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DNA Repair in Bacteriophage

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1. INTRODUCTION

Starting with the very first studies on DNA repair in 1947, most of the earliest work, involving recombinational repair, photoreactivation, and excision repair, were carried out with bacteriophage (phage) T4 (reviewed in *4,6*). Repair processes in phage appear to be similar to DNA repair processes in other organsims, and genes necessary for DNA repair in phage, such as phage T4 genes *denV* and *uvsX,* are homologous to repair genes in bacteria and eucaryotes (*see* Sub heading 7.). Thus, study of DNA repair processes in phage illuminates the mechanisms and adaptive functions of similar, but often more complex, processes in bacteria and eucaryotes.

The various phages differ in the extent to which they depend on host functions for repair of DNA damage. Two well-studied examples at opposite ends of the spectrum are phage T4 and phage λ. Phage T4 depends largely on gene products encoded by the phage genome itself, while phage λ depends to a great extent on bacterial host functions. In this review we have specifically focused on repair processes encoded by phage genomes. DNA repair processes of phage reliant on bacterial host gene products are ordinarily considered in the context of bacterial repair processes.

2. PATHWAYS OF REPAIR

The first two repair processes discussed in this section, DenV associated base-excision repair and photoreactivation, are specific to ultraviolet (UV)-induced pyrimidine dimers. The next repair process discussed, multiplicity reactivation (MR), is a general recombinational repair process that can act against a wide variety of lesions. It is highly effective against lesions caused by reactive oxygen species, which are likely to be important under natural circumstances. Double-strand break (DSB) repair also appears to be a recombinational repair process similar or identical to MR. Mismatch repair in phage T4 is a specialized process for resolving mismatched bases that can arise in heteroduplex DNA formed during recombination. In post-replication recombinational repair (PRRR), a DNA molecule with a single-strand damage replicates and forms two daughter molecules, one of which has a single-strand gap opposite the damaged template strand. This gap can then be filled in by recombination with the intact daughter chromosome. This process does not directly remove the damage, but rather allows

From: *DNA Damage and Repair, Vol. 3: Advances from Phage to Humans*
Edited by: J. A. Nickoloff and M. F. Hoekstra © Humana Press Inc., Totowa, NJ

accurate bypass of the damage. The Luria-Latarjet effect appears to be a consequence of both base-excision repair and recombinational repair. Error-prone repair may reflect lower accuracy of the DNA synthesis associated with a recombinational repair process compared to the DNA synthesis associated with normal chromosome replication. Finally, replication repair occurs by an unknown mechanism that appears to be independent of base-excision repair and recombinational repair.

2.1 Base-Excision Repair

In phage T4, excision repair processes appear to be limited to the pathway of base-excision repair of UV-induced pyrimidine dimers in which the first enzyme to act is DNA endonuclease V (DenV, the product of T4 gene *denV*). T4 DenV is a bifunctional enzyme with both a pyrimidine-dimer specific glycosylase activity and an apurinic/apyrimidinic (AP) lyase activity. The pathway of DenV mediated base-excision repair has been extensively studied (the early work is reviewed in ref. [6] and more recent studies are reviewed by Valerie [67]). The first step in the pathway is the cleavage of the N-glycosyl bond at the 5′-side of the pyrimidine dimer by the action of the glycosylase activity of the DenV protein, resulting in an AP site. This is followed by incision of the sugar phosphate backbone on the 3′ side of the AP site by the AP lyase activity of DenV. Then a second incision on the 5′ side of the AP site is made, presumably by the AP lyase activity of T4 Gp30 DNA ligase (*11;* Subheading 3.11.). The next step is thought to be removal of the remaining portion of the pyrimidine dimer by the 5′→3′ exonuclease activity of the host *Escherichia coli* DNA polymerase I. Concomitant with this removal, the single-strand gap created by the removal is filled in by the polymerizing activity of DNA polymerase I. The average patch size is between 4 and 7 nucleotides. Repair is completed by DNA ligase (Gp30), which seals the last phosphodiester bond.

2.2. Photoreactivation

Photoreactivation is the enzyme-mediated light-dependent monomerization of pyrimidine dimers, resulting in repair of DNA. In T-even phage, photoreactivation is likely owing to a host enzyme, since this process occurs in phage T2 as early as 10 seconds after adsorption. Photoreactivation appears to be a major mode of repair of UV-induced lethal damage in phage T4 (reviewed in *4,6*). Photoreactivation also has been studied in phages that infect *Vibrio cholerae.* UV-induced DNA damage in phages of different morphological and serological groups is efficiently photoreactivated *(3,55,61).*

2.3. Multiplicity Reactivation

When phage are treated by a DNA damaging agent (causing reduced survival), it is often found that allowing multiple phage to infect each cell gives a level of survival of infective centers that is substantially greater than would be expected from the presence of multiple independent targets. This phenomenon, referred to as multiplicity reactivation (MR), implies that when more than one phage chromosome are present within a host cell, a DNA repair process is available that is not active when there is only one phage chromosome per cell. A substantial amount of evidence (reviewed in *4,6*) indicates that MR is a recombinational repair process. First, recombination is implicated because, by definition, the process requires two or more chromosomes. Second, for phage T4 damaged by UV, nitrous acid (HNO_2) or mitomycin C (MMC), MR depends

on several gene functions required for normal levels of spontaneous recombination (e.g., Gp32, Gp46, Gp47, UvsX, and UvsY). Third, under conditions where MR occurs, the frequency of genetic recombination increases. Fourth, a mutation in gene *46* or *47* reduces or eliminates both MR of HNO_2-damaged phage and the increased genetic recombination caused by HNO_2 damage. Hydrogen peroxide (H_2O_2) and its free-radical product, the hydroxyl radical (OH•) are ubiquitous major sources of DNA damage in living organisms. The lethal DNA damages caused by H_2O_2 in phage T4 were repaired by MR more efficiently than most other types of damages studied *(14).* MR has also been found in phage T7 inactivated by methyl methanesulfonate (MMS) *(35).*

2.4. Double-Strand Break Repair

DSBs are produced by X-rays and by the decay of incorporated ^{32}P. Other lesions are also produced in both cases, but inactivation of the phage is correlated with DSBs (reviewed in *43*). Thus, the MR observed after damage caused by either ^{32}P decay or X-irradiation is inferred to involve DSB repair. Other studies suggest that a special protein-linked form of DSB can also be recombinationally repaired. The anti-tumor acridine *m*-AMSA inhibits the growth of phage T4 by targeting the phage-encoded type II DNA topoisomerase *(31).* Topoisomerase-deficient mutants were shown to be resistant to *m*-AMSA, indicating that *m*-AMSA inhibits growth by blocking the cleavage complex from being further processed, rather than by inhibiting enzyme activity. The inhibitor traps a reaction intermediate consisting of a covalent protein-DNA complex in which both 5′ ends of a DSB are linked, via phosphotyrosine bonds, to the topoisomerase. This complex appears to be subject to recombinational repair on the basis of evidence that mutations in genes necessary for recombination (*uvsX, uvsY, 46/47,* and *59*) each increase sensitivity to *m*-AMSA, and *m*-AMSA stimulates recombination during T4 infection *(54).* Sensitivity to *m*-AMSA was also increased by a mutation in *uvsW (72).* Furthermore, a mutation in the *rnh* gene (RNase H and 5′ to 3′ exonuclease) also increased sensitivity to *m*-AMSA, and to UV as well, suggesting that the Rnh protein may be involved in recombinational repair *(72).*

Topoisomerase I (TOP1)-mediated DNA damage induced by camptothecin in the presence of active transcription was studied using purified calf-thymus TOP1 and phage T7 RNA polymerase *(73).* Transcription elongation processed reversible TOP1-camptothecin-DNA cleavable complexes into irreversible strand breaks on the template, but not on the nontemplate strand, within the transcribed region. This suggests a model in which collision between the TOP1-cleavable complexes located on the template strand and the elongating RNA polymerase results in transcription arrest and conversion of TOP1 cleavable complexes into strand breaks.

DSB repair in phage T4 was also investigated using a physical assay that involved a plasmid substrate with two inverted repeat DNA segments. A DSB introduced into one repeat during a T4 infection induces efficient DSB repair using the second repeat as a template. This reaction was coupled to plasmid replication, was frequently associated with exchange of flanking DNA, and had an absolute requirement for the products of genes *uvsX, uvsY, 32, 46,* and *47 (26).*

DSB repair was demonstrated in an in vitro phage T7 DNA replication and packaging system as well. It was found to be highly recombinogenic, indicating that a recombinational repair mechanism was involved *(48).*

2.5. Mismatch Repair

When two T4 phage, differing by mutation, infect the same host cell they may recombine with each other, thereby generating a heteroduplex region of DNA as an intermediate in recombination, with base mismatches. Base mismatches can be repaired—that is, converted to standard base pairs—during the recombination process. This repair appears to occur by a pathway that includes sequential action of endonuclease VII (Gp49), the $3' \rightarrow 5'$ exonuclease of the T4 DNA polymerase (Gp43), the DNA polymerizing activity of Gp43, and the DNA ligase (Gp30) (reviewed in *62,63*). In this proposed pathway, endonuclease VII cleaves a strand at a mismatched nucleotide producing a 3′-unpaired end, and T4 DNA polymerase excises the nonmatched single strand (with its $3' \rightarrow 5'$ exonuclease) and then fills in the resulting gap. Two mutants (defective in different genes) lacking mismatch repair have been isolated *(30).*

2.6. Post-Replication Recombinational Repair

The concept of post-replication recombinational repair (PRRR) was first proposed by Harm in 1964 for phage T4 and, at about the same time, by Howard Flanders for *E. coli* (reviewed in *6*). In phage, PRRR is a repair process that occurs in single infections (in contrast to MR). In phage T4, mutants defective in PRRR are identified by their (1) increased sensitivity to DNA-damaging agents in single infections compared to wild-type, and (2) reduced genetic recombination. By these criteria, the products of genes *32,46,47,59, uvsW, uvsX,* and *uvsY* are considered to be involved in PRRR (reviewed in *4,6*). A pathway for PRRR in phage T4 was proposed on the basis of the functions of the gene products that appear to be involved in the process, using the better understood PRRR in *E. coli* as a model *(6).*

2.7. Luria-Latarjet Effect

As reviewed by Hyman *(33),* Luria and Latarjet in 1947, using phage T2, carried out one of the earliest studies of DNA repair, although the result they described, later designated the Luria-Latarjet effect, was not interpreted in terms of DNA repair at the time of the study. The Luria-Latarjet effect is an increase in resistance of a virus to treatment by a DNA-damaging agent during the course of infection of host cells. Although first demonstrated in phage T2 using UV as the DNA-damaging agent, the Luria-Latarjet effect was later demonstrated with phages T4 and T5, and could be observed with X-rays or ^{32}P as the damaging agent (reviewed in *33*). Evidence was obtained, using a variety of phage DNA repair defective mutants, that the Luria-Latarjet effect is owing to three repair pathways; excision repair, PRRR, and MR *(33).* The Luria-Latarjet effect appears to develop in two stages. The first stage starts soon after infection and involves excision repair or PRRR. The second stage appears to begin after the first round of DNA replication is complete. DNA damage occurring at this stage can apparently be repaired by MR as well as the other two repair pathways.

2.8. Error-Prone Repair

In phage T4, the hypothesis that mutagenesis induced by UV and other DNA damaging agents occurs by error-prone repair is an attractive explanation for a number of observations (*see 4,6* for review of early work). A mutation in gene *30* (DNA ligase)

decreased UV and HNO_2 mutagenesis, and anti-mutator alleles of gene *43* (DNA polymerase) were found to reduce UV and psoralen-plus-UVA-light (PUVA) mutagenesis (reviewed in *4; see* also *22*). Mutations in other components of the replicative complex, i.e., Gps 32, 41, 44, and 45, increased UV-induced mutagenesis (reviewed in *4*). Mutagenesis induction by UV and ionizing radiation during single infections proceeds via a pathway that depends on UvsW, UvsX, and UvsY *(18).* Because these three gene products are necessary for recombinational repair, it appears that the DNA synthesis step(s) of a recombinational repair process (possibly PRRR) may be less accurate, or more inclined to trans-lesion synthesis, than the DNA synthesis associated with chromosome replication. In contrast, MR of UV-damaged phage appears to be an accurate process *(74).*

2.9. Replication Repair

As reviewed by Kreuzer and Drake *(43),* replication repair was initially identified by the finding of phage T4 mutants with increased sensitivity to MMS in a genetic background that was defective in recombinational repair. Later, mutants with increased UV sensitivity were found in a genetic background defective in both base-excision repair and recombinational repair. These mutations, which occurred in genes *32* or *41,* were thought to define a new repair pathway. Replication repair has not yet been identified in other organisms than phage T4, and its mechanism remains unknown.

3. ENZYMES EMPLOYED IN DNA REPAIR AND THEIR MECHANISMS OF ACTION

3.1. DenV of Phage T4

DNA endonuclease V, or DenV, is a small protein of 137 amino acids with N-glycosylase/β-lyase activity (reviewed in *44*). Its three-dimensional structure has been determined by X-ray crystallography *(51).* The enzyme scans nontarget DNA sequences by electrostatic interactions to search for damaged sites and, subsequently, specifically recognizes the pyrimidine dimer site. As can be seen from Table 1, the presence or absence of DenV does not alter susceptibility to lethal damage caused by agents other than UV light. The basic concave surface of endonuclease V interacts with double-stranded DNA that is sharply kinked at the pyrimidine dimer. There appear to be several specific interactions with phosphate groups on both strands occurring in the minor groove of the DNA. DenV is thought to act by flipping an adenine complementary to one of the thymines in the dimer into a cavity on the protein surface. The flipping may allow the enzyme to discriminate between damaged and normal DNA, and to generate an empty space within the DNA helix, to which catalytically important residues in the enzyme can gain access. Several of the specific amino acids of DenV essential for enzymatic activity have been identified (reviewed in *67,70*).

DenV appears to act by a mechanism common to that of other N-glycosylases that have an AP lyase activity such as *E. coli* endonuclease III, and formamidopyrimidine DNA glycosylase. All three enzymes have similar specificities in recognizing, and using as substrates, duplex oligonucleotides containing the base-lesion analogs O-methylhydroxylamine- and O-benzylhydroxylamine-modified abasic (AP) sites *(56).*

Table 1
Relative Numbers of Lethal Lesions Produced During Single Infections When Phage T4 Mutants are Defective in the Indicated Genes[a]

		Relative number of lethal lesions[b] produced by						
Gene defect	Function	UV	PUVA	MNNG	MMS	MMC	HNO_2	H_2O_2[c]
None		1.0	1.0	1.0	1.0	1.0	1.0	1.0
30	Ligase	1.2	1.2		2.9		1.0	
32	Single strand binding	1.4		2.6		1.1	1.3	0.7
41	Helicase	1.2			2.1			
43	Polymerase	1.1	1.2					
46	Exonuclease	1.4	1.5	3.0		1.4	1.2	0.6
47	Exonuclease	1.2	1.5	3.4		1.7	1.3	0.8
58–61	Primase	1.8						
59	Loads 41 helicase	1.6			2.8			
denV	Endonuclease	2.2	1.0	1.0	1.0	1.0	1.0	1.0
uvsX	Strand transfer	1.7	1.6	1.9	1.8	1.5	1.2	0.8
uvsY	Stabilize UvsX	1.7	1.6	1.6	1.9	1.5	1.2	
uvsW	Helicase	1.5	1.4		1.8			
39[d]	Topoisomerase		0.6			1.2		
52[d]	Topoisomerase		0.4			1.3		
60[d]	Topoisomerase	0.5			1.2			
rnh[e]	RNase, exonuclease	1.5						

[a] The values in the table were taken as the largest values reported in reviews by Bernstein *(4)* and Bernstein and *W*allace *(6),* except as indicated by superscripts.

[b] When applied to free phage or to phage-host complexes, all agents caused inactivation of the ability to produce infective centers. In general, when the log of the surviving fraction was plotted vs the dose of the agent, the survival curve was a straight line, although in the case of some UV irradiation curves there was a very small initial shoulder; that is, except for these small shoulders, survival curves represented killing with single-hit kinetics, following the equation $N/N_0 = e^{-kd}$, where N_0 is the number of phage present in an initially chosen population, N is the number surviving phage after treatment, d is the dose of the inactivating agent, and k is the number of lethal lesions introduced per unit dose. The numbers in the table represent $k_{mutant}/k_{wild\text{-}type}$.

[c] These values are from Chen and Bernstein *(14).*

[d] These values are calculated from data presented in Miskimins et al. *(50).*

[e] This value is from Woodworth and Kreuzer *(72).*

3.2. UvsX of Phage T4

The UvsX protein, in association with the products of genes *uvsY, dda,* and *32,* catalyzes homologous DNA pairing and an efficient in vitro strand-transfer reaction *(38,39,40,77,78).* These reactions are thought to be a central feature of any recombinational repair process. As discussed later (Subheading 7.2.), UvsX is a homolog of *E. coli* RecA *(23).* UvsX, like RecA, can assimilate linear single-stranded DNA into homologous superhelical duplexes to produce D-loops. A necessary prerequisite for UvsX protein-mediated pairing is the polymerization of this protein along the invading single strand, a process known as presynapsis. UvsY and the product of gene *32* are involved in this process as well *(34,38).* UvsX, UvsY, and Gp32 are all cooperative single-stranded DNA-binding proteins. UvsY binds specifically to both Gp32 and UvsX. These contacts allow UvsY to mediate binding of UvsX to Gp32-covered single-

stranded DNA. UvsY also acts to stabilize the filament. A series of events involving multiple protein-DNA and protein-protein interactions is required to mediate a transition from an initial Gp32-DNA complex to a mature presynaptic filament in which the UvsX and UvsY proteins are in contact with the DNA and each other, while most or all of the Gp32 is removed from the complex (reviewed in *34*).

The efficiency of UvsX protein-mediated joint molecule formation between supercoiled duplex DNA and oligonucleotides has a sharp dependence on the degree of homology *(60).* The reaction proceeds efficiently with oligonucleotides containing 32 homologous positions but not with oligonucleotides containing only 24 homologous bases. Even a single base-pair mismatch in the middle of a region of 40 homologous nucleotides had a detectable negative effect on the efficiency of pairing.

Mutants defective in the UvsX protein have increased sensitivity in single infections to inactivation by many agents including UV, PUVA, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), MMS, MMC, HNO_2, and H_2O_2 (*see* Table 1), suggesting that UvsX is necessary for a recombinational repair process that can occur in single infections, presumably PRRR (reviewed in *4,6*). Mutants defective in *uvsX* are also deficient in MR in response to damage caused by UV, HNO_2, and H_2O_2 (*14;* also reviewed in *4,6*).

3.3. UvsY of Phage T4

The UvsY protein is required for efficient recombination in T4 infected *E. coli.* It is an accessory protein for catalysis of strand exchange *(28).* UvsY stimulates the homologous pairing catalyzed by UvsX. UvsY accelerates loading of UvsX onto Gp32-covered DNA and stabilizes UvsX single-stranded DNA complexes (reviewed in *59*). The mechanism of filament stabilization seems to involve a slower loss of UvsX subunits. These presynaptic filaments are one of the early essential intermediates in the strand-exchange reaction between homologous single- and double-stranded DNAs. In general, mutants defective in *uvsY* are similar to mutants defective in *uvsX* in their pattern of increased sensitivity in single infection to a variety of DNA damaging agents (Table 1), and in their reduced ability to carry out MR (reviewed in *4,6*).

3.4. Gene Product 32 of Phage T4

T4 Gp32 protein is a helix-destabilizing protein that stimulates UvsX protein catalyzed synapsis *(78)* (*see* Subheading 3.2.). Gp32 appears to have a role in both the formation of joint molecules and in Gp41 helicase-catalyzed polar branch migration (*41; see* also Subheading 3.5.). Phage mutants defective in gene *32* have decreased recombination and increased sensitivity to various DNA damaging agents upon single infection (Table 1), implying that Gp32 is necessary for PRRR (reviewed in *4,6*). These mutants are also defective in MR of phage damaged by UV, HNO_2, MMC, and H_2O_2 *(14;* also reviewed in *4,6).*

Gp32 may also have a role in base-excision repair *(53).* This protein has a strong affinity for nicked AP DNA, suggesting that it may displace DenV from a damaged site once the enzyme has completed its enzymatic reaction, thus allowing DNA polymerase to fill the gap *(65).* The binding of Gp32 to nicked AP sites could also prevent resealing of the DNA by ligase before excision of the damage.

3.5. Gene Product 41 of Phage T4

The Gp41 helicase exhibits a single-stranded DNA-stimulated GTPase/ATPase activity and a 5′ to 3′ DNA helicase activity that is driven by ATP/GTP hydrolysis. Gp41 translocation is processive (and uni-directional) along DNA (reviewed in *21*). Although UvsY is required for homologous pairing, it strongly inhibits branch migration catalyzed by the UvsX protein. Polar branch migration is completely dependent on the gene *41* helicase. The helicase is delivered to the strand-exchange complex by the gene *59* accessory protein in a strand-specific fashion through direct interactions between Gp59 and Gp32 *(59).* The Gp41 helicase functions in both replication and recombination. However, a gene *41* mutation was isolated that specifically impairs recombination and not DNA replication, implying that the recombination function of Gp41 is at least partially separable from the replication function *(76).* Gene *41* mutants are deficient in MR of UV-damaged phage and have increased sensitivity to both UV and MMS in single infections, implying a defect in PRRR (reviewed in *4,6*) (also *see* Table 1).

3.6. Gene Product 59 of Phage T4

Phage T4 gene *59* encodes a protein that facilitates the loading of the T4 helicase (Gp 41) onto recombinational intermediates *(1,52).* Mutants defective in gene *59* have increased sensitivity to UV, MMS and X-rays in single infections, and also have reduced ability to carry out MR of UV-induced damages (reviewed in *4,6*).

3.7. Gene Products 46 and 47 of Phage T4

The products of genes *46* and *47* apparently form a recombinational exonuclease *(49),* but the enzyme has not been purified nor studied extensively. Gene *46* and *47* mutants are defective in both MR and PRRR subsequent to treatment with a wide range of agents (UV, HNO_2, MMC, ethyl methanesulfonate, MNNG, and H_2O_2) (*14;* also reviewed in *4,6*) (also *see* Table 1). Possible homologs of genes *46* and *47* have been found in phage T5 *(10).*

3.8. Junction Resolving Enzymes of Phages T4, T7, and T3

The latter stages of general recombination processes, including recombinational repair, are thought to require the resolution of four-way DNA junctions, also referred to as Holliday structures. This involves enzymes that can both recognize and manipulate these DNA structures. Such enzymes, called X-solvases or resolvases, have been isolated from a wide variety of sources including phage, eubacteria, yeast, mammals, and their viruses (reviewed in *27,36,71*).

Phage T4 endonuclease VII (Gp49), a 157 amino acid protein, is a well-studied example of this class of enzymes (reviewed in *36*). It cleaves four-way junctions by introducing symmetrical cuts in the two strands of like polarity. The nicks can subsequently be sealed by DNA ligase. T4 endonuclease VII has a broader range of substrate specificities than most X-solvases and, besides four-way junctions, it can also cleave three-way junctions, single-strand overhangs, nicks, gaps, heteroduplex loops, base mismatches, curved DNA, and bulky adducts (reviewed in *9*). Endonuclease VII has several domains *(27).* Towards the N-terminal end of the protein lies a section of polypeptide in which four cysteine residues, distributed in a CxxC—CxxC pattern, coordinate one atom of zinc; and in the C-terminal region lies a 31 amino acid sequence

that is 48% identical with a sequence found in the DNA repair protein DenV (*see* Subheading 3.1.). This sequence of endonuclease VII can be replaced with the corresponding sequence from T4 DenV with no change in the pattern of cleavage of four-way junctions *(27).* A dimerization domain has also been identified *(9).*

In phage T4 infections, another four-way resolvase may be present that can partially compensate for loss of endonuclease VII. Mutations in gene *49* were found to reduce genetic recombination, but not MR *(32),* and repair of DSBs was only partially reduced by gene *49* mutations *(26).* Endonuclease VII appears to play a key role in mismatch repair (*see* Subheading 2.5. above) and may therefore contribute to gene conversion, which is thought to occur by heteroduplex repair during genetic recombination *(63).*

Phage T7 undergoes genetic recombination during infection, and this is reduced by mutants defective in T7 endonuclease I (Gp3) (reviewed in *36,71*). This enzyme has been shown to cleave branched DNA species including four-way junctions. The junction-resolving enzymes from T7 and T4 are functionally quite similar. At the amino acid-sequence level, however, there is little similarity between the two, except for a region of about 45 bases where they share 32% identity. Phage T3 encodes an enzyme that is required for genetic recombination and is very similar in sequence and function to T7 endonuclease I.

3.9. The dda Gene of Phage T4

The T4 Dda protein is a DNA helicase that is required to move the T4 replication complex past DNA template-bound proteins in vitro. This helicase also allows the phage-recombination machinery to drive the branch-migration reaction, which the UvsX protein catalyzes, through a RNA polymerase promoter complex *(39,58).* Mutants defective in *dda* are not grossly radiation-sensitive and recombination-deficient, suggesting that another helicase may substitute for the Dda protein, if necessary. (It may be speculated that Gp41 helicase complexed with Gp59 might provide this function.) *(58).*

3.10. UvsW of Phage T4

Mutants of phage T4 defective in *uvsW* have reduced spontaneous recombination and increased sensitivity to UV, PUVA, and MMS (Table 1). UvsW is necessary for normal levels of both MR and PRRR (reviewed in *4,6*). Analysis of the *uvsW* gene *(20),* indicates that the promoter region contains a sequence resembling the consensus for T4 late promoters, and that *uvsW* is expressed as a late gene. UvsW appears to be a helicase that catalyzes branch migration and dissociation of RNA-DNA hybrids *(12).* UvsW was suggested to be the key regulatory factor in the switch from early to late DNA replication *(20).*

3.11. Ligase

Mutants defective in DNA ligase (Gp30) have increased sensitivity to UV and MMS in single-infections (reviewed in *4,6*). The final step in excision repair and recombinational repair is thought to involve the sealing of a phosphodiester bond by DNA ligase. The mechanism of action of DNA ligase involves covalent modification of the enzyme by adenylation, transfer of the AMP residue in a phosphoanhydride linkage to the 5′-phosphate of nicked DNA, and then resealing of the DNA strand using the energy of

AMP hydrolysis. In the absence of ATP, T4 DNA ligase can also act as an AP lyase to catalyze a β-elimination reaction that leads to the removal of an abasic 5′-dRP residue *(11).* The first step in base-excision repair of a pyrimidine dimer in phage T4 infection is the generation of an AP site by the glycosylase activity of DenV, (*see* Subheading 2.1.). The combined action of the AP endonuclease activity of DenV (on the 3′ side of the AP site) and the AP lyase activity of DNA ligase (on the 5′ side of the AP site) removes the AP site leaving a one-nucleotide gap.

3.12. The Gene 2.5 Single-Strand Binding Protein and the Gene 4 Helicase of Phage T7

The gene *4* helicase of phage T7, like the gene *41* helicase of phage T4, mediates DNA-strand transfer between homologous DNA molecules. In phage T7 infected *E. coli,* the gene *4* helicase-mediated strand-exchange reaction is thought to provide the major pathway for recombinational repair *(41).* In phage T7-infected cells, recombinational repair of DSBs is very efficient (reviewed in *42*). The gene *4* helicase acts together with the gene *2.5* single-stranded binding protein to promote the annealing of homologous regions of two DNA partners to form a joint molecule and then strand transfer. In this reaction T7 Gp2.5 is essential for the formation of a joint molecule, but it is not required for the further T7 Gp4-mediated strand transfer *(41).* T7 gene *4* helicase alone is able to mediate strand transfer, provided that a joint molecule is available. Strand transfer can proceed at a normal rate even if thymine dimers are present in both partners *(42).* Short nonhomologous inserts in either partner can also be tolerated.

4. MUTAGENESIS AS A SIDE-EFFECT OF REPAIR

As pointed out by Holmquist *(29),* a major function of base-excision repair is to minimize mutation resulting from endogenous lesions. However, glycosylases employed in base-excision repair, such as DenV, are not completely specific for damaged sites, but at some frequency they remove normal bases, producing AP sites unnecessarily. There is an error frequency for repairing AP sites, which generates spontaneous mutations. As Holmquist notes, the mutation rate in phages T2 and T4 is maintained by evolutionary pressure to be at about the same rate as in other haploid microorganisms, i.e., about 0.003 mutations per genome per replication. This implies selective pressure to maintain DenV at a balanced level that is sufficient for repair but not so high as to generate excessive mutations.

5. EFFECTIVENESS OF DIFFERENT REPAIR MODES

In Table 1, we summarize rates of lethal hits delivered to mutants relative to lethal hits delivered to wild-type phage T4 during single infections. The largest relative inactivation rate in Table 1 is 3.4 for a gene *47* mutant treated with MNNG. It can then be calculated that when the gene *47* product is functional, (3.4–1.0)/3.4, or 71% of the lethal MNNG lesions present during a mutant infection are repaired by the gene *47* product in a wild-type infection. This calculation may underestimate the fraction of lethal lesions that can be repaired in a single infection, since other repair pathways, not involving gene *47* function, might remove additional lesions caused by this agent. In the cases of UV and MMS damage there are three pathways of repair that operate during a single infection (reviewed in *6*). In these cases we can use $K_{\text{triple-mutant}}/K_{\text{wild-type}}$ values of

Table 2
Measured Levels of Repair During Single and Multiple Infections[a]

	Singe infections	Multiple infections	
Agent	Percent lesions repaired[b]	MR factor	Shoulder repair
UV	82	4.2	18
MNNG	71	2.9	0
MMS	64	—	—
MMC	41	36.0	0
X rays	41	4.0	27
PUVA	—	11.0	0
HNO_2	23	5.0	0
^{32}P	—	4.6	0
H_2O_2	—	8.3	2

[a] Reviewed in *(6, 14).*

[b] These values were calculated using $k_{mutant}/k_{wild\text{-}type}$ values from Table 1 except for UV and MMS. For UV and MMS the calculations are described in the text.

mutants defective in all three pathways. These triple mutant values are 5.5 for UV and 2.8 for MMS. In Table 2, we give the calculated percentages of lethal lesions known to be repaired in single infections for the agents listed, using values from multiple pathways where known.

Data from MR experiments are presented by plotting log of surviving infective-center-forming ability of multiply infected cells versus dose of an inactivating agent. These MR survival curves are usually declining straight lines, or declining straight lines with an initial shoulder. Over the declining straight line region of the curve, the inactivation kinetics can be represented in a form similar to the inactivation kinetics of singly infected cells, as $N/N_0 = e^{-kd}$, where d is the dose of agent applied and k is the inactivation constant, indicating the lethal hits delivered per unit dose of agent. To compare the survival of singly infected cells and multiply infected cells, an MR factor can be calculated. The MR factor is given by k_{mono}/k_{multi}, where the k values are taken from the straight line declining portions of the curves. Figure 1 shows a hypothetical set of curves where the MR factor is 4.0. This hypothetical set of curves also shows a hypothetical single infection by mutant phage where the relative number of lethal lesions delivered to the mutant infected cells, compared to wild-type infected cells, is 2.0. A shoulder is shown in the survival curve representing the multiply-infected cells (designated wt MR). The length of the shoulder is defined by the dose of inactivating agent needed to reach the transition to the straight line declining portion of the curve, and in this figure the shoulder is set at 4.0. Such shoulders, which occur in MR curves obtained with a number of agents, appear to reflect a type of saturable recombinational repair. As reviewed in Chen and Bernstein *(14),* for most agents, the shoulder on an MR curve is removed in the presence of mutations causing defects in recombination. Together, the MR factor and the shoulder repair indicate the additional repair available in multicomplexes compared with monocomplexes.

Table 2 lists the MR factors and shoulder repair values that have been found for several agents. MR is a very powerful form of repair. For agents that give rise to large MR factors or large shoulder-repair factors, such as UV, MMC, X rays, and H_2O_2, (similar

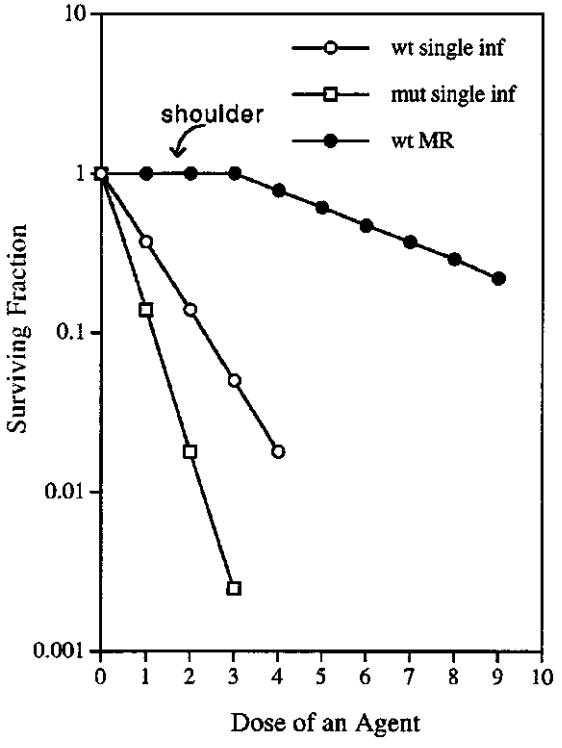

Fig. 1. Hypothetical inactivation curves to illustrate relative survival of infected cells where the infecting phage are: wild-type, infecting cells singly (open circles, designated wt single inf); mutant, infecting cells singly (open squares, designated mut single inf); or wild-type, infecting cells with a multiplicity greater than or equal to 2.0 (filled circles, designated wt MR). Shoulders are often found on wild-type MR curves, as is indicated here.

to the hypothetical agent in Fig. 1), cells singly infected by wild-type phage and treated by the agent may give survival of less than 0.1% when multiply infected cells give survival near 100%.

6. COMPLEMENTATION OF REPAIR DEFECTS IN OTHER SPECIES BY *DENV*

The phage T4 *denV* gene, which codes for endonuclease V, has been introduced into *E. coli, Saccharomyces cerevisiae,* mouse and human cells. In *E. coli, denV* complements nucleotide excision-repair mutants defective in *uvrA, uvrB, uvrC,* and *uvrD* as well as recombination-repair mutants defective in *recA, recB,* and *recC (69).*

In immortalized repair-proficient mammalian cells, endonuclease V activity significantly increases the rate and overall extent of pyrimidine dimer removal *(37,46).* When murine epithelial cells were transfected with the *denV* gene, pyrimidine dimer repair was enhanced two-to threefold *(45).* In control cells, not transfected with *denV,* the patch size for excision repair of DNA photoproducts was estimated to be 34 nucleotides

per photoproduct removed. However in the *denV*-transfected cells, a smaller average patch size of 10–16 nucleotides per photoproduct removed was found *(45).* Thus, endonuclease V activity appears to alter not only the extent, but also the nature of excision repair in UV-exposed mammalian epithelial cells. In humans, *denV* partially complemented the nucleotide excision-repair defect in xeroderma pigmentosum mutant cells (complementation groups A, C, and E) in terms of restoration of colony-forming ability and excision-repair synthesis after UV irradiation *(17,68).* DenV, when topically applied within liposomes to the skin of XP patients, allowed fewer cyclobutylpyrimidine dimers to accumulate in DNA, and reduced erythema *(75).* Thus DenV appears to have therapeutic potential for XP patients.

Cockayne syndrome (CS) is an autosomol recessive disorder characterized by hypersensitivity to UV light and a defect in the preferential repair of UV-induced lesions in transcriptionally active DNA by the nucleotide excision-repair pathway. Expression of *denV* in CS (complementation group A) cells resulted in partial correction of the UV-sensitive phenotype in assays of gene-specific repair and cell viability, while correction of CS (complementation group B) cells in the same assays was minimal or nonexistant *(24).* As described earlier, DenV is a glycosylase that is specific for cyclobutane-pyrimidine dimers, and DenV-incised lesions are believed to be processed via the base excision-repair pathway. The inability of DenV to complement the nucleotide excision-repair defect in CS-B cells to normal levels, suggests that the CS-B gene may have a role in base-excision repair *(24).*

Transgenic tobacco plants expressing phage T4 *denV* exhibited varing degrees of increased, rather than decreased, sensitivity to UV-C light and the alkylating agent dimethyl sulfate *(47).* This suggests that a defect arises when the plants try to repair AP sites. AP sites should be introduced into UV-irradiated DNA by the DenV glycosylase, and into alkylated DNA by the spontaneous elimination of alkyl-purines. They are the only lesions generated by both agents. These may be further cleaved by the AP lyase activity of DenV, but in a manner that produces a cleavage product poorly adapted to pre-existing tobacco base-excision repair pathways *(47).*

7. HOMOLOGIES OF PHAGE DNA REPAIR PROTEINS WITH PROTEINS OF BACTERIA AND EUKARYOTES

7.1. DenV of Phage T4

As reviewed in Furuta et al. *(25)* and Krokan et al. *(44),* DenV homologs have been found in *E. coli, Micrococcus luteus,* and *S. cerevisiae,* although the sizes and amino acid sequences of the enzymes from these organisms differ substantially from those of DenV. Recently, however, a fairly close homolog of DenV was found in the virus PBCV-1. This homolog has 41% amino acid identity to DenV *(25).* The virus PBCV-1 replicates in certain eukaryotic chlorella-like green algae present as endosymbionts in some isolates of *Paramecium bursaria.* Furuta et al. *(25),* using a probe that hybridizes to the *denV* homolog in PBCV-1, assessed 42 other viruses of chlorella, and found that their probe hybridized strongly to the DNA of 37 chlorella viruses and weakly to the other five virus DNAs. The *denV* homolog from PBCV-1 was also able to complement the DNA damage-repair defect of an *E. coli* mutant defective in *uvrA* and *recA*.

7.2. Gene Product UvsX of Phage T4

The *E. coli* RecA protein is unable to complement mutants defective in UvsX *(77).* Nevertheless, analysis of the primary sequence relationships of UvsX to the three-dimensional structure of RecA from *E. coli* suggests that UvsX is a structural homolog of the bacterial RecA protein *(64).* By similar criteria, the Dmc1 protein from *S. cerevisiae* also appears to be a structural homolog of RecA *(64).* These analyses argue that proteins in this group are members of a single family that diverged from a common ancestor that existed prior to the divergence of prokaryotes and eukaryotes. Dmc1 has a meiosis-specific function required for meiotic recombination, and thus the study of UvsX function may illuminate aspects of meiosis.

7.3. Gene Products 46 and 47 of Phage T4

The *rad52* gene of *S. cerevisiae,* necessary for recombinational repair, was found to complement phage T4 mutants defective in genes 46 and 47, implying similarity of function of the yeast and phage gene products *(15).*

7.4. Ligases of Phage

DNA ligases fall into two broad classes, those that are NAD-dependent and those that are ATP-dependent. These two classes differ in the source of the AMP molecule that becomes linked to the ε amino group of lysine in the ligase, as the first step in the ligation reaction. Whereas the NAD-dependent ligases are found exclusively in eubacteria, the ATP-dependent ligases are found ubiquitously in eukaryotes and archaea *(16).* The ATP-dependent DNA ligases are also found in the T-even phages T4 and T6, the T-odd phages T3 and T7, in eukaryotic DNA viruses and, recently, in *Haemophilus influenzae (16).*

8. RECOGNITION OF DAMAGED SITES IN DNA

8.1. DenV

The co-crystal structure of T4 endonuclease V in complex with a DNA duplex containing a thymine dimer has been reported *(70).* The three-dimensional structure of the complex refined at 2.75 angstrom resolution revealed the unique structure of the bound DNA duplex. Contrary to what one might expect, there was no interaction of the enzyme with the cross-linked moiety of the pyrimidine rings. On the other hand, there was extensive interaction between the amino acid side-chains of DenV and the deformed phosphate backbones in the vicinity of the pyrimidine dimer.

8.2. Gp32

Gp32 binds with high specificity to single-stranded DNA. It also binds more efficiently to double-stranded DNA modified either with cis-diaminodichloroplatinum(II) or with aminofluorene derivatives than to native DNA *(65).* This increased affinity is related to the formation of locally unpaired regions, which are strong binding sites for the single-strand binding protein. On the other hand, Gp32 has the same low affinity for native DNA and DNA containing methylated purines and other types of damage that do not induce sufficient structural change to allow Gp32 binding. Despite its cooperative mode of binding to single-stranded DNA, Gp32 alone is not able to melt damaged DNA. Therefore only a limited number of protein monomers bind to each damaged site *(66).*

8.3. DNA Polymerase of Phage T4

Damaged sites in the template strand of DNA may be recognized by blockage of the movement of DNA polymerase during replication. DNA adducts of the environmental carcinogen benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) interact stereospecifically with prokaryotic and eukaryotic polymerases in vitro. Of six different DNA polymerases tested for their ability to replicate past different diastereomers of BPDE at specific sites in the DNA, the T4 DNA polymerase (Gp43) was most strongly inhibited *(13).* However T4 DNA polymerase can replicate past O^6-methylguanine residues in the presence of Gp45 accessory protein that clamps the polymerase to the DNA template *(57).*

9. CONCLUDING REMARKS: ADAPTIVE CONSEQUENCES OF DNA REPAIR

We have reviewed the main processes of DNA repair encoded by phage genomes. Phage T4 was emphasized because most of the experimental work on DNA repair in phage was performed with this organism. The repair processes in phage T4 are of two kinds. The first kind, including DenV mediated base-excision repair and photoreactivation, is specific for one type of damage, pyrimidine dimers. The second kind, including MR, double-strand break repair and PRRR, are recombinational repair processes. In principle, it is only when two or more genomes (or portions thereof) are present that the important class of double-strand damage can be accurately repaired. As reviewed by Bernstein and Wallace *(6),* these recombinational repair processes appear to reflect a common underlying mechanism mediated by a common set of gene products that can repair a wide variety of DNA damages. As described in Subheading 5, MR (or the sexual mating process of multiplicity reactivation) is a particularly powerful form of recombinational repair.

UvsX, a RecA/Dmc1 homolog, plays a central role in all of the recombinational repair processes. The function of recombination during meiosis in eucaryotes and analogous processes in procaryotes is currently a hotly debated issue in the context of the adaptive advantage of sex, a major unsolved problem in biology. There are two opposing views on this issue. The first is that recombination, and hence sex, is primarily an adaptation for promoting allelic variation (reviewed in *2*). The second view is that recombination is primarily an adaptation for DNA repair and that allelic variation is a byproduct (reviewed in *7,8*). Cox *(19)* has argued, from an enzymological perspective, that the RecA protein evolved as the central component of a recombinational DNA repair system, with the generation of genetic diversity as a sometimes beneficial byproduct. One of the arguments presented by Cox is that RecA protein binding, which initiates recombination, is targeted to perturbed, or underwound regions of duplex DNA that can result when DNA is damaged, a characteristic expected if recombination is primarily an adaptation for repair. In phage T4, the similar UvsX recombination protein depends on Gp32, which (*see* Subheading 8.2.) is targeted to DNA damage. An experiment was performed using multiple infection in phage T4 as a model of sexual interaction, to test the concept that the adaptive advantage of sex is promotion of recombinational repair *(5).* When an undamage phage T4 injects its DNA into a host cell, it establishes a barrier to infection by a second phage T4 within 2 min. This promotes asexual reproduction and the preservation of the host cell as a resource solely for the first phage. However if the first phage is

treated with UV, its barrier to superinfection is reduced. Thus, damage to a first-infecting phage shifts reproduction from an asexual mode towards a sexual mode, as would be expected if mating is an adaptation for repair. Furthermore, genes from the damaged first-infecting phage were shown to have a much enhanced survival owing to the shift towards sexual reproduction. Overall in phage T4, there is strong experimental support for the concept that recombination is primarily an adaptation for DNA repair, a process vital for immediate survival, and little, if any, evidence that the generation of recombinational variation is of substantial benefit.

ACKNOWLEDGMENT

This work was supported by NIH Program Project Grant #CA72008.

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2

Post-Replication Repair

A New Perspective Focusing on the Coordination Between Recombination and DNA Replication

Steven J. Sandler

1. INTRODUCTION

The repair of DNA is crucial to the survival of every organism. Organisms have evolved many biochemical pathways for detecting and repairing DNA damage with high fidelity. Failure to repair DNA with high fidelity leads to a high mutation frequency. This in turn is correlated with a high risk of cancer in humans.

The type of DNA damage usually dictates the type of DNA repair pathway used by the cell. This article focuses on post-replication repair (PRR) of DNA. This process was first noted while using ultraviolet (UV)-irradiation as a source of DNA damage. While there are several types of UV-induced lesions (i.e., pyrimidine dimers), most are removed by the nucleotide-excision repair (NER) and photoreactivation repair (PR) pathways. However, when a replication fork encounters one of these noncoding lesions (not removed by NER or PR), a special type of recombinational repair pathway is available to repair the DNA damage. This type of repair has been referred to both daughter-strand gap repair (DSGR) and PRR. In recent years PRR has become synonymous with the RecF pathway of recombination in *Escherichia coli.* The RecF pathway of recombinational DNA repair operates on gapped DNA substrates that presumably arise after the replication fork has partially replicated past a noncoding lesion in the template DNA (leaving a gap opposite the noncoding lesion). The missing information (forming the gap) across from the noncoding lesion is then supplied by the other daughter duplex DNA in a process requiring RecA. In this process, information from the fully replicated daughter, complementary to the lesion, is placed across from the lesion. Thus the damage is now in a DNA duplex and can be repaired either NER or PR.

Recently PRR has been integrated into a broader paradigm that explains the coordination of DNA repair, DNA replication, and recombination in the cell. This paradigm, called CPR (*c*oordinated *p*rocessing of damaged *r*eplication forks) *(19,20,80),* combines many other aspects of DNA metabolism and cell division. CPR emphasizes that these processes are used by the cell in a housekeeping sense (without the occurrence of

From: *DNA Damage and Repair, Vol. 3: Advances from Phage to Humans*
Edited by: J. A. Nickoloff and M. F. Hoekstra © Humana Press Inc., Totowa, NJ

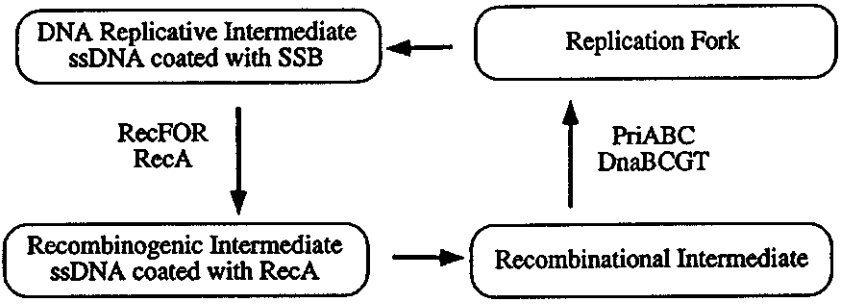

Fig. 1. Proposed relationship between DNA replication and recombination intermediates. The gene names along the vertical arrows indicate the proteins that perform multiple steps in the conversion between substrates. The reactions between boxes separated by the horizontal arrows are less well-defined. Once again they indicate conversion between one recombination or replication structure and another.

extra DNA damage). In a simple sense, CPR is the process by which collapsed or arrested replication forks are repaired by recombinational processes and then restarted. It is thought that restart is an essential process. It is important to emphasize that some of these repair and restart processes may be *recA*-independent since *recA* mutants are viable. Other aspects of CPR include chromosome partitioning (*dif*locus and *XerCD*) *(91)* and cell division (*ftsK*) *(91).*

Thus this review will explain PRR in terms of CPR and will focus on two groups of proteins thought to catalyze opposite reactions in the cell (Fig. 1). The first group includes the RecF, RecO, and RecR (RecFOR) proteins and are thought to catalyze presynaptic steps in PRR that convert a DNA replicative intermediate, ssDNA coated with SSB to a recombinogenic intermediate, ssDNA coated with RecA *(14,78).* The assembly of a RecA-ssDNA filament is critical for molecular healing of the damaged replication fork by recombination. The second group of *E. coli* proteins were initially characterized as elements required in vitro DNA replication of ΦX174 ssDNA phage. Their role in *E. coli* however, has only recently begun to be appreciated. These proteins, collectively called the primosome assembly proteins, include PriA, PriB, PriC, DnaT, DnaC, DnaB, and DnaG (reviewed in *34,57,58,81*). They are thought to restart DNA replication forks at recombinational intermediates and to be essential for normal vegetative growth. Thus they complete the transition in PRR, allowing a collapsed replication fork that has been repaired by the action of RecFOR, RecA, and the process of homologous recombination to be restarted.

The reader is also referred to other articles that review aspects of PRR *(37,38),* the homologous recombination machinery in general *(14,35,46,48), recFOR* specifically *(72),* CPR *(20,57,80),* and the primosome assembly proteins *(34,57,58,81).*

2. AN OVERVIEW OF HOMOLOGOUS RECOMBINATION

Recombination is often thought of as occurring in three distinct stages. The first stage is called pre-synapsis. In this stage, one of the two interacting duplexes of DNA is tailored so that it can be bound by the RecA protein. This tailoring often involves the generation of ssDNA through the action of helicases that unwind duplex DNA or sin-

gle-stranded exonulceases that selectively degrade one strand of DNA. Once the ssDNA is generated, it can be bound by RecA to create a protein-DNA helical filament. It is this filament that is thought to be the active agent that searches for a homologous region of duplex DNA. How RecA performs this search is still an unsolved aspect of RecA biochemistry. Several models have been proposed to explain RecA strand pairing, recognition of homologous regions, and strand exchange *(8,51,86).* Once a homologous region is found, RecA can catalyze the invasion of the ssDNA into the duplex DNA displacing the identical strand. This DNA structure is often called a displacement loop or D-loop. This structure includes a crossover or Holliday junction *(90)* that can undergo branch migration in which the Holliday junction diffuses along the length of the DNA molecule. The Holliday structures may then be resolved enzymatically in *E. coli* by RuvC or Rus *(87).* These enzymes can cleave two strands of the Holliday structure either vertically or horizontally. The position and way in which these structures are resolved determines the structure and phenotypes of the recombinants. Traditionally, the process of recombination has been thought to end after the post-synaptic steps described. However as will be discussed later, it is now necessary to invoke other post-synaptic steps that include the assembly of a DNA replication fork at a recombination intermediate in order to attain viable recombinants.

2.1. Substrates for Homologous Recombination

When thinking about the molecular process of recombination, it is often instructive to focus on the different types of DNA substrates before introducing the gene products that operate on them. To a large degree, the type of DNA substrate dictates that set of gene products will be needed to perform the recombination event. In this sense recombination is thought to be substrate-limited. The field has largely divided the many different types of DNA substrates into two varieties: duplex molecules with double-strand ends and duplex molecules with regions of ssDNA (gaps). These substrates are operated on by the RecBCD (*see* Chapter 8 in Volume *1*) and RecF pathways of recombination respectively and the latter is described in more detail later. One way to think about these two pathways is that they describe sets of pre-synaptic enzymes that funnel the many different DNA substrates into substrates that can be bound by RecA (Fig. 2). Historically, the RecF pathway genes also included gene products involved in branch migration (*ruvA* and *ruvB*) and Holliday junction resolution *(ruvC).* Now however, these enzyme are thought only to play a post-synaptic role. The roles of *recJ, recN,* and *recQ,* also commonly referred to as RecF pathway genes, in pre- and or post-synapsis are presently unclear.

2.2. Pathways of Homologous Recombination

Conceptually, a recombinational pathway for DNA is very much like a biochemical pathway for the biosynthesis of an amino acid. One has substrates that are acted on by enzymes that produce products. Genetically, if a mutation is introduced that blocks that pathway (when there is only one pathway), a phenotype will be seen. If the cell has multiple pathways that can provide the same end product, a phenotype will only be seen if genes in both pathways are mutated. For recombination, the phenotype seen is often a decrease in recombination frequency. This is often measured by the production of a physically or genetically scoreable recombinant normalized against experimental para-

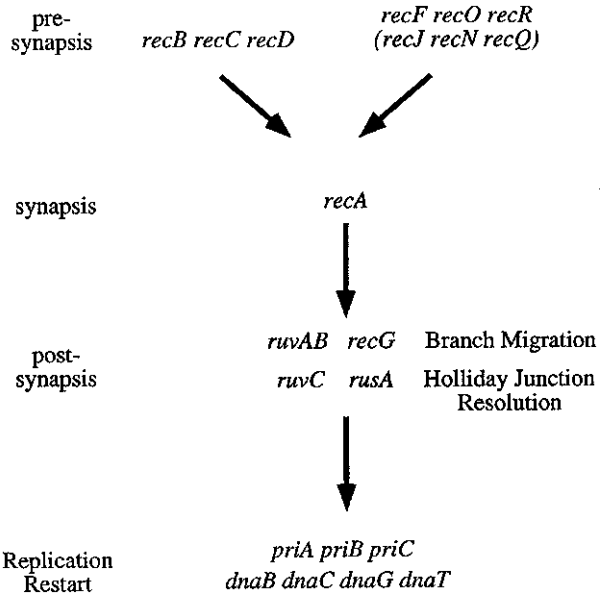

Fig. 2. Genes needed for RecBCD and RecF pathways of recombination in *E. coli.* The genes are grouped to correspond with their different roles in the stages of recombination. The genes in parentheses encode functions whose role is not yet clear. Although the genes involving replication restart have been placed after the post-synaptic steps of branch migration and Holliday junction resolution, this has not yet been demonstrated experimentally. These genes define a minimal set. Other genes know to have an affect on these processes include *ssb, lig,* mismatch repair, and genes encoding the different topoisomerases.

meters. For some years, the use of different recombinational substrates introduced by different protocols (i.e., conjugation, transduction, inter- and intra-plasmidic recombination, and substrates with direct or inverted repeats) caused some confusion. With the recognition that these substrates are physically different and need a different set of recombinases to be processed by the cell, illumination has come to the field.

In *E. coli,* there are commonly thought to be two main pathways for recombination. These have been alluded to earlier and are diagrammed in different ways in Figs. 2 and 3. The major differences between these pathways are in the pre-synaptic steps; the two pathways are named after the genes used in these initial steps. Figure 3 illustrates that the differences in recombination pathways are only in the pre-synaptic steps and that the substrates are processed to common intermediates. Although there is only one synaptic protein listed, the post-synaptic steps of branch migration and Holliday junction resolution require multiple enzymes. The reason for this redundancy is not clear.

The examples in Fig. 3 use replicative DNA substrates in which the DNA damage could result from housekeeping functions or additional insult to the cell. The left side of Fig. 2 shows a standard model for RecBCD mediated double-strand break repair *(88).* In the RecBCD pathway, a duplex DNA with a double-stranded end is produced when the replication fork encounters a nick. In short, the dsDNA end is tailored so that RecA can use it in strand invasion to produce a D-loop. Branch migration and Holliday junction resolution can leave a substrate that is then ready for restarting DNA replication. For additional details, *see* Chapter 8 in Volume 1.

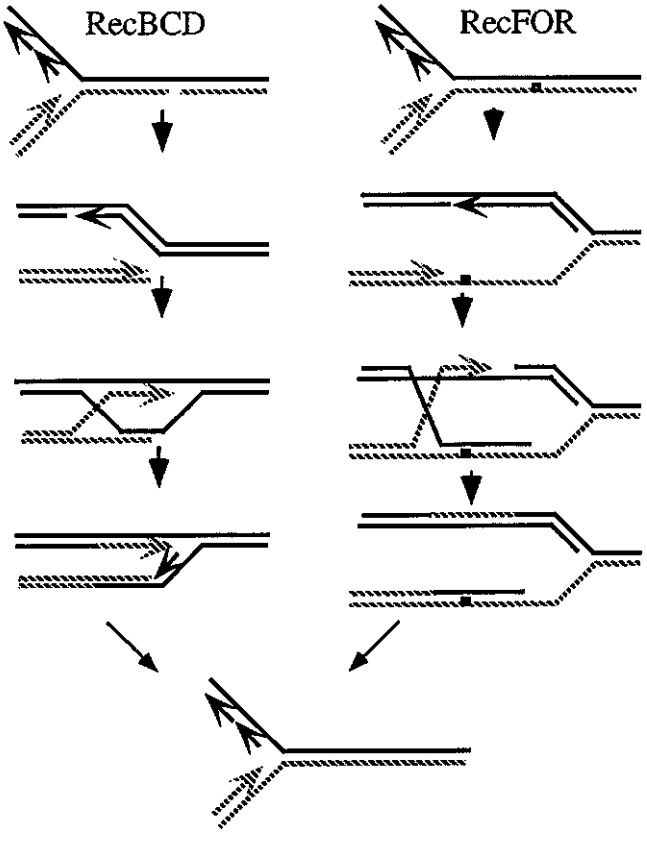

Fig. 3. How the RecBCD and RecFOR pathways of recombination may repair collapsed replication forks and then produce a structure that is suitable for restart of DNA replication. The structures shown include the starting substrate, replication-fork collapse, a synapsis/post-synapsis intermediate, Holliday junction intermediates, a substrate for replication fork restart and the restarted replication fork.

The right side of Fig. 3 shows PRR by the RecFOR pathway of a noncoding lesion. This process conjures up several theoretical problems and questions. How does the replication machinery replicate past the noncoding lesion on only one strand? How far past the lesion does it go? What is the molecular signal for this procedure? What are the required proteins? How large are the gaps produced? Is the process different if the damage is encountered by the lagging or leading strand polymerases? Although clear answers to none of these question are available, the events after production of the proposed gap have been addressed in some detail later.

To complement this broad overview of recombination, PRR and CPR, the remainder of the article will focus on *recF, recO,* and *recR (recFOR)* genes and proteins and then introduce the less well-known and -studied primosome assembly genes.

3. GENETICS OF RecFOR

3.1. Isolation and Characterization of the recFOR Genes

The first mutation found in *recF, recF143,* was identified by its ability to cause extreme recombination deficiency (Rec$^-$ as measured by conjugal recombination) and

UV sensitivity (UV^S) in a *recB recC sbcB sbcC* strain *(28).* Unlike *recA, recB,* and *recC* single mutations that cause both Rec^- and UV^S phenotypes, *recF143* single mutations caused only UV^S. The ability to affect DNA repair and not recombination has been both the hallmark and the riddle of *recF* function in DNA metabolism. Mutations in *recO* and *recR* were subsequently shown to produce virtually identical phenotypes *(26,43,107).* It has been proposed that *recF, recO,* and *recR (recFOR),* act in a biochemical pathway that identifies and converts a DNA replicative intermediate (ssDNA coated with single-stranded DNA binding protein [SSB]) to a recombinogenic one (ssDNA coated with RecA) by helping RecA displace SSB on the proper DNA substrate *(14,78)* (Fig. 1). However, it is still unclear how RecFOR orchestrates the transition from DNA replication to recombination. Research suggests that subcomplexes of RecOR *(85,97),* RecFR *(105,106)* and RecF may also exist in addition to RecFOR *(27)* and may catalyze different parts of the complete reaction or they may have other roles in the cell. RecFOR homologs have only been found in bacteria. Nonetheless, their function appears to be maintained in other evolutionarily diverged systems. For example, yeast genes *RAD52, RAD55,* and *RAD57* have been proposed to have a function analogous to *E. coli* RecFOR *(92,93).*

3.1.1. Phenotypes of recFOR Mutants

Single mutations in the *recFOR* genes cause UV sensitivity *(28,33,55),* attenuation of UV-induction of the SOS response *(26,107),* and decreased plasmid recombination *(15,21,33).* For UV sensitivity, the three genes are epistatic *(13,44,45,47,55).* Other phenotypes of *recF* mutants include defects in mutagenesis of ssDNA phages *(12)* and induction of the adaptive response *(101).* These latter phenotypes have not been tested for *recO* and *recR* mutants. The effect of *recF, recO,* and *recR* mutations in other mutant backgrounds is of great interest. For example, it has been shown in *uvrA (96)* and *priA (73)* mutant strains, that *recF* mutations either decrease the UV resistance or viability of the strain. This argues that *RecF* function is needed in pathways that operate in the absence of these other gene products.

Perhaps the study of *recFOR* has been given less emphasis historically than the RecBCD pathway because these genes were originally perceived to act in an alternate or secondary pathway of recombination in *E. coli.* This is certainly not the case; single mutations in *recFOR* have distinctive phenotypes (e.g., UV^S). The importance of the RecF pathway and its difference from the RecBCD pathway of recombination lies in the types of substrates that it handles. The RecBCD enzyme acts at the ends of linear DNA molecules, whereas the *recFOR* proteins have been associated with recombination in the middle of DNA molecules. Thus, *recB* or *recC* single mutations have major effects on conjugal recombination (linear DNA substrates with ends) and single mutations in *recFOR* affect recombination with plasmid substrates *(15,21,33),* P22 transduction and chromosomal recombination in *Salmonella typhimurium (24,60).* This may not be the only difference from the RecBCD pathway since UV-induced SOS expression by the RecF pathway requires DNA replication *(83,84).* It should also be noted that the RecF pathway is flexible and can be adopted to function on linear DNA substrates during conjugal recombination. Thus in a *recBC sbcBC* strain, *recFOR* mutations have dramatic effects on conjugal recombination *(28).*

Phenotypic studies on the overexpression of *recFOR* genes have revealed interesting and useful information about these proteins. Overexpression of the *recO* protein can

Table 1
Biochemistry of RecFOR proteins and Interactions DNA, SSB, and RecA

	RecO, 26 kDa	RecF, – 40 kDa	RecR, 22 kDa
O	1. Monomer in solution 2. Binds both ssDNA and ds DNA 3. Promotes renaturation of complementary ssDNA, requires Mg^{+2}, inhibited by 160 mM NaCl – ATP independent. *(50)* 4. Strand assimilates short oligo and supercoiled DNA, requires Mg^{+2}, ATP independent. *(49).*		
F	1. RecF co-precipitates RecO. This is reversed in presence of ATP *(27).* 2. RecF interacts with RecO even if SSB is present *(27)* .	1. Binds ssDNA, ATP independent *(25, 52).* 2. Inhibits RecA joint molecule formation and RecA ATPase activity *(52).* 3. Binds ATP, dsDNA binding is ATP or ATPγS dependent *(53)* Binding is stronger for ATPγS than ATP. 4. Binding ssDNA or dsDNA or dsDNA enhances ATP binding *(53).* 5. ATPase activity with dsDNA (105). 6. Binding dsDNA with ATPγS – 1 monomer per 4–6 bp *(105).*	
R	1. RecR and RecO overcome SSB inhibition of RecA catalyzed joint molecule formation *(97)* 2. RecOR-SSB ssDNA complex helps RecA to nulceate on DNA *(98)* 3. Stabilzes 5′ end-dependent dissociation of RecA filament *(85)*	1. RecR stablizes RecF on dsDNA in presence of ATP – 1:1 molar ratio of proteins coats dsDNA (105). 2. ATPase activity is stimulated by RecR *(105).* 3. RecR-RecF complex limits extension of RecA filament from ssDNA in a gap to the dsDNA *(106).*	1. *E. coli* protein – No reported activity by itself on dsDNA *(105).* 2. *B. subtilis* protein – Binds ssDNA and ds DNA – binding enhanced by presence of damage in DNA, ATP and divalent metal ions *(3).* Binding of RecR multimers associated with DNA loops in EM *(7).*

partially suppress the UV^S caused by *recA* mutations *(49)*. This correlates with the finding that RecO can catalyze a similar reaction to RecA in vitro (see Subheading 4.2; Table 1; and *49*). Wild-type *recF* overexpression causes inhibition of UV-induced SOS expression, decreased UV^R and cell viability *(79)*. All known mutant *recF* genes show the same phenotypes as a *recF* null mutant when in single copy on the chromosome, but each displays only a subset of the overexpression phenotypes, indicating that the different mutations remove different subsets of RecF activities. Hence, analysis of overexpression phenotypes is a useful method to define and correlate in vivo and in vitro activities of RecF. The *recF4115* mutation is the only *recF* missense protein where all overexpression phenotypes have been eliminated *(74)*.

3.2. Evolutionary Conservation of recFOR Genes

Chromosomal mutations in either the *recF, recO,* and *recR* genes have been most intensively studied in *E. coli* (FOR) (for example, *see [78]*), *S. typhimurium* (F) *(24,60)* and *Bacillus subtilis* (FR) *(2–4)*. Several missense mutations have been isolated in *recF (recF143, recF4101, recF4104, recF4115) (74,79),* insertion mutations (*recF400::kan [94]* and *recF332::Tn3* [9]) and deletions *(recF349) (79)*. Only insertion mutations have been reported so far for *recR (55)* and *recO (33)*.

recF, recO, or *recR* homologs have been reported in 48 different types of bacteria spanning the kingdom of *Proteobacteria* to *Aquicales* (*see* Table 2). Many organisms have homologs of all three genes. In several of the completed genomic sequences only *recF* and *recR* homologs are reported. However, *Helicobacter pylori* and *Aquifex aeolicus* contain only a *recR* homolog and *Borrelia burgdorferi* and *Mycoplasma genitalium* lack *recFOR* homologs. It is worth equal note that while *Treponema pallidum* contains both *recF* and *recR* homologs, this organism has no *recBCD* homologs (and *Borrelia burgdorferi* has the converse:, only *recBCD* homologs and no *recFOR* homologs) *(22,23)*. These findings raise many questions. For example: Do organisms that only have *recF* and *recR* contain only a subset of RecFOR activities? If not, do they have an evolutionary nonhomologous (but functional analogous) *recO* gene? Are organisms without a full RecFOR complement more sensitive to DNA damage than ones with a full complement? It is also noteworthy that only two groups of proteobacteria contain *recO* homologs. From this observation and given the phylogeny of 16S rRNA, it is tempting to speculate that *recO* genes were a late acquisition in the evolution of bacterial-repair systems. Answers to these questions may take some time because little is known about how these diverse organisms repair, replicate, and recombine their DNA.

No *recFOR* evolutionary homologs have been reported in the *Archaea* or *Eucarya.* Rad52, Rad55, and Rad57 in yeast are reported to assist the yeast RecA homolog, Rad51 compete for ssDNA coated with RPA (SSB analog) *(92,93)*. Hence it is possible that while the *recFOR* homologs are not found in eucaryotes, their function is conserved.

3.3. Location and Regulation of E. coli recF, recO, and recR Genes

In bacteria, clues to biological functions of some genes can be suggested by the function of neighboring genes and patterns of transcriptional regulation. In *E. coli,* both *recF* and *recR* are in groups of genes that are needed for DNA replication. The *recO* gene is found downstream of the *era* gene. This gene of unknown function is essential

Table 2
Organisms Containing *recF, recO* and *recR* Homologs

Bacterial grouping	Organism	*recF*	*recR*	*recO*
α *Proteobacteria*	*Caulobacter crescentus*	U37793		
	Rickettsia prowazekii[a]		X[b]	
β *Proteobacteria*	*Neisseria gonorrhoeae*	X	X	X
	Neisseria meningitidis		X	X
	Bordetella pertussis		X	X
	Thiobacillus ferrooxidan	X	X	
γ *Proteobacteria*	*Escherichia coli*[a]	K02179	M38777	U36841
	Haemophilus influenzae Rd[a]	U32780	U32727	U32718
	Haemophilus ducreyi		AF017750	
	Pseudomonas aeruginosa	X	X	X
	Pseudomonas putida	X62504		
	Salmonella typhimurium	X62505	X	U48415
	Proteus mirabilis	M58352		
	Actinobacillus pleuropneumoniae	X63626		
	Actinobacillus actinomycetemcomitans	X	X	X
	Yersinia pestis	X	X	X
	Shewanella putrefaciens	X	X	X
	Pasteurella multocida	X	X	X
	Klebsiella pneumoniae	X	X	X
	Vibrio cholerae	X	X	X
	Azotobacter vinelandii	X86404		
	Coxiella burnetii			L27436
ε *Proteobacteria*	*Helicobacter pylori*[a]	AE000602		
	Campylobacter jejuni	X		
Firmicutes (Low GC gram positive)	*Bacillus subtilis*[a]	X02369	X17014	
	Lactococcus lactis	X89367		
	Staphylococcus aureus	X71437	X	
	Clostridium difficile	X	X	
	Clostridium acetobutylicum	X	X	
	Enterococcus faecalis	X	X	
	Streptococcus mutans		X	
	Streptococcus pneumoniae	X	X	
	Streptococcus thermophilus		P96053	
	Streptococcus pyogenes	U07342		
Actinomycetes (High GC gram positive)	*Mycobacterium smegmatis*	X92503		
	Mycobacterium tuberculosis[a]	Z80233	AL022121	
	Mycobacterium leprae[a]	Z70722	AL023596	
	Mycobacterium bovis	X	X	
	Streptomyces coelicolor	L27063		
Green sulfur	*Chlorobium tepidum*	X	X	

(Continues)

Table 2
Continued

Bacterial grouping	Organism	*recF*	*recR*	*recO*
Cyanobacteria	*Synechocystis sp. PCC6803*[a]	D90907	D90916	
Spirochaetales	*Treponema pallidum*[a]	AE001185	AE001268	
Cytophagales	*Porphyromonas gingivalis*	X	X	
Chlamydiales	*Chlamydia trachomatis*[a]	AE001282	AE001297	
	Chlamydia pneumoniae[a]	X	X	
Thermus/Deinococcus	*Deinococcus radiodurans*	X	X	
Aquificales	*Aquifex aeolicus*[a]		AE000742	

[a] Completed genome sequence.
"X" indicates either a partial or full sequence is available in the uncompleted genome database as of August 1999. Genbank accession numbers are given where known.

for growth. As will be expanded upon later, the association of *recF* and *recR* with DNA replication genes is suggestive that these two proteins (and possibly *recO* as well) may interact with the replication machinery.

Understanding of the transcriptional regulation of the three groups of genes encoding *recFOR* is at a rudimentary level. Most is known about the transcriptional regulation of the *recF* gene. Transcriptional studies using different fragments of the *dnaA-dnaN-recF* region fused to *lacZ* on both plasmids and chromosomes have identified several sequences that act as promoters and transcriptional terminator *(5,6,68).* These results suggest that this region of the chromosome is under complex regulation and that *recF* transcriptional regulation is part of a larger network involving *dnaA* and *dnaN (11,29).* It has been recently shown that *dnaN* and *recF* promoters are induced greater than 40-fold during entry into stationary phase *(100).* Studies on the transcriptional regulation of the *recO* and *recR* genes have not yet been reported.

The level of the *recFOR* proteins in the cell is thought to be quite low, although it has not been precisely determined. This speculation is based on several observations. First an upper limit of the amount of RecF has been estimated at less than 190 molecules per cell *(52).* Second, several processes that inhibit *recF* overexpression were identified when *recF* was overexpressed from a plasmid *(76,77).* Third, the codon usage of *recF* is similar to that of other poorly expressed *E. coli* genes *(9).* Although overexpression of *recR* is not problematic like *recF,* expression of *recR* in maxicells is quite low *(56).* Another interesting observation is that the *recF, recO,* and *recR* genes all overlap with the genes in upstream of them. This type of arrangement, called translational coupling *(67),* is used by the cell to ensure equal levels of expression of two proteins that interact. In the case of *recF* and *recR,* however, a mechanism opposite of translational coupling seems to operate because much less of the *recF* and *recR* gene products are seen relative to the *dnaN* and *orf12* gene products, respectively *(9,55).* A common mechanism acting either at the level of transcription or translation to regulate or coordinate levels of *recFOR* gene expression remains to be elucidated.

4. BIOCHEMICAL INTERACTIONS BETWEEN RecFOR AND OTHER PROTEINS

4.1. Interactions with SSB and RecA

The idea that *recFOR* modulates the binding of SSB and RecA to ssDNA derives from both genetic and biochemical data. Moureau *(61)* showed that overexpression of SSB yielded phenotypes similar to those of *recF* mutants. Volkert and colleagues *(102,104)* isolated several suppressors of *recF* mutations that mapped in *recA* (e.g., *recA803*). Others showed that these suppressors would also suppress *recO* and *recR* mutations *(52,103).* Biochemical analysis showed that RecA803 protein could make joint molecules under conditions where SSB was inhibitory to RecA-catalyzed reactions *(54).* Umezu et al. *(97)* showed that RecO and RecR were sufficient to overcome the SSB inhibition in RecA-catalyzed joint molecule reactions. The unresolved finding in this report was that RecF had no role in the in vitro reaction. This was in contradiction to the in vivo reaction, where *recF* has a definite role. At least one aspect of the reaction conditions in these experiments did not mimic the in vivo conditions as very high levels of RecO and RecR were used in vitro and only low amounts of RecOR are thought to present in vivo. This prompted further testing, which showed that overexpression of *recOR* could suppress *recF* mutations and provided a reason for why RecF was not needed in the in vitro reactions *(78).* These findings led to the molecular matchmaker model for RecFOR function in *E. coli (14,78).* This model proposes that RecF acts as a molecular matchmaker identifying and binding to a specific DNA structure: a gapped DNA intermediate left by DNA replication where SSB was bound to ssDNA. RecF would then help to load RecOR, which in turn helped to modify the SSB-ssDNA so that RecA could bind *(14,78).*

4.2. RecFOR Biochemistry

Biochemical analysis of the RecFOR proteins has been extremely difficult for at least three reasons: (1) There are no known enzymatic activities to follow during purification. Hence the proteins are purified on the criteria of solubility and electrophoretic purity. (2) The structure of the DNA substrate on which RecFOR operates is not known. It is, however, hypothesized to be a gapped DNA molecule produced by DNA replication. (3) Because DNA replication is required for RecF-dependent SOS induction *(84),* it is likely that in vitro visualization of RecFOR activity will require components of the DNA replication machinery. In spite of these difficulties, significant achievements have been made in understanding the biochemical properties of these proteins. All three proteins have been overproduced and purified. Table 2 lists the known activities of the proteins both singly and in combination.

Singly, the *E. coli* RecO and RecF and *B. subtilis* RecR proteins display properties of proteins that are likely to be involved with DNA metabolism. They bind both ssDNA and dsDNA and this binding is modulated by divalent metal cations and nucleotide cofactors. RecO has two activities that could be specifically associated with recombination: renaturation of ssDNA and strand assimilation of an oligonucleotide with a homologous dsDNA circular supercoiled DNA substrate. Like RecO, the yeast *RAD52* protein also has an ATP-independent strand-transfer activity *(66).*

Early in its study, RecF activity was defined in terms of its ability to inhibit RecA catalyzed reactions *(52).* Although these are interesting reactions in their own right, one

needs to remember that in vivo there is usually a large excess of RecA over RecF. Therefore, these reactions may not be physiologically relevant. More recently, conditions for an ATPase activity have been identified for RecF in the presence of dsDNA *(105).* It is likely that this activity is physiologically relevant and important. Evidence supporting this idea includes: (1) *RecF4101,* a mutant in the phosphate binding hole, is defective in RecF activity in vivo *(75).* (2) RecF4101 does not display ATPase activity in vitro *(105).* The RecF ATPase activity is also stimulated in the presence of RecR *(105).*

RecOR and RecFR display activities that modulate the activity of RecA protein. RecOR help RecA load onto ssDNA coated with SSB, as previously mentioned. They also help to stabilize RecA filaments and prevent end-dependent dissociation *(85).* These two mechanisms may be related. It is easy to visualize how these two activities would be useful in the early stages of RecA filament formation. The effect of RecFR on RecA protein filaments was discovered by first looking at the effect of RecR on RecF binding to dsDNA. RecF binds to dsDNA in a sequence-independent fashion. Binding is stronger in the presence of ATPγS than ATP. RecR stabilizes RecF binding to dsDNA in the presence of ATP *(105).* When RecA binds to ssDNA on a gapped DNA substrate, eventually RecA filament formation will extend beyond the ssDNA into the dsDNA region. If RecFR is present, it will halt growth of the RecA filament *(106).*

Madiraju and colleagues have defined complexes of RecFOR and SSB proteins in the absence of DNA using immunoprecipitation and different types of chromatography *(27).* They find that RecO interacts with RecF, RecR, and SSB. In these assays, RecO can bind either RecR or SSB but not both. On the other hand, Umezu and Kolodner found using BIAcore sensor chips that RecO can bind both RecR and SSB and that SSB binds RecO with higher affinity than RecR *(98).* The former group found that RecO can bind RecF and SSB at the same time and that the addition of ATP abolishes the RecFO interaction. They also see complexes between RecFOR and RecFOR-SSB depending on the order of addition.

In summary, it appears that the RecFOR proteins are capable of a variety of activities in vitro either singly or in combination that could be useful in recombination. Whether any of these activities is used by these proteins in vivo remains to be proven.

5. A MODEL FOR THE ROLE OF RecFOR IN THE CELL

It is arguable that the main function of recombination is to help restart stalled replication forks *(17,18,36,99).* The process can be envisioned in the following steps: detection of DNA damage by the replication machinery, production of a gap in the DNA behind the replication fork, PRR substitution of DNA not replicated because of damage (e.g., RecA, RecFOR), and then restarting of the replication machinery. Several observations mentioned previously are consistent with RecFOR having an important role in the repair of collapsed and/or stalled replication forks by recombinational DNA repair. It is conceivable that RecF (OR) persists during the entire repair process and is not necessary only during the early pre-synaptic phase.

Figure 4 shows a model that combines several of the ideas represented in the literature *(14,17,27,78).* The model focuses on the role of the RecFOR, RecA, and SSB proteins in PRR. The model assumes that the replication fork encounters a noncoding lesion on the template for the leading strand. The replication fork leaves a gap and pauses or stalls at least one Okazaki fragment upstream. The ssDNA is first covered

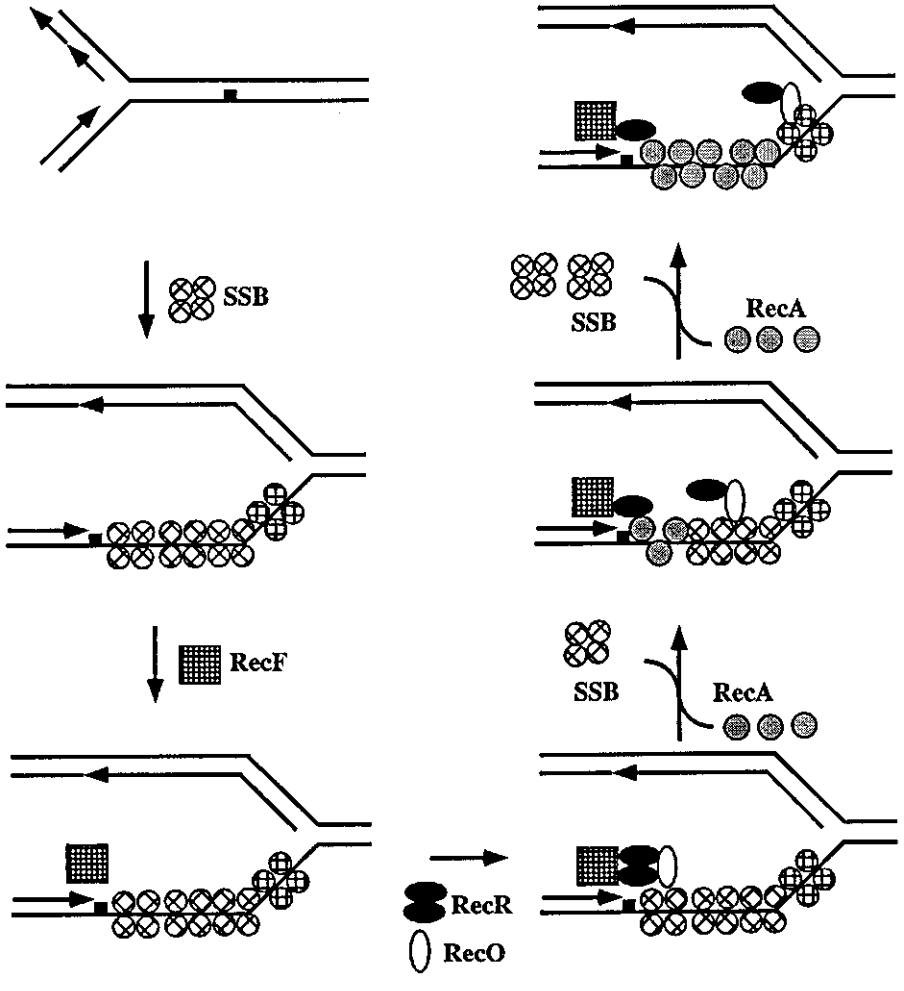

Fig. 4. Model of RecFOR in PRR. In the first panel, a replication fork approaching a noncoding lesion (small black box on the DNA) is diagrammed. In the second panel, the replication fork has translocated one Okazaki fragment past the noncoding lesion. No replication has occurred on the template strand containing the noncoding lesion. The remainder of the diagram is explained in the text.

with SSB. RecF then recognizes the DNA-protein structure (assumed in this diagram to be at the left edge of the gap) and then helps to load RecO and RecR. RecR is shown as a dimer at this point. After RecFOR assembly, the complex splits. One subunit of RecR goes with each RecO and the other RecR subunit remains with RecF. The RecOR complex is free to interact with SSB and help load RecA. When it reaches a dsDNA section, it stops and anchors the 5′ end of the RecA filament preventing dissociation. The RecFR complex left behind prevents the RecA filament from extending in to the dsDNA and focuses the RecA filament in the region of ssDNA. The RecA filament then searches the other daughter duplex for a region of homology and places the noncoding lesion across from its complement. The DNA lesion is now ready for removal by an excision repair reaction.

This model incorporates many of the observations mentioned in this article. However, it does not indicate how RecOR moves along the DNA removing the SSB, helping RecA to bind to the ssDNA or define when the RecF ATPase activity is needed. It also has RecA loading in a discontinuous fashion (3′ to 5′) *(69).* An alternative scheme (not shown) that does not require RecOR to migrate along the DNA suggests RecR protein stabilizes a loop of ssDNA (as has been shown for the *B. subtilis* RecR protein) such that ends of the ssDNA region are close together. The complex can then split into RecFR and RecOR subcomplexes attached to each end of the gap and function as explained earlier. This scheme also has the advantage that RecA can load continuously in the 5′ to 3′ direction from the RecOR nucleating point.

5.1. A Molecular Connection Between RecF(OR) and DNA Replication

The position of *recF* and *recR* on the chromosome in the middle of operons encoding DNA-replication proteins and the observation that UV-induction of the SOS response by *recF* requires DNA replication suggests a connection between *recF, recR,* and DNA replication. Rothman and Clark *(70)* showed that after UV irradiation, DNA synthesis in a *recF143* mutant was greatly reduced relative to wild-type. Courcelle et al. *(16)* have shown that both *recF* and *recR* mutations lead to much greater amounts of degradation of newly synthesized DNA after UV irradiation and a decreased ability to complete ongoing rounds of DNA replication than wild-type.

A role for *recF* in DNA replication has been suggested by overlapping activity with *priA* in UV-induction of the SOS response (pre-synaptic role) and cell viability (post-synaptic role) *(73).* PriA, originally isolated as a component of the ΦX174 in vitro DNA replication system (a model for primosome assembly and synthesis of RNA primers during lagging strand DNA synthesis at a replication fork [58]) has been shown to be essential for DNA repair and homologous recombination *(32,82).* Interestingly, the overlapping roles of *priA* and *recF* are not shared by *recR* and *recO.* This is the only example of a phenotype where *recF* is different from that of *recO* and *recR* (where all three have been tested). It should be noted that the proposed post-synaptic role for *recF* is highly speculative.

Three additional observations support a role for *recF* in DNA replication and or cell viability. The first is that *recF143* causes a decrease in UV-mutagenesis with ssDNA phages *(12).* This function may be overlapping with *priA.* The second is that *recF* is necessary for stable DNA replication and cell viability in a *rnh-102 recA200 rin-15* and *rnh-102 recA200 rin-15 dnaA508* strains, respectively *(95).* The third situation in which *recF* may have a role in DNA replication is that it is needed for viability in a *recA200 polA25::spc lexA71::Tn5* strain *(10).* Although a common thread between the latter three mutant strains is not apparent, all have a defect in DNA replication (and or cell viability) that is suppressed by some mutation (either *rin-15* or *lexA71::Tn5*) and this new situation is then dependent on the *recF* gene product. It is not clear, however, if *recF* participates in a pathway that is active to a small degree in wild-type cells and this becomes the major pathway in the mutant cells, or if the pathway only becomes active in these "suppressed" states. The dependence of *recO* and *recR* in these strains has not been addressed.

6. THE ROLE OF THE PRIMOSOME ASSEMBLY PROTEINS IN RESTARTING REPLICATION FORKS

The role of the primosome assembly proteins (PriA, PriB, PriC, DnaB, DnaC, DnaG, DnaT) in the cell is beginning to become clear (reviewed in *[57,58,80,81]*). These *E.*

coli proteins were originally discovered as host proteins required in the ΦX174 in vitro DNA replication system. The biochemical properties of these proteins suggested that they were involved in the synthesis of RNA primers on the lagging strand at a replication fork. Although this is still possible, it now appears that these proteins have different or additional roles. It is now believed that these proteins help to restart replication forks that have stalled or collapsed and were repaired by recombinational and RecA-independent processes.

6.1. Primosome Assembly Proteins in DNA Replication In Vitro

Although the ΦX174 in vitro DNA replication system may not be the best model of the in vivo function of the primosome assembly proteins, this system provides the clearest picture at the biochemical level and provides a platform for discussing their in vivo functions. A key feature of the assembly process is the DNA substrate to which the proteins are loaded. This is the primosome assembly site (PAS) of the ΦX174 chromosome. Although other ssDNA phages and some plasmids have these sites, they have never been found on the *E. coli* chromosome. It is thought that both sequence and secondary structure are important for PAS *(1,89).* It is suspected that the PAS is a structure that some phages and plasmids have evolved to take advantage of the *E. coli* host system for their replication. Figure 5 shows the order of assembly of the primosomal proteins onto the PAS *(62,63).* The PriA protein binds to PAS and serves as a platform for the loading of the other proteins. PriA is a multifunctional protein with helicase, ATPase, and translocase activities that are genetically separate from its ability to assemble primosomes *(109).* The PriB protein binds to PriA-PAS. It is thought that PriB stabilizes PriA at PAS *(41).* DnaT then loads onto the PriA-PriB-PAS complex. These three proteins form a protein-nucleic acid complex that serves as an entry point for DnaC to load DnaB, the replicative helicase. DnaC is the only primosome assembly protein not part of the final primosome. Using some DNA substrates with PAS, there is an optimizing requirement for PriC before this step. However, the role of PriC is unknown. The PriABC-DnaTB complex is called the pre-primosome and DnaG (Primase) can interact with it in a distributive *(108)* fashion to synthesize RNA primers that are competent to be extended by Pol III holoenzyme.

6.2. Primosome Assembly Proteins and E. coli Replication Restart

Many questions were raised that eventually helped to clarify the role of the primosome assembly proteins in *E. coli.* The first is based on the observation that no PAS sites have been found on the *E. coli* chromosome. If true, then what is the natural substrate for PriA? Also, no slow or fast stop mutants of *priA, priB,* or *priC* have ever been isolated and none of these proteins are needed in an in vitro system that mimic initiation of DNA replication at *oriC.* This raised questions about how and when PriA PriB, DnaT, and PriC become associated with a replication fork. Surprisingly, a *priA* null mutant was unexpectedly found to be viable, deficient in recombination and DNA repair, and had high basal levels of SOS expression *(39,65).* Hence PriA was a DNA replication protein that also had roles in recombination. Tokio Kogoma *(30–32)* proposed that the pathway of primosome assembly might be used by *E. coli* to load replication forks at recombinational intermediates. This hypothesis has led to many advances in understanding the biochemistry of PriA. Several studies *(40,59,64)* have now shown that PriA binds to D-loops, a key recombinational intermediate.

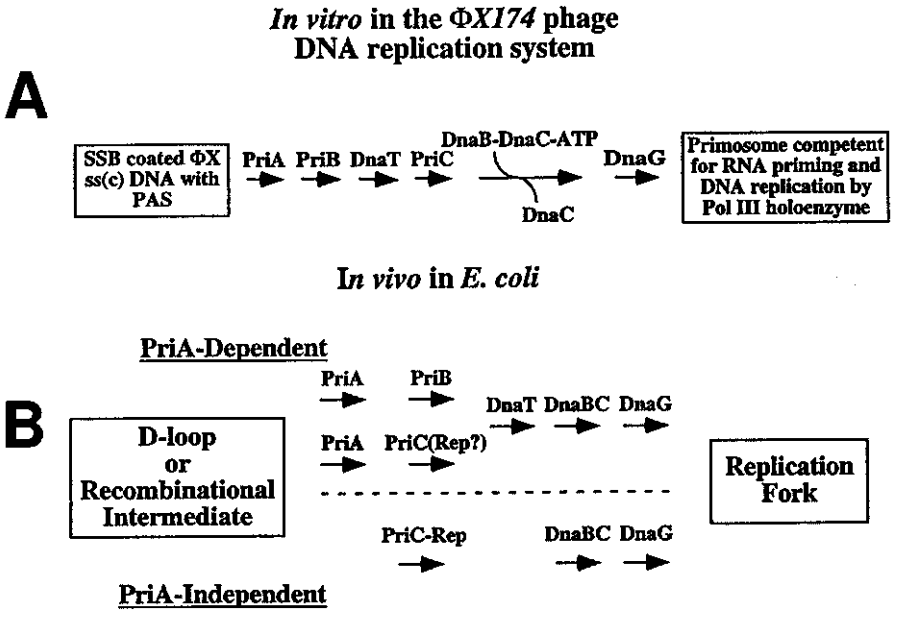

Fig. 5. Two models for the action of the primosome assembly proteins. In **A,** the in vitro assembly of the primosome assembly proteins on the ssDNA phage is shown. In the **B,** is a model for two pathways of primosome assembly as a prelude to the loading of a DNA replication fork. One should note that in the bottom half, although the substrate is referred to as a recombinational intermediate like a D-loop, in actuality this substrate has yet to be identified in vivo. The Rep protein in the PriA-dependent pathway is listed with a question mark because its role is currently not clear.

Understanding the genetics of the primosome assembly genes have been of intense interest to our lab. Almost every simple prediction of ΦX174 model so far has not proved true for *E. coli.* For instance, one would predict that *priB* and to lesser extent, *priC,* should have the same mutant phenotypes as *priA* mutants, but this was not the case. Null mutants of *priB* and *priC* are not readily distinguishable from wild-type *(81).* Yet the *priB priC* double mutant is inviable, suggesting that these proteins have a redundant and essential role in *E. coli.* Extragenic suppressors of *priA* mutations have been found and mapped in *dnaC (82).* These are thought to load DnaB at the correct DNA substrate in the absence of PriA, PriB, PriC and DnaT. While these *dnaC* suppressors fully suppress *priA* mutant phenotypes, they only partially suppress *priB priC* mutant phenotypes *(81).* In vitro, the PriA suppressor DnaC810 can load Pol III holoenzyme (via DnaB) at a D-loop *(42).* Hence the biochemistry is beginning to agree with the in vivo data.

Finally, a lingering question has been why *priA* mutations are not lethal, as it would seem that restarting replication forks at recombinational intermediates is essential. One idea is that there are multiple pathways for restarting replication forks. Evidence for this has come from synthetic lethality studies of pairs of primosome assembly mutants *(71).* Figure 5 shows a diagram of one model that explains how some gene products may be involved in these multiple pathways. It is noteworthy that the *priC* protein, which has not found a secure home in the ΦX174 model, is essential for the PriA-independent path-

way. Also, another orphan DNA replication gene, *rep,* is essential for this pathway. Additional experiments show that the *dnaC809* suppression pathway of *priA* mutant phenotypes occurs by the elevation or modulation of the PriC-Rep pathway *(71,80).*

Why would *E. coli* two pathways to restart replication? Although there are several possible reasons, the most appealing is that there are different DNA substrates that need to be processed into replication forks. These could arise by different mechanisms such as replication fork arrest or collapse. One might expect that repair of these two situations would lead to different DNA structures with different complements of proteins and thus may be optimally restarted by two different systems. This idea is analogous to the RecBCD and RecF pathways of recombination, which act preferentially on different types of substrates (double-strand ends or gaps) to repair and recombine DNA.

ACKNOWLEDGMENTS

This work was supported by start-up funds from the University of Massachusetts and grant RPG-99-194-01-GMC from the American Cancer Society.

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3

Abasic Site Repair in Higher Eukaryotes

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1. INTRODUCTION

Base-excision repair (BER) refers to a repair pathway that generates and repairs abasic sites in double-stranded (ds) DNA (Fig. 1) *(101,134,222,264).* BER is important not only in maintaining the integrity of nuclear DNA but also in protecting mitochondrial DNA against oxidative onslaught from $FADH_2$ and NADH and the reactive oxygen species generated during O_2 reduction *(42).* Estimates of the number of abasic sites generated per mammalian cell per day run as high as 10^6/cell/d *(88).* Abasic sites are unstable, degrading spontaneously into DNA strand-breaks by β-elimination *(132)* that retard DNA polymerases *(43,44,50,66,91,237).* They are highly mutagenic because of nontemplated DNA *(59,108,273)* and RNA *(66,216,217,283)* synthesis. Moreover, abasic sites engage in suicide reactions with topoisomerase I, leading to permanent DNA damage and premature cell death *(196)* and can form covalent complexes with topoisomerase II that cause DNA double-strand breaks *(107),* which can bind poly (ADP-ribose) polymerase *(2,152,153).* Despite the large number of abasic sites generated per cell per day, the number of resulting mutations is extremely low. The difference reflects the elaborate mechanisms that the cell has devised to repair abasic sites *(134).*

An abasic site can be created by spontaneous base loss as in depurination, by DNA oxidation *(133,134,250),* or by the action of DNA glycosylases *(45a,66,115,159),* which recognize and cleave nonbulky base lesions in DNA *(115).* Some glycosylases remove the altered base without cleaving the DNA backbone. Others not only remove the base and but also cleave the abasic site 3′ to the sugar residue (Fig. 1). Cleavage 3′ to the sugar residue occurs by a lyase or β-elimination mechanism *(115,159).* In either case, once the abasic site has formed, it must be nicked on the 5′ side of the dRP in order to generate the free 3′ hydroxyl group that is required by the repair polymerase to insert the correct nucleotide(s). Depending on which polymerase inserts the correct nucleotide(s), the strand downstream of the abasic site may remain undisturbed or it may be subject to strand displacement. Subsequently, the dRP residue and any displaced DNA are removed so that the newly reconstructed strand can be ligated.

The enzyme that appears to be common to all the branches of the BER pathway is AP endonuclease (AP endo). AP endo cleavage generates the critical free 3′ hydroxyl for repair DNA synthesis *(58).* Because all the repair mechanisms converge at the aba-

From: *DNA Damage and Repair, Vol. 3: Advances from Phage to Humans*
Edited by: J. A. Nickoloff and M. F. Hoekstra

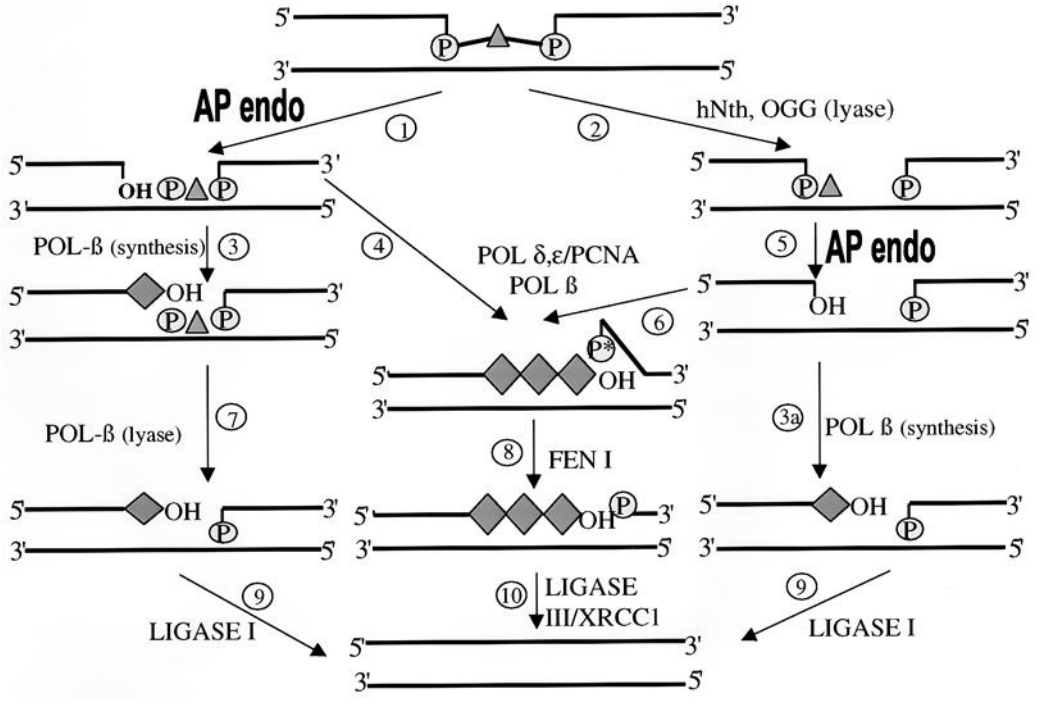


Fig. 1. Repair of abasic sites diverge after the generation of the 3′-hydroxyl required for replacement synthesis. The various enzymatic reactions are numbered for reference to the text. P, phosphate; Δ, deoxyribose; ◇, newly inserted nucleotide; P*, 5′-deoxyribose phosphate. The product of displacement synthesis (reactions 4 and 6) can be 2–6 nucleotides in length but is shown as 3 nucleotides for clarity. Reactions 9 and 10 may be interchangeable. Short-patch repair is comprised of steps 1 or 2, 3 or 5, 7 or 3a, and 9 or 10. Long-patch repair is comprised of steps 1 or 2 and 5, 4 or 6, 8, and 9 or 10.

sic site and because the repair processes diverge after cleavage of the abasic site 5′ to the phosphodeoxyribose, we suggest that this repair pathway is better termed abasic site repair (ASR). Henceforth, we will refer to the pathway as ASR.

This review will present a detailed biochemical picture of the overall ASR pathways in higher eukaryotes and then review the enzymes and the auxiliary proteins involved in the individual steps. After presenting the evidence for interactions among the components involved in ASR, we discuss subcellular locations of the pathway and, finally, probable interactions with other DNA repair systems. We shall briefly discuss the different DNA glycosylases without going into detail, because they are involved in generating the abasic site but not repairing it and because they have been reviewed extensively elsewhere *(45,45a,115,159).* Studies on yeast are omitted because the various enzymatic activities found in higher eukaryotes, while present, may be grouped differently and are reviewed elsewhere *(4,98,219,279).* The major emphasis will be on studies appearing in the last several years.

2. OVERVIEW

Under normal circumstances, DNA undergoes depurination at a measurable rate such that some 10,000 abasic sites/mammalian cell/d are generated *(66,133,134).* In

addition, a variety of lesions in DNA arise by spontaneous deamination of cytosine, from errors occurring during replication including insertion of damaged bases or of uracil, from reactions with endogenous reactive oxygen species *(50,66,162)* or from exogenous exposure to toxic species in food and/or the environment *(66,162).* The lesions include, among others, uracil, 3-methyladenine, 8-oxoguanine, and thymine glycol. Nonbulky lesions are generally recognized by the DNA glycosylases that remove the modified base and leave an abasic site (Fig. 1). Higher eukaryotic cells have two classes of DNA glycosylases, those that remove the base only and those that remove the base and nick the DNA on the 3′ side of the dRP residue. Enzymes in the first category include uracil DNA glycosylase (UDG), G/T glycosylase, thymine glycol-DNA glycosylase, thymine DNA glycosylase, and 3-methyladenine-DNA glycosylase *(115).* UDG is by far the most abundant and active of these, because the presence of uracil in DNA occurs at high rates. Under normal circumstances estimates range as high as one deoxyuridine out of every 200 or 300 thymidine residues inserted during replication *(112).* Another major source of uracil in DNA is the spontaneous deamination of cytosine *(66).* The second set of glycosylases include 8-oxoguanine DNA glycosylase (OGG), hNth1 *(92),* N-methylpurine/DNA glycosylase *(66),* and adenine-specific DNA glycosylases *(112).* N-glycosylases in eukaryotic cells are not abundant *(115,222).* To complicate matters further, at least one DNA glycosylase is able to remove normal bases in a random fashion, albeit at a slow rate *(15).*

In most instances, apurinic/apyrimidinic endonuclease (AP endo) initiates the repair process of an abasic site (Fig. 1, reaction 1). AP endo cleaves an intact abasic site 5′ to the phosphodeoxyribose (dRP) and generates the 3′ hydroxyl to be used by the DNA polymerase that will insert the correct nucleotide. The dRP moiety remaining attached to the downstream strand will be removed in subsequent steps. If a glycosylase/lyase has already nicked the abasic site 3′ to the dRP (reaction 2), AP endo has been proposed as the enzyme that facilitates removal of the 3′ phosphodeoxyribose residue (reaction 5) and generates the all-important 3′ hydroxyl for subsequent polymerase replacement of the nucleotide (reaction 3a) (but see section on AP endo later).

If the glycosylase has not nicked the abasic site 3′ to the dRP residue, DNA polymerase β (pol β) fills the gap with the correct nucleotide (reaction 3) and then excises the dRP from the downstream cleaved strand (reaction 7). Because synthesis is limited to a single nucleotide, this pathway is commonly called "short-patch" or "single-nucleotide" BER. We shall refer to it as short-patch ASR. Ligase I or possibly ligase III together with XRCC1 (*X*-ray Cross-Complementation protein 1) then seals the two ends together (reaction 9). In the alternate pathway (reactions 4, 6, 8, 10), pol β *(55)* or DNA polymerase ε or δ in conjunction with Proliferating Cell Nuclear Antigen (PCNA) replaces the missing nucleotide plus 2–6 nucleotides downstream of the initial abasic site (reaction 4 or 6). The displaced strand is cleaved by flap endonuclease (FEN1) (reaction 8). Because 2–6 nucleotides are inserted after the 3′ hydroxyl, this pathway has been referred to as "long-patch" or "alternate" BER *(47,57,109,200)* in contrast to short-patch repair where only the one nucleotide gap is filled. After long-patch repair, ligase III in conjunction with XRCC1 or possibly ligase I seals the ends of the DNA strand together (reaction 10). Some reports indicate that the ligases (ligase I and ligase III/XRCC1) may be interchangeable *(109).*

The biochemical mechanism for deciding which pathway is used for insertion of nucleotides and rejoining is speculative at this time. Long-patch repair in some cells is

observed when pol β is deficient *(16,63)* or when the dRP residue cannot be cleaved by lyase activity, as occurs when the abasic site is reduced *(109)* (*see* below). In other pol β-deficient cells, long-patch repair is not detected *(215)* unless a closed circular substrate is employed *(55).* To complicate matters further, antibody against pol β inhibits long-patch repair in cell-free extracts *(109).* In a reconstituted system described by Fortini et al. *(64) (see Subheading 2.4.),* the choice of pathway depended on the type of lesion. Repair of lesions requiring a DNA glycosylase/lyase was restricted to short-patch ASR, whereas repair of lesions requiring a DNA glycosylase without lyase activity could be repaired by either long- or short-patch ASR. Whether these are the only situations where each pathway occurs is not known.

The various reaction sequences of ASR have been measured in extracts from a variety of higher eukaryotic cells including *Xenopus laevis* oocytes *(154,156),* bovine testis *(225),* mouse fibroblasts *(36,227),* and human cell lines *(52,54,56,109,175).* Apparently, short-patch ASR predominates in human and mouse cells when the substrate is a ds oligonucleotide or plasmid substrate containing a G/U mispair *(16,67,109,136,227),* because the inactivation of pol β by blocking antibody prevents the completion of repair *(109,227)* and because knockout mouse cells lacking pol β are unable to perform ASR on a short oligonucleotide *(227)* (but *see* above Subheading 2.2.2.). In addition, extracts from fibroblasts that respond to lipopolysaccharide treatment with increased BER fail to respond if the cells lack pol β *(36).* These results would imply that in extracts from these cells at least the long-patch pathway is lacking, inefficient, or not increased. Reconstitution of long-patch repair by human cell extracts has also been accomplished. The substrate was ds closed circular DNA containing a single uracil *(54,55).* In this case, RPA stimulated PCNA-dependent repair. The two cofactors were additive; neither could substitute for the other.

The reaction sequences for the short- and long-patch pathways have now been reconstituted with purified proteins *(64,109,116,177,229).* Components in the former pathway most often include UDG, AP endo, pol β, and DNA ligase I or DNA ligase III/XRCC1. The substrate is often a defined oligonucleotide containing a G/U pair, with substrate and enzymes being present in approximately equimolar concentrations. The rate-determining step for the short-patch pathway on a 51-mer ds oligonucleotide with a single G/U pair at position 22, i.e., one involving generation of the abasic site by UDG, single nucleotide gap filling by pol β, and ligation by DNA ligase I, has been identified *(229).* The insertion of the new nucleotide can occur prior to removal of the dRP moiety. The enzymatic efficiency (k_{cat}/K_m) of the individual components ranges from 420 $\mu M^{-1}s^{-1}$ for UDG to 0.15 $\mu M^{-1}s^{-1}$ for the dRP lyase activity of β-pol. Indeed, the number of molecules per cell for pol β is about 10% that of AP endo (3.5×10^5 for human fibroblasts and 7×10^6 for HeLa cells) *(34).* In other words, not only is pol β an inefficient enzyme, it is present at ~10% the level of AP endo. Because the slowest step in the pathway is the dRPase activity and because this rate reflects the overall rate of the pathway, the dRPase activity of pol-β is likely to be the rate-limiting step *(229).*

Components in the long-patch pathway reconstituted from purified or recombinant proteins may include UDG, FEN1, PCNA, pol δ and/or pol ε, ligase I or III, RPA or RFC, and XRCC1 *(47,71,109,116,158,165).* Omission of PCNA causes accumulation of pre-excision reaction intermediates following strand displacement, which is overcome by the addition of PCNA *(71).* A PCNA mutant unable to bind FEN1 is unable to stimulate excision *(71).* On the other hand, if pol β performs long-patch repair, as might

occur when its dRPase cannot remove the dRP residue (for example, if the abasic site were created by tetrahydrofuran or had been stabilized by reduction), then FEN1 can still cleave the displaced DNA strand without the assistance of PCNA *(71).* In most cases, RFC is required and RPA will not substitute. However, the choice of factor may depend on the starting substrate. As of this writing, there are no data available for the kinetic measurements of individual components in the long patch pathway.

An interesting series of experiments using mammalian cell extracts examined whether pol β is the *only* polymerase that can perform short patch ASR *(53).* The substrate was a closed circular DNA containing a single 8-oxoguanine at a defined site. Extracts from pol β deficient mouse cells resulted in extension of the repair gap by long-patch repair only 50% of the time. The remainder was accomplished through replacement of a single nucleotide. Thus, in cases where the pathway is initiated via a glycosylase/lyase reaction BOTH long-patch and short-patch repair can occur, *even in the absence of pol* β. Clearly this is a different case than the situation where ASR is initiated through removal of a uracil owing to the action of UDG and suggests that polymerase(s) other than pol β are capable of filling the single nucleotide gap through short-patch repair. Because pol δ is not known to be able to insert single nucleotides, is it possible that under these circumstances pol ε, which is involved in maturation of Okazaki fragments (*see* below Subheading 2.2.2.2.), inserts a single nucleotide, thus performing short-patch repair? Or, in cell extracts such as these, could the polymerase be DNA polymerase α or one of the newly discovered or as yet undiscovered DNA polymerases not previously associated with ASR?

2.1. The Abasic Site

Several NMR studies have examined the structure of the abasic site in solution when the abasic site is located in a ds oligonucleotide *(11,43,44,130A,131,147,253).* In general, the presence of an abasic site increases flexibility of the DNA and concomitantly increases the diffusion constant *(147).* However, the solution structure of the oligonucleotide depends on the base opposite the abasic site and the sequence context. An apurinic site differs from an apyrimidinic site. The former has α and β hemiacetal forms in approximately equal amounts, whereas the latter is predominantly the β hemiacetal. Sequence context is also important, because the NMR structure of an abasic site located in the context of curved DNA (dA tract) is different from the ones just described *(253).* In addition, the structures of the both α and β hemiacetals differ from those where the DNA oligonucleotide is not bent.

In a study investigating the effects of an abasic site on DNA conformation, when a tetrahydrofuran was incorporated into a family of duplex 13-mers, the global B-form conformation did not change. However, the presence of the lesion induced enthalpic destabilization of the duplex with the magnitude of the effect being dependent on the sequence context *(74).* In a second study using a ds 10-mer with a tetrahydrofuran inserted into the center of one of the strands, the stem region of the duplex adopted a right-handed helical structure with the abasic site excluded from the helix *(131).* The effect of the presence of a second abasic site on the contralateral strand is described in Subheading 2.2

Despite local thermodynamic and structural changes in DNA containing an abasic site or an abasic site analog, AP endo, the enzyme responsible for recognizing abasic sites, appears to have little sequence preference *(28,263;* Mckenzie and Strauss, unpublished data). Consequently, it is likely that the enzyme itself is capable of imposing a

Table 1
Proteins Involved in ASR[a]

Repair protein	Subunit	Molecular mass (kDa)	Chromosomal location (Human)
AP endonuclease		35.5	14q11.2–12 *(208)*
The polymerases			
Pol β		39.0	8p11–12 *(27)*
Pol δ	Catalytic	125	19q13.3 *(103)*
	Small	50	7 *(280)*
Pol ε	Catalytic	261	12q24.3 *(235)*
	Small	55	14q13–q21 *(129)*
Pol γ		136	15q24–q26 *(210,252,286)*
Associated enzymes			
FEN1		42	11q12 *(86)*
Ligase I		125	19q13.2–13.3 *(287)*
Ligase IIIa		922	17q11–12 *(35,259)*
Ligase IIIb			" "
Mitochondrial ligase			" "*(121)*
Auxilliary proteins			
PCNA		29	20p12–p13 *(205,245)*
$p21^{Cip1/Waf1}$		21	
RPA	hRPA70	70	17p13.3 *(288)*
	hRPA32	32	1P35 *(288)*
	hRPA14	14	7P22 *(288)*
RFC	p140	140	4P13–p14 *(142)*
	p40	40	7q11.23 *(184)*
	p38	38	13q12.3–q13 *(184)*
p37	37	3Q27 (184)	
	p36	36	12q24.2–q24.3 *(184)*
PARP		122	1q42 *(84)*
XRCC1		70	19q13.2 *(122)*

[a] The individual proteins with their molecular masses and human chromosomal location are listed. The numbers in parentheses refer to the literature citation.

transition state conformation that is independent of the initial conformation of the abasic site-containing oligonucleotide. (*See* also the description of domain mapping and co-crystal studies described in Subheading 2.2.1.)

2.2. The Enzymes

The enzymes and auxiliary proteins that participate in ASR are listed in Table 1 that also includes their subunits, molecular masses, and chromosomal locations in the human genome. A more complete description of each protein follows.

2.2.1. AP Endonuclease

The enzyme that initiates ASR is AP endo *(93).* The enzyme is highly conserved from man to *E. coli,* where the homologous enzyme is exonuclease III (Exo III) *(7,51,58,209,223).* The active site is also structurally similar in these two enzymes.

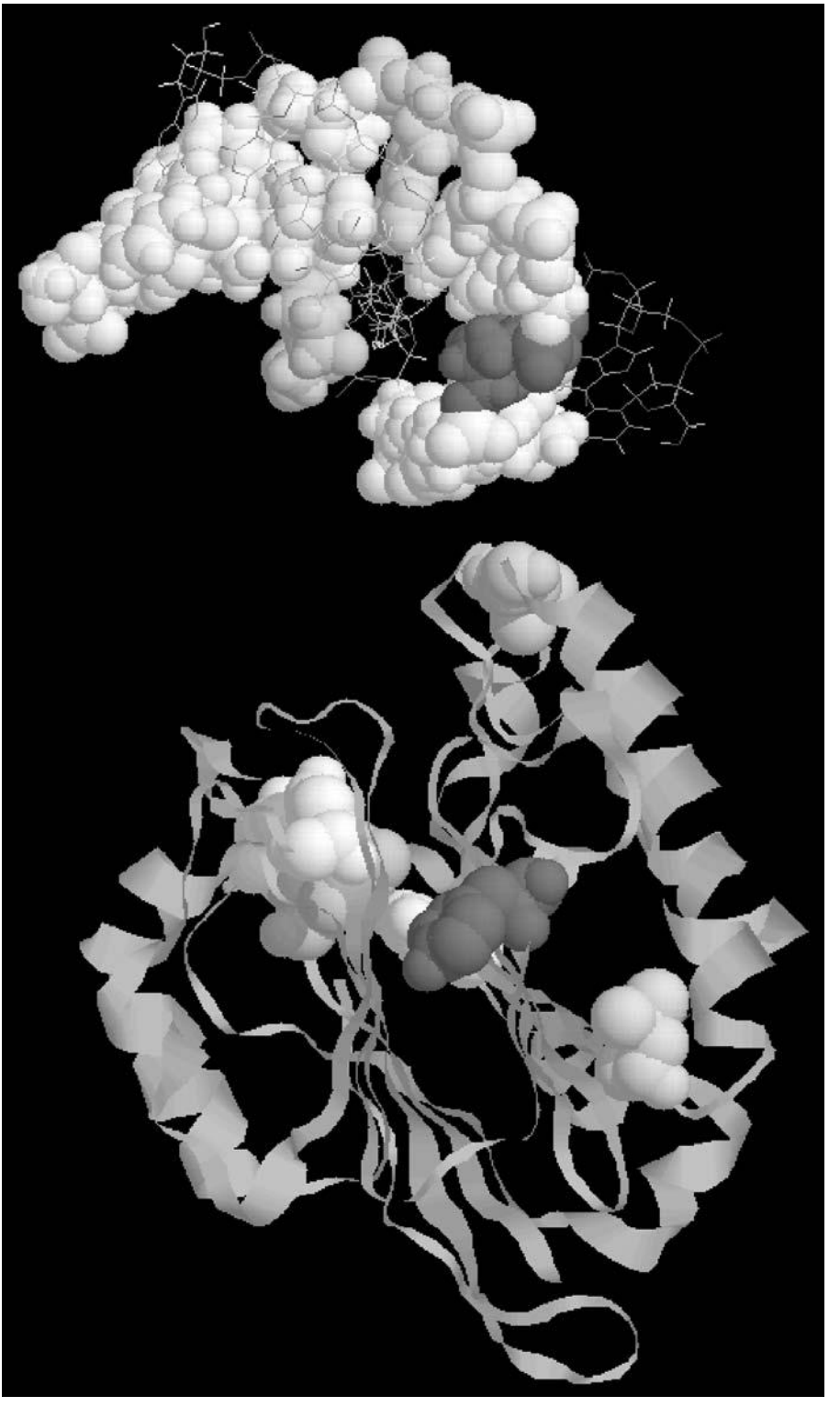
The human enzyme consists of 318 amino acids with a molecular mass of 35.5 kDa. The crystal structure of the enzyme lacking the first 43 N-terminal residues has been reported *(76)* and is shown in Fig. 2. The enzyme consists of two portions, the nonordered amino terminus that extends to amino acid 36 and is missing in the enzyme from prokaryotes, and an ordered globular domain comprising the remainder of the molecule *(231).* The unconserved first 36 amino acids include 11 positively charged residues, 8 negatively charged residues, and 4 proline and glycine residues that discourage the formation of alpha helices. The function of the amino terminus for DNA repair is unknown, because its removal does not appear to alter the ability of the enzyme to nick DNA *(7,95).* However, the amino terminus contains two potential nuclear localization sequences, one at residue 2 (PKRGKKG) and one at residue 21 (PEAKKSK) *(85)* and a potential mitochondrial transit peptide sequence at residue 14 *(173).* Although the function of the N-terminus in the nicking reaction is unclear, this portion of the protein is apparently involved in the redox activity described in Subheading 2.2.1.

The major enzymatic activity of AP endo is cleavage of the abasic site on the 5′ side of the phosphodeoxyribose in ds DNA *(58,82,231).* This activity requires a divalent cation with Mg^{2+} being preferred. Mn^{2+} will not substitute, although in some site-directed mutants, Ca^{2+} restores the activity to that of the wild-type enzyme *(151).* The nicking activity requires residues from amino acid 62 through the carboxyl terminus *(95).* Deletion mutants beyond NΔ61 are unable to nick abasic site-containing oligonucleotides nor provide resistance to methyl methane sulfonate in *E. coli* xth^{-} nfo^{-} double mutants *(95).*

Other enzymatic activities found in Exo III are markedly diminished in the human enzyme. These include a 3′ exonuclease activity, 3′ dRPase activity, 3′ phosphodiesterase activity *(34,50,58,234),* and an RNase H activity *(7,209).* Although the turnover number for the 3′ phosphodiesterase is 0.05 s^{-1} *(266)* and its activity is present at ~1% of the nicking activity, a strong case can be made for its importance under oxidative-stress conditions. The level of AP endo expression is increased when cells are exposed to nontoxic levels of a variety of ROS *(204)* that generate the need for the 3′ end removing activity. Izumi et al. *(93)* point out that the level of expression of AP endo remains unchanged by treatment with alkylating agents or ultraviolet (UV) light, where there is apparently sufficient number of molecules to perform the standard nicking reaction. In the case of oxidative stress, the 3′ phosphodiesterase activity may be rate-limiting. Although the protein is abundant, the necessary activity is low and the amount of enzyme is insufficient to meet the enhanced need.

Detailed kinetic analysis of the AP endo DNA strand scission reaction was accomplished using single-turnover kinetics. In this type of analysis, the enzyme undergoes one round of catalysis. When it dissociates from either substrate or product, it is prevented from undergoing a second catalytic round. Single-turnover analysis was made possible when it was demonstrated that the β-elimination product of a ds oligonucleotide containing an abasic site (HDP) acts as a powerful inhibitor of the nicking activity of the enzyme *(230).* In order to perform single-turnover studies, enzyme was allowed to bind to substrate in the absence of divalent cation. One round of catalysis by enzyme already bound to substrate was initiated by the addition of Mg^{2+} in the presence of HDP + heparin.

These studies made it possible to determine the kinetic binding and dissociation constants for substrate of the wild-type and several mutant enzymes and to demonstrate that the kinetic scheme followed by the wild-type enzyme is best described by a Briggs-



Haldane mechanism *(230).* The turnover number for the wild-type enzyme is 10 s^{-1}, whereas the catalytic efficiency is 1.0×10^8 $M^{-1}s^{-1}$ and the K_d is 0.8 nM. These constants serve as reference points for all subsequent studies.

AP endo has another activity that is ostensibly unrelated to ASR and that resides in the N-terminal portion of the molecule. The enzyme is able to promote DNA binding by p53 *(96)* as well as by the AP-1 transcription factors, Fos/Jun heterodimers or Jun/Jun homodimers, in vitro, in the absence of reducing agents. Binding was demonstrated by electromobility shift assay (EMSA) *(210A,270,271).* Activity for stimulating DNA-binding of Fos/Jun dimers as well as NFκB, Myb, AP-1 proteins, members of ATF/CREB family, and HIFα (hypoxia inducible factor) *(87,90,258,262)* is reported to require Cys^{65} because site-directed mutagenesis of AP endo Cys^{65} to alanine results in loss of the redox activity. Initially, Cys^{65} was hypothesized to form a disulfide bridge with Cys^{93}, which together could provide the oxidative function. Although these residues were initially conceived as being readily accessible to the surface of the enzyme *(254),* both domain-mapping and X-ray crystallography demonstrated that they are not *(76,231).* Because there are no disulfide bridges in AP endo, at least none stable enough to be found in the crystal structure *(76),* the two residues themselves may not be involved in redox activity; rather, they might maintain a conformation that allows other residues that are surface accessible such as the amino terminus to perform this function *(96).*

Although physical evidence for redox function of intact AP endo is not in hand, nuclear magnetic resonance (NMR) studies reveal that when a peptide comprising residues 59–71 of AP endo is mixed with human thioredoxin, the AP endo peptide binds in a crescent-shaped groove on the surface of thioredoxin *(201).* One study indicates that AP endo may be involved in regulating gene expression in response to hypoxic conditions *(261).* Smooth-muscle cells respond to oxygen deprivation by inducing the expression of heme oxygenase (HO-1) *(277).* In response to hypoxic conditions, thioredoxin becomes localized in the nucleus. Thioredoxin treatment of cells co-transfected with AP endo and the enhancer region for HO-1 enhanced gene expression of HO-1 threefold.

As stated earlier, in the crystal structure lacking the first 43 N-terminal residues, the enzyme is globular. The globular portion consists of two six-stranded β-sheets surrounded by α-helices, which together form a four-layered α/β sandwich arranged in a nuclease fold, a feature characteristic of many endonucleases. The cocrystal reveals that the enzyme inserts loops into both major and minor grooves, binding the flipped out AP

Fig. 2. Three-dimensional structure of human AP endonuclease *(76)* and a 12-mer oligonucleotide containing a single abasic site *(11).* Although abasic site-containing DNA does not fit spatially into the crystal structure, which was obtained in the absence of DNA, the oligonucleotide is shown to provide a sense of scale and to illustrate results described in the text. Active-site residues of AP endo are His^{309} (yellow), Asp^{283} (white), Asp^{308} (white), and Glu^{96} (white). Residues identified by domain mapping *(217)* as protease accessible but blocked in the presence of substrate/product are Leu^{179} (green), Tyr^{144} (red), and Leu^{205} (blue). The structure of the 12-mer with the single abasic site, obtained by NMR *(11),* is aligned to permit the viewer to visualized potential interactions with the active site and the groove along which the blocked sites lie. The abasic site is indicated as green space-filled atoms in a wireframe chain. The complementary strand is shown as space-filled, where the position opposite the abasic site is blue, the position located at -1 is identified in yellow and the position located at -3 is shown in red.

site in a pocket. The orphan base across from the flipped out AP site hydrogen bonds with the base 5′ to the AP site, which results in a kink in the DNA *(163a).* The active site is proposed to include His^{309} flanked spatially by two aspartate residues, Asp^{283} and Asp^{308} (Fig. 2). The importance of these residues for catalysis and substrate binding has been confirmed kinetically *(141)* and by EMSA *(150).* Kinetic binding studies provide the precise binding constants that are related to enzyme function, i.e., enzymatic cleavage, whereas EMSA studies provide data on structural binding of the enzyme to its substrate independent of enzymatic activity. Differences in results obtained by the two protocols occur frequently (176A, 210A, Fattal and Strauss, unpublished data) and may reflect sensitivity of EMSA to forces imposed by an electrical field as well as shear force from the supporting gel during electrophoresis, or may reflect a two-step binding process involving conformational change and/or the destabilization that occurs during electrophoresis. Asp^{283} and Asp^{308} are apparently involved in maintaining the spatial conformation of the active site histidine *(141)* and in providing a negatively charged environment that permits interaction with the abasic site. Kinetic studies have shown that mutation of either active-site aspartate diminishes binding of the abasic-site substrate to the enzyme by two orders of magnitude and enhances dissociation. Mutation of both active-site aspartates diminishes kinetic binding by four orders of magnitude *(141).* Mutation of His^{309} to asparagine decreased k_{cat} by 30,000-fold, while attempts to measure binding under single-turnover conditions were unsuccessful owing to weak enzyme-substrate interaction. Despite the striking differences in enzymatic cleavage and kinetic binding, the two aspartate mutations, alone or together, and the histidine mutant had near wild-type binding behavior on EMSA analysis *(150).* Other site-directed mutagenesis studies have also shown that residues required for shifts in electrophoretic mobility are not necessarily the ones required for nicking *(8,150).* These data argue that at least some of the residues involved in AP endo complex stability under EMSA conditions are not the same as those involved in catalysis.

In addition to kinetic and EMSA studies, molecular modeling indicates that replacement of either aspartate with alanine probably results in movement of other key residues in the neighborhood of the active site. Many of the residues that move and the degree of shifting are different for the two aspartates, again implying that the two residues are not strictly equivalent *(141).* Apparently these small, computer-predicted changes in conformation may result in large changes in enzymatic behavior but do not affect the behavior of the enzyme during gel-shift assays.

A number of other mutations at highly conserved residues have been prepared and examined for nicking activity under steady-state enzymatic conditions or for complementation by phenotypic screening in *Escherichia coli.* These include Glu^{96} *(7,8,9,94),* Asp^{70}, Asp^{90} Arg^{177} *(210a),* Asp^{210} *(61,210a),* Asp^{219}, Met^{270}(163A), Met^{271} *(163a),* Asp^{308} and His^{255} *(8),* Asn^{212} *(211),* Glu^{96}/Lys^{98} *(94),* Phe^{266} and Trp^{267} *(60),* and Tyr^{171} *(61).* Although all of the residues examined except Asp^{70} showed some deficiency in nicking, none show the deficiency demonstrated by conversion of His^{309} to alanine described earlier. Although Glu^{96} is essential for complementation in *E. coli,* the E96A mutation is suppressed by conversion of Lys^{98} to arginine *(94).* Finally, cleavage is enhanced in the R177A mutant *(163a).* In addition, AP endo variants that have been identified in the human population include L104R, E126D, R237A, D283G, D148E, G306A, and G241R *(80a).* The first three variants exhibited 40–60% reduction in incision activity, while the

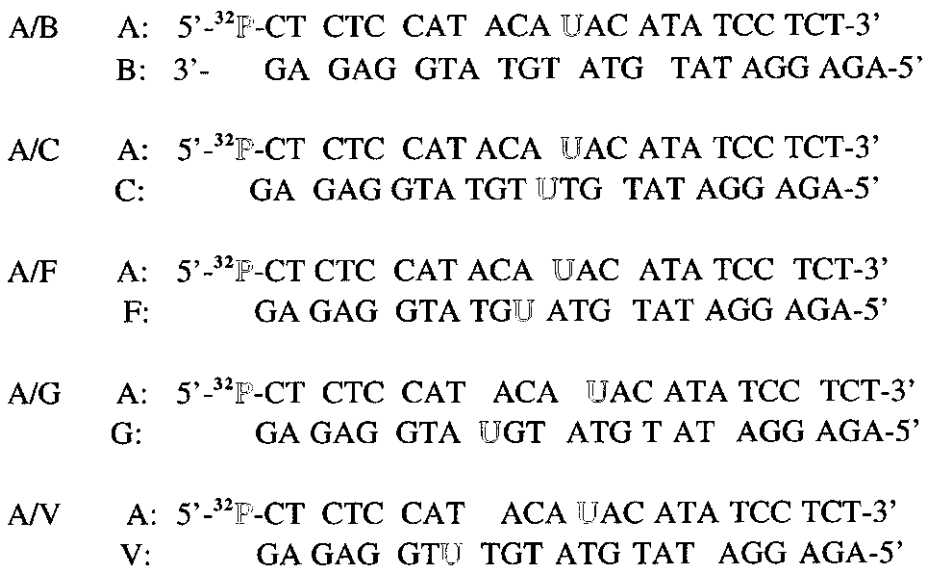


Fig. 3. Bistranded abasic site-containing substrates.

fourth, D283G, behaved similarly to D283A *(141,150).* Although neither D14E nor G306A affected cleavage activity, G241R had slightly *increased* activity.

On the basis of the crystal structure of AP endo and by analogy with other nucleases where a co-crystal has been obtained, Gorman et al. *(76)* have proposed a mechanism by which AP endo might bind to and nick an abasic site (*see* Fig. 2). The human enzyme requires at least 4 bp 5′ and at least 3 bp 3′ of an abasic site for incision and makes contacts within both the minor and major groove and with both strands of DNA around the abasic site *(262,263).* Five residues interact with the phosphate that precedes the one in the abasic site; another six residues interact with the phosphate in the dRP site. An aromatic residue such as Phe^{266} or Trp^{212} might interact with the abasic site itself. Note that Erzberger et al. *(60,61)* report that F266A and W267A retain considerable activity, while the nicking ability of Y171A, which is not remarked upon by Gorman et al., is greatly reduced. In the mechanism proposed by Gorman et al. *(76),* Asp^{283} extracts a proton from $His^{309,}$ which then hydrogen-bonds with water. The oxygen from the water molecule then attacks the phosphate on the 5′ side of the abasic site, which was made more electropositive because of the polarization of the metal ion complexed to $Glu^{96.}$ This mechanism implies that substitution of Asp^{283} with alanine should leave a catalytically inactive protein, which it does not *(141,151).* However, mutagenesis studies indicate that *either* Asp^{283} or Asp^{308} might extract the proton from $His^{309.}$

AP endo binds specifically around the abasic site in DNA and causes a pronounced distortion at the abasic site in a preincision complex *(263).* In light of this observation, what is the minimal DNA structure that AP endo requires in order to recognize an abasic site? Because the enzyme acts efficiently on substrate where the abasic site has been reduced with $NaBH_4$ or replaced by tetrahydrofuran, the mutarotation that a deoxyribose is expected to undergo is not necessary for enzymatic cleavage *(263).* In addition, the enzyme maintains considerable activity when the abasic site is replaced by propane-

diol. No activity is retained when the abasic site is replaced by 2-(aminobutyl)-1,3-propanediol, implying that the DNA backbone is critical but that the remainder of the deoxyribose is not. However, positioning a phosphorothioate ester immediately 5′ to the abasic site was highly inhibitory, with the Sp isomer being far more inhibitory (>10,000-fold) than the Rp isomer (~20-fold). Hence, the molecular spacing from the nucleotide preceding the abasic site to the one following the abasic site needs to be maintained, as does the electronic configuration around the 5′ phosphate. Furthermore, enzymatic cleavage requires at least 4 bp 5′ to an abasic site *(263)*

The configuration of the DNA just upstream from the abasic site is of particular importance, as shown by the effects that a second abasic site has on cleavage of the first *(32,33)* (Fig. 3). If the second abasic site is located downstream to the abasic site in question and on the opposite strand, it seems to have little effect on nicking the first abasic site. On the other hand, if the second abasic site is located upstream and on the opposite strand as the first abasic site (for example, A/V in Fig. 3), it can have a major impact on the ability of the enzyme to nick the first abasic site. In interpreting the data, it is not possible to distinguish whether the presence of the second abasic site introduces a kink in the substrate such that it can no longer bind nor position the first abasic site for cleavage or whether the base opposite the second abasic site is able to "swing out" of the helix and interrupt binding of the enzyme in a position to cleave the first site. Physical studies of oligonucleotides with more than a single abasic site examined the impact of two tetrahydrofuran residues opposite each other *(73)* or displaced by one base in either the 5′ or the 3′ directing. *(130a)* When the abasic sites are opposite each other, the lesion has minimal impact on the duplex transition enthalpy, but decreases the melting temperature (T_m) by 12°C. Although the data could have been interpreted as leaving the sugar phosphate backbone essentially unchanged with solvent providing the hydrogen bonding between the two strands, the authors preferred to interpret their data in the following fashion: the two tetrahydrofuranylphosphate moieties rotate out away from the helix, thereby allowing the 5′ and 3′ flanking base pairs to collapse on one another to form a stack similar to that seen within the control duplex. When two abasic sites are located one on each strand in a 5′ or 3′ orientation to each other, the thermal stability of the duplexes is severely reduced, especially in the 5′ orientation. In the 5′ orientation the abasic sites take up an extrahelical location, which contrasts with their smooth alignment along the sugar-phosphate backbone in the 3′ orientation *(130a).* The one thing that is clear is that conformation of the DNA helix for about one half of a helical turn upstream of the abasic site is important for recognition and cleavage.

The aforementioned data become even more relevant in the context of whether enzyme and/or substrate might undergo conformational changes during binding and cleavage. In domain-mapping experiments, substrate with an abasic site bound to the enzyme slows proteolysis at three chymotrypsin-cleavage sites *(231).* To conduct these experiments a divalent cation was required in order for proteolysis to proceed. In order to perform these experiments, both substrate and enzyme had to be present in high, stoichiometric amounts. Hence, there was a mixture of both substrate and product by the end of the proteolytic period. If product binds to AP endo differently than substrate, as is likely from the EMSA studies presented earlier, then a mixture of structures will become apparent during the domain mapping. The three blocked sites visualized during domain mapping form a spherical triangle on the surface of the molecule on one side of

the nuclease fold (Fig. 2). The distances correspond to approximately one turn of a DNA helix for the first two blocked pairs and one half a turn of the helix for the third pair. Given the distances between the three residues and their steric relationships, it is unlikely that a single oligonucleotide could block all three sites simultaneously. We have proposed that the disturbance in the structure upstream from the abasic site is relieved when the enzyme nicks the abasic site. Hence, substrate and product probably bind differently to the enzyme and almost certainly undergo a conformational change as nicking proceeds. Despite the fact that the enzyme in the presence or absence of substrate or product shows little difference in tertiary structure *(163a),* we cannot exclude the possibility that it undergoes a conformational change as it executes the nicking reaction, so that if substrate and product could bind simultaneously to the same molecule, they would abut and block the three nucleotide residues upstream from the abasic site.

Finally the question arises as to how AP endo locates an abasic site in a long DNA molecule. Many DNA metabolizing enzymes do not dissociate from DNA between successive rounds of catalysis, whether the reaction is insertion of nucleotides, e.g., a polymerase, or cleavage, e.g., a nuclease *(79)* or a DNA glycosylase *(13).* Such enzymes are described as processive in contrast to distributive enzymes that dissociate after each catalytic round *(112).* Processive enzymes can remain associated with substrate for thousands of rounds of catalysis, as in the case of DNA polymerases involved in replication; for 100–200 nucleotides as in the case of UDG or *Eco R1,* or for 4–6 nucleotides as in the case of pol β. AP endo falls into the second category and remains associated with DNA for a minimum of 175–200 nucleotides before dissociating from a multi-abasic site containing concatemer. In short, AP endo, like UDG and *EcoR1,* is processive *(28).*

AP endo not only scans DNA for abasic sites, but it can also displace glycosylases that dissociate slowly from their product *(257).* For instance, in the case of thymine DNA glycosylase, the dissociation rate (k_{off}) of the glycosylase is so slow that on a substrate with a G/T mismatch, the half-life of the complex is 5–10 h. The presence of AP endo reduces the half-time to 30–60 min. Although physical evidence of interaction between AP endo and any of the glycosylases remains elusive, these kinetic data provide support for direct interactions of this DNA glycosylase and AP endo.

Very little is known regarding post-translational regulation of AP endo. AP endo has potential phosphorylation sites for at least six protein kinases including casein kinase II, casein kinase I, and protein kinase C(PKC). Indeed, AP endo is subject to phosphorylation by all three of these kinases to varying extents *(276)* (*see* Fig. 4A, lanes 1 and 2). However, only phosphorylation owing to casein kinase II is reported to be associated with loss of nicking activity *(263).*

Because AP endo is subject to phosphorylation by a variety of protein kinases, there has been speculation that it might also be phosphorylated by DNA-dependent protein kinase (DNA-PK). DNA-PK is involved in repair of damage from ionizing radiation and double-strand break repair. It is also involved in V(D)J recombination that occurs during lymphoblast differentiation. It requires the ends of double-stranded DNA, although in the test tube DNA constructs containing single-strand to double-stranded transitions also activate DNA-PK in vitro *(30,164).* Would DNA containing an abasic site activate DNA-PK and would an activated DNA-PK recognize and phosphorylate AP endo? Alternatively, would AP endo in the presence of its substrate be more or less susceptible to phosphorylation? Figure 4B, lanes 3–5, shows the activation and phos-

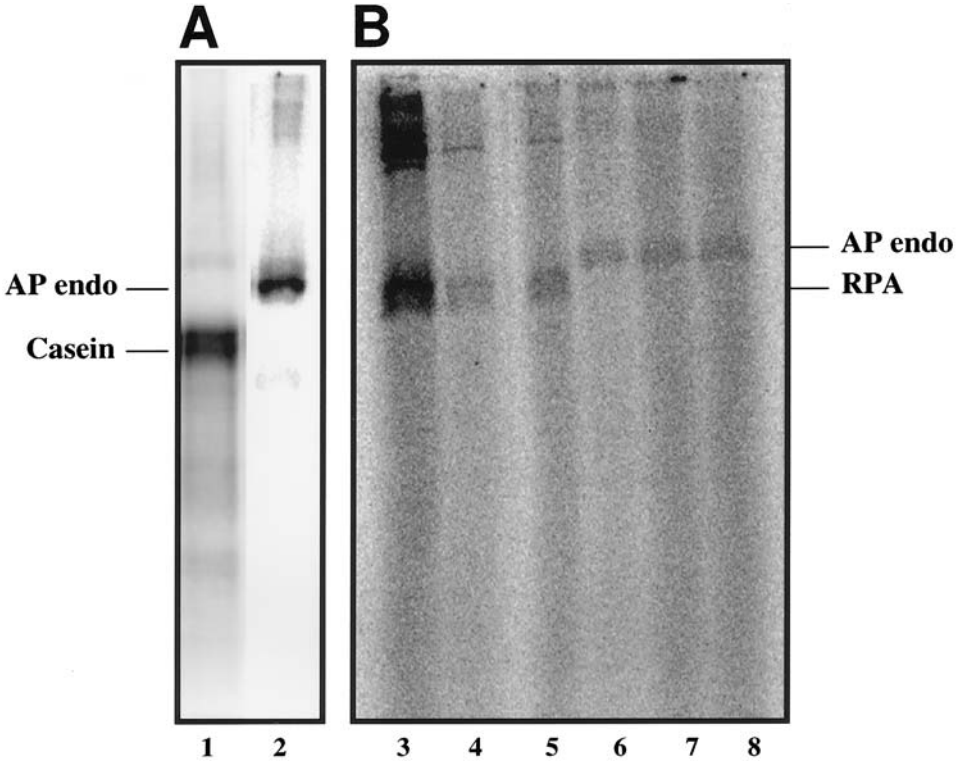


Fig. 4. AP endo is a substrate for casein kinase II but not for DNA protein kinase (DNA-PK). **(A)** Casein (12 pmoles) (lane 1) or AP endo (19 pmoles) (lane 2) was phosphorylated by casein kinase (500 U) as described by Yacoub et al. (276) and resolved by SDS-PAGE (12.5% gel). **(B)** RPA (72 pmoles)(lanes 3–5) or AP endo (36 pmoles)(lanes 6–8) was subjected to phosphorylation by DNA-PK (88 ng) as described by Chan and Lees-Miller *(30).* In lanes 3 and 6, the kinase was activated by 5 μg/mL sonicated calf thymus DNA; in lanes 4 and 7 the kinase was activated by the same amount of 45-mer oligonucleotide containing a U at position 21; in lanes 5 and 8 the kinase was activated by the same amount of 45-mer oligonucleotide in which the U had been removed to create an abasic site by means of uracil DNA glycosylase. Reaction volumes were 25 μL for casein kinase and 20 μL for DNA-PK.

phorylation of RPA, the traditional substrate for DNA-PK (*see* section on RPA under Auxiliary Proteins). Although sonicated calf thymus DNA activates the kinase towards RPA, oligonucleotide with or without an abasic site is unable to stimulate phosphorylation of RPA. When the protein to be phosphorylated is AP endo (Fig. 4B, lanes 6–8), poor phosphorylation is seen with all three sources of DNA. Thus, we conclude that AP endo is not a good target protein for DNA-PK.

AP endo was once thought of strictly as a housekeeping gene, because it is present in all cells and tissues. However, the level of expression varies with tissue type, developmental stage, degree of malignancy *(39,102,206,265,274),* Ca^{2+} concentration *(183),* and in the response of colon cancer cells to hypoxia *(275).* Furthermore, AP endo apparently interacts with p53, the tumor-suppressor gene most commonly associated with human cancer and that is responsible for cell-cycle arrest in G1 or G2 when cells are exposed to

γ-irradiation *(69,160,181).* Some groups report that expression of AP endo is activated in human and rodent cells by sublethal levels of ROS but not other genotoxic agents including alkylating agents *(93).* It is also activated by exposure to asbestos *(68).* The importance of AP endo in fetal development is clear from the fact that homozygous embryos do not survive beyond day E6.5 *(272).* As of this writing, a viable homozygous null cell line has not been established (B. Demple, personal communication)

2.2.2. The Polymerases

2.2.2.1. DNA Polymerase β

The Polymerase Activity The major DNA polymerase thought to be responsible for short-patch repair is DNA polymerase β (pol β) *(16,67,109,227).* Pol β is capable not only of DNA synthesis but also of 5′ -dRP lyase activity, as described in next paragraph. Not only has pol β no proofreading capability, but also it has the lowest fidelity of all the DNA polymerases *(119,120)* and may be responsible for a large part of the errors attributable to BER *(88).* Pol β shows maximal efficiency and maximal fidelity on 5′-phosphorylated 1-nucleotide gapped DNA *(31,189).* The average pol β error rate is ~6×10^{-4} *(119,120),* which is much higher than that of other DNA polymerases. Indeed, overexpression of pol β in CHO cells has been reported to give rise to a mutator phenotype with decreased sensitivity to cisplatin *(26).*

This 39 kDa protein has two major domains *(117,118):* the amino terminal 8 kDa (residues 1–75) and the carboxyl 31 kDa (residues 87–334) separated by a protease-sensitive hinge region *(190).* Pol β can use either Mg^{2+} or Mn^{2+}. The crystal structure is available in several different conformations with and without template primer and substrate dNTP *(189,190,220,221).* The current model for binding of pol β to a gapped DNA substrate requires a 90° bend in the single-stranded template, which probably enhances nucleotide selectivity during DNA synthesis. Thus, the series of X-ray crystallographic structures provides evidence for an induced-fit mechanism in terms of binding the incoming dNTP *(221).* Both domains are involved in recognition of substrate and catalysis as shown by cross-linking studies *(40,228),* and X-ray crystallography *(221).* The hinge region can influence fidelity, as shown by site-directed mutagenesis followed by fidelity studies *(185).* For example mutation of a single tyrosine in the hinge region results in an increase in both base substitution and frame-shift errors. Stopped-flow fluorescence studies reveal that there are multiple conformational changes during the catalytic cycle *(97,203,282).* Pre-steady state analysis has been used to determine theoretical levels of processivity for polymerization by pol β, which are low in comparison with other eukaryotic polymerases *(260).* Both chemistry and a conformational change are rate-limiting for nucleotide incorporation *(260).*

The 8 kDa domain contains the dRP lyase activity *(157,197).* A Schiff base is formed between Lys^{72} and the dRP residue. Mutation of this residue diminishes dRPase activity by ~90% while leaving DNA binding unaffected *(197).* Processive gap filling up to six nucleotides requires a 5′-phosphate group that is recognized by the 8 kDa domain *(199).* Pol β is processive in that it is capable of inserting up to six residues without dissociating from the template *(1,226).* In terms of fidelity, the accuracy of inserting one nucleotide into a single nucleotide gap exceeds fidelity of repairing other substrates *(31).* Although processive filling of a 5-nucleotide gap results in a similar base substitution fidelity as distributive synthesis along a longer template, closely spaced base sub-

stitutions are produced at a rate more than 60-fold higher than for distributive filling of gaps >300-nucleotides *(188)*. Whether the enzyme exhibits processivity in locating nearby gaps on the same DNA molecule, as might occur with damage spaced by nucleosomes, is not known at this time.

Site-directed mutagenesis has been used extensively to study structural and functional aspects of pol β enzymatic activity. Despite initial thoughts to the contrary, site-directed mutagenesis of several residues in pol β show that hydrogen bonding to the incoming dNTP or terminal primer nucleotide is *not* required for either high catalytic efficiency or nucleotide discrimination. Hydrogen bonding, however, is required between the enzyme and the template strand *(10,187)*. In other cases at least one mutation in the carboxyl end of the protein acts as a dominant negative and enhances mutation rates *(38)*.

The importance of pol β during embryogenesis is demonstrated by the fact that there are no viable homozygous knockout offspring and embryos die at midgestation day E10.5. Cells deficient in pol β through knockout mutation are hypersensitive to induction of apoptosis and chromosomal breakage by methylating agents and cross-linking antineoplastic drugs such as mitomycin C and mafosfamide *(180,227)*. They are not hypersensitive to agents that cause oxidative damage *(227)*. Pol β is inactivated by in vitro phosphorylation with PKC *(240)*. It is also a substrate for poly(ADP-ribosyl) polymerase (PARP) *(182)*. The physiological relevance of these observations is not understood at this time.

The Lyase Activity Evidence that the end-trimming or tailoring activity (lyase function) required to remove the 5′ dRP residue might be a function of pol β was first presented in 1995 *(155)*. Since that time, the classic imino intermediate found for all lyase endonucleases was demonstrated *(191)*. The lyase activity resides in the 8 kDa domain with Lys^{72} forming a Schiff base *(62,157,197,198)*. Conversion of Lys^{72} to alanine abolishes most of the activity. A preincised site is preferred, because k_{cat} for lyase activity on an intact abasic site is ~1/200 that of the site previously cleaved with AP endo *(198)*. Finally, lyase activity does not require divalent cation *(198)*.

Other proteins that also perform dRPase activity have been described, leading to speculation that pol β may not be the only protein to perform the step in ASR. For instance, both T4 DNA ligase *(19)* and *Drosophila* ribosomal protein S3 *(218)* have lyase activity. In mitochondria, which lack pol β, DNA polymerase γ (pol γ) performs the lyase function *(139,198)*. The physiologically relevant dRPase is not clear or perhaps this activity is redundant. Of particular interest is that FEN1 does NOT have an associated lyase activity *(46)*.

2.2.2.2. Replicative DNA Polymerases from the Nucleus

Two DNA polymerases involved in replication of nuclear DNA have been implicated in long-patch repair. They may also be involved in short-patch ASR when pol β is missing *(16,63,158)*. DNA polymerase δ (pol δ) and/or DNA polymerase ε (pol ε) can participate in long-patch BER in vitro. In some cases PCNA and RFC (Replication Factor C) but not RPA are required *(232)* but others report that RPA but not RFC is necessary *(47)*. Although both polymerases are capable of replacing a single nucleotide at the lesion site, the repair reaction is delayed compared to the rate of single nucleotide replacement by pol β.

Human pol δ is comprised of two subunits of 125 kDa and 50 kDa *(20).* Pol δ has 3′ to 5′ exo activity that can correct a terminal mismatched base. The error rate is $<1/10^6$ base insertions. Pol δ functions as a dimer in replication, where the structure is likely to coordinate the synthesis of leading and lagging strands. Its processivity depends on PCNA, which leads to a reduction in K_m and an increase in V_{max}. Thus, PCNA may enhance processivity by increasing both the residence time of pol δ on the DNA template-primer and the rate at which individual nucleotides are incorporated *(176).*

The second nuclear DNA polymerase that may be involved in long-patch ASR is pol ε, which is comprised of a 261 kDa catalytic subunit with a 55 kDa tightly associated peptide *(20).* The error frequency for synthesis by pol ε is 8×10^{-4} *(238).* Pol ε is probably involved in Okazaki fragment maturation, because it functions more efficiently as gap size decreases *(167)* and its processivity does not require PCNA at low-salt concentrations *(122,233,278).* Despite this fact, pol ε is able to drive PCNA/RFC dependent processive DNA synthesis under physiological salt concentrations (>0.1 *M* NaCl) *(126,149,278).* Pol ε has 3′ to 5′ exonuclease proofreading capability and binds ss DNA but not ds DNA *(126).*

2.2.2.3. Replicative DNA Polymerase from the Mitochondrion

The large quantities of reactive oxygen species generated by mitochondria mean that oxidative damage to mitochondrial DNA is a constant hazard for the cell (*see* Subheading 2.6.). The major polymerase involved in replicating mitochondrial DNA also plays an important part in protecting mitochondrial DNA from oxidative damage. Mitochondria have a unique DNA polymerase, DNA polymerase gamma (pol γ), and do not contain pol β, pol δ, or pol ε. Although the catalytic protein is comprised of a single 136 kDa subunit *(78,140)* with polymerase and 3′ to 5′ exonuclease activity *(140)* as well as the dRPase activity necessary for successful ASR *(139),* the polymerase functions as a dimer.

Replication by pol γ is by a strand-displacement mechanism involving mitochondrial single-stranded DNA-binding protein that stimulates processivity rather than stimulating primer recognition. Binding of pol γ to single-stranded (ss) DNA blocks action of its 3′ to 5′ exonuclease proofreading function. Careful analysis of catalytic steps in incorporation of dNTPs into mitochondrial DNA demonstrates a maximum polymerization rate of 3.5 s^{-1}, which could sustain replication of the mitochondrial genome in a physiologically relevant time-frame *(78).* Pol γ can be stimulated by PCNA *(236).*

2.2.3. Flap Endonuclease 1

Flap endonuclease 1 (FEN1), a 42 kDa protein *(86),* is a member of the structure-specific endonuclease family and repairs nicked double-stranded DNA substrates that have the 5′-end of the nick expanded into a single-stranded tail *(104).* FEN1 has both exonucleolytic and endonucleolytic activities. During lagging strand synthesis, it removes RNA primers from Okazaki fragments *(5,128),* leaving a nick or a single nucleotide gap. With a 5′ flap, FEN1 slides from the 5′ end of the DNA to the point of annealing to perform cleavage *(171).* It is able to cleave an unannealed flap with a dRP at the 5′ end but it has no dRpase activity *per se (46).*

FEN1 has been used to reconstitute long-patch ASR pathway by several groups *(105,109,134,158).* In particular, in a reconstituted system with proteins derived from *Xenopus laevis,* FEN1 was able to excise the 5′-incised abasic site in the PCNA-dependent pathway. DNA synthesis was not required for this activity if PCNA and a replica-

tion factor C-containing fraction were present. The pol β dependent pathway could also use FEN1 for excision of the synthetic abasic sites, which were not susceptible to β-elimination and which had been cleaved on the 5′ side by AP endo. In the latter case, PCNA and replication factor C were unnecessary but DNA synthesis was required *(71)*.

2.2.4. The Ligases

The two ligases known to participate in ASR are ligases I and III *(241,242)* and a newly discovered mitochondrial ligase *(181),* which is a variant of ligase III *(121)*. All ligases require ATP as a cofactor in order to join DNA *(135)*. The enzymes first form a covalent bond with AMP, transfer the AMP to the 5′ -phosphate terminus of the nick to be sealed in the DNA, and then seal the nick while they remove the AMP. The ligases are differentiated by structural elements, substrate range, gene locus, and subcellular location.

Ligase I is 125 kDa with an 85 kDa C-terminal catalytic domain. After binding to the site requiring ligation through the action of PCNA, ligase I is cleaved to generate the catalytically active C terminal fragment. In terms of synthetic substrates, ligase I ligates oligo dT•poly dA and oligo rA•poly dT, but not oligo dT•poly rA. In terms of physiological substrates, ligase I normally joins Okazaki fragments during DNA replication and is likely to be involved in strand breaks resulting from alkylating agents and ionizing irradiation. Ligase I interacts directly with pol β *(57,200)*. Furthermore, ligase I inhibits strand displacement of pol δ whether or not PCNA and RFC are present. Ligase I stimulates DNA pol ε in a PCNA-dependent manner *(166)*. Stimulation occurs when PCNA is present in low concentrations but at higher concentrations of PCNA, stimulation no longer occurs. DNA ligase I knockout mice are embryonic lethals at day E6.5 *(14)*

DNA ligase IIIa is a polypeptide of 922 amino acids that ligates all three synthetic substrates described earlier. The amino terminus is responsible for binding to the PCNA homotrimer, whereas the carboxyl end of the molecule is able to bind XRCC1 via BRCT motifs.

Mitochondrial ligase is a 100 kDa protein that forms an enzyme-adenylate intermediate consistent with the known mode of action of other ligases. This ligase, which can ligate oligo (dT) strands annealed to poly(rA), is a variant of ligase III *(121,192)*.

The genes for DNA ligases I and III have been cloned and the sites on the human chromosome have been identified. Human ligase I, LIG1, is ubiquitously expressed, although expression is highest in thymus with elevated levels in testis. Expression increases after UV irradiation. Human ligase III, LIG3, has two gene products differentiated by alternative splicing. The mRNA species encoding ligase III-α is ubiquitously expressed but the DNA ligase III-β mRNA only occurs in the testis. Consequently, the two may play distinct roles in germ-cell metabolism *(242)*. LIG3 also encodes mitochondrial ligase *(121)*.

2.3 The Auxiliary Proteins

2.3.1 Proliferating Cell Nuclear Antigen

PCNA was first discovered as an autoantigen in patients with lupus erythematosus and was later shown to be a component of both replication and repair *(100)*. PCNA is a sliding clamp composed of three 29 kDa subunits (258 amino acids each) that functions as a processivity device by clamping a DNA polymerase to DNA. The presence of PCNA increases processivity as much as 100-fold *(245)*.

The crystal structure shows a closed circular ring formed from tight association between three monomers *(111).* Each PCNA monomer is composed of two domains that fold to form a "quasi-six-fold symmetry" in the PCNA trimer. The trimer is the functional unit that acts as a clamp enabling DNA polymerases δ and ε to interact efficiently with DNA and proceed in a processive fashion. PCNA is itself assembled onto DNA by the clamp-loading replication factor (RFC; *see* Subheading 2.3.4.). PCNA interacts with a multiplicity of DNA associated proteins including but not limited to DNA polymerases δ and ε, clamp-loader replication factor (RFC), FEN1 *(128),* DNA ligase I *(127),* and $p21^{Cip1/Waf1}$ *(251),* as reviewed by Tsurimoto *(245).* The interaction with pol δ is via the small subunit *(285).* Site-directed mutagenesis has been used to identify the residues involved in binding many of these factors *(99).* In particular, a hydrophobic pocket is formed that is important for interacting with pol δ, p21, FEN1, and ligase I. In order for PCNA to stimulate the FEN1 activity, PCNA must be below the 5′ flap *(99).* The fact that PCNA is loaded onto DNA in a fixed orientation relative to the direction of DNA permits discrimination of newly synthesized DNA strands from parental strands during replication. It may also have a role in orienting the repair process, at least in mismatch repair *(245)* (*see* section on interactions with other DNA repair systems).

Not surprisingly, PCNA *promotes misincorporation* catalyzed by pol δ *(168)* by decreasing the off rate of the pol δ•template-primer complex, e.g., by increasing stability. In fact, it stimulates bypass synthesis by pol δ 53-fold past an abasic site *(169).* Incorporation is primarily dA in accordance with "A rule." $p21^{Cip1/Waf1}$, an inhibitor of PCNA-dependent DNA replication, inhibits PCNA-stimulated synthesis in vitro past model abasic template sites. (Y. Matsumoto, personal communication).

PCNA is involved in both nucleotide excision repair and the long-patch pathway of ASR. In particular, involvement with the latter pathway was shown by reconstitution with AP endo, RFC, PCNA, FEN1, pol δ, and DNA ligase I. Neither RPA nor Ku protein complex enhanced the repair activity in this system *(232).* Others have reconstituted long-patch BER with RPA instead of RFC *(47).* In a recent study using human cell extracts, RPA and PCNA complemented each other; neither was able to replace the other *(54).*

2.3.2. $p21^{Cip1/waf1}$

$p21^{Cip1/waf1}$ is a cyclin-dependent kinase inhibitor that binds to PCNA and subsequently modifies its activity. In fact, p21 and FEN1 bind in a mutually exclusive fashion to PCNA. When p21 is bound to PCNA, it prevents PCNA from participating in DNA replication and possible repair. The protein induces G1 arrest and can block the onset of S phase in response to DNA damage *(70).* It also inhibits repair of DNA by pol δ, DNA ligase, RFC, PCNA, and FEN 1 (Kim et al., personal communication). However, it fails to inhibit pol β-dependent repair of abasic sites. Both DNA synthesis and abasic-site excision by FEN1 are suppressed by p21 in vitro. Loading of PCNA on circular DNA containing an abasic site is also blocked by p21, but preloaded PCNA is relatively resistant to p21. Consequently, it appears that p21 suppresses PCNA-dependent ASR by blocking loading of PCNA on DNA *(106).*

2.3.3. Replication Protein A

RPA (replication protein A, also known as replication factor A or RFA) is a heterotrimer ss DNA binding protein *(267).* It is the most abundant of single-stranded

DNA binding proteins. hRPA comprises 3 subunits, 70, 32, and 14 kDa, known as hRPA70, hRPA32, hRPA14, respectively.

During replication, RPA is required for DNA synthesis reconstituted from purified proteins and is part of the large 17-S multiprotein complex that includes pol δ and/or ε. RPA binds ssDNA nonspecifically. When bound to ssDNA, which occurs during S phase and after DNA damage, the 32 kDa subunit is phosphorylated by DNA-PK. Phosphorylation occurs in a cell-cycle dependent fashion or in response to DNA damage induced by ionizing radiation or UV light *(21,29,137)* and leads to enhanced unwinding ability *(29,137).* The RPA·ssDNA complex is very stable (binding constant = 10^9) and can tolerate urea up to 6 *M* and guanidine.HCl up to 2 *M.* The two ssDNA binding domains reside in the RPA70 subunit, although the C terminus of hRPA70 is not itself involved in binding *(75).* The complex of hRPA32 and hRPA14 also has a single-strand DNA-binding domain *(18).* The protein prefers binding to pyrimidine tracts over purine tracts.

RPA stimulates completion of long-patch ASR *(47)* in a salt and PCNA-independent fashion, which means the stimulation is independent of its ability to unwind dsDNA. However, involvement in DNA repair may require recognition of single-stranded regions. RPA also stimulates BER in PCNA-dependent repair of abasic sites *(54).* Although the repair proteins with which RPA interacts are largely those required for damage recognition and excision in nucleotide excision repair, e.g., XPA, XPG, and ERCC-1/XPF, RPA also interacts with the RAD52 protein, which is itself essential for double-strand DNA-break repair and with XPG *(72).* The BER proteins with which RPA interacts include UDG *(170,172)* and FEN1 *(17),* which it stimulates. RPA is also ADP-ribosylated, the significance of which remains unclear at this time *(268).*

2.3.4. Replication Factor C

RFC is a five-subunit protein complex (p140,p40,p38,p37, and p36) that acts as a clamp loader for PCNA onto DNA. Either pol δ or pol ε then associates with DNA at the primer terminus, at which time RFC dissociates from the complex *(195).* ATP hydrolysis is required for loading. Human RFC has been reconstituted from its five subunits simultaneously expressed in baculovirus-infected cells *(22).* Apparently all five subunits constitute the ATPase activity required for loading *(23,194).*

The large subunit contains the DNA and PCNA binding domains *(65).* DNA primer-end recognition and PCNA binding activities are located in the C-terminal half of p140, whereas the N-terminal half of the protein is not required for RFC complex formation, replication activity, and PCNA loading *(246).* The large subunit is also a substrate for phosphorylation by Ca^{2+} calmodulin-dependent protein kinase II. RFC is inactive upon phosphorylation *(146).* RFC has been used as an important component in reconstituting long-patch BER when pol δ or pol ε are involved *(232).*

2.3.5. Poly(ADP-ribose) Polymerase

PARP is a 122 kDa protein with an amino terminal DNA-binding domain, a central automodification domain and a C-terminal catalytic domain. The enzyme ADP ribosylates nuclear proteins either in a straight chain or in a branched chain at the site of a DNA-strand break *(48,49,212).* The proteins that it targets are those involved in chromatin architecture and DNA metabolism or it can auto-ribosylate itself. PARP molecules carrying long chains of branched ADP-ribose polymers lose their affinity for DNA and become inactivated. Ribosylated PARP then loses its poly(ADP-ribose) through the action of poly(ADP-ribose)glycohydrolase, which degrades protein-bound polymers down to the

protein-proximal ADP-ribose residue. The first residue is removed by ADP-ribosyl protein lyase so that PARP is ready for a new round of nick-binding and automodification.

Two observations make it likely that PARP is involved in activating proteins involved in repair of single-strand breaks in DNA. First, nuclear proteins activated with PARP bind to single-strand DNA breaks, and, second, knockout mice homozygous for the PARP deletion are extremely sensitive to methyl nitrosourea (MNU) and to γ-irradiation. Cells from the knockout mice are highly sensitive to methylmethane sulfonate, which causes growth retardation, G2/M arrest, and chromosome instability. Although they are delayed in DNA break resealing, cell viability is restored after transient expression of the PARP gene. PARP ribosylates pol β *(182)* and binds XRCC1. Thus, PARP is likely to play an important role in activating ASR in vivo *(244).*

2.3.6. XRCC1

X-ray Cross Complementation Protein 1 (XRCC1) is a 70 kDa protein with 633 amino acids *(239)* whose function is best described as a "nick sensor" *(24).* The protein binds with pol β, ligase III, and PARP. Knockout mice for XRCC1 do not survive embryogenesis. XRCC1 is detected in all tissues but is most prevalent in testis *(284).* XRCC1 functions as a homodimer, interacting via a BRCT domain in the C terminus. The BRCT site provides the site for interaction with ligase III as determined by far Western and affinity-precipitation analyses *(174,281).* The solution structure of the N-terminal domain is now available, revealing that the terminal domain binds both gapped and nicked single-stranded DNA and a gapped DNA-pol β complex *(148).*

The gene for XRCC1 complements a defect in CHO mutant EM9 cells, which are 10-fold more sensitive to ethylmethanesulfonate and ~twofold more sensitive to ionizing radiation. These cells have reduced ability to rejoin single-strand DNA breaks and a 10-fold elevated level of sister chromatid exchange compared with the CHO parental line *(239);* EM9 is also hypersensitive to camptothecin, an inhibitor of topoisomerase I. The last effect is independent of DNA replication *(6).*

In vitro studies have shown that XRCC1 interacts with one of the two forms of DNA ligase III *(25,174),* with pol β and possibly with PARP *(24).* The interaction with PARP, which was demonstrated by the yeast two-hybrid system, occurs via the central region (amino acids 301–402), which contains a BRCA1 C-terminus. Overexpression of XRCC1 in COS cells decreases PARP activity in vivo *(149).*

The CHO EM-C11 cell line has greatly reduced levels of XRCC1. Extracts from these cells are partially defective in ligation of BER repair patches in comparison to WT CHO-9 extracts. Treatment of CHO EM-C11 with alkylating agents results in an altered spectrum of mutations in comparison with parent CHO-9 line, consistent with the hypothesis that reduced ligation efficiency of single-strand breaks generated during ASR owing to XRCC1 levels may lead to deletions *(186)* When added to an in vitro BER system, XRCC1 suppresses strand displacement by pol β *(116),* an observation that suggests that strand displacement is not the optimal repair mechanism, provided that pol β is available for repair synthesis.

2.4. Interactions Between Different Components of the ASR Pathway

That the different components in the BER pathways must recruit or interact with one another is intuitively obvious but experimentally difficult to demonstrate. The most compelling evidence for recruitment is provided by Fortini et al. *(64),* where the selec-

tion of the repair route depends on the initial lesion, implying that the first enzyme to recognize the damage controls the selection of the entire pathway. Other clues to interactions include data showing that in the yeast two-hybrid system pol β interacts with AP endo. Furthermore, the two proteins cause an EMSA supershift when the substrate is abasic site-containing DNA *(12).* Prasad et al. *(200)* demonstrated that pol β and ligase I not only co-immunoprecipitate but also migrate together on glycerol gradient centrifugation analysis. Waters et al. *(244)* also demonstrated AP endo can enhance the turnover of several glycosylases. XRCC1 has binding sites for PARP, ligase III, and pol β, leading to speculation that XRCC1 is a scaffolding on which ASR takes place *(148).*

On the other hand, some authors feel that the nature of the gap and the form and location of the abasic-site deoxyribose determine which repair pathway occurs. Short-patch repair is the mode of choice because AP endo and pol β are such efficient enzymes. However, if the 5′-deoxyribose has been reduced or altered so that pol β cannot cleave it from the downstream strand, if the 5′-deoxyribose is missing as occurs with a gap or if both the 5′-deoxyribose and the next 5′-phosphate are missing, then long-patch repair is more likely to occur. Because long-patch repair requires the presence of PCNA, any substrate that cannot retain PCNA, e.g., a relatively short oligonucleotide, will not undergo long-patch repair. Consequently, so long as the substrate is a short oligonucleotide, BER will proceed poorly by either mechanism in extracts from pol β –/– cells. This line of thought does not take into account those observations where the pathway appears to be determined largely by the initial lesion.

2.5. Molecular Interactions with other DNA Repair Systems

Recently, a number of findings have demonstrated that in humans, ASR and other major DNA-repair systems of the cell share many of their molecular components (*see* Fig. 5). Experimental data suggest that an essential nuclease in nucleotide excision repair (NER) plays a role in ASR of at least one form of oxidative damage. In addition a protein complex, central to the mismatch repair process in human cells, also acts to couple the excision by BER of an oxidative lesion to transcription. PCNA and the polymerases δ and ε are essential parts of the DNA replication machinery and are shared among several of the major repair systems, while AP endo and PARP may have overlapping roles in both ASR and double-strand break repair (DSBR).

2.5.1. XPG Plays a Role in NER and BER

NER is the pathway by which several helix-distorting lesions, including those caused by UV light and certain carcinogens, are removed from DNA. It is a complex multiprotein process involving dual incision on either side of the lesion to be excised and subsequent removal of the oligonucleotide containing the damage *(215).* Two different structure-specific nucleases are used to create the dual incision. In mammalian cells, these are the XPG protein, which makes the 3′ incision, and the ERCC1-XPF complex, which makes the 5′ incision *(268).*

Defects in NER are generally nonlethal but mutations in any of the seven genes involved in the early steps of the process (XPA through XPG) result in the hereditary disease xeroderma pigmentosum (XP). XP patients exhibit acute sun sensitivity, marked skin changes in exposed areas, susceptibility to skin cancer, and frequently progressive neurological degeneration.

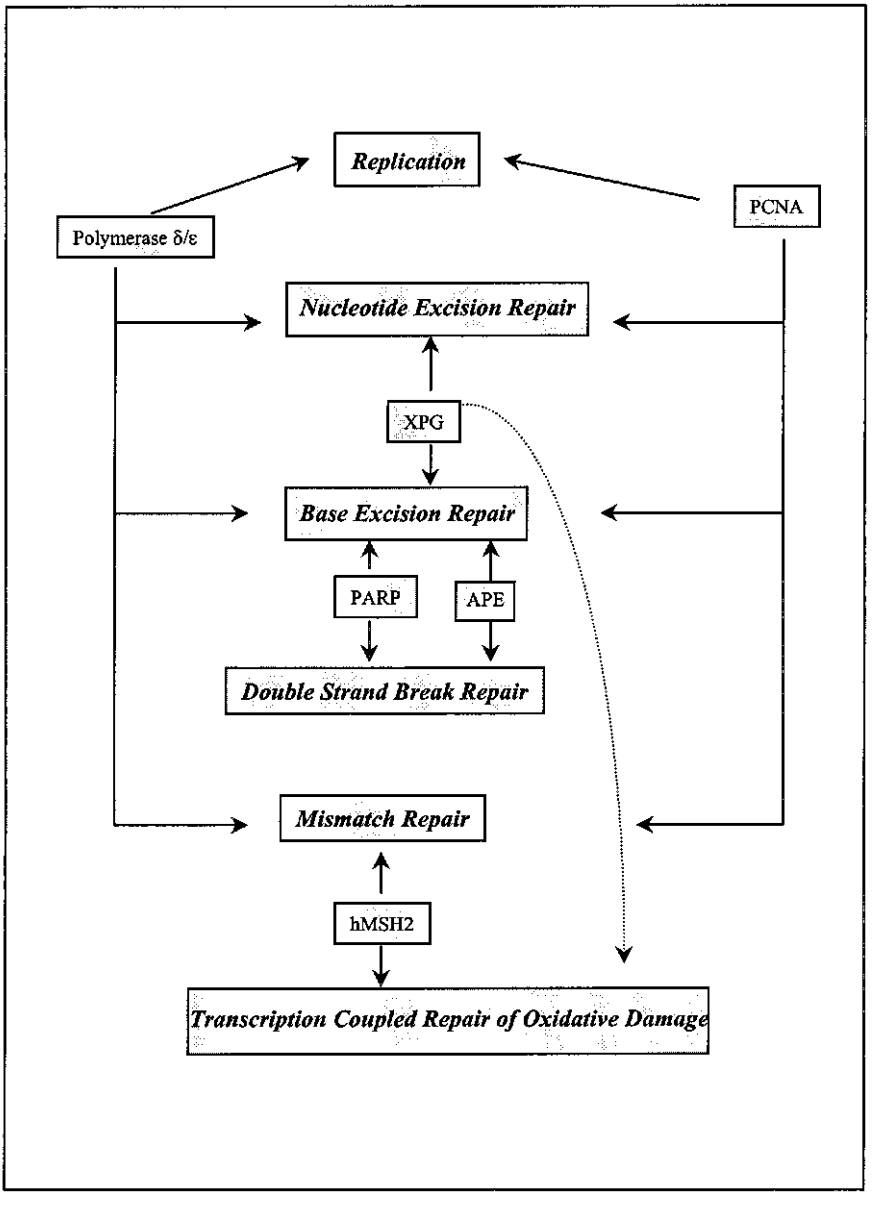


Fig. 5. Abasic site repair shares molecular partners with other DNA repair and replication systems. PCNA and polymerase δ/ε are essential components of replication and are shared among three of the major DNA repair systems including mismatch repair, base excision repair, and nucleotide excision repair. XPG is an essential nuclease of nucleotide excision repair and in addition plays a role in the global and transcription-coupled repair of oxidative damage facilitated by the enzymes of base excision repair. HMSH2, a required component of mismatch repair, may also help couple the repair of oxidative lesions to transcription. PARP and AP endo may play a role in both base excision repair and double-strand break repair.

Cockayne's syndrome (CS) is also a rare human photosensitive disease with a recessive inheritance pattern. CS patients suffer from developmental and neurological abnormalities. Death results from progressive neurological degradation before the age of 20 years. CS patients display an increased photosensitivity of the skin, but unlike XP patients, do not develop skin tumors *(248).* Cells from patients with CS are defective in the preferential removal of lesions from the transcribed strands of active genes by a transcription-coupled repair (TCR) process, including removal of specific lesions targeted by ASR, as discussed later. Classic CS is caused by mutations in either the CSA or CSB genes *(243).* CS and XP are usually clinically and genetically different but complementation studies have assigned some CS patients to the XP groups B, D, or G *(89,249).* Recently it was demonstrated that three CS patients had mutations in XPG that would produce severely truncated protein. In contrast, two sibling XPG patients without CS were found to have a missense mutation in XPG that inactivated its function in NER but produced full-length protein *(179).* Such findings have led to the conclusion that the clinical presentation of CS in patients with XPG mutations is not related to the incision function of XPG in NER but entails a second function for the enzyme that requires full length protein.

The NER/Cockayne's connection has now been extended to BER. Oxidatively damaged bases such as thymine glycol (Tg) are among the most abundant lesions resulting from ionizing radiation and other processes that generate reactive oxygen species *(256).* The removal of Tg lesions from DNA by the BER pathway is initiated by hNth1, a bifunctional enzyme that acts both as a DNA glycosylase to remove the altered base and also as an AP lyase by cleaving the DNA backbone. CS patients from the XP complementation group G who produce severely truncated XPG protein exhibit a reduced ability to remove Tg from their DNA after exposure to ionizing radiation *(41).* This observation has led to the hypothesis that certain domains of XPG may be essential for removal of Tg lesions in DNA.

The repair of oxidatively damaged DNA through BER has been reconstituted using purified human proteins, hNth1, AP endo, pol β, and DNA ligase III-XRCC1 in conjunction with substrates containing oxidized forms of pyrimidines, i.e., Tg and dihydrouracil *(110).* The initial step of the reaction was found to be strongly stimulated by purified human XPG, which promotes binding of hNth1 to oligonucleotide substrates containing damaged DNA. XPG proteins carrying mutations that disable nuclease function but yield full-length protein were found to stimulate hNth1 activity in a similar fashion to wild-type enzyme. These findings suggest that XPG activates the BER of oxidative DNA damage by promoting the binding of hNth1 to its DNA target and this activation is independent of its role as a nuclease in NER. Development of CS in XP-G patients may therefore be related to inefficient removal of endogenous oxidative damage by BER. Whether XPG also acts to enhance the repair of other DNA lesions that feed into the BER or ASR pathway is unknown at this time.

2.5.2. TCR of Oxidative Damage removed by the BER Pathway

Tg lesions have been shown to block ongoing transcription *(83,113,123)* and in normal cells are removed more rapidly from the transcribed strand of DNA than from the nontranscribed strand *(124).* The removal of Tg from DNA, facilitated by the enzymes of the BER pathway, is thus thought to be a transcription-coupled process.

Mutations which severely truncate the XPG protein, such as those seen in CS patients from XP group G, impair not only global, but also transcription-coupled removal of Tg lesions from DNA *(41).* In addition to activation of hNth1 glycosylase, XPG might interact with proteins required for TCR of oxidative lesions such as Tg. Other genes that encode proteins thought to be involved in TCR of Tg include CSA and CSB *(125);* BRCA1, the breast and ovarian cancer susceptibility gene *(77);* and hMSH2, a central component of the mismatch repair process.

Mismatch repair (MMR) is a DNA repair process that specifically ensures genetic stability by correcting DNA biosynthetic errors and by preventing recombination between divergent DNA sequences *(111,163,202).* In human cells, repair is initiated by one of two heterodimers: MutSα and MutSβ. The former, comprising hMSH2 and hMSH6, recognizes single mispairs, single-base loops and loops of two base pairs, whereas the latter, comprising hMSH2 and hMSH3, recognizes primarily three and four base-pair loops. When bound to repair targets in DNA, MutSα or MutSβ recruit a third heterodimer, MutLα, containing the proteins hMLH1 and hPMS2, to initiate repair *(136).* Cells defective in the hMSH2 gene lack MMR activity and are deficient in the removal of Tg from the transcribed strand of an active gene without an apparent decrease in overall genomic repair or an increased sensitivity to ionizing radiation *(124).* Conversely, cells defective in the hMLH1 gene show normal levels of TCR. Thus hMSH2 but not hMLH1 plays a role in the TCR of oxidative damage in DNA facilitated by the enzymes of ASR.

2.5.3. AP Endo and Poly(ADP-ribose)Polymerase in BER and DSBR

As discussed earlier, AP endo is a central component of the BER pathway. Recent evidence suggests that it may also play a role in the repair of certain double-strand breaks in DNA. Oxidative damage to the sugar moiety of DNA results in strand breaks containing a 3′-blocking group. The 3′-phosphodiesterase activity of human AP endo may function in the removal of such blocked termini at double-strand break ends. Additional factors may be required for the repair of some damaged 3′-termini, in particular those on 3′ overhangs *(234).*

PARP is an abundant nuclear protein that binds to single-strand interruptions in DNA that result from treatment with ionizing radiation or alkylating agents *(48).* PARP functions in DSBR when it stimulates DNA-PK, which is required for the rejoining of double-stranded DNA breaks *(213).* In terms of ASR, PARP contains a distinct binding site for the XRCC1 protein: a component of BER that binds both pol β and ligase III. By interacting directly with XRCC1, PARP may be involved in recruiting BER components such as pol β and ligase III to the site of DNA strand breaks *(149).* (Note that AP endo is not itself a substrate for DNA-PK; *see* Subheading 2.2.1.) The interaction between PARP and AP endo that function as components of both BER and DSBR further extends the ever-emerging list of shared molecular partners in DNA repair.

2.5.4. PCNA is Involved in all Three Major DNA Repair Pathways

Overlapping function for components of BER and other DNA-repair systems extend beyond removal of oxidative DNA damage to components involved in replication such as PCNA and pol δ/ε *(99).* In addition to its function in replication, PCNA has been found to play a part in several of the major cellular DNA repair systems including long-patch BER, NER, and MMR.

FEN1 is a structure-specific nuclease that recognizes and cleaves 5′ overhang or flap DNA structures *(81,143,144,207).* As reviewed earlier, FEN1 nuclease removes the 5′ reaction intermediate generated during long-patch BER. PCNA binds directly to FEN 1 and stimulates its endonucleolytic activity at branched structures and its exonucleolytic activity at nicked and gapped structures *(37,128,269).* Human PCNA was found to stimulate long-patch BER either through its direct interaction with the FEN1 nuclease or through its association with high molecular-weight polymerases such as δ or ε, which incorporate several nucleotides during strand displacement as part of the long-patch BER process *(128).*

PCNA also plays a role in other repair pathways. In NER, for example, DNA synthesis that follows excision of damaged DNA is mediated by pol δ or ε holoenzyme and requires PCNA *(178,224).* Furthermore, purified human PCNA interacts directly with expressed human XPG endonuclease, which interestingly shares homologous regions, including the predicted nuclease domain and a PCNA-binding region, with the FEN1 nuclease *(72).* A conserved arginine in XPG (Arg^{992}) is critical for PCNA binding and R992A and R992E mutant forms of XPG fail to reconstitute fully NER activity in vivo. The specific function of a PCNA-XPG interaction is not clear. The complex might provide a mechanism by which excision and resynthesis of NER could be interconnected. Could a PCNA-XPG complex also play a role in the BER of thymine glycol?

Recently it was demonstrated that pol δ is required for human MMR in vitro and that the resynthesis step of the pathway is PCNA-dependent *(138).* When MMR was examined in human cell extracts using an assay that did not require DNA synthesis, repair activity was inhibited by addition of $p21^{Cip1/Waf1}$ or a p21 peptide known to sequester PCNA *(247).* These data suggest DNA repair that PCNA plays a role in an early step of MMR, one that precedes synthesis.

More recent work has demonstrated that human MSH2, MLH1, PMS2, and PCNA can be co-immunoprecipitated, suggesting formation of a repair-initiation complex among these proteins *(80).* How PCNA functions in the early steps of MMR is not known but it has been suggested that it may help determine which strand of DNA should be repaired *(247).*

PCNA is a central player in all three of the major human repair systems, either as part of the resynthesis machinery, post-excision of damaged or mispaired bases in DNA, or as an integral part or the repair processes themselves. Indeed, sharing of common molecular partners such as PCNA, FEN1, and pol δ/ε between repair and replication-associated chain elongation might form the basis of a mechanism to allow coordination of DNA replication and repair processes in vivo *(255).* Is it possible that competition for PCNA between replication and repair systems following DNA damage could facilitate the stalling of replication and allow for repair of damage?

2.6. ASR Occurs in the Mitochondrion as well as in the Nucleus

Eukaryotic cells synthesize the bulk of their ATP in mitochondria through oxidative phosphorylation. The presence of large amounts of $FADH_2$ and NADH as well as reactive oxygen species including hydrogen peroxide *(214)* arising during the conversion of O_2 to H_2O are a clear liability to the integrity of biologically important molecules in the mitochondrion. Consequently, eukaryotic mitochondria are rich in the enzymes

involved in ASR *(192).* An in vitro system purified from *Xenopus laevis* ovary mitochondria repaired a single abasic site via mitochondrial AP endo, DNA polymerase γ, the DNA polymerase located in the mitochondrion, and a mitochondrial ligase, which is structurally related to DNA ligase III. Furthermore, a mitochondrial UDG was able to initiate repair of an oligonucleotide containing a single uracil. The dRPase was located on pol γ *(138).* Interestingly, when oxidative damage was induced by photoactivated methylene blue and the rate of repair examined in regions of the mitochondrial genome undergoing transcription, there was no difference in the rate of repair between strands or between two different regions of the genome that differ with regard to transcriptional activity *(3).* Consequently, it would appear that mitochondria are rich in enzymes involved in ASR and that repair is not transcription-coupled.

2.7. Unanswered Questions

In the quest to accurately depict the extent of knowledge about abasic site repair, this review leaves many unanswered questions. These include:

1. Is long-patch repair with polymerases other than pol β significant in normal cell physiology?
2. Given that the only physical biochemical evidence for interaction between different pairs of molecular partners is between ligase I and pol β, how do the molecules involved in ASR communicate with each other? How do they recruit one another?
3. Which protein or proteins is (are) responsible in the cell for dRPase activity when the phosphodeoxyribose remains associated on the upstream side of the lesion? AP endo can perform this function, but the turnover number is low and the activity is inefficient.
4. Which protein or proteins is (are) responsible in the cell for dRPase activity when the dRP remains associated on the downstream side of the lesion? Although pol γ performs this function in mitochondria, is it always pol β in nuclear ASR?
5. What regulates which pathway is chosen for repair? Is it the form of the substrate molecule independent of the lesion, e.g., short linear vs long linear vs circular DNA molecules? Is it the lesion itself or the glycosylase that is attracted to the lesion? If the glycosylase determines the pathway for repair, then there must be some way for the glycosylase to communicate with the appropriate DNA polymerase or with a co-factor that will bind to that DNA polymerase. In the case of long-patch repair, is the requirement for RFC as opposed to RPA a function of substrate (covalently closed circles with a single abasic site *[105,232]* vs a linear oligomer nucleotide with a single abasic site *[47]*)?
6. Once repair is in progress, can the pathway be switched? Although it is unlikely that long-patch repair might switch to short-patch repair, the possibility that the phosphodeoxyribose might be damaged after incision with AP endo would require the switch from short-patch to long-patch repair.
7. Are there proteins in ASR that remain to be discovered?
8. What regulates the distribution of different enzymes and pathways to different organelles?
9. Are there genotoxic insults from the environment in addition to oxidizing agents that might stimulate or decrease ASR?
10. We have no understanding at all of how these repair pathways will function on DNA packaged into chromatin or whether the type of chromatin (euchromatin or heterochromatin) will matter.
11. Are there cell-cycle effects that have gone undetected?

ACKNOWLEDGMENTS

We thank Dr. Susan Lees-Miller for providing reagents to examine the activity of DNA-PK on AP endo. Drs. Carlos de los Santos, Paul Doetsch, Eugenia Dogliotti, Mark Kelley, Thomas Lindahl, Yoshihiro Matsumoto, Sancar Mitra, John Tainer, Michael Weinfeld and David Wilson III provided manuscripts prior to publication. Drs. William Beard and Paul Doetsch provided numerous helpful comments and critical reading of the manuscript.

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