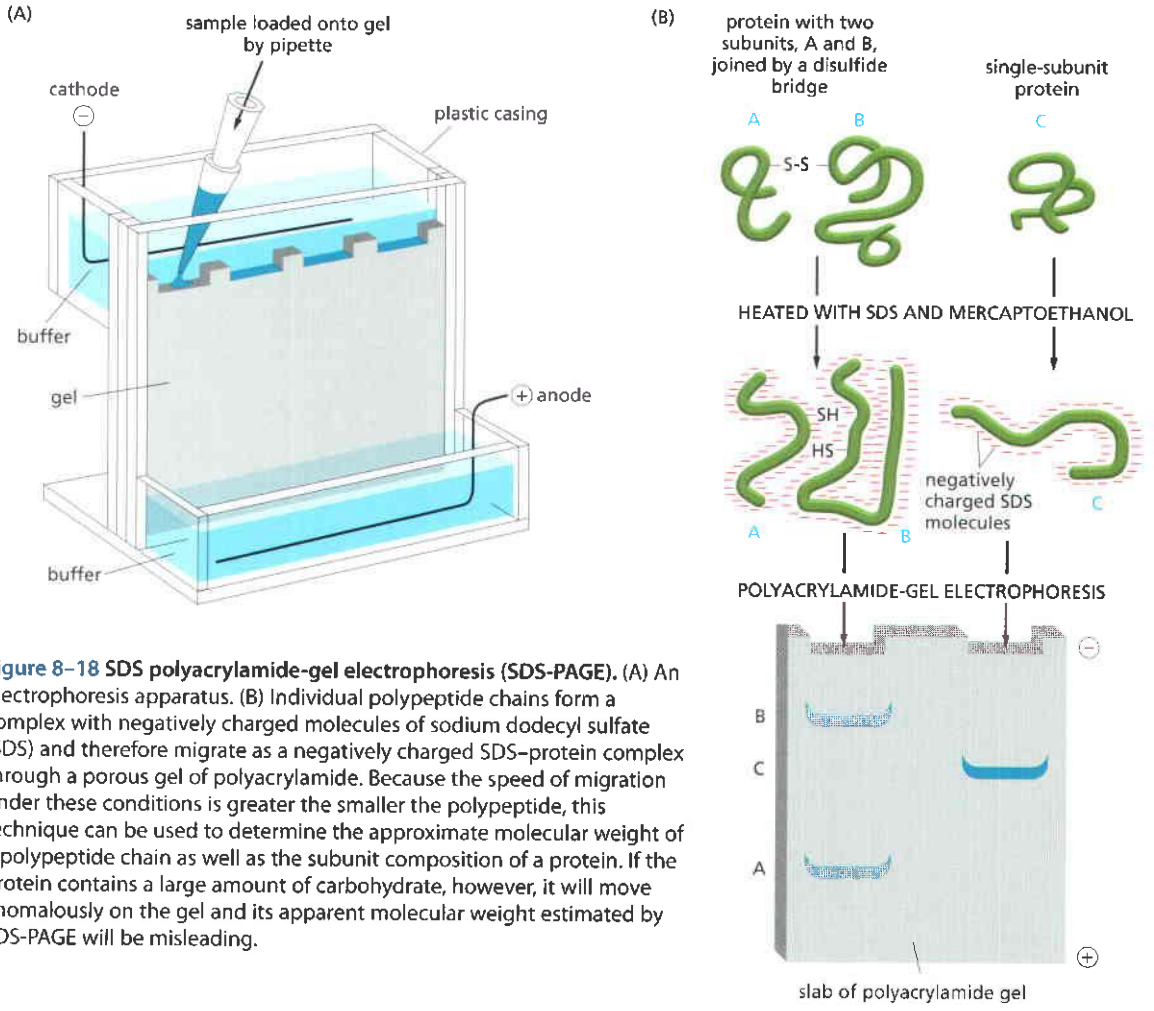


Figure 8–18 SDS polyacrylamide-gel electrophoresis (SDS-PAGE). (A) An electrophoresis apparatus. (B) Individual polypeptide chains form a complex with negatively charged molecules of sodium dodecyl sulfate (SDS) and therefore migrate as a negatively charged SDS–protein complex through a porous gel of polyacrylamide. Because the speed of migration under these conditions is greater the smaller the polypeptide, this technique can be used to determine the approximate molecular weight of a polypeptide chain as well as the subunit composition of a protein. If the protein contains a large amount of carbohydrate, however, it will move anomalously on the gel and its apparent molecular weight estimated by SDS-PAGE will be misleading.

Specific Proteins Can Be Detected by Blotting with Antibodies

A specific protein can be identified after its fractionation on a polyacrylamide gel by exposing all the proteins present on the gel to a specific antibody that has been coupled to a radioactive isotope, to an easily detectable enzyme, or to a fluorescent dye. For convenience, this procedure is normally carried out after transferring (by "blotting") all of the separated proteins present in the gel onto a sheet of nitrocellulose paper or nylon membrane. Placing the membrane over the gel and driving the proteins out of the gel with a strong electric field transfers the protein onto the membrane. The membrane is then soaked in a solution of labeled antibody to reveal the protein of interest. This method of detecting proteins is called **Western blotting**, or **immunoblotting** (Figure 8–20).

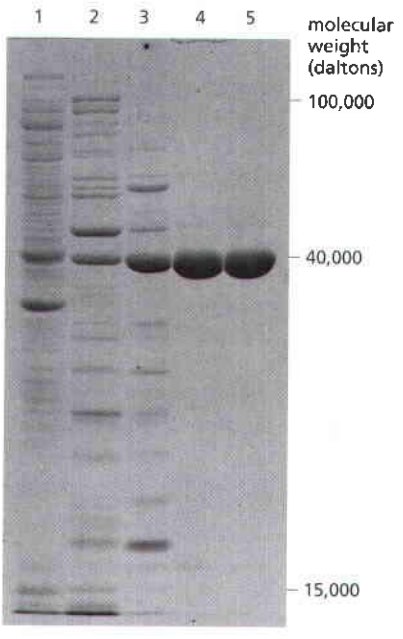
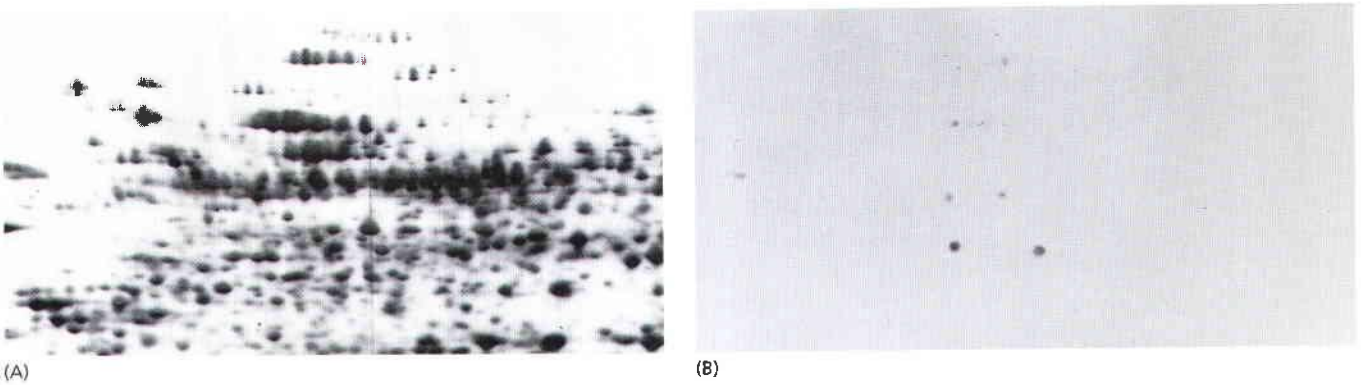


Figure 8–19 Analysis of protein samples by SDS polyacrylamide-gel electrophoresis. The photograph shows a Coomassie-stained gel that has been used to detect the proteins present at successive stages in the purification of an enzyme. The leftmost lane (lane 1) contains the complex mixture of proteins in the starting cell extract, and each succeeding lane analyzes the proteins obtained after a chromatographic fractionation of the protein sample analyzed in the previous lane (see Figure 8–14). The same total amount of protein (10 μg) was loaded onto the gel at the top of each lane. Individual proteins normally appear as sharp, dye-stained bands; a band broadens, however, when it contains too much protein. (From T. Formosa and B.M. Alberts, *J. Biol. Chem.* 261:6107–6118, 1986.)



(A) (B)

Figure 8–20 Western blotting. All the proteins from dividing tobacco cells in culture are first separated by two-dimensional polyacrylamide-gel electrophoresis (described in Figure 8–23). In (A), the positions of the proteins are revealed by a sensitive protein stain. In (B), the separated proteins on an identical gel were then transferred to a sheet of nitrocellulose and exposed to an antibody that recognizes only those proteins that are phosphorylated on threonine residues during mitosis. The positions of the dozen or so proteins that are recognized by this antibody are revealed by an enzyme-linked second antibody. This technique is also known as immunoblotting (or Western blotting). (From J.A. Traas et al., *Plant J.* 2:723–732, 1992. With permission from Blackwell Publishing.)

Mass Spectrometry Provides a Highly Sensitive Method for Identifying Unknown Proteins

A frequent problem in cell biology and biochemistry is the identification of a protein or collection of proteins that has been obtained by one of the purification procedures discussed in the preceding pages (see, for example, Figure 8–16). Because the genome sequences of most common experimental organisms are now known, catalogues of all the proteins produced in those organisms are available. The task of identifying an unknown protein (or collection of unknown proteins) thus reduces to matching some of the amino acid sequences present in the unknown sample with known catalogued genes. This task is now performed almost exclusively by using mass spectrometry in conjunction with computer searches of databases.

Charged particles have very precise dynamics when subjected to electrical and magnetic fields in a vacuum. Mass spectrometry exploits this principle to separate ions according to their mass-to-charge ratio. It is an enormously sensitive technique. It requires very little material and is capable of determining the precise mass of intact proteins and of peptides derived from them by enzymatic or chemical cleavage. Masses can be obtained with great accuracy, often with an error of less than one part in a million. The most commonly used form of the technique is called *matrix-assisted laser desorption ionization–time-of-flight spectrometry (MALDI-TOF)*. In this approach, the proteins in the sample are first broken into short peptides. These peptides are mixed with an organic acid and then dried onto a metal or ceramic slide. A laser then blasts the sample, ejecting the peptides from the slide in the form of an ionized gas, in which each molecule carries one or more positive charges. The ionized peptides are accelerated in an electric field and fly toward a detector. Their mass and charge determines the time it takes them to reach the detector: large peptides move more slowly, and more highly charged molecules move more quickly. By analyzing those ionized peptides that bear a single charge, the precise masses of peptides present in the original sample can be determined. MALDI-TOF can also be used to accurately measure the mass of intact proteins as large as 200,000 daltons. This information is then used to search genomic databases, in which the masses of all proteins and of all their predicted peptide fragments have been tabulated from the genomic sequences of the organism (**Figure 8–21**A). An unambiguous match to a particular open reading frame can often be made by knowing the mass of only a few peptides derived from a given protein.

MALDI-TOF provides accurate molecular weight measurements for proteins and peptides. Moreover, by employing two mass spectrometers in tandem (an arrangement known as MS/MS), it is possible to directly determine the

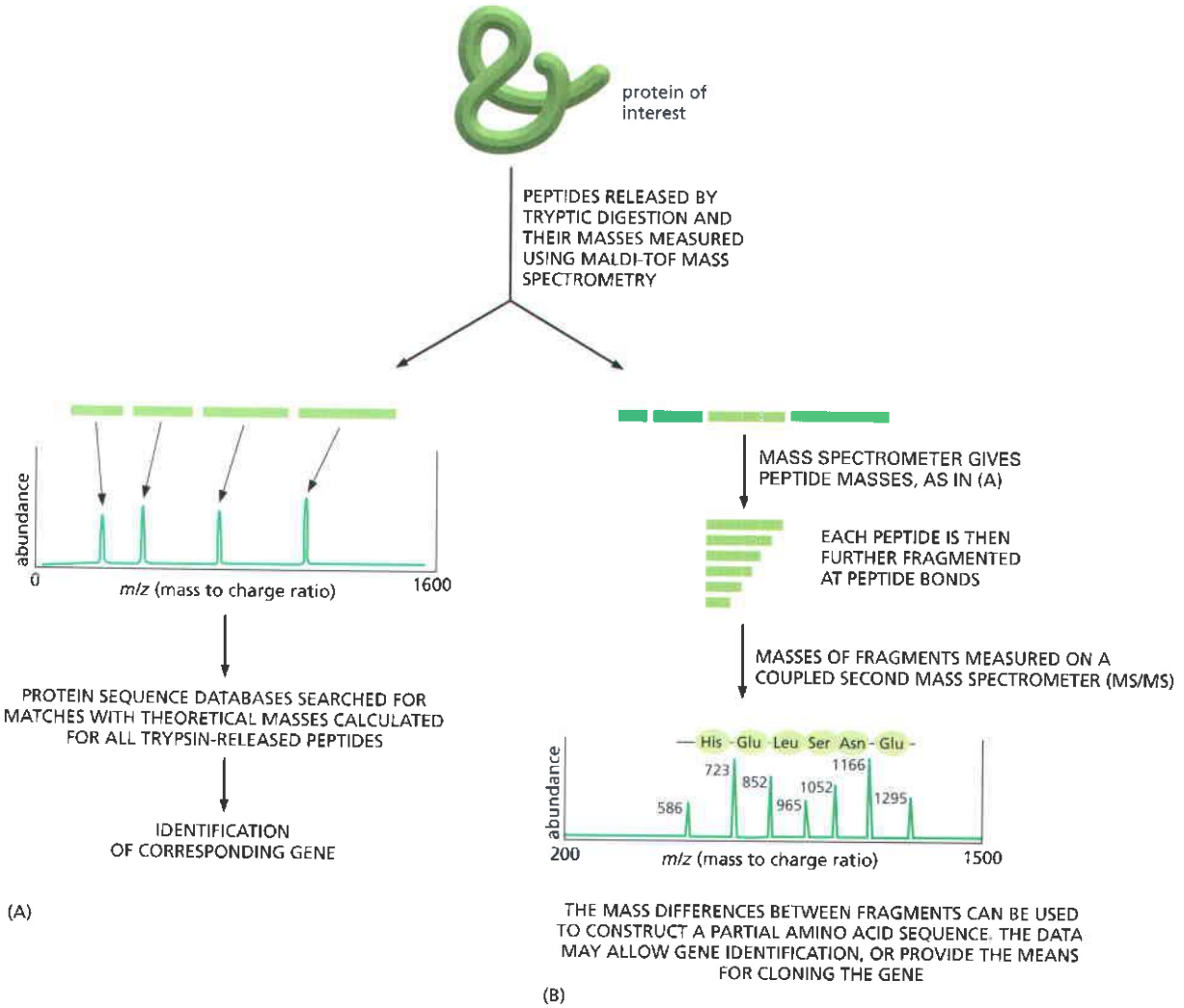


Figure 8–21 Use of mass spectrometry to identify proteins and to sequence peptides. An isolated protein is digested with trypsin and the peptide fragments are then loaded into the mass spectrometer. Two different approaches can then be used to identify the protein. (A) In the first method, peptide masses are measured precisely using MALDI-TOF mass spectrometry. Sequence databases are then searched to find the gene that encodes a protein whose calculated tryptic digest profile matches these values. (B) Mass spectrometry can also be used to determine directly the amino acid sequence of peptide fragments. In this example, tryptic peptides are first separated based on mass within a mass spectrometer. Each peptide is then further fragmented, primarily by cleaving its peptide bonds. This treatment generates a nested set of peptides, each differing in size by one amino acid. These fragments are fed into a second coupled mass spectrometer, and their masses are determined. The difference in masses between two closely related peptides can be used to deduce the "missing" amino acid. By repeated applications of this procedure, a partial amino acid sequence of the original protein can be determined. For simplicity, the analysis shown begins with a single species of purified protein. In reality, mass spectrometry is usually carried out on mixtures of proteins, such as those obtained for affinity chromatography experiments (see Figure 8–16), and can identify all the proteins present in the mixtures. As explained in the text, mass spectrometry can also detect post-translational modifications of proteins.

amino acid sequences of individual peptides in a complex mixture. As described above, the protein sample is first broken down into smaller peptides, which are separated from each other by mass spectrometry. Each peptide is then further fragmented through collisions with high-energy gas atoms. This method of fragmentation preferentially cleaves the peptide bonds, generating a ladder of fragments, each differing by a single amino acid. The second mass spectrometer then separates these fragments and displays their masses. The amino acid sequence of a peptide can then be deduced from these differences in mass (Figure 8–21B).

MS/MS is particularly useful for detecting and precisely mapping post-translational modifications of proteins, such as phosphorylations or acetylations. Because these modifications impart a characteristic mass increase to an amino acid, they are easily detected by mass spectrometry. As described in

Chapter 3, proteomics, a general term that encompasses many different experimental techniques, is the characterization of all proteins in the cell, including all protein–protein interactions and all post-translational modifications. In combination with the rapid purification techniques discussed in the last section, mass spectrometry has emerged as the most powerful method for mapping both the post-translational modifications of a given protein and the proteins that remain associated with it during purification.

Two-Dimensional Separation Methods are Especially Powerful

Because different proteins can have similar sizes, shapes, masses, and overall charges, most separation techniques such as SDS polyacrylamide-gel electrophoresis or ion-exchange chromatography cannot typically display all the proteins in a cell or even in an organelle. In contrast, **two-dimensional gel electrophoresis**, which combines two different separation procedures, can resolve up to 2000 proteins—the total number of different proteins in a simple bacterium—in the form of a two-dimensional protein map.

In the first step, the proteins are separated by their intrinsic charges. The sample is dissolved in a small volume of a solution containing a nonionic (uncharged) detergent, together with β-mercaptoethanol and the denaturing reagent urea. This solution solubilizes, denatures, and dissociates all the polypeptide chains but leaves their intrinsic charge unchanged. The polypeptide chains are then separated in a pH gradient by a procedure called *isoelectric focusing*, which takes advantage of the variation in the net charge on a protein molecule with the pH of its surrounding solution. Every protein has a characteristic isoelectric point, the pH at which the protein has no net charge and therefore does not migrate in an electric field. In isoelectric focusing, proteins are separated electrophoretically in a narrow tube of polyacrylamide gel in which a gradient of pH is established by a mixture of special buffers. Each protein moves to a position in the gradient that corresponds to its isoelectric point and remains there (**Figure 8–22**). This is the first dimension of two-dimensional polyacrylamide-gel electrophoresis.

In the second step, the narrow gel containing the separated proteins is again subjected to electrophoresis but in a direction that is at a right angle to the direction used in the first step. This time SDS is added, and the proteins separate according to their size, as in one-dimensional SDS-PAGE: the original narrow gel is soaked in SDS and then placed on one edge of an SDS polyacrylamide-gel slab, through which each polypeptide chain migrates to form a discrete spot. This is the second dimension of two-dimensional polyacrylamide-gel electrophoresis. The only proteins left unresolved are those that have both identical sizes and identical isoelectric points, a relatively rare situation. Even trace amounts of each polypeptide chain can be detected on the gel by various staining procedures—or by autoradiography if the protein sample was initially labeled with a radioisotope (**Figure 8–23**). The technique has such great resolving power that it can distinguish between two proteins that differ in only a single charged amino acid.

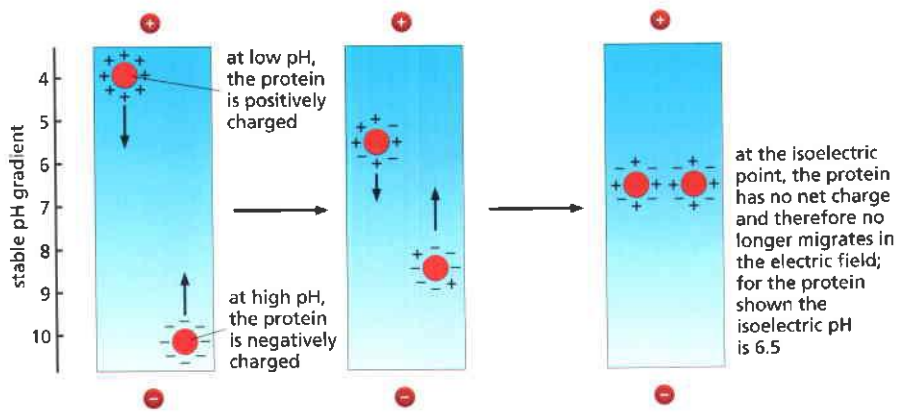


Figure 8–22 Separation of protein molecules by isoelectric focusing. At low pH (high H^+ concentration), the carboxylic acid groups of proteins tend to be uncharged (–COOH) and their nitrogen-containing basic groups fully charged (for example, $-NH_3^+$), giving most proteins a net positive charge. At high pH, the carboxylic acid groups are negatively charged ($-COO^-$) and the basic groups tend to be uncharged (for example, $-NH_2$), giving most proteins a net negative charge. At its *isoelectric pH*, a protein has no net charge since the positive and negative charges balance. Thus, when a tube containing a fixed pH gradient is subjected to a strong electric field in the appropriate direction, each protein species present migrates until it forms a sharp band at its isoelectric pH, as shown.

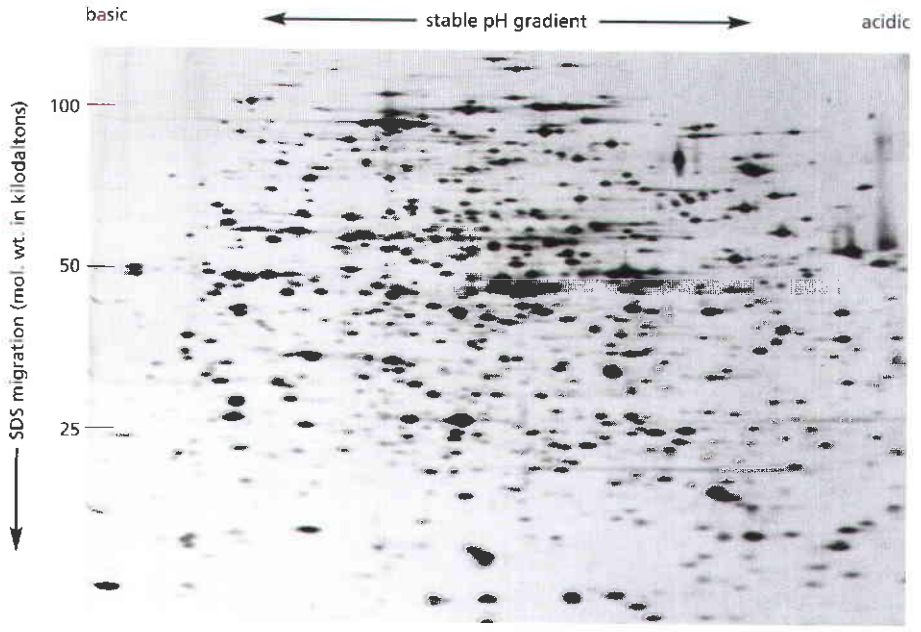


Figure 8–23 Two-dimensional polyacrylamide-gel electrophoresis. All the proteins in an *E. coli* bacterial cell are separated in this gel, in which each spot corresponds to a different polypeptide chain. The proteins were first separated on the basis of their isoelectric points by isoelectric focusing from left to right. They were then further fractionated according to their molecular weights by electrophoresis from top to bottom in the presence of SDS. Note that different proteins are present in very different amounts. The bacteria were fed with a mixture of radioisotope-labeled amino acids so that all of their proteins were radioactive and could be detected by autoradiography (see pp. 602–603). (Courtesy of Patrick O'Farrell.)

A different, even more powerful, "two-dimensional" technique is now available when the aim is to determine all of the proteins present in an organelle or another complex mixture of proteins. Because the technique relies on mass spectroscopy, it requires that the proteins be from an organism with a completely sequenced genome. First, the mixture of proteins present is digested with trypsin to produce short peptides. Next, these peptides are separated by a series of automated liquid chromatography steps. As the second dimension, each separated peptide is fed directly into a tandem mass spectrometer (MS/MS) that allows its amino acid sequence, as well as any post-translational modifications, to be determined. This arrangement, in which a tandem mass spectrometer (MS/MS) is attached to the output of an automated liquid chromatography (LC) system, is referred to as LC-MS/MS. It is now becoming routine to subject an entire organelle preparation to LC-MS/MS analysis and to identify hundreds of proteins and their modifications. Of course, no organelle isolation procedure is perfect, and some of the proteins identified will be contaminating proteins. These can often be excluded by analyzing neighboring fractions from the organelle purification and "subtracting" them out from the peak organelle fractions.

Hydrodynamic Measurements Reveal the Size and Shape of a Protein Complex

Most proteins in a cell act as part of larger complexes, and knowledge of the size and shape of these complexes often leads to insights regarding their function. This information can be obtained in several important ways. Sometimes, a complex can be directly visualized using electron microscopy, as described in Chapter 9. A complementary approach relies on the hydrodynamic properties of a complex, that is, its behavior as it moves through a liquid medium. Usually, two separate measurements are made. One measure is the velocity of a complex as it moves under the influence of a centrifugal field produced by an ultracentrifuge (see Figure 8–11A). The sedimentation constant (or S-value) obtained depends on both the size and the shape of the complex and does not, by itself, convey especially useful information. However, once a second hydrodynamic measurement is performed—by charting the migration of a complex through a gel-filtration chromatography column (see Figure 8–13B)—both the approximate shape of a complex and its molecular weight can be calculated.

Molecular weight can also be determined more directly by using an analytical ultracentrifuge, a complex device that allows protein absorbance measurements

to be made on a sample while it is subjected to centrifugal forces. In this approach, the sample is centrifuged until it reaches equilibrium, where the centrifugal force on a protein complex exactly balances its tendency to diffuse away. Because this balancing point is dependent on a complex's molecular weight but not on its particular shape, the molecular weight can be directly calculated, as needed to determine the stoichiometry of each protein in a protein complex.

Sets of Interacting Proteins Can Be Identified by Biochemical Methods

Because most proteins in the cell function as part of complexes with other proteins, an important way to begin to characterize the biological role of an unknown protein is to identify all of the other proteins to which it specifically binds.

One method for identifying proteins that bind to one another tightly is *co-immunoprecipitation*. In this case, an antibody recognizes a specific target protein; reagents that bind to the antibody and are coupled to a solid matrix then drag the complex out of solution to the bottom of a test tube. If the original target protein is associated tightly enough with another protein when it is captured by the antibody, the partner precipitates as well. This method is useful for identifying proteins that are part of a complex inside cells, including those that interact only transiently—for example, when extracellular signal molecules stimulate cells (discussed in Chapter 15). Another method frequently used to identify a protein's binding partners is protein affinity chromatography (see Figure 8–13C). To employ this technique to capture interacting proteins, a target protein is attached to polymer beads that are packed into a column. When the proteins in a cell extract are washed through this column, those proteins that interact with the target protein are retained by the affinity matrix. These proteins can then be eluted and their identity determined by mass spectrometry.

In addition to capturing protein complexes on columns or in test tubes, researchers are developing high-density protein arrays to investigate protein interactions. These arrays, which contain thousands of different proteins or antibodies spotted onto glass slides or immobilized in tiny wells, allow one to examine the biochemical activities and binding profiles of a large number of proteins at once. For example, if one incubates a fluorescently labeled protein with arrays containing thousands of immobilized proteins, the spots that remain fluorescent after extensive washing each contain a protein to which the labeled protein specifically binds.

Protein–Protein Interactions Can Also Be Identified by a Two-Hybrid Technique in Yeast

Thus far, we have emphasized biochemical approaches to the study of protein–protein interactions. However, a particularly powerful strategy, called the **two-hybrid system**, relies on exploiting the cell's own mechanisms to reveal protein–protein interactions.

The technique takes advantage of the modular nature of gene activator proteins (see Figure 7–45). These proteins both bind to specific DNA sequences and activate gene transcription, and these activities are often performed by two separate protein domains. Using recombinant DNA techniques, two such protein domains are used to create separate "bait" and "prey" fusion proteins. To create the "bait" fusion protein, the DNA sequence that codes for a target protein is fused with DNA that encodes the DNA-binding domain of a gene activator protein. When this construct is introduced into yeast, the cells produce the fusion protein, with the target protein attached to this DNA-binding domain (**Figure 8–24**). This fusion protein binds to the regulatory region of a reporter gene, where it serves as "bait" to fish for proteins that interact with the target protein. To search for potential binding partners (potential prey for the bait), the candidate proteins also have to be constructed as fusion proteins: DNA encoding the activation domain of a gene activator protein is fused to a large

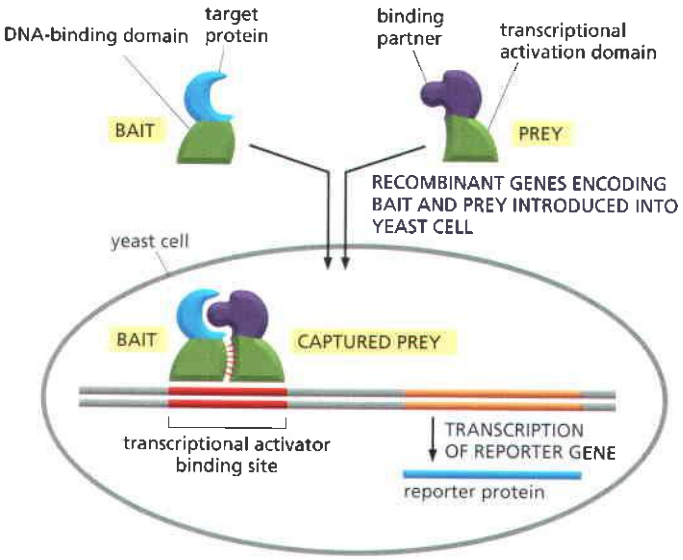


Figure 8–24 The yeast two-hybrid system for detecting protein–protein interactions. The target protein is fused to a DNA-binding domain that directs the fusion protein to the regulatory region of a reporter gene as "bait." When this target protein binds to another specially designed protein in the cell nucleus ("prey"), their interaction brings together two halves of a transcriptional activator, which then switches on the expression of the reporter gene.

number of different genes. Members of this collection of genes—encoding potential "prey"—are introduced individually into yeast cells containing the bait. If the yeast cell receives a DNA clone that expresses a prey partner for the bait protein, the two halves of a transcriptional activator are united, switching on the reporter gene (see Figure 8–24).

This ingenious technique sounds complex, but the two-hybrid system is relatively simple to use in the laboratory. Although the protein–protein interactions occur in the yeast cell nucleus, proteins from every part of the cell and from any organism can be studied in this way. The two-hybrid system has been scaled up to map the interactions that occur among all of the proteins an organism produces. In this case, a set of bait and prey fusions is produced for every cell protein, and every bait/prey combination can be monitored. In this way protein interaction maps have been generated for most of the proteins in yeast, *C. elegans,* and *Drosophila.*

Combining Data Derived from Different Techniques Produces Reliable Protein-Interaction Maps

As previously discussed in Chapter 3, extensive protein-interaction maps can be very useful for identifying the functions of proteins (see Figure 3–82). For this reason, both the two-hybrid method and the biochemical technique discussed earlier known as tap-tagging (see pp. 515–516) have been automated to determine the interactions between thousands of proteins. Unfortunately, different results are found in different experiments, and many of the interactions detected in one laboratory are not detected in another. Therefore, the most useful protein-interaction maps are those that combine data from many experiments, requiring that each interaction in the map be confirmed by more than one technique.

Optical Methods Can Monitor Protein Interactions in Real Time

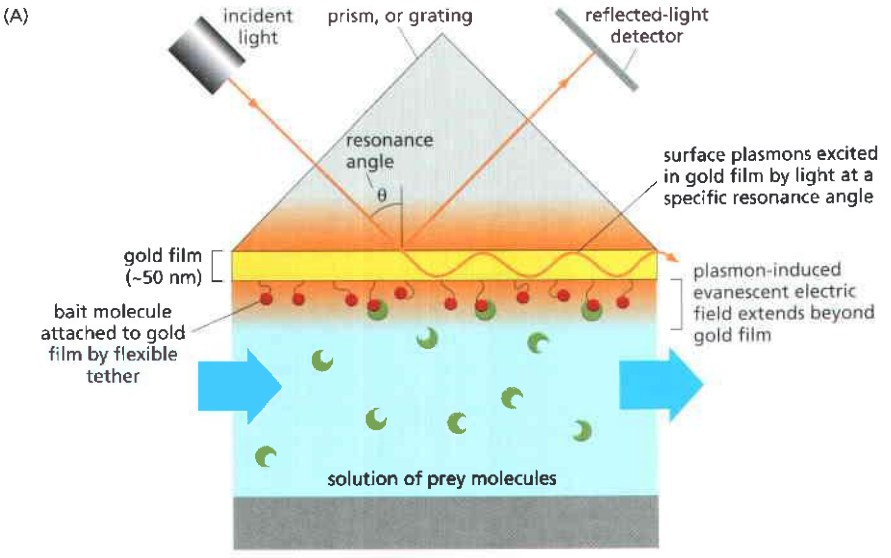
Once two proteins—or a protein and a small molecule—are known to associate, it becomes important to characterize their interaction in more detail. Proteins can associate with each other more or less permanently (like the subunits of RNA polymerase or the proteosome), or engage in transient encounters that may last only a few milliseconds (like a protein kinase and its substrate).

To understand how a protein functions inside a cell, we need to determine how tightly it binds to other proteins, how rapidly it dissociates from them, and how covalent modifications, small molecules, or other proteins influence these interactions. Such studies of protein dynamics often employ optical methods.

Certain amino acids (for example, tryptophan) exhibit weak fluorescence that can be detected with sensitive fluorimeters. In many cases, the fluorescence intensity, or the emission spectrum of fluorescent amino acids located in a protein–protein interface, will change when the proteins associate. When this change can be detected by fluorimetry, it provides a sensitive and quantitative measure of protein binding.

A particularly useful method for monitoring the dynamics of a protein's binding to other molecules is called **surface plasmon resonance** (**SPR**). The SPR method has been used to characterize a wide variety of molecular interactions, including antibody-antigen binding, ligand-receptor coupling, and the binding of proteins to DNA, carbohydrates, small molecules, and other proteins.

SPR detects binding interactions by monitoring the reflection of a beam of light off the interface between an aqueous solution of potential binding molecules and a biosensor surface carrying an immobilized bait protein. The bait protein is attached to a very thin layer of metal that coats one side of a glass prism (**Figure 8–25**). A light beam is passed through the prism; at a certain angle, called the *resonance angle*, some of the energy from the light interacts with the cloud of electrons in the metal film, generating a plasmon—an oscillation of the electrons at right angles to the plane of the film, bouncing up and down between its upper and lower surfaces like a weight on a spring. The plasmon, in turn, generates an electrical field that extends a short distance—about the wavelength of the light—above and below the metal surface. Any change in the composition of



(B) The binding of prey molecules to bait molecules increases the refractive index of the surface layer. This alters the resonance angle for plasmon induction, which can be measured by a detector.

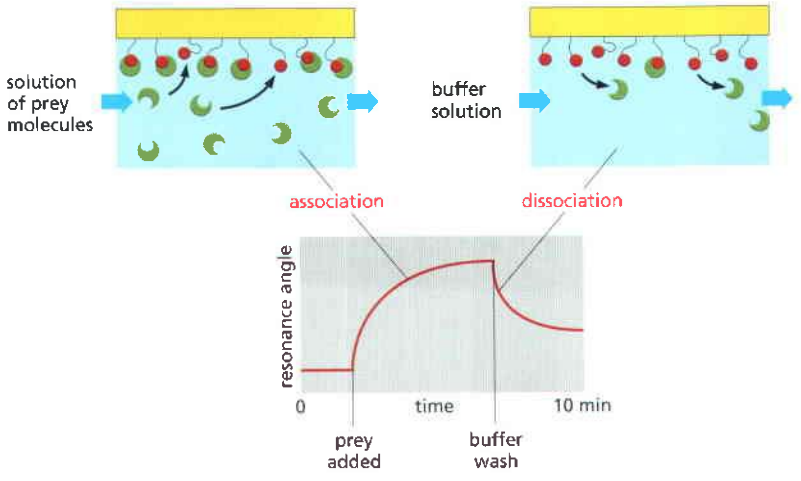


Figure 8–25 Surface plasmon resonance. (A) SPR can detect binding interactions by monitoring the reflection of a beam of light off the interface between an aqueous solution of potential binding molecules *(green)* and a biosensor surface coated with an immobilized bait protein *(red)*. (B) A solution of prey proteins is allowed to flow past the immobilized bait protein. Binding of prey molecules to the bait protein produces a measurable change in the resonance angle, as does their dissociation when a buffer solution washes them off. These changes, monitored in real time, reflect the association and dissociation of the molecular complexes.

the environment within the range of the electrical field will cause a measurable change in the resonance angle.

To measure binding, a solution containing proteins (or other molecules) that might interact with the immobilized bait protein is allowed to flow past the biosensor surface. Proteins binding to the bait change the composition of the molecular complexes on the metal surface, causing a change in the resonance angle (see Figure 8–25). The changes in the resonance angle are monitored in real time and reflect the kinetics of the association—or dissociation—of molecules with the bait protein. The association rate (k_{on}) is measured as the molecules interact, and the dissociation rate (k_{off}) is determined as buffer washes the bound molecules from the sensor surface. A binding constant (K) is calculated by dividing k_{off} by k_{on}. In addition to determining the kinetics, SPR can be used to determine the number of molecules that are bound in each complex: the magnitude of the SPR signal change is proportional to the mass of the immobilized complex.

The SPR method is particularly useful because it requires only small amounts of the protein, the protein does not have to be labeled in any way, and the interactions of the protein with other molecules can be monitored in real time.

A third optical method for probing protein interactions uses *green fluorescent protein* (discussed in detail below) and its derivatives of different colors. In this application, two proteins of interest are each labeled with a different fluorochrome, such that the emission spectrum of one fluorochrome overlaps the absorption spectrum of the second fluorochrome. If the two proteins—and their attached fluorochromes—come very close to each other (within about 1–10 nm), the energy of the absorbed light is transferred from one fluorochrome to the other. The energy transfer, called **fluorescence resonance energy transfer (FRET)**, is determined by illuminating the first fluorochrome and measuring emission from the second (**Figure 8–26**). This technique is especially powerful because, when combined with fluorescence microscopy, it can be used to characterize protein-protein interactions at specific locations inside living cells.

Some Techniques Can Monitor Single Molecules

The biochemical methods described so far in this chapter are used to study large populations of molecules, a limitation that reflects the small size of typical biological molecules relative to the sensitivity of the methods to detect them. However, the recent development of highly sensitive and precise measurement methods has created a new branch of biophysics—the study of single molecules. Single-molecule studies are particularly important in cell biology because many processes rely on the activities of only a few critical molecules in the cell.

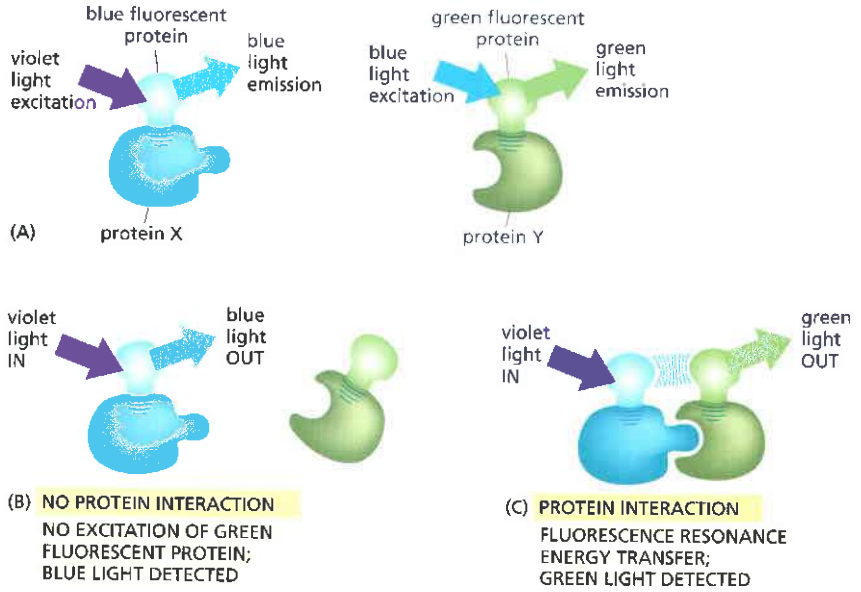


Figure 8–26 Fluorescence resonance energy transfer (FRET). To determine whether (and when) two proteins interact inside a cell, the proteins are first produced as fusion proteins attached to different color variants of green fluorescent protein (GFP). (A) In this example, protein X is coupled to a blue fluorescent protein, which is excited by violet light (370–440 nm) and emits blue light (440–480 nm); protein Y is coupled to a green fluorescent protein, which is excited by blue light and emits green light (510 nm). (B) If protein X and Y do not interact, illuminating the sample with violet light yields fluorescence from the blue fluorescent protein only. (C) When protein X and protein Y interact, FRET can now occur. Illuminating the sample with violet light excites the blue fluorescent protein, whose emission in turn excites the green fluorescent protein, resulting in an emission of green light. The fluorochromes must be quite close together—within about 1–10 nm of one another—for FRET to occur. Because not every molecule of protein X and protein Y is bound at all times, some blue light may still be detected. But as the two proteins begin to interact, emission from the donor GFP falls as the emission from the acceptor GFP rises.

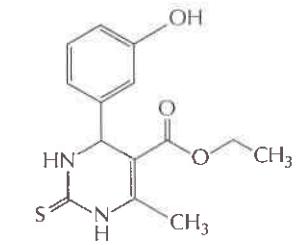
The first example of a technique for studying the function of single protein molecules was the use of a patch electrode to measure current flow through single ion channels (see Figure 11–33). Another approach is to attach the protein to a larger structure, such as a polystyrene bead, which can then be observed by conventional microscopy. This strategy has been particularly useful in measuring the movements of motor proteins. For example, molecules of the motor protein kinesin (discussed in Chapter 16) can be attached to a bead, and by observing the kinesin-attached bead moving along a microtubule, the step size of the motor (that is, the distance moved for each ATP molecule hydrolyzed) can be measured. As we will see in Chapter 9, optical microscopes have a limited resolution due to the diffraction of light, but computational and optical methods can be used to determine the position of a bead to a much finer precision than the resolution limit of the microscope. Using such techniques, extremely small movements—on the order of nanometers—can easily be detected and quantified.

Another advantage of attaching molecules to large beads is that these beads can serve as "handles" by which the molecules can be manipulated. This allows forces to be applied to the molecules, and their response observed. For example, the speed or step size of a motor can be measured as a function of the force it is pulling against. As discussed in the next chapter, a focused laser beam can be used as "optical tweezers" to generate a mechanical force on a bead, allowing motor proteins to be studied under an applied force (see Figure 9–35). <CGCG> Beads can also be manipulated using magnetic fields, a technology known as "magnetic tweezers." If multiple beads are present in a magnetic field, they will all experience the same force, potentially allowing large numbers of beads to be manipulated in parallel in a single experiment.

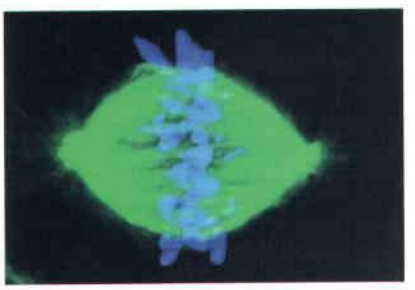
While beads can be used as markers to track protein movements, it is clearly preferable to be able to visualize the proteins themselves. In the next chapter, we shall see that recent refinements in microscopy have now made this possible.

Protein Function Can Be Selectively Disrupted With Small Molecules

Chemical inhibitors have contributed to the development of cell biology. For example, the microtubule inhibitor colchicine is routinely used to test whether microtubules are required for a given biological process; it also led to the first purification of tubulin several decades ago. In the past, these small molecules were usually natural products; that is, they were synthesized by living creatures. Although, as a whole, natural products have been extraordinarily useful in science and medicine (see, for example, Table 6–4, p. 385), they acted on a limited number of biological processes. However, the recent development of methods to synthesize hundreds of thousands of small molecules and to carry out large-scale automated screens holds the promise of identifying chemical inhibitors for virtually any biological process. In such approaches, large collections of small chemical compounds are simultaneously tested, either on living cells or in cell-free assays. Once an inhibitor is identified, it can be used as a probe to identify, through affinity chromatography (see Figure 8–13C) or other means, the protein to which the inhibitor binds. This general strategy, often called **chemical biology**, has successfully identified inhibitors of many proteins that carry out key processes in cell biology. The kinesin protein that functions in mitosis, for example, was identified by this method (**Figure 8–27**). Chemical inhibitors give the cell biologist great control over the timing of inhibition, as drugs can be rapidly added to or removed from cells, allowing protein function to be switched on or off quickly.



(A) monastrol


(B) 5 μm

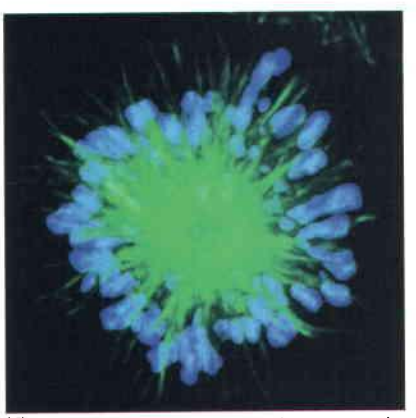
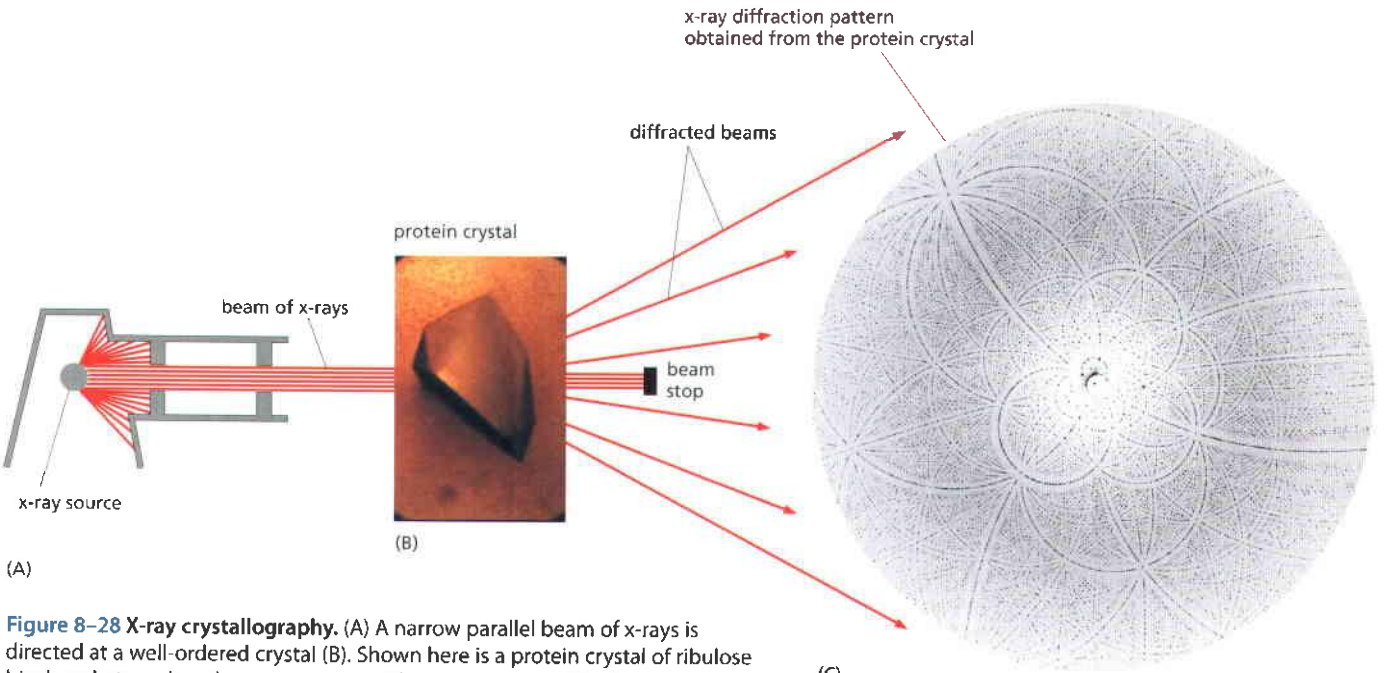

(C) 5 μm

Figure 8–27 Small-molecule inhibitors for manipulating living cells. (A) Chemical structure of monastrol, a kinesin inhibitor identified in a large-scale screen for small molecules that disrupt mitosis. (B) Normal mitotic spindle seen in an untreated cell. The microtubules are stained *green* and chromosomes *blue*. (C) Monopolar spindle that forms in cells treated with monastrol. (B and C, from T.U. Mayer et al., *Science* 286:971–974, 1999. With permission from AAAS.)

Protein Structure Can Be Determined Using X-Ray Diffraction

The main technique that has been used to discover the three-dimensional structure of molecules, including proteins, at atomic resolution is **x-ray crystallography**. X-rays, like light, are a form of electromagnetic radiation, but they have a much shorter wavelength, typically around 0.1 nm (the diameter of a hydrogen



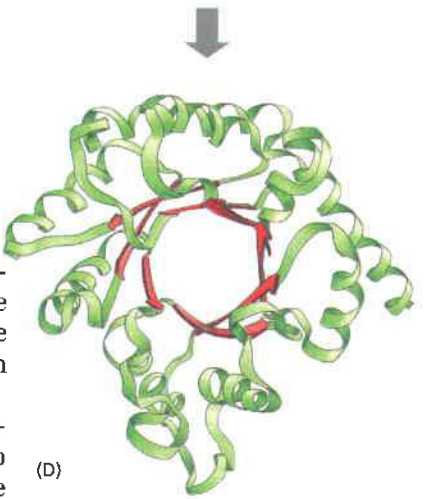


Figure 8–28 X-ray crystallography. (A) A narrow parallel beam of x-rays is directed at a well-ordered crystal (B). Shown here is a protein crystal of ribulose bisphosphate carboxylase, an enzyme with a central role in CO_2 fixation during photosynthesis. The atoms in the crystal scatter some of the beam, and the scattered waves reinforce one another at certain points and appear as a pattern of diffraction spots (C). This diffraction pattern, together with the amino acid sequence of the protein, can be used to produce an atomic model (D). The complete atomic model is hard to interpret, but this simplified version, derived from the x-ray diffraction data, shows the protein's structural features clearly (α helices, *green*; β strands, *red*). The components pictured in A to D are not shown to scale. (B, courtesy of C. Branden; C, courtesy of J. Hajdu and I. Andersson; D, adapted from original provided by B. Furugren.)

atom). If a narrow parallel beam of x-rays is directed at a sample of a pure protein, most of the x-rays pass straight through it. A small fraction, however, are scattered by the atoms in the sample. If the sample is a well-ordered crystal, the scattered waves reinforce one another at certain points and appear as diffraction spots when recorded by a suitable detector (**Figure 8–28**).

The position and intensity of each spot in the x-ray diffraction pattern contain information about the locations of the atoms in the crystal that gave rise to it. Deducing the three-dimensional structure of a large molecule from the diffraction pattern of its crystal is a complex task and was not achieved for a protein molecule until 1960. But in recent years x-ray diffraction analysis has become increasingly automated, and now the slowest step is likely to be the generation of suitable protein crystals. This step requires large amounts of very pure protein and often involves years of trial and error to discover the proper crystallization conditions; the pace has greatly accelerated with the use of recombinant DNA techniques to produce pure proteins and robotic techniques to test large numbers of crystallization conditions.

Analysis of the resulting diffraction pattern produces a complex three-dimensional electron-density map. Interpreting this map—translating its contours into a three-dimensional structure—is a complicated procedure that requires knowledge of the amino acid sequence of the protein. Largely by trial and error, the sequence and the electron-density map are correlated by computer to give the best possible fit. The reliability of the final atomic model depends on the resolution of the original crystallographic data: 0.5 nm resolution might produce a low-resolution map of the polypeptide backbone, whereas a resolution of 0.15 nm allows all of the non-hydrogen atoms in the molecule to be reliably positioned.

A complete atomic model is often too complex to appreciate directly, but simplified versions that show a protein's essential structural features can be readily derived from it (see Panel 3–2, pp. 132–133). The three-dimensional

structures of about 20,000 different proteins have now been determined by x-ray crystallography or by NMR spectroscopy (see below)—enough to begin to see families of common structures emerging. These structures or protein folds often seem to be more conserved in evolution than are the amino acid sequences that form them (see Figure 3–13).

X-ray crystallographic techniques can also be applied to the study of macromolecular complexes. In a recent triumph, the method was used to determine the structure of the ribosome, a large and complex machine made of several RNAs and more than 50 proteins (see Figure 6–64). The determination required the use of a synchrotron, a radiation source that generates x-rays with the intensity needed to analyze the crystals of such large macromolecular complexes. <GGCC>

NMR Can Be Used to Determine Protein Structure in Solution

Nuclear magnetic resonance (NMR) spectroscopy has been widely used for many years to analyze the structure of small molecules. This technique is now also increasingly applied to the study of small proteins or protein domains. Unlike x-ray crystallography, NMR does not depend on having a crystalline sample. It simply requires a small volume of concentrated protein solution that is placed in a strong magnetic field; indeed, it is the main technique that yields detailed evidence about the three-dimensional structure of molecules in solution.

Certain atomic nuclei, particularly hydrogen nuclei, have a magnetic moment or spin: that is, they have an intrinsic magnetization, like a bar magnet. The spin aligns along the strong magnetic field, but it can be changed to a misaligned, excited state in response to applied radiofrequency (RF) pulses of electromagnetic radiation. When the excited hydrogen nuclei return to their aligned state, they emit RF radiation, which can be measured and displayed as a spectrum. The nature of the emitted radiation depends on the environment of each hydrogen nucleus, and if one nucleus is excited, it influences the absorption and emission of radiation by other nuclei that lie close to it. It is consequently possible, by an ingenious elaboration of the basic NMR technique known as two-dimensional NMR, to distinguish the signals from hydrogen nuclei in different amino acid residues, and to identify and measure the small shifts in these signals that occur when these hydrogen nuclei lie close enough together to interact. Because the size of such a shift reveals the distance between the interacting pair of hydrogen atoms, NMR can provide information about the distances between the parts of the protein molecule. By combining this information with a knowledge of the amino acid sequence, it is possible in principle to compute the three-dimensional structure of the protein (**Figure 8–29**).

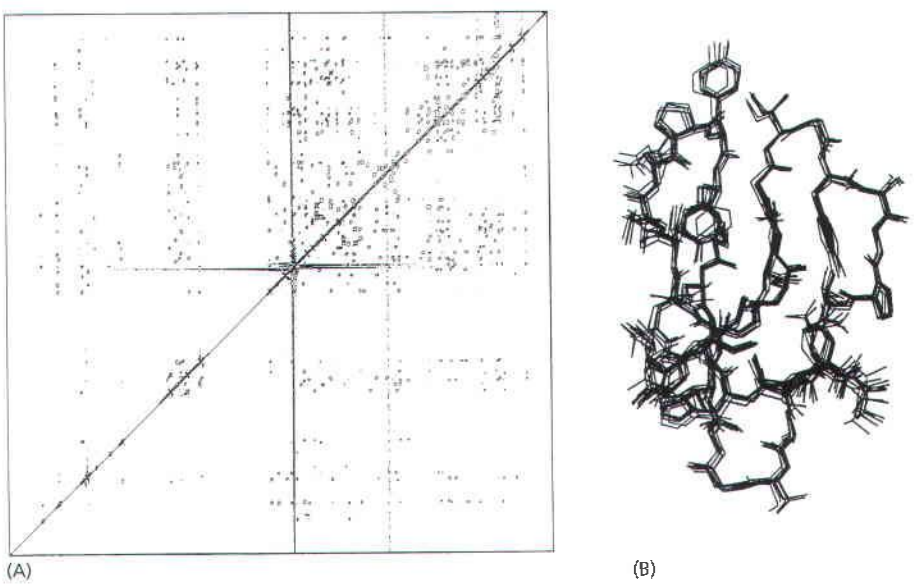


Figure 8–29 NMR spectroscopy. (A) An example of the data from an NMR machine. This two-dimensional NMR spectrum is derived from the C-terminal domain of the enzyme cellulase. The spots represent interactions between hydrogen atoms that are near neighbors in the protein and hence reflect the distance that separates them. Complex computing methods, in conjunction with the known amino acid sequence, enable possible compatible structures to be derived. (B) Ten structures of the enzyme, which all satisfy the distance constraints equally well, are shown superimposed on one another, giving a good indication of the probable three-dimensional structure. (Courtesy of P. Kraulis.)

For technical reasons the structure of small proteins of about 20,000 daltons or less can be most readily determined by NMR spectroscopy. Resolution decreases as the size of a macromolecule increases. But recent technical advances have now pushed the limit to about 100,000 daltons, thereby making the majority of proteins accessible for structural analysis by NMR.

Because NMR studies are performed in solution, this method also offers a convenient means of monitoring changes in protein structure, for example during protein folding or when the protein binds to another molecule. NMR is also used widely to investigate molecules other than proteins and is valuable, for example, as a method to determine the three-dimensional structures of RNA molecules and the complex carbohydrate side chains of glycoproteins.

Some landmarks in the development of x-ray crystallography and NMR are listed in Table 8–2.

Protein Sequence and Structure Provide Clues About Protein Function

Having discussed methods for purifying and analyzing proteins, we now turn to a common situation in cell and molecular biology: an investigator has identified a gene important for a biological process but has no direct knowledge of the biochemical properties of its protein product.

Thanks to the proliferation of protein and nucleic acid sequences that are catalogued in genome databases, the function of a gene—and its encoded protein—can often be predicted by simply comparing its sequence with those of previously characterized genes (see Figure 3–14). Because amino acid sequence

Table 8–2 Landmarks in the Development of X-ray Crystallography and NMR and Their Application to Biological Molecules

1864	**Hoppe-Seyler** crystallizes, and names, the protein hemoglobin.
1895	**Röntgen** observes that a new form of penetrating radiation, which he names x-rays, is produced when cathode rays (electrons) hit a metal target.
1912	**Von Laue** obtains the first x-ray diffraction patterns by passing x-rays through a crystal of zinc sulfide. **W.L. Bragg** proposes a simple relationship between an x-ray diffraction pattern and the arrangement of atoms in a crystal that produce the pattern.
1926	**Summer** obtains crystals of the enzyme urease from extracts of jack beans and demonstrates that proteins possess catalytic activity.
1931	**Pauling** publishes his first essays on 'The Nature of the Chemical Bond,' detailing the rules of covalent bonding.
1934	**Bernal and Crowfoot** present the first detailed x-ray diffraction patterns of a protein obtained from crystals of the enzyme pepsin.
1935	**Patterson** develops an analytical method for determining interatomic spacings from x-ray data.
1941	**Astbury** obtains the first x-ray diffraction pattern of DNA.
1946	**Block** and **Purcell** describe NMR.
1951	**Pauling and Corey** propose the structure of a helical conformation of a chain of L-amino acids—the α helix—and the structure of the β sheet, both of which were later found in many proteins.
1953	**Watson and Crick** propose the double-helix model of DNA, based on x-ray diffraction patterns obtained by **Franklin and Wilkins.**
1954	**Perutz** and colleagues develop heavy-atom methods to solve the phase problem in protein crystallography.
1960	**Kendrew** describes the first detailed structure of a protein (sperm whale myoglobin) to a resolution of 0.2 nm, and **Perutz** presents a lower-resolution structure of the larger protein hemoglobin.
1966	**Phillips** describes the structure of lysozyme, the first enzyme to have its structure analyzed in detail.
1971	**Jeener** proposes the use of two-dimensional NMR, and **Wuthrich** and colleagues first use the method to solve a protein structure in the early 1980s.
1976	**Kim and Rich** and **Klug** and colleagues describe the detailed three-dimensional structure of tRNA determined by x-ray diffraction.
1977–1978	**Holmes and Klug** determine the structure of tobacco mosaic virus (TMV), and **Harrison** and **Rossman** determine the structure of two small spherical viruses.
1985	**Michel, Deisenhofer** and colleagues determine the first structure of a transmembrane protein (a bacterial reaction center) by x-ray crystallography. **Henderson** and colleagues obtain the structure of bacteriorhodopsin, a transmembrane protein, by high-resolution electron-microscopy methods between 1975 and 1990.

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 Score =  399 bits (1025), Expect = e-111
 Identities = 198/290 (68%), Positives = 241/290 (82%), Gaps = 1/290

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Sbjct: 241  FPRWQAQDLATVVPNLDPAGLDLLSKMLRYEPSKRITARQALEHEYFKDL 290
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Figure 8–30 Results of a BLAST search. Sequence databases can be searched to find similar amino acid or nucleic acid sequences. Here, a search for proteins similar to the human cell-cycle regulatory protein Cdc2 *(Query)* locates maize Cdc2 *(Sbjct)*, which is 68% identical (and 82% similar) to human Cdc2 in its amino acid sequence. The alignment begins at residue 57 of the Query protein, suggesting that the human protein has an N-terminal region that is absent from the maize protein. The *green* blocks indicate differences in sequence, and the *yellow* bar summarizes the similarities: when the two amino acid sequences are identical, the residue is shown; conservative amino acid substitutions are indicated by a plus sign (+). Only one small gap has been introduced—indicated by the *red arrow* at position 194 in the Query sequence—to align the two sequences maximally. The alignment score *(Score)*, which is expressed in two different types of units, takes into account penalties for substitutions and gaps; the higher the alignment score, the better the match. The significance of the alignment is reflected in the *Expectation* (E) value, which specifies how often a match this good would be expected to occur by chance. The lower the E value, the more significant the match; the extremely low value here (e^{-111}) indicates certain significance. E values much higher than 0.1 are unlikely to reflect true relatedness. For example, an E value of 0.1 means there is a 1 in 10 likelihood that such a match would arise solely by chance.

determines protein structure, and structure dictates biochemical function, proteins that share a similar amino acid sequence usually have the same structure and usually perform similar biochemical functions, even when they are found in distantly related organisms. In modern cell biology, the study of a newly discovered protein usually begins with a search for previously characterized proteins that are similar in their amino acid sequences.

Searching a collection of known sequences for homologous genes or proteins is typically done over the World Wide Web, and it simply involves selecting a database and entering the desired sequence. A sequence alignment program—the most popular are BLAST and FASTA—scans the database for similar sequences by sliding the submitted sequence along the archived sequences until a cluster of residues falls into full or partial alignment (**Figure 8–30**). The results of even a complex search—which can be performed on either a nucleotide or an amino acid sequence—are returned within minutes. Such comparisons can predict the functions of individual proteins, families of proteins, or even most of the protein complement of a newly sequenced organism.

As was explained in Chapter 3, many proteins that adopt the same conformation and have related functions are too distantly related to be identified as clearly homologous from a comparison of their amino acid sequences alone (see Figure 3–13). Thus, an ability to reliably predict the three dimensional structure of a protein from its amino acid sequence would improve our ability to infer protein function from the sequence information in genomic databases. In recent years, major progress has been made in predicting the precise structure of a protein. These predictions are based, in part, on our knowledge of tens of thousands of protein structures that have already been determined by x-ray crystallography and NMR spectroscopy and, in part, on computations using our knowledge of the physical forces acting on the atoms. However, it remains a substantial and important challenge to predict the structures of proteins that are large or have multiple domains, or to predict structures at the very high levels of resolution needed to assist in computer-based drug discovery.

While finding homologous sequences and structures for a new protein will provide many clues about its function, it is usually necessary to test these insights through direct experimentation. However, the clues generated from sequence comparisons typically point the investigator in the correct experimental direction, and their use has therefore become one of the most important strategies in modern cell biology.

Summary

Most proteins function in concert with other proteins, and many methods exist for identifying and studying protein–protein interactions. Small-molecule inhibitors allow the functions of proteins they act upon to be studied in living cells. Because proteins with similar structures often have similar functions, the biochemical activity of a

protein can often be predicted by searching databases for previously characterized proteins that are similar in their amino acid sequences.

ANALYZING AND MANIPULATING DNA

Until the early 1970s, DNA was the most difficult biological molecule for the biochemist to analyze. Enormously long and chemically monotonous, the string of nucleotides that forms the genetic material of an organism could be examined only indirectly, by protein or RNA sequencing or by genetic analysis. Today, the situation has changed entirely. From being the most difficult macromolecule of the cell to analyze, DNA has become the easiest. It is now possible to isolate a specific region of almost any genome, to produce a virtually unlimited number of copies of it, and to determine the sequence of its nucleotides in a few hours. At the height of the Human Genome Project, large facilities with automated machines were generating DNA sequences at the rate of 1000 nucleotides per second, around the clock. By related techniques, an isolated gene can be altered (engineered) at will and transferred back into the germ line of an animal or plant, so as to become a functional and heritable part of the organism's genome.

These technical breakthroughs in **genetic engineering**—the ability to manipulate DNA with precision in a test tube or an organism—have had a dramatic impact on all aspects of cell biology by facilitating the study of cells and their macromolecules in previously unimagined ways. **Recombinant DNA technology** comprises a mixture of techniques, some newly developed and some borrowed from other fields such as microbial genetics (Table 8–3). Central to the technology are the following key techniques:

1. Cleavage of DNA at specific sites by restriction nucleases, which greatly facilitates the isolation and manipulation of individual genes.
2. DNA ligation, which makes it possible to design and construct DNA molecules that are not found in nature.
3. DNA cloning through the use of either cloning vectors or the polymerase chain reaction, in which a portion of DNA is repeatedly copied to generate many billions of identical molecules.
4. Nucleic acid hybridization, which makes it possible to find a specific sequence of DNA or RNA with great accuracy and sensitivity on the basis of its ability to selectively bind a complementary nucleic acid sequence.
5. Rapid determination of the sequence of nucleotides of any DNA (even entire genomes), making it possible to identify genes and to deduce the amino acid sequence of the proteins they encode.
6. Simultaneous monitoring of the level of mRNA produced by every gene in a cell, using nucleic acid microarrays, in which tens of thousands of hybridization reactions take place simultaneously.

In this section, we describe each of these basic techniques, which together have revolutionized the study of cell biology.

Restriction Nucleases Cut Large DNA Molecules into Fragments

Unlike a protein, a gene does not exist as a discrete entity in cells, but rather as a small region of a much longer DNA molecule. Although the DNA molecules in a cell can be randomly broken into small pieces by mechanical force, a fragment containing a single gene in a mammalian genome would still be only one among a hundred thousand or more DNA fragments, indistinguishable in their average size. How could such a gene be purified? Because all DNA molecules consist of an approximately equal mixture of the same four nucleotides, they cannot be readily separated, as proteins can, on the basis of their different charges and binding properties.

The solution to all of these problems began to emerge with the discovery of **restriction nucleases**. These enzymes, which can be purified from bacteria, cut the DNA double helix at specific sites defined by the local nucleotide sequence, thereby cleaving a long double-stranded DNA molecule into fragments of

Table 8–3 Some Major Steps in the Development of Recombinant DNA and Transgenic Technology

1869	**Miescher** first isolates DNA from white blood cells harvested from pus-soaked bandages obtained from a nearby hospital.
1944	**Avery** provides evidence that DNA, rather than protein, carries the genetic information during bacterial transformation.
1953	**Watson and Crick** propose the double-helix model for DNA structure based on x-ray results of **Franklin and Wilkins.**
1955	**Kornberg** discovers DNA polymerase, the enzyme now used to produce labeled DNA probes.
1961	**Marmur and Doty** discover DNA renaturation, establishing the specificity and feasibility of nucleic acid hydridization reactions.
1962	**Arber** provides the first evidence for the existence of DNA restriction nucleases, leading to their purification and use in DNA sequence characterization by **Nathans and H. Smith.**
1966	**Nirenberg, Ochoa,** and **Khorana** elucidate the genetic code.
1967	**Gellert** discovers DNA ligase, the enzyme used to join DNA fragments together.
1972–1973	DNA cloning techniques are developed by the laboratories of **Boyer, Cohen, Berg,** and their colleagues at Stanford University and the University of California at San Francisco.
1975	**Southern** develops gel-transfer hybridization for the detection of specific DNA sequences.
1975–1977	**Sanger and Barrell** and **Maxam and Gilbert** develop rapid DNA-sequencing methods.
1981–1982	**Palmiter and Brinster** produce transgenic mice; **Spradling and Rubin** produce transgenic fruit flies.
1982	**GenBank,** NIH's public genetic sequence database, is established at Los Alamos National Laboratory.
1985	**Mullis** and co-workers invent the polymerase chain reaction (PCR).
1987	**Capecchi** and **Smithies** introduce methods for performing targeted gene replacement in mouse embryonic stem cells.
1989	**Fields and Song** develop the yeast two-hybrid system for identifying and studying protein interactions.
1989	**Olson** and colleagues describe sequence-tagged sites, unique stretches of DNA that are used to make physical maps of human chromosomes.
1990	**Lipman** and colleagues release BLAST, an algorithm used to search for homology between DNA and protein sequences.
1990	**Simon** and colleagues study how to efficiently use bacterial artificial chromosomes, BACs, to carry large pieces of cloned human DNA for sequencing.
1991	**Hood and Hunkapillar** introduce new automated DNA sequence technology.
1995	**Venter** and colleagues sequence the first complete genome, that of the bacterium *Haemophilus influenzae.*
1996	**Goffeau** and an international consortium of researchers announce the completion of the first genome sequence of a eucaryote, the yeast *Saccharomyces cerevisiae.*
1996–1997	**Lockhart** and colleagues and **Brown and DeRisi** produce DNA microarrays, which allow the simultaneous monitoring of thousands of genes.
1998	**Sulston and Waterston** and colleagues produce the first complete sequence of a multicellular organism, the nematode worm *Caenorhabditis elegans.*
2001	Consortia of researchers announce the completion of the draft human genome sequence.
2004	Publication of the "finished" human genome sequence.

strictly defined sizes. Different restriction nucleases have different sequence specificities, and it is relatively simple to find an enzyme that can create a DNA fragment that includes a particular gene. The size of the DNA fragment can then be used as a basis for partial purification of the gene from a mixture.

Different species of bacteria make different restriction nucleases, which protect them from viruses by degrading incoming viral DNA. Each bacterial nuclease recognizes a specific sequence of four to eight nucleotides in DNA. These sequences, where they occur in the genome of the bacterium itself, are protected from cleavage by methylation at an A or a C nucleotide; the sequences in foreign DNA are generally not methylated and so are cleaved by the restriction nucleases. Large numbers of restriction nucleases have been purified from various species of bacteria; several hundred, most of which recognize different nucleotide sequences, are now available commercially.

Some restriction nucleases produce staggered cuts, which leave short single-stranded tails at the two ends of each fragment (**Figure 8–31**). Ends of this type are known as *cohesive ends,* as each tail can form complementary base pairs with the tail at any other end produced by the same enzyme (**Figure 8–32**). The cohesive ends generated by restriction enzymes allow any two DNA fragments to be easily joined together, as long as the fragments were generated with the same restriction nuclease (or with another nuclease that produces the same cohesive ends). DNA molecules produced by splicing together two or more DNA fragments are called **recombinant DNA** molecules.

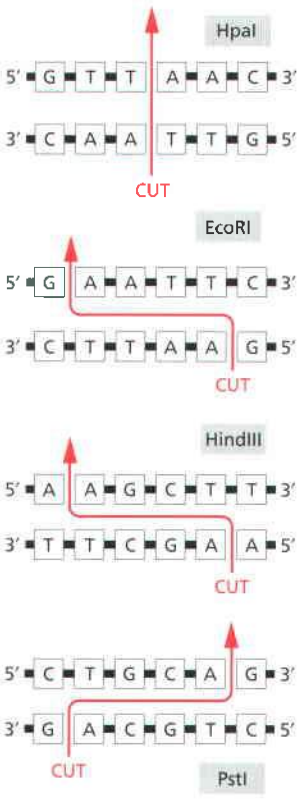


Figure 8–31 The DNA nucleotide sequences recognized by four widely used restriction nucleases. As in the examples shown, such sequences are often six base pairs long and "palindromic" (that is, the nucleotide sequence is the same if the helix is turned by 180 degrees around the center of the short region of helix that is recognized). The enzymes cut the two strands of DNA at or near the recognition sequence. For the genes encoding some enzymes, such as HpaI, the cleavage leaves blunt ends; for others, such as EcoRI, HindIII, and PstI, the cleavage is staggered and creates cohesive ends. Restriction nucleases are obtained from various species of bacteria: HpaI is from *Haemophilus parainfluenzae*, EcoRI is from *Escherichia coli*, HindIII is from *Haemophilus influenzae*, and PstI is from *Providencia stuartii*.

Gel Electrophoresis Separates DNA Molecules of Different Sizes

The same types of gel electrophoresis methods that have proved so useful in the analysis of proteins can determine the length and purity of DNA molecules. The procedure is actually simpler than for proteins: because each nucleotide in a nucleic acid molecule already carries a single negative charge (on the phosphate group), there is no need to add the negatively charged detergent SDS that is required to make protein molecules move uniformly toward the positive electrode. For DNA fragments less than 500 nucleotides long, specially designed polyacrylamide gels allow the separation of molecules that differ in length by as little as a single nucleotide (**Figure 8–33A**). The pores in polyacrylamide gels, however, are too small to permit very large DNA molecules to pass; to separate these by size, the much more porous gels formed by dilute solutions of agarose (a polysaccharide isolated from seaweed) are used (Figure 8–33B). These DNA separation methods are widely used for both analytical and preparative purposes.

A variation of agarose-gel electrophoresis, called *pulsed-field gel electrophoresis*, makes it possible to separate even extremely long DNA molecules. Ordinary gel electrophoresis fails to separate such molecules because the steady electric field stretches them out so that they travel end-first through the gel in snakelike configurations at a rate that is independent of their length. In pulsed-field gel electrophoresis, by contrast, the direction of the electric field changes periodically, which forces the molecules to reorient before continuing to move snakelike through the gel. This reorientation takes much more time for larger molecules, so that longer molecules move more slowly than shorter ones. As a consequence, even entire bacterial or yeast chromosomes separate into discrete bands in pulsed-field gels and so can be sorted and identified on the basis of their size (Figure 8–33C). Although a typical mammalian chromosome of 10^8 base pairs is too large to be sorted even in this way, large segments of these chromosomes are readily separated and identified if the chromosomal DNA is first cut with a restriction nuclease selected to recognize sequences that occur only rarely (once every 10,000 or more nucleotide pairs).

The DNA bands on agarose or polyacrylamide gels are invisible unless the DNA is labeled or stained in some way. One sensitive method of staining DNA is to expose it to the dye *ethidium bromide*, which fluoresces under ultraviolet light when it is bound to DNA (see Figure 8–33B,C). An even more sensitive detection method incorporates a radioisotope into the DNA molecules before electrophoresis; ^{32}P is often used as it can be incorporated into DNA phosphates and emits an energetic β particle that is easily detected by autoradiography, as in Figure 8–33. (For a discussion of radioisotopes, see p. 601).

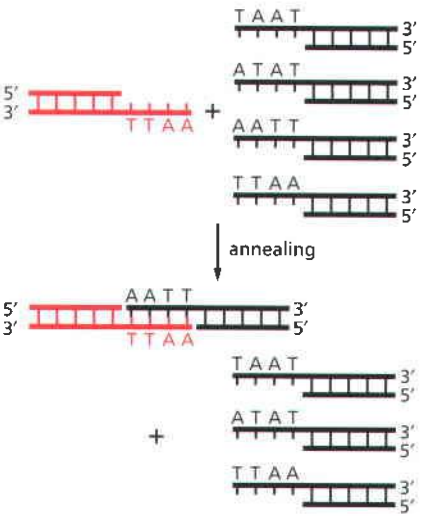


Figure 8–32 The use of restriction nucleases to produce DNA fragments that can be easily joined together. Fragments with the same cohesive ends can readily join by complementary base-pairing between their cohesive ends, as illustrated. The two DNA fragments that join in this example were both produced by the EcoRI restriction nuclease, whereas the three other fragments were produced by different restriction nucleases that generated different cohesive ends (see Figure 8–31). Blunt-ended fragments, like those generated by HpaI (see Figure 8–31), can be spliced together with more difficulty.

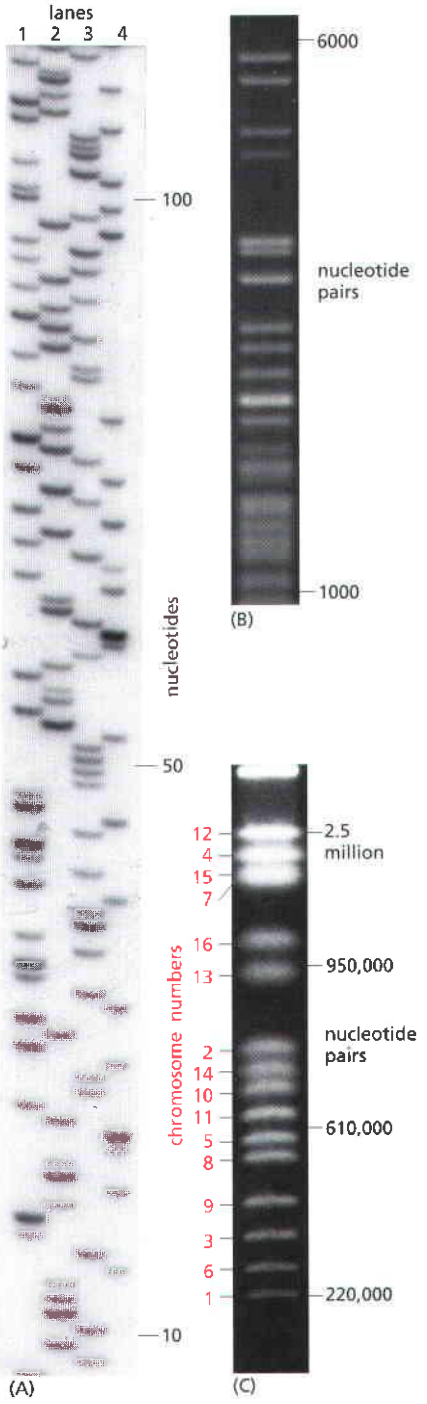


Figure 8–33 Gel electrophoresis techniques for separating DNA molecules by size. In the three examples shown, electrophoresis is from top to bottom, so that the largest—and thus slowest-moving—DNA molecules are near the top of the gel. (A) A polyacrylamide gel with small pores was used to fractionate single-stranded DNA. In the size range 10 to 500 nucleotides, DNA molecules that differ in size by only a single nucleotide can be separated from each other. In the example, the four lanes represent sets of DNA molecules synthesized in the course of a DNA-sequencing procedure. The DNA to be sequenced has been artificially replicated from a fixed start site up to a variable stopping point, producing a set of partial replicas of differing lengths. (Figure 8–50 explains how such sets of partial replicas are synthesized.) Lane 1 shows all the partial replicas that terminate in a G, lane 2 all those that terminate in an A, lane 3 all those that terminate in a T, and lane 4 all those that terminate in a C. Since the DNA molecules used in these reactions were radiolabeled, their positions can be determined by autoradiography, as shown.

(B) An agarose gel with medium-sized pores was used to separate double-stranded DNA molecules. This method is most useful in the size range 300 to 10,000 nucleotide pairs. These DNA molecules are fragments produced by cleaving the genome of a bacterial virus with a restriction nuclease, and they have been detected by their fluorescence when stained with the dye ethidium bromide. (C) The technique of pulsed-field agarose gel electrophoresis was used to separate 16 different yeast *(Saccharomyces cerevisiae)* chromosomes, which range in size from 220,000 to 2.5 million nucleotide pairs. The DNA was stained as in (B). DNA molecules as large as 10^7 nucleotide pairs can be separated in this way. (A, courtesy of Leander Lauffer and Peter Walter; B, courtesy of Ken Kreuzer; C, from D. Vollrath and R.W. Davis, *Nucleic Acids Res.* 15:7865–7876, 1987. With permission from Oxford University Press.)

Purified DNA Molecules Can Be Specifically Labeled with Radioisotopes or Chemical Markers *in vitro*

Two procedures are widely used to label isolated DNA molecules. In the first method, a DNA polymerase copies the DNA in the presence of nucleotides that are either radioactive (usually labeled with ^{32}P) or chemically tagged (**Figure 8–34**A). In this way, "DNA probes" containing many labeled nucleotides can be produced for nucleic acid hybridization reactions (discussed below). The second procedure uses the bacteriophage enzyme polynucleotide kinase to transfer a single ^{32}P-labeled phosphate from ATP to the 5′ end of each DNA chain (Figure 8–34B). Because only one ^{32}P atom is incorporated by the kinase into each DNA strand, the DNA molecules labeled in this way are often not radioactive enough to be used as DNA probes; because they are labeled at only one end, however, they have been invaluable for other applications, including DNA footprinting, as discussed in Chapter 7.

Radioactive labeling methods are being replaced by labeling with molecules that can be detected chemically or through fluorescence. To produce such non-radioactive DNA molecules, specially modified nucleotide precursors are used (Figure 8–34C). A DNA molecule made in this way is allowed to bind to its complementary DNA sequence by hybridization, as discussed in the next section, and is then detected with an antibody (or other ligand) that specifically recognizes its modified side chain (**Figure 8–35**).

Nucleic Acid Hybridization Reactions Provide a Sensitive Way to Detect Specific Nucleotide Sequences

When an aqueous solution of DNA is heated at 100°C or exposed to a very high pH (pH ≥ 13), the complementary base pairs that normally hold the two strands of the double helix together are disrupted and the double helix rapidly dissociates into two single strands. This process, called *DNA denaturation*, was for many years thought to be irreversible. In 1961, however, it was discovered that complementary single strands of DNA readily re-form double helices by a process called **hybridization** (also called *DNA renaturation*) if they are kept for a

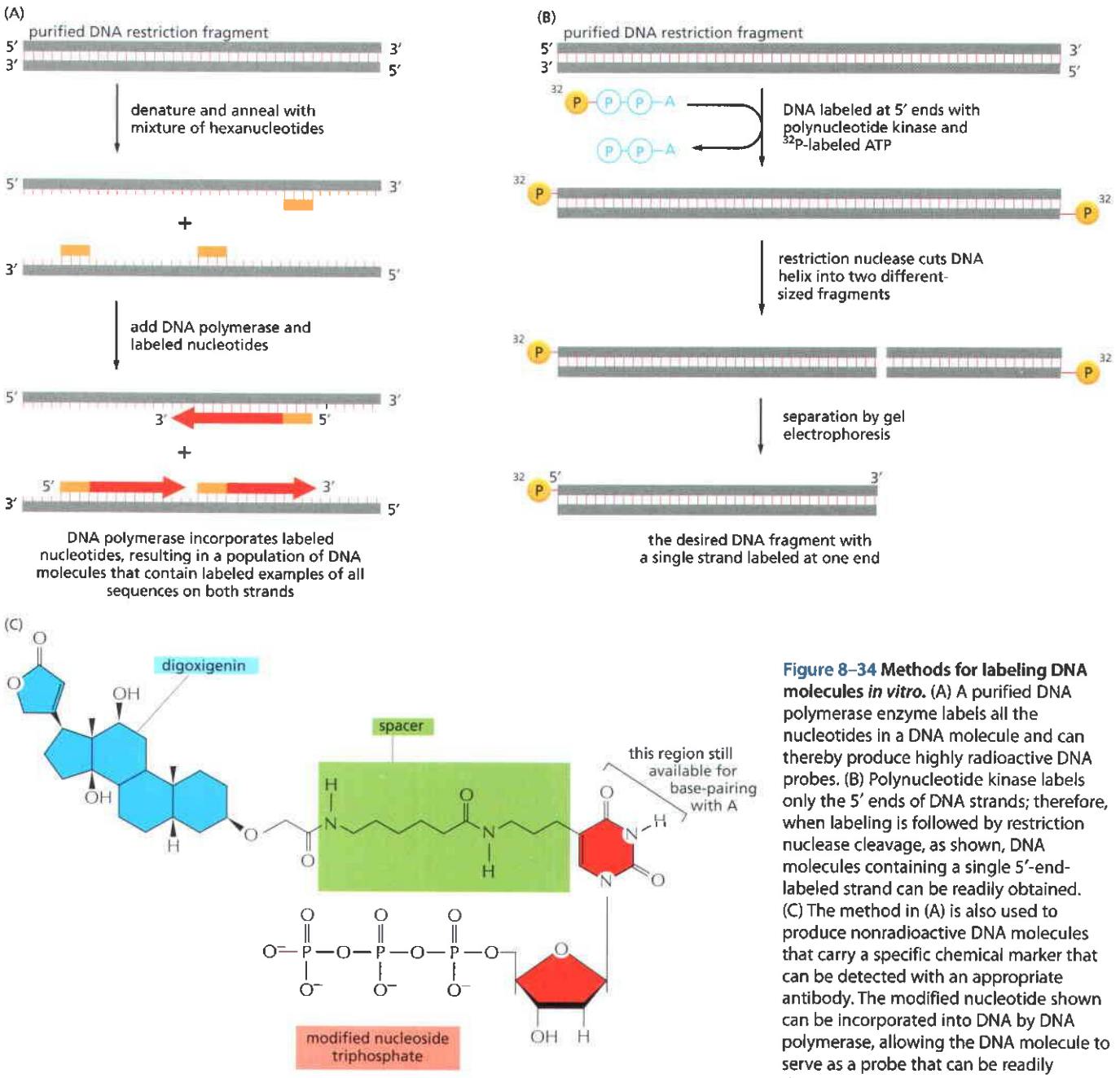


Figure 8–34 Methods for labeling DNA molecules *in vitro*. (A) A purified DNA polymerase enzyme labels all the nucleotides in a DNA molecule and can thereby produce highly radioactive DNA probes. (B) Polynucleotide kinase labels only the 5′ ends of DNA strands; therefore, when labeling is followed by restriction nuclease cleavage, as shown, DNA molecules containing a single 5′-end-labeled strand can be readily obtained. (C) The method in (A) is also used to produce nonradioactive DNA molecules that carry a specific chemical marker that can be detected with an appropriate antibody. The modified nucleotide shown can be incorporated into DNA by DNA polymerase, allowing the DNA molecule to serve as a probe that can be readily detected. The base on the nucleoside triphosphate shown is an analog of thymine, in which the methyl group on T has been replaced by a spacer arm linked to the plant steroid digoxigenin. An anti-digoxygenin antibody coupled to a visible marker such as a fluorescent dye is used to visualize the probe. Other chemical labels such as biotin can be attached to nucleotides and used in essentially the same way.

prolonged period at 65°C. Similar hybridization reactions can occur between any two single-stranded nucleic acid chains (DNA/DNA, RNA/RNA, or RNA/DNA), provided that they have complementary nucleotide sequences. These specific hybridization reactions are widely used to detect and characterize specific nucleotide sequences in both RNA and DNA molecules.

Single-stranded DNA molecules used to detect complementary sequences are known as **probes**; these molecules, which carry radioactive or chemical markers to facilitate their detection, can range from fifteen to thousands of nucleotides long. Hybridization reactions using DNA probes are so sensitive and selective that they can detect complementary sequences present at a concentration as low as one molecule per cell. It is thus possible to determine how many copies of any DNA sequence are present in a particular DNA sample. The same technique can be used to search for related but nonidentical genes. To find a gene of interest in an organism whose genome has not yet been sequenced, for example, a portion of a known gene can be used as a probe (**Figure 8–36**).

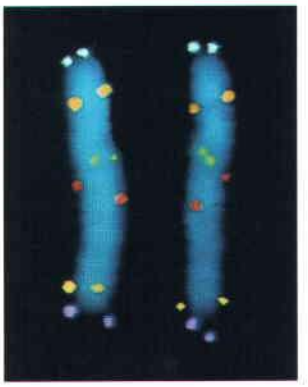


Figure 8–35 *In situ* hybridization to locate specific genes on chromosomes. Here, six different DNA probes have been used to mark the locations of their respective nucleotide sequences on human chromosome 5 at metaphase. The probes have been chemically labeled and detected with fluorescent antibodies. Both copies of chromosome 5 are shown, aligned side by side. Each probe produces two dots on each chromosome, since a metaphase chromosome has replicated its DNA and therefore contains two identical DNA helices. (Courtesy of David C. Ward.)

Alternatively, DNA probes can be used in hybridization reactions with RNA rather than DNA to find out whether a cell is expressing a given gene. In this case a DNA probe that contains part of the gene's sequence is hybridized with RNA purified from the cell in question to see whether the RNA includes nucleotide sequences matching the probe DNA and, if so, in what quantities. In somewhat more elaborate procedures, the DNA probe is treated with specific nucleases after the hybridization is complete, to determine the exact regions of the DNA probe that have paired with the RNA molecules. One can thereby determine the start and stop sites for RNA transcription, as well as the precise boundaries of the intron and exon sequences in a gene (**Figure 8–37**).

Today, the positions of intron/exon boundaries are usually determined by sequencing the *complementary DNA (cDNA)* sequences that represent the mRNAs expressed in a cell and comparing them with the nucleotide sequence of the genome. We describe later how cDNAs are prepared from mRNAs.

The hybridization of DNA probes to RNAs allows one to determine whether or not a particular gene is being transcribed; moreover, when the expression of a gene changes, one can determine whether the change is due to transcriptional or post-transcriptional controls (see Figure 7–92). These tests of gene expression were initially performed with one DNA probe at a time. *DNA microarrays* now allow the simultaneous monitoring of hundreds or thousands of genes at a time, as we discuss later. Hybridization methods are in such wide use in cell biology today that it is difficult to imagine how we could study gene structure and expression without them.

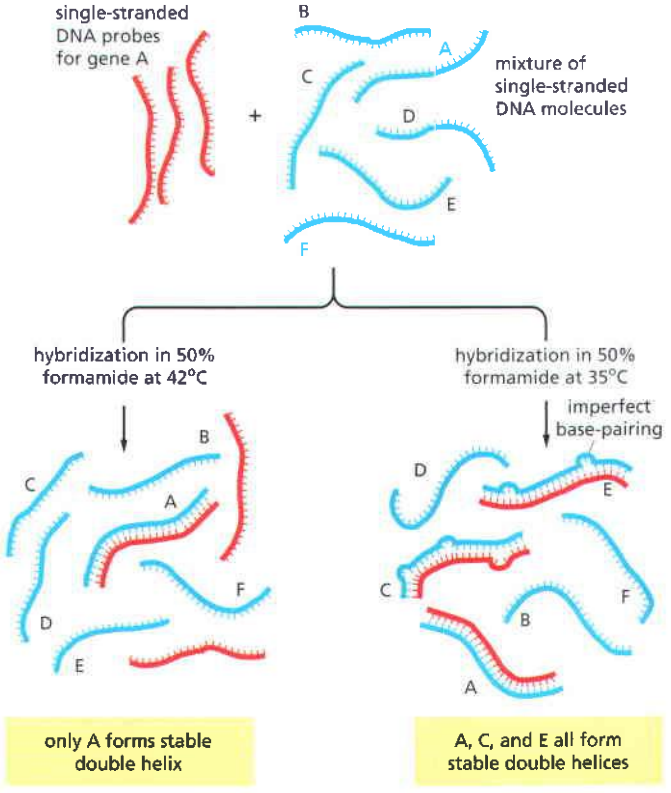


Figure 8–36 Stringent versus nonstringent hybridization conditions. To use a DNA probe to find an identical match, stringent hybridization conditions are used; the reaction temperature is kept just a few degrees below that at which a perfect DNA helix denatures in the solvent used (its *melting temperature*), so that all imperfect helices formed are unstable. When a DNA probe is being used to find DNAs with related, as well as identical, sequences, less stringent conditions are used; hybridization is performed at a lower temperature, which allows even imperfectly paired double helices to form. Only the lower-temperature hybridization conditions can be used to search for genes that are nonidentical but related to gene A (C and E in this example).

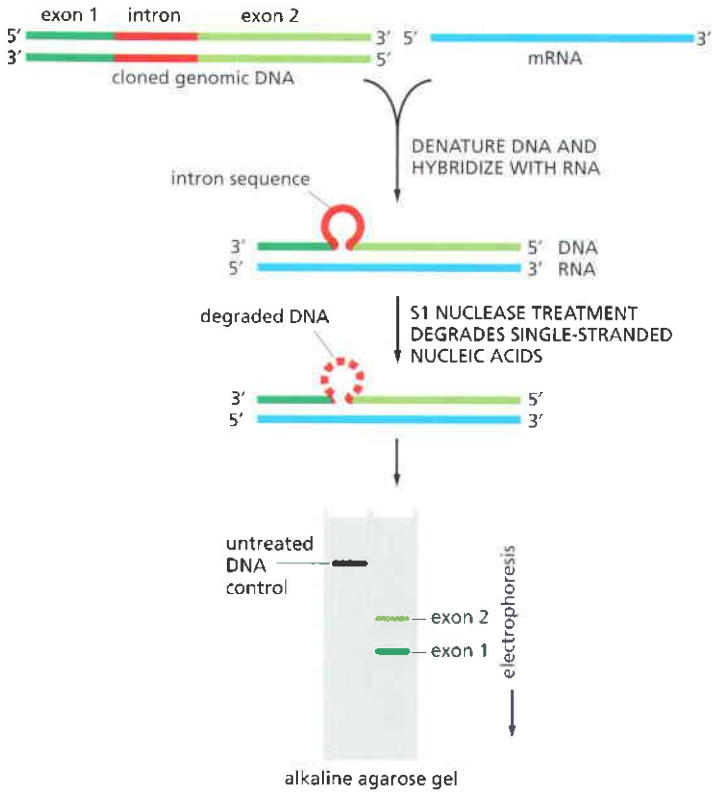


Figure 8–37 The use of nucleic acid hybridization to determine the region of a cloned DNA fragment that is present in an mRNA molecule. The method shown requires a nuclease that cuts the DNA chain only where it is not base-paired to a complementary RNA chain. The positions of the introns in eucaryotic genes are mapped by the method shown. For this type of analysis, the DNA is electrophoresed through a denaturing agarose gel, which causes it to migrate as single-stranded molecules. The location of each end of an RNA molecule can be determined using similar methods.

Northern and Southern Blotting Facilitate Hybridization with Electrophoretically Separated Nucleic Acid Molecules

In a complex mixture of nucleic acids, DNA probes are often used to detect only those molecules with sequences that are complementary to all or part of the probe. Gel electrophoresis can be used to fractionate the many different RNA or DNA molecules in a crude mixture according to their size before the hybridization reaction is performed; if the probe binds to molecules of only one or a few sizes, one can be certain that the hybridization was indeed specific. Moreover, the size information obtained can be invaluable in itself. An example illustrates this point.

Suppose that one wishes to determine the nature of the defect in a mutant mouse that produces abnormally low amounts of albumin, a protein that liver cells normally secrete into the blood in large amounts. First, one collects identical samples of liver tissue from mutant and normal mice (the latter serving as controls) and disrupts the cells in a strong detergent to inactivate nucleases that might otherwise degrade the nucleic acids. Next, one separates the RNA and DNA from all of the other cell components: the proteins present are completely denatured and removed by repeated extractions with phenol—a potent organic solvent that is partly miscible with water; the nucleic acids, which remain in the aqueous phase, are then precipitated with alcohol to separate them from the small molecules of the cell. Then, one separates the DNA from the RNA by their different solubilities in alcohols and degrades any contaminating nucleic acid of the unwanted type by treatment with a highly specific enzyme—either an RNase or a DNase. The mRNAs are typically separated from bulk RNA by retention on a chromatography column that specifically binds the poly-A tails of mRNAs.

To analyze the albumin-encoding mRNAs, a technique called **Northern blotting** is used. First, the intact mRNA molecules purified from mutant and control liver cells are fractionated on the basis of their sizes into a series of bands by gel electrophoresis. Then, to make the RNA molecules accessible to DNA probes, a replica of the pattern of RNA bands on the gel is made by transferring ("blotting") the fractionated RNA molecules onto a sheet of nitrocellulose or nylon paper. The paper is then incubated in a solution containing a labeled DNA probe, the sequence of which corresponds to part of the template strand that

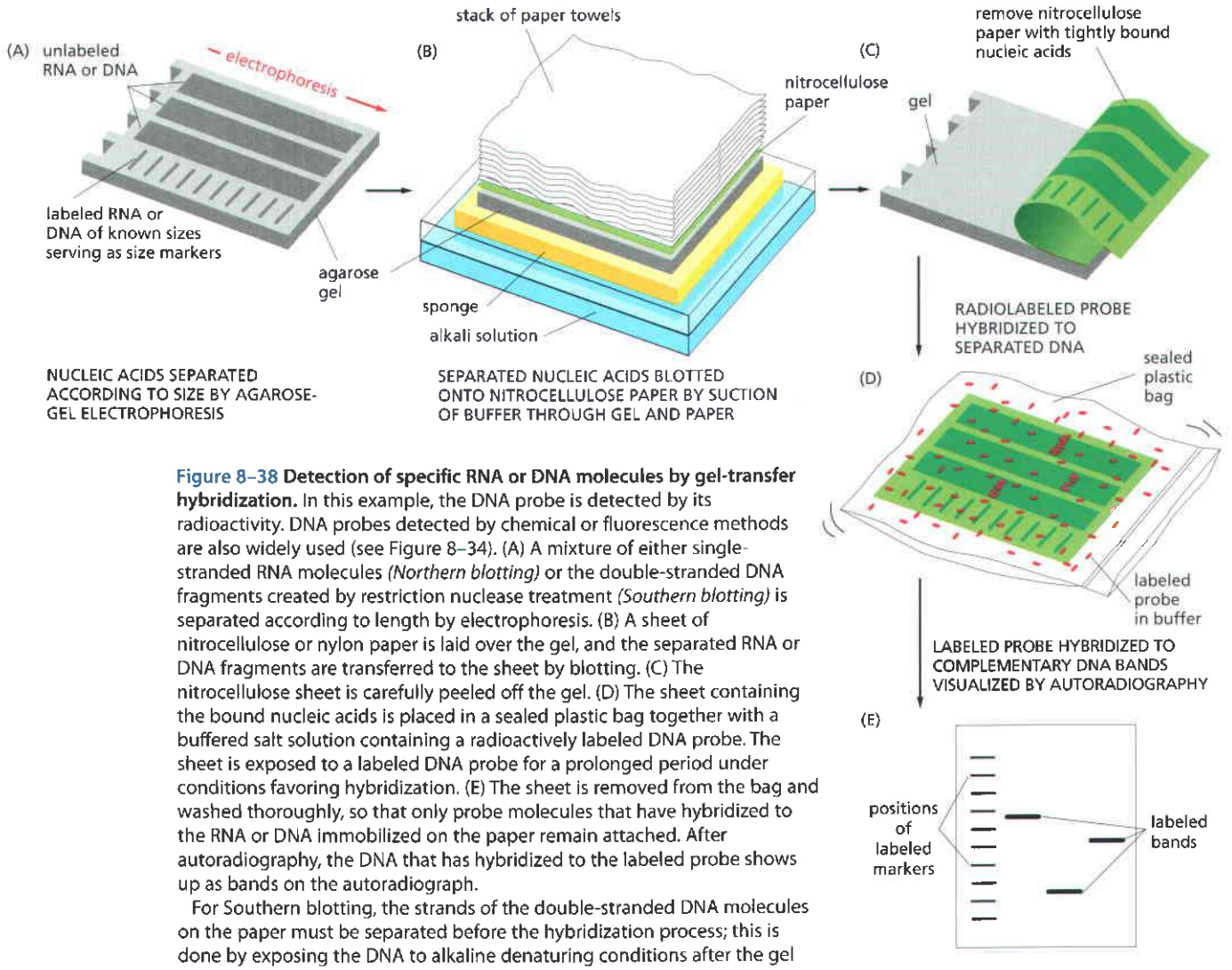


Figure 8–38 Detection of specific RNA or DNA molecules by gel-transfer hybridization. In this example, the DNA probe is detected by its radioactivity. DNA probes detected by chemical or fluorescence methods are also widely used (see Figure 8–34). (A) A mixture of either single-stranded RNA molecules *(Northern blotting)* or the double-stranded DNA fragments created by restriction nuclease treatment *(Southern blotting)* is separated according to length by electrophoresis. (B) A sheet of nitrocellulose or nylon paper is laid over the gel, and the separated RNA or DNA fragments are transferred to the sheet by blotting. (C) The nitrocellulose sheet is carefully peeled off the gel. (D) The sheet containing the bound nucleic acids is placed in a sealed plastic bag together with a buffered salt solution containing a radioactively labeled DNA probe. The sheet is exposed to a labeled DNA probe for a prolonged period under conditions favoring hybridization. (E) The sheet is removed from the bag and washed thoroughly, so that only probe molecules that have hybridized to the RNA or DNA immobilized on the paper remain attached. After autoradiography, the DNA that has hybridized to the labeled probe shows up as bands on the autoradiograph.

For Southern blotting, the strands of the double-stranded DNA molecules on the paper must be separated before the hybridization process; this is done by exposing the DNA to alkaline denaturing conditions after the gel has been run (not shown).

produces albumin mRNA. The RNA molecules that hybridize to the labeled DNA probe on the paper (because they are complementary to part of the normal albumin gene sequence) are then located by detecting the bound probe by autoradiography or by chemical means (**Figure 8–38**). The sizes of the hybridized RNA molecules can be determined by reference to RNA standards of known sizes that are electrophoresed side by side with the experimental sample. In this way, one might discover that liver cells from the mutant mice make albumin mRNA in normal amounts and of normal size; alternatively, you might find that they make it in normal size but in greatly reduced amounts. Another possibility is that the mutant albumin mRNA molecules are abnormally short; in this case the gel blot could be retested with a series of shorter DNA probes, each corresponding to small portions of the gene, to reveal which part of the normal RNA is missing.

The original gel-transfer hybridization method, called **Southern blotting**, analyzes DNA rather than RNA. (It was named after its inventor, and the Northern and Western blotting techniques were named with reference to it.) Here, isolated DNA is first cut into readily separable fragments with restriction nucleases. The double-stranded fragments are then separated on the basis of size by gel electrophoresis, and those complementary to a DNA probe are identified by blotting and hybridization, as just described for RNA (see Figure 8–38). To characterize the structure of the albumin gene in the mutant mice, an albumin-specific DNA probe would be used to construct a detailed *restriction map* of the genome in the region of the albumin gene (such a map consists of the pattern of DNA fragments produced by various restriction nucleases). From this map one

could determine if the albumin gene has been rearranged in the defective animals—for example, by the deletion or the insertion of a short DNA sequence; most single-base changes, however, could not be detected in this way.

Genes Can Be Cloned Using DNA Libraries

Any DNA fragment can be cloned. In molecular biology, the term **DNA cloning** is used in two senses. In one sense, it literally refers to the act of making many identical copies of a DNA molecule—the amplification of a particular DNA sequence. However, the term also describes the isolation of a particular stretch of DNA (often a particular gene) from the rest of a cell's DNA, because this isolation is greatly facilitated by making many identical copies of the DNA of interest. As discussed earlier in this chapter, cloning, particularly when used in the context of developmental biology, can also refer to the generation of many genetically identical cells starting from a single cell or even to the generation of genetically identical organisms. In all cases, cloning refers to the act of making many genetically identical copies; in this section, we will use the term cloning (or DNA cloning or gene cloning) to refer to methods designed to generate many identical copies of a segment of nucleic acid.

DNA cloning in its most general sense can be accomplished in several ways. The simplest involves inserting a particular fragment of DNA into the purified DNA genome of a self-replicating genetic element—generally a virus or a plasmid. A DNA fragment containing a human gene, for example, can be joined in a test tube to the chromosome of a bacterial virus, and the new recombinant DNA molecule can then be introduced into a bacterial cell, where the inserted DNA fragment will be replicated along with the DNA of the virus. Starting with only one such recombinant DNA molecule that infects a single cell, the normal replication mechanisms of the virus can produce more than 10^{12} identical virus DNA molecules in less than a day, thereby amplifying the amount of the inserted human DNA fragment by the same factor. A virus or plasmid used in this way is known as a *cloning vector*, and the DNA propagated by insertion into it is said to have been *cloned*.

To isolate a specific gene, one often begins by constructing a *DNA library*—a comprehensive collection of cloned DNA fragments from a cell, tissue, or organism. This library includes (one hopes) at least one fragment that contains the gene of interest. Libraries can be constructed with either a virus or a plasmid vector and are generally housed in a population of bacterial cells. The principles underlying the methods used for cloning genes are the same for either type of cloning vector, although the details may differ. Today, most cloning is performed with plasmid vectors.

The **plasmid vectors** most widely used for gene cloning are small circular molecules of double-stranded DNA derived from larger plasmids that occur naturally in bacterial cells. They generally account for only a minor fraction of the total host bacterial cell DNA, but they can easily be separated owing to their small size from chromosomal DNA molecules, which are large and precipitate as a pellet upon centrifugation. For use as cloning vectors, the purified plasmid DNA circles are first cut with a restriction nuclease to create linear DNA molecules. The genomic DNA to be used in constructing the library is cut with the same restriction nuclease, and the resulting restriction fragments (including those containing the gene to be cloned) are then added to the cut plasmids and annealed via their cohesive ends to form recombinant DNA circles. These recombinant molecules containing foreign DNA inserts are then covalently sealed with the enzyme DNA ligase (**Figure 8–39**).

In the next step in preparing the library, the recombinant DNA circles are introduced into bacterial cells that have been made transiently permeable to DNA. These bacterial cells are now said to be *transfected* with the plasmids. As the cells grow and divide, doubling in number every 30 minutes, the recombinant plasmids also replicate to produce an enormous number of copies of DNA circles containing the foreign DNA (**Figure 8–40**). Many bacterial plasmids carry genes for antibiotic resistance (discussed in Chapter 24), a property that can be

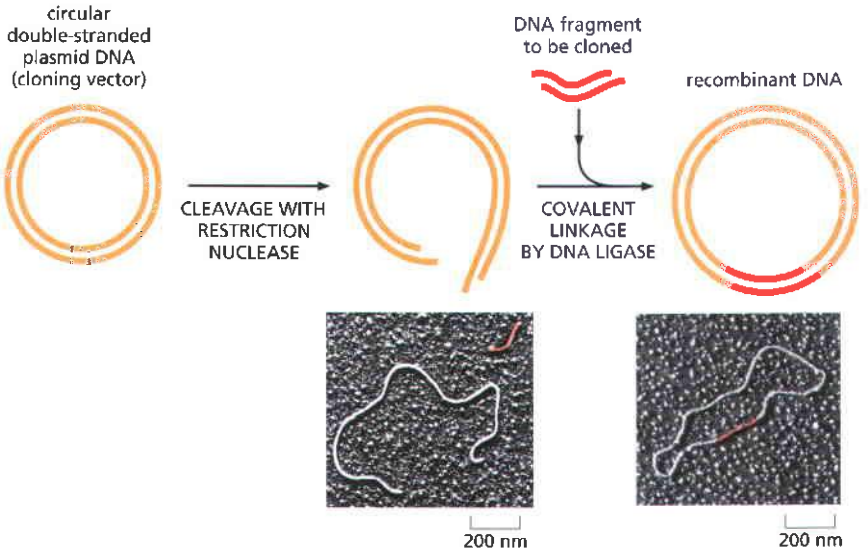


Figure 8–39 The insertion of a DNA fragment into a bacterial plasmid with the enzyme DNA ligase. The plasmid is cut open with a restriction nuclease (in this case one that produces cohesive ends) and is mixed with the DNA fragment to be cloned (which has been prepared with the same restriction nuclease). DNA ligase and ATP are added. The cohesive ends base-pair, and DNA ligase seals the nicks in the DNA backbone, producing a complete recombinant DNA molecule. (Micrographs courtesy of Huntington Potter and David Dressler.)

exploited to select those cells that have been successfully transfected; if the bacteria are grown in the presence of the antibiotic, only cells containing plasmids will survive. Each original bacterial cell that was initially transfected contains, in general, a different foreign DNA insert; this insert is inherited by all of the progeny cells of that bacterium, which together form a small colony in a culture dish.

For many years, plasmids were used to clone fragments of DNA of 1000 to 30,000 nucleotide pairs. Larger DNA fragments are more difficult to handle and were harder to clone. Then researchers began to use *yeast artificial chromosomes* (*YACs*), which could accommodate very large pieces of DNA (**Figure 8–41**). Today, new plasmid vectors based on the naturally occurring F plasmid of *E. coli* are used to clone DNA fragments of 300,000 to 1 million nucleotide pairs. Unlike smaller bacterial plasmids, the F plasmid—and its derivative, the **bacterial artificial chromosome** (**BAC**)—is present in only one or two copies per *E. coli* cell. The fact that BACs are kept in such low numbers in bacterial cells may contribute to their ability to maintain large cloned DNA sequences stably: with only a few BACs present, it is less likely that the cloned DNA fragments will become scrambled by recombination with sequences carried on other copies of the plasmid. Because of their stability, ability to accept large DNA inserts, and ease of handling, BACs are now the preferred vector for building DNA libraries of complex organisms—including those representing the human and mouse genomes.

Two Types of DNA Libraries Serve Different Purposes

Cleaving the entire genome of a cell with a specific restriction nuclease and cloning each fragment as just described produces a very large number of DNA fragments—on the order of a million for a mammalian genome. The fragments are distributed among millions of different colonies of transfected bacterial cells.

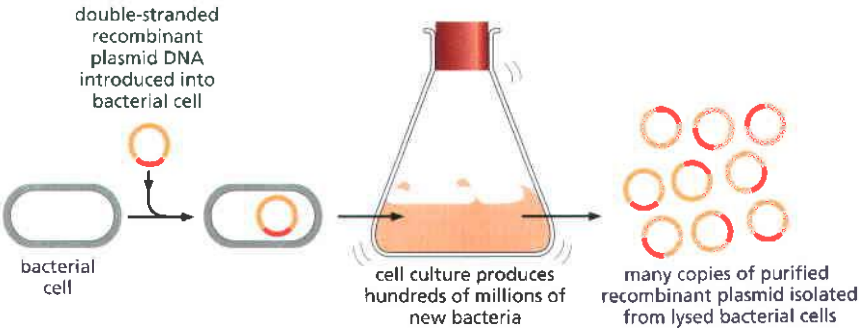


Figure 8–40 The amplification of the DNA fragments inserted into a plasmid. To produce large amounts of the DNA of interest, the recombinant plasmid DNA in Figure 8–39 is introduced into a bacterium by transfection, where it will replicate many millions of times as the bacterium multiplies.

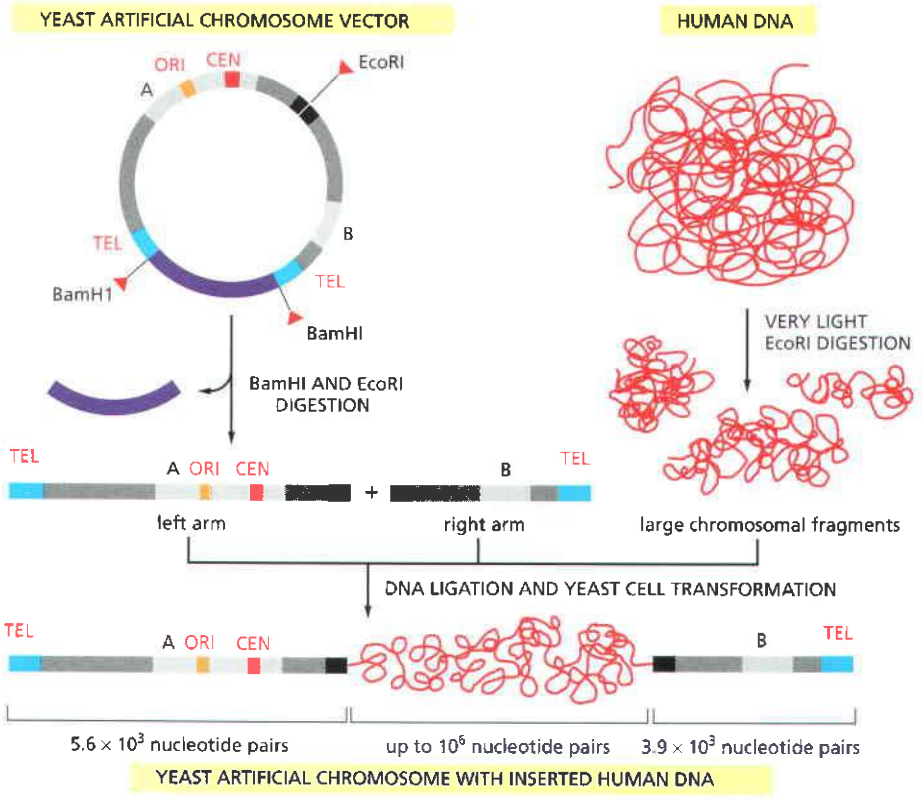


Figure 8–41 The making of a yeast artificial chromosome (YAC). A YAC vector allows the cloning of very large DNA molecules. TEL, CEN, and ORI are the telomere, centromere, and origin of replication sequences, respectively, for the yeast *Saccharomyces cerevisiae;* all of these are required to propagate the YAC. BamHI and EcoRI are sites where the corresponding restriction nucleases cut the DNA double helix. The sequences denoted A and B encode enzymes that serve as selectable markers to allow the easy isolation of yeast cells that have taken up the artificial chromosome. Because bacteria divide more rapidly than yeasts, most large-scale cloning projects now use *E. coli* as the means for amplifying DNA. (Adapted from D.T. Burke, G.F. Carle and M.V. Olson, *Science* 236:806–812, 1987. With permission from AAAS.)

When working with BACs rather than typical plasmids, larger fragments can be inserted, and so fewer transfected bacterial cells are required to cover the genome. In either case, each of the colonies is composed of a clone of cells derived from a single ancestor cell, and therefore harbors many copies of a particular stretch of the fragmented genome (Figure 8–42). Such a plasmid is said to contain a **genomic DNA clone**, and the entire collection of plasmids is called a **genomic DNA library**. But because the genomic DNA is cut into fragments at random, only some fragments contain genes. Many of the genomic DNA clones obtained from the DNA of a higher eucaryotic cell contain only noncoding DNA, which, as we discussed in Chapter 4, makes up most of the DNA in such genomes.

An alternative strategy is to begin the cloning process by selecting only those DNA sequences that are transcribed into mRNA and thus are presumed to correspond to protein-encoding genes. This is done by extracting the mRNA from cells and then making a DNA copy of each mRNA molecule present—a so-called *complementary DNA,* or *cDNA.* The copying reaction is catalyzed by the reverse transcriptase enzyme of retroviruses, which synthesizes a complementary DNA chain on an RNA template. The single-stranded cDNA molecules synthesized by the reverse transcriptase are converted into double-stranded cDNA molecules by DNA polymerase, and these molecules are inserted into a plasmid or virus vector and cloned (Figure 8–43). Each clone obtained in this way is called a **cDNA clone**, and the entire collection of clones derived from one mRNA preparation constitutes a **cDNA library**.

Figure 8–44 illustrates some important differences between genomic DNA clones and cDNA clones. Genomic clones represent a random sample of all of the DNA sequences in an organism and, with very rare exceptions, are the same regardless of the cell type used to prepare them. By contrast, cDNA clones contain only those regions of the genome that have been transcribed into mRNA. Because the cells of different tissues produce distinct sets of mRNA molecules, a distinct cDNA library is obtained for each type of cell used to prepare the library.

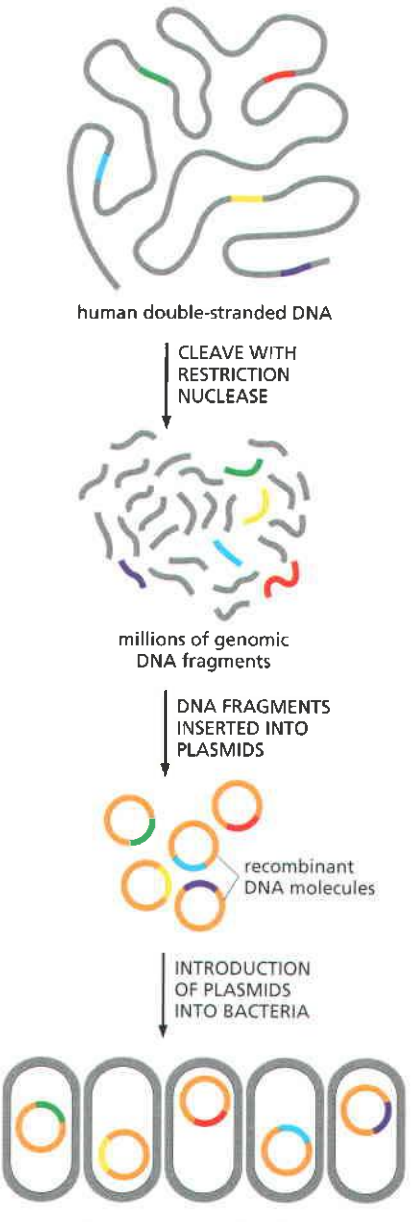


Figure 8–42 Construction of a human genomic DNA library. A genomic library is usually stored as a set of bacteria, each bacterium carrying a different fragment of human DNA. For simplicity, cloning of just a few representative fragments *(colored)* is shown. In reality, all of the *gray* DNA fragments would also be cloned.

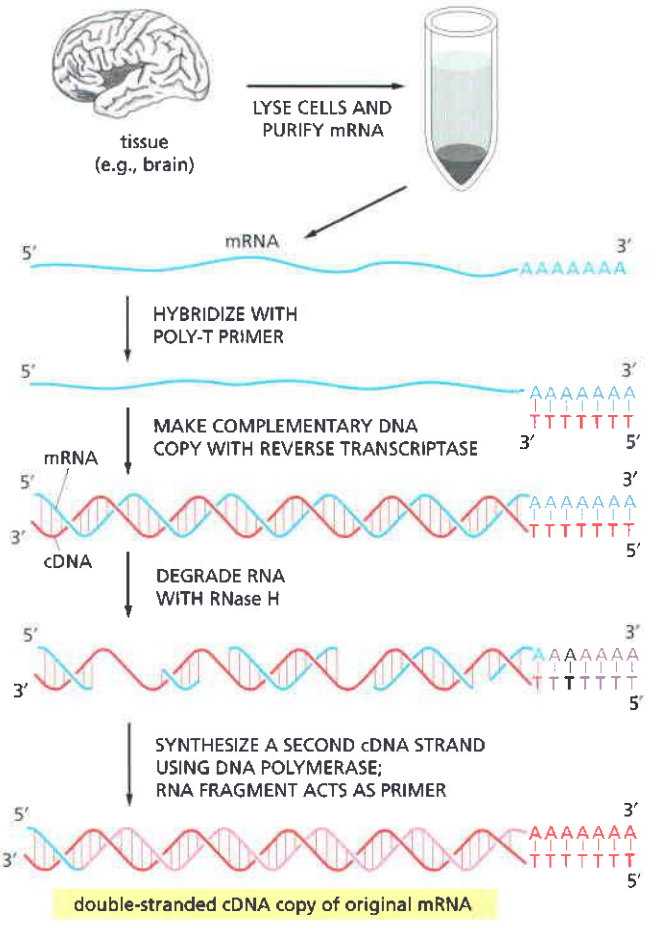


Figure 8–43 The synthesis of cDNA. Total mRNA is extracted from a particular tissue, and the enzyme reverse transcriptase produces DNA copies (cDNA) of the mRNA molecules (see p. 320). For simplicity, the copying of just one of these mRNAs into cDNA is illustrated. A short oligonucleotide complementary to the poly-A tail at the 3′ end of the mRNA (discussed in Chapter 6) is first hybridized to the RNA to act as a primer for the reverse transcriptase, which then copies the RNA into a complementary DNA chain, thereby forming a DNA/RNA hybrid helix. Treating the DNA/RNA hybrid with RNase H (see Figure 5–12) creates nicks and gaps in the RNA strand. The enzyme DNA polymerase then copies the remaining single-stranded cDNA into double-stranded cDNA. The fragment of the original mRNA is the primer for this synthesis reaction, as shown. Because the DNA polymerase used to synthesize the second DNA strand can synthesize through the bound RNA molecules, the RNA fragment that is base-paired to the 3′ end of the first DNA strand usually acts as the primer for the final product of the second strand synthesis. This RNA is eventually degraded during subsequent cloning steps. As a result, the nucleotide sequences at the extreme 5′ ends of the original mRNA molecules are often absent from cDNA libraries.

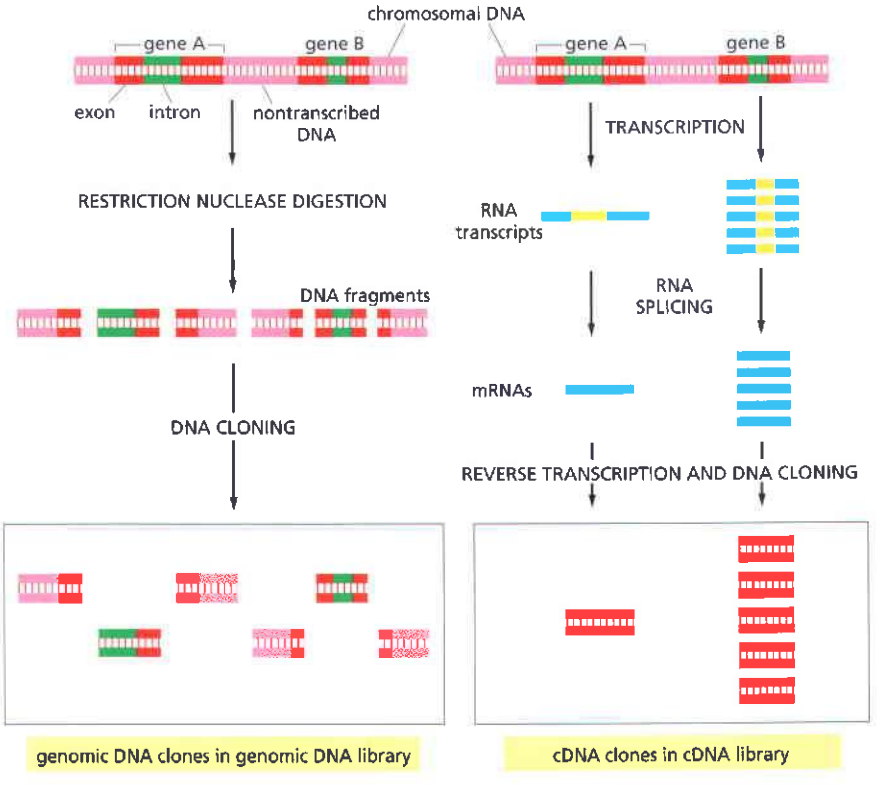


Figure 8–44 The differences between cDNA clones and genomic DNA clones derived from the same region of DNA. In this example, gene A is infrequently transcribed, whereas gene B is frequently transcribed, and both genes contain introns *(green)*. In the genomic DNA library, both the introns and the nontranscribed DNA *(pink)* are included in the clones, and most clones contain, at most, only part of the coding sequence of a gene *(red)*. In the cDNA clones, the intron sequences *(yellow)* have been removed by RNA splicing during the formation of the mRNA *(blue)*, and a continuous coding sequence is therefore present in each clone. Because gene B is transcribed more frequently than gene A in the cells from which the cDNA library was made, it is represented much more frequently than A in the cDNA library. In contrast, A and B are in principle represented equally in the genomic DNA library.

cDNA Clones Contain Uninterrupted Coding Sequences

There are several advantages in using a cDNA library for gene cloning. First, specialized cells produce large quantities of some proteins. In this case, the mRNA encoding the protein is likely to be produced in such large quantities that a cDNA library prepared from the cells is highly enriched for the cDNA molecules encoding the protein, greatly reducing the problem of identifying the desired clone in the library (see Figure 8–44). Hemoglobin, for example, is made in large amounts by developing erythrocytes (red blood cells); for this reason the globin genes were among the first to be cloned.

By far the most important advantage of cDNA clones is that they contain the uninterrupted coding sequence of a gene. As we have seen, eucaryotic genes usually consist of short coding sequences of DNA (exons) separated by much longer noncoding sequences (introns); the production of mRNA entails the removal of the noncoding sequences from the initial RNA transcript and the splicing together of the coding sequences. Neither bacterial nor yeast cells will make these modifications to the RNA produced from a gene of a higher eucaryotic cell. Thus, when the aim of the cloning is either to deduce the amino acid sequence of the protein from the DNA sequence or to produce the protein in bulk by expressing the cloned gene in a bacterial or yeast cell, it is much preferable to start with cDNA. cDNA libraries have an additional use: as described in Chapter 7, many mRNAs from humans and other complex organisms are alternatively spliced, and a cDNA library often represents many, if not all, of the alternatively spliced mRNAs produced from a given cell line or tissue.

Genomic and cDNA libraries are inexhaustible resources, which are widely shared among investigators. Today, many such libraries are also available from commercial sources.

Genes Can Be Selectively Amplified by PCR

Now that so many genome sequences are available, genes can be cloned directly without the need to first construct DNA libraries. A technique called the **polymerase chain reaction** (**PCR**) makes this rapid cloning possible. Starting with an entire genome, PCR allows the DNA from a selected region to be amplified several billionfold, effectively "purifying" this DNA away from the remainder of the genome.

To begin, a pair of DNA oligonucleotides, chosen to flank the desired nucleotide sequence of the gene, are synthesized by chemical methods. These oligonucleotides are then used to prime DNA synthesis on single strands generated by heating the DNA from the entire genome. The newly synthesized DNA is produced in a reaction catalyzed *in vitro* by a purified DNA polymerase, and the primers remain at the 5′ ends of the final DNA fragments that are made (**Figure 8–45**A).

Nothing special is produced in the first cycle of DNA synthesis; the power of the PCR method is revealed only after repeated rounds of DNA synthesis. Every cycle doubles the amount of DNA synthesized in the previous cycle. Because each cycle requires a brief heat treatment to separate the two strands of the template DNA double helix, the technique requires the use of a special DNA polymerase, isolated from a thermophilic bacterium, that is stable at much higher temperatures than normal so that it is not denatured by the repeated heat treatments. With each round of DNA synthesis, the newly generated fragments serve as templates in their turn, and within a few cycles the predominant product is a single species of DNA fragment whose length corresponds to the distance between the two original primers (see Figure 8–45B).

In practice, effective DNA amplification requires 20–30 reaction cycles, with the products of each cycle serving as the DNA templates for the next—hence the term polymerase "chain reaction." A single cycle requires only about 5 minutes, and the entire procedure can be easily automated. PCR thereby makes possible the "cell-free molecular cloning" of a DNA fragment in a few hours, compared with the several days required for standard cloning procedures. This technique

is now used routinely to clone DNA from genes of interest directly—starting either from genomic DNA or from mRNA isolated from cells (**Figure 8–46**).

The PCR method is extremely sensitive; it can detect a single DNA molecule in a sample. Trace amounts of RNA can be analyzed in the same way by first transcribing them into DNA with reverse transcriptase. The PCR cloning technique has largely replaced Southern blotting for the diagnosis of genetic diseases and for the detection of low levels of viral infection. It also has great promise in forensic medicine as a means of analyzing minute traces of blood or other tissues—

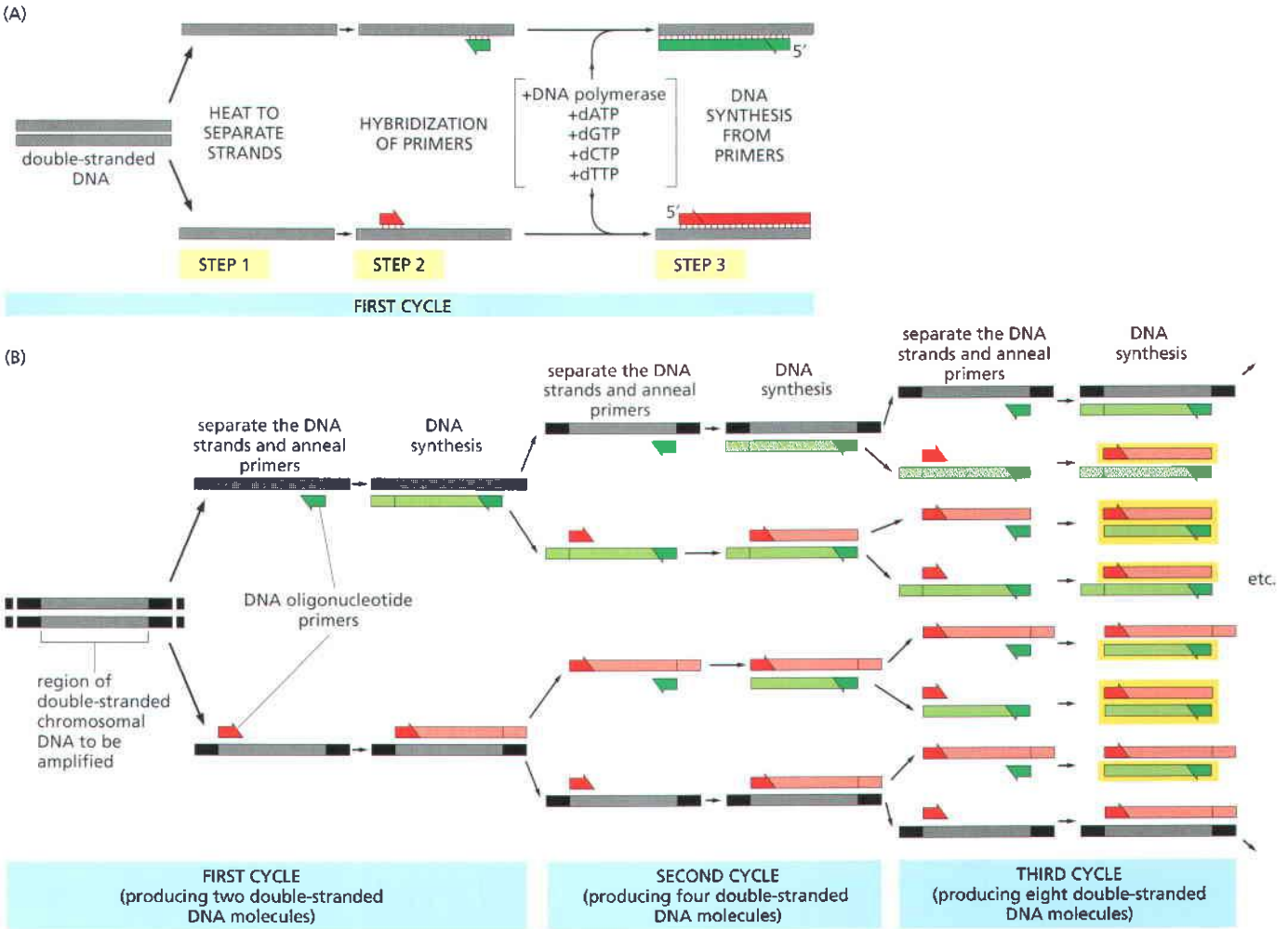


Figure 8–45 Amplification of DNA by the PCR technique. <TACG> Knowledge of the DNA sequence to be amplified is used to design two synthetic, primer DNA oligonucleotides. One primer is complementary to the sequence on one strand of the DNA double helix, and one is complementary to the sequence on the other strand, but at the opposite end of the region to be amplified. These oligonucleotides serve as primers for *in vitro* DNA synthesis, which is performed by a DNA polymerase, and they determine the segment of the DNA to be amplified. (A) PCR starts with a double-stranded DNA, and each cycle of the reaction begins with a brief heat treatment to separate the two strands (step 1). After strand separation, cooling of the DNA in the presence of a large excess of the two primer DNA oligonucleotides allows these primers to hybridize to complementary sequences in the two DNA strands (step 2). This mixture is then incubated with DNA polymerase and the four deoxyribonucleoside triphosphates to synthesize DNA, starting from the two primers (step 3). The entire cycle is then begun again by a heat treatment to separate the newly synthesized DNA strands. (B) As the procedure is performed over and over again, the newly synthesized fragments serve as templates in their turn, and within a few cycles the predominant DNA is identical to the sequence bracketed by and including the two primers in the original template. Of the DNA put into the original reaction, only the sequence bracketed by the two primers is amplified because there are no primers attached anywhere else. In the example illustrated in (B), three cycles of reaction produce 16 DNA chains, 8 of which *(boxed in yellow)* are the same length as and correspond exactly to one or the other strand of the original bracketed sequence shown at the far left; the other strands contain extra DNA downstream of the original sequence, which is replicated in the first few cycles. After four more cycles, 240 of the 256 DNA chains correspond exactly to the original bracketed sequence, and after several more cycles, essentially all of the DNA strands have this unique length.

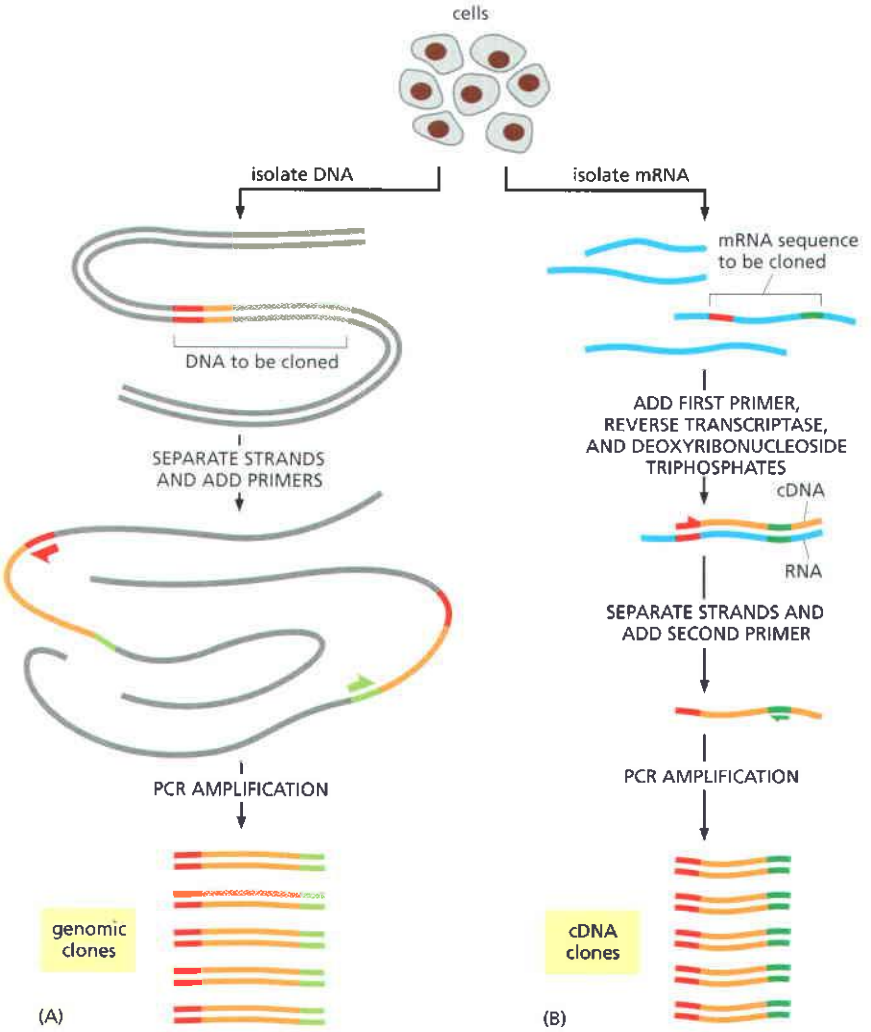


Figure 8–46 Use of PCR to obtain a genomic or cDNA clone. (A) To obtain a genomic clone using PCR, chromosomal DNA is first purified from cells. PCR primers that flank the stretch of DNA to be cloned are added, and many cycles of the reaction are completed (see Figure 8–45). Since only the DNA between (and including) the primers is amplified, PCR provides a way to obtain a short stretch of chromosomal DNA selectively in a virtually pure form. (B) To use PCR to obtain a cDNA clone of a gene, mRNA is first purified from cells. The first primer is then added to the population of mRNAs, and reverse transcriptase is used to make a complementary DNA strand. The second primer is then added, and the single-stranded cDNA molecule is amplified through many cycles of PCR, as shown in Figure 8–45. For both types of cloning, the nucleotide sequence of at least part of the region to be cloned must be known beforehand.

even as little as a single cell—and identifying the person from whom the sample came by his or her genetic "fingerprint" (**Figure 8–47**).

Cells Can Be Used As Factories to Produce Specific Proteins

The vast majority of the thousands of different proteins in a cell, including many with crucially important functions, are present in very small amounts. In the past, for most of them, it has been extremely difficult, if not impossible, to obtain more than a few micrograms of pure material. One of the most important contributions of DNA cloning and genetic engineering to cell biology is that they have made it possible to produce any of the cell's proteins in nearly unlimited amounts.

Large amounts of a desired protein are produced in living cells by using **expression vectors** (**Figure 8–48**). These are generally plasmids that have been designed to produce a large amount of a stable mRNA that can be efficiently translated into protein in the transfected bacterial, yeast, insect, or mammalian cell. To prevent the high level of the foreign protein from interfering with the transfected cell's growth, the expression vector is often designed to delay the synthesis of the foreign mRNA and protein until shortly before the cells are harvested and lysed (**Figure 8–49**).

Because the desired protein made from an expression vector is produced inside a cell, it must be purified away from the host-cell proteins by chromatography after cell lysis; but because it is such a plentiful species in the cell lysate (often 1–10% of the total cell protein), the purification is usually easy to accomplish in only a few steps. As we saw above, many expression vectors have been

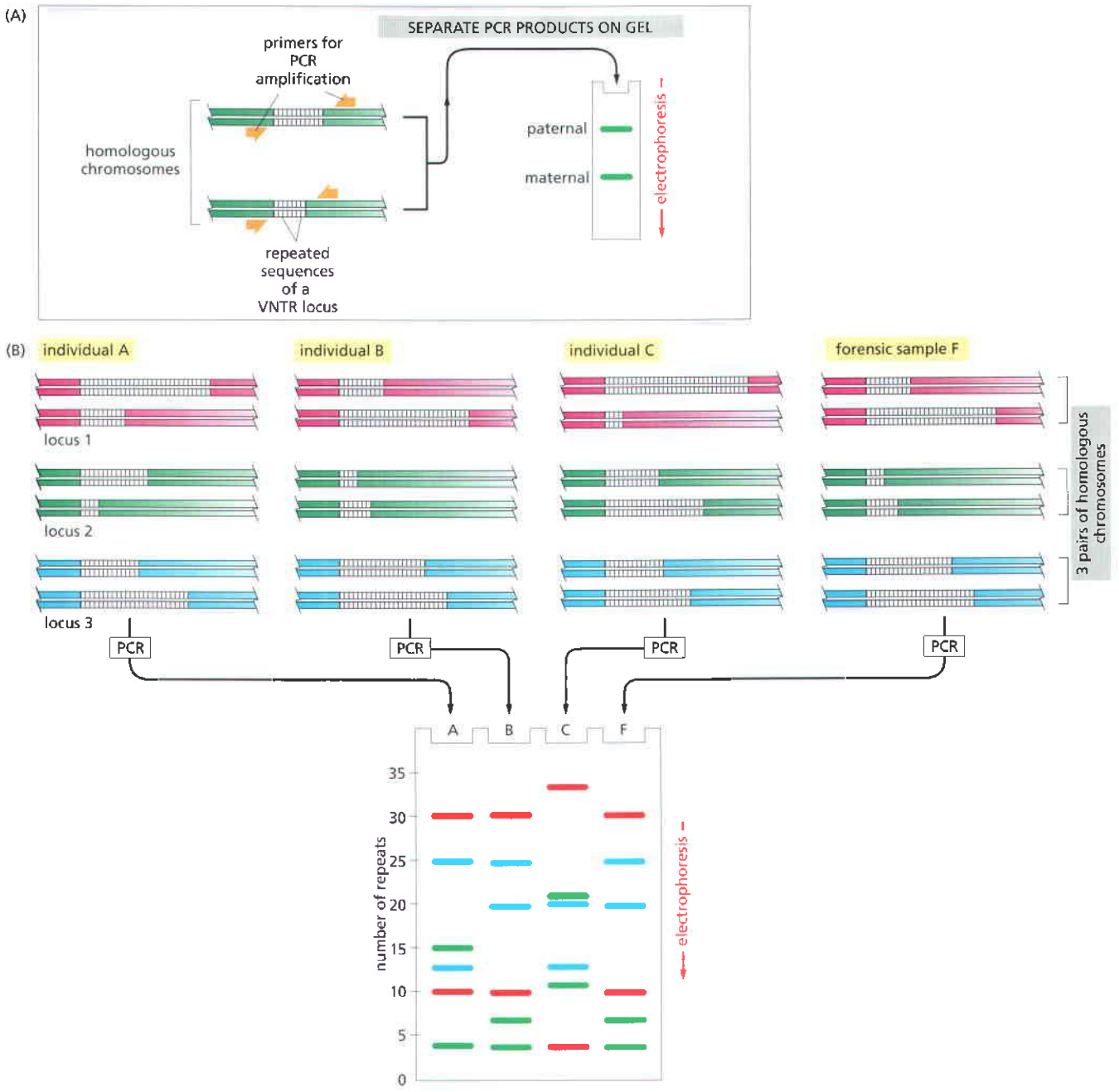


Figure 8–47 How PCR is used in forensic science. (A) The DNA sequences that create the variability used in this analysis contain runs of short, repeated sequences, such as CACACA . . ., which are found in various positions (loci) in the human genome. The number of repeats in each run can be highly variable in the population, ranging from 4 to 40 in different individuals. A run of repeated nucleotides of this type is commonly referred to as a *hypervariable microsatellite* sequence—also known as a VNTR *(variable number of tandem repeat)* sequence. Because of the variability in these sequences at each locus, individuals usually inherit a different variant from their mother and from their father; two unrelated individuals therefore do not usually contain the same pair of sequences. A PCR analysis using primers that bracket the locus produces a pair of bands of amplified DNA from each individual, one band representing the maternal variant and the other representing the paternal variant. The length of the amplified DNA, and thus the position of the band it produces after electrophoresis, depends on the exact number of repeats at the locus. (B) In the schematic example shown here, the same three VNTR loci are analyzed (requiring three different pairs of specially selected oligonucleotide primers) from three suspects (individuals A, B, and C), producing six DNA bands for each person after polyacrylamide-gel electrophoresis. Although some individuals have several bands in common, the overall pattern is quite distinctive for each. The band pattern can therefore serve as a "fingerprint" to identify an individual nearly uniquely. The fourth lane (F) contains the products of the same reactions carried out on a forensic sample. The starting material for such a PCR can be a single hair or a tiny sample of blood that was left at the crime scene. When examining the variability at 5–10 different VNTR loci, the odds that two random individuals would share the same genetic pattern by chance can be approximately 1 in 10 billion. In the case shown here, individuals A and C can be eliminated from further enquiries, whereas individual B remains a clear suspect for committing the crime. A similar approach is now routinely used for paternity testing.

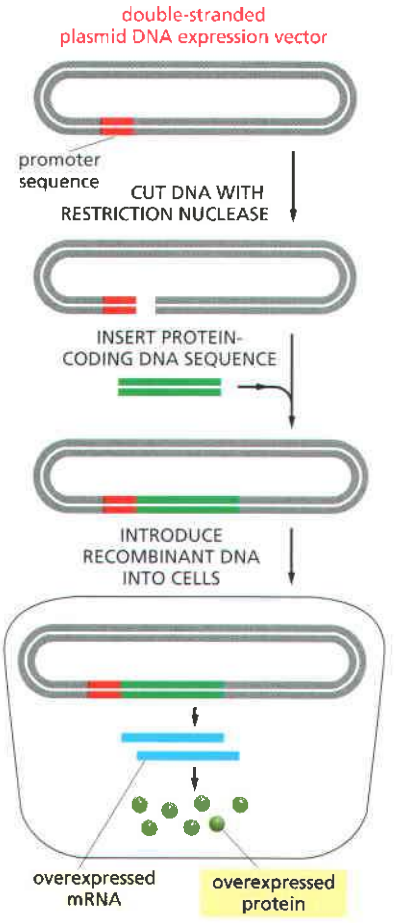


Figure 8–48 Production of large amounts of a protein from a protein-coding DNA sequence cloned into an expression vector and introduced into cells. A plasmid vector has been engineered to contain a highly active promoter, which causes unusually large amounts of mRNA to be produced from an adjacent protein-coding gene inserted into the plasmid vector. Depending on the characteristics of the cloning vector, the plasmid is introduced into bacterial, yeast, insect, or mammalian cells, where the inserted gene is efficiently transcribed and translated into protein.

designed to add a molecular tag—a cluster of histidine residues or a small marker protein—to the expressed protein to allow easy purification by affinity chromatography (see Figure 8–16). A variety of expression vectors are available, each engineered to function in the type of cell in which the protein is to be made. In this way, cells can be induced to make vast quantities of medically useful proteins—such as human insulin and growth hormone, interferon, and viral antigens for vaccines. More generally, these methods make it possible to produce every protein—even those that may be present in only a few copies per cell—in large enough amounts to be used in the kinds of detailed structural and functional studies that we discussed earlier.

DNA technology also can produce large amounts of any RNA molecule whose gene has been isolated. Studies of RNA splicing, protein synthesis, and RNA-based enzymes, for example, are greatly facilitated by the availability of pure RNA molecules. Most RNAs are present in only tiny quantities in cells, and they are very difficult to purify away from other cell components—especially from the many thousands of other RNAs present in the cell. But any RNA of interest can be synthesized efficiently *in vitro* by transcription of its DNA sequence (produced by one of the methods just described) with a highly efficient viral RNA polymerase. The single species of RNA produced is then easily purified away from the DNA template and the RNA polymerase.

Proteins and Nucleic Acids Can Be Synthesized Directly by Chemical Reactions

Chemical reactions have been devised to synthesize directly specific sequences of amino acids or nucleic acids. These methodologies provide direct sources of biological molecules and do not rely on any cells or enzymes. Chemical synthesis is the method of choice for obtaining nucleic acids in the range of 100 nucleotides or fewer, which are particularly useful in the PCR-based approaches discussed above. Chemical synthesis is also routinely used to produce specific peptides that, when chemically coupled to other proteins, are used to generate antibodies against the peptide.

DNA Can Be Rapidly Sequenced

Methods that allow the nucleotide sequence of any DNA fragment to be determined simply and quickly have made it possible to determine the DNA sequences of tens of thousands of genes, and many complete genomes (see Table 1–1, p. 18). The volume of DNA sequence information is now so large (many tens of billions of nucleotides) that powerful computers must be used to store and analyze it.

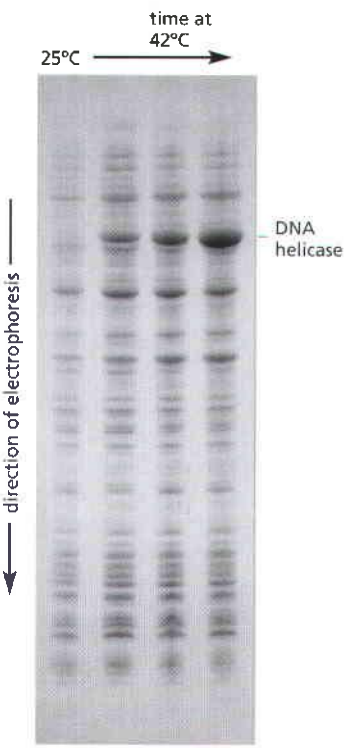


Figure 8–49 Production of large amounts of a protein by using a plasmid expression vector. In this example, bacterial cells have been transfected with the coding sequence for an enzyme, DNA helicase; transcription from this coding sequence is under the control of a viral promoter that becomes active only at temperatures of 37°C or higher. The total cell protein has been analyzed by SDS polyacrylamide-gel electrophoresis, either from bacteria grown at 25°C (no helicase protein made) or after a shift of the same bacteria to 42°C for up to 2 hours (helicase protein has become the most abundant protein species in the lysate). (Courtesy of Jack Barry.)

Large-volume DNA sequencing was made possible through the development in the mid-1970s of the **dideoxy method** for sequencing DNA, which is based on *in vitro* DNA synthesis performed in the presence of chain-terminating dideoxyribonucleoside triphosphates (**Figure 8–50**).

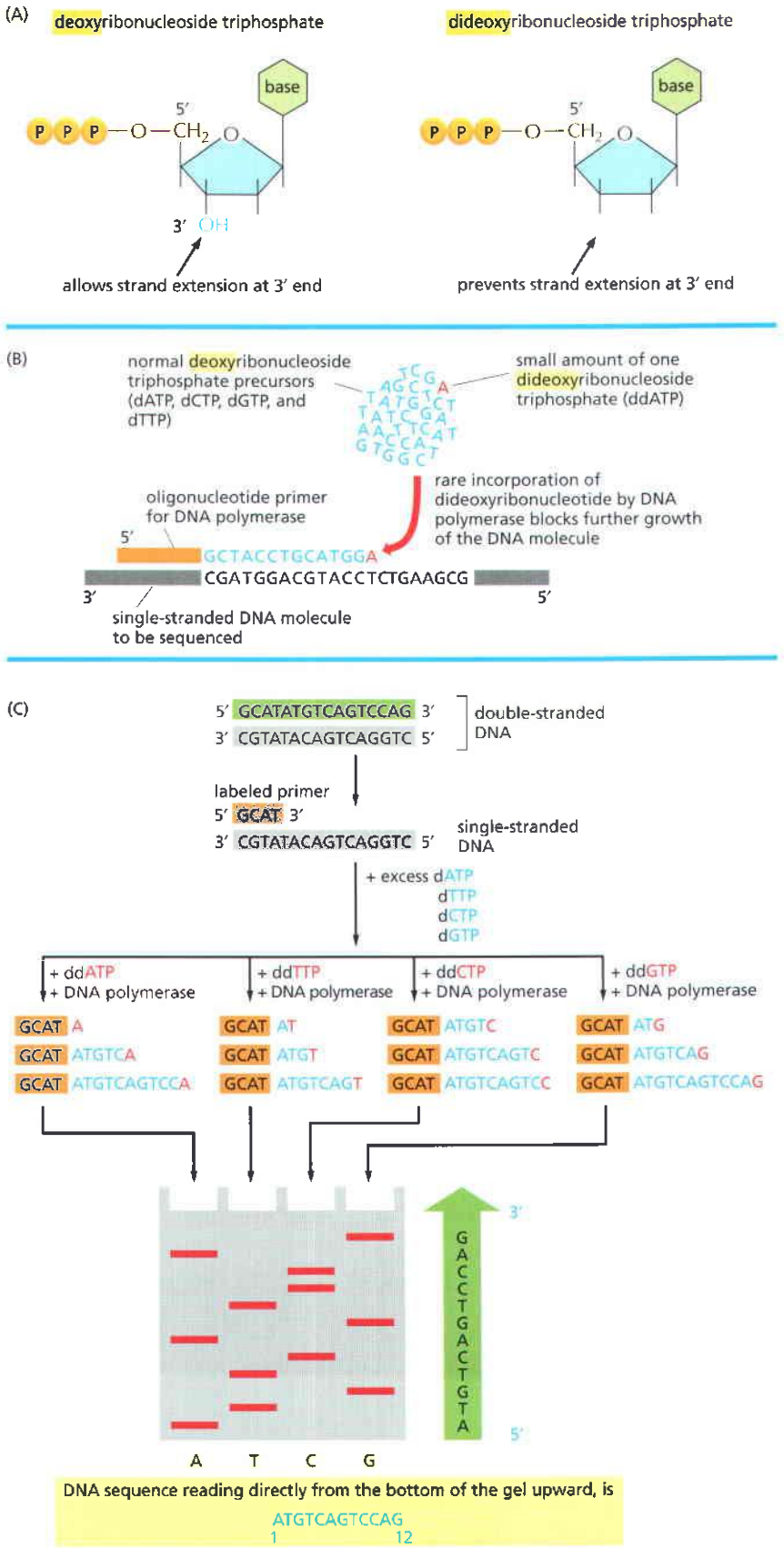


Figure 8–50 The enzymatic—or dideoxy—method of sequencing DNA. (A) This method relies on the use of dideoxyribonucleoside triphosphates, derivatives of the normal deoxyribonucleoside triphosphates that lack the 3′ hydroxyl group. (B) Purified DNA is synthesized *in vitro* in a mixture that contains single-stranded molecules of the DNA to be sequenced *(gray)*, the enzyme DNA polymerase, a short primer DNA *(orange)* to enable the polymerase to start DNA synthesis, and the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP: *blue* A, C, G, and T). If a dideoxyribonucleotide analog *(red)* of one of these nucleotides is also present in the nucleotide mixture, it can become incorporated into a growing DNA chain. Because this chain now lacks a 3′ OH group, the addition of the next nucleotide is blocked, and the DNA chain terminates at that point. In the example illustrated, a small amount of dideoxyATP (ddATP, symbolized here as a *red* A) has been included in the nucleotide mixture. It competes with an excess of the normal deoxyATP (dATP, *blue* A), so that ddATP is occasionally incorporated, at random, into a growing DNA strand. This reaction mixture will eventually produce a set of DNAs of different lengths complementary to the template DNA that is being sequenced and terminating at each of the different As. The exact lengths of the DNA synthesis products can then be used to determine the position of each A in the growing chain. (C) To determine the complete sequence of a DNA fragment, the double-stranded DNA is first separated into its single strands and one of the strands is used as the template for sequencing. Four different chain-terminating dideoxyribonucleoside triphosphates (ddATP, ddCTP, ddGTP, ddTTP, again shown in *red)* are used in four separate DNA synthesis reactions on copies of the same single-stranded DNA template *(gray)*. Each reaction produces a set of DNA copies that terminate at different points in the sequence. The products of these four reactions are separated by electrophoresis in four parallel lanes of a polyacrylamide gel (labeled here A, T, C, and G). The newly synthesized fragments are detected by a label (either radioactive or fluorescent) that has been incorporated either into the primer or into one of the deoxyribonucleoside triphosphates used to extend the DNA chain. In each lane, the bands represent fragments that have terminated at a given nucleotide (e.g., A in the leftmost lane) but at different positions in the DNA. By reading off the bands in order, starting at the bottom of the gel and working across all lanes, the DNA sequence of the newly synthesized strand can be determined. The sequence is given in the *green arrow* to the right of the gel. This sequence is complementary to the template strand *(gray)* from the original double-stranded DNA molecule, and identical to a portion of the *green* 5′-to-3′ strand.

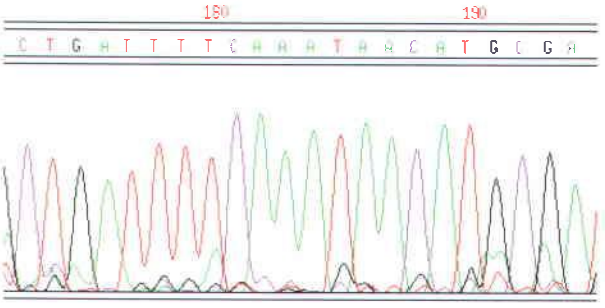
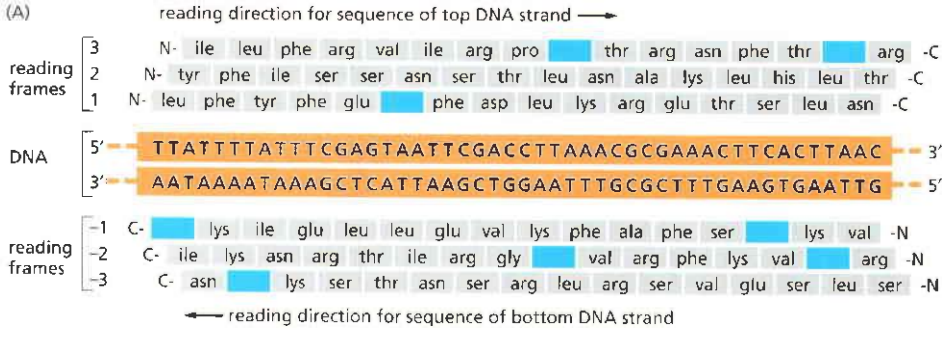


Figure 8–51 Automated DNA sequencing. Shown at the bottom is a tiny part of the raw data from an automated DNA-sequencing run as it appears on the computer screen. Each prominant colored peak represents a nucleotide in the DNA sequence—a clear stretch of nucleotide sequence can be read here between positions 173 and 194 from the start of the sequence. The small peaks along the baseline represent background "noise" and, as long as they are much lower than the "signal" peaks, they are ignored. This particular example is taken from the international project that determined the complete nucleotide sequence of the genome of the plant *Arabidopsis*. (Courtesy of George Murphy.)

Although the same basic method is still used today, many improvements have been made. DNA sequencing is now completely automated: robotic devices mix the reagents and then load, run, and read the order of the nucleotide bases from the gel. Chain-terminating nucleotides that are each labeled with a different colored fluorescent dye facilitate these tasks; in this case, all four synthesis reactions can be performed in the same tube, and the products can be separated in a single lane of a gel. A detector positioned near the bottom of the gel reads and records the color of the fluorescent label on each band as it passes through a laser beam (**Figure 8–51**). A computer then reads and stores this nucleotide sequence. Some modern systems dispense with the traditional gel entirely, separating nucleic acids by capillary electrophoresis, a method that facilitates rapid automation.

Nucleotide Sequences Are Used to Predict the Amino Acid Sequences of Proteins

Now that DNA sequencing is so rapid and reliable, it has become the preferred method for determining, indirectly, the amino acid sequences of most proteins. Given a nucleotide sequence that encodes a protein, the procedure is quite straightforward. Although in principle there are six different reading frames in which a DNA sequence can be translated into protein (three on each strand), the correct one is generally recognizable as the only one lacking frequent stop codons (**Figure 8–52**). As we saw when we discussed the genetic code in Chap-



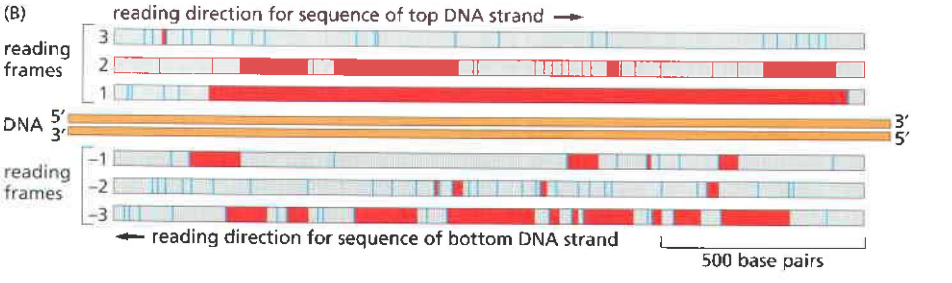


Figure 8–52 Finding the regions in a DNA sequence that encode a protein. (A) Any region of the DNA sequence can, in principle, code for six different amino acid sequences, because any one of three different reading frames can be used to interpret the nucleotide sequence on each strand. Note that a nucleotide sequence is always read in the 5′-to-3′ direction and encodes a polypeptide from the N-terminus to the C-terminus. For a random nucleotide sequence read in a particular frame, a stop signal for protein synthesis is encountered, on average, about once every 20 amino acids. In this sample sequence of 48 base pairs, each such signal *(stop codon)* is colored *blue*, and only reading frame 2 lacks a stop signal. (B) Search of a 1700 base-pair DNA sequence for a possible protein-encoding sequence. The information is displayed as in (A), with each stop signal for protein synthesis denoted by a *blue line*. In addition, all of the regions between possible start and stop signals for protein synthesis (see p. 381) are displayed as *red bars*. Only reading frame 1 actually encodes a protein, which is 475 amino acid residues long.

ter 6, a random sequence of nucleotides, read in frame, will encode a stop signal for protein synthesis about once every 20 amino acids. Nucleotide sequences that encode a stretch of amino acids much longer than this are candidates for presumptive exons, and they can be translated (by computer) into amino acid sequences and checked against databases for similarities to known proteins from other organisms. If necessary, a limited amount of amino acid sequence can then be determined from the purified protein to confirm the sequence predicted from the DNA.

The problem comes, however, in determining which nucleotide sequences—within a whole genome—represent genes that encode proteins. Identifying genes is easiest when the DNA sequence is from a bacterial or archaeal chromosome, which lacks introns, or from a cDNA clone. The location of genes in these nucleotide sequences can be predicted by examining the DNA for certain distinctive features (discussed in Chapter 6). Briefly, these genes that encode proteins are identified by searching the nucleotide sequence for *open reading frames (ORFs)* that begin with an initiation codon, usually ATG, and end with a termination codon, TAA, TAG, or TGA. To minimize errors, computers used to search for ORFs are often directed to count as genes only those sequences that are longer than, say, 100 codons in length.

For more complex genomes, such as those of animals and plants, the presence of large introns embedded within the coding portion of genes complicates the process. In many multicellular organisms, including humans, the average exon is only 150 nucleotides long. Thus one must also search for other features that signal the presence of a gene, for example, sequences that signal an intron/exon boundary or distinctive upstream regulatory regions. Recent efforts to solve the exon prediction problem have turned to artificial intelligence algorithms, in which the computer learns, based on known examples, what sets of features are most indicative of an exon boundary.

A second major approach to identifying the coding regions in chromosomes is through the characterization of the nucleotide sequences of the detectable mRNAs (using the corresponding cDNAs). The mRNAs (and the cDNAs produced from them) lack introns, regulatory DNA sequences, and the nonessential "spacer" DNA that lies between genes. It is therefore useful to sequence large numbers of cDNAs to produce a very large database of the coding sequences of an organism. These sequences are then readily used to distinguish the exons from the introns in the long chromosomal DNA sequences that correspond to genes.

The Genomes of Many Organisms Have Been Fully Sequenced

Owing in large part to the automation of DNA sequencing, the genomes of many organisms have been fully sequenced; these include plant chloroplasts and animal mitochondria, large numbers of bacteria, and archaea, and many of the model organisms that are studied routinely in the laboratory, including many yeasts, a nematode worm, the fruit fly *Drosophila*, the model plant *Arabidopsis*, the mouse, dog, chimpanzee, and, last but not least, humans. Researchers have also deduced the complete DNA sequences for a wide variety of human pathogens. These include the bacteria that cause cholera, tuberculosis, syphilis, gonorrhea, Lyme disease, and stomach ulcers, as well as hundreds of viruses—including smallpox virus and Epstein–Barr virus (which causes infectious mononucleosis). Examination of the genomes of these pathogens provides clues about what makes them virulent and will also point the way to new and more effective treatments.

Haemophilus influenzae (a bacterium that can cause ear infections and meningitis in children) was the first organism to have its complete genome sequence—all 1.8 million nucleotide pairs—determined by the *shotgun sequencing method*, the most common strategy used today. In the shotgun method, long sequences of DNA are broken apart randomly into many shorter fragments. Each fragment is then sequenced and a computer is used to order these pieces into a whole chromosome or genome, using sequence overlap to guide the assembly. The shotgun method is the technique of choice for

sequencing small genomes. Although larger, more repetitive genome sequences are more challenging to assemble, the shotgun method—in combination with the analysis of large DNA fragments cloned in BACs—has played a key role in their sequencing as well.

With new sequences appearing at a steadily accelerating pace in the scientific literature, comparison of the complete genome sequences of different organisms allows us to trace the evolutionary relationships among genes and organisms, and to discover genes and predict their functions (discussed in Chapters 3 and 4). Assigning functions to genes often involves comparing their sequences with related sequences from model organisms that have been well characterized in the laboratory, such as the bacterium *E. coli*, the yeasts *S. cerevisiae* and *S. pombe*, the nematode worm *C. elegans*, and the fruit fly *Drosophila* (discussed in Chapter 1).

Although the organisms whose genomes have been sequenced share many biochemical pathways and possess many proteins that are homologous in their amino acid sequence or structure, the functions of a very large number of newly identified proteins remain unknown. Depending on the organism, some 15–40% of the proteins encoded by a sequenced genome do not resemble any protein that has been studied biochemically. This observation underscores a limitation of the emerging field of genomics: although comparative analysis of genomes reveals a great deal of information about the relationships between genes and organisms, it often does not provide immediate information about how these genes function, or what roles they have in the physiology of an organism. Comparison of the full gene complement of several thermophilic bacteria, for example, does not reveal why these bacteria thrive at temperatures exceeding 70°C. And examination of the genome of the incredibly radioresistant bacterium *Deinococcus radiodurans* does not explain how this organism can survive a blast of radiation that can shatter glass. Further biochemical and genetic studies, like those described in the other sections of this chapter, are required to determine how genes, and the proteins they produce, function in the context of living organisms.

Summary

DNA cloning allows a copy of any specific part of a DNA or RNA sequence to be selected from the millions of other sequences in a cell and produced in unlimited amounts in pure form. DNA sequences can be amplified after cutting chromosomal DNA with a restriction nuclease and inserting the resulting DNA fragments into the chromosome of a self-replicating genetic element such as a virus or a plasmid. Plasmid vectors are generally used, and the resulting "genomic DNA library" is housed in millions of bacterial cells, each carrying a different cloned DNA fragment. Individual cells from this library that are allowed to proliferate produce large amounts of a single cloned DNA fragment. The polymerase chain reaction (PCR) allows DNA cloning to be performed directly with a thermostable DNA polymerase—provided that the DNA sequence of interest is already known.

The procedures used to obtain DNA clones that correspond in sequence to mRNA molecules are the same except that a DNA copy of the mRNA sequence, called cDNA, is first made. Unlike genomic DNA clones, cDNA clones lack intron sequences, making them the clones of choice for analyzing the protein product of a gene.

Nucleic acid hybridization reactions provide a sensitive means of detecting a gene or any other nucleotide sequence of interest. Under stringent hybridization conditions (a combination of solvent and temperature at which even a perfect double helix is barely stable), two strands can pair to form a "hybrid" helix only if their nucleotide sequences are almost perfectly complementary. The enormous specificity of this hybridization reaction allows any single-stranded sequence of nucleotides to be labeled with a radioisotope or chemical and used as a probe to find a complementary partner strand, even in a cell or cell extract that contains millions of different DNA and RNA sequences. Probes of this type are widely used to detect the nucleic acids corresponding to specific genes, both to facilitate their purification and characterization, and to localize them in cells, tissues, and organisms.

The nucleotide sequence of DNA can be determined rapidly and simply by using highly automated techniques based on the dideoxy method for sequencing DNA. This technique has made it possible to determine the complete DNA sequences of the genomes of many organisms. Comparison of the genome sequences of different organisms allows us to trace the evolutionary relationships among genes and organisms, and it has proved valuable for discovering new genes and predicting their functions.

Taken together, these techniques for analyzing and manipulating DNA have made it possible to identify, isolate, and sequence genes from any organism of interest. Related technologies allow scientists to produce the protein products of these genes in the large quantities needed for detailed analyses of their structure and function, as well as for medical purposes.

STUDYING GENE EXPRESSION AND FUNCTION

Ultimately, one wishes to determine how genes—and the proteins they encode—function in the intact organism. Although it may seem counterintuitive, one of the most direct ways to find out what a gene does is to see what happens to the organism when that gene is missing. Studying mutant organisms that have acquired changes or deletions in their nucleotide sequences is a time-honored practice in biology and forms the basis of the important field of **genetics**. Because mutations can disrupt cell processes, mutants often hold the key to understanding gene function. In the classical genetic approach, one begins by isolating mutants that have an interesting or unusual appearance: fruit flies with white eyes or curly wings, for example. Working backward from the **phenotype**—the appearance or behavior of the individual—one then determines the organism's **genotype**, the form of the gene responsible for that characteristic (**Panel 8–1**).

Today, with numerous genome sequences available, the exploration of gene function often begins with a DNA sequence. Here, the challenge is to translate sequence into function. One approach, discussed earlier in the chapter, is to search databases for well-characterized proteins that have similar amino acid sequences to the protein encoded by a new gene, and from there employ some of the methods described in the previous section to explore the gene's function further. But to determine directly a gene's function in a cell or organism, the most effective approach involves studying mutants that either lack the gene or express an altered version of it. Determining which cell processes have been disrupted or compromised in such mutants will usually shed light on a gene's biological role.

In this section, we describe several approaches to determining a gene's function, starting from a DNA sequence or an organism with an interesting phenotype. We begin with the classical genetic approach, which starts with a *genetic screen* for isolating mutants of interest and then proceeds toward identification of the gene or genes responsible for the observed phenotype. We then describe the set of techniques that are collectively called *reverse genetics*, in which one begins with a gene or gene sequence and attempts to determine its function. This approach often involves some intelligent guesswork—searching for homologous sequences and determining when and where a gene is expressed—as well as generating mutant organisms and characterizing their phenotype.

Classical Genetics Begins by Disrupting a Cell Process by Random Mutagenesis

Before the advent of gene cloning technology, most genes were identified by the abnormalities produced when the gene was mutated. This classical genetic approach—identifying the genes responsible for mutant phenotypes—is most easily performed in organisms that reproduce rapidly and are amenable to genetic manipulation, such as bacteria, yeasts, nematode worms, and fruit flies. Although spontaneous mutants can sometimes be found by examining extremely large populations—thousands or tens of thousands of individual

GENES AND PHENOTYPES

Gene: a functional unit of inheritance, usually corresponding to the segment of DNA coding for a single protein.

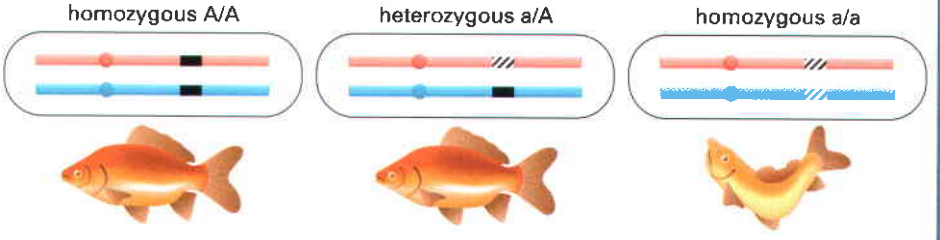
Genome: all of an organism's DNA sequences.

Wild-type: the normal, naturally occurring type

Mutant: differing from the wild-type because of a genetic change (a mutation)

GENOTYPE: the specific set of alleles forming the genome of an individual

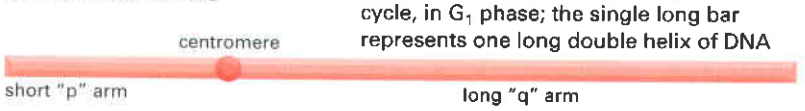
PHENOTYPE: the visible character of the individual



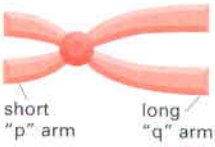
allele A is dominant (relative to a); allele a is recessive (relative to A)

In the example above, the phenotype of the heterozygote is the same as that of one of the homozygotes; in cases where it is different from both, the two alleles are said to be co-dominant.

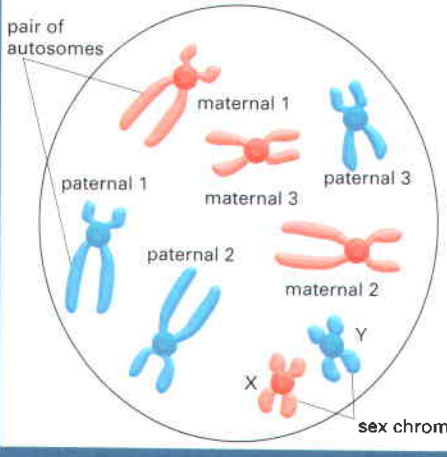
CHROMOSOMES



a chromosome at the beginning of the cell cycle, in G_1 phase; the single long bar represents one long double helix of DNA

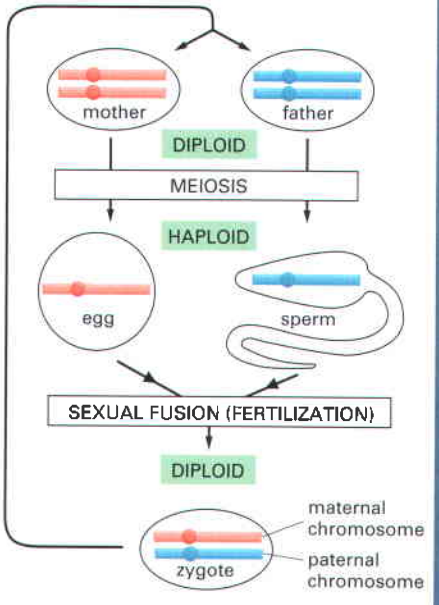


a chromosome near the end of the cell cycle, in metaphase; it is duplicated and condensed, consisting of two identical sister chromatids (each containing one DNA double helix) joined at the centromere.



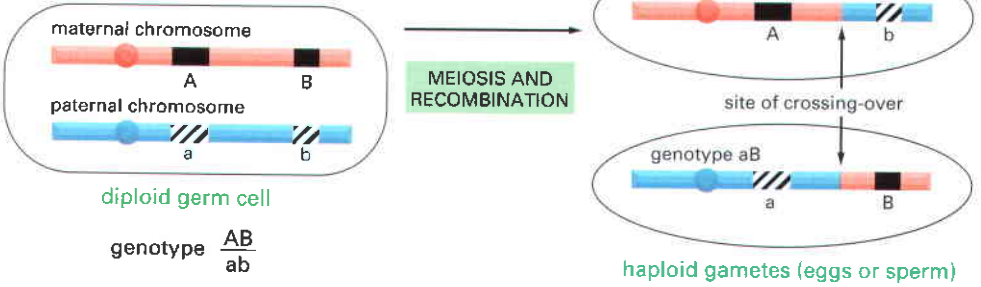
A normal diploid chromosome set, as seen in a metaphase spread, prepared by bursting open a cell at metaphase and staining the scattered chromosomes. In the example shown schematically here, there are three pairs of autosomes (chromosomes inherited symmetrically from both parents, regardless of sex) and two sex chromosomes—an X from the mother and a Y from the father. The numbers and types of sex chromosomes and their role in sex determination are variable from one class of organisms to another, as is the number of pairs of autosomes.

THE HAPLOID–DIPLOID CYCLE OF SEXUAL REPRODUCTION



For simplicity, the cycle is shown for only one chromosome/chromosome pair.

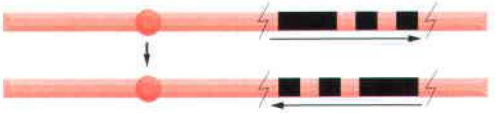
MEIOSIS AND GENETIC RECOMBINATION



The greater the distance between two loci on a single chromosome, the greater is the chance that they will be separated by crossing over occurring at a site between them. If two genes are thus reassorted in x% of gametes, they are said to be separated on a chromosome by a genetic map distance of x map units (or x centimorgans).

TYPES OF MUTATIONS

POINT MUTATION: maps to a single site in the genome, corresponding to a single nucleotide pair or a very small part of a single gene



INVERSION: inverts a segment of a chromosome

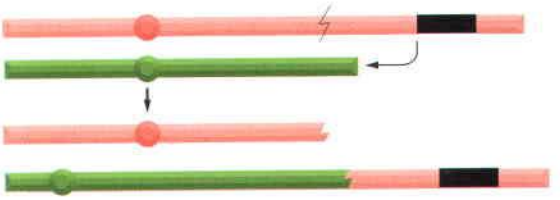
lethal mutation: causes the developing organism to die prematurely.

conditional mutation: produces its phenotypic effect only under certain conditions, called the *restrictive* conditions. Under other conditions—the *permissive* conditions—the effect is not seen. For a *temperature-sensitive* mutation, the restrictive condition typically is high temperature, while the permissive condition is low temperature.

loss-of-function mutation: either reduces or abolishes the activity of the gene. These are the most common class of mutations. Loss-of-function mutations are usually *recessive*—the organism can usually function normally as long as it retains at least one normal copy of the affected gene.

null mutation: a loss-of-function mutation that completely abolishes the activity of the gene.

DELETION: deletes a segment of a chromosome



TRANSLOCATION: breaks off a segment from one chromosome and attaches it to another

gain-of-function mutation: increases the activity of the gene or makes it active in inappropriate circumstances; these mutations are usually *dominant*.

dominant-negative mutation: dominant-acting mutation that blocks gene activity, causing a loss-of-function phenotype even in the presence of a normal copy of the gene. This phenomenon occurs when the mutant gene product interferes with the function of the normal gene product.

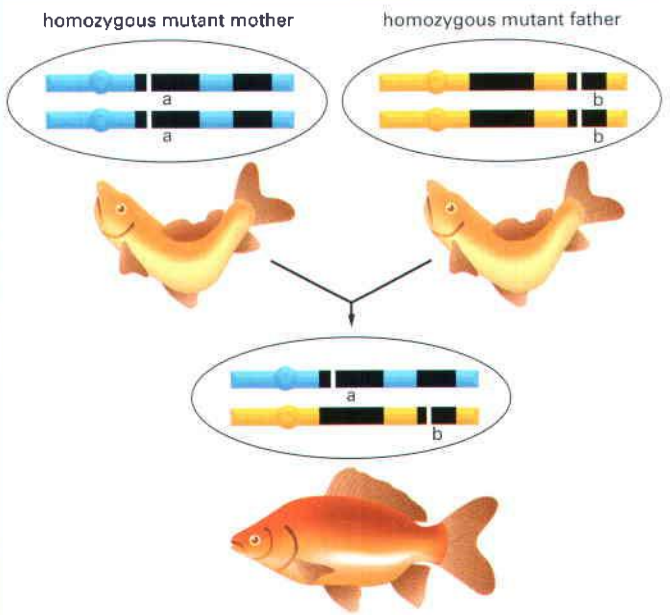
suppressor mutation: suppresses the phenotypic effect of another mutation, so that the double mutant seems normal. An *intragenic* suppressor mutation lies within the gene affected by the first mutation; an *extragenic* suppressor mutation lies in a second gene—often one whose product interacts directly with the product of the first.

TWO GENES OR ONE?

Given two mutations that produce the same phenotype, how can we tell whether they are mutations in the same gene? If the mutations are recessive (as they most often are), the answer can be found by a complementation test.

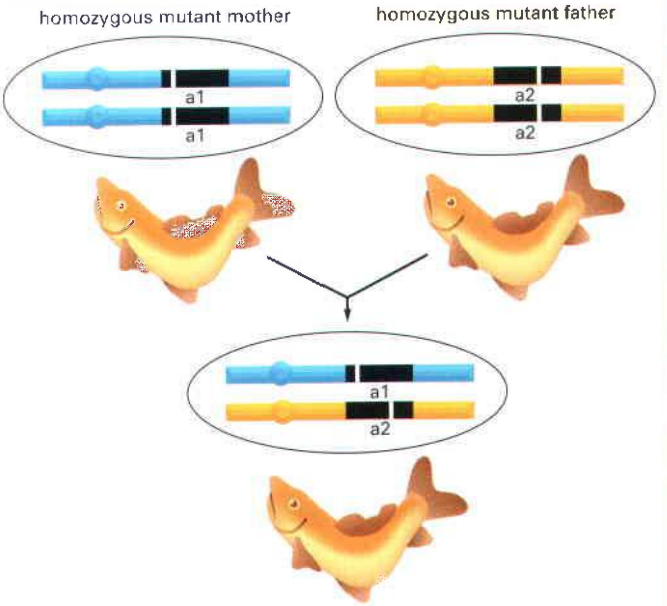
In the simplest type of complementation test, an individual who is homozygous for one mutation is mated with an individual who is homozygous for the other. The phenotype of the offspring gives the answer to the question.

COMPLEMENTATION: MUTATIONS IN TWO DIFFERENT GENES



hybrid offspring shows normal phenotype: one normal copy of each gene is present

NONCOMPLEMENTATION: TWO INDEPENDENT MUTATIONS IN THE SAME GENE



hybrid offspring shows mutant phenotype: no normal copies of the mutated gene are present

organisms—isolating mutant individuals is much more efficient if one generates mutations with chemicals or radiation that damage DNA. By treating organisms with such mutagens, very large numbers of mutant individuals can be created quickly and then screened for a particular defect of interest, as we discuss shortly.

An alternative approach to chemical or radiation mutagenesis is called *insertional mutagenesis*. This method relies on the fact that exogenous DNA inserted randomly into the genome can produce mutations if the inserted fragment interrupts a gene or its regulatory sequences. The inserted DNA, whose sequence is known, then serves as a molecular tag that aids in the subsequent identification and cloning of the disrupted gene (**Figure 8–53**). In *Drosophila*, the use of the transposable P element to inactivate genes has revolutionized the study of gene function in the fly. Transposable elements (see Table 5–3, p. 318) have also been used to generate mutations in bacteria, yeast, mice, and the flowering plant *Arabidopsis*.

Such classical genetic studies are well suited for dissecting biological processes in experimental organisms, but how can we study gene function in humans? Unlike the genetically accessible organisms we have been discussing, humans do not reproduce rapidly, and they cannot be intentionally treated with mutagens. Moreover, any human with a serious defect in an essential process, such as DNA replication, would die long before birth.

There are two main ways that we can study human genes. First, because genes and gene functions have been so highly conserved throughout evolution, the study of less complex model organisms reveals critical information about similar genes and processes in humans. The corresponding human genes can then be studied further in cultured human cells. Second, many mutations that are not lethal—tissue-specific defects in lysosomes or cell-surface receptors, for example—have arisen spontaneously in the human population. Analyses of the phenotypes of the affected individuals, together with studies of their cultured cells, have provided many unique insights into important human cell functions. Although such mutations are rare, they are very efficiently discovered because of a unique human property: the mutant individuals call attention to themselves by seeking special medical care.

Figure 8–53 Insertional mutant of the snapdragon, Antirrhinum. A mutation in a single gene coding for a regulatory protein causes leafy shoots to develop in place of flowers. The mutation allows cells to adopt a character that would be appropriate to a different part of the normal plant. The mutant plant is on the *left*, the normal plant on the *right*. (Courtesy of Enrico Coen and Rosemary Carpenter.)

Genetic Screens Identify Mutants with Specific Abnormalities

Once a collection of mutants in a model organism such as yeast or fly has been produced, one generally must examine thousands of individuals to find the altered phenotype of interest. Such a search is called a **genetic screen**, and the larger the genome, the less likely it is that any particular gene will be mutated. Therefore, the larger the genome of an organism, the bigger the screening task becomes. The phenotype being screened for can be simple or complex. Simple phenotypes are easiest to detect: one can screen many organisms rapidly, for example, for mutations that make it impossible for the organism to survive in the absence of a particular amino acid or nutrient.

More complex phenotypes, such as defects in learning or behavior, may require more elaborate screens (**Figure 8–54**). But even genetic screens that are used to dissect complex physiological systems should be as simple as possible in design, and, if possible, should permit the simultaneous examination of large numbers of mutants. As an example, one particularly elegant screen was designed to search for genes involved in visual processing in zebrafish. The basis of this screen, which monitors the fishes' response to motion, is a change in behavior. Wild-type fish tend to swim in the direction of a perceived motion, whereas mutants with defects in their visual processing systems swim in random directions—a behavior that is easily detected. One mutant discovered in this screen is called *lakritz*, which is missing 80% of the retinal ganglion cells that help to relay visual signals from the eye to the brain. As the cellular organization of the zebrafish retina is similar to that of all vertebrates, the study of such mutants should also provide insights into visual processing in humans.

Because defects in genes that are required for fundamental cell processes—RNA synthesis and processing or cell-cycle control, for example—are usually

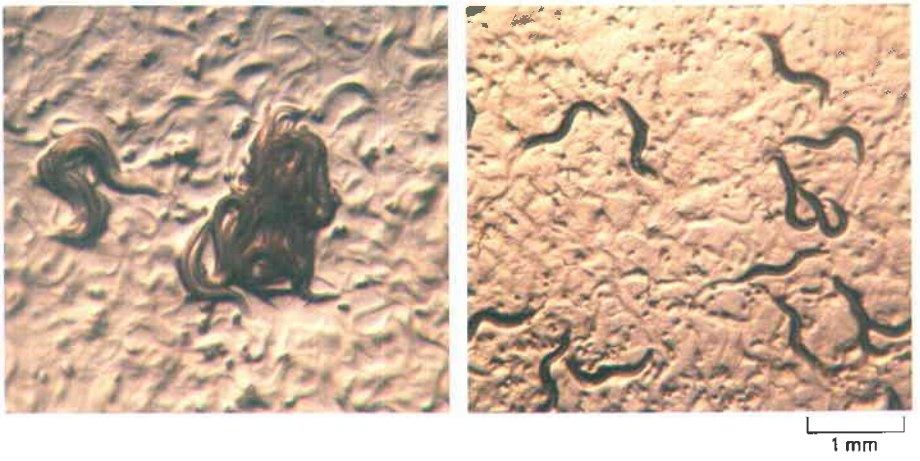


Figure 8–54 A behavioral phenotype detected in a genetic screen. (A) Wild-type *C. elegans* engage in social feeding. The worms migrate around until they encounter their neighbors and commence feeding on bacteria. (B) Mutant animals feed by themselves. (Courtesy of Cornelia Bargmann, *Cell* 94: cover, 1998. With permission from Elsevier.)

lethal, the functions of these genes are often studied in individuals with **conditional mutations**. The mutant individuals function normally as long as "permissive" conditions prevail, but demonstrate abnormal gene function when subjected to "nonpermissive" (restrictive) conditions. In organisms with *temperature-sensitive mutations,* for example, the abnormality can be switched on and off experimentally simply by changing the temperature; thus, a cell containing a temperature-sensitive mutation in a gene essential for survival will die at a nonpermissive temperature but proliferate normally at the permissive temperature (**Figure 8–55**). The temperature-sensitive gene in such a mutant usually contains a point mutation that causes a subtle change in its protein product.

Many temperature-sensitive mutations were found in the bacterial genes that encode the proteins required for DNA replication. The mutants were identified by screening populations of mutagen-treated bacteria for cells that stop making DNA when they are warmed from 30°C to 42°C. These mutants were later used to identify and characterize the corresponding DNA replication proteins (discussed in Chapter 5). Similarly, screens for temperature-sensitive mutations led to the identification of many proteins involved in regulating the cell cycle, as well as many proteins involved in moving proteins through the secretory pathway in yeast (see Panel 13–1). Related screening approaches demonstrated the function of enzymes involved in the principal metabolic pathways of bacteria and yeast (discussed in Chapter 2) and identified many of the gene products responsible for the orderly development of the *Drosophila* embryo (discussed in Chapter 22).

Mutations Can Cause Loss or Gain of Protein Function

Gene mutations are generally classed as "loss of function" or "gain of function." A loss of function mutation results in a gene product that either does not work or works too little; thus, it reveals the normal function of the gene. A gain of function mutation results in a gene product that works too much, works at the wrong time or place, or works in a new way (**Figure 8–56**).

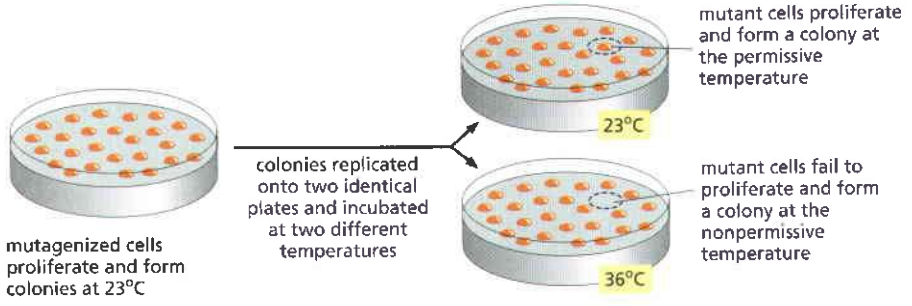


Figure 8–55 Screening for temperature-sensitive bacterial or yeast mutants. Mutagenized cells are plated out at the permissive temperature. They divide and form colonies, which are transferred to two identical Petri dishes by replica plating. One of these plates is incubated at the permissive temperature, the other at the nonpermissive temperature. Cells containing a temperature-sensitive mutation in a gene essential for proliferation can divide at the normal, permissive temperature but fail to divide at the elevated, nonpermissive temperature.

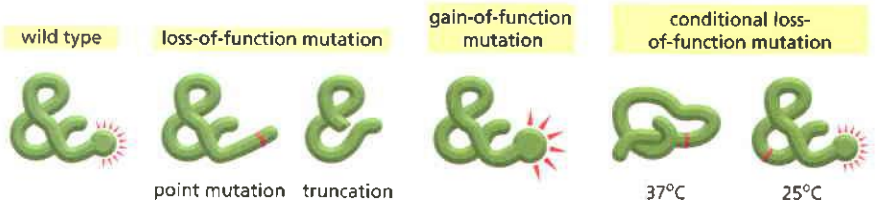


Figure 8–56 Gene mutations that affect their protein product in different ways. In this example, the wild-type protein has a specific cell function denoted by the *red rays*. Mutations that eliminate this function, increase the function, or render the function sensitive to higher temperatures are shown. The temperature-sensitive conditional mutant protein carries an amino acid substitution *(red)* that prevents its proper folding at 37°C, but allows the protein to fold and function normally at 25°C. Such conditional mutations are especially useful for studying essential genes; the organism can be grown under the permissive condition and then moved to the nonpermissive condition to study the function of the gene.

An important early step in the genetic analysis of any mutant cell or organism is to determine whether the mutation causes a loss or a gain of function. A standard test is to determine whether the mutation is *dominant* or *recessive*. A dominant mutation is one that still causes the mutant phenotype, in the presence of a single copy of the wild-type gene. A recessive mutation is one that is no longer able to cause the mutant phenotype in the presence of a single wild-type copy of the gene. Although cases have been described in which a loss-of-function mutation is dominant or a gain-of-function mutation is recessive, in the vast majority of cases, recessive mutations are loss of function, and dominant mutations are gain of function. It is easy to determine if a mutation is dominant or recessive. One simply mates a mutant with a wild-type to obtain diploid cells or organisms. The progeny from the mating will be heterozygous for the mutation. If the mutant phenotype is no longer observed, one can conclude that the mutation is recessive and is very likely to be a loss-of-function mutation.

Complementation Tests Reveal Whether Two Mutations Are in the Same Gene or Different Genes

A large-scale genetic screen can turn up many different mutations that show the same phenotype. These defects might lie in different genes that function in the same process, or they might represent different mutations in the same gene. Alternative forms of a gene are known as **alleles**. The most common difference between alleles is a substitution of a single nucleotide pair, but different alleles can also bear deletions, substitutions, and duplications. How can we tell, then, whether two mutations that produce the same phenotype occur in the same gene or in different genes? If the mutations are recessive—if, for example, they represent a loss of function of a particular gene—a **complementation test** can be used to ascertain whether the mutations fall in the same or in different genes. To test complementation in a diploid organism, an individual that is homozygous for one mutation—that is, it possesses two identical alleles of the mutant gene in question—is mated with an individual that is homozygous for the other mutation. If the two mutations are in the same gene, the offspring show the mutant phenotype, because they still will have no normal copies of the gene in question (see Panel 8–1). If, in contrast, the mutations fall in different genes, the resulting offspring show a normal phenotype, because they retain one normal copy (and one mutant copy) of each gene; the mutations thereby complement one another and restore a normal phenotype. Complementation testing of mutants identified during genetic screens has revealed, for example, that 5 genes are required for yeast to digest the sugar galactose, 20 genes are needed for *E. coli* to build a functional flagellum, 48 genes are involved in assembling bacteriophage T4 viral particles, and hundreds of genes are involved in the development of an adult nematode worm from a fertilized egg.

Genes Can Be Ordered in Pathways by Epistasis Analysis

Once a set of genes involved in a particular biological process has been identified, the next step is often to determine in which order the genes function. Gene order is perhaps easiest to explain for metabolic pathways, where, for example, enzyme A is necessary to produce the substrate for enzyme B. In this case, we would say that the gene encoding enzyme A acts before (upstream of) the gene encoding enzyme B in the pathway. Similarly, where one protein regulates the

activity of another protein, we would say that the former gene acts before the latter. Gene order can, in many cases, be determined purely by genetic analysis without any knowledge of the mechanism of action of the gene products involved.

Suppose we have a biosynthetic process consisting of a sequence of steps, such that performance of a step B is conditional on completion of the preceding step A; and suppose *gene A* is required for step A, and *gene B* is required for step B. Then a null mutation (a mutation that abolishes function) in gene *A* will arrest the process at step A, regardless of whether gene *B* is functional or not, whereas a null mutation in gene *B* will cause arrest at step B only if gene *A* is still active. In such a case, gene *A* is said to be *epistatic* to gene *B*. By comparing the phenotypes of the different combinations of mutations, we can therefore discover the order in which the genes act. This type of analysis is called **epistasis analysis**. As an example, the pathway of protein secretion in yeast has been analyzed in this way. Different mutations in this pathway cause proteins to accumulate aberrantly in the endoplasmic reticulum (ER) or in the Golgi apparatus. When a yeast cell is engineered to carry both a mutation that blocks protein processing in the ER *and* a mutation that blocks processing in the Golgi apparatus, proteins accumulate in the ER. This indicates that proteins must pass through the ER before being sent to the Golgi before secretion (**Figure 8–57**). Strictly speaking, an epistasis analysis can only provide information about gene order in a pathway when both mutations are null alleles. When the mutations retain partial function, their epistasis interactions can be difficult to interpret.

Sometimes, a double mutant will show a new or more severe phenotype than either single mutant alone. This type of genetic interaction is called a *synthetic* phenotype, and if the phenotype is death of the organism, it is called *synthetic lethality*. In most cases, a synthetic phenotype indicates that the two genes act in two different parallel pathways, either of which is capable of mediating the same cell process. Thus, when both pathways are disrupted in the double mutant, the process fails altogether, and the synthetic phenotype is observed.

Genes Identified by Mutations Can Be Cloned

Once the mutant organisms are produced in a genetic screen, the next task is identifying the gene or genes responsible for the altered phenotype. If the phenotype has been produced by insertional mutagenesis, locating the disrupted gene is fairly simple. DNA fragments containing the insertion (a transposon or a retrovirus, for example) are collected and amplified by PCR, and the nucleotide sequence of the flanking DNA is determined. Genome databases can then be searched for open reading frames containing this flanking sequence.

If a DNA-damaging chemical was used to generate the mutations, identifying the inactivated gene is often more laborious, but it can be accomplished by several different approaches. In one, the first step is to experimentally determine the gene's location in the genome. To map a newly discovered gene, its rough chromosomal location is first determined by assessing how far the gene lies from other known genes in the genome. Estimating the distance between genetic loci is usually done by *linkage analysis*, a technique that relies on the tendency for genes that lie near one another on a chromosome to be inherited together. Even closely linked genes, however, can be separated by

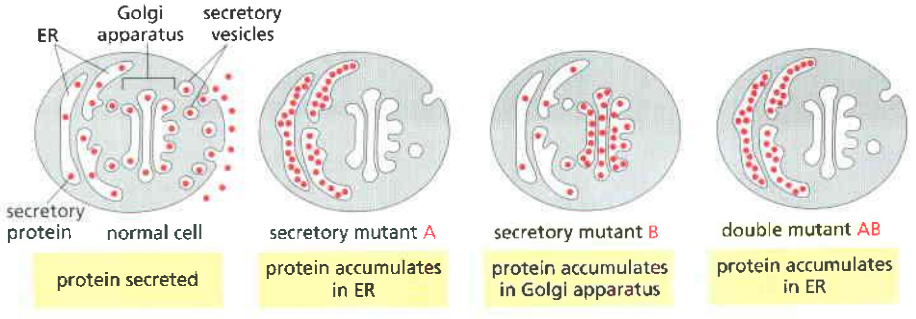


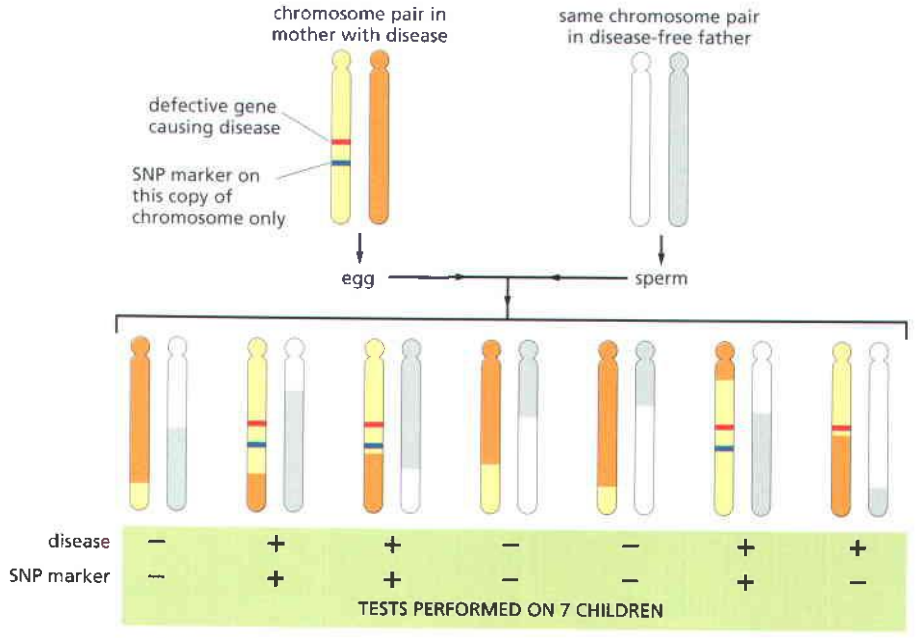
Figure 8–57 Using genetics to determine the order of function of genes. In normal cells, secretory proteins are loaded into vesicles, which fuse with the plasma membrane to secrete their contents into the extracellular medium. Two mutants, A and B, fail to secrete proteins. In mutant A, secretory proteins accumulate in the ER. In mutant B, secretory proteins accumulate in the Golgi. In the double mutant AB, proteins accumulate in the ER; this indicates that the gene defective in mutant A acts before the gene defective in mutant B in the secretory pathway.

recombination during meiosis. The larger the distance between two genetic loci, the greater the chance that they will be separated by a crossover (see Panel 8–1). By calculating the recombination frequency between two genes, the approximate distance between them can be determined. If the position of one gene in the genome is known, that of the second gene can thereby be estimated.

Because genes are not always located close enough to one another to allow a precise pinpointing of their position, linkage analyses often rely on physical markers along the genome for estimating the location of an unknown gene. These markers are generally short stretches of nucleotides, with a known sequence and genome location, that can exist in at least two allelic forms. The simplest markers are *single-nucleotide polymorphisms (SNPs)*, short sequences that differ by one nucleotide pair among individuals in a population. SNPs can be detected by hybridization techniques. Many such physical markers, distributed all along the length of chromosomes, have been collected for a variety of organisms. If the distribution of these markers is sufficiently dense, one can, through a linkage analysis that tests for the tight co-inheritance of one or more SNPs with the mutant phenotype, narrow the potential location of a gene to a chromosomal region that may contain only a few gene sequences. These are then considered candidate genes, and their structure and function can be tested directly to determine which gene is responsible for the original mutant phenotype.

Human Genetics Presents Special Problems and Opportunities

Although genetic experimentation on humans is considered unethical and is legally banned, humans do suffer from a large variety of genetic disorders. The linkage analysis described above can be used to identify the genes responsible for these heritable conditions. Such studies require DNA samples from a large number of families affected by the disease. These samples are examined for the presence of physical markers such as SNPs that seem to be closely linked to the disease gene, in that they are always inherited by individuals who have the disease and not by their unaffected relatives. The disease gene is then located as described above (**Figure 8–58**). The genes for cystic fibrosis and Huntington's disease, for example, were discovered in this way.



CONCLUSION: gene causing disease is co-inherited with SNP marker from diseased mother in 75% of the diseased progeny. If this same correlation is observed in other families that have been examined, the gene causing disease is mapped to this chromosome close to the SNP. Note that an SNP that is either far away from the gene on the same chromosome or located on a different chromosome from the gene of interest will be co-inherited only 50% of the time.

Figure 8–58 Genetic linkage analysis using physical markers on DNA to find a human gene. In this example, the co-inheritance of a specific human phenotype (here a genetic disease) with an SNP marker. If individuals who inherit the disease nearly always inherit a particular SNP marker, then the gene causing the disease and the SNP are likely to be close together on the chromosome, as shown here. To prove that an observed linkage is statistically significant, hundreds of individuals may need to be examined. Note that the linkage will not be absolute unless the SNP marker is located in the gene itself. Thus, occasionally the SNP will be separated from the disease gene by crossing over during meiosis in the formation of the egg or sperm: this has happened in the case of the chromosome pair on the far right. When working with a sequenced genome, this procedure would be repeated with SNPs located on either side of the initial SNP, until a 100% co-inheritance is found.

Note that the egg and sperm will each contribute only one chromosome of each pair from the parent to the child.

Human Genes Are Inherited in Haplotype Blocks, Which Can Aid in the Search for Mutations That Cause Disease

With the complete human genome sequence in hand, we can now study human genetics in a way that was impossible only a few years ago. For example, we can begin to identify those DNA differences that distinguish one individual from another. No two humans (with the exception of identical twins) have the same genome. Each of us carries a set of polymorphisms—differences in nucleotide sequence—that make us unique. These polymorphisms can be used as markers for building genetic maps and performing genetic analyses to link particular polymorphisms with specific diseases or predispositions to disease.

The problem is that any two humans typically differ by about 0.1% in their nucleotide sequences (approximately one nucleotide difference every 1000 nucleotides). This translates to about 3 million differences between one person and another. Theoretically, one would need to search through all 3 million of those polymorphisms to identify the one or two that are responsible for a particular heritable disease or disease predisposition. To reduce the number of polymorphisms we need to examine, researchers are taking advantage of the recent discovery that human genes tend to be inherited in blocks.

The human species is relatively young, and it is thought that we are descended from a relatively small population of individuals who lived in Africa about 10,000 years ago. Because only a few hundred generations separate us from this ancestral population, large segments of human chromosomes have passed from parent to child unaltered by the recombination events that occur in meiosis. In fact, we observe that certain sets of alleles (including SNPs) are inherited in large blocks within chromosomes. These ancestral chromosome segments—sets of alleles that have been inherited in clusters with little genetic rearrangement across the generations—are called **haplotype blocks**. Like genes, SNPs, and other genetic markers—which exist in different alleleic forms—haplotype blocks also come in a limited number of "flavors" that are common in the human population, each of which represents an allele combination passed down from a shared ancestor long ago.

Researchers are now constructing a human genome map based on these haplotype blocks—called a **haplotype map** (**hapmap**). Geneticists hope that the human haplotype map will make the search for disease-causing and disease-susceptibility genes a much more manageable task. Instead of searching through each of the many millions of SNPs in the human population, one need only search through a considerably smaller set of selected SNPs to identify the haplotype block that appears to be inherited by individuals with the disease. (These searches still involve DNA samples from large numbers of people, and SNPs are now typically scored using robotic technologies.) If a specific haplotype block is more common among people with the disease than in unaffected individuals, the mutation linked to that disease will likely be located in that same segment of DNA (**Figure 8–59**). Researchers can then zero in on the specific region within the block to search for the specific gene associated with the disease. This approach should, in principle, allow one to analyze the genetics of those common diseases in which multiple genes confer susceptibility.

A detailed examination of haplotype blocks can even tell us whether a particular allele has been favored by natural selection. As a rule, when a new allele of a gene arises that does not confer a selective advantage on the individual, it will take a long time for that allele to become common in the population. The more common—and therefore older—such an allele is, the smaller should be the haplotype block that surrounds it, because it will have had many chances of being separated from its neighboring variations by the recombination events that occur in meiosis generation after generation.

A new allele may quickly spread in a population, however, if it confers some dramatic advantage on the organism. For example, mutations or variations that make an organism more resistant to an infection might be selected for because organisms with this variation would be more likely to survive and pass the

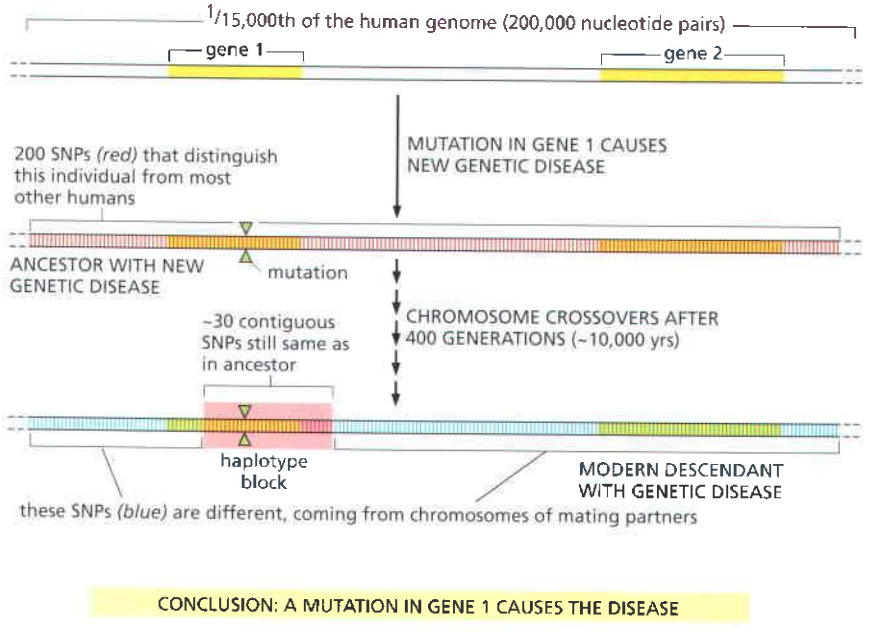


Figure 8–59 Tracing the inheritance of SNPs within haplotype blocks to reveal the location of a disease-causing gene. An ancestor who acquires a disease-causing mutation in gene 1 will pass that mutation along to his or her descendants. Part of this gene is embedded within a haplotype block *(red shading)*—a cluster of variations (about 30 SNPs) that have been passed along from the ancestor in a continuous chunk. In the 400 generations that separate the ancestor from modern descendants with the disease, SNPs located over most of the ancestral 200,000-nucleotide-pair region shown have been shuffled by meiotic recombination in the descendant genome *(blue)*. (Note that the overlap of yellow and red is seen as *orange*, and the overlap of yellow and blue is seen as *green*.) The 30 SNPs within the haplotype block, however, have been inherited as a group, as no crossover events have yet separated them. To locate a gene that causes the inherited disease, the SNP patterns in a number of people who have the disease need to be analyzed. An individual with the disease will retain the ancestral pattern of SNPs located within the haplotype block shown, revealing that the disease-causing mutation is likely to lie within that haplotype block—thus in gene 1. The beauty of using haplotype maps for this type of linkage analysis is that only a fraction of the total SNPs need to be examined: one should be able to locate genes after searching through only about 10% of the 3 million useful SNPs present in the human genome.

mutation on to their offspring. Working with haplotype maps of individual genes, researchers have detected such positive selection for two human genes that confer resistance to malaria. The alleles that confer resistance are widespread in the population, but they are embedded in unusually large haplotype blocks, suggesting that they rose to prominence recently in the human gene pool (**Figure 8–60**).

In revealing the paths along which humans evolved, the human haplotype map provides a new window into our past; in helping us discover the genes that make us susceptible or resistant to disease, the map may also provide a rough guide to our individual futures.

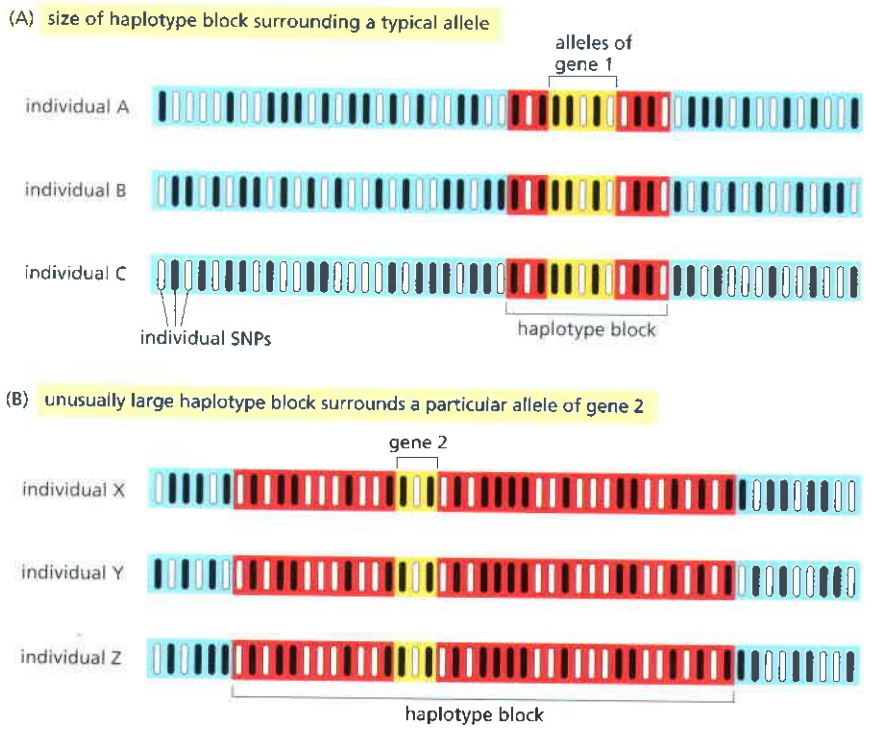


Figure 8–60 Identification of alleles that have been selected for in fairly recent human history by the unusually large haplotype blocks in which they are embedded. The SNPs are indicated in this diagram by vertical bars, which are shown as *white* or *black* according to their DNA sequence. Haplotype blocks are shaded in *red*, genes in *yellow*, and the rest of the chromosome in *blue*. These data suggest that this particular allele of gene 2 arose relatively recently in human history.

Complex Traits Are Influenced by Multiple Genes

A concert pianist might have an aunt who plays the violin. In another family, the parents and the children might all be fat. In a third family, the grandmother might be an alcoholic, and her grandson might abuse drugs. To what extent are such characteristics—musical ability, obesity, and addiction—inherited genetically? This is a very difficult question to answer. Some traits or diseases "run in families" but appear in only a few relatives or with no easily discernible pattern.

Characteristics that do not follow simple (sometimes called Mendelian) patterns of inheritance but have a genetically inherited component are termed **complex traits**. These traits are often **polygenic**; that is, they are influenced by multiple genes, each of which makes a small contribution to the phenotype in question. The effects of these genes are additive, which means that, together, they produce a continuum of varying features within the population. Individually, the genes that contribute to a polygenic trait are distributed to offspring in simple patterns, but because they all influence the phenotype, the pattern of traits inherited by offspring is often highly complex.

A simple example of a polygenic trait is eye color, which is determined by enzymes that control the distribution and production of the pigment melanin: the more melanin produced, the darker the eye color. Because numerous genes contribute to the formation of melanin, eye color in humans shows enormous variation, from the palest gray to a dark chocolate brown.

Although diseases based on mutations in single genes (for example, sickle-cell anemia and hemophilia) were some of the earliest recognized human inherited phenotypes, only a small fraction of human traits are dictated by single genes. The most obvious human phenotypes—from height, weight, eye color, and hair color to intelligence, temperament, sociability, and humor—arise from the interaction of many genes. Multiple genes also almost certainly underlie a propensity for the most common human diseases: diabetes, heart disease, high blood pressure, allergies, asthma, and various mental illnesses, including major depression and schizophrenia. Researchers are exploring new strategies—including the use of the haplotype maps discussed earlier—to understand the complex interplay between genes that act together to determine many of our most "human" traits.

Reverse Genetics Begins with a Known Gene and Determines Which Cell Processes Require Its Function

As we have seen, classical genetics starts with a mutant phenotype (or, in the case of humans, a range of characteristics) and identifies the mutations (and consequently the genes) responsible for it. Recombinant DNA technology, in combination with genome sequencing, has made possible a different type of genetic approach. Instead of beginning with a mutant organism and using it to identify a gene and its protein, an investigator can start with a particular gene and proceed to make mutations in it, creating mutant cells or organisms so as to analyze the gene's function. Because this approach reverses the traditional direction of genetic discovery—proceeding from genes to mutations, rather than vice versa—it is commonly referred to as **reverse genetics**.

Reverse genetics begins with a cloned gene, a protein with interesting properties that has been isolated from a cell, or simply a genome sequence. If the starting point is a protein, the gene encoding it is first identified and, if necessary, its nucleotide sequence is determined. The gene sequence can then be altered *in vitro* to create a mutant version. This engineered mutant gene, together with an appropriate regulatory region, is transferred into a cell where it can integrate into a chromosome, becoming a permanent part of the cell's genome. All of the descendants of the modified cell will now contain the mutant gene.

If the original cell used for the gene transfer is a fertilized egg, whole multicellular organisms can be obtained that contain the mutant gene, provided that the mutation does not cause lethality. In some of these animals, the altered gene

will be incorporated into the germ cells—a *germ-line mutation*—allowing the mutant gene to be passed on to their progeny.

Genes Can Be Engineered in Several Ways

We have seen that mutant organisms lacking a particular gene may quickly reveal the function of the protein it encodes. For this reason, a gene "knockout"—in which both copies of the gene in a diploid organism have been inactivated or deleted—is a particularly useful type of mutation. However, there are many more types of genetic alterations available to the experimenter. For example, by altering the regulatory region of a gene before it is reintegrated into the genome, one can create mutant organisms in which the gene product is expressed at abnormally high levels, in the wrong tissue, or at the wrong time in development (**Figure 8–61**). By placing the gene under the control of an *inducible promoter*, the gene can be switched on or off at any time, and the effects observed. Inducible promoters that function in only a specific tissue can be used to monitor the effects of shutting the gene off (or turning it on) in that particular tissue. Finally, *dominant-negative* mutations are often employed particularly in those organisms in which it is simpler to add an altered gene to the genome than to replace the endogenous genes with it. The dominant-negative strategy exploits the fact that most proteins function as parts of larger protein complexes. The inclusion of just one nonfunctional component can often inactivate such complexes. Therefore, by designing a gene that produces large quantities of a mutant protein that is inactive but still able to assemble into the complex, it is often possible to produce a cell in which all the complexes are inactivated despite the presence of the normal protein (**Figure 8–62**).

As noted in the earlier discussion of classical genetics, if a protein is required for the survival of the cell (or the organism), a dominant-negative mutant will be inviable, making it impossible to test the function of the protein. To avoid this problem in reverse genetics, one can couple the mutant gene to an inducible promoter in order to produce the faulty gene product only on command—for example, in response to an increase in temperature or to the presence of a specific signal molecule.

In studying the action of a gene and the protein it encodes, one does not always wish to make drastic changes—flooding cells with huge quantities of the protein or eliminating a gene product entirely. It is sometimes useful to make slight changes in a protein's structure so that one can begin to dissect which portions of a protein are important for its function. The activity of an enzyme, for example, can be studied by changing a single amino acid in its active site. Special techniques are required to alter genes (and thus their protein products) in such subtle ways. The first step is often the chemical synthesis of a short DNA molecule containing the desired altered portion of the gene's nucleotide sequence. This synthetic DNA oligonucleotide is hybridized with single-stranded plasmid DNA that contains the DNA sequence to be altered, using conditions that allow imperfectly matched DNA strands to pair. The synthetic oligonucleotide will now serve as a primer for DNA synthesis by DNA polymerase, thereby generating a DNA double helix that incorporates the altered

Figure 8–61 Ectopic misexpression of Wnt, a signaling protein that affects development of the body axis in the early *Xenopus* embryo. In this experiment, mRNA coding for Wnt was injected into the ventral vegetal blastomere, inducing a second body axis (discussed in Chapter 22). (From S. Sokol et al., *Cell* 67:741–752, 1991. With permission from Elsevier.)

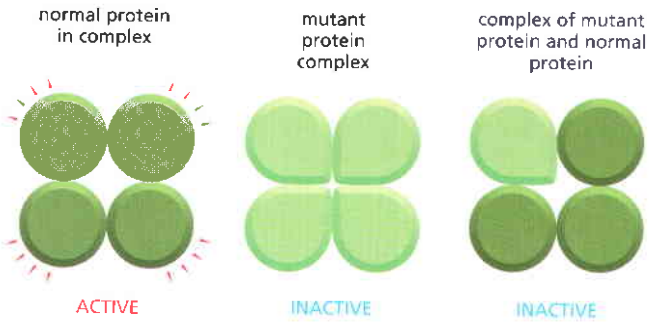


Figure 8–62 A dominant-negative effect of a protein. Here, a gene is engineered to produce a mutant protein that prevents the normal copies of the same protein from performing their function. In this simple example, the normal protein must form a multisubunit complex to be active, and the mutant protein blocks function by forming a mixed complex that is inactive. In this way, a single copy of a mutant gene located anywhere in the genome can inactivate the normal products produced by other gene copies.

sequence into one of its two strands. After transfection, plasmids that carry the fully modified gene sequence are obtained. The modified DNA is then inserted into an expression vector so that the redesigned protein can be produced in the appropriate type of cells for detailed studies of its function. By changing selected amino acids in a protein in this way—a technique called **site-directed mutagenesis**—one can determine exactly which parts of the polypeptide chain are important for such processes as protein folding, interactions with other proteins, and enzymatic catalysis (**Figure 8–63**).

Engineered Genes Can Be Inserted into the Germ Line of Many Organisms

Altered genes can be introduced into cells in a variety of ways. DNA can be microinjected into mammalian cells with a glass micropipette or introduced by a virus that has been engineered to carry foreign genes. In plant cells, genes are frequently introduced by a technique called particle bombardment: DNA samples are painted onto tiny gold beads and then literally shot through the cell wall with a specially modified gun. *Electroporation* is the method of choice for introducing DNA into bacteria and some other cells. In this technique, a brief electric shock renders the cell membrane temporarily permeable, allowing foreign DNA to enter the cytoplasm.

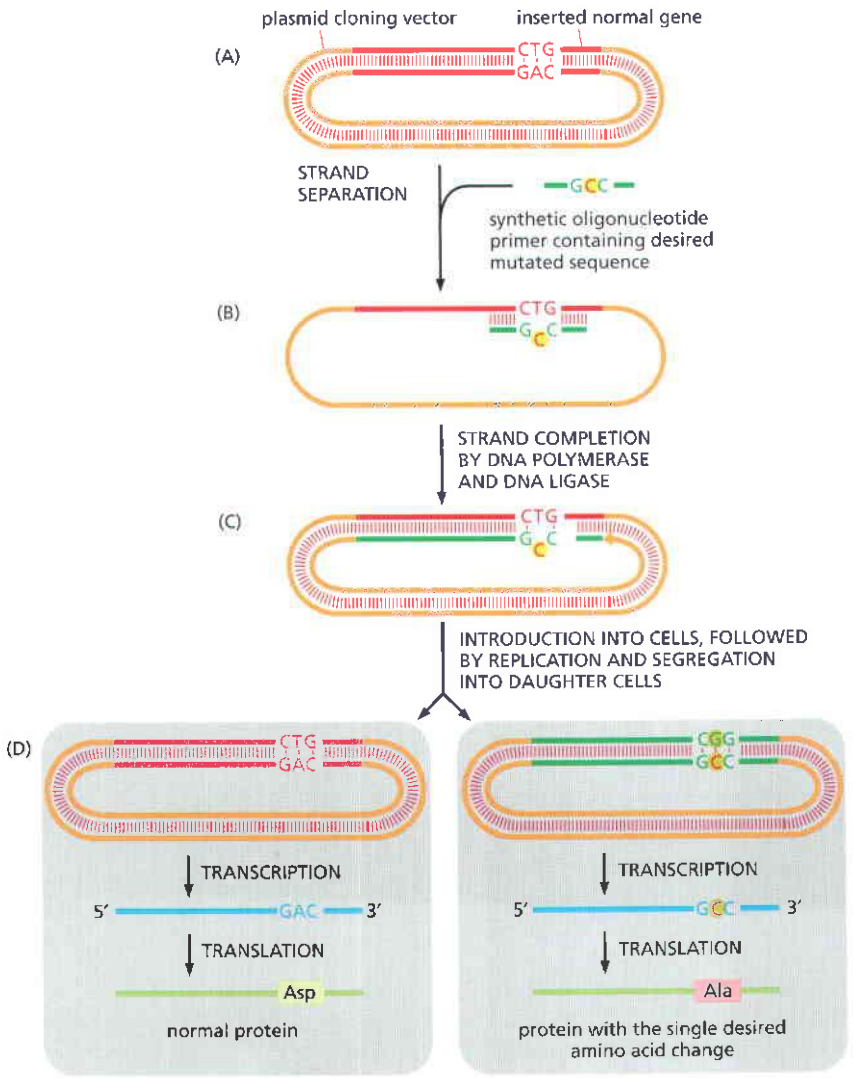


Figure 8–63 The use of a synthetic oligonucleotide to modify the protein-coding region of a gene by site-directed mutagenesis. (A) A recombinant plasmid containing a gene insert is separated into its two DNA strands. A synthetic oligonucleotide primer corresponding to part of the gene sequence but containing a single altered nucleotide at a predetermined point is added to the single-stranded DNA under conditions that permit imperfect DNA hybridization (see Figure 8–36). (B) The primer hybridizes to the DNA, forming a single mismatched nucleotide pair. (C) The recombinant plasmid is made double-stranded by *in vitro* DNA synthesis (starting from the primer) followed by sealing by DNA ligase. (D) The double-stranded DNA is introduced into a cell, where it is replicated. Replication using one strand of the template produces a normal DNA molecule, but replication using the other strand (the one that contains the primer) produces a DNA molecule carrying the desired mutation. Only half of the progeny cells will end up with a plasmid that contains the desired mutant gene. However, a progeny cell that contains the mutated gene can be identified, separated from other cells, and cultured to produce a pure population of cells, all of which carry the mutated gene. Only one of the many changes that can be engineered in this way is shown here. With an oligonucleotide of the appropriate sequence, more than one amino acid substitution can be made at a time, or one or more amino acids can be inserted or deleted. Although not shown in this figure, it is also possible to create a site-directed mutation by using the appropriate oligonucleotides and PCR (instead of plasmid replication) to amplify the mutated gene.

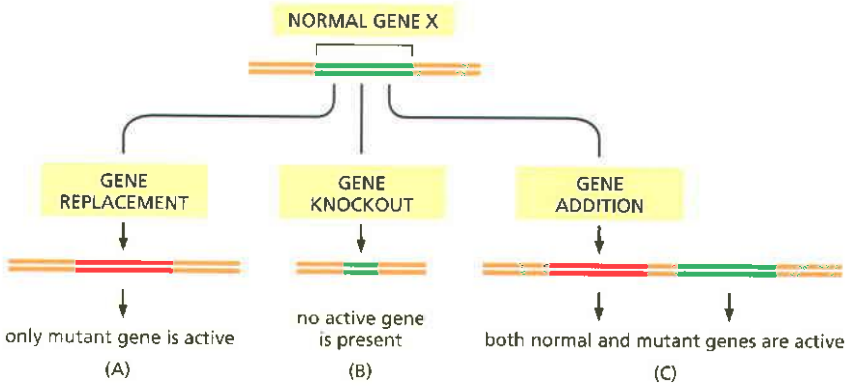


Figure 8–64 Gene replacement, gene knockout, and gene addition. A normal gene can be altered in several ways to produce a transgenic organism. (A) The normal gene *(green)* can be completely replaced by a mutant copy of the gene *(red)*. This provides information on the activity of the mutant gene without interference from the normal gene, and thus the effects of small and subtle mutations can be determined. (B) The normal gene can be inactivated completely, for example, by making a large deletion in it. (C) A mutant gene can simply be added to the genome. In some organisms this is the easiest type of genetic engineering to perform. This approach can provide useful information when the introduced mutant gene overrides the function of the normal gene, as with a dominant-negative mutation (see Figure 8–62).

Unlike higher eucaryotes (which are multicellular and diploid), bacteria, yeasts, and the cellular slime mold *Dictyostelium* generally exist as haploid single cells. In these organisms, an artificially introduced DNA molecule carrying a mutant gene can, with a relatively high frequency, replace the single copy of the normal gene by homologous recombination; it is therefore easy to produce cells in which the mutant gene has replaced the normal gene (**Figure 8–64**A). In this way, cells can be made in order to miss a particular protein or produce an altered form of it. The ability to perform direct gene replacements in lower eucaryotes, combined with the power of standard genetic analyses in these haploid organisms, explains in large part why studies in these types of cells have been so important for working out the details of those cell processes that are shared by all eucaryotes.

Animals Can Be Genetically Altered

Gene additions and replacements are also possible, but more difficult to perform, in animals and plants. Animals and plants that have been genetically engineered by either gene insertion, gene deletion, or gene replacement are called **transgenic organisms**, and any foreign or modified genes that are added are called **transgenes**. We concentrate our discussion on transgenic mice, as enormous progress is being made in this area. If a DNA molecule carrying a mutated mouse gene is transferred into a mouse cell, it usually inserts into the chromosomes at random, but about once in a thousand times, it replaces one of the two copies of the normal gene by homologous recombination. By exploiting these rare "gene targeting" events, any specific gene can be altered or inactivated in a mouse cell by a direct gene replacement. In the special case in which both copies of the gene of interest is completely inactivated or deleted, the resulting animal is called a **"knockout" mouse**.

The technique works as follows. In the first step, a DNA fragment containing a desired mutant gene (or a DNA fragment designed to interrupt a target gene) is inserted into a vector and then introduced into cultured embryonic stem (ES) cells (see Figure 8–5), which are capable of producing cells of many different types. After a period of cell proliferation, the rare colonies of cells in which a homologous recombination event is likely to have caused a gene replacement to occur are isolated. The correct colonies among these are identified by PCR or by Southern blotting: they contain recombinant DNA sequences in which the inserted fragment has replaced all or part of one copy of the normal gene. In the second step, individual ES cells from the identified colony are taken up into a fine micropipette and injected into an early mouse embryo. The transfected ES cells collaborate with the cells of the host embryo to produce a normal-looking mouse; large parts of this chimeric animal, including—in favorable cases—cells of the germ line, often derive from the transfected ES cells (**Figure 8–65**).

The mice with the transgene in their germ line are then bred to produce both a male and a female animal, each heterozygous for the gene replacement (that is, they have one normal and one mutant copy of the gene). When these two

mice are mated, one-fourth of their progeny will be homozygous for the altered gene. Studies of these homozygotes allow the function of the altered gene—or the effects of eliminating the gene's activity—to be examined in the absence of the corresponding normal gene.

The ability to prepare transgenic mice lacking a known normal gene has been a major advance, and the technique is now being used to determine the functions of all mouse genes (**Figure 8–66**). A special technique is used to produce conditional mutants, in which a selected gene becomes disrupted in a specific tissue at a certain time in development. The strategy takes advantage of a site-specific recombination system to excise—and thus disable—the target gene in a particular place or at a particular time. The most common of these recombination systems, called **Cre/lox**, is widely used to engineer gene replacements in mice and in plants (see Figure 5–79). In this case, the target gene in ES cells is replaced by a fully functional version of the gene that is flanked by a pair of the

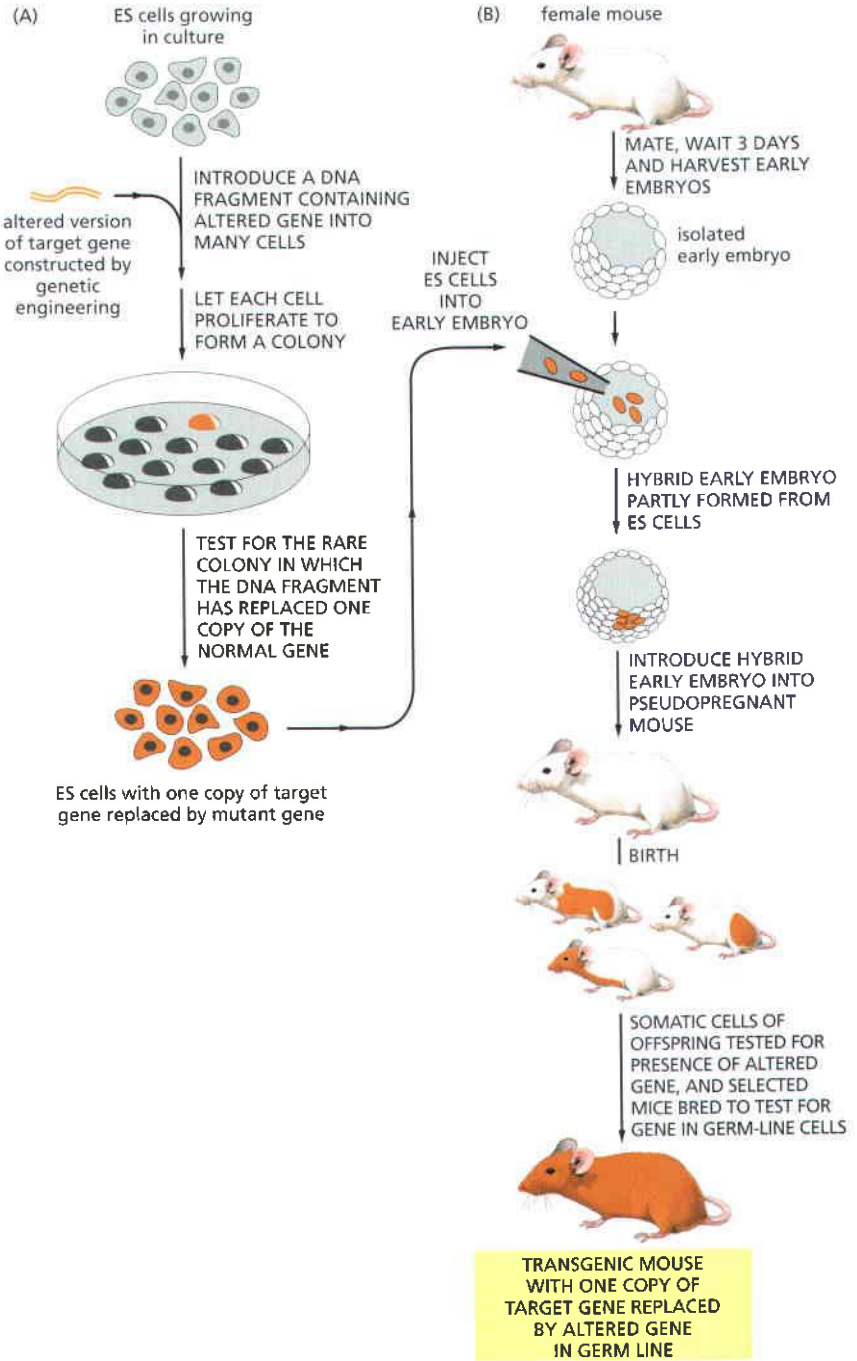


Figure 8–65 Summary of the procedures used for making gene replacements in mice. In the first step (A), an altered version of the gene is introduced into cultured ES (embryonic stem) cells. Only a few rare ES cells will have their corresponding normal genes replaced by the altered gene through a homologous recombination event. Although the procedure is often laborious, these rare cells can be identified and cultured to produce many descendants, each of which carries an altered gene in place of one of its two normal corresponding genes. In the next step of the procedure (B), these altered ES cells are injected into a very early mouse embryo; the cells are incorporated into the growing embryo, and a mouse produced by such an embryo will contain some somatic cells (indicated by *orange*) that carry the altered gene. Some of these mice will also contain germ-line cells that contain the altered gene; when bred with a normal mouse, some of the progeny of these mice will contain one copy of the altered gene in all of their cells. If two such mice are bred (not shown), some of the progeny will contain two altered genes (one on each chromosome) in all of their cells.

If the original gene alteration completely inactivates the function of the gene, these homozygous mice are known as knockout mice. When such mice are missing genes that function during development, they often die with specific defects long before they reach adulthood. These lethal defects are carefully analyzed to help determine the normal function of the missing gene.

(A) (B)

Figure 8–66 Transgenic mice engineered to express a mutant DNA helicase show premature aging. The helicase, encoded by the *Xpd* gene, is involved in both transcription and DNA repair. Compared with a wild-type mouse of the same age (A), a transgenic mouse that expresses a defective version of *Xpd* (B) exhibits many of the symptoms of premature aging, including osteoporosis, emaciation, early graying, infertility, and reduced life-span. The mutation in *Xpd* used here impairs the activity of the helicase and mimics a mutation that in humans causes trichothiodystrophy, a disorder characterized by brittle hair, skeletal abnormalities, and a very reduced life expectancy. These results indicate that an accumulation of DNA damage can contribute to the aging process in both humans and mice. (From J. de Boer et al., *Science* 296:1276–1279, 2002. With permission from AAAS.)

short DNA sequences, called *lox sites*, that are recognized by the *Cre recombinase* protein. The transgenic mice that result are phenotypically normal. They are then mated with transgenic mice that express the Cre recombinase gene under the control of an inducible promoter. In the specific cells or tissues in which Cre is switched on, it catalyzes recombination between the lox sequences—excising a target gene and eliminating its activity. Similar recombination systems are used to generate conditional mutants in *Drosophila* (see Figure 22–49).

Transgenic Plants Are Important for Both Cell Biology and Agriculture

A damaged plant can often repair itself by a process in which mature differentiated cells "dedifferentiate," proliferate, and then redifferentiate into other cell types. In some circumstances, the dedifferentiated cells can even form an apical meristem, which can then give rise to an entire new plant, including gametes. This remarkable developmental plasticity of plant cells can be exploited to generate transgenic plants from cells growing in culture.

When a piece of plant tissue is cultured in a sterile medium containing nutrients and appropriate growth regulators, many of the cells are stimulated to proliferate indefinitely in a disorganized manner, producing a mass of relatively undifferentiated cells called a *callus*. By carefully manipulating the nutrients and growth regulators, one can induce the formation of shoot and then root apical meristems within the callus, and, in many species, regenerate a whole new plant.

Callus cultures can also be mechanically dissociated into single cells, which will grow and divide as a suspension culture. In several plants—including tobacco, petunia, carrot, potato, and *Arabidopsis*—a single cell from such a suspension culture can be grown into a small clump (a clone) from which a whole plant can be regenerated. Such a cell, which has the ability to give rise to all parts of the organism, is considered **totipotent**. Just as mutant mice can be derived by the genetic manipulation of ES cells in culture, so transgenic plants can be created from single totipotent plant cells that have been transfected with DNA in culture (**Figure 8–67**).

The ability to produce transgenic plants has greatly accelerated progress in many areas of plant cell biology. It has had an important role, for example, in isolating receptors for growth regulators and in analyzing the mechanisms of morphogenesis and of gene expression in plants. It has also opened up many new possibilities in agriculture that could benefit both farmer and consumer. It has made it possible, for example, to modify the lipid, starch, and protein stored in seeds, to impart pest and virus resistance to plants, and to create modified plants that tolerate extreme habitats such as salt marshes or water-stressed soil.

Many of the major advances in understanding animal development have come from studies on the fruit fly *Drosophila* and the nematode worm *C. elegans*, which are amenable to classical genetic analysis, as well as to experimental manipulation. Progress in plant developmental biology has, in the past, been relatively slow by comparison. Many of the plants that have proved most amenable to genetic analysis—such as maize and tomato—have long life cycles and very large genomes, making both classical and molecular genetic analysis very time-consuming. Increasing attention is consequently being paid to a fast-growing small weed, the common wall cress *(Arabidopsis thaliana)*, which has

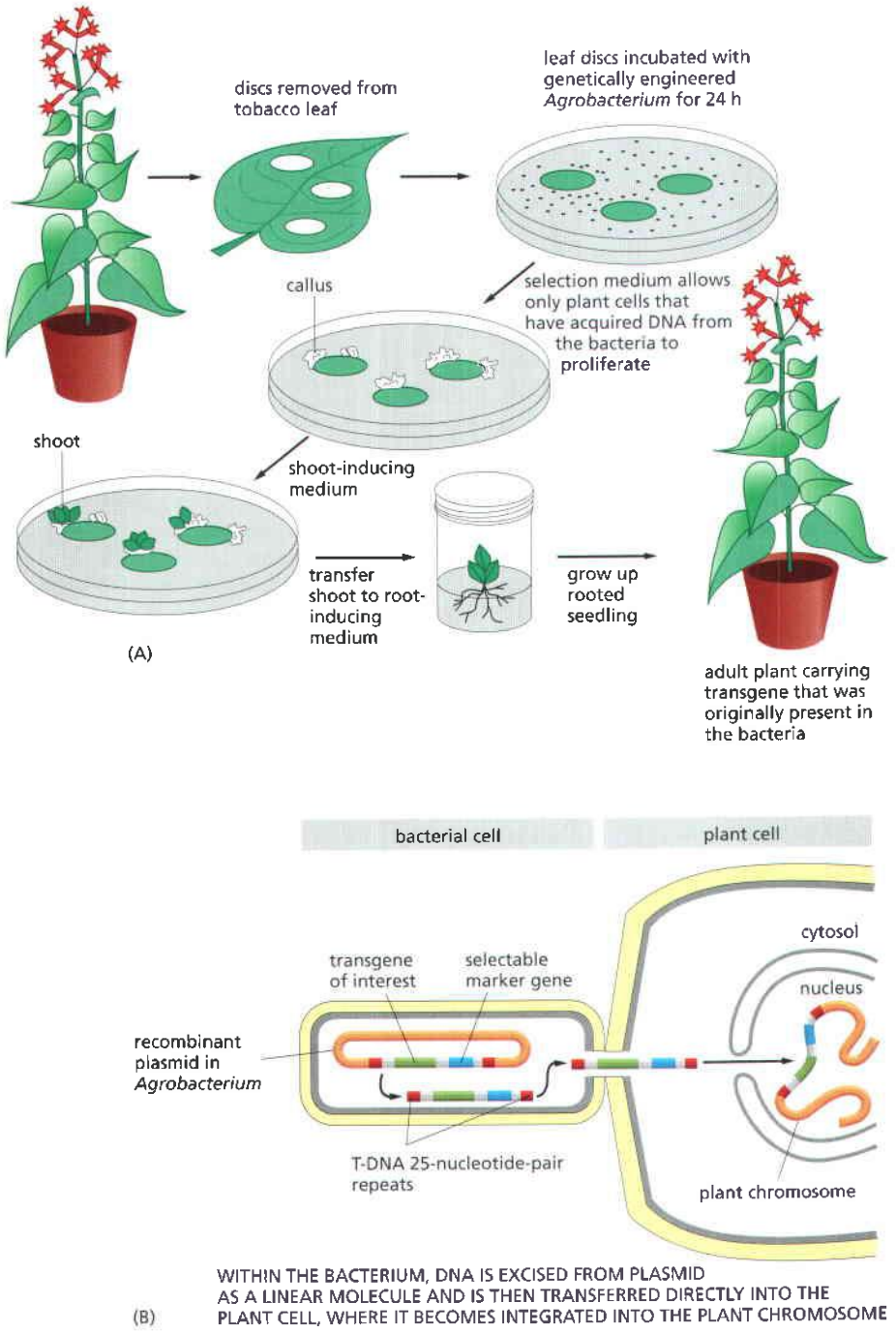


Figure 8–67 A procedure used to make a transgenic plant. (A) Outline of the process. A disc is cut out of a leaf and incubated in culture with *Agrobacterium* cells that carry a recombinant plasmid that contains both a selectable marker gene and a desired transgene. The wounded cells at the edge of the disc release substances that attract the *Agrobacterium* cells and cause them to inject DNA into these cells. Only those plant cells that take up the appropriate DNA and express the selectable marker gene survive to proliferate and form a callus. The manipulation of growth regulators and nutrients supplied to the callus induces it to form shoots, which subsequently root and grow into adult plants carrying the transgene. (B) The preparation of the recombinant plasmid and its transfer to plant cells. An *Agrobacterium* plasmid that normally carries the T-DNA sequence is modified by substituting a selectable marker gene (such as the kanamycin-resistance gene) and a desired transgene between the 25-nucleotide-pair T-DNA repeats. When the *Agrobacterium* recognizes a plant cell, it efficiently passes a DNA strand that carries these sequences into the plant cell, using the special machinery that normally transfers the plasmid's T-DNA sequence.

several major advantages as a "model plant" (see Figures 1–46 and 22–112). The relatively small *Arabidopsis* genome was the first plant genome to be completely sequenced, and the pace of research on this organism now rivals that of the model animals.

Large Collections of Tagged Knockouts Provide a Tool for Examining the Function of Every Gene in an Organism

Extensive collaborative efforts are underway to assemble comprehensive libraries of mutations in a variety of model organisms, including *S. cerevisiae, C. elegans, Drosophila, Arabidopsis,* and the mouse. The ultimate aim in each case is to produce a collection of mutant strains in which every gene in the organism has been systematically deleted or altered in such a way that it can be conditionally disrupted. Collections of this type will provide an invaluable resource for investi-

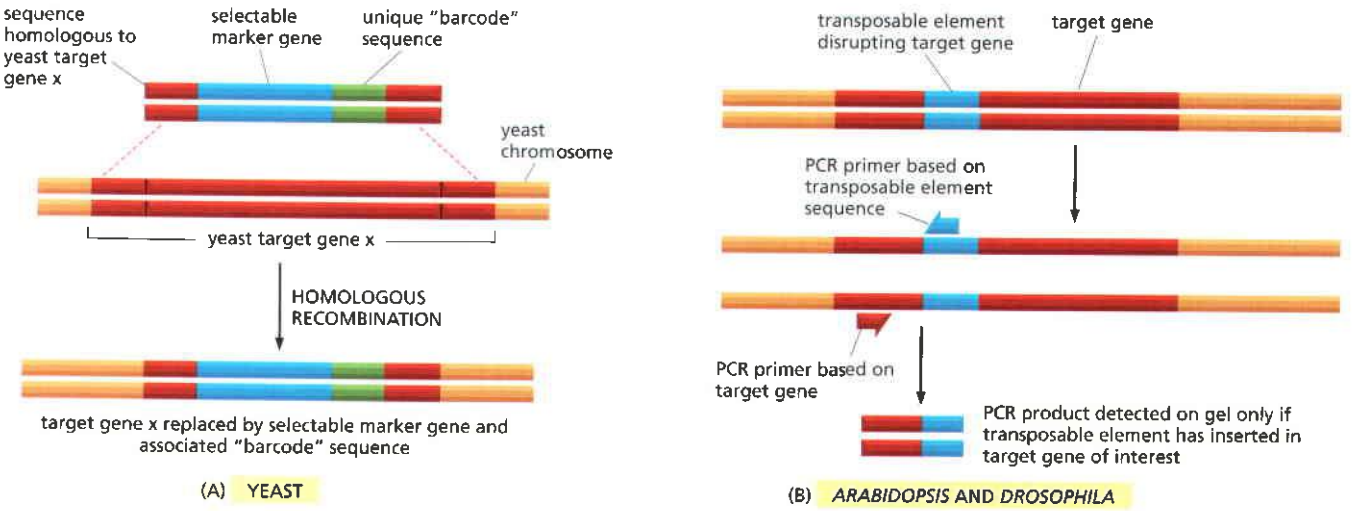


Figure 8–68 Making collections of mutant organisms. (A) A deletion cassette for use in yeast contains DNA sequences *(red)* homologous to each end of a target gene x, a selectable marker gene *(blue)*, and a unique "barcode" sequence approximately 20 nucleotide pairs in length *(green)*. This DNA is introduced into yeast cells, where it readily replaces the target gene by homologous recombination. By using a collection of such cassettes, each specific for one gene, a library of yeast mutants can be constructed containing a mutant for every gene. (B) A similar approach can be taken to prepare tagged knockout mutants in *Arabidopsis* and *Drosophila*. In this case, mutations are generated by the accidental insertion of a transposable element into a target gene. The total DNA from the resulting organism can be collected and quickly screened for disruption of a gene of interest by using PCR primers that bind to the transposable element and to the target gene. A PCR product is detected on the gel only if the transposable element has inserted into the target gene (see Figure 8–45).

gating gene function on a genomic scale. In some cases, each of the individual mutations within the collection will express a distinct molecular tag—in the form of a unique DNA sequence—designed to make identification of the altered gene rapid and routine.

In *S. cerevisiae*, the task of generating a complete set of 6000 mutants, each missing only one gene, is made simpler by yeast's propensity for homologous recombination. For each gene, a "deletion cassette" is prepared. The cassette consists of a special DNA molecule that contains 50 nucleotides identical in sequence to each end of the targeted gene, surrounding a selectable marker. In addition, a special "barcode" sequence tag is embedded in this DNA molecule to facilitate the later rapid identification of each resulting mutant strain (**Figure 8–68**). A large mixture of such gene knockout mutants can then be grown under various selective test conditions—such as nutritional deprivation, a temperature shift, or the presence of various drugs—and the cells that survive can be rapidly identified by their unique sequence tags. By assessing how well each mutant in the mixture fares, one can begin to assess which genes are essential, useful, or irrelevant for growth under the various conditions.

The challenge in deriving information from the study of such yeast mutants lies in deducing a gene's activity or biological role based on a mutant phenotype. Some defects—an inability to live without histidine, for example—point directly to the function of the wild-type gene. Other connections may not be so obvious. What might a sudden sensitivity to cold indicate about the role of a particular gene in the yeast cell? Such problems are even greater in organisms that are more complex than yeast. The loss of function of a single gene in the mouse, for example, may affect many different tissue types at different stages of development—whereas the loss of other genes may have no obvious effect. Adequately characterizing mutant phenotypes in mice often requires a thorough examination, along with extensive knowledge of mouse anatomy, histology, pathology, physiology, and complex behavior.

The insights generated by examination of mutant libraries, however, will be great. For example, studies of an extensive collection of mutants in *Mycoplasma genitalium*—the organism with the smallest known genome—have identified the minimum complement of genes essential for cellular life. Analysis of the mutant pool suggests that growth under laboratory conditions requires about three-quarters of the 480 protein-coding genes in *M. genitalium*. Approximately

100 of these essential genes are of unknown function, which suggests that a surprising number of the basic molecular mechanisms that underlie life have yet to be discovered.

RNA Interference Is a Simple and Rapid Way to Test Gene Function

Although knocking out a gene in an organism and studying the consequences is perhaps the most powerful approach for understanding the functions of the gene, a much easier way to inactivate genes has been recently discovered. Called **RNA interference** (**RNAi**, for short), this method exploits a natural mechanism used in many plants, animals, fungi, and protozoa to protect themselves against certain viruses and transposable elements (see Figure 7–115). The technique introduces into a cell or organism a double-stranded RNA molecule whose nucleotide sequence matches that of part of the gene to be inactivated. After the RNA is processed, it hybridizes with the mRNA produced by the target gene and directs its degradation. The cell subsequently uses small fragments of this degraded RNA to produce more double-stranded RNA, which directs the continued elimination of the target mRNA. Because these short RNA fragments can be passed on to progeny cells, RNAi can cause heritable changes in gene expression. But, as we saw in Chapter 7, there is a second mechanism through which RNAi can stably inactivate genes. RNA fragments produced by degradation in the cytosol can enter the nucleus and interact with the target gene itself, directing its packaging into a transcriptionally repressed form of chromatin. This dual mode of controlling gene expression makes RNAi an especially effective tool for shutting down genes, one at a time.

RNAi is frequently used to inactivate genes in *Drosophila* and mammalian cell culture lines. Indeed, sets of 15,000 *Drosophila* RNAi molecules (one for every gene) allow researchers, in several months, to test the role of every fly gene in any process that can be monitored using cultured cells. Soon, it will be possible to carry out the same type of analysis with the 25,000 mouse and human genes. RNAi has also been widely used to study gene function in the nematode, *C. elegans*. When working with worms, introducing the double-stranded RNA is quite simple: the RNA can be injected directly into the intestine of the animal, or the worm can be fed with *E. coli* engineered to produce the RNA (**Figure 8–69**). The RNA is distributed throughout the body of the worm, where it inhibits expression of the target gene in different tissue types. Because the entire genome of *C. elegans* has been sequenced, RNAi is being used to help in assigning functions to the entire complement of worm genes.

More recently, a related technique has also been widely applied to mice. In this case, the RNAi is not injected or fed to the mouse; rather, recombinant DNA techniques are used to make transgenic animals that express the RNAi under the control of an inducible promoter. Often this is a specially designed RNA that can fold back on itself and, through base pairing, produce a double-stranded region

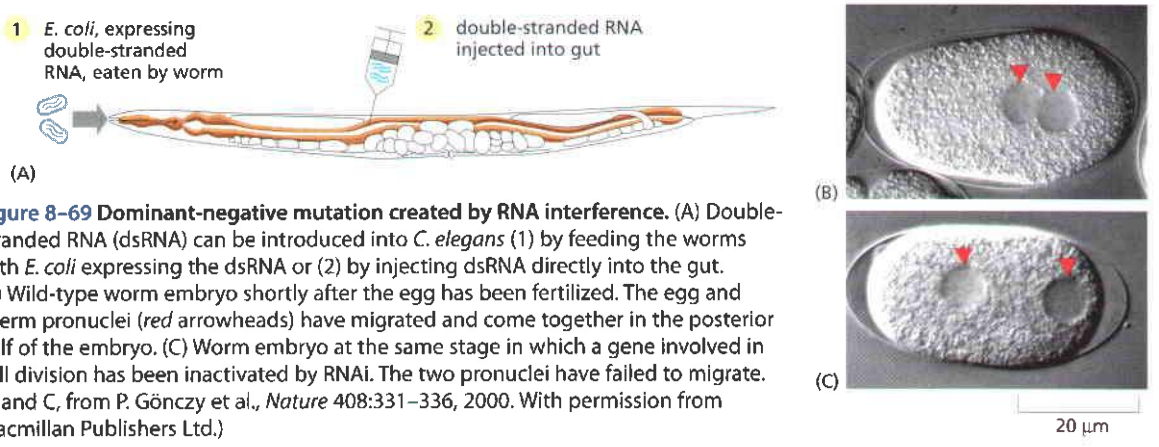


Figure 8–69 Dominant-negative mutation created by RNA interference. (A) Double-stranded RNA (dsRNA) can be introduced into *C. elegans* (1) by feeding the worms with *E. coli* expressing the dsRNA or (2) by injecting dsRNA directly into the gut. (B) Wild-type worm embryo shortly after the egg has been fertilized. The egg and sperm pronuclei (*red* arrowheads) have migrated and come together in the posterior half of the embryo. (C) Worm embryo at the same stage in which a gene involved in cell division has been inactivated by RNAi. The two pronuclei have failed to migrate. (B and C, from P. Gönczy et al., *Nature* 408:331–336, 2000. With permission from Macmillan Publishers Ltd.)

that is recognized by the RNAi machinery. The process inactivates only the genes that exactly match the RNAi sequence. Depending on the inducible promoter used, the RNAi can be produced only in a specified tissue or only at a particular time in development, allowing the functions of the target genes to be analyzed in elaborate detail.

RNAi has made reverse genetics simple and efficient in many organisms, but it has several potential limitations compared with true genetic knockouts. For unknown reasons, RNAi does not efficiently inactivate all genes. Moreover, within whole organisms, certain tissues may be resistant to the action of RNAi (for example, neurons in nematodes). Another problem arises because many organisms contain large gene families, the members of which exhibit sequence similarity. RNAi therefore sometimes produces "off-target" effects, inactivating related genes in addition to the targeted gene. One strategy to avoid such problems is to use multiple small RNA molecules matched to different regions of the same gene. Ultimately, the results of any RNAi experiment must be viewed as a strong clue to, but not necessarily a proof of, normal gene function.

Reporter Genes and *In Situ* Hybridization Reveal When and Where a Gene Is Expressed

Important insights into gene function can often be obtained by examining when and where a gene is expressed in the cell or in the whole organism. Determining the pattern and timing of gene expression can be accomplished by replacing the coding portion of the gene under study with a reporter gene. In most cases, the expression of the reporter gene is then monitored by tracking the fluorescence or enzymatic activity of its protein product (see Figures 9–26 and 9–27).

As discussed in detail in Chapter 7, regulatory DNA sequences, located upstream or downstream of the coding region, control gene expression. These regulatory sequences, which determine exactly when and where the gene is expressed, can be easily studied by placing a reporter gene under their control and introducing these recombinant DNA molecules into cells (**Figure 8–70**).

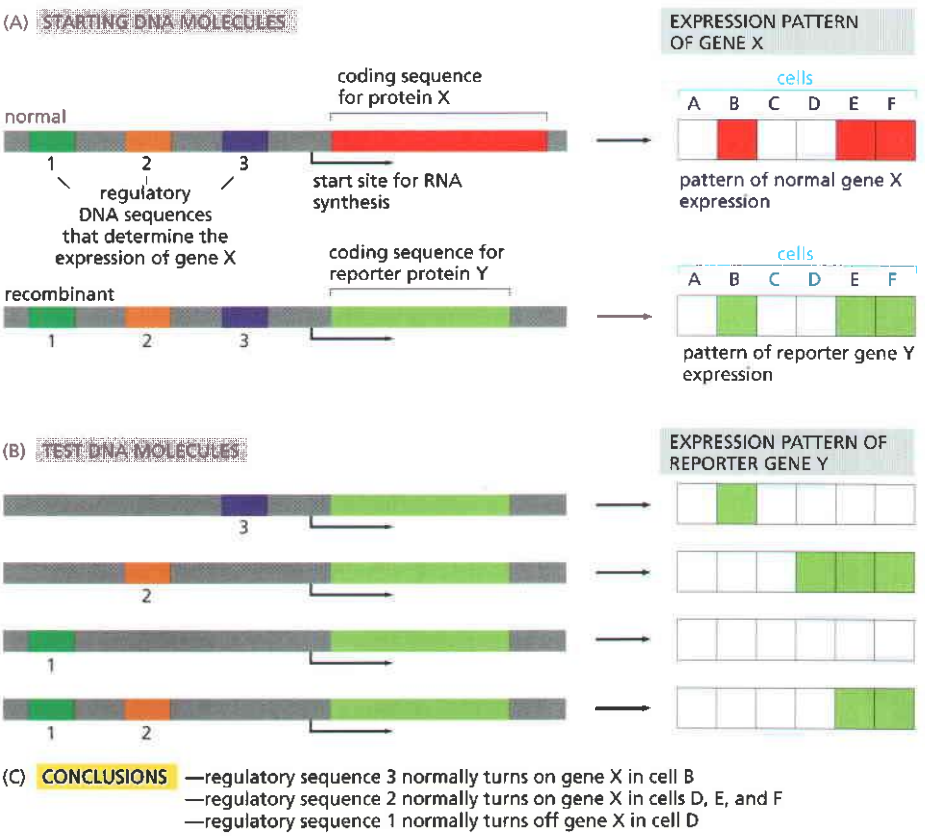


Figure 8–70 Using a reporter protein to determine the pattern of a gene's expression. (A) In this example, the coding sequence for protein X is replaced by the coding sequence for reporter protein Y. The expression pattern for X and Y are the same. (B) Various fragments of DNA containing candidate regulatory sequences are added in combinations to produce test DNA molecules encoding reporter gene Y. These recombinant DNA molecules are then tested for expression after their transfection into a variety of different types of mammalian cells. The results are summarized in (C).

For experiments in eucaryotic cells, two commonly used reporter proteins are the enzyme β-galactosidase *(β-gal)* (see Figure 7–55B) and green fluorescent protein or GFP (see Figure 9–26). Figure 7–55B shows an example in which the *β-gal* gene is used to monitor the activity of the *Eve* gene regulatory sequence in a *Drosophila* embryo.

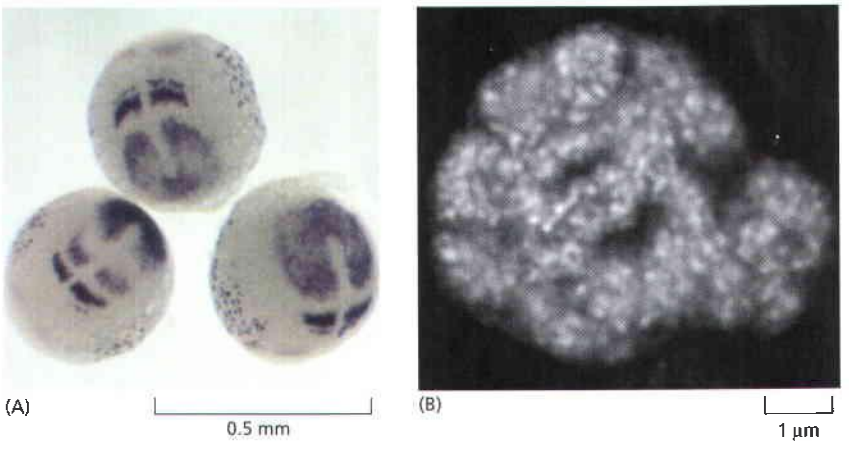


Figure 8–71 *In situ* hybridization for RNA localization. (A) Expression pattern of *DeltaC* mRNA in the early zebrafish embryo. This gene codes for a ligand in the Notch signaling pathway (discussed in Chapter 15), and the pattern shown here reflects its role in the development of somites—the future segments of the vertebrate trunk and tail. (B) High-resolution RNA *in situ* localization reveals the sites within the nucleolus of a pea cell where ribosomal RNA is synthesized. The sausage-like structures, 0.5–1 μm in diameter, correspond to the loops of chromosomal DNA that contain the genes encoding rRNA. Each small white spot represents transcription of a single rRNA gene. (A, courtesy of Yun-Jin Jiang; B, courtesy of Peter Shaw.)

It is also possible to directly observe the time and place that the mRNA product of a gene is expressed. Although this strategy often provides the same general information as the reporter gene approaches discussed above, there are instances where it provides additional information; for example, when the gene is transcribed but the mRNA is not immediately translated, or when the gene's final product is RNA rather than protein. This procedure, called ***in situ* hybridization**, relies on the principles of nucleic acid hybridization described earlier. Typically, tissues are gently fixed so that their RNA is retained in an exposed form that can hybridize with a labeled complementary DNA or RNA probe. In this way, the patterns of differential gene expression can be observed in tissues, and the location of specific RNAs in cells can be determined (**Figure 8–71**). In the *Drosophila* embryo, for example, such patterns have provided new insights into the mechanisms that create distinctions between cells in different positions during development (described in Chapter 22).

Using similar approaches, it is also possible to visualize specific DNA sequences in cells. In this case, tissue, cell, or even chromosome preparations are briefly exposed to high pH to disrupt their nucleotide pairs, and nucleic acid probes are added, allowed to hybridize with the cells' DNA, and then visualized (see Figure 8–35).

Expression of Individual Genes Can Be Measured Using Quantitative RT-PCR

Although reporter genes and *in situ* hybridization reveal patterns of gene expression, it is often desirable to quantitate gene expression by directly measuring mRNA levels in cells. Although Northern blots (see Figure 8–38) can be adapted to this purpose, a more accurate method is based on the principles of PCR (**Figure 8–72**). This method, called **quantitative RT-PCR** (reverse transcription-polymerase chain reaction), begins with the total population of mRNA molecules purified from a tissue or a cell culture. It is important that no DNA be present in the preparation; it must be purified away or enzymatically degraded. Two DNA primers that specifically match the gene of interest are added, along with reverse transcriptase, DNA polymerase, and the four deoxynucleoside triphosphates needed for DNA synthesis. The first round of synthesis is the reverse transcription of the mRNA into DNA using one of the primers. Next, a series of heating and cooling cycles allows the amplification of that DNA strand by conventional PCR (see Figure 8–45). The quantitative part of this method relies on a direct relationship between the rate at which the PCR product is generated and the original concentration of the mRNA species of interest. By adding chemical dyes to the PCR reaction that fluoresce only when bound to double-stranded DNA, a simple fluorescence measurement can be used to track the progress of the reaction and thereby accurately deduce the starting concentration of the mRNA that is amplified (see Figure 8–72). Although it seems complicated, this quantitative RT-PCR technique (sometimes called *real time PCR*) is relatively fast and simple

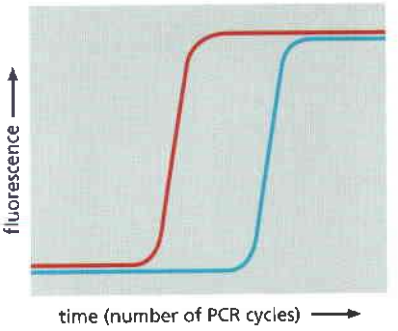


Figure 8–72 RNA levels can be measured by quantitative RT-PCR. The fluorescence measured is generated by a dye that fluoresces only when bound to the double-stranded DNA products of the RT-PCR reaction (see Figure 8–46B). The red sample has a higher concentration of the mRNA being measured than does the blue sample, since it requires fewer PCR cycles to reach the same half-maximal concentration of double-stranded DNA. Based on this difference, the relative amounts of the mRNA in the two samples can be precisely determined.

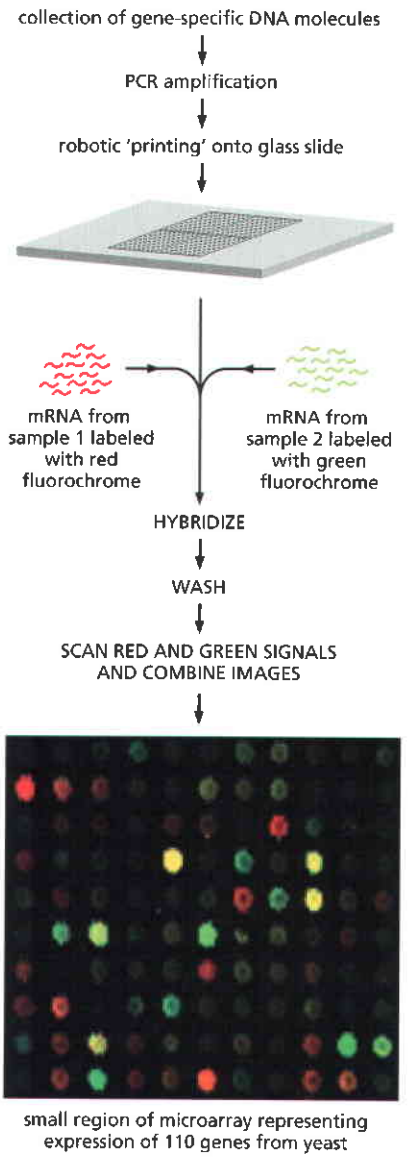


Figure 8–73 Using DNA microarrays to monitor the expression of thousands of genes simultaneously. To prepare the microarray, DNA fragments—each corresponding to a gene—are spotted onto a slide by a robot. Prepared arrays are also widely available commercially. In this example, mRNA is collected from two different cell samples for a direct comparison of their relative levels of gene expression; the two samples, for example, could be from cells treated with a hormone and untreated cells of the same type. These samples are converted to cDNA and labeled, one with a red fluorochrome, the other with a green fluorochrome. The labeled samples are mixed and then allowed to hybridize to the microarray. After incubation, the array is washed and the fluorescence scanned. In the portion of a microarray shown, which represents the expression of 110 yeast genes, *red* spots indicate that the gene in sample 1 is expressed at a higher level than the corresponding gene in sample 2; *green* spots indicate that expression of the gene is higher in sample 2 than in sample 1. *Yellow* spots reveal genes that are expressed at equal levels in both cell samples. Dark spots indicate little or no expression in either sample of the gene whose fragment is located at that position in the array. (Microarray courtesy of J.L. DeRisi et al., *Science* 278:680–686, 1997. With permission from AAAS.)

to perform in the laboratory; it has displaced Northern blotting as the method of choice for quantifying mRNA levels from any given gene.

Microarrays Monitor the Expression of Thousands of Genes at Once

So far we have discussed techniques that can be used to monitor the expression of only a single gene (or relatively few genes) at a time. Developed in the 1990s, **DNA microarrays** have revolutionized the analysis of gene expression by monitoring the RNA products of thousands of genes at once. By examining the expression of so many genes simultaneously, we can now begin to identify and study the gene expression patterns that underlie cell physiology: we can see which genes are switched on (or off) as cells grow, divide, differentiate, or respond to hormones or to toxins.

DNA microarrays are little more than glass microscope slides studded with a large number of DNA fragments, each containing a nucleotide sequence that serves as a probe for a specific gene. The most dense arrays may contain tens of thousands of these fragments in an area smaller than a postage stamp, allowing thousands of hybridization reactions to be performed in parallel (**Figure 8–73**). Some microarrays are prepared from large DNA fragments that have been generated by PCR and then spotted onto the slides by a robot. Others contain short oligonucleotides that are synthesized on the surface of the glass wafer with techniques similar to those that are used to etch circuits onto computer chips. In either case, the exact sequence—and position—of every probe on the chip is known. Thus, any nucleotide fragment that hybridizes to a probe on the array can be identified as the product of a specific gene simply by detecting the position at which it is bound.

To use a DNA microarray to monitor gene expression, mRNA from the cells being studied is first extracted and converted to cDNA (see Figure 8–43). The cDNA is then labeled with a fluorescent probe. The microarray is incubated with this labeled cDNA sample and hybridization is allowed to occur (see Figure 8–73). The array is then washed to remove cDNA that is not tightly bound, and the positions in the microarray to which labeled DNA fragments have bound are identified by an automated scanning-laser microscope. The array positions are then matched to the particular gene whose sample of DNA was spotted in this location.

Typically the fluorescent DNA from the experimental samples (labeled, for example, with a red fluorescent dye) are mixed with a reference sample of cDNA fragments labeled with a differently colored fluorescent dye (green, for example). Thus, if the amount of RNA expressed from a particular gene in the cells of interest is increased relative to that of the reference sample, the resulting spot is red. Conversely, if the gene's expression is decreased relative to the reference sample, the spot is green. If there is no change compared to the reference sample, the spot

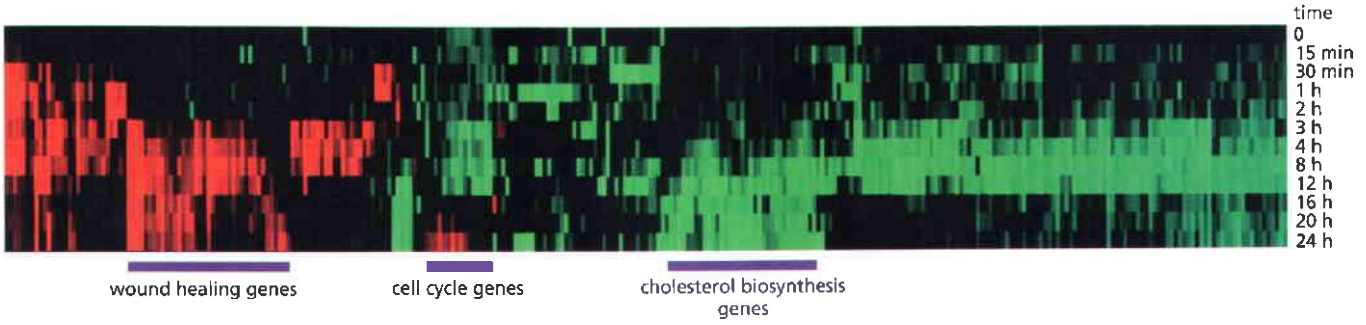


Figure 8–74 Using cluster analysis to identify sets of genes that are coordinately regulated. Genes that belong to the same cluster may be involved in common pathways or processes. To perform a cluster analysis, microarray data are obtained from cell samples exposed to a variety of different conditions, and genes that show coordinate changes in their expression pattern are grouped together. In this experiment, human fibroblasts were deprived of serum for 48 hours; serum was then added back to the cultures at time 0 and the cells were harvested for microarray analysis at different time points. Of the 8600 genes analyzed on the DNA microarray, just over 300 showed threefold or greater variation in their expression patterns in response to serum re-introduction. Here, *red* indicates an increase in expression; *green* is a decrease in expression. On the basis of the results of many microarray experiments, the 8600 genes have been grouped in clusters based on similar patterns of expression. The results of this analysis show that genes involved in wound healing are turned on in response to serum, while genes involved in regulating cell cycle progression and cholesterol biosynthesis are shut down. (From M.B. Eisen et al., *Proc. Natl Acad. Sci. U.S.A.* 95:14863–14868, 1998. With permission from National Academy of Sciences.)

is yellow. Using such an internal reference, gene expression profiles can be tabulated with great precision.

So far, DNA microarrays have been used to examine everything from the changes in gene expression that make strawberries ripen to the gene expression "signatures" of different types of human cancer cells (see Figure 7–3); or from changes that occur as cells progress through the cell cycle to those made in response to sudden shifts in temperature. Indeed, because microarrays allow the simultaneous monitoring of large numbers of genes, they can detect subtle changes in a cell, changes that might not be manifested in its outward appearance or behavior.

Comprehensive studies of gene expression also provide an additional layer of information that is useful for predicting gene function. Earlier, we discussed how identifying a protein's interaction partners can yield clues about that protein's function. A similar principle holds true for genes: information about a gene's function can be deduced by identifying genes that share its expression pattern. Using a technique called *cluster analysis*, one can identify sets of genes that are coordinately regulated. Genes that are turned on or turned off together under different circumstances are likely to work in concert in the cell: they may encode proteins that are part of the same multiprotein machine, or proteins that are involved in a complex coordinated activity, such as DNA replication or RNA splicing. Characterizing a gene whose function is unknown by grouping it with known genes that share its transcriptional behavior is sometimes called "guilt by association." Cluster analyses have been used to analyze the gene expression profiles that underlie many interesting biological processes, including wound healing in humans (**Figure 8–74**).

In addition to monitoring the level of mRNA corresponding to every gene in a genome, DNA microarrays have many other uses. For example, they can be used to monitor the progression of DNA replication in a cell (see Figure 5–32) and, when combined with immunoprecipitation, can pinpoint every position in the genome occupied by a given gene regulatory protein (see Figure 7–32). Microarrays can also be used to quickly identify disease-causing microbes by hybridizing DNA from infected tissues to an array containing genomic DNA sequences from large collections of pathogens.

Single-Cell Gene Expression Analysis Reveals Biological "Noise"

The methods for monitoring mRNAs just described give average expression levels for each mRNA across a large population of cells. By using a fluorescent

reporter protein whose expression is under the control of a promoter of interest, it is also possible to accurately measure expression levels in individual cells. These new approaches have revealed a startling amount of variability, often called *biological noise*, between the individual cells in a homogeneous population of cells. These studies have also revealed the presence of distinct subpopulations of cells whose existence would be masked if only the average across a whole population were considered. For example, a bimodal distribution of expression levels would indicate that the cells can exist in two distinct states (**Figure 8–75**), with the average expression level of the population being somewhere between them. The behavior of individual cells has important implications for understanding biology, for example, by revealing that some cells constantly and rapidly switch back and forth between two states.

Currently, there are two approaches for monitoring gene expression in individual cells. In the imaging approach, live cells are mounted on a slide and viewed through a fluorescence microscope. This method has the advantage that a given cell can be followed over time, allowing temporal changes in expression to be measured. The second approach, flow cytometry, works by streaming a dilute suspension of cells past an illuminator and measuring the fluorescence of individual cells as they flow past the detector (see Figure 8–2). Although it has the advantage that the expression levels of very large numbers of cells can be measured with precision, flow cytometry does not allow a given cell to be tracked over time; hence, it is complementary to the imaging methods.

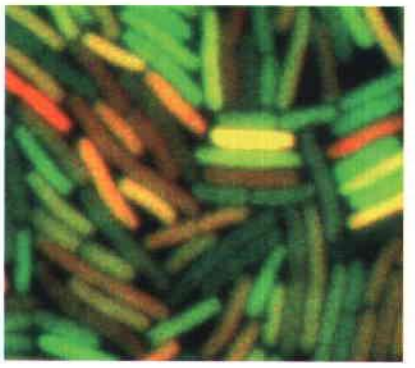


Figure 8–75 Different levels of gene expression in individual cells within a population of *E. coli* bacteria. For this experiment, two different reporter proteins (one fluorescing *green*, the other *red*) controlled by a copy of the same promoter, have been introduced into all of the bacteria. When illuminated, some cells express only one gene copy, and so appear either *red* or *green*, while others express both gene copies, and so appear *yellow*. This experiment also reveals variable levels of fluorescence, indicating variable levels of gene expression within an apparently uniform population of cells. (From M.B. Elowitz, A.J. Levine, E.O. Siggia and P.S. Swain, *Science* 297:1183–1186, 2002. With permission from AAAS.)

Summary

Genetics and genetic engineering provide powerful tools for the study of gene function in both cells and organisms. In the classical genetic approach, random mutagenesis is coupled with screening to identify mutants that are deficient in a particular biological process. These mutants are then used to locate and study the genes responsible for that process.

Gene function can also be ascertained by reverse genetic techniques. DNA engineering methods can be used to alter genes and to re-insert them into a cell's chromosomes so that they become a permanent part of the genome. If the cell used for this gene transfer is a fertilized egg (for an animal) or a totipotent plant cell in culture, transgenic organisms can be produced that express the mutant gene and pass it on to their progeny. Especially important for cell biology is the ability to alter cells and organisms in highly specific ways—allowing one to discern the effect on the cell or the organism of a designed change in a single protein or RNA molecule.

Many of these methods are being expanded to investigate gene function on a genome-wide scale. The generation of mutant libraries in which every gene in an organism has been systematically deleted or disrupted provides invaluable tools for exploring the role of each gene in the elaborate molecular collaboration that gives rise to life. Technologies such as DNA microarrays can monitor the expression of thousands of genes simultaneously, providing detailed, comprehensive snapshots of the dynamic patterns of gene expression that underlie complex cell processes.

PROBLEMS

Which statements are true? Explain why or why not.

8–1 Because a monoclonal antibody recognizes a specific antigenic site (epitope), it binds only to the specific protein against which it was made.

8–2 Given the inexorable progress of technology, it seems inevitable that the sensitivity of detection of molecules will ultimately be pushed beyond the yoctomole level (10^{-24} mole).

8–3 Surface plasmon resonance (SPR) measures association (k_{on}) and dissociation (k_{off}) rates between molecules in real time, using small amounts of unlabeled molecules, but it does not give the information needed to determine the binding constant (K).

8–4 If each cycle of PCR doubles the amount of DNA synthesized in the previous cycle, then 10 cycles will give a 10^3-fold amplification, 20 cycles will give a 10^6-fold amplification, and 30 cycles will give a 10^9-fold amplification.

Discuss the following problems.

8–5 A common step in the isolation of cells from a sample of animal tissue is to treat it with trypsin, collagenase, and EDTA. Why is such a treatment necessary, and what

does each component accomplish? And why does this treatment not kill the cells?

8–6 Do you suppose it would be possible to raise an antibody against another antibody? Explain your answer.

8–7 Distinguish between velocity sedimentation and equilibrium sedimentation. For what general purpose is each technique used? Which do you suppose might be best suited for separating two proteins of different size?

8–8 Tropomyosin, at 93 kd, sediments at 2.6 S, whereas the 65-kd protein, hemoglobin, sediments at 4.3 S. (The sedimentation coefficient S is a linear measure of the rate of sedimentation: both increase or decrease in parallel.) These two proteins are shown as α-carbon backbone models in **Figure Q8–1**. How is it that the bigger protein sediments more slowly than the smaller one? Can you think of an analogy from everyday experience that might help you with this problem?

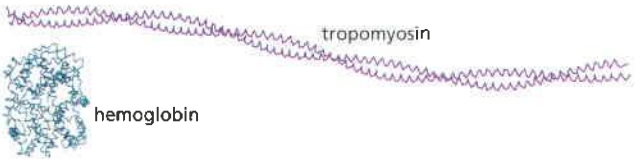


Figure Q8–1 Backbone models of tropomyosin and hemoglobin (Problem 8–8).

8–9 In the classic paper that demonstrated the semi-conservative replication of DNA, Meselson and Stahl began by showing that DNA itself will form a band when subjected to equilibrium sedimentation. They mixed randomly fragmented *E. coli* DNA with a solution of CsCl so that the final solution had a density of 1.71 g/mL. As shown in **Figure Q8–2**, with increasing length of centrifugation at 70,000 times gravity, the DNA, which was initially dispersed throughout the centrifuge tube, became concentrated over time into a discrete band in the middle.

A. Describe what is happening with time and explain why the DNA forms a discrete band.

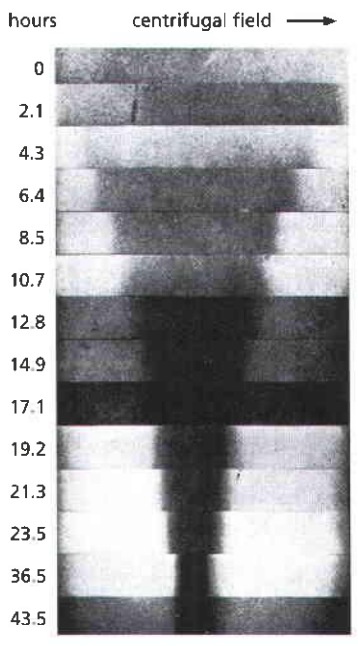


Figure Q8–2 Ultraviolet absorption photographs showing successive stages in the banding of *E. coli* DNA (Problem 8–9). DNA, which absorbs UV light, shows up as *dark regions* in the photographs. The bottom of the centrifuge tube is on the *right*. (From M. Meselson and F.W. Stahl, *Proc. Natl Acad. Sci. U.S.A.* 44:671–682, 1958. With permission from National Academy of Sciences.)

B. What is the buoyant density of the DNA? (The density of the solution at which DNA "floats" at equilibrium defines the "buoyant density" of the DNA.)

C. Even if the DNA were centrifuged for twice as long—or even longer—the width of the band remains about what is shown at the bottom of Figure Q8–2. Why does the band not become even more compressed? Suggest some possible reasons to explain the thickness of the DNA band at equilibrium.

8–10 Hybridoma technology allows one to generate monoclonal antibodies to virtually any protein. Why is it then that tagging proteins with epitopes is such a commonly used technique, especially since an epitope tag has the potential to interfere with the function of the protein?

8–11 How many copies of a protein need to be present in a cell in order for it to be visible as a band on a gel? Assume that you can load 100 μg of cell extract onto a gel and that you can detect 10 ng in a single band by silver staining. The concentration of protein in cells is about 200 mg/mL, and a typical mammalian cell has a volume of about 1000 μm^3 and a typical bacterium a volume of about 1 μm^3. Given these parameters, calculate the number of copies of a 120-kd protein that would need to be present in a mammalian cell and in a bacterium in order to give a detectable band on a gel. You might try an order-of-magnitude guess before you make the calculations.

8–12 You want to amplify the DNA between the two stretches of sequence shown in **Figure Q8–3**. Of the listed primers choose the pair that will allow you to amplify the DNA by PCR.

DNA to be amplified

5′-GACCTGTGGAAGC——————CATACGGGATTGA-3′
3′-CTGGACACCTTCG——————GTATGCCCTAACT-5′

primers

(1) 5′-GACCTGTCCAAGC-3′
(2) 5′-CTGGACACCTTCG-3′
(3) 5′-CGAAGGTGTCCAG-3′
(4) 5′-GCTTCCACAGGTC-3′
(5) 5′-CATACGGGATTGA-3′
(6) 5′-GTATGCCCTAACT-3′
(7) 5′-TGTTAGGGCATAC-3′
(8) 5′-TCAATCCCGTATG-3′

Figure Q8–3 DNA to be amplified and potential PCR primers (Problem 8–12).

8–13 In the very first round of PCR using genomic DNA, the DNA primers prime synthesis that terminates only when the cycle ends (or when a random end of DNA is encountered). Yet, by the end of 20 to 30 cycles—a typical amplification—the only visible product is defined precisely by the ends of the DNA primers. In what cycle is a double-stranded fragment of the correct size first generated?

8–14 Explain the difference between a gain-of-function mutation and a dominant-negative mutation. Why are both these types of mutation usually dominant?

8–15 Discuss the following statement: "We would have no idea today of the importance of insulin as a regulatory hormone if its absence were not associated with the devastating human disease diabetes. It is the dramatic consequences of its absence that focused early efforts on the identification of insulin and the study of its normal role in physiology."

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9

Visualizing Cells

In This Chapter

Because cells are small and complex, it is hard to see their structure, hard to discover their molecular composition, and harder still to find out how their various components function. The tools at our disposal determine what we can learn about cells, and the introduction of new techniques has frequently resulted in major advances in cell biology. To understand contemporary cell biology, therefore, it is necessary to know something of its methods.

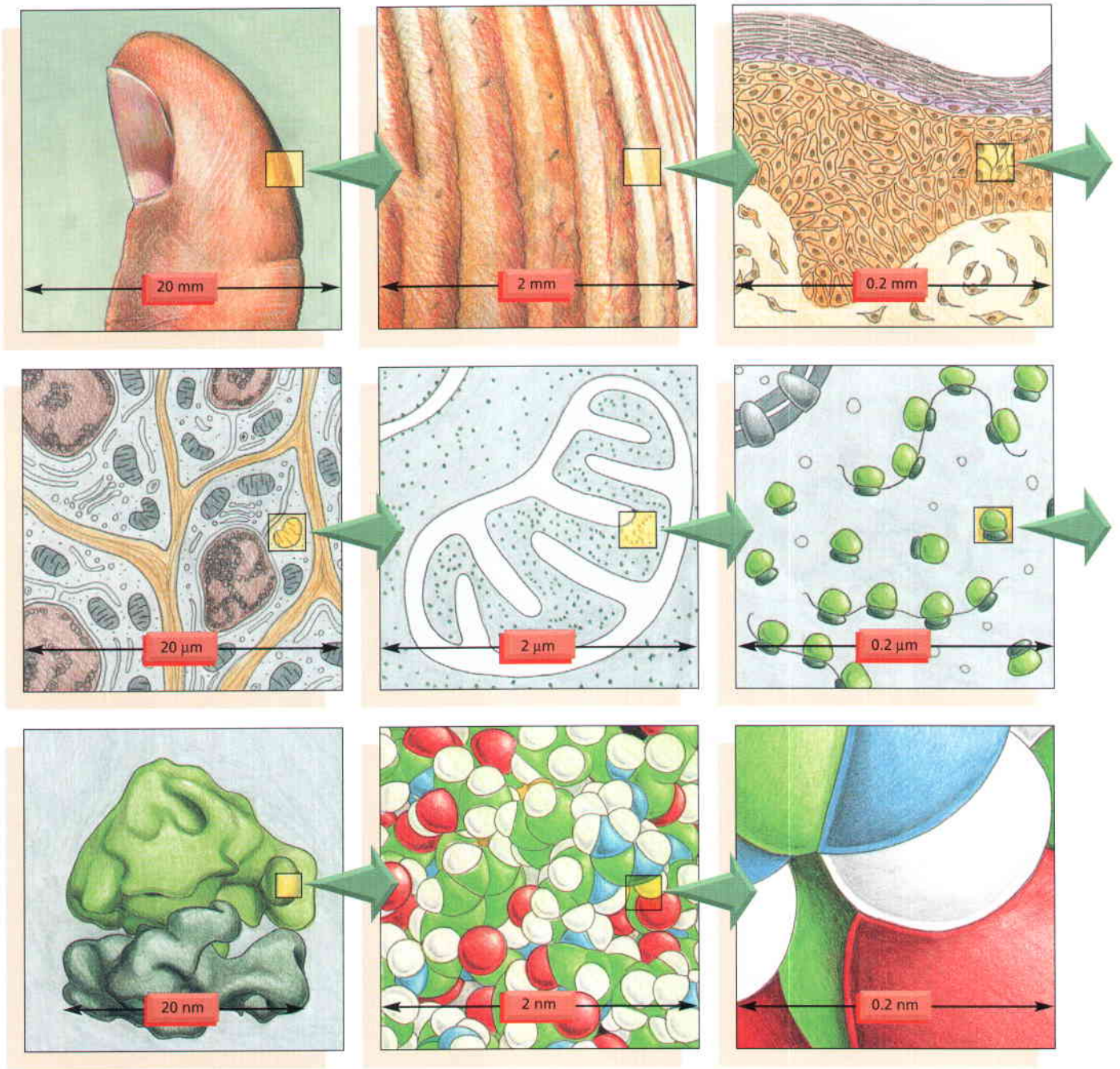
In this chapter, we briefly describe some of the principal microscopy methods used to study cells. Understanding the structural organization of cells is an essential prerequisite for learning how cells function. Optical microscopy will be our starting point because cell biology began with the light microscope, and it is still an essential tool. In recent years optical microscopy has become ever more important, largely owing to the development of methods for the specific labeling and imaging of individual cellular constituents and the reconstruction of their three-dimensional architecture. An important advantage of optical microscopy is that light is relatively nondestructive. By tagging specific cell components with fluorescent probes, such as intrinsically fluorescent proteins, we can thus watch their movement, dynamics, and interactions in living cells. Optical microscopy is limited in resolution by the wavelength of visible light. By using a beam of electrons instead, electron microscopy can image the macromolecular complexes within cells at almost atomic resolution, and in three dimensions.

Although optical microscopy and electron microscopy are important methods, it is what they have enabled scientists to discover about the structural architecture of the cell that makes them interesting. Use this chapter as a reference and read it in conjunction with the later chapters of the book rather than viewing it as an introduction to them.

LOOKING AT CELLS IN THE LIGHT MICROSCOPE

A typical animal cell is 10–20 μm in diameter, which is about one-fifth the size of the smallest particle visible to the naked eye. Only after good light microscopes became available in the early part of the nineteenth century did Schleiden and Schwann propose that all plant and animal tissues were aggregates of individual cells. Their discovery in 1838, known as the **cell doctrine**, marks the formal birth of cell biology.

Animal cells are not only tiny, but they are also colorless and translucent. Consequently, the discovery of their main internal features depended on the development, in the latter part of the nineteenth century, of a variety of stains that provided sufficient contrast to make those features visible. Similarly, the far more powerful electron microscope introduced in the early 1940s required the development of new techniques for preserving and staining cells before the full complexities of their internal fine structure could begin to emerge. To this day, microscopy relies as much on techniques for preparing the specimen as on the performance of the microscope itself. In the following discussions, we therefore consider both instruments and specimen preparation, beginning with the light microscope.



The series of images in Figure 9–1 illustrate an imaginary progression from a thumb to a cluster of atoms. Each successive image represents a tenfold increase in magnification. The naked eye could see features in the first two panels, the resolution of the light microscope would extend to about the fourth panel, and the electron microscope to between about the seventh and eighth panel. Figure 9–2 shows the sizes of various cellular and subcellular structures and the ranges of size that different types of microscopes can visualize.

Figure 9–1 A sense of scale between living cells and atoms. Each diagram shows an image magnified by a factor of ten in an imaginary progression from a thumb, through skin cells, to a ribosome, to a cluster of atoms forming part of one of the many protein molecules in our body. Atomic details of macromolecules, as shown in the last two panels, are usually beyond the power of the electron microscope.

The Light Microscope Can Resolve Details 0.2 µm Apart

A fundamental limitation of all microscopes is that a given type of radiation cannot be used to probe structural details much smaller than its own wavelength. The ultimate limit to the resolution of a light microscope is therefore set by the wavelength of visible light, which ranges from about 0.4 µm (for violet) to 0.7 µm

(for deep red). In practical terms, bacteria and mitochondria, which are about 500 nm (0.5 μm) wide, are generally the smallest objects whose shape we can clearly discern in the **light microscope**; smaller details than this are obscured by effects resulting from the wavelike nature of light. To understand why this occurs, we must follow the path of a beam of light waves as it passes through the lenses of a microscope (**Figure 9–3**).

Because of its wave nature, light does not follow exactly the idealized straight ray paths that geometrical optics predict. Instead, light waves travel through an optical system by several slightly different routes, so that they interfere with one another and cause *optical diffraction* effects. If two trains of waves reaching the same point by different paths are precisely *in phase*, with crest matching crest and trough matching trough, they will reinforce each other so as to increase brightness. In contrast, if the trains of waves are *out of phase*, they will interfere with each other in such a way as to cancel each other partly or entirely (**Figure 9–4**). The interaction of light with an object changes the phase relationships of the light waves in a way that produces complex interference effects. At high magnification, for example, the shadow of an edge that is evenly illuminated with light of uniform wavelength appears as a set of parallel lines (**Figure 9–5**), whereas that of a circular spot appears as a set of concentric rings. For the same reason, a single point seen through a microscope appears as a blurred disc, and two point objects close together give overlapping images and may merge into one. No amount of refinement of the lenses can overcome this limitation imposed by the wavelike nature of light.

The limiting separation at which two objects appear distinct—the so-called **limit of resolution**—depends on both the wavelength of the light and the *numerical aperture* of the lens system used. The numerical aperture is a measure of the width of the entry pupil of the microscope, scaled according to its distance from the object; the wider the microscope opens its eye, so to speak, the more sharply it can see (**Figure 9–6**). Under the best conditions, with violet light

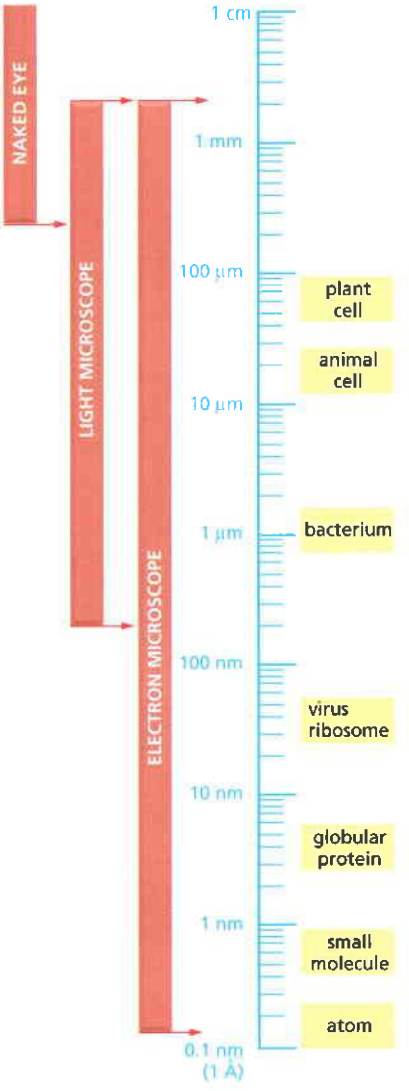


Figure 9–2 Resolving power. Sizes of cells and their components are drawn on a logarithmic scale, indicating the range of objects that can be readily resolved by the naked eye and in the light and electron microscopes. The following units of length are commonly employed in microscopy:
μm (micrometer) = 10^{-6} m
nm (nanometer) = 10^{-9} m
Å (Ångström unit) = 10^{-10} m

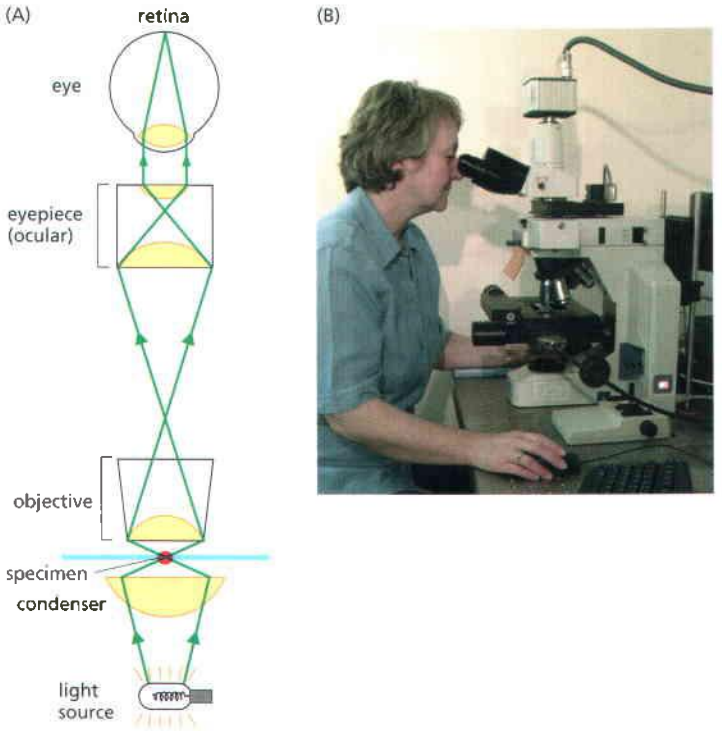


Figure 9–3 A light microscope. (A) Diagram showing the light path in a compound microscope. Light is focused on the specimen by lenses in the condenser. A combination of objective lenses and eyepiece lenses are arranged to focus an image of the illuminated specimen in the eye. (B) A modern research light microscope. (B, courtesy of Andrew Davies.)

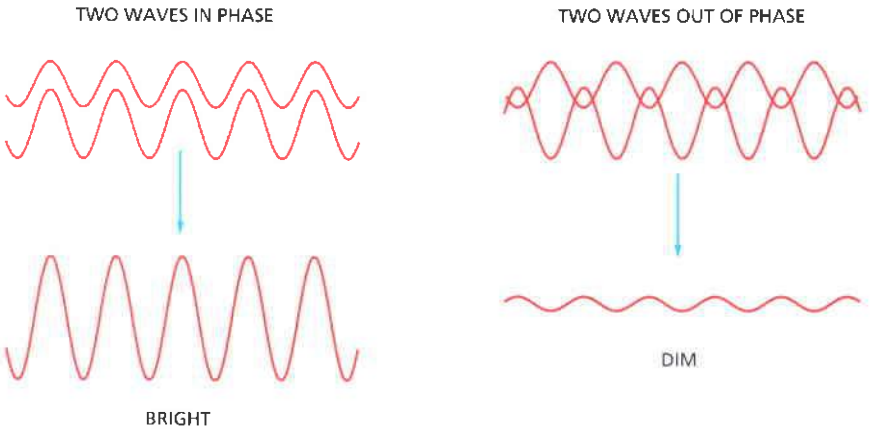


Figure 9–4 Interference between light waves. When two light waves combine in phase, the amplitude of the resultant wave is larger and the brightness is increased. Two light waves that are out of phase cancel each other partly and produce a wave whose amplitude, and therefore brightness, is decreased.

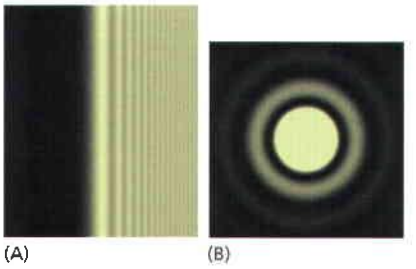


Figure 9–5 Images of an edge and of a point of light. (A) The interference effects, or fringes, seen at high magnification when light of a specific wavelength passes the edge of a solid object placed between the light source and the observer. (B) The image of a point source of light. Diffraction spreads this out into a complex, circular pattern, whose width depends on the numerical aperture of the optical system: the smaller the aperture the bigger (more blurred) the diffracted image. Two point sources can be just resolved when the center of the image of one lies on the first dark ring in the image of the other: this defines the limit of resolution.

(wavelength = 0.4 μm) and a numerical aperture of 1.4, the light microscope can theoretically achieve a limit of resolution of just under 0.2 μm. Microscope makers at the end of the nineteenth century achieved this resolution and it is only rarely matched in contemporary, factory-produced microscopes. Although it is possible to *enlarge* an image as much as we want—for example, by projecting it onto a screen—it is never possible to resolve two objects in the light microscope that are separated by less than about 0.2 μm; they will appear as a single object. Notice the difference between *resolution*, discussed above, and *detection*. If a small object, below the resolution limit, itself emits light, then we may still be able to see or detect it. Thus, we can see a single fluorescently labeled microtubule even though it is about ten times thinner than the resolution limit of the light microscope. Diffraction effects, however, will cause it to appear blurred and at least 0.2 μm thick (see Figure 9–17). Because of the bright light they emit we can detect or see the stars in the night sky, even though they are far below the angular resolution of our unaided eyes. They all appear as similar points of light,

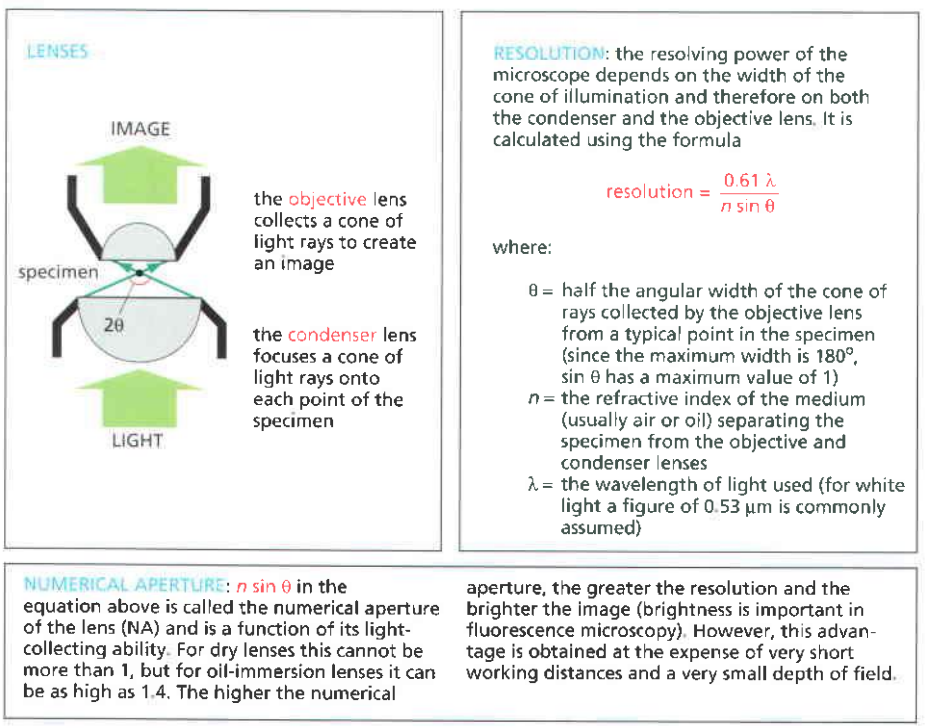


Figure 9–6 Numerical aperture. The path of light rays passing through a transparent specimen in a microscope illustrates the concept of numerical aperture and its relation to the limit of resolution.

differing only in their color or brightness. Using sensitive detection methods, we can detect and follow the behavior of even a single fluorescent protein molecule with a light microscope.

We see next how we can exploit interference and diffraction to study unstained cells in the living state.

Living Cells Are Seen Clearly in a Phase-Contrast or a Differential-Interference-Contrast Microscope

Microscopists have always been challenged by the possibility that some components of the cell may be lost or distorted during specimen preparation. The only certain way to avoid the problem is to examine cells while they are alive, without fixing or freezing. For this purpose, light microscopes with special optical systems are especially useful.

When light passes through a living cell, the phase of the light wave is changed according to the cell's refractive index: a relatively thick or dense part of the cell, such as a nucleus, retards light passing through it. The phase of the light, consequently, is shifted relative to light that has passed through an adjacent thinner region of the cytoplasm. The **phase-contrast microscope** and, in a more complex way, the **differential-interference-contrast microscope** exploit the interference effects produced when these two sets of waves recombine, thereby creating an image of the cell's structure (**Figure 9–7**). Both types of light microscopy are widely used to visualize living cells. <TCAA>

A simpler way to see some of the features of a living cell is to observe the light that is scattered by its various components. In the **dark-field microscope**, the illuminating rays of light are directed from the side so that only scattered light enters the microscope lenses. Consequently, the cell appears as a bright object against a dark background. With a normal **bright-field microscope**, light passing through a cell in culture forms the image directly. **Figure 9–8** compares images of the same cell obtained by four kinds of light microscopy.

Phase-contrast, differential-interference-contrast, and dark-field microscopy make it possible to watch the movements involved in such processes as mitosis and cell migration. Since many cellular motions are too slow to be seen in real time, it is often helpful to make time-lapse movies. Here, the camera records successive frames separated by a short time delay, so that when the resulting picture series is played at normal speed, events appear greatly speeded up.

Images Can Be Enhanced and Analyzed by Digital Techniques

In recent years electronic, or digital, imaging systems, and the associated technology of **image processing**, have had a major impact on light microscopy. Certain practical limitations of microscopes, relating to imperfections in the optical

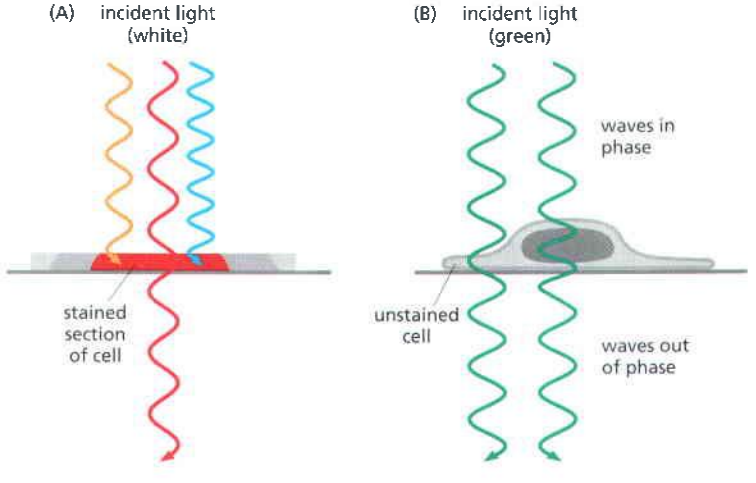


Figure 9–7 Two ways to obtain contrast in light microscopy. (A) The stained portion of the cell will absorb light of some wavelengths, which depend on the stain, but will allow other wavelengths to pass through it. A colored image of the cell is thereby obtained that is visible in the normal bright-field light microscope. (B) Light passing through the unstained, living cell experiences very little change in amplitude, and the structural details cannot be seen even if the image is highly magnified. The phase of the light, however, is altered by its passage through either thicker or denser parts of the cell, and small phase differences can be made visible by exploiting interference effects using a phase-contrast or a differential-interference-contrast microscope.

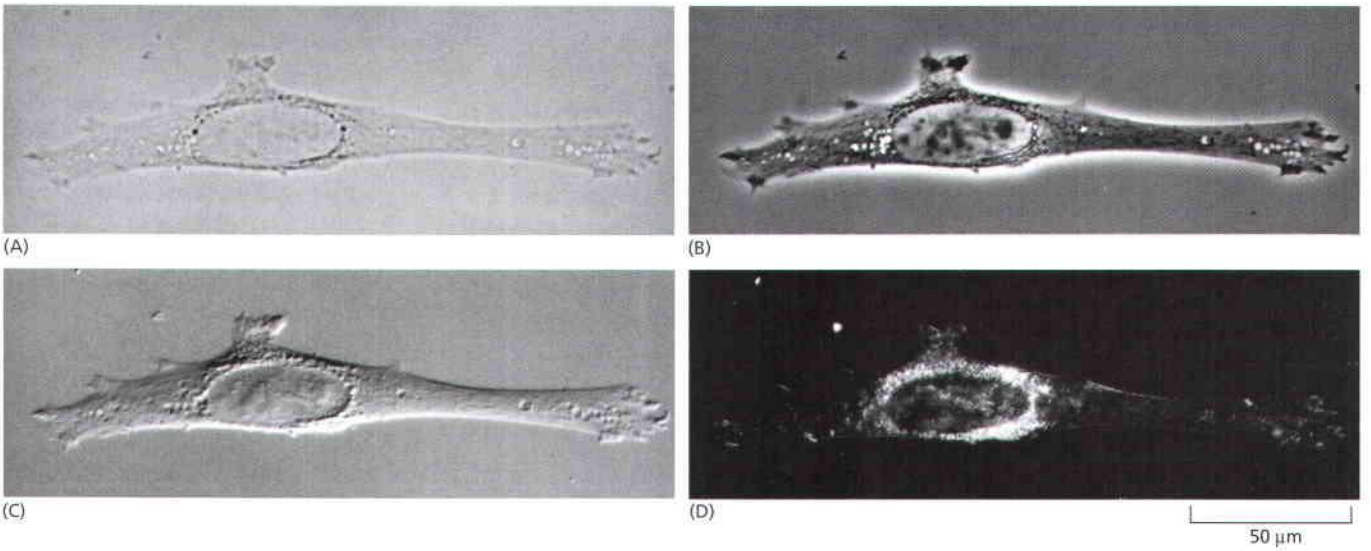


Figure 9–8 Four types of light microscopy. Four images are shown of the same fibroblast cell in culture. All images can be obtained with most modern microscopes by interchanging optical components. (A) Bright-field microscopy. (B) Phase-contrast microscopy. (C) Nomarski differential-interference-contrast microscopy. (D) Dark-field microscopy.

system have been largely overcome. Electronic imaging systems have also circumvented two fundamental limitations of the human eye: the eye cannot see well in extremely dim light, and it cannot perceive small differences in light intensity against a bright background. To increase our ability to observe cells in low light conditions, we can attach a sensitive digital camera to a microscope. These cameras contain a charge-coupled device (CCD), similar to those found in consumer digital cameras. Such CCD cameras are often cooled to reduce image noise. It is then possible to observe cells for long periods at very low light levels, thereby avoiding the damaging effects of prolonged bright light (and heat). Such low-light cameras are especially important for viewing fluorescent molecules in living cells, as explained below.

Because images produced by CCD cameras are in electronic form, they can be readily digitized, fed to a computer, and processed in various ways to extract latent information. Such image processing makes it possible to compensate for various optical faults in microscopes to attain the theoretical limit of resolution. Moreover, by digital image processing, contrast can be greatly enhanced to overcome the eye's limitations in detecting small differences in light intensity. Although this processing also enhances the effects of random background irregularities in the optical system, digitally subtracting an image of a blank area of the field removes such defects. This procedure reveals small transparent objects that were previously impossible to distinguish from the background.

The high contrast attainable by computer-assisted differential-interference-contrast microscopy makes it possible to see even very small objects such as single microtubules (**Figure 9–9**), which have a diameter of 0.025 μm, less than one-tenth the wavelength of light. Individual microtubules can also be seen in a fluorescence microscope if they are fluorescently labeled (see Figure 9–15). In both cases, however, the unavoidable diffraction effects badly blur the image so that the microtubules appear at least 0.2 μm wide, making it impossible to distinguish a single microtubule from a bundle of several microtubules.

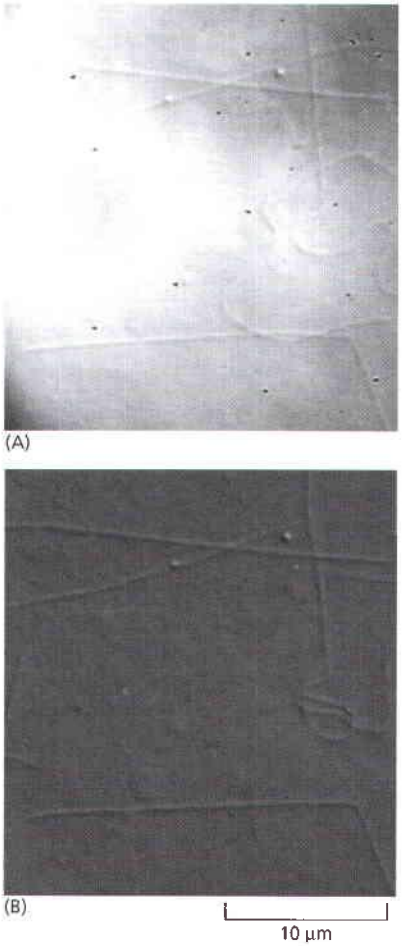


Figure 9–9 Image processing. (A) Unstained microtubules are shown here in an unprocessed digital image, captured using differential-interference-contrast microscopy. (B) The image has now been processed, first by digitally subtracting the unevenly illuminated background, and second by digitally enhancing the contrast. The result of this image processing is a picture that is easier to interpret. Note that the microtubules are dynamic and some have changed length or position between the before-and-after images. (Courtesy of Viki Allan.)

Intact Tissues Are Usually Fixed and Sectioned before Microscopy

Because most tissue samples are too thick for their individual cells to be examined directly at high resolution, they must be cut into very thin transparent slices, or *sections*. To first immobilize, kill, and preserve the cells within the tissue they must be treated with a *fixative*. Common fixatives include formaldehyde and glutaraldehyde, which form covalent bonds with the free amino groups of proteins, cross-linking them so they are stabilized and locked into position.

Because tissues are generally soft and fragile, even after fixation, they need to be embedded in a supporting medium before sectioning. The usual embedding media are waxes or resins. In liquid form these media both permeate and surround the fixed tissue; they can then be hardened (by cooling or by polymerization) to form a solid block, which is readily sectioned with a microtome. This is a machine with a sharp blade that operates like a meat slicer (**Figure 9–10**). The sections (typically 1–10 μm thick) are then laid flat on the surface of a glass microscope slide.

There is little in the contents of most cells (which are 70% water by weight) to impede the passage of light rays. Thus, most cells in their natural state, even if fixed and sectioned, are almost invisible in an ordinary light microscope. There are three main approaches to working with thin tissue sections that reveal the cells themselves or specific components within them.

First, and traditionally, sections can be stained with organic dyes that have some specific affinity for particular subcellular components. The dye *hematoxylin*, for example, has an affinity for negatively charged molecules and therefore reveals the distribution of DNA, RNA, and acidic proteins in a cell (**Figure 9–11**). The chemical basis for the specificity of many dyes, however, is not known.

Second, sectioned tissues can be used to visualize specific patterns of differential gene expression. *In situ* hybridization, discussed earlier (p. 573), reveals the cellular distribution and abundance of specific expressed RNA molecules in sectioned material or in whole mounts of small organisms or organs (**Figure 9–12**). A third and very sensitive approach, generally and widely applicable for localizing proteins of interest, depends on using fluorescent probes and markers, as we explain next.

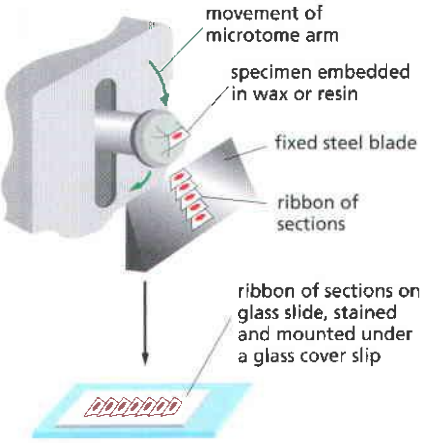


Figure 9–10 Making tissue sections. This illustration shows how an embedded tissue is sectioned with a microtome in preparation for examination in the light microscope.

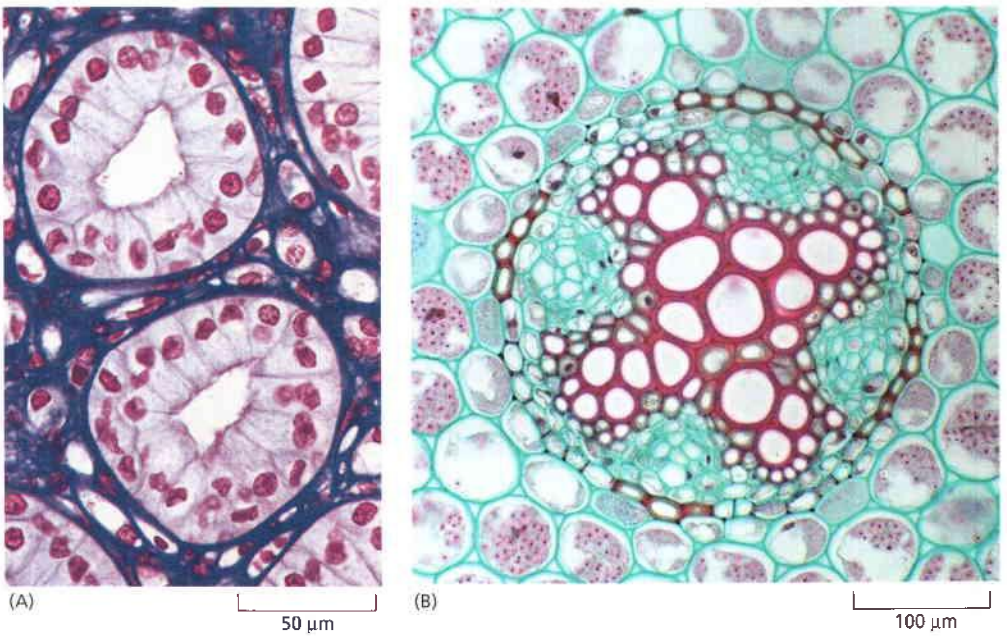


Figure 9–11 Staining of cellular components. (A) This section of cells in the urine-collecting ducts of the kidney was stained with a combination of dyes, hematoxylin and eosin, commonly used in histology. Each duct is made of closely packed cells (with nuclei stained *red)* that form a ring. The ring is surrounded by extracellular matrix, stained *purple*. (B) This section of a young plant root is stained with two dyes, safranin and fast green. The fast green stains the cellulosic cell walls while the safranin stains the lignified xylem cell walls bright red. (A, from P.R. Wheater et al., Functional Histology, 2nd ed. London: Churchill Livingstone, 1987; B, courtesy of Stephen Grace.)

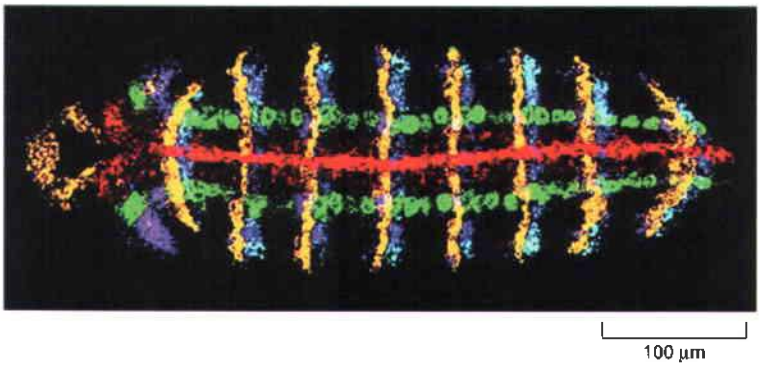


Figure 9–12 RNA *in situ* hybridization. As described in chapter 8 (see Figure 8–71), it is possible to visualize the distribution of different RNAs in tissues using *in situ* hybridization. Here, the transcription pattern of five different genes involved in patterning the early fly embryo is revealed in a single embryo. Each RNA probe has been fluorescently labeled in a different way, some directly and some indirectly, and the resulting images false-colored and combined to see each individual transcript most clearly. The genes whose expression pattern is revealed here are *wingless (yellow)*, *engrailed (blue)*, *short gastrulation (red)*, *intermediate neuroblasts defective (green)*, and *muscle specific homeobox (purple)*. (From D. Kosman et al., *Science* 305:846, 2004. With permission from AAAS.)

Specific Molecules Can Be Located in Cells by Fluorescence Microscopy

Fluorescent molecules absorb light at one wavelength and emit it at another, longer wavelength. If we illuminate such a compound at its absorbing wavelength and then view it through a filter that allows only light of the emitted wavelength to pass, it will glow against a dark background. Because the background is dark, even a minute amount of the glowing fluorescent dye can be detected. The same number of molecules of an ordinary stain viewed conventionally would be practically invisible because the molecules would give only the faintest tinge of color to the light transmitted through this stained part of the specimen.

The fluorescent dyes used for staining cells are visualized with a **fluorescence microscope**. This microscope is similar to an ordinary light microscope except that the illuminating light, from a very powerful source, is passed through two sets of filters—one to filter the light before it reaches the specimen and one to filter the light obtained from the specimen. The first filter passes only the wavelengths that excite the particular fluorescent dye, while the second filter blocks out this light and passes only those wavelengths emitted when the dye fluoresces (**Figure 9–13**).

Fluorescence microscopy is most often used to detect specific proteins or other molecules in cells and tissues. A very powerful and widely used technique is to couple fluorescent dyes to antibody molecules, which then serve as highly specific and versatile staining reagents that bind selectively to the particular macromolecules they recognize in cells or in the extracellular matrix. Two fluorescent dyes that have been commonly used for this purpose are *fluorescein*, which emits an intense green fluorescence when excited with blue light, and

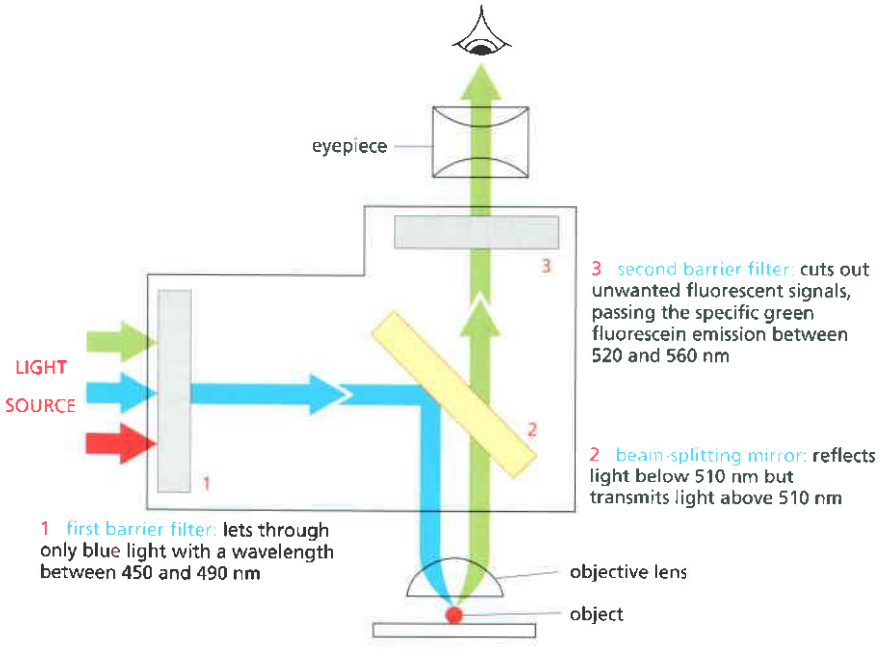


Figure 9–13 The optical system of a fluorescence microscope. A filter set consists of two barrier filters (1 and 3) and a dichroic (beam-splitting) mirror (2). This example shows the filter set for detection of the fluorescent molecule fluorescein. High-numerical-aperture objective lenses are especially important in this type of microscopy because, for a given magnification, the brightness of the fluorescent image is proportional to the fourth power of the numerical aperture (see also Figure 9–6).

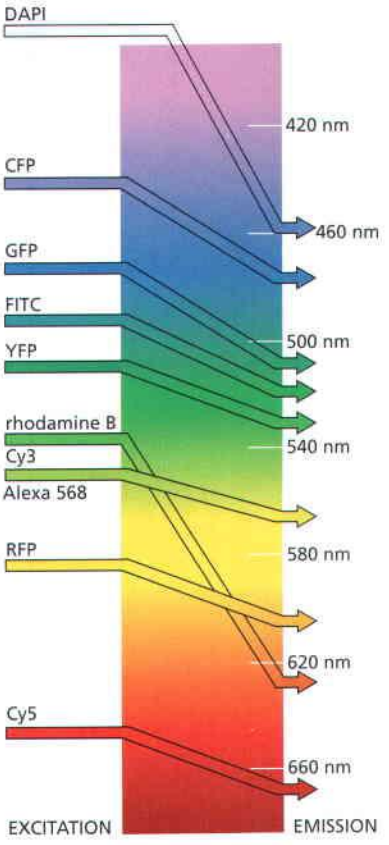


Figure 9–14 Fluorescent probes. The maximum excitation and emission wavelengths of several commonly used fluorescent probes are shown in relation to the corresponding colors of the spectrum. The photon emitted by a fluorescent molecule is necessarily of lower energy (longer wavelength) than the photon absorbed and this accounts for the difference between the excitation and emission peaks. CFP, GFP, YFP and RFP are cyan, green, yellow and red fluorescent proteins respectively. These are not dyes, and are discussed in detail later in the chapter. DAPI is widely used as a general fluorescent DNA probe, which absorbs UV light and fluoresces bright blue. FITC is an abbreviation for fluorescence isothiocyanate, a widely used derivative of fluorescein, which fluoresces bright green. The other probes are all commonly used to fluorescently label antibodies and other proteins.

rhodamine, which emits a deep red fluorescence when excited with green–yellow light (**Figure 9–14**). By coupling one antibody to fluorescein and another to rhodamine, the distributions of different molecules can be compared in the same cell; the two molecules are visualized separately in the microscope by switching back and forth between two sets of filters, each specific for one dye. As shown in **Figure 9–15**, three fluorescent dyes can be used in the same way to distinguish between three types of molecules in the same cell. Many newer fluorescent dyes, such as Cy3, Cy5, and the Alexa dyes, have been specifically developed for fluorescence microscopy (see Figure 9–14). These organic fluorochromes have some disadvantages. They are excited only by light of precise, but different, wavelengths, and additionally they fade fairly rapidly when continuously illuminated. More stable inorganic fluorochromes have recently been developed, however. Tiny crystals of semiconductor material, called nanoparticles, or *quantum dots*, can all be excited to fluoresce by a broad spectrum of blue light. Their emitted light has a color that depends on the exact size of the nanocrystal, between 2 and 10 nm in diameter, and additionally the fluorescence fades only slowly with time (**Figure 9–16**). These nanoparticles, when coupled to other probes such as antibodies, are therefore ideal for tracking molecules over time. If introduced into a living cell, in an embryo for example, the progeny of that cell can be followed many days later by their fluorescence, allowing cell lineages to be tracked.

Fluorescence microscopy methods, discussed later in the chapter, can be used to monitor changes in the concentration and location of specific molecules inside *living* cells (see p. 592).

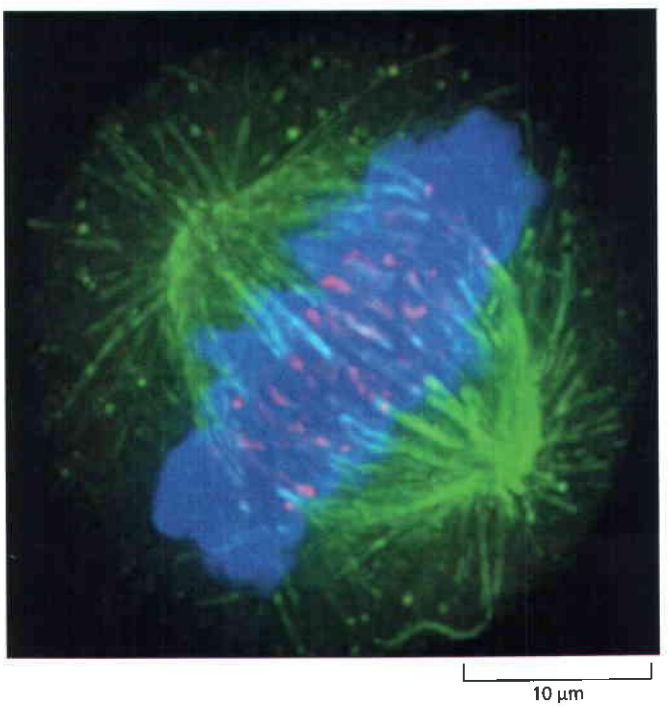


Figure 9–15 Multiple-fluorescent-probe microscopy. In this composite micrograph of a cell in mitosis, three different fluorescent probes have been used to stain three different cellular components. <GTCT> The spindle microtubules are revealed with a *green* fluorescent antibody, centromeres with a *red* fluorescent antibody and the DNA of the condensed chromosomes with the *blue* fluorescent dye DAPI. (Courtesy of Kevin F. Sullivan.)

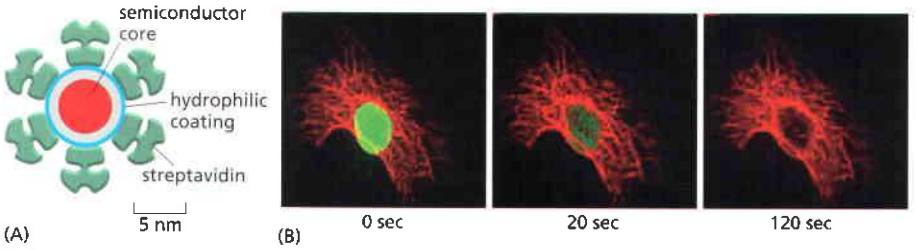


Figure 9–16 Fluorescent nanoparticles or quantum dots. Quantum dots are tiny nanoparticles of cadmium selenide, a semiconductor, with a coating to make them water-soluble (A). They can be coupled to protein probes such as antibodies or streptavidin and, when introduced into a cell, will bind to a protein of interest. Different-sized quantum dots emit light of different colors—the larger the dot the longer the wavelength—but they are all excited by the same blue light. (B) Quantum dots can keep shining for weeks, unlike most fluorescent organic dyes. In this cell, a nuclear protein is labeled *(green)* with an organic fluorescent dye (Alexa 488), while microtubules are stained *(red)* with quantum dots bound to streptavidin. On continuous exposure to blue light the fluorescent dye fades quickly while the quantum dots continue to fluoresce. (B, from X. Wu et al., *Nat. Biotechnol.* 21:41–46, 2003. With permission from Macmillan Publishers Ltd.)

Antibodies Can Be Used to Detect Specific Molecules

Antibodies are proteins produced by the vertebrate immune system as a defense against infection (discussed in Chapter 24). They are unique among proteins because they are made in billions of different forms, each with a different binding site that recognizes a specific target molecule (or *antigen*). The precise antigen specificity of antibodies makes them powerful tools for the cell biologist. When labeled with fluorescent dyes, antibodies are invaluable for locating specific molecules in cells by fluorescence microscopy (**Figure 9–17**); labeled with electron-dense particles such as colloidal gold spheres, they are used for similar purposes in the electron microscope (discussed below).

When we use antibodies as probes to detect and assay specific molecules in cells we frequently amplify the fluorescent signal they produce by chemical methods. For example, although a marker molecule such as a fluorescent dye can be linked directly to an antibody used for specific recognition—the *primary antibody*—a stronger signal is achieved by using an unlabeled primary antibody and then detecting it with a group of labeled *secondary antibodies* that bind to it (**Figure 9–18**). This process is called *indirect immunocytochemistry.*

The most sensitive amplification methods use an enzyme as a marker molecule attached to the secondary antibody. The enzyme alkaline phosphatase, for example, in the presence of appropriate chemicals, produces inorganic phosphate that in turn leads to the local formation of a colored precipitate. This reveals the location of the secondary antibody and hence the location of the antibody–antigen complex. Since each enzyme molecule acts catalytically to generate many thousands of molecules of product, even tiny amounts of antigen can be detected. An enzyme-linked immunosorbent assay (ELISA) based on this principle is frequently used in medicine as a sensitive test—for pregnancy or for various types of infections, for example. Although the enzyme amplification makes enzyme-linked methods very sensitive, diffusion of the colored precipitate away from the enzyme limits the spatial resolution of this method for

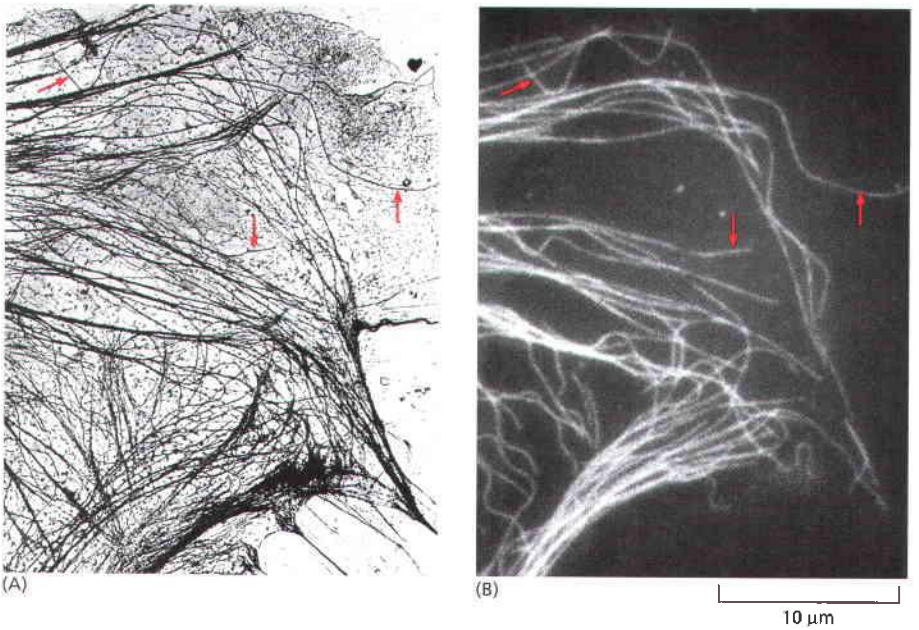


Figure 9–17 Immunofluorescence. (A) A transmission electron micrograph of the periphery of a cultured epithelial cell showing the distribution of microtubules and other filaments. (B) The same area stained with fluorescent antibodies against tubulin, the protein that assembles to form microtubules, using the technique of indirect immunocytochemistry (see Figure 9–18). *Red arrows* indicate individual microtubules that are readily recognizable in both images. Note that, because of diffraction effects, the microtubules in the light microscope appear 0.2 μm wide rather than their true width of 0.025 μm. (From M. Osborn, R. Webster and K. Weber, *J. Cell Biol.* 77:R27–R34, 1978. With permission from The Rockefeller University Press.)

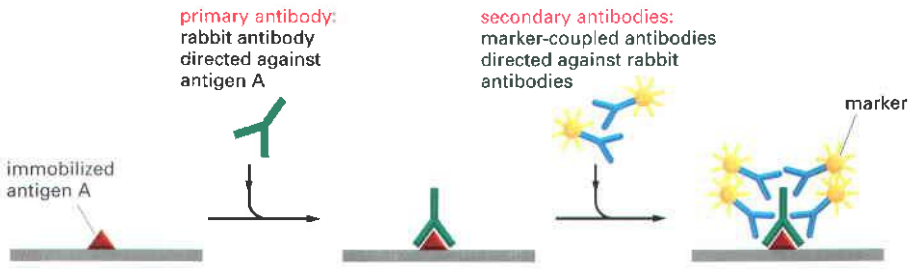


Figure 9–18 Indirect immunocytochemistry. This detection method is very sensitive because many molecules of the secondary antibody recognize each primary antibody. The secondary antibody is covalently coupled to a marker molecule that makes it readily detectable. Commonly used marker molecules include fluorescent dyes (for fluorescence microscopy), the enzyme horseradish peroxidase (for either conventional light microscopy or electron microscopy), colloidal gold spheres (for electron microscopy), and the enzymes alkaline phosphatase or peroxidase (for biochemical detection).

microscopy, and fluorescent labels are usually used for the most precise optical localization.

Antibodies are made most simply by injecting a sample of the antigen several times into an animal such as a rabbit or a goat and then collecting the antibody-rich serum. This *antiserum* contains a heterogeneous mixture of antibodies, each produced by a different antibody-secreting cell (a B lymphocyte). The different antibodies recognize various parts of the antigen molecule (called an antigenic determinant, or epitope), as well as impurities in the antigen preparation. Removing the unwanted antibody molecules that bind to other molecules sharpens the specificity of an antiserum for a particular antigen; an antiserum produced against protein X, for example, when passed through an affinity column of antigens X, will bind to these antigens, allowing other antibodies to pass through the column. Purified anti-X antibody can subsequently be eluted from the column. Even so, the heterogeneity of such antisera sometimes limits their usefulness. The use of monoclonal antibodies largely overcomes this problem (see Figure 8–8). However, monoclonal antibodies can also have problems. Since they are single-antibody protein species, they show almost perfect specificity for a single site or epitope on the antigen, but the accessibility of the epitope, and thus the usefulness of the antibody, may depend on the specimen preparation. For example, some monoclonal antibodies will react only with unfixed antigens, others only after the use of particular fixatives, and still others only with proteins denatured on SDS polyacrylamide gels, and not with the proteins in their native conformation.

Imaging of Complex Three-Dimensional Objects Is Possible with the Optical Microscope

For ordinary light microscopy, as we have seen, a tissue has to be sliced into thin sections to be examined; the thinner the section, the crisper the image. The process of sectioning loses information about the third dimension. How, then, can we get a picture of the three-dimensional architecture of a cell or tissue, and how can we view the microscopic structure of a specimen that, for one reason or another, cannot first be sliced into sections? Although an optical microscope is focused on a particular focal plane within complex three-dimensional specimens, all the other parts of the specimen, above and below the plane of focus, are also illuminated and the light originating from these regions contributes to the image as "out-of-focus" blur. This can make it very hard to interpret the image in detail and can lead to fine image structure being obscured by the out-of-focus light.

Two distinct but complementary approaches solve this problem: one is computational, the other is optical. These three-dimensional microscopic imaging methods make it possible to focus on a chosen plane in a thick specimen while rejecting the light that comes from out-of-focus regions above and below that plane. Thus one sees a crisp, thin *optical section*. From a series of such optical sections taken at different depths and stored in a computer, it is easy to reconstruct a three-dimensional image. The methods do for the microscopist what the CT scanner does (by different means) for the radiologist investigating a human body: both machines give detailed sectional views of the interior of an intact structure.

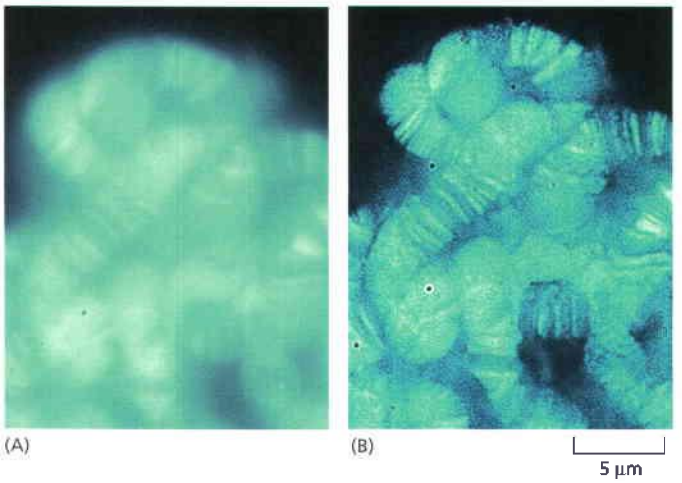


Figure 9–19 Image deconvolution. (A) A light micrograph of the large polytene chromosomes from *Drosophi* stained with a fluorescent DNA-bindin dye. (B) The same field of view after image deconvolution clearly reveals th banding pattern on the chromosomes Each band is about 0.25 µm thick, approaching the resolution limit of th light microscope. (Courtesy of the Joh Sedat Laboratory.)

The computational approach is often called *image deconvolution.* To understand how it works, remember that the wavelike nature of light means that the microscope lens system produces a small blurred disc as the image of a point light source (see Figure 9–5), with increased blurring if the point source lies above or below the focal plane. This blurred image of a point source is called the *point spread function.* An image of a complex object can then be thought of as being built up by replacing each point of the specimen by a corresponding blurred disc, resulting in an image that is blurred overall. For deconvolution, we first obtain a series of (blurred) images, usually with a cooled CCD camera, focusing the microscope in turn on a series of focal planes—in effect, a (blurred) three-dimensional image. The stack of digital images is then processed by computer to remove as much of the blur as possible. Essentially the computer program uses the microscope's point spread function to determine what the effect of the blurring would have been on the image, and then applies an equivalent "deblurring" (deconvolution), turning the blurred three-dimensional image into a series of clean optical sections. The computation required is quite complex, and used to be a serious limitation. However, with faster and cheaper computers, the image deconvolution method is gaining in power and popularity. **Figure 9–19** shows an example.

The Confocal Microscope Produces Optical Sections by Excluding Out-of-Focus Light

The confocal microscope achieves a result similar to that of deconvolution, but does so by manipulating the light before it is measured; thus it is an analog technique rather than a digital one. The optical details of the **confocal microscope** are complex, but the basic idea is simple, as illustrated in **Figure 9–20**, and the results are far superior to those obtained by conventional light microscopy (**Figure 9–21**).

The microscope is generally used with fluorescence optics (see Figure 9–13), but instead of illuminating the whole specimen at once, in the usual way, the optical system at any instant focuses a spot of light onto a single point at a specific depth in the specimen. It requires a very bright source of pinpoint illumination that is usually supplied by a laser whose light has been passed through a pinhole. The fluorescence emitted from the illuminated material is collected and brought to an image at a suitable light detector. A pinhole aperture is placed in front of the detector, at a position that is *confocal* with the illuminating pinhole—that is, precisely where the rays emitted from the illuminated point in the specimen come to a focus. Thus, the light from this point in the specimen converges on this aperture and enters the detector.

By contrast, the light from regions out of the plane of focus of the spotlight is also out of focus at the pinhole aperture and is therefore largely excluded from the detector (see Figure 9–20). To build up a two-dimensional image, data from

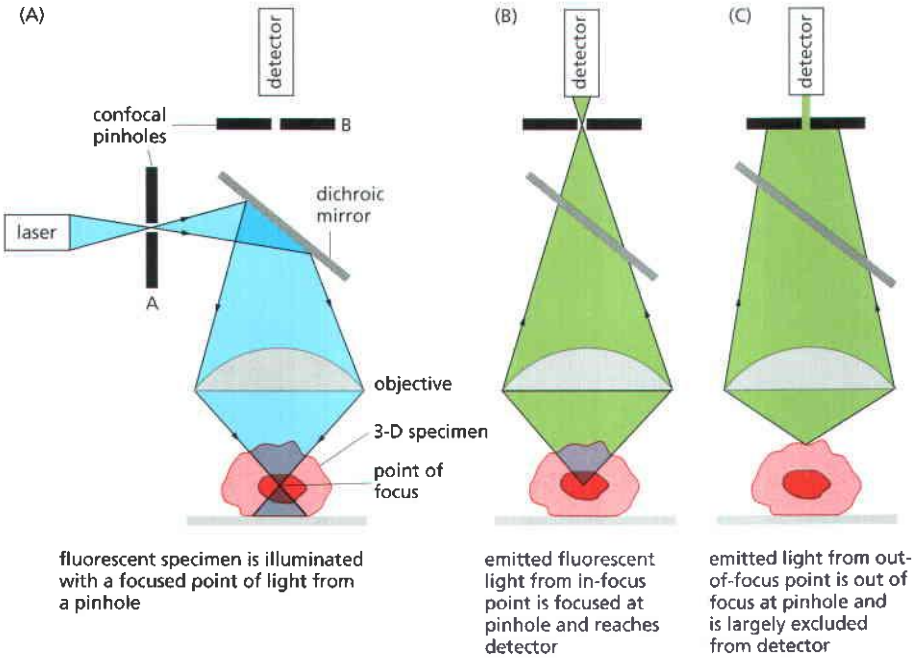


Figure 9–20 The confocal fluorescence microscope. This simplified diagram shows that the basic arrangement of optical components is similar to that of the standard fluorescence microscope shown in Figure 9–13, except that a laser is used to illuminate a small pinhole whose image is focused at a single point in the specimen (A). Emitted fluorescence from this focal point in the specimen is focused at a second (confocal) pinhole (B). Emitted light from elsewhere in the specimen is not focused at the pinhole and therefore does not contribute to the final image (C). By scanning the beam of light across the specimen, a very sharp two-dimensional image of the exact plane of focus is built up that is not significantly degraded by light from other regions of the specimen.

each point in the plane of focus are collected sequentially by scanning across the field in a raster pattern (as on a television screen) and are displayed on a video screen. Although not shown in Figure 9–20, the scanning is usually done by deflecting the beam with an oscillating mirror placed between the dichroic mirror and the objective lens in such a way that the illuminating spotlight and the confocal pinhole at the detector remain strictly in register.

The confocal microscope has been used to resolve the structure of numerous complex three-dimensional objects (**Figure 9–22**), including the networks of cytoskeletal fibers in the cytoplasm and the arrangements of chromosomes and genes in the nucleus.

The relative merits of deconvolution methods and confocal microscopy for three-dimensional optical microscopy are still the subject of debate. Confocal microscopes are generally easier to use than deconvolution systems and the final optical sections can be seen quickly. In contrast, the cooled CCD (charge-coupled device) cameras used for deconvolution systems are extremely efficient at collecting small amounts of light, and they can be used to make detailed three-dimensional images from specimens that are too weakly stained or too easily damaged by the bright light used for confocal microscopy.

Both methods, however, have another drawback; neither is good at coping with thick specimens. Deconvolution methods quickly become ineffective any deeper than about 40 μm into a specimen, while confocal microscopes can only

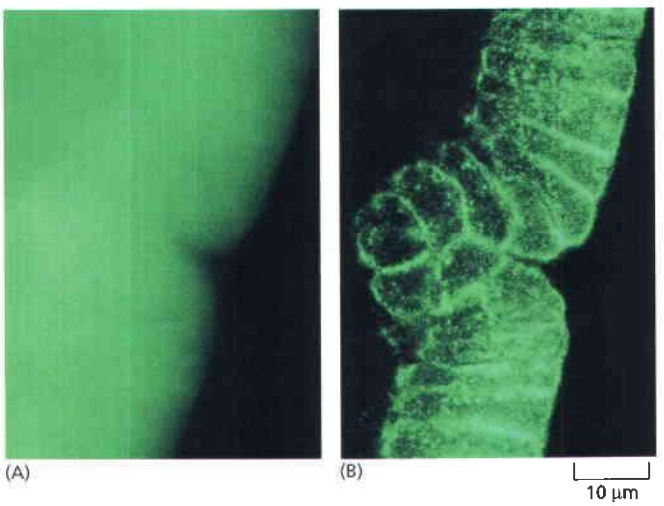


Figure 9–21 Conventional and confocal fluorescence microscopy compared. These two micrographs are of the same intact gastrula-stage *Drosophila* embryo that has been stained with a fluorescent probe for actin filaments. (A) The conventional, unprocessed image is blurred by the presence of fluorescent structures above and below the plane of focus. (B) In the confocal image, this out-of-focus information is removed, resulting in a crisp optical section of the cells in the embryo. (Courtesy of Richard Warn and Peter Shaw.)

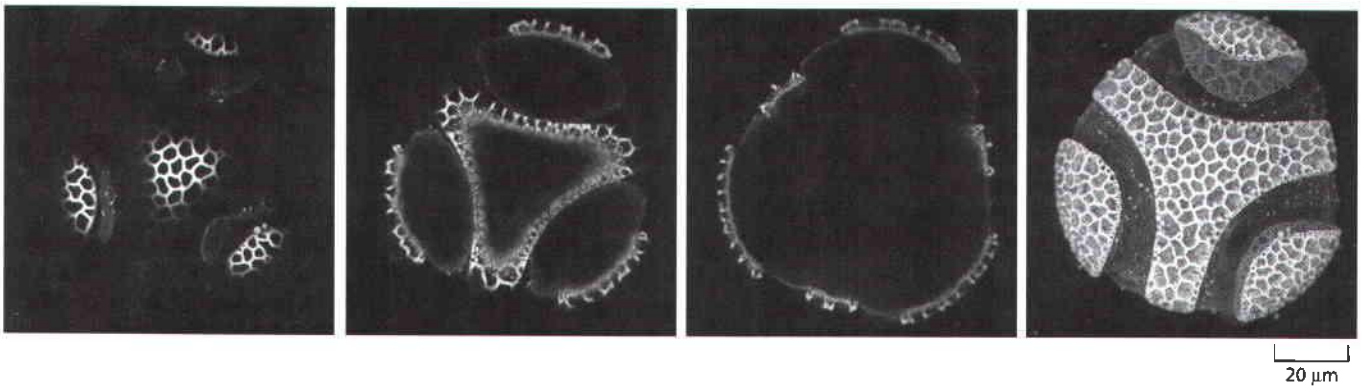


Figure 9–22 Three-dimensional reconstruction from confocal microscope images. Pollen grains, in this case from a passion flower, have a complex sculptured cell wall that contains fluorescent compounds. Images obtained at different depths through the grain, using a confocal microscope, can be recombined to give a three-dimensional view of the whole grain, shown on the right. Three selected individual optical sections from the full set of 30, each of which shows little contribution from its neighbors, are shown on the left. (Courtesy of Brad Amos.)

obtain images up to a depth of about 150 μm. Special confocal microscopes can now take advantage of the way in which fluorescent molecules are excited, to probe even deeper into a specimen. Fluorescent molecules are usually excited by a single high-energy photon, of shorter wavelength than the emitted light, but they can in addition be excited by the absorption of two (or more) photons of lower energy, as long as they both arrive within a femtosecond or so of each other. The use of this longer-wavelength excitation has some important advantages. In addition to reducing background noise, red or near infrared light can penetrate deeper within a specimen. Multiphoton confocal microscopes, constructed to take advantage of this "**two-photon**" effect, can typically obtain sharp images even at a depth of 0.5 mm within a specimen. This is particularly valuable for studies of living tissues, notably in imaging the dynamic activity of synapses and neurons just below the surface of living brains (**Figure 9–23**).

Fluorescent Proteins Can Be Used to Tag Individual Proteins in Living Cells and Organisms

Even the most stable cellular structures must be assembled, disassembled, and reorganized during the cell's life cycle. Other structures, often enormous on the molecular scale, rapidly change, move, and reorganize themselves as the cell conducts its internal affairs and responds to its environment. Complex, highly organized pieces of molecular machinery move components around the cell, controlling traffic into and out of the nucleus, from one organelle to another, and into and out of the cell itself.

Various techniques have been developed to make specific components of living cells visible in the microscope. Most of these methods use fluorescent proteins, and they require a trade-off between structural preservation and efficient labeling. All of the fluorescent molecules discussed so far are made outside the cell and then artificially introduced into it. Now new opportunities have been opened up by the discovery of genes coding for protein molecules that are themselves inherently fluorescent. Genetic engineering then enables the creation of lines of cells or organisms that make their own visible tags and labels, without the introduction of foreign molecules. These cellular exhibitionists display their inner workings in glowing fluorescent color.

Foremost among the fluorescent proteins used for these purposes by cell biologists is the **green fluorescent protein** (**GFP**), isolated from the jellyfish *Aequoria victoria*. This protein is encoded in the normal way by a single gene that can be cloned and introduced into cells of other species. The freshly translated protein is not fluorescent, but within an hour or so (less for some alleles of

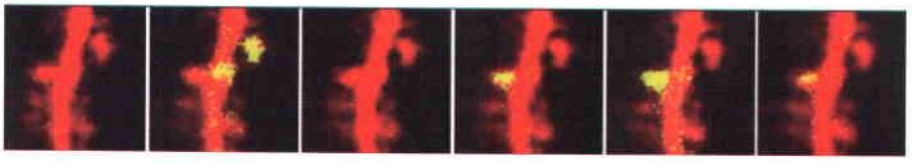


Figure 9–23 Multi-photon imaging. Infrared laser light causes less damage to living cells and can also penetrate further, allowing microscopists to peer deeper into living tissues. The two-photon effect, in which a fluorochrome can be excited by two coincident infrared photons instead of a single high-energy photon, allows us to see nearly 0.5 mm inside the cortex of a live mouse brain. A dye, whose fluorescence changes with the calcium concentration, reveals active synapses *(yellow)* on the dendritic spines *(red)* that change as a function of time. (Courtesy of Karel Svoboda.)

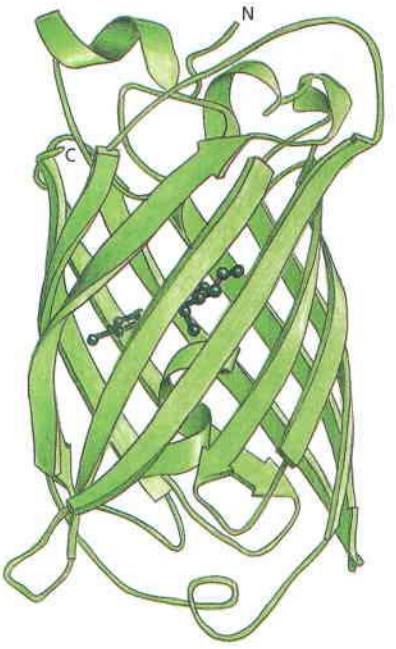


Figure 9–24 Green fluorescent protein (GFP). The structure of GFP, shown here schematically, highlights the eleven β strands that form the staves of a barrel. Buried within the barrel is the active chromophore *(dark green)* that is formed post-translationally from the protruding side chains of three amino acid residues. (Adapted from M. Ormö et al., *Science* 273:1392–1395, 1996. With permission from AAAS.)

the gene, more for others) it undergoes a self-catalyzed post-translational modification to generate an efficient and bright fluorescent center, shielded within the interior of a barrel-like protein (**Figure 9–24**). Extensive site-directed mutagenesis performed on the original gene sequence has resulted in useful fluorescence in organisms ranging from animals and plants to fungi and microbes. The fluorescence efficiency has also been improved, and variants have been generated with altered absorption and emission spectra in the blue–green–yellow range. Recently a family of related fluorescent proteins discovered in corals, has extended the range into the red region of the spectrum (see Figure 9–14).

One of the simplest uses of GFP is as a reporter molecule, a fluorescent probe to monitor gene expression. A transgenic organism can be made with the GFP-coding sequence placed under the transcriptional control of the promoter belonging to a gene of interest, giving a directly visible readout of the gene's expression pattern in the living organism (**Figure 9–25**). In another application, a peptide location signal can be added to the GFP to direct it to a particular cellular compartment, such as the endoplasmic reticulum or a mitochondrion, lighting up these organelles so they can be observed in the living state (see Figure 12–35B).

The GFP DNA-coding sequence can also be inserted at the beginning or end of the gene for another protein, yielding a chimeric product consisting of that protein with a GFP domain attached. In many cases, this GFP-fusion protein behaves in the same way as the original protein, directly revealing its location and activities by means of its genetically encoded contrast (**Figure 9–26**). <TAAT> It is often possible to prove that the GFP-fusion protein is functionally equivalent to the untagged protein, for example by using it to rescue a mutant lacking that protein. GFP tagging is the clearest and most unequivocal way of showing the distribution and dynamics of a protein in a living organism (**Figure 9–27**). <TTCT>

Protein Dynamics Can Be Followed in Living Cells

Fluorescent proteins are now exploited, not just to see where in a cell a particular protein is located, but also to uncover its kinetic properties and to find out whether it might interact with other proteins. We now describe three techniques in which GFP and its relatives are used in this way.

The first is the monitoring of interactions between one protein and another by **fluorescence resonance energy transfer (FRET)**. In this technique, whose principles have been described earlier (see Figure 8–26), the two molecules of interest are each labeled with a different fluorochrome, chosen so that the emission spectrum of one fluorochrome overlaps with the absorption spectrum of the other. If the two proteins bind so as to bring their fluorochromes into very close proximity (closer than about 5 nm), one fluorochrome transfers the energy of the absorbed light directly to the other. Thus, when the complex is illuminated at the excitation wavelength of the first fluorochrome, fluorescent light is pro-

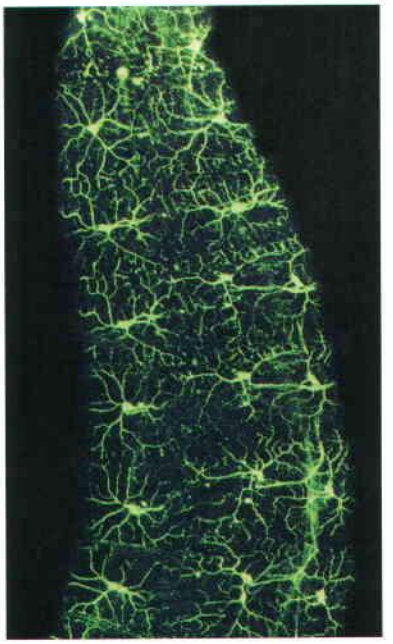


Figure 9–25 Green fluorescent protein (GFP) as a reporter. For this experiment, carried out in the fruit fly, the GFP gene was joined (using recombinant DNA techniques) to a fly promoter that is active only in a specialized set of neurons. This image of a live fly embryo was captured by a fluorescence microscope and shows approximately 20 neurons, each with long projections (axons and dendrites) that communicate with other (nonfluorescent) cells. These neurons are located just under the surface of the animal and allow it to sense its immediate environment. (From W.B. Grueber et al., *Curr. Biol.* 13:618–626, 2003. With permission from Elsevier.)

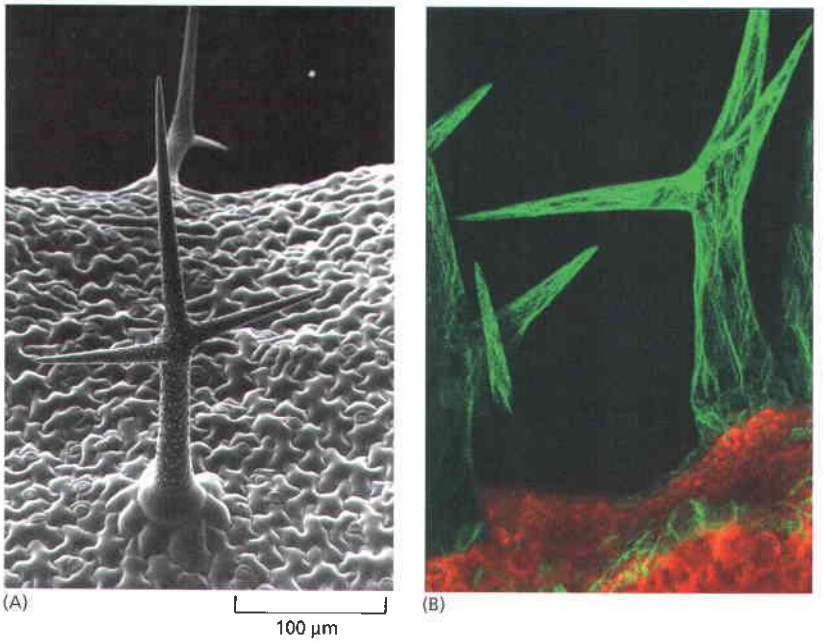


Figure 9–26 GFP-tagged proteins. (A) The upper surface of the leaves of *Arabidopsis* plants are covered with huge branched single-cell hairs that rise up from the surface of the epidermis. These hairs, or trichomes, can be imaged in the scanning electron microscope. (B) If an *Arabidopsis* plant is transformed with a DNA sequence coding for talin (an actin-binding protein), fused to a DNA sequence coding for GFP, the fluorescent talin protein produced binds to actin filaments in all the living cells of the transgenic plant. Confocal microscopy can reveal the dynamics of the entire actin cytoskeleton of the trichome *(green)*. The red fluorescence arises from chlorophyll in cells within the leaf below the epidermis. (A, courtesy of Paul Linstead; B, courtesy of Jaideep Mathur.)

duced at the emission wavelength of the second. This method can be used with two different spectral variants of GFP as fluorochromes to monitor processes such as the interaction of signaling molecules with their receptors, or proteins in macromolecular complexes (**Figure 9–28**).

The complexity and rapidity of many intracellular processes, such as the actions of signaling molecules or the movements of cytoskeletal proteins, make them difficult to study at a single-cell level. Ideally, we would like to be able to introduce any molecule of interest into a living cell at a precise time and location and follow its subsequent behavior, as well as the response of the cell to that molecule. Microinjection is limited by the difficulty of controlling the place and time of delivery. A more powerful approach involves synthesizing an inactive form of the fluorescent molecule of interest, introducing it into the cell, and then activating it suddenly at a chosen site in the cell by focusing a spot of light on it. This process is referred to as **photoactivation**. Inactive photosensitive precursors of this type, often called *caged molecules*, have been made for many fluorescent molecules. A microscope can be used to focus a strong pulse of light from a laser on any tiny region of the cell, so that the experimenter can control exactly where and when the fluorescent molecule is photoactivated.

One class of caged fluorescent proteins is made by attaching a photoactivatable fluorescent tag to a purified protein. It is important that the modified protein remain biologically active: labeling with a caged fluorescent dye adds a bulky group to the surface of a protein, which can easily change the protein's properties. A satisfactory labeling protocol is usually found by trial and error. Once a biologically active labeled protein has been produced, it needs to be introduced into the living cell (see Figure 9–34), where its behavior can be followed. Tubulin, labeled with caged fluorescein for example, when injected into a dividing cell, can be incorporated into microtubules of the mitotic spindle.

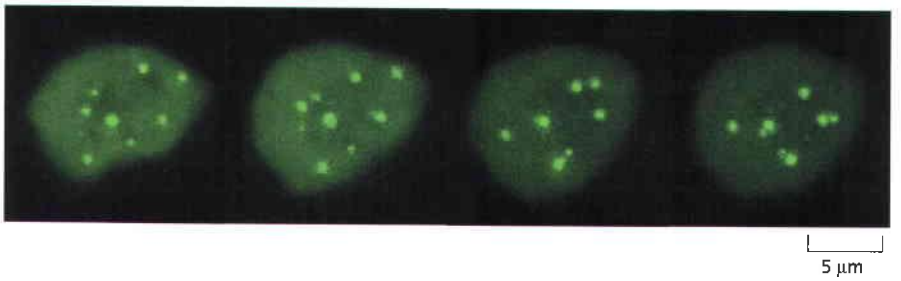


Figure 9–27 Dynamics of GFP tagging. This sequence of micrographs shows a set of three-dimensional images of a living nucleus taken over the course of an hour. Tobacco cells have been stably transformed with GFP fused to a spliceosomal protein that is concentrated in small nuclear bodies called Cajal bodies (see Figure 6–48). The fluorescent Cajal bodies, easily visible in a living cell with confocal microscopy, are dynamic structures that move around within the nucleus. (Courtesy of Kurt Boudonck, Liam Dolan, and Peter Shaw.)

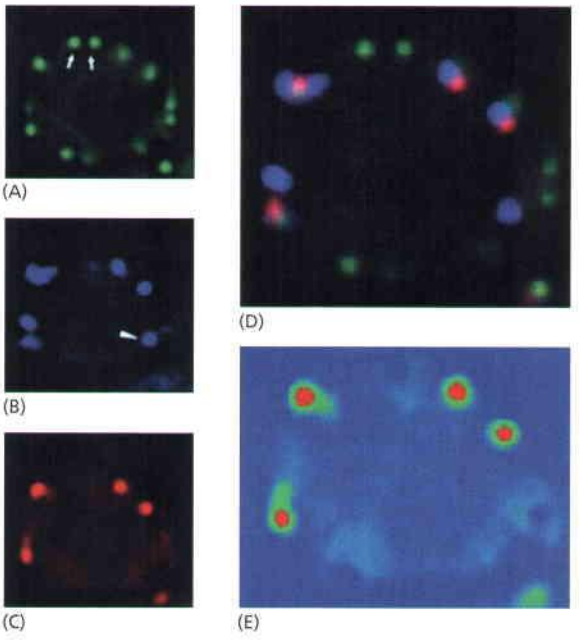


Figure 9–28 Fluorescence resonance energy transfer (FRET) imaging. This experiment shows that a protein called Sla1p can interact tightly with another protein, called Abp1p, which is involved in cortical actin attachment at the surface of a budding yeast cell. Sla1p is expressed in the yeast cell (A) as a fusion protein with a yellow variant of GFP (YFP), while Abp1p is expressed as a fusion protein (B) with a cyan variant of GFP (CFP). The FRET signal (see also Figure 8–26), displayed here in *red* (C), is obtained by exciting the CFP but recording only the fluorescence emitted from the YFP, which will occur only when the two molecules are tightly associated (within 0.5 nm). The spots at the cortex (D), seen when (A), (B), and (C) are superimposed, are of three sorts, those where Sla1p is found alone *(arrows* in A), those where Abp1p is found alone *(arrowhead* in B), and those where they are closely associated and generate a FRET signal, shown in the false-colored and corrected image (E). Since Sla1p was already known to form part of the endocytic machinery, this experiment physically connects that process with the process of actin attachment to the cell cortex. (From D.T. Warren et al., *J. Cell Sci.* 115:1703–1715, 2002. With permission from The Company of Biologists.)

When a small region of the spindle is illuminated with a laser, the labeled tubulin becomes fluorescent, so that its movement along the spindle microtubules can be readily followed (**Figure 9–29**).

A more recent development in photoactivation is the discovery that the genes encoding GFP and related fluorescent proteins can be mutated to produce protein variants, usually with a single amino acid change, that fluoresce only weakly under normal excitation conditions, but can be induced to fluoresce strongly by activating them with a strong pulse of light at a different wavelength. In principle the microscopist can then follow the local *in vivo* behavior of any protein that can be expressed as a fusion with one of these GFP variants. These genetically encoded, photoactivateable fluorescent proteins thus avoid the need to introduce the probe into the cell, and allow the lifetime and behaviour of any protein to be studied independently of other newly synthesized proteins (**Figure 9–30**).

A third way to exploit GFP fused to a protein of interest is to use a strong focussed beam of light from a laser to extinguish the GFP fluorescence in a specified region of the cell. By analyzing the way in which the remaining fluorescent protein molecules move into the bleached area as a function of time, we can obtain information about the protein's kinetic parameters. This technique, usually carried out with a confocal microscope, is known as **fluorescence recovery after photobleaching (FRAP)** and, like photoactivation, can deliver valuable quantitative data about the protein of interest, such as diffusion coefficients <ATGT>, active transport rates, or binding and dissociation rates from other proteins (**Figure 9–31**).

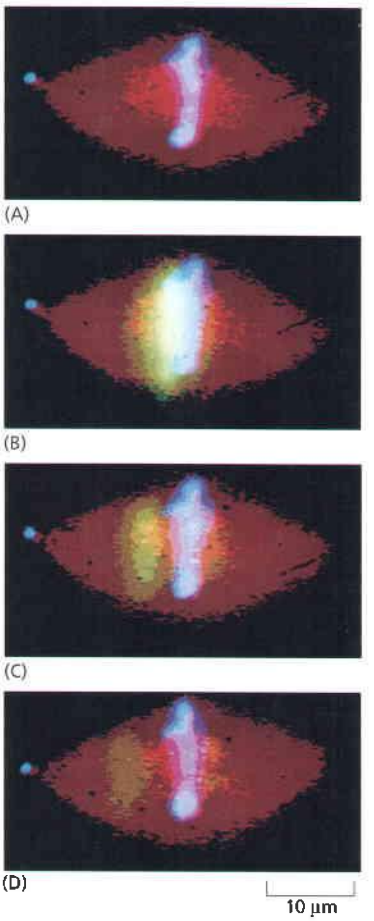


Figure 9–29 Determining microtubule flux in the mitotic spindle with caged fluorescein linked to tubulin. (A) A metaphase spindle formed *in vitro* from an extract of *Xenopus* eggs has incorporated three fluorescent markers: rhodamine-labeled tubulin *(red)* to mark all the microtubules, a *blue* DNA-binding dye that labels the chromosomes, and caged-fluorescein-labeled tubulin, which is also incorporated into all the microtubules but is invisible because it is nonfluorescent until activated by ultraviolet light. (B) A beam of UV light uncages the caged-fluorescein-labeled tubulin locally, mainly just to the left side of the metaphase plate. Over the next few minutes (after 1.5 minutes in C, after 2.5 minutes in D), the uncaged-fluorescein–tubulin signal moves toward the left spindle pole, indicating that tubulin is continuously moving poleward even though the spindle (visualized by the *red* rhodamine-labeled tubulin fluorescence) remains largely unchanged. (From K.E. Sawin and T.J. Mitchison, *J. Cell Biol.* 112:941–954, 1991. With permission from The Rockefeller University Press.)

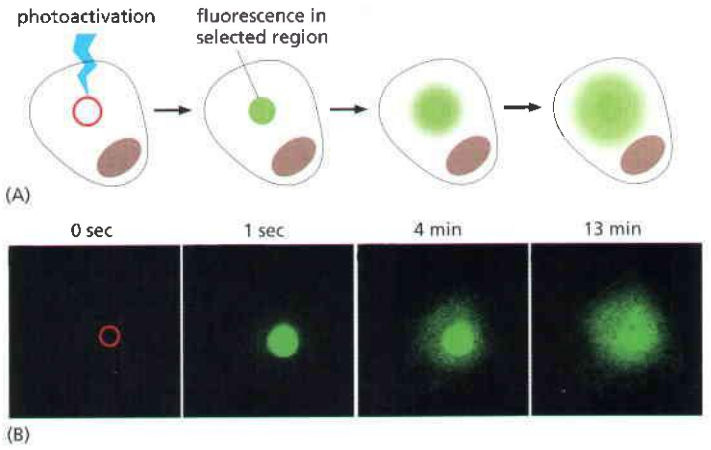


Figure 9–30 Photoactivation. Photoactivation is the light-induced activation of an inert molecule to an active state. In this experiment a photoactivatable variant of GFP is expressed in a cultured animal cell. Before activation (time 0), little or no GFP fluorescence is detected in the selected region *(red circle)* when excited by blue light at 488 nm. After activation of the GFP however, using a UV laser pulse at 413 nm, it rapidly fluoresces brightly in the selected region *(green)*. The movement of GFP, as it diffuses out of this region, can be measured. Since only the photoactivated proteins are fluorescent within the cell, the trafficking, turnover and degradative pathways of proteins can be monitored. (B, from J. Lippincott-Schwartz and G.H. Patterson, *Science* 300:87–91, 2003. With permission from AAAS.)

Light-Emitting Indicators Can Measure Rapidly Changing Intracellular Ion Concentrations

One way to study the chemistry of a single living cell is to insert the tip of a fine, glass, ion-sensitive **microelectrode** directly into the cell interior through the plasma membrane. This technique is used to measure the intracellular concentrations of common inorganic ions, such as H^+, Na^+, K^+, Cl^-, and Ca^{2+}. However, ion-sensitive microelectrodes reveal the ion concentration only at one point in a cell, and for an ion present at a very low concentration, such as Ca^{2+}, their responses are slow and somewhat erratic. Thus, these microelectrodes are not ideally suited to record the rapid and transient changes in the concentration of cytosolic Ca^{2+} that have an important role in allowing cells to respond to extracellular signals. Such changes can be analyzed with **ion-sensitive indicators**, whose light emission reflects the local concentration of the ion. Some of these indicators are luminescent (emitting light spontaneously), while others are fluorescent (emitting light on exposure to light).

Aequorin is a luminescent protein isolated from a marine jellyfish; it emits light in the presence of Ca^{2+} and responds to changes in Ca^{2+} concentration in the range of 0.5–10 μM. If microinjected into an egg, for example, aequorin emits a flash of light in response to the sudden localized release of free Ca^{2+} into the cytoplasm that occurs when the egg is fertilized (**Figure 9–32**). Aequorin has also been expressed transgenically in plants and other organisms to provide a method of monitoring Ca^{2+} in all their cells without the need for microinjection, which can be a difficult procedure.

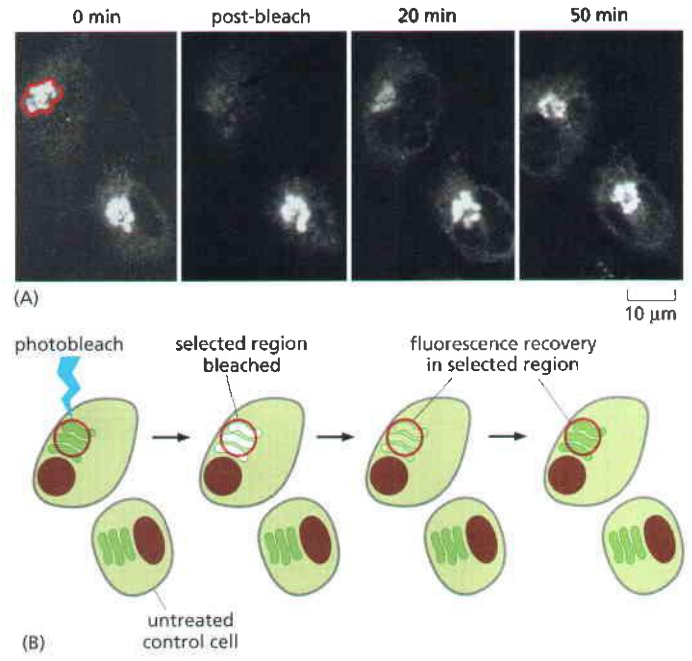


Figure 9–31 Fluorescence recovery after photobleaching (FRAP). A strong focused pulse of laser light will extinguish, or bleach, the fluorescence of GFP. By selectively photobleaching a set of fluorescently tagged protein molecules within a defined region of a cell, the microscopist can monitor recovery over time, as the remaining fluorescent molecules move into the bleached region. The experiment shown in (A) uses monkey cells in culture that express galactosyltransferase, an enzyme that constantly recycles between the Golgi apparatus and the endoplasmic reticulum. The Golgi apparatus in one of the two cells is selectively photobleached, while the production of new fluorescent protein is blocked by treating the cells with cycloheximide. The recovery, resulting from fluorescent enzyme molecules moving from the ER to the Golgi, can then be followed over a period of time. (B) Schematic diagram of the experiment shown in (A). (A, from J. Lippincott-Schwartz et al., *Histochem. Cell Biol.* 116:97–107, 2001. With permission from Springer-Verlag.)

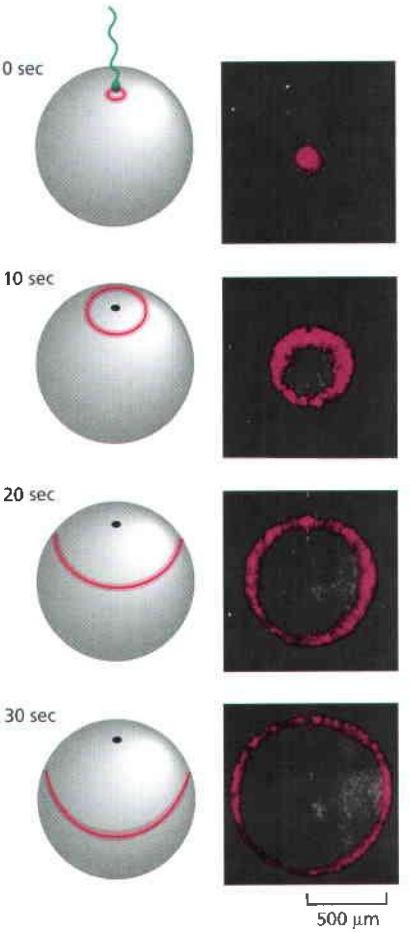


Figure 9–32 Aequorin, a luminescent protein. The luminescent protein aequorin emits light in the presence of free Ca^{2+}. Here, an egg of the medaka fish has been injected with aequorin, which has diffused throughout the cytosol, and the egg has then been fertilized with a sperm and examined with the help of a very sensitive camera. The four photographs were taken looking down on the site of sperm entry at intervals of 10 seconds and reveal a wave of release of free Ca^{2+} into the cytosol from internal stores just beneath the plasma membrane. This wave sweeps across the egg starting from the site of sperm entry, as indicated in the diagrams on the *left*. (Photographs reproduced from J.C. Gilkey, L.F. Jaffe, E.B. Ridgway and G.T. Reynolds, *J. Cell Biol.* 76:448–466, 1978. With permission from The Rockefeller University Press.)

Bioluminescent molecules like aequorin emit tiny amounts of light—at best, a few photons per indicator molecule—that are difficult to measure. Fluorescent indicators produce orders of magnitude more photons per molecule; they are therefore easier to measure and can give better spatial resolution. Fluorescent Ca^{2+} indicators have been synthesized that bind Ca^{2+} tightly and are excited by or emit light at slightly different wavelengths when they are free of Ca^{2+} than when they are in their Ca^{2+}-bound form. By measuring the ratio of fluorescence intensity at two excitation or emission wavelengths, we can determine the concentration ratio of the Ca^{2+}-bound indicator to the Ca^{2+}-free indicator, thereby providing an accurate measurement of the free Ca^{2+} concentration. <CGTC> Indicators of this type are widely used for second-by-second monitoring of changes in intracellular Ca^{2+} concentrations in the different parts of a cell viewed in a fluorescence microscope (**Figure 9–33**). <AGGA>

Similar fluorescent indicators measure other ions; some detect H^+, for example, and hence measure intracellular pH. Some of these indicators can enter cells by diffusion and thus need not be microinjected; this makes it possible to monitor large numbers of individual cells simultaneously in a fluorescence microscope. New types of indicators, used in conjunction with modern image-processing methods, are leading to similarly rapid and precise methods for analyzing changes in the concentrations of many types of small molecules in cells.

Several Srategies Are Available by Which Membrane-Impermeant Substances Can Be Introduced into Cells

It is often useful to introduce membrane-impermeant molecules into a living cell, whether they are antibodies that recognize intracellular proteins, normal cell proteins tagged with a fluorescent label, or molecules that influence cell behavior. One approach is to microinject the molecules into the cell through a glass micropipette.

When microinjected into a cell, antibodies can block the function of the molecule that they recognize. Anti-myosin-II antibodies injected into a fertilized sea urchin egg, for example, prevent the egg cell from dividing in two, even though nuclear division occurs normally. This observation demonstrates that this myosin has an essential role in the contractile process that divides the cytoplasm during cell division, but that it is not required for nuclear division.

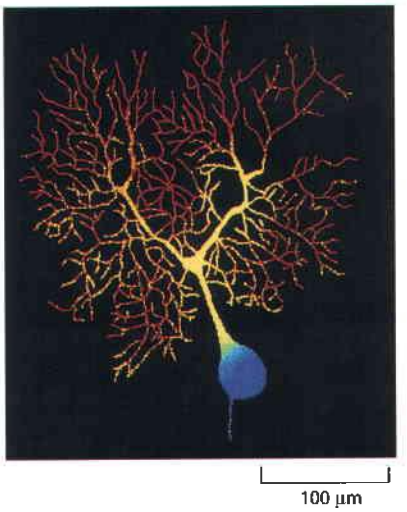


Figure 9–33 Visualizing intracellular Ca^{2+} concentrations by using a fluorescent indicator. The branching tree of dendrites of a Purkinje cell in the cerebellum receives more than 100,000 synapses from other neurons. The output from the cell is conveyed along the single axon seen leaving the cell body at the bottom of the picture. This image of the intracellular Ca^{2+} concentration in a single Purkinje cell (from the brain of a guinea pig) was taken with a low-light camera and the Ca^{2+}-sensitive fluorescent indictor fura-2. The concentration of free Ca^{2+} is represented by different colors, *red* being the highest and *blue* the lowest. The highest Ca^{2+} levels are present in the thousands of dendritic branches. (Courtesy of D.W. Tank, J.A. Connor, M. Sugimori and R.R. Llinas.)

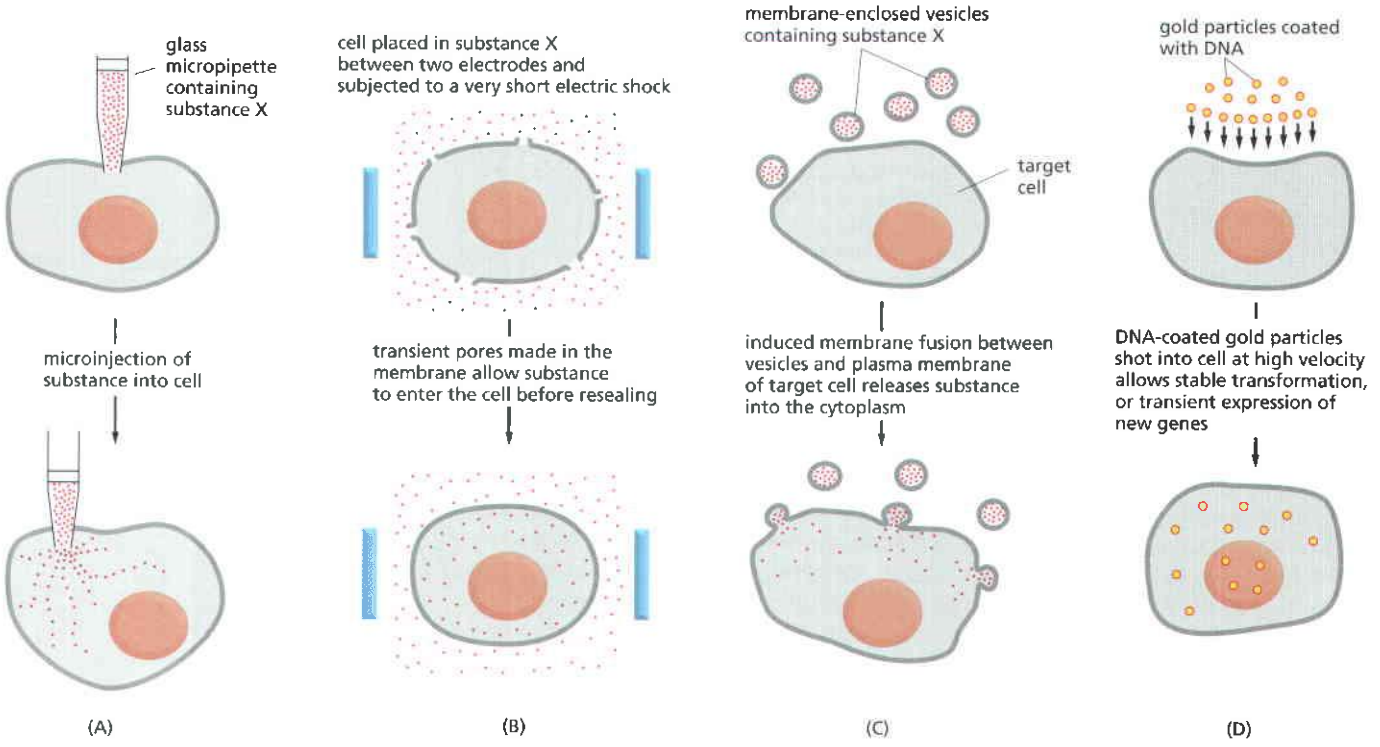


Figure 9–34 Methods of introducing a membrane-impermeant substance into a cell. (A) The substance is injected through a micropipette, either by applying pressure or, if the substance is electrically charged, by applying a voltage that drives the substance into the cell as an ionic current (a technique called *iontophoresis*). (B) The cell membrane is made transiently permeable to the substance by disrupting the membrane structure with a brief but intense electric shock (2000 V/cm for 200 μsec, for example).(C) Membrane-enclosed vesicles are loaded with the desired substance and then induced to fuse with the target cells. (D) Gold particles coated with DNA are used to introduce a novel gene into the nucleus.

Microinjection, although widely used, demands that each cell be injected individually; therefore, it is possible to study at most only a few hundred cells at a time. Other approaches allow large populations of cells to be permeabilized simultaneously. Partly disrupting the structure of the cell plasma membrane, for example, makes it more permeable; this is usually accomplished by using a powerful electric shock or a chemical such as a low concentration of detergent. The electrical technique has the advantage of creating large pores in the plasma membrane without damaging intracellular membranes. Depending on the cell type and the size of the electric shock, the pores allow even macromolecules to enter (and leave) the cytosol rapidly. This process of *electroporation* is valuable also in molecular genetics, as a way of introducing DNA molecules into cells. With a limited treatment, a large fraction of the cells repair their plasma membrane and survive.

A third method for introducing large molecules into cells is to cause membrane-enclosed vesicles that contain these molecules to fuse with the cell's plasma membrane thus delivering their cargo. Thus method is used routinely to deliver nucleic acids into mammalian cells, either DNA for transfection studies or RNA for RNAi experiments (discussed in Chapter 8). In the medical field it is also being explored as a method for the targeted delivering of new pharmaceuticals.

Finally, DNA and RNA can also be physically introduced into cells by simply blasting them in at high velocity, coated onto tiny gold particles. Living cells, shot with these nucleic-acid-coated gold particles (typically less than 1 μm in diameter) can successfully incorporate the introduced RNA (used for transient expression studies or RNAi, for example) or DNA (for stable transfection). All four of these methods, illustrated in **Figure 9–34**, are used widely in cell biology.

Light Can Be Used to Manipulate Microscopic Objects As Well As to Image Them

Photons carry a small amount of momentum. This means that an object that absorbs or deflects a beam of light experiences a small force. With ordinary light sources, this radiation pressure is too small to be significant. But it is important on a cosmic scale (helping prevent gravitational collapse inside stars), and, more

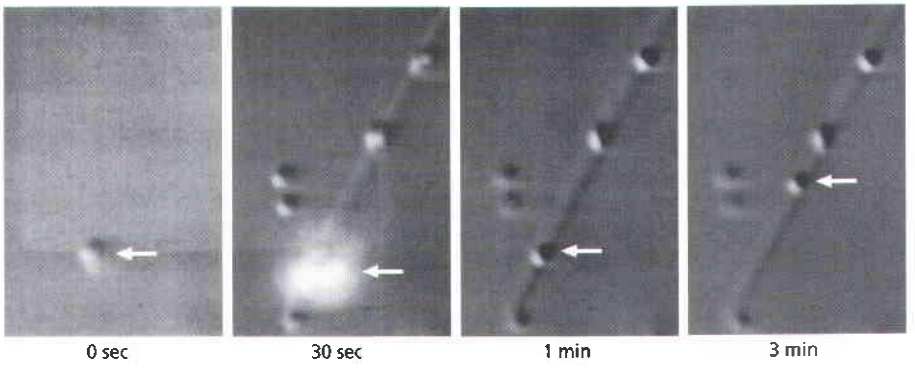


Figure 9–35 Optical tweezers. A focused laser beam can be used to trap microscopic particles and move them about at will. <CGCG> In this experiment, such optical tweezers are used to pick up a small silica bead (0.2 nm, *arrow*), coated with few kinesin molecules (0 sec), and place it on an isolated ciliary axoneme that is built from microtubules (30 sec). The bright halo seen here is the reflection of the laser at the interface between the water and the coverslip. The kinesin on the released bead (1 min) couples ATP hydrolysis to movement along the microtubules of the axoneme, and powers the transport of the bead along it (3 min). (From S.M. Block et al., *Nature* 348:348–352, 1990. With permission from Macmillan Publishers Ltd.)

modestly, in the cell biology lab, where an intense focused laser beam can exert large enough forces to push small objects around inside a cell. If the laser beam is focused on an object having a higher refractive index than its surroundings, the beam is refracted, causing very large numbers of photons to change direction. The pattern of photon deflection holds the object at the focus of the beam; if it begins to drift away from this position, radiation pressure pushes it back by acting more strongly on one side than the other. Thus, by steering a focused laser beam, usually an infrared laser, which is minimally absorbed by the cellular constituents, one can create "**optical tweezers**" to move subcellular objects like organelles and chromosomes around. This method, sometimes referred to as laser tweezers <CGCG> <CACA>, has been used to measure the forces exerted by single actin–myosin molecules, by single microtubule motors, and by RNA polymerase (**Figure 9–35**).

Intense focused laser beams that are more strongly absorbed by biological material can also be used more straightforwardly as optical knives—to kill individual cells, to cut or burn holes in them, or to detach one intracellular component from another. In these ways, optical devices can provide a basic toolkit for cellular microsurgery.

Single Molecules Can Be Visualized by Using Total Internal Reflection Fluorescence Microscopy

While beads can be used as markers to track protein movements, it is clearly preferable to be able to visualize the proteins themselves. In principle this can be accomplished by labeling the protein with a fluorescent molecule, either by chemically attaching a small fluorescent molecule to isolated protein molecules or by expressing fluorescent protein fusion constructs (see p. 593). In ordinary microscopes, however, single fluorescent molecules cannot be reliably detected. The limitation has nothing to do with the resolution limit, but instead arises from the interference of light emitted by out-of-focus molecules that tends to blot out the fluorescence from the particular molecule of interest. This problem can be solved by the use of a specialized optical technique called total internal reflectance fluorescence (TIRF) microscopy. In a TIRF microscope, laser light shines onto the coverslip surface at the precise critical angle at which total internal reflection occurs (**Figure 9–36**A). Because of total internal reflection, the light does not enter the sample, and the majority of fluorescent molecules are not, therefore, illuminated. However, electromagnetic energy does extend, as an evanescent field, for a very short distance beyond the surface of the coverslip and into the specimen, allowing just those molecules in the layer closest to the surface to become excited. When these molecules fluoresce, their emitted light is no longer competing with out-of-focus light from the overlying molecules, and can now be detected. TIRF has allowed several dramatic experiments, for instance imaging of single motor proteins moving along microtubules or single actin filaments forming and branching, although at present the technique is restricted to a thin layer within only 100–200 nm of the cell surface (Figure 9–36B and C).

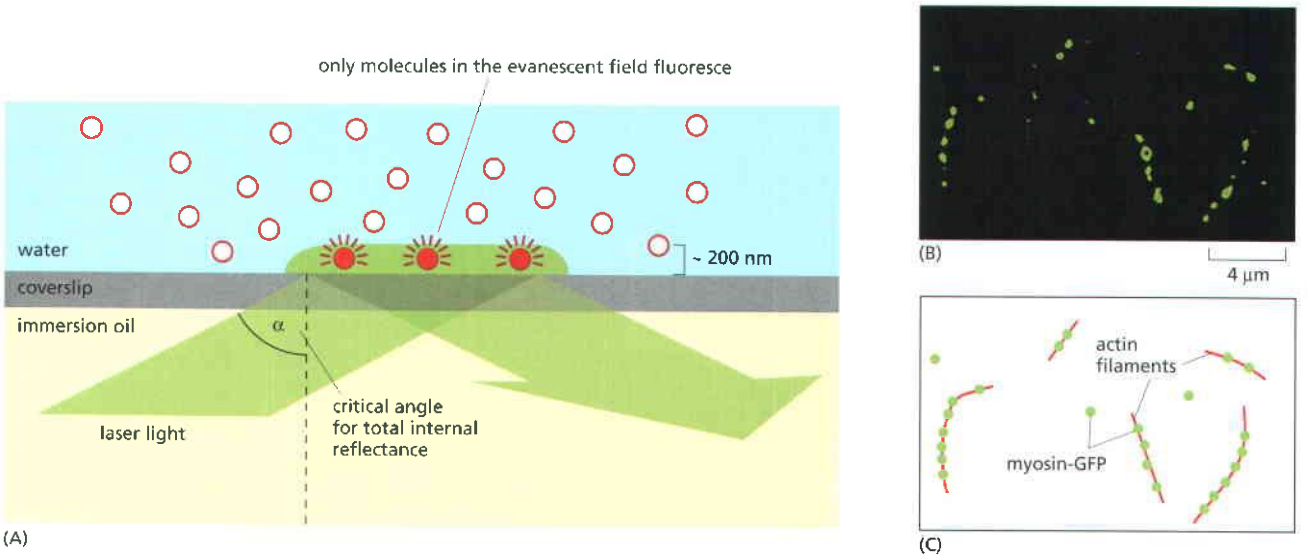


Figure 9–36 TIRF microscopy allows the detection of single fluorescent molecules. (A) TIRF microscopy uses excitatory laser light to illuminate the coverslip surface at the critical angle at which all the light is reflected by the glass–water interface. Some electromagnetic energy extends a short distance across the interface as an evanescent wave that excites just those molecules that are very close to the surface. (B) TIRF microscopy is used here to image individual myosin-GFP molecules *(green dots)* attached to non-fluorescent actin filaments (C), which are invisible but stuck to the surface of the coverslip. (Courtesy of Dmitry Cherny and Clive R. Bagshaw.)

Individual Molecules Can Be Touched and Moved Using Atomic Force Microscopy

While TIRF allows single molecules to be visualized, it is strictly a passive observation method. In order to probe molecular function, it is ultimately useful to be able to manipulate individual molecules themselves, and atomic force microscopy (AFM) provides a method to do just that. In an AFM device, an extremely small and sharply pointed tip, of silicon or silicon nitride, is made using nanofabrication methods similar to those used in the semiconductor industry. The tip of the AFM is attached to a springy cantilever arm mounted on a highly precise positioning system that allows it to be moved over very small distances. In addition to this precise movement capability, the AFM is able to measure the mechanical force felt by its tip as it moves over the surface (**Figure 9–37**A). When AFM was first developed, it was intended as an imaging technology to measure molecular-scale features on a surface. When used in this mode, the probe is scanned over the surface, moving up and down as necessary to maintain a constant interaction force with the surface, thus revealing any objects such as proteins that might be present on the otherwise flat surface (see Figures 10–14 and 10–32). AFM is not limited to simply imaging surfaces, however, and can also be used to pick up and move single molecules, in a molecular-scale version of the optical tweezers described above. Using this technology, the mechanical properties of individual protein molecules can be measured in detail. For example, AFM has been used to unfold a single protein molecule in order to measure the energetics of domain folding (Figure 9–37B). The full potential to probe proteins mechanically, as well as to assemble individual proteins into defined arrangements using AFM, is only now starting to be explored, but it seems likely that this tool will become increasingly important in the future.

Molecules Can Be Labeled with Radioisotopes

As we have just seen, in cell biology it is often important to determine the quantities of specific molecules and to know where they are in the cell and how their level or location changes in response to extracellular signals. The molecules of interest range from small inorganic ions, such as Ca^{2+} or H^+, to large macromolecules, such as specific proteins, RNAs, or DNA sequences. We have so far described how sensitive fluorescence methods can be used for assaying these types of molecules, as well as for following the dynamic behavior of many of them in living cells. In ending this section, we describe how radioisotopes are used to trace the path of specific molecules through the cell.

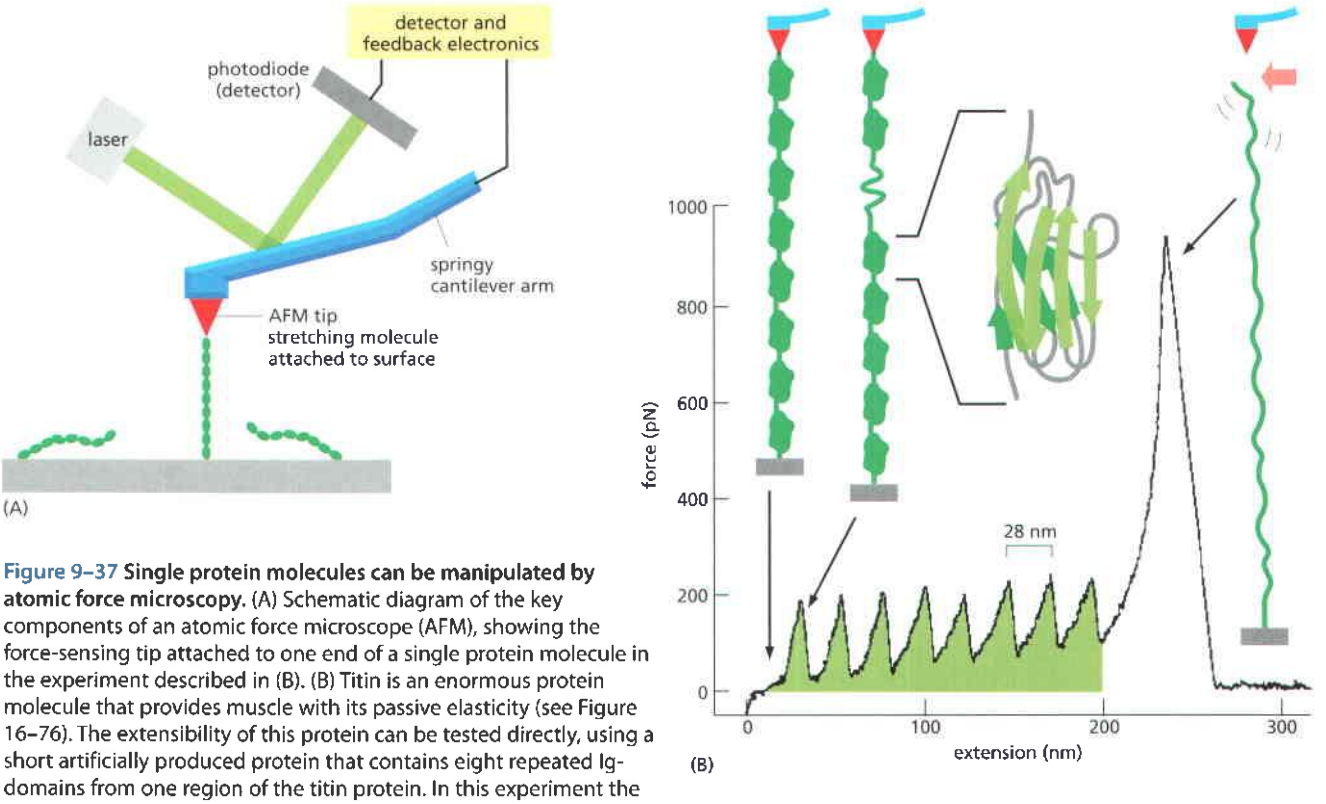


Figure 9–37 Single protein molecules can be manipulated by atomic force microscopy. (A) Schematic diagram of the key components of an atomic force microscope (AFM), showing the force-sensing tip attached to one end of a single protein molecule in the experiment described in (B). (B) Titin is an enormous protein molecule that provides muscle with its passive elasticity (see Figure 16–76). The extensibility of this protein can be tested directly, using a short artificially produced protein that contains eight repeated Ig-domains from one region of the titin protein. In this experiment the tip of the AFM is used to pick up, and progressively stretch, a single molecule until it eventually ruptures. As force is applied, each Ig-domain suddenly begins to unfold, and the force needed in each case (about 200 pN) can be recorded. The region of the force–extension curve shown in *green* records the sequential unfolding event for each of the eight protein domains. (Adapted from W.A. Linke et al., *J. Struct. Biol.* 137:194–205, 2002. With permission from Elsevier.)

Most naturally occurring elements are a mixture of slightly different isotopes. These differ from one another in the mass of their atomic nuclei, but because they have the same number of protons and electrons, they have the same chemical properties. In radioactive isotopes, or radioisotopes, the nucleus is unstable and undergoes random disintegration to produce a different atom. In the course of these disintegrations, either energetic subatomic particles, such as electrons, or radiations, such as gamma-rays, are given off. By using chemical synthesis to incorporate one or more radioactive atoms into a small molecule of interest, such as a sugar or an amino acid, the fate of that molecule (and of specific atoms in it) can be traced during any biological reaction.

Although naturally occurring radioisotopes are rare (because of their instability), they can be produced in large amounts in nuclear reactors, where stable atoms are bombarded with high-energy particles. As a result, radioisotopes of many biologically important elements are readily available (Table 9–1). The radiation they emit is detected in various ways. Electrons (β particles) can be detected in a Geiger counter by the ionization they produce in a gas, or they can be measured in a scintillation counter by the small flashes of light they induce in a scintillation fluid. These methods make it possible to measure accurately the quantity of a particular radioisotope present in a biological specimen. Using either light or electron microscopy, it is also possible to determine the location of a radioisotope in a specimen by autoradiography, as we describe below. All of these methods of detection are extremely sensitive: in favorable circumstances, nearly every disintegration—and therefore every radioactive atom that decays—can be detected.

Table 9–1 Some Radioisotopes in Common Use in Biological Research

ISOTOPE	HALF-LIFE
^{32}P	14 days
^{131}I	8.1 days
^{35}S	87 days
^{14}C	5570 years
^{45}Ca	164 days
^{3}H	12.3 years

The isotopes are arranged in decreasing order of the energy of the β radiation (electrons) they emit. ^{131}I also emits γ radiation. The half-life is the time required for 50% of the atoms of an isotope to disintegrate.

Radioisotopes Are Used to Trace Molecules in Cells and Organisms

One of the earliest uses of radioactivity in biology was to trace the chemical pathway of carbon during photosynthesis. Unicellular green algae were maintained in an atmosphere containing radioactively labeled CO_2 ($^{14}CO_2$), and at various times after they had been exposed to sunlight, their soluble contents were separated by paper chromatography. Small molecules containing ^{14}C atoms derived from CO_2 were detected by a sheet of photographic film placed over the dried paper chromatogram. In this way most of the principal components in the photosynthetic pathway from CO_2 to sugar were identified.

Radioactive molecules can be used to follow the course of almost any process in cells. In a typical experiment the cells are supplied with a precursor molecule in radioactive form. The radioactive molecules mix with the preexisting unlabeled ones; both are treated identically by the cell as they differ only in the weight of their atomic nuclei. Changes in the location or chemical form of the radioactive molecules can be followed as a function of time. The resolution of such experiments is often sharpened by using a pulse-chase labeling protocol, in which the radioactive material (the pulse) is added for only a very brief period and then washed away and replaced by nonradioactive molecules (the chase). Samples are taken at regular intervals, and the chemical form or location of the radioactivity is identified for each sample (**Figure 9–38**). Pulse-chase experiments, combined with autoradiography, have been important, for example, in elucidating the pathway taken by secreted proteins from the ER to the cell exterior.

Radioisotopic labeling is a uniquely valuable way of distinguishing between molecules that are chemically identical but have different histories—for example, those that differ in their time of synthesis. In this way, for example, it was shown that almost all of the molecules in a living cell are continually being degraded and replaced, even when the cell is not growing and is apparently in a steady state. This "turnover," which sometimes takes place very slowly, would be almost impossible to detect without radioisotopes.

Today, nearly all common small molecules are available in radioactive form from commercial sources, and virtually any biological molecule, no matter how complicated, can be radioactively labeled. Compounds can be made with radioactive atoms incorporated at particular positions in their structure, enabling the separate fates of different parts of the same molecule to be followed during biological reactions (**Figure 9–39**).

As mentioned previously, one of the important uses of radioactivity in cell biology is to localize a radioactive compound in sections of whole cells or tissues by autoradiography. In this procedure, living cells are briefly exposed to a pulse of a specific radioactive compound and then incubated for a variable period—to allow them time to incorporate the compound—before being fixed and processed for light or electron microscopy. Each preparation is then overlaid with a thin film of photographic emulsion and left in the dark for several days, during which the radioisotope decays. The emulsion is then developed, and the position of the radioactivity in each cell is indicated by the position of the developed silver grains (see Figure 5–29). If cells are exposed to ^3H-thymidine, a radioactive precursor of DNA, for example, it can be shown that DNA is made in the nucleus

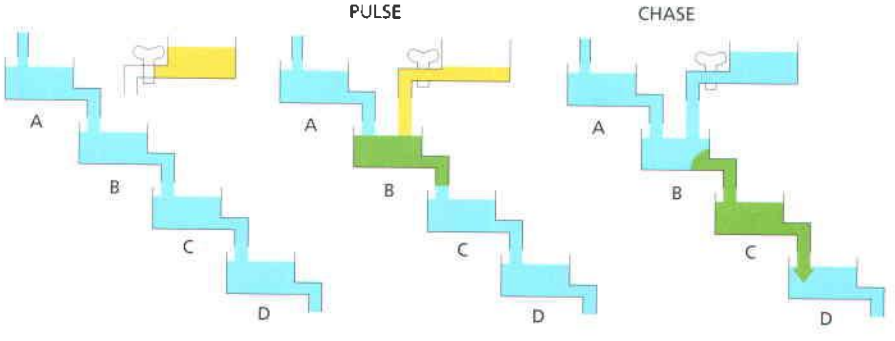


Figure 9–38 The logic of a typical pulse-chase experiment using radioisotopes. The chambers labeled A, B, C, and D represent either different compartments in the cell (detected by autoradiography or by cell-fractionation experiments) or different chemical compounds (detected by chromatography or other chemical methods).

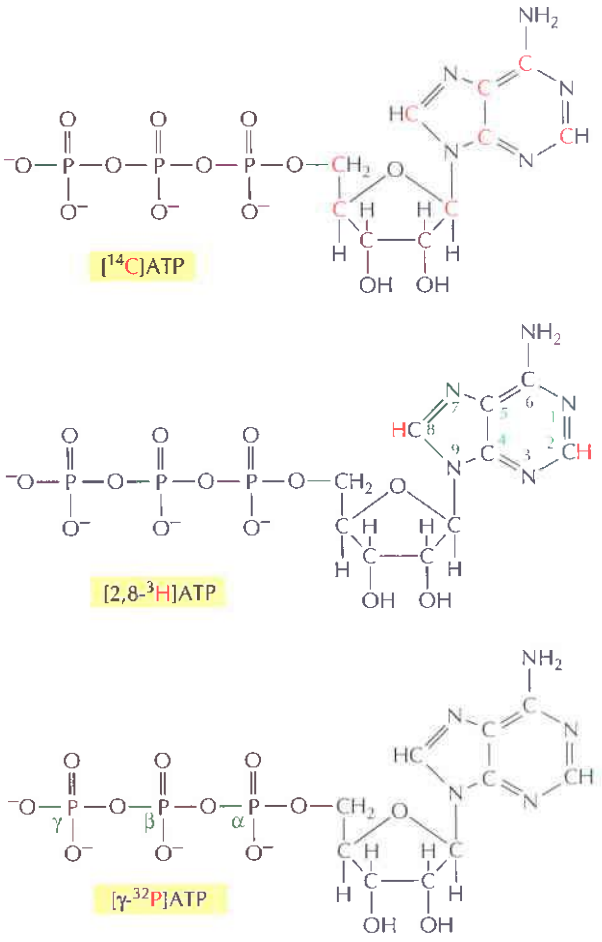


Figure 9–39 Radioisotopically labeled molecules. Three commercially available radioactive forms of ATP, with the radioactive atoms shown in *red*. The nomenclature used to identify the position and type of the radioactive atoms is also shown.

and remains there (**Figure 9–40**). By contrast, if cells are exposed to ^{3}H-uridine, a radioactive precursor of RNA, it is found that RNA is initially made in the nucleus (see Figure 4–62) and then moves rapidly into the cytoplasm. Radiolabeled molecules can also be detected by autoradiography after they are separated from other molecules by gel electrophoresis: the positions of both proteins (see Figure 8–23) and nucleic acids (see Figure 8–33A) are commonly detected on gels in this way.

Summary

Many light-microscope techniques are available for observing cells. Cells that have been fixed and stained can be studied in a conventional light microscope, whereas antibodies coupled to fluorescent dyes can be used to locate specific molecules in cells in a fluorescence microscope. Living cells can be seen with phase-contrast, differential-interference-contrast, dark-field, or bright-field microscopes. All forms of light microscopy are facilitated by digital image-processing techniques, which enhance sensitivity and refine the image. Confocal microscopy and image deconvolution both provide thin optical sections and can be used to reconstruct three-dimensional images.

Techniques are now available for detecting, measuring, and following almost any desired molecule in a living cell. Fluorescent indicator dyes can be introduced to measure the concentrations of specific ions in individual cells or in different parts of a cell. Fluorescent proteins are especially versatile probes that can be attached to other proteins by genetic manipulation. Virtually any protein of interest can be genetically engineered as a fluorescent-fusion protein, and then imaged in living cells by fluorescence microscopy. The dynamic behavior and interactions of many molecules can now be followed in living cells by variations on the use of fluorescent protein tags, in some cases at the level of single molecules. Radioactive isotopes of various elements can also be used to follow the fate of specific molecules both biochemically and microscopically.

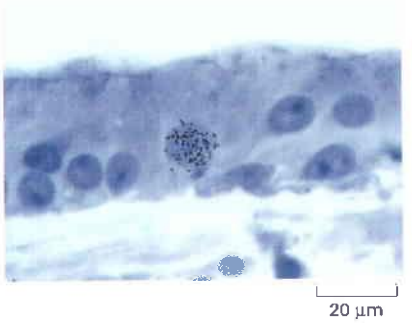


Figure 9–40 Autoradiography. This tissue has been exposed for a short period to ^{3}H-thymidine. Cells that are replicating their DNA incorporate this radioactively labeled DNA precursor into their nuclei and can subsequently be visualized by autoradiography. The silver grains, seen here as *black dots* in the photographic emulsion over the section, reveal which cell was making new DNA. The labeled nucleus shown here is in the sensory epithelium from the inner ear of a chicken. (Courtesy of Mark Warchol and Jeffrey Corwin.)

LOOKING AT CELLS AND MOLECULES IN THE ELECTRON MICROSCOPE

Light microscopy is limited in the fineness of detail that it can reveal. Microscopes using other types of radiation—in particular, electron microscopes—can resolve much smaller structures than is possible with visible light. This higher resolution comes at a cost: specimen preparation for electron microscopy is much more complex and it is harder to be sure that what we see in the image corresponds precisely to the actual structure being examined. It is now possible, however, to use very rapid freezing to preserve structures faithfully for electron microscopy. Digital image analysis can be used to reconstruct three-dimensional objects by combining information either from many individual particles or from multiple tilted views of a single object. Together these approaches are extending the resolution and scope of electron microscopy to the point at which we can begin to faithfully image the structures of individual macromolecules and the complexes they form.

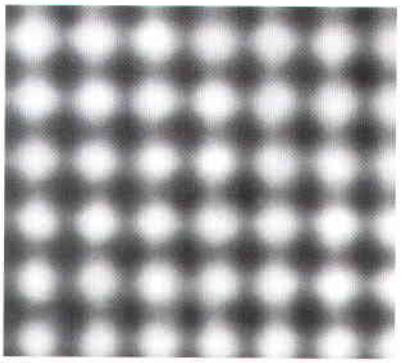


Figure 9–41 The limit of resolution of the electron microscope. This transmission electron micrograph of a thin layer of gold shows the individual files of atoms in the crystal as bright spots. The distance between adjacent files of gold atoms is about 0.2 nm (2 Å). (Courtesy of Graham Hills.)

The Electron Microscope Resolves the Fine Structure of the Cell

The relationship between the limit of resolution and the wavelength of the illuminating radiation (see Figure 9–6) holds true for any form of radiation, whether it is a beam of light or a beam of electrons. With electrons, however, the limit of resolution can be made very small. The wavelength of an electron decreases as its velocity increases. In an **electron microscope** with an accelerating voltage of 100,000 V, the wavelength of an electron is 0.004 nm. In theory the resolution of such a microscope should be about 0.002 nm, which is 100,000 times that of the light microscope. Because the aberrations of an electron lens are considerably harder to correct than those of a glass lens, however, the practical resolving power of most modern electron microscopes is, at best, 0.1 nm (1 Å) (**Figure 9–41**). This is because only the very center of the electron lenses can be used, and the effective numerical aperture is tiny. Furthermore, problems of specimen preparation, contrast, and radiation damage have generally limited the normal effective resolution for biological objects to 1 nm (10 Å). This is nonetheless about 200 times better than the resolution of the light microscope. Moreover, in recent years, the performance of electron microscopes has been improved by the development of electron illumination sources called field emission guns. These very bright and coherent sources can substantially improve the resolution achieved.

In overall design the transmission electron microscope (TEM) is similar to a light microscope, although it is much larger and "upside down" (**Figure 9–42**).

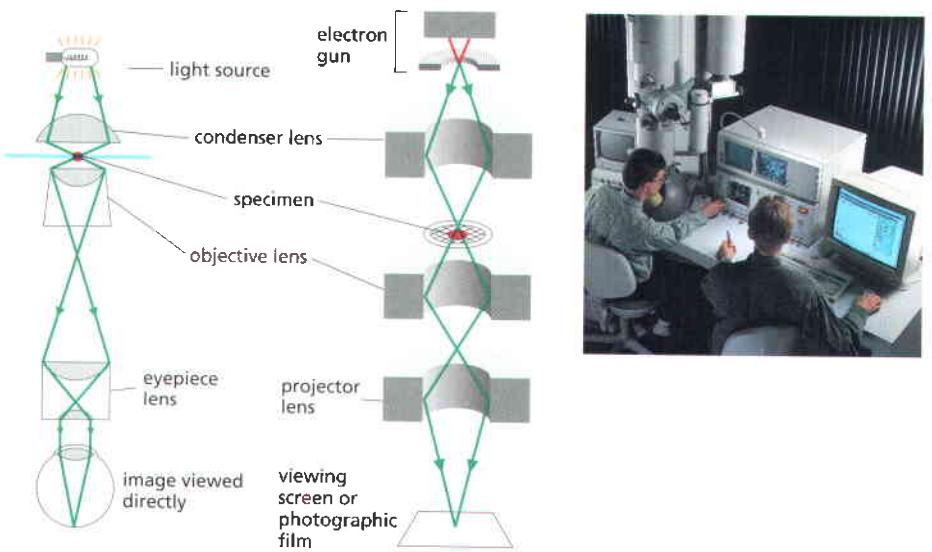


Figure 9–42 The principal features of a light microscope and a transmission electron microscope. These drawings emphasize the similarities of overall design. Whereas the lenses in the light microscope are made of glass, those in the electron microscope are magnetic coils. The electron microscope requires that the specimen be placed in a vacuum. The inset shows a transmission electron microscope in use. (Photograph courtesy of FEI Company Ltd.)

The source of illumination is a filament or cathode that emits electrons at the top of a cylindrical column about 2 m high. Since electrons are scattered by collisions with air molecules, air must first be pumped out of the column to create a vacuum. The electrons are then accelerated from the filament by a nearby anode and allowed to pass through a tiny hole to form an electron beam that travels down the column. Magnetic coils placed at intervals along the column focus the electron beam, just as glass lenses focus the light in a light microscope. The specimen is put into the vacuum, through an airlock, into the path of the electron beam. As in light microscopy, the specimen is usually stained—in this case, with *electron-dense* material, as we see in the next section. Some of the electrons passing through the specimen are scattered by structures stained with the electron-dense material; the remainder are focused to form an image, in a manner analogous to the way an image is formed in a light microscope. The image can be observed on a phosphorescent screen or recorded, either on a photographic plate or with a high-resolution digital camera. Because the scattered electrons are lost from the beam, the dense regions of the specimen show up in the image as areas of reduced electron flux, which look dark.

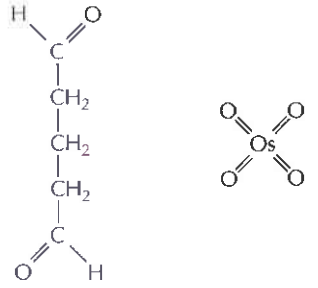
Biological Specimens Require Special Preparation for the Electron Microscope

In the early days of its application to biological materials, the electron microscope revealed many previously unimagined structures in cells. But before these discoveries could be made, electron microscopists had to develop new procedures for embedding, cutting, and staining tissues.

Since the specimen is exposed to a very high vacuum in the electron microscope, living tissue is usually killed and preserved by fixation—first with *glutaraldehyde*, which covalently cross-links protein molecules to their neighbors, and then with *osmium tetroxide*, which binds to and stabilizes lipid bilayers as well as proteins (**Figure 9–43**). Because electrons have very limited penetrating power, the fixed tissues normally have to be cut into extremely thin sections (50–100 nm thick, about 1/200 the thickness of a single cell) before they are viewed. This is achieved by dehydrating the specimen and permeating it with a monomeric resin that polymerizes to form a solid block of plastic; the block is then cut with a fine glass or diamond knife on a special microtome. These *thin sections*, free of water and other volatile solvents, are placed on a small circular metal grid for viewing in the microscope (**Figure 9–44**). <AACA>

The steps required to prepare biological material for viewing in the electron microscope have challenged electron microscopists from the beginning. How can we be sure that the image of the fixed, dehydrated, resin-embedded specimen finally seen bears any relation to the delicate aqueous biological system that was originally present in the living cell? The best current approaches to this problem depend on rapid freezing. If an aqueous system is cooled fast enough to a low enough temperature, the water and other components in it do not have time to rearrange themselves or crystallize into ice. Instead, the water is supercooled into a rigid but noncrystalline state—a "glass"—called vitreous ice. This state can be achieved by slamming the specimen onto a polished copper block cooled by liquid helium, by plunging it into or spraying it with a jet of a coolant such as liquid propane, or by cooling it at high pressure.

Some frozen specimens can be examined directly in the electron microscope using a special, cooled specimen holder. In other cases the frozen block can be fractured to reveal interior surfaces, or the surrounding ice can be sublimed away to expose external surfaces. However, we often want to examine thin sections, and stain them to yield adequate contrast in the electron microscope image (discussed further below). A compromise is therefore to rapid-freeze the tissue, then replace the water, maintained in the vitreous (glassy) state, by organic solvents, and finally embed the tissue in plastic resin, cut sections, and stain. Although technically still difficult, this approach stabilizes and preserves the tissue in a condition very close to its original living state (**Figure 9–45**).



glutaraldehyde osmium tetroxide

Figure 9–43 Two common chemical fixatives used for electron microscopy. The two reactive aldehyde groups of glutaraldehyde enable it to cross-link various types of molecules, forming covalent bonds between them. Osmium tetroxide forms cross-linked complexes with many organic compounds, and in the process becomes reduced. This reaction is especially useful for fixing cell membranes, since the C=C double bonds present in many fatty acids react with osmium tetroxide.

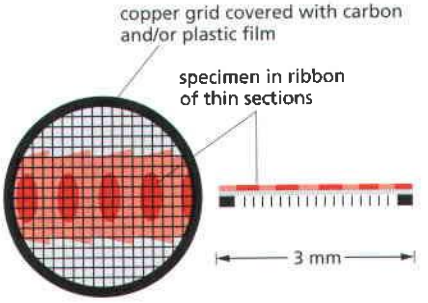


Figure 9–44 The copper grid that supports the thin sections of a specimen in a TEM.

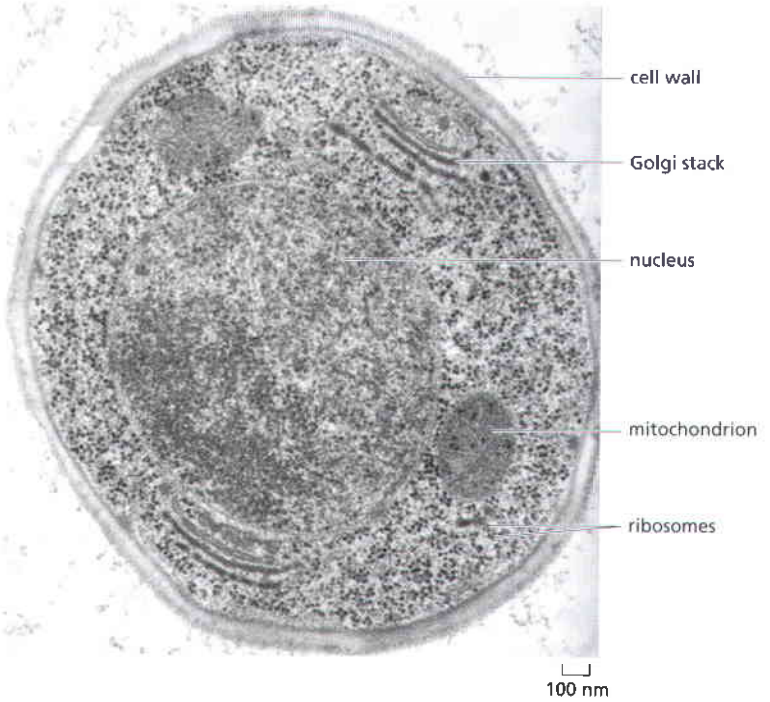


Figure 9–45 Thin section of a cell. This thin section is of a yeast cell that has been very rapidly frozen and the vitreous ice replaced by organic solvents and then by plastic resin. The nucleus, mitochondria, cell wall, Golgi stacks, and ribosomes can all be readily seen in a state that is presumed to be as life-like as possible. (Courtesy of Andrew Staehelin.)

Contrast in the electron microscope depends on the atomic number of the atoms in the specimen: the higher the atomic number, the more electrons are scattered and the greater the contrast. Biological tissues are composed of atoms of very low atomic number (mainly carbon, oxygen, nitrogen, and hydrogen). To make them visible, they are usually impregnated (before or after sectioning) with the salts of heavy metals such as uranium and lead. The degree of impregnation, or "staining," with these salts reveals different cellular constituents with various degrees of contrast. Lipids, for example, tend to stain darkly after osmium fixation, revealing the location of cell membranes.

Specific Macromolecules Can Be Localized by Immunogold Electron Microscopy

We have seen how antibodies can be used in conjunction with fluorescence microscopy to localize specific macromolecules. An analogous method—**immunogold electron microscopy**—can be used in the electron microscope. The usual procedure is to incubate a thin section with a specific primary antibody, and then with a secondary antibody to which a colloidal gold particle has been attached. The gold particle is electron-dense and can be seen as a black dot in the electron microscope (**Figure 9–46**).

Thin sections often fail to convey the three-dimensional arrangement of cellular components in the TEM and can be very misleading: a linear structure such as a microtubule may appear in section as a pointlike object, for example, and a section through protruding parts of a single irregularly shaped solid body may give the appearance of two or more separate objects. The third dimension can be reconstructed from serial sections (**Figure 9–47**), but this is still a lengthy and tedious process.

Even thin sections, however, have a significant depth compared with the resolution of the electron microscope, so they can also be misleading in an opposite way. The optical design of the electron microscope—the very small aperture used—produces a large depth of field, so the image seen corresponds to a superimposition (a projection) of the structures at different depths. A further complication for immunogold labeling is that the antibodies and colloidal gold particles do not penetrate into the resin used for embedding; therefore, they detect antigens only at the surface of the section. This means that first, the sensitivity of detection is low, since antigen molecules present in the deeper parts of the

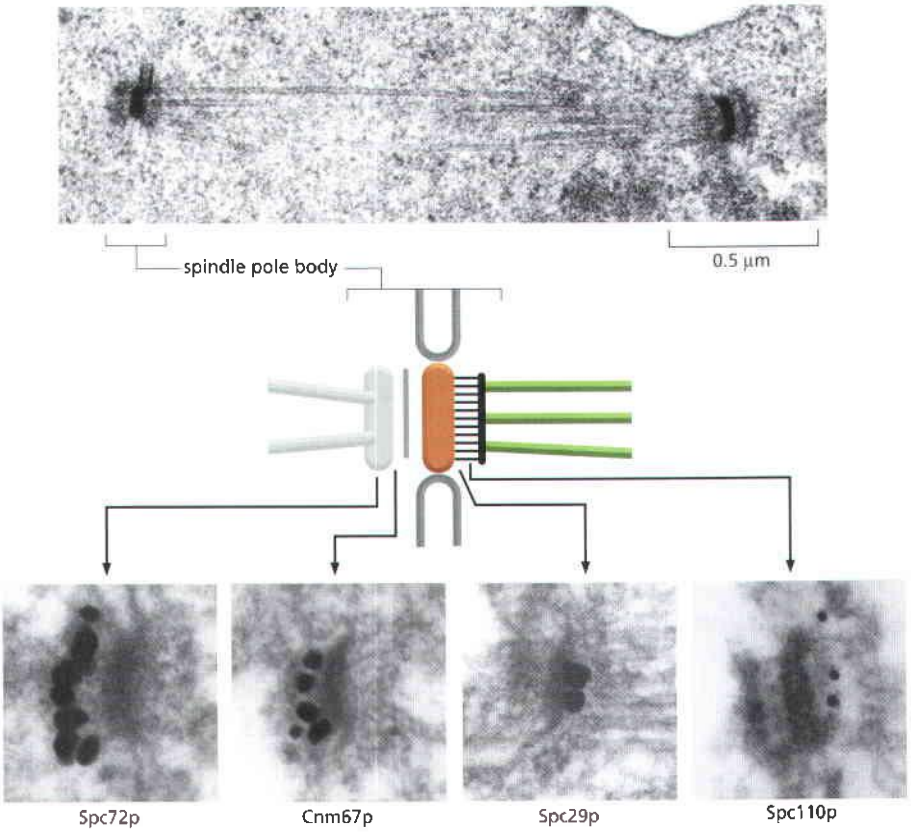


Figure 9–46 Localizing proteins in the electron microscope. Immunogold electron microscopy is used here to localize four different protein components to particular locations within the spindle pole body of yeast. At the top is a thin section of a yeast mitotic spindle showing the spindle microtubules that cross the nucleus, and connect at each end to spindle pole bodies embedded in the nuclear envelope. A diagram of the components of a single spindle pole body is shown below. Antibodies against four different proteins of the spindle pole body are used, together with colloidal gold particles *(black dots)*, to reveal where within the complex structure each protein is located. (Courtesy of John Kilmartin.)

section are not detected, and second, we may get a false impression of which structures contain the antigen and which do not. A solution to this problem is to label the specimen before embedding it in plastic, when cells and tissues are still fully accessible to labeling reagents. Extremely small gold particles, about 1 nm in diameter, work best for this procedure. Such small gold particles are usually not directly visible in the final sections, so additional silver or gold is nucleated around the tiny 1 nm gold particles in a chemical process very much like photographic development.

Images of Surfaces Can Be Obtained by Scanning Electron Microscopy

A **scanning electron microscope (SEM)** directly produces an image of the three-dimensional structure of the surface of a specimen. The SEM is usually a smaller, simpler, and cheaper device than a transmission electron microscope. Whereas the TEM uses the electrons that have passed through the specimen to form an

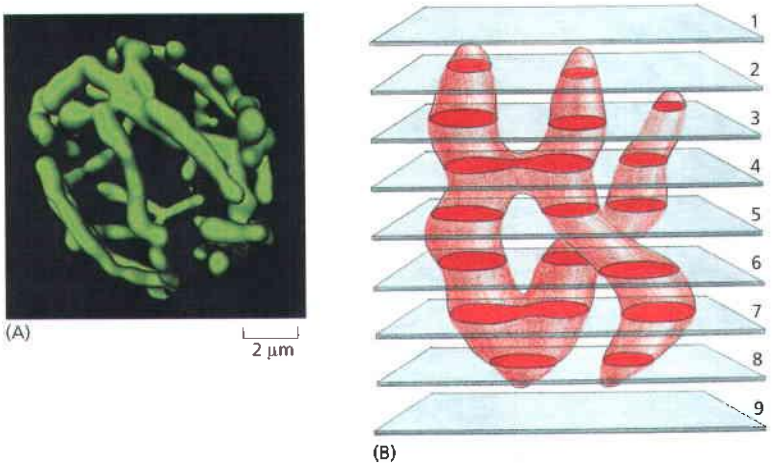


Figure 9–47 A three-dimensional reconstruction from serial sections. (A) A three-dimensional reconstruction of the mitochondrial compartment of a live yeast cell, assembled from a stack of optical sections, shows its complex branching structure. Single thin sections of such a structure in the electron microscope sometimes give misleading impressions. In this example (B), most sections through a cell containing a branched mitochondrion seem to contain two or three separate mitochondria (compare Figure 9–45). Sections 4 and 7, moreover, might be interpreted as showing a mitochondrion in the process of dividing. The true three-dimensional shape, however, can be reconstructed from serial sections. (A, courtesy of Stefan Hell.)

image, the SEM uses electrons that are scattered or emitted from the specimen's surface. The specimen to be examined is fixed, dried, and coated with a thin layer of heavy metal. Alternatively, it can be rapidly frozen, and then transferred to a cooled specimen stage for direct examination in the microscope. Often an entire plant part or small animal can be put into the microscope with very little preparation (**Figure 9–48**). The specimen, prepared in any of these ways, is then scanned with a very narrow beam of electrons. The quantity of electrons scattered or emitted as this primary beam bombards each successive point of the metallic surface is measured and used to control the intensity of a second beam, which moves in synchrony with the primary beam and forms an image on a television screen. In this way, a highly enlarged image of the surface as a whole is built up (**Figure 9–49**).

The SEM technique provides great depth of field; moreover, since the amount of electron scattering depends on the angle of the surface relative to the beam, the image has highlights and shadows that give it a three-dimensional appearance (see Figure 9–48 and **Figure 9–50**). Only surface features can be examined, however, and in most forms of SEM, the resolution attainable is not very high (about 10 nm, with an effective magnification of up to 20,000 times). As a result, the technique is usually used to study whole cells and tissues rather than subcellular organelles. <AACG> Very high-resolution SEMs have, however, been developed with a bright coherent-field emission gun as the electron source. This type of SEM can produce images that rival TEM images in resolution (**Figure 9–51**).

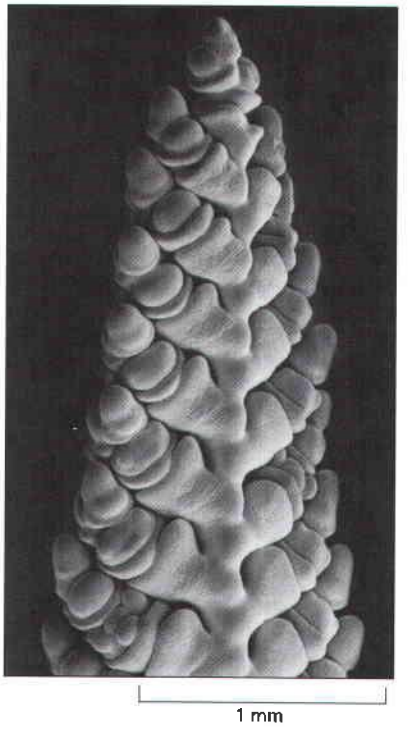


Figure 9–48 A developing wheat flower, or spike. This delicate flower spike was rapidly frozen, coated with a thin metal film, and examined in the frozen state in a SEM. This micrograph, which is at a low magnification, demonstrates the large depth of focus of the SEM. (Courtesy of Kim Findlay.)

Metal Shadowing Allows Surface Features to Be Examined at High Resolution by Transmission Electron Microscopy

The TEM can also be used to study the surface of a specimen—and generally at a higher resolution than in the SEM—to reveal the shape of individual macromolecules for example. As in scanning electron microscopy, a thin film of a heavy metal such as platinum is evaporated onto the dried specimen. In this case, however, the metal is sprayed from an oblique angle so as to deposit a coat-

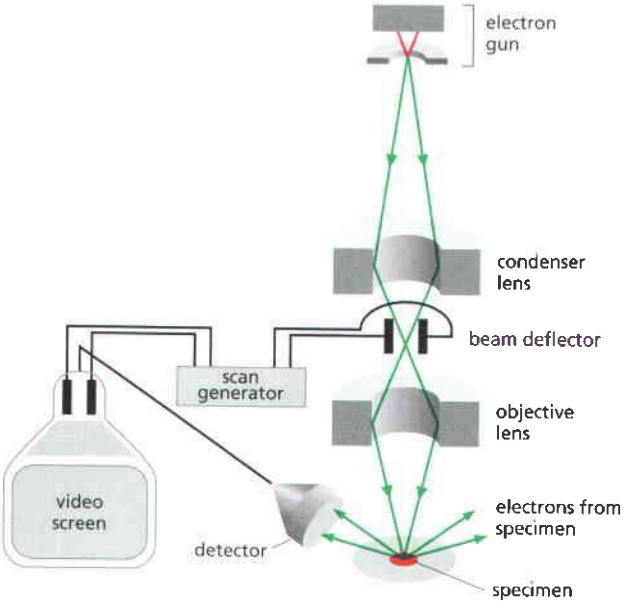


Figure 9–49 The scanning electron microscope. In a SEM, the specimen is scanned by a beam of electrons brought to a focus on the specimen by the electromagnetic coils that act as lenses. The detector measures the quantity of electrons scattered or emitted as the beam bombards each successive point on the surface of the specimen and controls the intensity of successive points in an image built up on a video screen. The SEM creates striking images of three-dimensional objects with great depth of focus and a resolution between 3 nm and 20 nm depending on the instrument. (Photograph courtesy of Andrew Davies.)

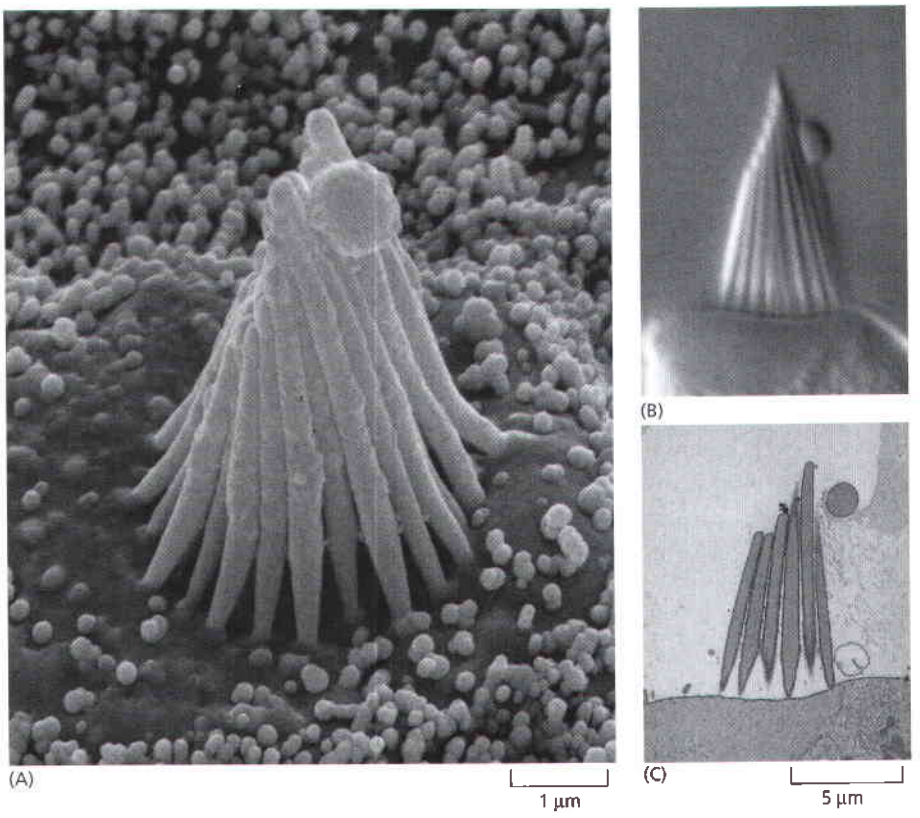


Figure 9–50 Scanning electron microscopy. (A) A scanning electron micrograph of the stereocilia projecting from a hair cell in the inner ear of a bullfrog. <CATA> For comparison, the same structure is shown by (B) differential-interference-contrast light microscopy and (C) thin-section transmission electron microscopy. (Courtesy of Richard Jacobs and James Hudspeth.)

ing that is thicker in some places than others—a process known as *metal shadowing* because a shadow effect is created that gives the image a three-dimensional appearance.

Some specimens coated in this way are thin enough or small enough for the electron beam to penetrate them directly. This is the case for individual molecules, macromolecular complexes, and viruses—all of which can be dried down, before shadowing, onto a flat supporting film made of a material that is relatively transparent to electrons, such as carbon or plastic. The internal structure of cells can also be imaged using metal shadowing. In this case samples are very rapidly frozen (as described above) and then cracked open with a knife blade. The ice level at the fractured surface is lowered by the sublimation of ice in a vacuum as the temperature is raised—in a process called freeze-drying. The parts of the cell exposed by this *etching* process are then shadowed as before to make a metal replica. The organic material of the cell remains must be dissolved away after shadowing to leave only the thin metal *replica* of the surface of the specimen. The replica is then reinforced with a film of carbon so it can be placed on a grid and examined in the transmission electron microscope in the ordinary way (**Figure 9–52**). This technique exposes structures in the interior of the cell and can reveal their three-dimensional organization with exceptional clarity (**Figure 9–53**).

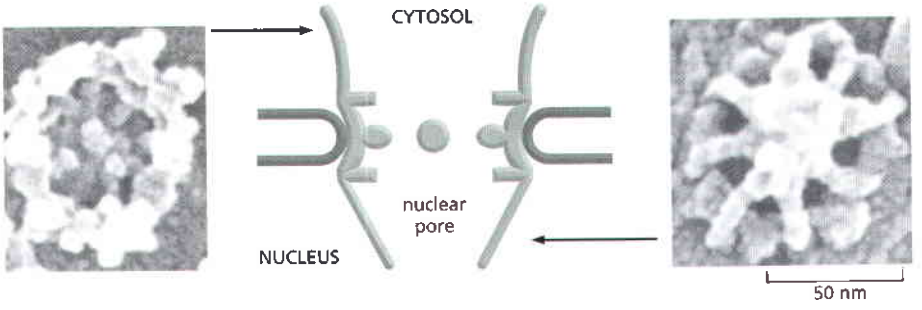


Figure 9–51 The nuclear pore. Rapidly frozen nuclear envelopes were imaged in a high-resolution SEM, equipped with a field emission gun as the source of electrons. These views of each side of a nuclear pore represent the limit of resolution of the SEM, and should be compared with Figure 12–9. (Courtesy of Martin Goldberg and Terry Allen.)

Negative Staining and Cryoelectron Microscopy Both Allow Macromolecules to Be Viewed at High Resolution

Although isolated macromolecules, such as DNA or large proteins, can be visualized readily in the electron microscope if they are shadowed with a heavy metal to provide contrast, finer detail can be seen by using **negative staining**. In this technique, the molecules, supported on a thin film of carbon, are mixed with a solution of a heavy-metal salt such as uranyl acetate. After the sample has dried, a very thin film of metal salt covers the carbon film everywhere except where it has been excluded by the presence of an adsorbed macromolecule. Because the macromolecule allows electrons to pass through it much more readily than does the surrounding heavy-metal stain, a reversed or negative image of the molecule is created. Negative staining is especially useful for viewing large macromolecular aggregates such as viruses or ribosomes, and for seeing the subunit structure of protein filaments (Figure 9–54).

Shadowing and negative staining can provide high-contrast surface views of small macromolecular assemblies, but the size of the smallest metal particles in the shadow or stain used limits the resolution of both techniques. Recent methods provide an alternative that has allowed us to visualize directly at high resolution even the interior features of three-dimensional structures such as viruses and organelles. In this technique, called **cryoelectron microscopy**, rapid freezing to form vitreous ice is again the key. A very thin (about 100 nm) film of an aqueous suspension of virus or purified macromolecular complex is prepared on a microscope grid. The specimen is then rapidly frozen by plunging it into a coolant. A special sample holder is used to keep this hydrated specimen at −160°C in the vacuum of the microscope, where it can be viewed directly without fixation, staining, or drying. Unlike negative staining, in which what we see is the envelope of stain exclusion around the particle, hydrated cryoelectron microscopy produces an image from the macromolecular structure itself. However, to extract the maximum amount of structural information, special image-processing techniques must be used, as we describe next.

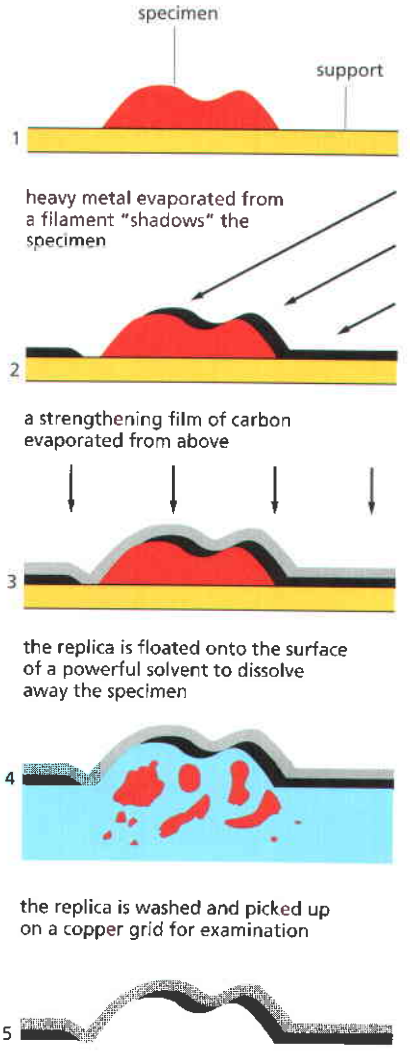


Figure 9–52 The preparation of a metal-shadowed replica of the surface of a specimen. Note that the thickness of the metal reflects the surface contours of the original specimen.

Multiple Images Can Be Combined to Increase Resolution

Any image, whether produced by an electron microscope or by an optical microscope, is made by particles—electrons or photons—striking a detector of some sort. But these particles are governed by quantum mechanics, so the numbers reaching the detector are predictable only in a statistical sense. In the limit of very large numbers of particles, the distribution at the detector is accurately determined by the imaged specimen. However, with smaller numbers of particles, this underlying structure in the image is obscured by the statistical fluctuations in the numbers of particles detected in each region. The term *noise* describes the random variability that confuses the underlying image of the spec-

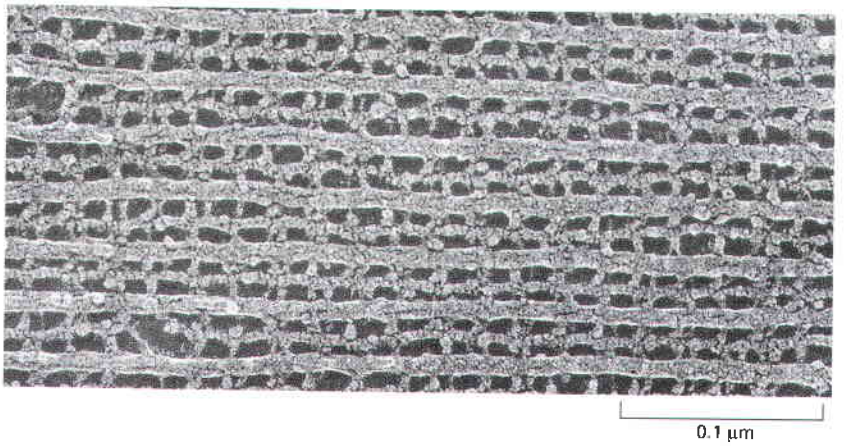


Figure 9–53 A regular array of protein filaments in an insect muscle. To obtain this image, the muscle cells were rapidly frozen to liquid helium temperature, fractured through the cytoplasm, and subjected to deep etching. A metal-shadowed replica was then prepared and examined at high magnification. (Courtesy of Roger Cooke and John Heuser.)

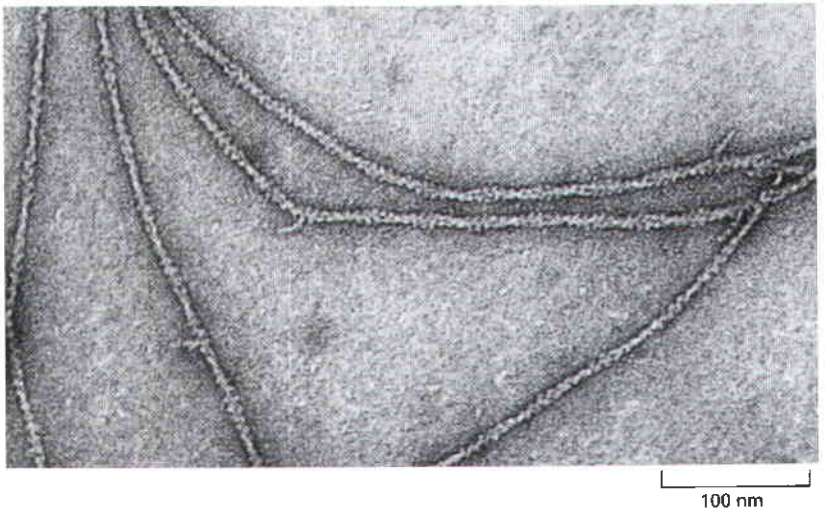


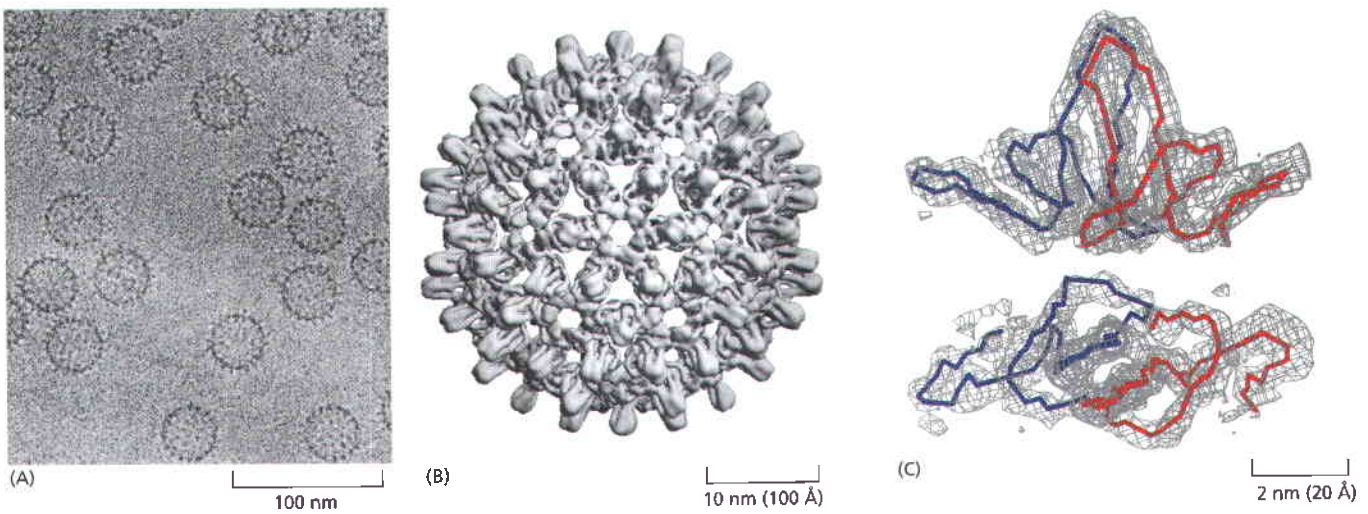
Figure 9–54 Negatively stained actin filaments. In this transmission electron micrograph, each filament is about 8 nm in diameter and is seen, on close inspection, to be composed of a helical chain of globular actin molecules. (Courtesy of Roger Craig.)

imen itself. Noise is important in light microscopy at low light levels, but it is a particularly severe problem for electron microscopy of unstained macromolecules. A protein molecule can tolerate a dose of only a few tens of electrons per square nanometer without damage, and this dose is orders of magnitude below what is needed to define an image at atomic resolution.

The solution is to obtain images of many identical molecules—perhaps tens of thousands of individual images—and combine them to produce an averaged image, revealing structural details that were hidden by the noise in the original images. This procedure is called **single-particle reconstruction**. Before combining all the individual images, however, they must be aligned with each other. Sometimes it is possible to induce proteins and complexes to form crystalline arrays, in which each molecule is held in the same orientation in a regular lattice. In this case, the alignment problem is easily solved, and several protein structures have been determined at atomic resolution by this type of electron crystallography. In principle, however, crystalline arrays are not absolutely required. With the help of a computer, the digital images of randomly distributed and unaligned molecules can be processed and combined to yield high-resolution reconstructions. <TATT> Although structures that have some intrinsic symmetry make the task of alignment easier and more accurate, this technique has has also been used for objects, like ribosomes, with no symmetry. **Figure 9–55** shows the structure of an icosahedral virus that has been determined at high resolution by the combination of many particles and multiple views.

With well-ordered crystalline arrays, a resolution of 0.3 nm has been achieved by electron microscopy—enough to begin to see the internal atomic

Figure 9–55 Single-particle reconstruction. Spherical protein shells of the hepatitis B virus are preserved in a thin film of ice (A) and imaged in the transmission electron microscope. Thousands of individual particles were combined by single-particle reconstruction to produce the three-dimensional map of the icosahedral particle shown in (B). The two views of a single protein dimer (C), forming the spikes on the surface of the shell, show that the resolution of the reconstruction (0.74 nm) is sufficient to resolve the complete fold of the polypeptide chain. (A, courtesy of B. Böttcher, S.A. Wynne, and R.A. Crowther; B and C, from B. Böttcher, S.A. Wynne, and R.A. Crowther, *Nature* 386:88–91, 1997. With permission from Macmillan Publishers Ltd.)



arrangements in a protein and to rival x-ray crystallography in resolution. With single-particle reconstruction, the present limit is about 0.5 nm, enough to identify protein subunits and domains, and limited protein secondary structure. Although electron microscopy is unlikely to supersede x-ray crystallography (discussed in Chapter 8) as a method for macromolecular structure determination, it has some very clear advantages. First, it does not absolutely require crystalline specimens. Second, it can deal with extremely large complexes—structures that may be too large or too variable to crystallize satisfactorily.

The analysis of large and complex macromolecular structures is helped considerably if the atomic structure of one or more of the subunits is known, for example from x-ray crystallography. Molecular models can then be mathematically "fitted" into the envelope of the structure determined at lower resolution using the electron microscope. Figure 9–56 shows the structure of a ribosome with the location of a bound release factor displayed in this way (see also Figures 6–74 and 6–75).

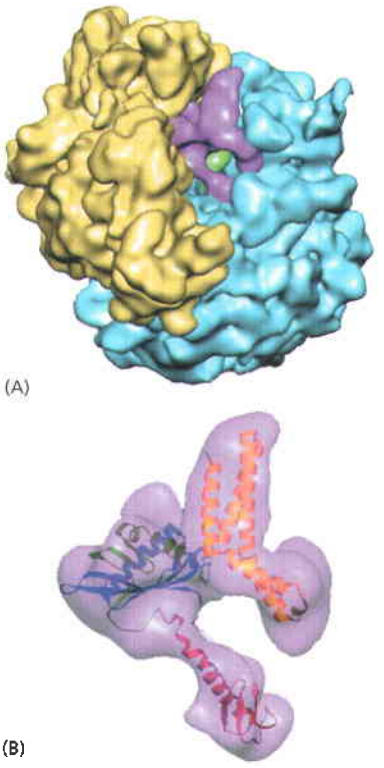


Figure 9–56 Single-particle reconstruction and molecular model fitting. Bacterial ribosomes, with and without the release factor required for peptide release from the ribosome, were used here to derive high-resolution three-dimensional cryo-EM maps at a resolution of better than 1 nm. Images of nearly 20,000 separate ribosomes, preserved in ice, were used to produce single particle reconstructions. In (A) the 30S ribosomal subunit *(yellow)* and the 50S subunit *(blue)* can be distinguished from the additional electron density that can be attributed to the release factor RF2 *(pink)*. The known molecular structure of RF2 has then been modeled into this electron density (B). (From U.B.S. Rawat et al., *Nature* 421:87–90, 2003. With permission from Macmillan Publishers Ltd.)

Different Views of a Single Object Can Be Combined to Give a Three-dimensional Reconstruction

The detectors used to record images from electron microscopes produce two-dimensional pictures. Because of the large depth of field of the microscope, all the parts of the three-dimensional specimen are in focus, and the resulting image is a projection of the structure along the viewing direction. The lost information in the third dimension can be recovered if we have views of the same specimen from many different directions. The computational methods for this technique were worked out in the 1960s, and they are widely used in medical computed tomography (CT) scans. In a CT scan, the imaging equipment is moved around the patient to generate the different views. In **electron-microscope (EM) tomography**, the specimen holder is tilted in the microscope, which achieves the same result. In this way, we can arrive at a three-dimensional reconstruction, in a chosen standard orientation, by combining a set of different views of a single object in the microscope's field of view. Each individual view will be very noisy, but by combining them in three dimensions and taking an average, the noise can be largely eliminated, yielding a clear view of the molecular structure. Starting with thick plastic sections of embedded material, three-dimensional reconstructions, or *tomograms*, <ATCC> <CGAT> are used extensively to describe the detailed anatomy of small regions of the cell, such as the Golgi apparatus (Figure 9–57) or the cytoskeleton. Increasingly, however, microscopists are applying EM tomography to unstained frozen hydrated sections, and even to rapidly frozen whole cells or organelles (Figure 9–58). Electron microscopy now provides a robust bridge between the scale of the single molecule and that of the whole cell.

Summary

Determining the detailed structure of the membranes and organelles in cells requires the higher resolution attainable in a transmission electron microscope. Specific macromolecules can be localized with colloidal gold linked to antibodies. Three-dimensional views of the surfaces of cells and tissues are obtained by scanning electron microscopy. The shapes of isolated macromolecules that have been shadowed with a heavy metal or outlined by negative staining can also be readily determined by electron microscopy. Using computational methods, either multiple images or views from different directions can be combined to produce detailed reconstructions of macromolecules and molecular complexes through the techniques of electron tomography and single-particle reconstruction, often applied to cryo-preserved specimens. The resolution obtained with these methods means that atomic structures of individual macromolecules can often be "fitted" to the images derived by electron microscopy, and that the TEM is increasingly able to completely bridge the gap between structures determined by x-ray crystallography and those determined in the light microscope.

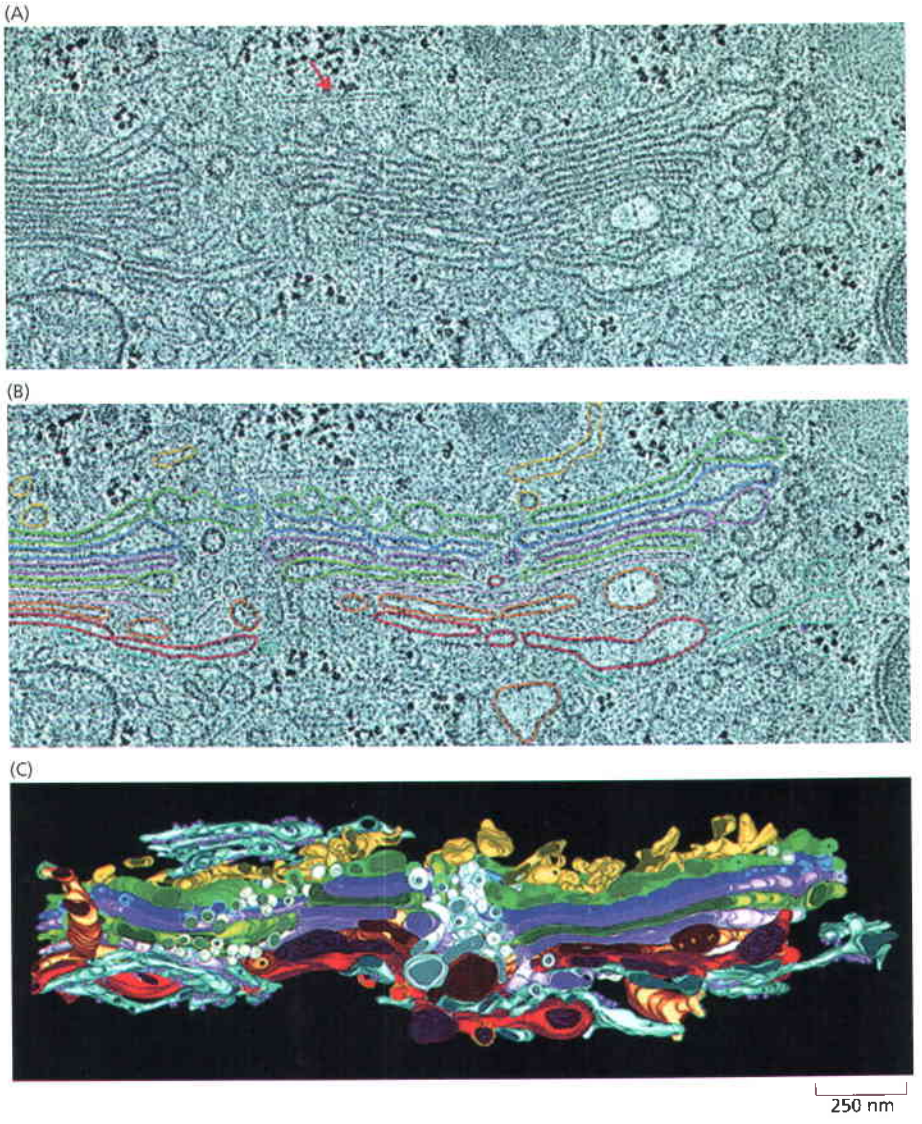


Figure 9–57 Electron microscope (EM) tomography. Samples that have been rapidly frozen, and then freeze-substituted and embedded in plastic, preserve their structure in a condition that is very close to their original living state. This experiment shows an analysis of the three-dimensional structure of the Golgi apparatus from a rat kidney cell prepared in this way. Several thick sections (250 nm) of the cell have been tilted in a high-voltage electron microscope, along two different axes, and about 160 different views recorded. The digital data were combined using EM tomography methods to produce a final three-dimensional reconstruction at a resolution of about 7 nm. The computer can then present very thin slices of the complete three-dimensional data set, or tomogram, and two serial slices, each only 4 nm thick, are shown here (A) and (B). Very little changes from one slice to the next, but using the full data set, and by manually color coding the membranes (B), a full three-dimensional picture of the complete Golgi complex and its associated vesicles can be presented (C). (From M.S. Ladinsky et al., *J.Cell Biol.* 144:1135–1149, 1999. With permission from The Rockefeller University Press.)

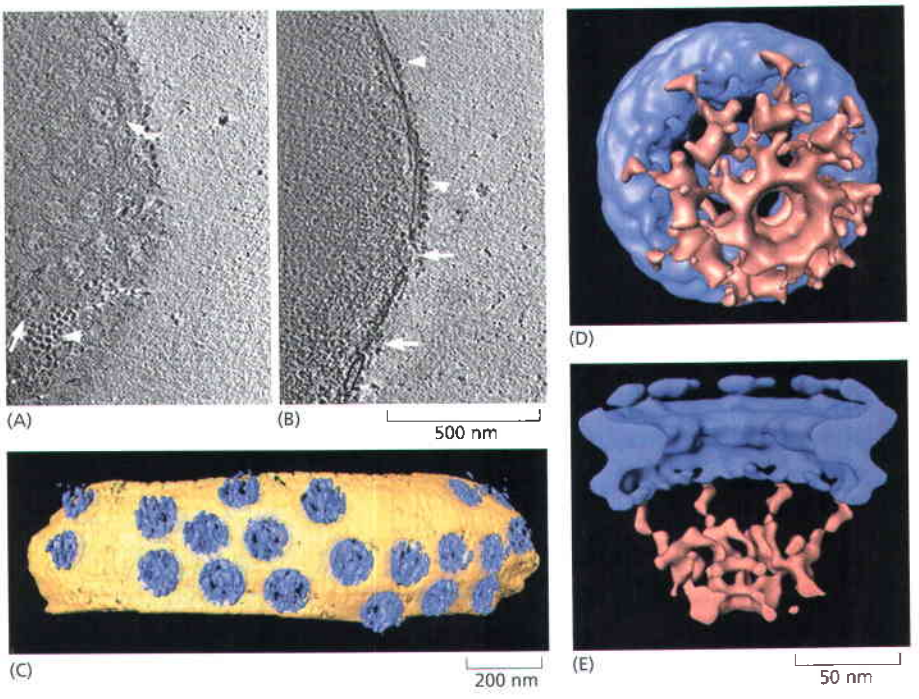


Figure 9–58 Combining cryo-EM tomography and single-particle reconstruction. In addition to sections, the technique of EM tomography may also be applied to small unfixed specimens that are rapidly frozen and examined, while still frozen, using a tilting stage in the microscope. In this experiment the small nuclei of *Dictyostelium* are gently isolated and then very rapidly frozen before a series of tilted views of them is recorded. These different digital views are combined by EM tomography methods to produce a three-dimensional tomogram. Two thin digital slices (10 nm) through this tomogram are shown, in which top views (A) and side views (B) of individual nuclear pores can be seen. In the three-dimensional model (C), a surface rendering of the pores *(blue)* can be seen embedded in the nuclear envelope *(yellow)*. From a series of tomograms it was possible to extract data sets for nearly 300 separate nuclear pores, whose structures could then be averaged using the techniques of single particle reconstruction. The surface-rendered view of one of these reconstructed pores is shown from the nuclear face in (D) and in section in (E) and should be compared with Figure 12–10. The pore complex is colored *blue* and the nuclear basket *brown*. (From M. Beck et al., *Science* 306:1387–1390, 2004. With permission from AAAS.)

PROBLEMS

Which statements are true? Explain why or why not.

9–1 Because the DNA double helix is only 10 nm wide—well below the resolution of the light microscope—it is impossible to see chromosomes in living cells without special stains.

9–2 A fluorescent molecule, having absorbed a single photon of light at one wavelength, always emits it at a longer wavelength.

9–3 Caged molecules can be introduced into a cell and then activated by a strong pulse of laser light at the precise time and cellular location chosen by the experimenter.

Discuss the following problems.

9–4 The diagrams in **Figure Q9–1** show the paths of light rays passing through a specimen with a dry lens and with an oil-immersion lens. Offer an explanation for why oil-immersion lenses should give better resolution. Air, glass, and oil have refractive indices of 1.00, 1.51, and 1.51, respectively.

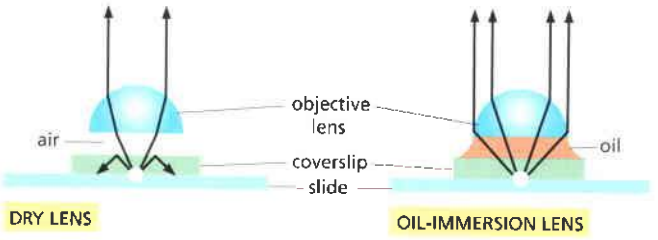


Figure Q9–1 Paths of light rays through dry and oil-immersion lenses (Problem 9–4). The *white circle* at the origin of the light rays is the specimen.

9–5 **Figure Q9–2** shows a diagram of the human eye. The refractive indices of the components in the light path are: cornea 1.38, aqueous humor 1.33, crystalline lens 1.41, and vitreous humor 1.38. Where does the main refraction—the main focusing—occur? What role do you suppose the lens plays?

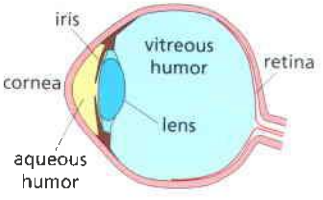


Figure Q9–2 Diagram of the human eye (Problem 9–5).

9–6 Why do humans see so poorly underwater? And why do goggles help?

9–7 Explain the difference between resolution and magnification.

9–8 Antibodies that bind to specific proteins are important tools for defining the locations of molecules in cells. The sensitivity of the primary antibody—the antibody that reacts with the target molecule—is often enhanced by using labeled secondary antibodies that bind to it. What are the advantages and disadvantages of using secondary antibodies that carry fluorescent tags versus those that carry bound enzymes?

9–9 **Figure Q9–3** shows a series of modified GFPs that emit light in a range of colors. How do you suppose the exact same chromophore can fluoresce at so many different wavelengths?

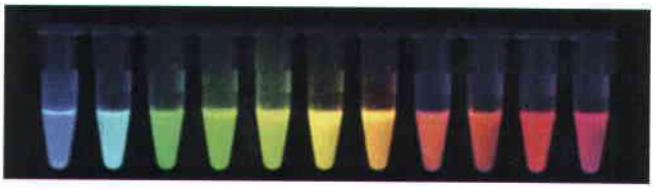


Figure Q9–3 A rainbow of colors produced by modified GFPs (Problem 9–9). (From R.F. Service, *Science* 306:1457, 2004. With permission from AAAS.)

9–10 Consider a fluorescent detector designed to report the cellular location of active protein tyrosine kinases. A blue (cyan) fluorescent protein (CFP) and a yellow fluorescent protein (YFP) were fused to either end of a hybrid protein domain. The hybrid protein segment consisted of a substrate peptide recognized by the Abl protein tyrosine kinase and a phosphotyrosine binding domain (**Figure Q9–4A**). Stimulation of the CFP domain does not cause emission by the YFP domain when the domains are separated. When the CFP and YFP domains are brought close together, however, fluorescence resonance energy transfer (FRET) allows excitation of CFP to stimulate emission by YFP. FRET shows up experimentally as an increase in the ratio of emission at 526 nm versus 476 nm (YFP/CFP) when CFP is excited by 434-nm light.

Incubation of the reporter protein with Abl protein tyrosine kinase in the presence of ATP gave an increase in YFP/CFP emission (Figure Q9–4B). In the absence of ATP or the Abl protein, no FRET occurred. FRET was also eliminated by addition of a tyrosine phosphatase (Figure Q9–4B). Describe as best you can how the reporter protein detects active Abl protein tyrosine kinase.

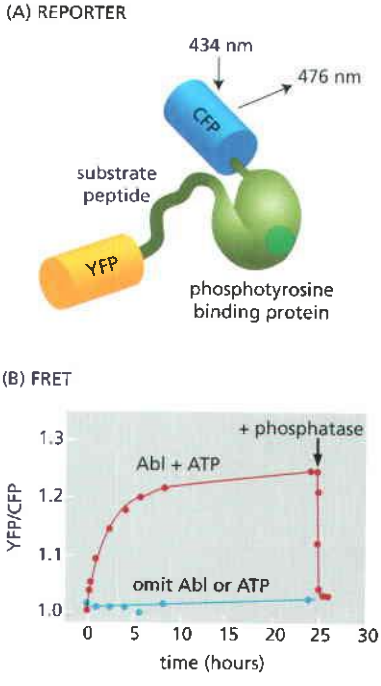


Figure Q9–4 Fluorescent reporter protein designed to detect tyrosine phosphorylation (Problem 9–10). (A) Domain structure of reporter protein. Four domains are indicated: CFP, YFP, tyrosine kinase substrate peptide, and a phosphotyrosine-binding domain. (B) FRET assay. YFP/CFP is normalized to 1.0 at time zero. The reporter was incubated in the presence (or absence) of Abl and ATP for the indicated times. *Arrow* indicates time of addition of a tyrosine phosphatase. (From A.Y. Ting, K.H. Klain, R.L. Klemke and R.Y. Tsien, *Proc. Natl Acad. Sci. U.S.A.* 98:15003–15008, 2001. With permission from National Academy of Sciences.)

9–11 The practical resolving power of modern electron microscopes is around 0.1 nm. The major reason for this constraint is the small numerical aperture ($n \sin \theta$), which is

limited by θ (half the angular width of rays collected at the objective lens). Assuming that the wavelength (λ) of the electron is 0.004 nm and that the refractive index (n) is 1.0, calculate the value for θ. How does that value compare with a θ of 60°, which is typical for light microscopes?

$$\text{resolution} = \frac{0.61\,\lambda}{n \sin\theta}$$

9–12 It is difficult to tell bumps from pits just by looking at the pattern of shadows. Consider **Figure Q9–5**, which shows a set of shaded circles. In Figure Q9–5A the circles appear to be bumps; however, when the picture is simply turned upside down (Figure Q9–5B), the circles seem to be pits. This is a classic illusion. The same illusion is present in metal shadowing, as shown in the two electron micrographs in Figure Q9–5. In one the membrane appears to be covered in bumps, while in the other the membrane looks heavily pitted. Is it possible for an electron microscopist to be sure that one view is correct, or is it all arbitrary? Explain your reasoning.

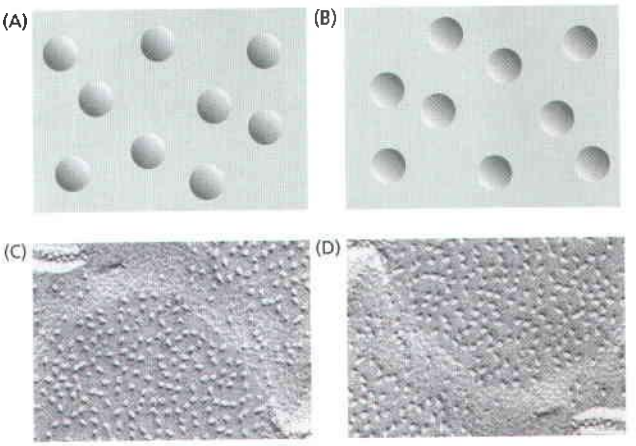


Figure Q9–5 Bumps and pits (Problem 9–12). **(A)** Shaded circles that look like bumps. **(B)** Shaded circles that look like pits. **(C)** An electron micrograph oriented so that it appears to be covered with bumps. **(D)** An electron micrograph oriented so that it appears to be covered with pits. (C and D, courtesy of Andrew Staehelin.)

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