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Replication of Damaged DNA and the Molecular Mechanism of Ultraviolet Light Mutagenesis*

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ABSTRACT: On UV irradiation of *Escherichia coli* cells, DNA replication is transiently arrested to allow removal of DNA damage by DNA repair mechanisms. This is followed by a resumption of DNA replication, a major recovery function whose mechanism is poorly understood. During the post-UV irradiation period the SOS stress response is induced, giving rise to a multiplicity of phenomena, including UV mutagenesis. The prevailing model is that UV mutagenesis occurs by the filling in of single-stranded DNA gaps present opposite UV lesions in the irradiated chromosome. These gaps can be formed by the activity of DNA replication or repair on the damaged DNA. The gap filling involves polymerization through UV lesions (also termed bypass synthesis or error-prone repair) by DNA polymerase III. The primary source of mutations is the incorporation of incorrect nucleotides opposite lesions. UV mutagenesis is a genetically regulated process, and it requires the SOS-inducible proteins RecA, UmuD, and UmuC. It may represent a minor repair pathway or a genetic program to accelerate evolution of cells under environmental stress conditions.

KEY WORDS: mutagenesis, replication, DNA, ultraviolet, SOS, *Escherichia coli*, carcinogenesis.

I. INTRODUCTION

Even under ordinary conditions, cellular DNA suffers repeated assaults from agents that impair its integrity by chemically modifying its structure.[114] There are at least four major sources that produce DNA lesions: (1) DNA-damaging agents that are present in the environment such as sunlight, ionizing radiation, and a variety of industrial wastes;[114,144,280] (2) cellular intermediates of metabolism such as oxidizing[110] or methylating[303] agents; (3) spontaneous chemical reactions of DNA such as depurination or deamination of cytosine;[208] and (4) incorporation into DNA of foreign or damaged nucleotides (e.g., dUTP,[178] 8-oxodGTP[234]).

Unrepaired DNA lesions pose a serious threat to the cell because they may block DNA replication or transcription, events that can potentially cause cell death. In addition, DNA lesions may signal incorrect coding information. This may lead to the synthesis of mutated proteins or to the incorrect activation of cellular pathways, events that may cause a biological catastrophe. To prevent these complications, essentially all living organisms have evolved several types of protective mechanisms. They function at two levels: (1) elimination of damaged or abnormal dNTPs from the cellular pool of DNA precursors and (2) repair of damaged DNA to restore the original nucleotide sequence. A major repair mechanism is excision repair (Figure 1). It involves the removal of a segment of the damaged DNA strand by a specific nuclease, followed by patching the DNA gap by DNA synthesis.[113,114,125,305,378,383] This ensures an accurate expression of genetic information, as far as DNA structure is involved, and enables stable inheritance of genetic traits. Cells have also developed tolerance mechanisms to cope with DNA damage that has escaped repair. This enables the progression of replication by bypass mechanisms that do not require the removal of the lesion. A typical example is recombinational repair in which the damaged area is patched by

* Dedicated to the memory of Harrison (Hatch) Echols, a friend and colleague.

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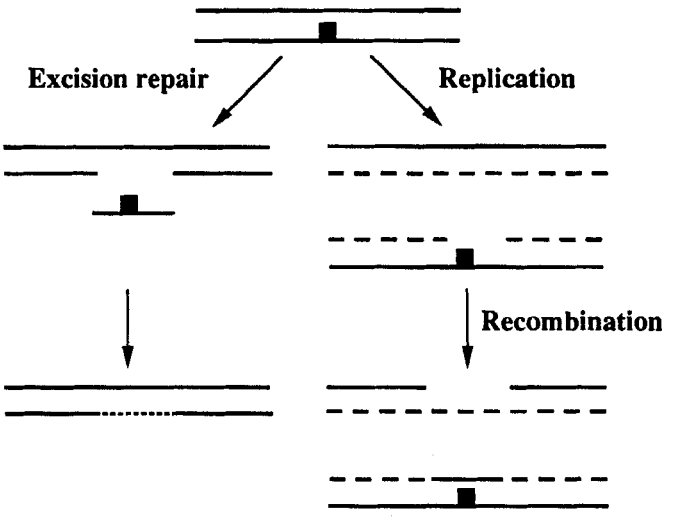

FIGURE 1. Mechanisms of repair and tolerance of DNA damage. Excision repair involves removal of a DNA segment carrying the lesion (shown as a black square), followed by repair synthesis (dotted line). Postreplication recombinational repair involves patching the gap in newly synthesized DNA (dashed line), using a segment of the parental DNA strand (plain line).

recombination using a complementary DNA segment originating from the fully replicated sister chromatid (Figure 1). Such tolerance mechanisms allow a second attempt of DNA repair later or else the lesion is diluted out by subsequent cell division cycles.[113,114]

Occasionally, DNA lesions that have escaped all elimination efforts undergo replication. It was once thought that in this situation, the DNA polymerase, being misled by the damaged nucleotide, incorporated an incorrect nucleotide opposite the lesion, thus carrying out the crucial misincorporation step of induced mutagenesis. This view of mutagenesis as a passive event was changed with the surprising discovery that ultraviolet (UV) mutagenesis in *Escherichia coli* was dependent on specific inducible gene products. This implies that UV mutagenesis is an active, genetically regulated process. Although it may still consist of replication through damaged DNA sites, it cannot occur efficiently without the assistance of specific inducible mutagenesis factors.[382,398] This raised the intriguing question of the role of this inducible form of mutagenesis: Is it a low-fidelity repair pathway or is it a genetic program to accelerate the evolution of cells under environmental stress conditions[93]?

It is now well established that UV mutagenesis is part of the global (SOS) stress response, induced in *E. coli* by a variety of DNA-damaging agents. Genes belonging to the SOS family comprise a regulon; they are usually not genetically linked, but they are negatively regulated by a common repressor, product of the *lexA* gene. Induction of the SOS response is effected by proteolytic cleavage of the LexA repressor, promoted by an activated form of the RecA protein.[211,382] This activation occurs as a result of binding of RecA to a ssDNA region in DNA, generated when replication is interrupted by a DNA lesion.[311] The induction of the SOS response leads to a multiplicity of phenomena, including inhibition of cell division, enhanced repair, induction of prophage, and mutagenesis.[382,398] It should be emphasized that some DNA lesions do cause mutations via simple miscoding, with no need to involve specialized functions (e.g., *O*-6 methyl guanine). A very large family of mutagens, however, including UV light, a major environmental carcinogen, as well as methylmethanesulfonate, 4-nitroquinoline 1-oxide, benzo(*a*)pyrenes, aflatoxin B1, neocarzinostatin, and angelicin plus near UV irradiation do require specialized SOS factors to form mutations.[98,108,165,245]

Accumulating evidence suggests that genetically regulated active mechanisms are important in other systems, including mammalian cells.[75,81,85,132,224,228,307,310] Because mutations play a central role in fundamental biological processes such as evolution,[121] carcinogenesis,[7,10,297,414] aging,[56,342,393] and the generation of somatic genetic diversity,[138] the phenomenon of induced mutagenesis stimulated a considerable research effort. Underlying it was the desire to understand the role of cellular functions that regulate and carry out the processing of DNA damage into mutation, a function that might be critical for the future of the cell or for the entire organism.

Although the basic ideas about the induced mutagenesis were laid out during the 1960s and 1970s,[398] its molecular mechanism has not been elucidated yet, even in the relatively simple *E. coli*. In this bacterium, the two major rescue operations dealing with lesions in DNA, that is, excision repair and postreplication recombinational repair, are largely error free and do not seem to be responsible for UV mutagenesis. A *recA* strain in which excision repair operates quite efficiently does not display UV mutagenesis,[260,396] arguing that by itself, excision repair is not mutagenic. In addition, excision-repair-deficient mutants are UV hypermutable, indicating that the repair nuclease is not required for UV mutagenesis.[42,134,394] On the other hand, strains carrying the *recB* and *recF* mutations, which inactivate the two major recombinational repair mechanisms, show normal UV mutagenesis. This indicates that UV mutagenesis can occur efficiently under severely reduced recombination.[164]

Analysis of DNA that was newly synthesized after UV irradiation revealed that it is of low molecular weight. Its average length was similar to the average distance between pyrimidine photodimers, the major UV lesions in DNA.[153,300] This suggested that DNA replication is interrupted at UV lesions, but can restart past the lesion. The gaps were repaired primarily by a recombinational mechanism,[115,301] which is believed to be largely error free, as mentioned earlier. However, a small fraction of gaps was not repaired when protein synthesis was inhibited by chloramphenicol. Under these conditions, UV mutagenesis was eliminated, raising the possibility that the mutagenic event involved a gap-filling reaction carried out by a DNA polymerase.[318] Such gap-filling polymerization, originally suggested by Witkin[395] and Bridges and co-workers,[39] is still the prevailing model for UV mutagenesis. The mutations arise, presumably because of the failure of the polymerase to read accurately damaged sites, leading to misincorporation opposite DNA lesions (Figure 2). The filling in of a DNA gap opposite a lesion by a DNA polymerase was termed bypass synthesis, trans-lesion DNA synthesis, or error-prone repair. The latter term refers to the repair of the ssDNA gap that constitutes a lesion in DNA rather than to the removal of the UV lesion itself, which presumably remains in DNA. According to this view, UV mutations are generated by a minor repair or tolerance pathway. Its big impact is not on the single cell but rather on a population of cells, driven from the fact that it tends to produce mutations at a high frequency.

This article focuses on UV mutagenesis in *E. coli*. UV radiation present in sunlight is a major environmental mutagen and carcinogen. Its impact on biological systems (e.g., skin cancer[32]) is a growing concern in recent years because of the ongoing depletion in the atmospheric ozone layer that shields Earth from solar UV radiation.[246,349] Being the classic example of an active and inducible mode of mutagenesis, UV mutagenesis in *E. coli* serves as a useful model for other organisms. In addition, it should be noted that most of the basic features of this process are common to mutagenesis pathways caused by many other mutagens and carcinogens in *E. coli*.

The article attempts to summarize and critically evaluate our present knowledge of UV mutagenesis in the context of two related processes: replication and repair of the damaged chromosome. Repair of damaged DNA has been the subject of numerous reviews.[113,114,123–125,209,305,306,324] In contrast, replication of damaged DNA has received much less attention. Therefore, the first part of this article addresses the impact of DNA damage, in particular UV radiation, on DNA replication. An attempt was made to provide a comprehensive and critical account of the current knowledge in this field, regardless of its direct connection to UV mutagenesis. The second part deals with the mechanism of UV mutagenesis. Although the article emphasizes biochemical studies,

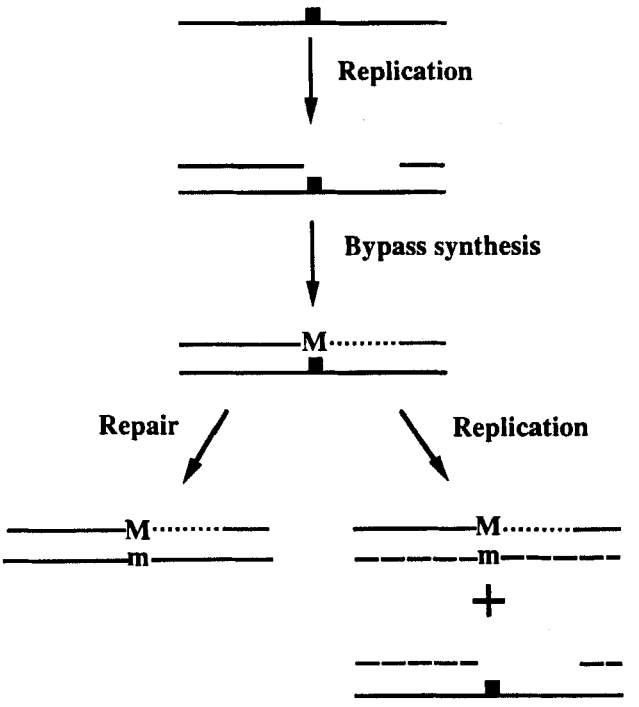

FIGURE 2. The bypass synthesis model of UV mutagenesis. Replication of damaged DNA leaves a gap opposite the lesion (shown as a black square). Filling in the gap by polymerization is likely to cause incorporation of an incorrect nucleotide opposite the lesion (M). Subsequent excision repair or an additional round of replication will establish the mutation in DNA.

an effort was made to integrate both genetic and biochemical data from *in vivo* and *in vitro* investigations. Several reviews that appeared in previous years addressed some of the issues discussed in this essay.[15,36,44,94,95,128,211,284,320,352,382,398]

II. DNA REPLICATION IN UV-IRRADIATED CELLS

A. Arrest of DNA Replication

UV radiation produces a multiplicity of lesions in DNA, of which only a few were chemically characterized. The major class of UV lesions involves the cyclobutyl-type pyrimidine dimers, also termed photodimers or pyrimidine dimers. They account for approximately 85% of the UV lesions in double-stranded DNA (dsDNA). Another class, responsible for approximately 10% of the UV lesions in dsDNA, contains the 6–4 adducts, which are formed on dimerization of adjacent pyrimidines via a 6–4′ bond.[280,293] Together, these two types of lesions account for 95% of UV lesions in DNA and are believed to be responsible for most of the biological effects of UV radiation.

When *E. coli* cells are UV-irradiated, the immediate response is a transient inhibition of DNA synthesis[89,90,329,347] that also leads to inhibition of cell division.[143,155,158,223,261,317,382] These inhibitions function to provide DNA repair with the opportunity to operate without interference from rapidly moving replication forks.

One of the first outcomes of UV irradiation of cells is the formation of UV lesions in DNA. Because both initiation and termination of replication in *E. coli* occur at specific sites on the chromosome, it is conceivable that the major effect of DNA lesions is at the elongation stage.

Indeed, analysis of DNA synthesized in cells after UV irradiation revealed short DNA products, whose length corresponded approximately to the average interdimer distance.[153,300] This suggested that chain elongation is interrupted by UV lesions but can resume past the lesions, leaving a ssDNA gap. Short-termination products were also observed by analyzing the *in vivo* replication products of UV-irradiated phages ϕX174[58] and λ.[83] The major cause responsible for the interruption of replication elongation is likely to be the inhibition of DNA polymerases by DNA lesions. This was suggested based on studies with a variety of purified DNA polymerases, including DNA polymerase I (pol I),[288,380] the major repair polymerase in *E. coli,* DNA polymerase II (pol II),[28,29] which has an unknown function, as well as DNA polymerase III (pol III) holoenzyme, the replicative polymerase of *E. coli.*[213,214,265] The encounter of DNA polymerases with UV lesions is a key event in the bypass synthesis model of UV mutagenesis and is discussed in detail in the second part of this article. Because this section addresses inhibition of chromosomal DNA replication, it concentrates on studies with pol III holoenzyme.

The simplest complete *in vitro* replication model system used for the study of replication of UV-irradiated DNA is the SS → RF system. It involves the replication of ssDNA from phages such as G4 or M13 to the duplex replicative form, RFII.[178] This reaction involves two basic stages: (1) initiation, in which an RNA primer is synthesized at the origin of replication on ssDNA coated with single-strand DNA binding protein (SSB) (in the case of M13, the primer is synthesized by RNA polymerase,[49,159] whereas in the case of G4 it is synthesized by DNA primase,[30]) and (2) elongation, which involves rapid and processive synthesis by pol III holoenzyme.[178] The M13 and G4 system can be visualized as models for leading strand DNA synthesis and have the advantage of being relatively simple. It was found that UV lesions are major blocks to DNA elongation in the SS → RF systems, leading to termination at a frequency of 80 to 90%.[214] It was shown that these terminations involve dissociation of the polymerase complex from DNA. The polymerase was able to replicate challenge DNA, implying that it dissociated fully active.[213] This means that the encounter with the lesion did not immobilize the multisubunit polymerase complex nor did it inactivate the polymerase activity. Thus, the same polymerase molecule that encountered the lesion, if translocated to a new primer past the lesion, might be able to continue polymerizing.

In contrast to the relatively simple priming of phages M13 and G4, the RNA primer for the replication of ssDNA from phage ϕX174 is synthesized by the *E. coli* primosome. It is a multiprotein complex composed of the PriA, PriB, PriC, DnaB, DnaC, DnaT (i protein), and DnaG (DNA primase) proteins.[3,178,237,252,391] The cellular function of the primosome is to synthesize the RNA primers for the Okazaki pieces that are polymerized on the lagging strand of the *E. coli* chromosome.[178] Although not yet used for that purpose, this system can be used as a useful model system for lagging strand replication of UV-irradiated DNA.

The *in vitro* replication of UV-irradiated duplex DNA was studied using the rolling-circle replication system of phage ϕX174.[332] In this system, multiple copies of phage ssDNA are synthesized using the (–) strand of a dsDNA molecule as a template. Replication proceeds via a rolling-circle mechanism and involves a simple replication fork.[97,149,195] This reaction is carried out by four purified proteins: DNA polymerase III holoenzyme, SSB, the ϕX174-encoded Gene A protein, and the host Rep DNA helicase.[178] As with the SS → RF replication, the RF → SS replication is inhibited by UV lesions in DNA.[332] The primary inhibitory target was the polymerization stage, whereas the unwinding of the duplex by the Rep helicase-Gene A complex was only slightly inhibited. In addition, it was shown that UV lesions introduced specifically on the (–) template strand inhibited replication much more severely than UV lesions introduced on the nonreplicated (+) strand.[332]

DNA replication of UV-irradiated DNA was severely inhibited also when assayed in crude protein extracts, leading to the production of short nascent DNA chains. This was shown for the replication of phage f1 ssDNA,[241] *oriC*-containing plasmids (Livneh, Z., unpublished), and pBR322-derivatives.[70,71]

The effects of UV lesions on replication proteins such as the primosome, DNA primase, RNA polymerase, and DNA helicases are less well

understood. The experiments with the Rep helicase would argue that replicative DNA helicases are not dramatically inhibited by the presence of UV lesions.[332] The same seems to be true for DnaB,[277] one of the major replicative helicases in *E. coli*;[178,200,243] however, this point deserves more detailed studies. Another unknown factor is the mutual effect of lesions present on the leading and lagging strands, when both are replicated simultaneously in a coordinated fashion. Does the stoppage of a polymerase on one strand cause stoppage on the other strand or does replication continue on the undamaged strand only? If replication does continue, is the blocked polymerase dragged along in the replisome complex or does it dissociate completely, leaving the replisome? The *in vitro* studies with ssDNA as a template show that the polymerase can disengage itself from the DNA at a blocking lesion. However, the behavior of the polymerase at a true replication fork is far from being understood.

Are factors other than direct elongation inhibition by UV lesions involved in replication shutoff following UV irradiation? One way to address this question is to examine post-UV DNA synthesis arrest in various mutants. It was reported that when cells were grown in rich medium, post-UV DNA synthesis arrest was not observed in a *recA* mutant, indicating involvement of RecA in the arrest of DNA synthesis after UV irradiation.[374] This conclusion was challenged by a more detailed study, with cells grown in minimal medium. In this case, an arrest in DNA synthesis was observed in both $RecA^+$ and $RecA^-$ strains.[167] The differences may result from the different UV doses used in these experiments: low doses of 0.5 to 1.5 Jm^{-2} were used in the former experiments, whereas 10 Jm^{-2} was used in the latter. In addition, the two studies were performed under different physiological conditions: in the rich medium, DNA replication proceeds via multiple forks and several chromosomes exist, whereas in poor medium there are fewer replication forks and a single chromosome.[178] Despite this apparent controversy, it seems likely that RecA protein is involved in some way in post-UV arrest of DNA synthesis; thus, it was reported that UV radiation caused a stronger inhibition of DNA synthesis in cells carrying the *recA718* mutation as compared with a $RecA^+$ strain (sixfold vs. threefold, respectively). In a strain carrying the *lexA*(Def) mutation along with *recA718*, a smaller inhibition of DNA synthesis of only twofold was observed after UV-irradiation.[403] These results imply that post-UV DNA synthesis is modulated by proteins in cell and, in particular, by the RecA protein. The rapid arrest response would suggest inhibition at the elongation level, but inhibition at the chromosome initiation level can also be important (see the following). Consistent with these results, it was shown that purified RecA protein inhibited the replication of ssDNA with pol III holoenzyme in the absence of SSB but not in its presence.[338] This may mean that the balance between SSB and RecA plays a role in replication arrest: the ssDNA gaps formed after irradiation may sequester free SSB, thus reducing its concentration to a point where RecA binding to DNA becomes inhibitory.[338] It is unknown whether other proteins are involved in the process.

An efficient replication arrest may require inhibition of new initiations from the origin of replication *(oriC)*, particularly because such initiation events often occur before the replication of the chromosome is completed. Indeed, it was found that UV irradiation inhibited *oriC*-dependent DNA replication of a λ phage carrying *oriC*.[379] A related earlier observation was that DNA replication of coliphage 186, but not that of the related phages λ or P2, was transiently inhibited when unirradiated phage infected UV-irradiated cells.[140] Replication of phage 186 (but not of phages λ or P2) requires the DnaA and DnaC proteins,[139] which are involved in *oriC* initiation.[178] The nature of this putative inhibitory factor is unknown, but a recently discovered specific inhibitor of *oriC* replication is a possible candidate. The protein, termed IciA (for inhibitor of chromosome initiation), has a mol wt of 33 kDa, behaves like a dimer, and binds specifically a three tandem repeat of a 13-mer in the replication origin of *E. coli*.[146,147] IciA binding blocks initiation by preventing the opening of the 13-mer region by the initiator DnaA protein. The *iciA* gene was located at 62.8 min on the chromosomal map.[373] Immunoblots with anti-IciA antibodies did not reveal an increase in the amount of IciA following UV irradiation or treatment with 4-nitroquinoline *N*-oxide (Skaliter et al.,

unpublished results). Thus, if the IciA protein is involved in post-UV DNA replication arrest by shutting off initiation from *oriC*, such an activity does not involve the induction of IciA. Alternatively, some other unknown *oriC* inhibitors may be responsible for this effect.

B. Post-UV Recovery of DNA Replication

Following the transient arrest, DNA synthesis recovers, regaining full rate at 30 to 45 min after irradiation.[167,403] Recovery of DNA synthesis requires protein synthesis as judged by its chloramphenicol sensitivity.[88,91] It occurs in *uvrA*, *recB*, or *umuC* mutants, but not in *recA* mutants, implying that the RecA protein is required. It is also largely rifampicin sensitive, suggesting the involvement of RNA polymerase.[167] It was proposed that at least one additional factor (termed induced replisome reactivation factor, IRR) is synthesized after irradiation and functions in the recovery process.[167]

The UmuD and UmuC proteins, which are required for UV mutagenesis (see later), are not required for recovery of DNA synthesis,[167] except for a particular case, that is, in strains carrying the *recA718* mutation.[403] These strains are recombination proficient, moderately UV sensitive, and UV hypermutable at lower UV doses. RecA718 protein requires DNA damage to become activated for SOS induction and expression when present at low baseline levels, but amplified levels of RecA718 protein are constitutively activated without DNA damage.[244,402] Strains that are *recA718umuC* are unable to recover DNA synthesis after UV radiation and are extremely UV sensitive.[403] This result was interpreted to mean that the Umu proteins may be able to perform a function in recovery of DNA synthesis that is usually performed by RecA.

The mechanism of recovery of DNA synthesis is unknown. It may involve resumption of elongation past blocking lesions (elongation reactivation) as well as reactivation of new chromosome initiation sites. Indeed, it was shown that UV irradiation activated chromosome initiation from a fixed alternative site,[20] but its mechanism is unknown. The evidence for recovery of preexisting forks (elongation reactivation) is based primarily on experiments with *dnaA* temperature-sensitive mutants. When these mutants were shifted to the restrictive temperature, initiation from *oriC* was inactivated, leading to a low level of DNA synthesis due to ongoing replication forks. This residual DNA synthesis was found to be inhibited by UV irradiation, implying that replication forks were indeed inhibited by UV lesions.[156,167] Even at the restrictive temperature, DNA synthesis did eventually recover,[156,167] a result interpreted as recovery of stalled replication forks.[167] This result, however, can be intepreted as a recovery due to the activation of secondary origins of replication, leading to *oriC*-independent initiation and, thus, to *dnaA* independence.[156] A replisome initiated at such origins may have a different composition and may be able to pass through DNA lesions. Indeed, alternative, *dnaA*-independent origins of replication have been shown to exist (see later). Thus, although cells might be able to reactivate stalled replication forks, more evidence is required to demonstrate such a phenomenon.

Reactivation of stalled replication forks may involve the synthesis of new primers past the blocking UV lesions to enable a restart of polymerization by pol III holoenzyme. Do such priming and polymerization reactions require inducible proteins? It is conceivable that priming can occur past UV lesions with the constitutive components of the replication apparatus, for example, on the lagging strand where frequent priming events are promoted by the primosome complex. If the encounter with the UV lesion does not lead to the disassembly of the primosome, then synthesizing a primer past the lesion may be readily accomplished. If, however, the primosome is disassembled, then priming may not occur until a new primosome assembly site (PAS) is encountered.[178] For priming to occur on the leading strand, or on the lagging strand at sites other than PAS, alternative priming pathways must exist, and they may require inducible proteins. Inducible factors may also be needed to overcome inhibitory activities. Thus, priming past UV lesions may be susceptible to digestion by RNaseH (see later), and polymerization by pol III holoenzyme may be inhibited by RecA.[338]

Both *in vivo* and *in vitro* studies on the replication of *oriC* plasmids revealed that RNaseH is a major specificity factor, which ensures initiation at *oriC*. It most likely acts by degrading RNA primers synthesized at sites other than *oriC*, thus aborting "false" initiations. Degradation of the RNA primers at *oriC* is probably prevented by the protection provided by the multiprotein assembly at this site.[141,176,207,275,276] This means that RNaseH may interfere with RNA priming past UV lesions and/or during activation of alternative origins of replication. The results of two studies are consistent with the idea that inhibition of RNaseH activity is important for maintaining RNA primers in the UV-irradiated cell. It was reported that overproduction of RNaseH from a plasmid inhibited post-UV DNA synthesis and caused increased UV sensitivity. These effects that can be explained by the degradation of RNA primers required for replication reactivation by the amplified RNaseH.[24] In a double mutant lacking RNaseH and having a high level of RecA (*sdrA224recAo^c; sdr* is allelic to *rnh*), recovery of DNA synthesis occurred, after a delay, without the need for additional SOS-inducible functions. This is consistent with a role of the SOS response in neutralizing the activity of RNaseH.[61]

Thus, resumption of post-UV DNA synthesis might require that the activity of RNaseH at the new origins or priming sites be neutralized. This may occur by inhibition of the synthesis of RNaseH, by the synthesis of an inhibitor of RNaseH, or by providing a shielding factor that would prevent the access of RNaseH to the RNA primers. Analysis of *rnh* mRNA transcripts and *rnh-lacZ* gene fusions revealed that the expression of *rnh* was inhibited by UV irradiation.[61,289] A direct assay of RNaseH activity in extracts prepared from SOS-induced cells, however, failed to detect a reduction in RNaseH activity.[19] Thus, the exact fate of RNaseH in the irradiated cells is not clear at this point. The role of a second RNaseH activity discovered recently in *E. coli*[150] is unknown.

Given the importance of priming in the recovery of DNA replication, it is likely to occur by more than one pathway. For example, a recombinational event may provide a DNA primer for elongation reactivation. As mentioned earlier, recombinational repair is operating on DNA gaps, that is, *after* resumption of polymerization past a lesion has occurred. However, this does not exclude the possibility of a recombinational event *preceding* priming past the lesion and providing a DNA primer to be used by the replication apparatus. Recombination-dependent initiation of replication was suggested to exist during the late stage of bacteriophage T4 infection.[180,222] Consistent with such a mechanism, a recombination-dependent replication reaction was demonstrated using purified phage T4 proteins. It was shown that a recombination event between homologous duplex and ssDNAs, catalyzed by the UvsX protein (the T4 analog of the *E. coli* RecA protein), generated a primer-template structure that could serve as a substrate for replication by the T4 DNA polymerase holoenzyme.[107] A related alternative mechanism, which bypasses the need to synthesize new primers, is strand switching (also termed "copy choice" mechanism), originally suggested by N. P. Higgins and B. S. Strauss.[351] According to this model, the polymerase bypasses the blocking lesion by copying the undamaged daughter strand instead of the blocked parental strand and switches back after clearing the lesion. Such a mechanism still remains to be proved.[95]

C. Stable DNA Replication

DNA replication in *E. coli* requires continuous protein synthesis, presumably because of the involvement in initiation at *oriC* of a protein with a short half-life. Stable DNA replication (SDR) is defined as a form of DNA replication that occurs in the absence of protein synthesis.[173] There appear to be two forms of SDR. Constitutive SDR (cSDR) involves an alternative chromosome initiation pathway in cells that lack RNaseH.[172] In such *rnh* mutants, DnaA, the *oriC*-initiator protein, is not required, and the *oriC* sequence can be deleted.[141,176,207,275,276] Chromosome initiation in these strains occurs at several other sites termed *oriK*.[82] cSDR requires the RecA protein and transcription by RNA polymerase, as judged by the rifampicin sensitivity of its initiation.[174] A model suggested for cSDR[178] is the initiation at promoter sites by RNA polymerase, forming RNA-DNA

hybrids that persist because of the absence of RNaseH. RecA protein interacts with the displaced strand of the R-loop, enabling the entry and assembly of the replication complex, similar to the activity of DnaA at *oriC*. Because the transcription for cSDR is stringently controlled (initiation is inhibited by amino acid starvation in RelA$^+$ but not in *relA* mutant strains), *oriK* sites might be stringently regulated promoters.

The relevance of SDR to post-UV recovery of DNA synthesis comes from the observation that UV radiation and other treatments that induce the SOS response also induce SDR. Inducible SDR (iSDR) depends on the replication proteins DnaB, DnaC, DnaE, and DnaG,[173] and DnaT.[196,240] In addition, it depends on activated RecA[175,401] and on RecB and RecC but not on RNA polymerase.[227] Do post-UV resumption of DNA synthesis and iSDR reflect the same process assayed in different ways? A major difference between these two phenomena is the dependence on RecBC. Whereas iSDR is RecBC-dependent, it was reported that post-UV resumption of DNA synthesis was not dependent on RecB. However, the significance of this difference is complicated by the fact that the two phenomena were assayed under different conditions. A dose of 30 Jm^{-2} was used in the SDR study, considerably higher than the 2.5 Jm^{-2} dose used in the study of post-UV DNA synthesis recovery. This difference is significant because it was noted that the *recB* strain was slower to recover as compared with the RecB$^+$ strain and recovery was affected by the UV dose.[167] Another difference is the rifampicin sensitivity: whereas iSDR is resistant to rifampicin, post-UV DNA synthesis seems to have both rifampicin-resistant and rifampicin-sensitive pathways.

A unifying view can be presented, in which iSDR, cSDR, and post-UV recovery of DNA synthesis represent overlapping phenomena belonging to a replication reactivation response to DNA damage. Under this response, perturbations to chromosome replication caused by DNA lesions are overcome by two basic strategies: the activation of alternative *dnaA*-independent origins of replication and reactivation of stalled forks (resumption of elongation). Usually, the induction of replication reactivation requires protein synthesis and is regulated by RecA and LexA, thus being part of the SOS response. New chromosome initiations and resumption of elongation share at least two common requirements: the RecA protein and a primer for elongation by DNA polymerase. RNA primers can be synthesized at alternative origins of replication, or past UV lesions by RNA polymerase (a rifampicin-sensitive pathway) or DNA primase (a rifampicin-resistant pathway). The functionality of these RNA primers depends on their protection from the activity of RNaseH, which would otherwise abort the recovery attempts by degrading the RNA primers. DNA primers, on the other hand, can be provided by a recombinational event, similar to the bacteriophage T4 system. RecA may be required for both recombinational priming events as well as for protection of RNA-DNA hybrids from RNaseH activity. This protection is not required in strains in which the *rnh* gene was inactivated by a mutation, but RecA is still required for initiation from the alternative chromosomal origins. It is possible that the participation of each of the different pathways of priming depends on the experimental conditions such as the UV dose given to the cells.

III. UV MUTAGENESIS

As discussed in the Introduction, the prevailing model for the mechanism of UV mutagenesis is the bypass synthesis of a lesion-containing ssDNA region. Despite a growing body of supporting evidence, this model has not yet been proved and the mechanistic details of the mutagenic reaction are not fully understood. In this section, we review the characteristics of UV mutagenesis, examine the data on its molecular mechanism, and try to relate it to the replication and repair of UV-irradiated DNA.

A. Specificity of UV Mutagenesis and the Identity of the Premutagenic Lesions

It is well established that most UV mutations are targeted to UV lesions. This was first suggested by studies showing that UV light produced a distinctive spectrum of mutations in the *lacI*

gene that was different from the spectrum of mutations produced by other DNA-damaging agents.[76] Later studies, using direct DNA sequence analysis, addressed the specificity of UV mutagenesis in a variety of systems, leading to similar conclusions (see later). It was found that UV mutations tend to appear in runs of two or more consecutive pyrimidines, sites that were shown to be hotspots for the formation of UV lesions.[47,120] With the development of techniques to construct DNA substrates with single lesions located at predetermined sites,[343] it was possible to demonstrate directly that individual UV lesions target mutations (see later). At the same time, evidence has accumulated for the existence of a minor pathway of mutagenesis that is apparently not associated with DNA lesions. This pathway, termed untargeted mutagenesis, is discussed later.

Considerable research effort was invested in determining the types of mutations that UV light produces in DNA. Mutational spectra were expected to provide information about the identity of the premutagenic lesions and help formulate guidelines for the prediction of the types of mutations produced by UV light in any given gene. The analysis of UV-mutagenesis spectra is complicated by at least four factors: (1) UV light produces a multiplicity of photoproducts in DNA, only some of which were chemically identified;[280,293] thus, some of the mutations may be formed at an unknown, relatively infrequent but highly mutagenic lesion in DNA; (2) mutational spectra in a given gene are usually biased by the mutant phenotype that can be assayed. As a result, each spectrum contains a subset of the mutations that the system is capable of producing; (3) a variety of cellular mechanisms, including replication, repair, recombination, and transcription, operate on the damaged DNA and may modify the ultimate mutagenesis specificity, depending on the genotype of the cell and on its physiological growth conditions; and (4) because of technical reasons, mutations were usually assayed on genes present on extrachromosomal elements, including F′ episome, lytic phage λ, the ssDNA phage M13, and plasmids. The particular life cycle of each extrachromosomal element may also affect its mutational spectra.

Despite these possible sources of variation, studies performed with the *lacI* gene carried on an F′,[76,202,256,312] the *cI* gene of phage λ, in both its lytic[408] and lysogenic states,[407] and the *lacZ′* gene segment carried on a recombinant M13 ssDNA phage,[203] gave qualitatively similar results (Table 1). It was found that UV radiation caused primarily base substitutions (75%; mostly GC → AT) and, in addition, frameshifts (15%), tandem double-base substitutions (4%), and deletions (7%, but this is probably an overestimate due to a

TABLE 1
Specificity of UV Mutagenesis

	Mutation type (%)					
Assay system	**Transition**	**Transversion**	**Frameshift**	**Tandem double**	**Deletion**	**Ref.**
1. *lacI*/F′	40	23	30	1	5	256
2. *lacI*/F′	45	14	11	3	27	312
3. *lacI*/F′	51	22	8	5	14	312
4. *lacI*/F′	43	39	16	1	1	202
5. *lacI*/F′	62	23	11	4	0	202
6. *lacZ′*/M13	51	23	20	6	0	203
7. *cI*/λ	66	13	11	5	0	408
Average	**51**	**23**	**15**	**4**	**7**	
8. *cI*/λ lysogen	43	38	7	5	2	407

Note: The spectra of UV mutations in the *lacI* gene were obtained under the following conditions: rows 1 and 2, an F′ in repair-proficient cells; 3, an F′ in a *uvrB* strain; 4, an F′ during vegetative growth; 5, an F′ during conjugation. The average values correspond to the mutagenesis data obtained using extrachromosomal replicons (rows 1–7).

specific deletion hot spot in *lacI*). If the data obtained for phages and episomes are averaged and compared with the data for *cI* in the chromosome, the spectra seem similar, with two differences: (1) in extrachromosomal genomes, transitions (51%) are twofold more frequent than transversions (23%), whereas in the chromosome, roughly equal numbers of transitions (43%) and transversions (38%) were obtained and (2) frameshifts were twofold less abundant in the chromosome (7%) when compared with extrachromosomal elements (15%). These differences are also observed when the lysogen *cI* mutations are compared with the phage *cI* mutations (Table 1), suggesting that they originate from real differences between the chromosome and extrachromosomal elements. However, the generality of this comparison must await more data on the specificity of chromosomal mutations.

The multiplicity of factors that affect mutations caused by UV radiation makes the evaluation of the importance of each lesion produced in DNA by UV radiation a complex question that is not easily answered. A common approach took advantage of the ability of visible light to activate the cellular DNA photolyase, resulting in specific repair of cyclobutane-type photodimers by direct reversal to the original pyrimidine-pyrimidine sequence.[306] These experiments have shown that most UV mutations in the *lacI* gene carried on an F′[187,199] or in the *cI* gene of phage λ[145] were eliminated by photoreactivation. This suggests that cyclobutane photodimers are the major source of UV mutations. However, the validity of this interpretation is undermined by the following arguments: (1) Cyclobutane photodimers comprise approximately 85% of the UV lesions in DNA. Thus, under normal conditions, the much smaller fraction of nondimer lesions has a better chance of escaping excision repair and being processed into mutations. If most of the photodimers are eliminated by photoreactivation, the remaining noncyclobutane dimer lesions are likely to be rapidly repaired by the cellular repair mechanisms, thus reducing the likelihood of mutagenic processing. Such minor but mutagenic lesions, known to be substrates for excision repair,[111] are the pyrimidine-pyrimidone 6–4 adducts.[31,119,201,293] One way to overcome this difficulty is to perform the experiments in excision repair-deficient strains. However, the mutagenesis processes in such strains might be different from those in wild-type cells and, even without excision repair, there might be an effect of recombinational repair. (2) UV mutagenesis may occur at two adjacent UV lesions, for example, at two closely opposed UV lesions rather than at a single lesion. Although two closely opposed UV lesions are rare, there is evidence to suggest that they are formed in DNA with a frequency much higher than the frequency calculated that is based purely on statistical arguments.[1,193,258] In such a case, the premutagenic lesion may involve a cyclobutane photodimer, but the misincorporation event may occur at the second closely opposed UV lesion, which need not necessarily be of the cyclobutane dimer type (e.g., 6–4 adducts).

The use of ssDNA molecules carrying a single defined lesion at a unique site has enabled a simple and straightforward examination of the mutagenicity of individual UV photoproducts by direct DNA sequence analysis with no need for selection. So far, three TT photoproducts were examined and yielded valuable information. The *cis-syn* cyclobutane TT dimer was found to have a low mutagenicity, giving rise to mutations in only 7% of the isolates. Interestingly, this photoproduct yielded 79% of T → A transversions and only 21% T → C transitions. All transversions as well as 85% of the transitions occurred at the 3′ thymine (the first to be encountered during DNA replication).[9] The TT (6–4) photoproduct gave a completely different result; it was highly mutagenic, producing mutations in 91% of the isolates, and among mutants there was a great predominance (93%) of T → C transitions. As in the case of the *cis-syn* cyclobutane dimer, essentially all mutations occurred at the 3′ thymine. Three percent of the mutations were tandem double mutations opposite the lesion.[201] The third photoproduct examined, the *trans-syn* TT cyclobutane dimer, is a minor UV lesions found in ssDNA but not in dsDNA.[18] It showed yet a different result: its mutagenicity was 11% and it yielded primarily single-T deletions (49% of the mutants) and T → A transversions (23%) at the 5′ thymine.[8] These results suggest that both *cis-syn* TT dimers and 6–4 TT adducts contribute to the final UV mutations. Although the 6–4 TT adducts are an order of magnitude more mutagenic (per lesion), TT

cyclobutane dimers formed an order of magnitude more frequently than the adducts,[280] making contributions of both lesions potentially important. In repair-proficient cells, the relative contributions may be modulated by the efficiencies of repair of these lesions. Notice that the majority of UV mutations occur at cytosines. As the same methodology is applied to cytosine-containing UV lesions, a more comprehensive picture is likely to emerge.

The use of phage ssDNA in the previous experiments has the advantage that there is no complication from DNA repair or recombination. Thus, this approach assays, presumably, the handling of a damaged site in DNA by the phage replication machinery under constitutive or SOS conditions. These advantages make such a model system useful. However, extrapolation of these data to chromosomal UV mutagenesis should be taken with caution for the following reasons: (1) Replication of the chromosome involves a multiprotein replisome that is more complex than the M13 replisome. The latter involves pol III holoenzyme and SSB for the synthesis of the complementary strand, whereas the chromosomal replisome involves a battery of proteins including at least DnaB, DnaC, PriA, PriB, PriC, DnaT, DnaG, and pol III holoenzyme. These proteins may alter the behavior of the polymerase as it encounters the lesion. (2) UV mutagenesis in wild-type *E. coli* cells may be associated with excision gaps rather than replication (see later), unlike the case of phage M13. (3) The structure of the *E. coli* chromosome is by far more complex than the structure of M13 ssDNA. Factors such as superhelicity, bending, and other higher order folding determinants may affect the mutagenicity of UV lesions. (4) Each lesion was examined in only one DNA sequence context. Thus, the generality of the findings is unknown. This is particularly important because the local DNA sequence affected both the abilities of polymerases to replicate through lesions and the specificity of nucleotides that were incorporated opposite the lesion (see later).

An additional factor that should be taken into account when attempting to assign a specific biological effect to a specific UV lesion is the possibility that photoproducts are changed by a secondary chemical reaction. An example is the spontaneous deamination of cytosine to uracil, which is facilitated by saturation of the 5–6 double bond of cystosine.[114,328,381] Because the 5–6 bonds are saturated in both pyrimidine cyclobutane dimers and in the 5′ pyrimidine of 6–4 adducts, the deamination of cytosine to form uracil-containing lesions may affect the mutagenic reaction. Given the great similarity of uracil and thymine, in particular in their base-pairing properties, it is conceivable that UT or TU cyclobutane photodimers have a low mutagenicity similar to TT photodimers. This implies that under certain conditions where deamination takes place, the "mutagenic" event occurs spontaneously on the template strand, that is, the deamination of C in a CT or TC dimer, whereas the incorporation opposite the lesion is accurate. Early attempts were made to evaluate this phenomenon by a protocol that involved UV irradiation of cells, followed by a heating period at 45°C to facilitate deamination, and finally photoreactivation to split the cyclobutane dimers.[327] Such a protocol gave only a slight number of mutations when compared with UV alone, most likely because most of the uracils were repaired by base-excision repair, initiated by uracil glycosylase,[114] that was unknown at that time. Indeed, when similar experiments were done with *ung* strains deficient in uracil glycosylase activity, it was possible to demonstrate the formation of mutations that could be explained by deamination of cytosine-containing cyclobutane dimers.[104,105] Such a process was implicated to be responsible for 70% of UV mutations in phage S13.[371] A major obstacle for the evaluation of the importance of deamination in UV mutagenesis is the lack of chemical data on the rate of deamination of cytosine-containing cyclobutane dimers and 6–4 adducts. Measurements based on biological activity led to conflicting estimates: a deamination rate of 10^{-3}/dimer/min was estimated for the *E. coli* chromosome, implying that full deamination of UV lesions would require 10 to 20 h.[104] Another study concluded that deamination proceeds via a sharp step kinetics and is completed within approximately 0.5 h in S13 ssDNA and within 1 h in λ DNA.[371] Most UV mutations in *E. coli* are fixed within less than 1 h. Thus, a direct chemical measurement of the

deamination rates of cytosine-containing photoproducts along with more biological studies is essential for the evaluation of the significance of deamination in UV mutagenesis. It is noted at this point that UV mutations occur also at thymidines where deamination cannot occur.

The Dewar pyrimidinone valence isomer of the 6–4 adduct is an example of a UV lesion that can be either primary or secondary, depending on the reaction conditions. It has been shown to be produced in DNA, presumably as a primary UV lesion, on irradiation with UV light at wavelengths between 280 and 360 nm, making it relevant to sunlight mutagenesis.[365,366] It can also be formed as a secondary lesion from the regular TT 6–4 adduct on irradiation with 313-nm UV light. When assayed as a single lesion in M13mp ssDNA, this lesion caused mutations at a frequency of 53%. Although most mutations (59%) occurred at the 3′ thymine, a significant portion (28%) occurred opposite the 5′ thymine. Twelve percent of the mutations were tandem double-base substitutions targeted to the lesion. The largest single type of mutation was T → C substitution at the 3′ thymine (46% of the mutations).[201]

B. Proteins Involved in UV Mutagenesis

Based on genetic analysis, UV mutagenesis requires the *recA*, *umuD*, *umuC*, and *dnaE* gene products. A summary follows of the underlying genetic evidence and the biochemical properties of the proteins encoded by these genes and by others that may be relevant to the mutagenic process. A more detailed description of the genes and their protein products, including comprehensive references, can be found in the reviews cited.

1. RecA Protein[78,179,292,298,386,399]

RecA is a protein containing 352 amino acids with a molecular weight of 38 kDa. It is encoded by the *recA* gene located at 58 min on the *E. coli* chromosome. Strains from which the *recA* gene was deleted are viable, implying that RecA is not an essential protein. Mutations in the *recA* gene show a complex and pleiotropic phenotype. It includes drastically reduced homologous recombination, extreme UV sensitivity, a deficiency in prophage induction, extensive DNA degradation following UV irradiation, and absence of inhibition of cell division after UV irradiation.[398] In addition, inactivation of *recA* essentially eliminates UV mutagenesis.[260,396] The diverse functions of the RecA protein are rather well understood, considering the two major activities of the protein in the cell. It is the activator of the SOS stress regulon and it is the major recombinase in *E. coli*, capable of catalyzing strand exchange between homologous DNA sequences. Extensive genetic studies have yielded a variety of mutants with distinctive phenotypes. These include *recA441* (formerly, *tif-1*) in which the protease function is heat inducible; *recA1*, which is defective in both recombination and protease function; *recA430*, which is recombination proficient but defective in protease function; *recA730*, which is constitutively activated for protease function; and *recA1203*, which is recombination deficient but proficient in protease function.[78,399]

The regulatory role of RecA in post-UV gene expression in *E. coli* is responsible for many of the phenotypes associated with RecA deficiency.[211] Following UV irradiation (or treatment with other agents), RecA is activated. This activation most likely involves binding of RecA to ssDNA regions generated when DNA replication is interrupted at DNA lesions.[311] The interaction of activated RecA with free LexA repressor leads to cleavage of the repressor. This cleavage shifts the equilibrium between DNA-bound LexA and free LexA toward the unbound state, leading to the derepression of the SOS regulon. Similarly, it inactivates the repressors of several phages such as λ. Both LexA and λ repressors were found to be autocleaved under basic pH (but not under neutral pH).[210] Thus, it seems that RecA acts to facilitate the autocleavage of the repressor rather than act directly as a protease, but it is absolutely required under physiological conditions. The protease activity of RecA is also required for the posttranslational activation of UmuD, a protein required for UV mutagenesis. The cleavage of UmuD, facilitated by activated RecA, yields a shorter protein, termed UmuD′, which is the active species in UV mutagenesis (see later). Several

studies have shown that RecA protein has yet a third role in UV mutagenesis. They are based on the observation that UV mutagenesis still depends on RecA in cells in which the SOS response has been constitutively turned on by inactivating the LexA repressor, and the UmuD′ and the UmuC proteins were supplied from a plasmid.[92,270,360] It has been suggested that the third role of RecA is a direct participation in the mutagenic reaction.[221] Consistent with this suggestion is the recent demonstration of a requirement for RecA in replicative bypass of an abasic site by pol III[294] (see later).

Purified RecA protein is a ssDNA- and dsDNA-binding protein, a DNA-dependent ATPase, and it self-assembles into filaments, both in the presence or absence of DNA. Its binding to DNA is stoichiometric, usually with a monomer covering 3–4 nucleotides on both ssDNA and dsDNA. The active RecA species is believed to be a nucleoprotein filament that covers a ssDNA or a gapped duplex DNA. Purified RecA catalyzes DNA strand exchange,[298] the key reaction in homologous recombination or recombinational repair, and this reaction proceeds past UV lesions.[215] Strand exchange reactions are unidirectional, require ATP, and can involve either three or four DNA strands. The role of ATP hydrolysis in RecA-promoted reactions is not clear, because both repressor cleavage and strand exchange can occur in the presence of ATPγS, a nonhydrolyzable analog of ATP. It has been suggested that coupling of ATP hydrolysis to strand exchange renders the reaction unidirectional and facilitates movement past structural and topological barriers, as required in recombinational repair.[298]

Binding of RecA to DNA[298] may be relevant to its role in UV mutagenesis. At pH 7 and above, binding to ssDNA is much faster than binding to dsDNA. In the absence of ATP, a "collapsed" RecA-ssDNA filament is observed in the electron microscope. Addition of ATP or ATPγS gives rise to a filament that is extended by 50 to 60% relative to B-form duplex DNA. In the presence of ATPγS, there are approximately 6 RecA monomers and 18 to 19 nucleotides per helical turn. RecA is bound to the phosphate backbone, with the bases accessible in the major helical grove of the filament. Binding is largely sequence-independent and is highly cooperative. RecA filament assembly occurs primarily in the 5′ → 3′ direction on ssDNA. Unlike binding to ssDNA, RecA-protein binding to duplex DNA exhibits an absolute requirement for ATP or ATP analogs such as ATPγS. When bound by RecA protein, duplex DNA is underwound by approximately 40%; however, this unwinding does not represent strand separation. Binding to duplex DNA is greatly facilitated by the presence of gaps of 50 nucleotides or more. On such substrates binding initiates in the gap, and filament proceeds rapidly to the flanking duplex. Assembly again proceeds 5′ → 3′ relative to the strand in the gap. There is a distinct asymmetry in the binding of the two strands of a duplex DNA, as revealed by DNase protection experiments using gapped duplex DNA. When RecA is bound, the initiating strand is protected from DNase cleavage two- to three-fold better than its complement. This implies distinct binding sites within the filament with different characteristics for the two strands.

It has been suggested that binding of RecA to UV lesion in duplex DNA[221,299] may provide an alternative route for activation of RecA and SOS induction. This suggestion is based on a correlation between the UV mutability of wild-type, *recA441*, and *recA430* mutants and the ability of RecA proteins isolated from these strains to bind UV-irradiated dsDNA and cleave LexA.[220] However, such a correlation can also be made with the ability of these mutant RecA proteins to use short ssDNA stretches as cofactors for repressor cleavage.[247,266,285] (UV lesions in DNA do not lead to the formation of single-strand regions in DNA, as indicated by its insensitivity to ssDNA nucleases.[162]) It has been shown that SOS induction *in vivo* requires DNA replication, arguing that ssDNA gaps are responsible for RecA activation.[311] Thus, the *in vivo* significance of RecA activation by UV lesions on duplex DNA is not clear; it may represent a minor pathway for SOS induction.[80]

Two other properties of RecA might be relevant to UV mutagenesis: (1) its inhibitory effect on replication with pol III holoenzyme in the absence of SSB,[338] a property discussed earlier in connection with post-UV inhibition of DNA synthesis; (2) RecA was found to inhibit the 3′ → 5′

exonuclease proofreading activity of pol III holoenzyme,[103,221,340] most likely by direct inhibition of the ε subunit of the polymerase[221] where the exonuclease active site resides. This aspect of RecA activity is discussed later.

2. Single-Strand DNA Binding Protein[62,219,254]

The *ssb* gene, located at 92 min on the *E. coli* map, encodes a protein of 177 amino acids with a molecular weight of 19 kDa. In solution, SSB is a homotetramer, and most of its activities are believed to be performed in this state. SSB is involved in all major aspects of DNA metabolism, including DNA replication, repair, and recombination, and thus *ssb* mutations usually cause a multiplicity of phenotypes. Most *ssb* mutations are temperature sensitive for DNA replication, UV sensitive, exhibit recombination deficiencies, and show excessive postirradiation DNA degradation. In addition, they are defective in the induction of the SOS response and, in particular, in RecA induction and induced mutagenesis.[122,205,206,387]

An insight into the involvement of SSB in UV mutagenesis came from experiments with *E. coli* B strains into which the *ssb-1* allele was transduced.[205,206] In these strains, when grown on nutrient agar (but not other media), the *ssb-1* mutation does not confer temperature sensitivity, thus allowing assessment of the effect of the SSB-1 protein on UV mutagenesis under conditions that allow cell growth. These strains showed moderate UV sensitivity and normal UV mutability at 30°C. At 42°C, these strains showed more extreme UV sensitivity and a fivefold reduction in UV mutability. By pretreating the cells with nalidixic acid before the temperature shift, UV mutagenesis could be restored. These results would argue that the reduction in UV mutagenesis in these mutants is due to the failure to induce the SOS response. Reduced UV mutagenesis was reported also for the *ssb-114* allele, whereas conflicting results were reported for the *ssb-113(exrB)* allele.

SSB binds ssDNA specifically and cooperatively. The number of nucleotides bound to an SSB tetramer depends on reaction conditions. Under presumably physiological conditions, a tetrameric SSB binds a ssDNA stretch of 65 nucleotides, representing binding, to all four DNA-binding sites. At high-SSB and low-ssDNA concentrations (which may exist in the cell) SSB binds 35 nucleotides, presumably representing binding to only two DNA-binding sites. The two types of binding of SSB to ssDNA have been visualized in the electron microscope. At low ratios of SSB to DNA, a beaded structure is observed in the electron microscope, representing a nucleosome-like structure in which the DNA is wrapped around the tetramer. The contour length of the DNA is drastically reduced to only 25% that of naked DNA under these conditions. With higher ratios of SSB to DNA, the morphology of the coated strand is smoother and more extended, with the contour length observed being 40% that of naked DNA. It was suggested that SSB binding to DNA can proceed via two types of cooperativity. Unlimited cooperativity reflects interaction on both sides of a bound SSB tetramer and leads to the formation of long SSB clusters. It is related to the $(SSB)_{35}$ binding mode and can lead to a single long cluster along the ssDNA ("smooth" binding). This type of cooperativity is seen with the phage T4 SSB, the gene 32 protein. The second type is limited cooperativity in which clustering is limited to two tetrameric SSB molecules, forming an octameric structure ("beaded" binding). This type of binding is related to the $(SSB)_{65}$ mode.[219] It is possible that SSB adopts different DNA-binding modes when participating in different cellular processes.

SSB affects many enzymatic activities by virtue of its tight binding to DNA and/or by direct protein-protein interactions. Many single-strand endonucleases and exonucleases are inhibited by SSB, providing protection for ssDNA regions, an effect that might have *in vivo* significance. The polymerization activity of pol III holoenzyme is greatly stimulated by SSB, making it an essential component of the replication machinery.[178] Interestingly, it was found that SSB inhibits the $3' \rightarrow 5'$ exonuclease activity of pol III holoenzyme under replication conditions but not in the absence of replication.[340] This was interpreted to suggest that SSB functions to improve the efficiency of

exonucleolytic proofreading by reducing the "wasteful" exonucleolytic degradation of correctly polymerized nucleotides. Polymerization by pol I or pol III core is inhibited by SSB, whereas pol II is stimulated.[28,178,264,341] SSB was found to increase fidelity of DNA synthesis, even with DNA polymerases that were inhibited by SSB and that contained no 3′ → 5′ proofreading exonuclease activity.[185,186] The effect of SSB on bypass synthesis is discussed later.

3. DNA Polymerases

a. DNA Polymerase I[178,204]

Pol I is encoded by the *polA* gene, located at 87 min on the *E. coli* map. It is composed of 928 amino acids and has a molecular weight of 103 kDa. Three catalytic activities reside on a single polypeptide chain: a 5′ → 3′ polymerase activity, a proofreading 3′ → 5′ exonuclease activity, and a 5′ → 3′ exonuclease activity. Proteolytic digestion of pol I separates the enzyme into two fragments. The large C-terminal fragment contains the polymerase and the 3′ → 5′ exonuclease activities (it is commonly termed the Klenow fragment). The small N-terminal fragment contains the 5′ → 3′ exonuclease activity. The X-ray structure of the Large Fragment has shown that the 3′ → 5′ exonuclease activity resides at its N-terminus in a domain separated from the polymerase domain. By preferential removal of mismatched base pairs, the 3′ → 5′ exonuclease activity increases the fidelity of polymerization 10- to 100-fold. Pol I is the major repair polymerase, responsible for resynthesis of short excision repair gaps. Consistent with such a role, pol I has a polymerization processivity of 15 to 20.[178] Processivity is defined as the number of nucleotides polymerized following a single initiation event before the polymerase dissociates. It reflects the affinity of the polymerase to the primer-template and provides a measure for the overall synthetic efficiency of a polymerase. Pol I functions also in DNA replication by excising and replacing RNA at the 5′ termini of nascent Okazaki fragments. Pol I is unique among the polymerases in its ability to use a nick on a duplex as a primer for DNA synthesis. This involves unwinding of the duplex beyond the nick and progressive strand displacement of the 5′ chain. When coupled with 5′ → 3′ exonuclease action, this leads to nick translation. Uncoupled from the exonuclease, the polymerase may use the displaced strand as a template (template switching).

Mutants lacking the *polA* gene are viable in minimal medium but not in rich medium. Both missense mutations and the deletion mutation do not decrease UV mutagenesis, implying that pol I is not essential for UV mutagenesis.[12,398] However, on induction of SOS conditions, pol I acquires a new form, termed DNA polymerase I* (pol I*), that shows reduced fidelity.[188,189] The new form of the polymerase may involve the association with another, yet unidentified, polypeptide, possibly a subunit of pol III holoenzyme.[302] pol I* may be involved in untargeted SOS mutagenesis (see later).

b. DNA Polymerase II[178,267,388,389]

Pol II is encoded by the *polB* gene, located at 2 min on the *E. coli* map. The gene has been cloned recently and sequenced by three groups.[27,63,64,151] Pol II is a single polypeptide of 782 amino acids with a total molecular weight of 90 kDa. Unlike pol I, pol II is unable to use a nick on a duplex as a primer-template and it is also less effective on long single-strand templates.[178,388,389] Its preferred substrate is gapped DNA, a reflection of its low processivity (5 nucleotides;[29]). The two polymerases differ also in their response to SSB: whereas pol I is either unaffected or even inhibited by SSB, pol II is stimulated 50- to 100-fold, bringing its activity to that observed with gapped DNA.[178] This is achieved presumably because of an increase in the processivity of the polymerase. Pol II (but not pol I) was also shown to be stimulated by the pol III subunits β and the γ complex,[29,142,391] leading to an increase in the processivity of the polymerase up to 1600 nucleotides per binding event.[29] Pol II showed limited bypass of an abasic site dependent on the presence of SSB and the β,γ complex.[29]

The physiological role of pol II is unknown, and cells with a mutated *polB* gene, or without the

polB gene, show no phenotype.[60,137,334] The *polB* has been shown to be allelic to *dinA*, a DNA damage-inducible gene controlled by the SOS response.[27,152] Indeed, the activity of pol II is increased seven-fold by SOS induction.[28] Somewhat surprisingly, although the *polB* gene is SOS-inducible, cells that do not carry the *polB* gene exhibit normal UV mutability and resistance.[334] This implies that *polB* is not essential for UV mutagenesis and does not have an important role in the repair of UV lesions. As mentioned earlier, however, in mutants deficient in pol I and pol III, pol II serves in the repair of UV lesions.[242,362]

c. DNA Polymerase III[178,250,251]

Pol III holoenzyme is the major replicative polymerase of *E. coli*. It is a complex composed of 10 different subunits, and dimerizes to form an assembly of twin polymerases with a total mass of 900 kDa. The holoenzyme can be biochemically resolved into a series of subassemblies with successively simpler compositions. The simplest form, termed pol III or pol III core, is composed of three subunits: α, ε, and θ.[248] The α subunit (130 kDa), product of the *dnaE(polC)* gene, carries the active site of the polymerase,[230] and the ε subunit (27.5 kDa) carries the 3′ → 5′ proofreading exonuclease responsible for removing mismatched base pairs.[315] The function of θ (10 kDa) is unknown. A highly processive polymerase could be constituted from purified polymerase subunits without θ, but not without ε.[357] Mixing purified α and ε subunits generates a 1:1 complex with twice the polymerase activity and 50- to 100-fold 3′ → 5′ exonuclease activity. This stimulation is achieved through increased affinity of ε to DNA as a result of its binding to DNA, as indicated by the 100-fold decrease in the apparent K_m.[231] As a polymerase, core has low catalytic activity and a low processivity of 10 nucleotides per binding event.[102] A cell contains 40 mol of pol III core.

The addition of the τ subunit (71 kDa) to pol III core generates a subassembly termed pol III′, which has a sixfold higher processivity in the presence of spermidine. Pol III′ is dimeric, $core_2\tau_2$, providing a basic dimeric polymerase unit that is also present in more complex assemblies of holoenzyme.[249,358] This indicates that the τ subunit is responsible for dimerization. The addition of the γ complex to pol III′ generates the 9-subunit pol III*, with an improved processivity of 200 nucleotides per binding event, in the presence of SSB.[101,392] The γ complex (also termed the γδ complex) is composed of 5 subunits: γ (47.5 kDa), δ (35 kDa), δ′ (33 kDa), χ (15 kDa), and ψ (12 kDa).[235,236] The γ (47.5 kDa) and τ (71 kDa) subunits, products of the *dnaX* gene, are encoded by the same reading frame with γ constituting the N-terminal 2/3 of τ. The γ subunit is generated by a −1 translational frameshift that allows the use of a UGA stop codon to terminate translation of γ.[23,106,375]

The final addition of the β subunit, a 40.6 kDa protein encoded by the *dnaN* gene, reconstitutes pol III holoenzyme.[54,154,278] In the presence of SSB, holoenzyme exhibits an enormous processivity of up to 100,000 nucleotides per binding event and it replicates ssDNA at a rate of 700 nucleotides per second, close to the estimated rate of DNA replication fork movement *in vivo* (1000 nucleotides per sec).[55,102,263,274] Unlike any of the other DNA polymerases, pol III holoenzyme is absolutely dependent on activation by ATP (or dATP) to form a highly stable polymerase-DNA initiation complex at the primer-template. It is this initial reaction that locks it to the DNA, ensuring high processivity.[55] The remarkable effect of the β subunit on the processivity of the polymerase is based on its ability to form a sliding clamp on DNA. The X-ray structure of a β dimer has shown that it forms a ring shape, with a hole large enough to accommodate duplex DNA,[177] as suggested previously based on biochemical analysis.[359] It is thought that once located on the DNA, the β subunit tethers the core to the DNA, providing a mechanism for the great processivity of the polymerase. Remarkably, the β subunit itself has no affinity for DNA, and it requires the activity of the γ complex to be loaded on DNA. The activity of the γ complex is catalytic, and it can load additional β subunits on other primer-templates.[236,273,390] Interestingly, the cell contains approximately 300 dimers of β, a 10 to 20 excess over the polymerase. This excess of β may serve to maintain pol III* in the holoenzyme form[197]

and it may endow processivity onto other pol III subassemblies or pol II.

The distribution of the 10 subunits between the two halves of the dimeric polymerase is unknown. It has been suggested that the polymerase is an asymmetric dimeric complex, composed of twin nonidentical polymerases. The leading strand polymerase contains pol III core complexed with τ and β for a highly processive polymerase unit. The lagging strand polymerase contains pol III, the γ complex, and β, and has a lower processivity.[232,251]

The *dnaE*, *dnaN*, and *dnaX* genes are essential. However, in a cell that contains a defective α subunit due to a conditionally lethal temperature-sensitive or nonsense mutation in *dnaE*, pol I can replace the α subunit, provided the cell carries a *pcbA1* mutation (an allele of *gyrB*). This must mean that pol I can interact with pol III subunits to become a replicase.[50,52,229] A related observation is the cosedimentation of pol I* activity with the τ, β, and ε subunits, and the immunoprecipitation of the τ subunit when pol I* was incubated with monoclonal antibodies against pol I.[302]

The importance of the *dnaQ* gene encoding the ε subunit is shown by its *mutD* allele, which causes an increase of up to 10^5 in the rate of spontaneous mutations.[77,100] This underlines the importance of the contribution of the ε subunit to the fidelity of the polymerase. The ε subunit isolated from such a mutator strain was shown to be defective in exonuclease activity.[84,96,316] A recently discovered mutation in the α subunit *(dnaE173)* was found to increase spontaneous mutagenesis 10^3- to 10^4-fold, demonstrating that the α subunit is also an important factor in replication fidelity.[233]

UV mutagenesis requires pol III. This dependence was demonstrated by showing that UV mutagenesis was reduced when cells carrying temperature sensitive alleles of the *dnaE* gene were shifted to the restrictive temperature after UV irradiation.[37,40,330] Moreover, no UV mutagenesis was observed at 42°C in *dnaE* strains in which the temperature sensitivity was suppressed by the *pcbA1* mutation.[50,126,127,229] Thus, although these strains could replicate and form colonies at the restrictive temperature, they did not show UV mutagenesis, most likely because of inactivation of the α subunit of pol III. Indeed, when a plasmid carrying the *dnaE* gene was introduced into these cells, UV mutagenesis was restored.[51,127]

4. UmuD and UmuC

The *umuC36* mutation was discovered by a brute-force approach in 1977 as a mutation that reduced UV and chemical mutagenesis without having a major effect on UV survival.[165,348] The fact that other SOS functions, such as prophage induction and postirradiation inhibition of cell division, were not affected implied a specific role in the mutagenic process itself and promoted extensive research on the role of this genetic locus. The cloning of the *umuC* locus revealed that it is composed of two genes arranged in an operon, with *umuD* followed by *umuC*.[99,336] The specific requirement for *umuDC* in UV mutagenesis[165] and its UV inducibility[4] further supported the notion of a regulated mutagenesis pathway, acting to enhance evolution under unfavorable environmental conditions.[93] Functionally homologous genes such as *mucAB*[283] and *impABC*[218] were found on naturally occurring conjugative plasmids, further supporting a role in genetic variation.

The *umuDC* operon, located at 26 min on the *E. coli* map, is under the SOS control.[4,168,282] *UmuD* encodes a protein of 139 amino acids with a molecular weight of 15 kDa. The *umuC* gene encodes a protein of 422 amino acids, with a molecular weight of 48 kDa.[168,282] The *umu* locus could be entirely removed without affecting cell viability.[409] It was estimated that UmuD is present at approximately 180 copies per uninduced cell, increasing up to 2400 copies in cells that lack a functional repressor. Induced levels of UmuC were found to be 12-fold lower with approximately 200 copies per cell.[411]

The amino acid sequences of UmuD and the plasmidic homologue MucA, deduced from the DNA sequence of the cloned genes,[168,282] revealed an unexpected homology to the LexA repressor at its site of cleavage by RecA.[282] Indeed, it was shown both *in vivo* and *in vitro* that UmuD is cleaved by activated RecA to yield a shorter polypeptide termed UmuD′.[53,270,335] The amino acid sequence of the N-terminus of purified UmuD′

revealed that the cleavage site was a Cys-Gly bond at amino acid 24 from the N-terminus. This was consistent with the site predicted based on the sequence homology with the LexA and λ cI repressors.[412] UmuD′ is the active species for UV mutagenesis, and a *umuD′C* artificial operon carried on a pBR322 vector fully substituted the original *umuDC* operon.[270] The same conclusion was reached later, using a low copy vector.[5]

The UmuD and UmuC proteins have been overproduced from plasmids and purified.[53,412] So far, no enzymatic activity has been associated with the Umu proteins. Recent evidence that UmuD′ and UmuC stimulate bypass synthesis by pol III at an abasic site[294] is discussed later. UmuC was shown to form complexes with both UmuD and UmuD′. The UmuD′-UmuC complex is thought to function in UV mutagenesis, whereas the UmuD-UmuC complex, which is inactive in UV mutagenesis, might have a negative regulatory role in the process.[412] A negative regulatory role for uncleaved UmuD was suggested earlier, based on the isolation of dominant negative mutations in *umuD*.[16] Overexpression of the UmuD′ protein was found to suppress the nonmutability of excision-deficient strains carrying the *umuC36* mutation (but not a more severe *umuC125* :: *Tn5* mutation). This was suggested as evidence that the mutant UmuC36 protein has reduced affinity for UmuD′ and that the effect could be compensated by an excess of UmuD′.[11] An interaction between UmuC and RecA was suggested by the retardation of UmuC on a column of immobilized activated RecA. UmuD′ was retained on the column only in the presence of UmuC, presumably through UmuD′-UmuC association.[112] Because the experiments were performed with cell extracts, however, an indirect interaction via a third factor could not be eliminated. A deficiency in the interaction of the mutant RecA1730 protein with UmuD′C *in vivo* was postulated to explain the nonmutability of the *recA1730* allele.[6] A similarity was found between UmuD and the gene 45 protein of bacteriophage T4 and between the UmuC protein and the gene 44 and gene 62 proteins.[14] These proteins function as accessory proteins for the T4 DNA polymerase, endowing it with high processivity.[272] Thus, UmuDC might have an activity similar to that of the T4 proteins. There is no experimental support for this hypothesis.

Overproduction of UmuD and UmuC by a multicopy plasmid in a *lexA51*(Def) background causes *E. coli* cells to become cold sensitive. They grow well at 42°C, but when shifted to 30°C, DNA synthesis stops immediately and ultimately the cells die.[239] This cold sensitivity may be caused by an interaction of the overproduced proteins with an essential component(s) of the replication machinery. The *umuC125* mutation was found to abolish cold sensitivity but not mutability.[238] Interestingly, this cold sensitivity is suppressed by mutations in the heat shock genes *groES* and *groEL*.[86] The proteins encoded by these genes belong to the ubiquitous family of molecular chaperones that play a fundamental role in the folding and secretion of certain proteins.[118] Under normal conditions, UV mutagenesis requires the GroE proteins, because both *groEL* and *groES* mutants are deficient in UV mutagenesis.[86,212] The effect of the GroE proteins is mediated via the stability of the UmuC protein. It has been estimated that the half-life of UmuC in a *groE*$^+$ strain is 17 min, whereas it is reduced to 6 min in a *groES* or *groEL* background.[86] Interestingly, the UV mutagenesis deficiency of *groE* mutants could be suppressed by coexpression of UmuD′ with UmuC, probably due to stabilization of UmuC in a UmuD′-UmuC complex.[87] Cold sensitivity due to overexpression of UmuD′-UmuC, unlike that imposed by UmuD-UmuC, was not suppressed by mutations in *groE*, supporting the proposed mode of involvement of *groE* in UV mutagenesis.

Mutagenesis by UV radiation and by other DNA-damaging agents such as methylmethanesulfonate, 4-nitroquinoline 1-oxide, benzo*(a)*-pyrenes, aflatoxin B1, neocarzinostatin, and angelicin plus near UV irradiation requires a functional *umu or umu*-like operon.[98,99,108,165,245] Other mutagens such as ethylmethanesulfonate or *N′*-methyl-*N*-nitronitrosoguanidine are largely *UmuDC*-independent,[314] whereas DNA-damaging agents such as γ radiation[308] and *N*-acetoxy *N*-2-acetylaminofluorene[171] have both *umu*-dependent and -independent pathways of mutagenesis. Even for UV mutagenesis, *umu* is not an absolute requirement and pathways of *umu*-independent mutagenesis exist. This was demon-

strated for UV mutagenesis of the F′, assaying for inactivation of the LacI repressor.[65] Under conditions where both mutants and wild-type colonies could grow, up to 30% of the normal yield of lacI⁻ mutations were found to be induced in a *uvrA6umuC122* :: *Tn5* strain. The specificity of mutations induced was similar to that of umuC⁺ cells. This suggested that UV mutagenesis in *umuC* cells might be, in general, similar to that in umuC⁺ cell, but occurs with reduced efficiency.[65] A pathway of mutagenesis that does not depend on *recA* or *umuC* was demonstrated with UV-irradiated phage S13 by assaying for the production of temperature-sensitive mutations.[369]

Homologues of *umuDC* were found to be much more widespread than originally thought.[48,321,376] In fact, based on hybridization to *umu* DNA probes from *E. coli* and *S. typhimurium* and on cross-reactivity with anti-UmuD antibodies, even species that displayed poor induced mutagenesis had *umu*-like genes and proteins.[321] *S. typhimurium* has a chromosomal *umuDC* homologue[346,372] and another homologue, termed *samAB*, present on a cryptic plasmid.[271] The *mucAB* operon is found on the drug-resistant plasmid pKM101 (a natural derivative of plasmid R46),[282,283] and the *impABC* operon is carried on plasmid TP110.[218,356] Recently, a *umu* locus was cloned from *Streptomyces coelicolor*.[259] Although these *umu*-like operons seem to function generally in a similar way,[283,322] they do exhibit features different from those of UmuDC. For instance, the *mucAB* operon was shown to be more effective than the *umuDC* operon in promoting UV mutagenesis,[22] and the *imp* operon contains three genes instead of two.[218] Unlike UmuD, MucA may also be active in UV mutagenesis in its unprocessed form.[333] Interestingly, although the *mucAB* operon restores UV mutagenesis in a *umuDC* mutant, the individual genes are not complementary. The *mucA* gene does not complement a *umuD* mutation, and the *mucB* gene does not complement a *umuC* mutation.

Under certain conditions, *umu* becomes an important survival function. It was found that *umuC* cells were inactivated four times more rapidly than *umuC*⁺ cells by angelicin (isopsoralen; a monofunctional psoralen) plus near-UV irradiation.[257] This suggests that the Umu proteins carry out important repair or tolerance functions for specific types of DNA damage. In certain genetic backgrounds, the introduction of a *umu* mutation leads to a decrease in UV survival. Thus, in the absence of excision repair, a *umuC* mutation mildly increased UV sensitivity.[163,165,257] The most striking case is the *recA718* mutation; when this mutation is present in the chromosome, an additional *umuC* mutation greatly sensitizes the cells to UV radiation due to the inability to resume DNA replication following UV irradiation. As mentioned earlier, it was suggested that this effect stems from the ability of UmuC to fulfill a function in resumption of DNA replication that is deficient in *recA718* cells.[403] The RuvA, RuvB, and RuvC proteins are involved in the resolution of recombination intermediates.[364] Mutations in the *ruvA* or *ruvB* genes cause UV sensitivity, most likely due to a deficiency in recombinational repair.[217] It was reported that in a background of the *ruvA200* mutation, the *umuC* mutation caused increased UV sensitivity.[309]

Two other phenomena, except for DNA-damage-induced mutagenesis and cell killing, were reported to be affected by the *umu* mutation. One is the alleviation of EcoK restriction of unmodified phage λ, or plasmids, observed during induction of the SOS response. It was found that *umuC* mutants showed a decrease in the ability to alleviate restriction as compared with a *umuC*⁺ strain, representing a function independent on mutagenesis.[136] The other is the ability of the *muc* and *umu* functions to stimulate spontaneous and UV-inducible precise excision of transposon Tn10 in *S. typhimurium*.[2,48]

C. Mechanism of UV Mutagenesis

1. Is UV Mutagenesis Initiated by Repair or Replication?

Although it is generally believed that UV mutagenesis is related to DNA replication, there is evidence to suggest the involvement of excision repair. If UV irradiation of *E. coli* cells is followed by an illumination with visible light, most UV mutations are prevented, presumably due to the repair of *cis-syn* cyclobutane

photodimers by DNA photolyase, before these lesions are processed into mutations. The kinetics of fixation of UV mutations can be determined by examining the time after UV irradiation at which these mutations cannot be further eliminated by photoreactivation. Using this approach, it was determined that UV mutations in wild-type cells are fixed within 20 min. This correlates well with the kinetics of excision repair, rather than DNA synthesis, which fully recovers after a longer period of 30 to 40 min.[41,268,269] Based on these observations, it was suggested that in wild-type cells, UV mutations are associated with excision gaps rather than DNA replication. These data, however, do not exclude the possibility of a limited replication-related event localized to the vicinity of the UV lesion. It might be uncoupled from the global-replication activity in the cell and occur before replication resumption. The involvement of the Uvr proteins, which are required for excision repair, could not be assayed directly, because *uvr* strains were found to be hypermutable by UV light.[42,134,394] When the kinetics of fixation of UV mutations was examined in these strains, it was found that fixation occurred later than in wild-type cells and correlated with the resumption of DNA-replication activity.[268,400] To explain these results, it was suggested that there exist at least two pathways of UV mutagenesis: a pathway operating at excision gaps and a pathway associated with DNA-replication gaps. Recently, direct evidence was obtained that supports a Uvr-dependent pathway of UV mutagenesis. Using an *in vitro* system, a pathway of UV mutagenesis that requires the UvrA, B, and C gene products was identified[71] (see later).

Excision repair normally operates to remove DNA lesions and is considered to be error free. The UvrABC nuclease, composed of the UvrA, UvrB, and UvrC proteins, introduces single-strand breaks 12–13 nucleotides apart at both sides of the lesion. Next, the damaged oligonucleotide is removed from DNA and replaced by a newly synthesized patch by the combined action of DNA helicase II (the UvrD protein) and pol I. DNA ligase finally seals the remaining nick. How can such a pathway create premutagenic sites? To explain this apparent paradox, it was suggested that premutagenic sites may be formed at locations of two closely opposed UV lesions.[33,319] The excision of one lesion by the UvrABC nuclease, followed by a possible gap enlargement by cellular exonucleases, will produce a ssDNA gap opposite the second UV lesion, a structure that cannot be repaired by excision repair (Figure 3). The filling in of such a gap by polymerization will

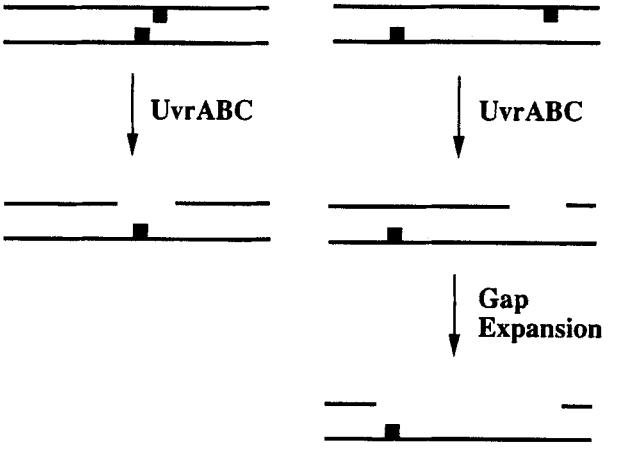

FIGURE 3. Excision repair can form premutagenic lesions in DNA. Excision of one of two closely opposed UV lesions initiated by the UvrABC repair nuclease will leave a lesion on a ssDNA region. A similar structure can be formed from nonadjacent opposing lesions if the UvrABC incisions are followed by gap expansion by exonucleases.

then produce a mutation opposite the remaining UV lesion.[33] Based on statistical consideration, it would appear that two closely opposed UV lesions should be extremely rare in DNA. It was found, however, that closely opposed photodimers are formed in DNA at a significant frequency of 1% of the total number of photodimers.[1,191,192,258] DNA-sequence mapping of the sites of formation of these lesions revealed that they tend to appear at sites of two closely opposed runs of polypyrimidine tracts.[193] Although the nicks produced by UvrABC at both sides of a UV lesion are 12 to 13 nucleotides apart,[125,305,378] the definition of two closely opposed UV lesions need not necessarily be limited to that distance. If for some reason a normal excision gap is extended by exonucleolytic degradation, it may expose a nonrepaired UV lesion farther than 12 to 13 nucleotides away, thus creating a nonrepairable lesion that may be a substrate for mutagenic gap filling (Figure 3). Closely opposed UV lesions were also suggested to cause replication arrest leading to the formation of overlapping daughter strand gaps in sister DNA molecules. Such gaps cannot be repaired by either excision or recombinational repair and might thus be substrates for error-prone filing by polymerization.[319] DNA gaps opposite UV lesions were suggested recently to be responsible for two related phenomena: the persistence of a stable SOS-inducing signal after excision repair of UV damage, in the absence of chromosomal replication, and the generation of UV mutations to streptomycin resistance in repair-proficient cells.[38]

A finding consistent with a role for excision gaps in UV mutagenesis is provided by the *mfd* mutation. Mutation frequency decline (MFD) is defined as the rapid and irreversible decrease in the frequency of certain damage-induced suppressor mutations that occurs when protein synthesis is transiently inhibited immediately after irradiation.[398] MFD is absent in a specific mutant termed *mfd* and *uvr* strains, indicating that it represents a specialized mode of excision repair.[116,117,394] Recent evidence[326] suggests that the *mfd* gene encodes a transcription-repair coupling factor that promotes preferential repair of the transcribed (template) strand by the UvrABC excision-repair system.[325] The UV survival of *mfd* strains is not different from wild-type, although the initial excision rate is one third that of wild-type cells.[116] This implies that although repair of potentially lethal lesions is slower, it is eventually as efficient as wild-type repair. Resumption of post-UV DNA synthesis is delayed in these mutants, very likely contributing to UV resistance by minimizing the number of UV lesions that DNA-replication forks encounter. Interestingly, UV mutability in *mfd* strains is fivefold higher than in wild-type. It thus appears that slow excision repair, which is characteristic of the *mfd* mutant, can cause elevated UV mutagenesis without affecting cell survival. It was pointed out[326] that it is unlikely that the slow excision would cause a larger proportion of UV lesions to become replicated before DNA repair is completed (as in *uvr* mutants). This is based on the observation that resumption of DNA synthesis is inhibited in *mfd* mutants and on the argument that an increase in the number of encounters between replication forks and UV lesions would predict not only an increase in UV mutations, but also decreased survival. It is possible that the slow excision-repair phenotype causes the persistence of excision gaps, which may be enlarged by exonucleolytic degradation until they expose another UV lesion, thus creating the substrate for mutagenic gap filling.

The vast majority of repair patches in wild-type cells was estimated to be 20 to 30 nucleotides long and involve pol I.[73,74] In the absence of pol I, pol II and pol III take over, leading to an increase patch size of about 100 nucleotides in length.[182,242,362,413] It was found that even in wild-type cells, some repair patches are at least 1500 nucleotides long. This long patch repair is dependent on UvrABC and on pol I, as is the short patch repair, but differs in its dependence on *recA* and *lexA*.[72,74] Extended excision gaps associated with such a pathway may be a source for premutagenic lesions. A related observation is a UV-inducible repair pathway that depends on the *uvrA*, *uvrB*, *lexA*, and *recA*. This pathway seems to be a tolerance mechanism rather than an excision-repair mechanism, because cyclobutyl pyrimidine dimers were found to persist in the DNA after recovery and replication. Interestingly, following recovery, the dimers became insensitive to the *M. luteus* dimer-specific nuclease but could be detected by

acid hydrolysis of radiolabeled chromosomal DNA followed by chromatographic analysis.[323]

2. Bypass Synthesis

As indicated earlier, the key step in UV mutagenesis is believed to be the replicative bypass of UV lesions (also termed bypass synthesis, or translesion DNA synthesis), originally suggested in the 1960s.[39,395] Support for the model came from experiments with ssDNA genomes that cannot undergo excision repair or recombination because of their single-stranded structure. The intracellular replication products of UV-irradiated phage ϕX174 were analyzed in cells that were UV irradiated before infection by UV-irradiated phage. It was found that UV irradiation of the cells induced a mechanism that allowed better replication of the phage ssDNA to the duplex form.[58] The simplest explanation for this observation is translesion polymerization, although it was not clear whether this induced DNA synthesis of the damaged DNA was accompanied by UV mutations.

A direct correlation between mutagenesis and the ability to replicate damaged nucleotides came from studies with M13mp ssDNA containing a single UV lesion at a defined site.[9,201] The first step in the replication cycle of phage M13 is the synthesis of the complementary strand by the cellular pol III holoenzyme.[178] Because essentially all DNA molecules contain the UV lesion, the production of a plaque implies that the UV lesion was bypassed. The efficiency of bypass synthesis can be measured by the ability of the transfected DNA to generate phage plaques. When such DNA constructs were used to transfect unirradiated cells, bypass synthesis was inefficient, as expected.[9,201] On transfection of UV-irradiated cells, the number of phage plaques increased considerably. This increased survival was associated with an increase in mutations opposite the site where the lesion was present.[9,201] The simplest interpretation of these results is that of bypass synthesis, the translesion polymerization by a DNA polymerase. However, one should bear in mind two points concerning this interpretation: (1) The model is based on the assumption that the lesion present on the ssDNA is not processed to any significant extent in the cell. Although this is certainly a reasonable assumption based on our current knowledge of DNA repair, one cannot rule out the possibility that the lesions were processed into mutations in the ssDNA prior to DNA replication by unknown enzymatic activities. (2) Mutagenesis in phage M13 may be different from chromosomal UV mutagenesis as discussed earlier.

The dependence of UV mutagenesis on pol III is consistent with the bypass synthesis model. The strict requirement for pol III, however, even under conditions in which another polymerase functions in replication (i.e., in *dnaE pcbA* mutants), is intriguing. The cell contains two other DNA polymerases that could have, in theory, performed bypass synthesis. Why would the cell recruit the replicative polymerase for the bypass? Would it not make more sense if the cell recruited for that purpose either pol I or pol II, which are repair enzymes, rather than the replicative polymerase? This is particularly intriguing considering the facts that pol II is UV inducible and all polymerases in purified form were shown to be able to bypass UV DNA lesions to some extent. Pol III holoenzyme in the presence of SSB was shown to bypass UV lesions in ssDNA[213,214] and dsDNA.[332] Pol I was shown to bypass UV lesions on a ssDNA[290] and a cyclobutane TT dimer on a synthetic oligonucleotide.[367] Pol II was shown to bypass abasic sites.[28,29] However, the genetic evidence as for the dispensability of pol I and pol II is unambiguous; mutants carrying a point mutation or a deletion in either *polA*[12,397] or *polB*[127,334] are proficient in UV mutagenesis. This does not necessarily mean that in a wild-type strain that contains all the three polymerases, neither pol I nor pol II plays a role in UV mutagenesis. It does, however, suggest that pol III has a unique role in UV mutagenesis that cannot be replaced by any of the other polymerases. Bypass synthesis without any assistance from SOS-induced proteins was suggested to be the basis for UV mutagenesis in two cases. One is the *umuC*- and *recA*-independent pathway of UV mutagenesis in the ssDNA phase S13,[369] and the other is the *umuC*-independent branch of UV mutagenesis in F′.[65]

The following are the most pronounced differences between pol III and the other two polymerases that might provide the biochemical grounds for the requirement for pol III in UV mutagenesis:

1. *Processivity.* As described earlier, pol III holoenzyme is highly processive (>100,000). In contrast, the processivities of pol I and pol II are 20 and 5 nucleotides, respectively. If UV mutagenesis involves filling in of gaps that are hundreds, or even thousands, nucleotides long, the requirement for pol III may have originated from its high processivity. This does not explain why in *dnaEpcbA1* cells, other DNA polymerases, which presumably become processive by interacting with accessory subunits of pol III,[29,142,302] cannot substitute for pol III.
2. *Separate exonuclease subunit.* Unlike pol I and pol II in which the polymerase and proofreading 3′ → 5′ exonuclease activities reside on the same polypeptide chains, pol III holoenzyme has a separate exonuclease subunit (ε). If the ε subunit interferes with UV mutagenesis,[109,157] then its exclusion from the polymerase complex provides a mechanism for eliminating this interference. Such a mechanism is impossible when the exonuclease is part of the polymerase polypeptide (although, of course, other inhibitory mechanisms can be used). At this point, it should be mentioned that in *in vitro* reconstitution experiments of pol III, the ε subunit (but not θ) was essential to form a processive polymerase.[357] However, the cell can tolerate the absence of the ε subunit, even for chromosomal replication, as indicated by the viability of *S. typhimurium* strains from which the *dnaQ* gene was removed.[194] The involvement of an epsilon-less pol III complex in UV mutagenesis was suggested by two groups.[35,286,410]
3. *Multiple protein-protein interactions.* Unlike the other two polymerases, pol III holoenzyme is composed of multiple subunits and it acts as part of the multiprotein replisome complex. This involves a multiplicity of protein-protein interaction, some of which may be vital for UV mutagenesis. It is possible that the SOS factors that are involved in UV mutagenesis have evolved from polymerase accessory proteins and were thus selected for an interaction with pol III.

The most likely candidate for carrying out bypass synthesis is a multiprotein assembly of DNA pol III, RecA, UmuD′, and UmuC.[94] The preferential binding of RecA to UV-damaged dsDNA[221,299] along with the evidence for an interaction between UmuC and RecA[112] led to the suggestion that RecA might be targeting the UmuD′C complex to UV lesions.[94] Under such conditions, the inhibition of the 3′ → 5′ exonuclease activity of the polymerase by RecA[103,221,340] might help in bypass synthesis. The function of UmuD and UmuC is unknown. It was suggested that they may form a specialized DNA clamp, whose function is to tether the polymerase to the lesion site.[13,216,339,340] The limited homology between these proteins and the processivity factors of phage T4 DNA polymerase is in agreement with such a role.[14] Recently, evidence has been presented that purified UmuD′, UmuC, and RecA proteins assist pol III holoenzyme in the replicative bypass of an abasic site.[294] This is the first direct evidence for UmuD′C- and RecA-stimulated bypass synthesis by pol III holoenzyme. As more studies are done using these purified proteins, a mechanistic picture of bypass synthesis is likely to emerge.

3. Factors That Determine Bypass Synthesis by DNA Polymerases

The development and the *in vivo* use of DNA constructs that contain a single defined lesion at a predetermined site made a significant contribution to the field of mutagenesis.[343] The use of such substrates for *in vitro* studies is in only its initial stages, primarily because of technical difficulties associated with obtaining large quantities of high-quality substrates. As a consequence, most *in vitro* studies on bypass of DNA lesions by purified DNA polymerases were performed with randomly damaged DNA substrates or with short synthetic nucleotides that contained a single defined lesion

at a predetermined site. Whereas the use of defined oligonucleotides eases considerably the analysis of bypass data, it suffers from the limitation that short oligonucleotides do not fully represent DNA. This is especially true for studies with pol III holoenzyme, which is very big and acts on SSB-coated DNA.

In analyzing the encounter of a lesion by a DNA polymerase during polymerization, one should consider at least four distinct kinetic steps (Figure 4): (1) polymerization opposite the damaged nucleotide (addition); (2) exonucleolytic removal of the nucleotide opposite the lesion (excision); (3) polymerization of the nucleotide(s) past the lesion (extension); and (4) dissociation of the polymerase from the DNA. Dissociation can occur after (or before) each of the "problematic" polymerization steps.

a. The Addition Step

Intuitively, the addition of a nucleotide opposite the lesion might be thought of as the kinetic barrier in translesion synthesis. However, polymerases usually have little trouble in performing this step, as indicated by the extensive turnover of dNTPs into dNMPs during DNA synthesis on damaged DNA. This turnover is the result of repeated cycles of addition, followed by excision of the newly incorporated nucleotide by the proofreading $3' \rightarrow 5'$ exonuclease of the polymerase (Figure 5). DNA damage-stimulated turnover of dNTPs was observed with pol I on UV-irradiated[380] or depurinated DNA templates[304] and with pol III holoenzyme opposite UV lesions[337] and apurinic sites.[133] Another line of evidence, indicating the relative ease of addition opposite lesions, was the finding that the terminal nucleotide of an interrupted DNA chain was occasionally positioned opposite the lesion.[352]

The kinetically slow step in the turnover reaction is the addition step.[337] Thus, the specificity of turnover represents the specificity of nucleotides added opposite the lesion. Assuming that incorporation of a nucleotide opposite a lesion represents the misincorporation step of mutagenesis, the turnover data can be used to predict muta-

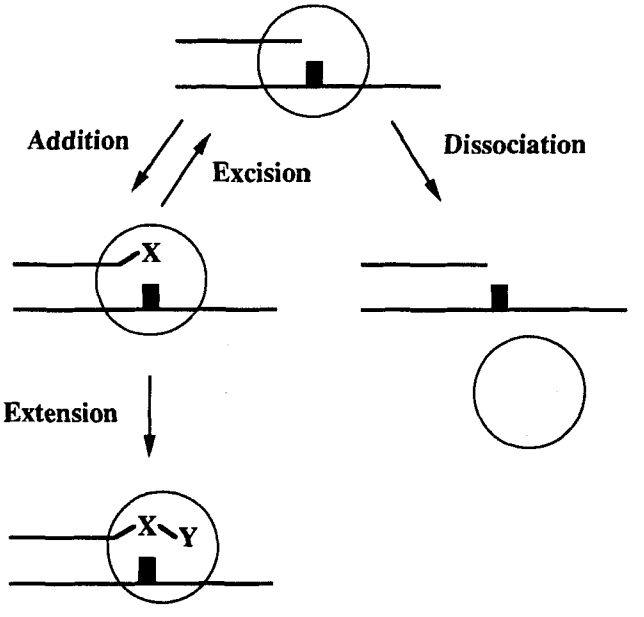

FIGURE 4. The major kinetic steps involved in bypass of DNA lesions by DNA polymerase. X and Y represent nucleotides polymerized opposite and past the lesion, respectively. The circle represents the polymerase, and the black square represents the DNA lesion.

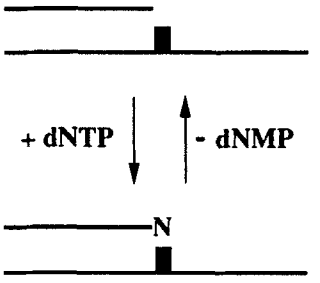

FIGURE 5. *Turnover of dNTPs to dNMPs during idling* of DNA polymerase stalled at a lesion-blocked primer-template. The turnover reaction results from repeated cycles of incorporation of a dNMP residue opposite the lesion, followed by its removal, in the form of free dNMP, by the exonuclease activity of the polymerase.

tional specificity. The predicted specificity of AP-site mutagenesis, based on *in vitro* turnover data obtained with pol III holoenzyme, turned out to be surprisingly accurate.[133] The relative abundance of mutations caused by AP sites, obtained by transfection of *E. coli* cells with depurinated M13*lac* ssDNA,[186] was strikingly similar to the relative levels of turnover of the 4 dNTPs on depurinated DNA.[133] This suggests that the *specificity* of AP-site mutagenesis is determined to a large extent by the intrinsic properties of the polymerase, with little influence by SOS-induced proteins. Moreover, it provides further support to the bypass synthesis model for AP-site mutagenesis. A similar correlation between turnover rates on UV-irradiated DNA and mutational specificity of UV lesions is harder to make because of the multiplicity of UV lesions on the substrate. However, if the data are taken to represent events occurring primarily at TT cyclobutane dimers, which are the most abundant UV lesions in DNA, then the turnover data[337] predict that T → A transversion will be the most frequent mutation at this lesion. Indeed, *in vivo* experiments with a ssDNA carrying a single TT dimer yielded primarily T → A transversions.[9]

Are there rules governing the specificity of the addition step? Based on *in vitro* studies, it was suggested that when DNA polymerases encounter noninstructional lesions, the default reaction is the preferential incorporation of dAMP residues opposite the lesions (the A rule;[350,353]). A similar suggestion was made based on *in vivo* studies.[368] For abasic sites, which probably represent the only purely noncoding lesion, the dAMP insertion preference was clearly demonstrated by several groups both *in vitro*[21,133,186,295,304,363] and *in vivo*.[198,313] Even for abasic sites, however, it is a preference rather than an absolute specificity. In a related reaction in which DNA polymerases were found to catalyze the end addition of nucleotides to duplex blunt ends, there was a preference for the addition of dAMP, even when there was no dTMP residue on the template strand of the duplex.[67] The basis for this tendency is not clear. A recent NMR study demonstrated that both adenine and guanine would fit into a double helix opposite an abasic site without distortion. However, therefore, the adenine-containing duplex was more stable, suggesting a thermodynamic basis for the adenine preference.[79] The rule may hold for truly noninformative lesions, but it appears that such lesions are rare. In most cases, the DNA polymerase can extract some coding information from a large diversity of DNA lesions. A striking example is the mutagenic specificity of the 6–4 TT adduct in ssDNA *in vivo*[201]: 85% of the progeny resulted in from incorporation of a G opposite the 3′ T, in violation of the A rule. This suggests that the polymerase is using information provided by the 6–4 adduct, and the same may be true for many other DNA lesions ([343,382] and references cited therein).

b. Role of the 3′ → 5′ Exonuclease

The extensive turnover observed at DNA lesions led to the suggestion that inhibition of the proofreading exonuclease activity of the polymerase under SOS conditions is the basis of UV mutagenesis.[380] The rationale for this suggestion was straightforward: a nucleotide incorporated opposite a lesion by the polymerase will be identified by its editing 3′ → 5′ exonuclease as a "mismatch" and will be excised rapidly. Inhibition of this editing activity was expected to allow bypass synthesis. This hypothesis is based on the assumption that it is the exonuclease activity that limits bypass, whereas other factors such as a slow extension step and the residence time of the polymerase on DNA were not taken into account.

In a direct test of the role of the 3′ → 5′ exonuclease activity in bypass synthesis, the replication with pol III holoenzyme of SSB-coated UV-irradiated ssDNA was studied under conditions in which the exonuclease activity of the polymerase was inhibited. Inhibition was achieved by four different methods[213,340]: (1) the use of dGMP, a competitive inhibitor of the exonuclease activity; (2) the addition of RecA protein that was shown to inhibit the exonuclease activity of pol III; (3) the use of a mutant DNA polymerase III prepared from a *mutD5* strain that is deficient in the exonuclease activity; and (4) the use of dTNPαS base analogs, which are efficiently polymerized into DNA but are excised very poorly by the exonucleolytic activity of pol III holoenzyme.[340] Under all conditions examined, inhibition of the 3′ → 5′ exonuclease activity of the polymerase was not sufficient to increase bypass synthesis. A similar conclusion was reached based on *in vivo* results with an *S. typhimurium* mutant that carried a deletion in *dnaQ*.[345] When the infectivity of UV-irradiated ϕX174 ssDNA phage was assayed in these cells, their survival was the same as in wild-type cells. This was interpreted to mean that the elimination of 3′ → 5′ exonuclease activity was not sufficient to enable replicative bypass of lesions. Consistent with this conclusion, *E. coli* cells carrying a *mutD5* mutation, which reduced the 3′ → 5′ exonuclease activity of pol III, did not show any enhancement of UV mutagenesis.[410]

It was shown recently that overproduction of the ε subunit of pol III reduced UV mutagenesis,[109,157] an observation taken to support the importance of inhibition of exonuclease activity for lesion bypass and UV mutagenesis. This, however, does not prove the point because it addressed a situation in which the ε subunit was likely present in large excess over the polymerase (the polymerase is present in no more than 10 to 20 copies per cell). Under these conditions, ε was likely to act uncoupled from the polymerase and might have interfered with the normal mutagenic bypass.[109]

Although inhibiting the 3′ → 5′ exonuclease activity of pol III holoenzyme is not sufficient to allow bypass of UV lesions or UV mutagenesis, it may still be necessary along with other changes in the polymerase properties for bypass synthesis. An alternative to inhibition of the exonuclease activity was the suggestion that the ε subunit is excluded from the polymerase complex that carries out the bypass reaction.[286,410]

c. The Extension Step

Regardless of the existence of a 3′ → 5′ exonuclease activity, the major kinetic barrier to polymerization for a variety of DNA lesions is the past-lesion extension step.[133,291,337,340,352] The extension step may be extremely slow because of the frayed nature of the base pair containing the lesion, the result of its great instability, as well as the instability of the polymerase-dNTP-DNA complex at the extension step. Indeed, a similarly slow extension step was found when the polymerase attempted to extend a mismatched base pair[281] where no base damage was involved. Not only might the first extension event be slow; it was found that at least five nucleotides must follow a mismatched base pair until the normal elongation rate is reached.[296] Increasing the concentration of dNTPs was found to increase the extent of bypass of a single TT cyclobutane dimer on a synthetic oligonucleotide by pol I, presumably by facilitating the extension step.[367]

d. Dissociation of the Polymerase

The presence of a lesion at the primer-template junction might destabilize the binding of the polymerase to the DNA, leading to rapid dissociation. Because the polymerase obviously needs to be on the DNA for bypass synthesis, the residence time on DNA at the damaged site is an important factor to consider. A long residence time will enable bypass even if the extension step is slow, whereas a very short residence may prevent bypass even when the extension step is fast. The analysis of dissociation kinetics from mismatches revealed that pol I dissociated from a mismatch much faster ($k = 3\ s^{-1}$) than from a correct base pair ($k = 0.2\ s^{-1}$).[181] Interestingly, the dissociation of T7 DNA polymerase from a mismatch ($k = 0.4\ s^{-1}$) was similar to that from a correct base pair ($k = 0.2\ s^{-1}$),[405] illustrating the different behavior of polymerases as they encoun-

ter an irregular primer-template. The residence time of the polymerase on DNA at lesion-blocked primer-templates has yet to be determined. It has been shown, however, that the stable complex that pol III holoenzyme forms at a regular primer-template site (residence time >90 min;[250]) is greatly destabilized by the presence of a lesion at that site, leading to dissociation of the polymerase.[337,340] Using a synthetic oligonucleotide carrying a single TT *cis-syn* cyclobutane photodimer, it was shown recently that bypass of the photodimer by pol I was dependent on the concentration of the enzyme.[367] This further supports the notion that binding affinity of the polymerase is an important factor in bypass synthesis.

The residence time at a lesion may modulate the effects of other factors on bypass synthesis. For a DNA polymerase that has a long residence time at a lesion, inhibition of the exonuclease activity may increase bypass by providing a better chance of extending the nucleotide opposite the lesion before dissociation. This may be the case for T7 DNA polymerase, whose exonuclease-deficient derivative, Sequenase, was found to bypass DNA lesions more efficiently than the wild-type enzyme.[354] An opposite example is pol III holoenzyme: one of the reasons that inhibition of its 3′ → 5′ exonuclease activity did not affect bypass synthesis may be the short residence time of this polymerase at UV lesions.[214,340]

e. Molecular Sensor Mechanisms

Why does the polymerase dissociate on encounter with a lesion? It could have been sitting on the DNA waiting for rescue or trying repeatedly to achieve a successful bypass event. This question is particularly valid for highly processive DNA polymerases such as pol III holoenzyme, T7 DNA polymerase, or T4 DNA polymerase holoenzyme. A possible answer to this question is that it may be beneficial to the cell to be able to regulate the bypass of lesions, switching between termination and bypass modes depending on the situation. As it first encounters a lesion, it is best for the replisome to stop, and allow time for a cleanup by DNA repair. It was thus suggested that the polymerase is equipped with a molecular sensor mechanism that promotes dissociation on encounter with a lesion.[331] As the polymerase replicates the DNA, it constantly probes the DNA structure. Any perturbation from an ordinary primer-template is read as a lesion and promotes dissociation.[331] The β subunit that tethers the polymerase to the DNA by forming a DNA sliding clamp might be the sensor. Thus, the prerequisite for a proper activity of the sliding clamp may be an ordinary primer-template terminus. A deformation or perturbation at this site may cause release of the clamp or weakening of its interaction with the polymerase, causing termination of elongation.[331] Alternatively, another subunit may serve as a sensor and transmit the signal to the β subunit to release the polymerase from DNA. Such a negative role for the β subunit in UV mutagenesis was supported by *in vivo* experiments that showed that overproduction of the β subunit caused a decrease in chromosomal UV mutagenesis. The explanation suggested for this effect was the sequestering of the polymerase in the sensor mode, programmed to stop at DNA lesions, thus preventing the formation of the bypass form capable of bypass synthesis.[361]

f. Accessory Proteins

These are defined as proteins that are not part of the polymerase but modify its activity by protein-protein interactions and/or by interacting with the DNA template. Such accessory proteins are essential for the full activity of replicative polymerases and may alter the properties of nonreplicative polymerases. The effect by accessory proteins is exemplified by SSB, which was found to be required for replicative bypass of UV lesion by pol III holoenzyme.[213] This could have resulted from making the damaged template more readily readable by the polymerase (i.e., stimulation of the extension step), or allowing longer residence time of the polymerase at the lesion. This effect of SSB could not have been detected using synthetic oligonucleotides, which are usually too short to allow proper binding of SSB. The same may be true for other accessory proteins, and long DNA templates may be needed to explore their effects.

Different DNA polymerases may be affected in different ways by accessory proteins, as exemplified by the effect of SSB. Bypass synthesis by pol III holoenzyme during replication of UV-irradiated ssDNA required SSB,[213] bypass of acetylaminofluorene adducts by T7 DNA polymerase was unaffected by SSB, whereas bypass of the same lesion by pol I was inhibited by SSB.[255] SSB also stimulated bypass of an abasic site by pol II.[29]

g. Elongation and Initiation Modes of Bypass

When considering the interaction of the polymerase with DNA at a lesion-blocked primer-template, at least two states of the polymerase should be considered: (1) the elongation mode for the polymerase encountering the lesion during normal processive polymerization and (2) the initiation mode for the polymerase trying to initiate polymerization at a primer terminus located at a lesion (Figure 6). The conformation and the properties of the polymerase in the initiation or elongation modes might be different, thus dictating different behavior at the lesion. Indeed, it has been shown that under elongation conditions, pol III holoenzyme in the presence of SSB is capable of bypass of pyrimidine photodimers[213,214] and AP sites,[133] whereas it is unable to do so under initiation condition, that is, when the first nucleotide to be polymerized should be opposite a lesion.[337,340] The polymerase could bind to the lesion blocked-primer template and perform cycles of polymerization-excision opposite the lesion, but it easily dissociated from the DNA. It was suggested that a major difference between the polymerase encountering a lesion in the elongation or initiation modes is the residence time on DNA. Because of the high processivity of the polymerase in the elongation mode reflecting extremely tight binding to DNA, the residence time of the polymerase at a lesion is high enough to enable a limited extent of bypass. Once dissociated from DNA, however, a subsequent bypass attempt implies binding of the polymerase in the initiation mode. In this situation, the residence time at a lesion is so short that the polymerase dissociates before ever being able to complete the slow extension step.[216,337,339,340] This is most likely due to the

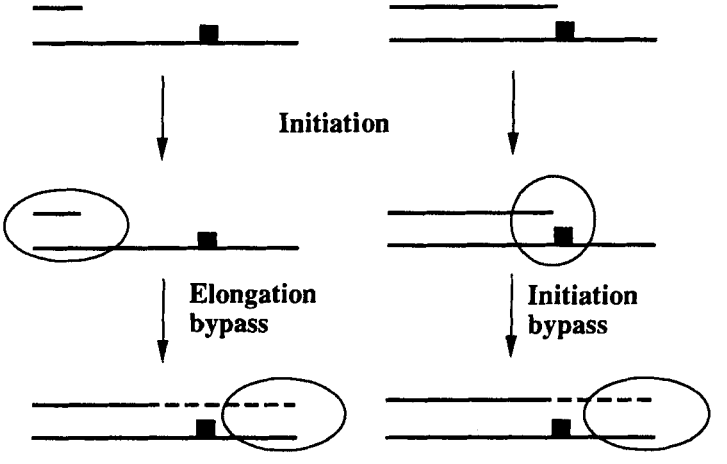

FIGURE 6. Elongation and initiation modes of bypass of lesions by DNA polymerase. When the polymerase initiates polymerization at a nondamaged primer-template upstream to the lesion, it encounters the lesion while it is in the elongation conformation (shown as an ellipse). When the primer is located at a template lesion, the polymerase attempts bypass while in the initiation conformation (shown as a circle).

inability of the polymerase to form a stable complex with the distorted primer-template at the site of the lesion. Such a complex can be stabilized by auxiliary proteins; however, another possible mechanism is the 3′ → 5′ exonucleolytic degradation of the primer terminus, for example, by the polymerase itself. This backward activity of the polymerase situates the primer terminus of the nascent DNA strand back in an undamaged region of the DNA and, thus, can enable the reformation of a stable polymerase-DNA initiation complex and a second bypass attempt.[340] Another source of difference between the two modes of bypass may stem from a difference in the polymerization-rate constants for the first nucleotide to be polymerized after binding of the polymerase to DNA, as compared with subsequent polymerization steps.

h. Effect of DNA Structure

The DNA structure affects bypass synthesis at at least three levels: the chemical structure of the lesion, its DNA sequence context, and higher DNA structure. Different DNA lesions are bypassed to different extents, depending on the effect of the particular lesion on the kinetic steps involved in bypass synthesis. This may be influenced by the DNA sequence near the lesion, as illustrated by the case of thymine glycol. Bypass synthesis of this lesion on a short oligonucleotide by the Klenow fragment of pol I was greatest with cytosine and guanine and least with adenine located on the template 5′ to the lesion.[68] Another aspect of sequence context effects is provided by the presence of sequences in DNA that cause pausing of DNA polymerases.[17,135,160,190,384] Imagine a lesion formed in such a pause sequence. It is likely to facilitate dissociation of the polymerase from DNA at that site and may, thus, affect bypass synthesis. The importance of the higher order structure of DNA is illustrated for the case of psoralen-modified DNA. It was shown that pol I could bypass psoralen monoadducts during nick translation on duplex DNA, but it failed to bypass the same DNA lesions on ssDNA.[287]

i. Fidelity of DNA Polymerases

Intuitively, it may be thought that reduced fidelity of a DNA polymerase will increase bypass synthesis. However, this is not necessarily the case. Although the bypass synthesis capability of a DNA polymerase and its fidelity are interrelated, they are two distinct properties, defined for two different situations. Fidelity of a DNA polymerase is defined by the error frequency of polymerization on an *undamaged* DNA substrate, whereas bypass synthesis involves the replication of damaged nucleotides. Thus, fidelity mechanisms are designed to address 12 specific mismatches at the primer-template site during polymerization, whereas bypass synthesis must be able to cope with the enormous diversity of DNA lesions. In some cases, reduced polymerization fidelity can be correlated with increased bypass synthesis, as exemplified by the manganese cation. This ion, which is known to decrease the fidelity of DNA polymerases,[129,169,377] was also found to stimulate bypass synthesis.[290] On the other hand, other treatments that reduce fidelity such as inhibition of the proofreading exonuclease activity do not necessarily affect bypass of lesions.

j. Summary of Mechanistic Bypass Considerations

In general, DNA polymerases can bypass DNA lesions. Bypass synthesis shows great variation, however, depending on the identity of the polymerase, inhibition of the proofreading 3′ → 5′ exonuclease activity, the chemical identity of the lesion, its DNA sequence context, the higher structure of the DNA, and the activity of accessory proteins.

The length of the residence time that is sufficient to allow bypass synthesis depends on the kinetic rates of addition, excision, and extension by the polymerase at the lesion. The addition step seems to be relatively easy for most polymerases, whereas the extension step is a major obstacle in bypass, because, generally, it is extremely slow. Inhibition of the exonuclease activity might help because it will provide a better

chance to carry out the slow extension step. This may be particularly true for polymerases that do not easily dissociate from DNA on encountering DNA lesions but may make no difference for polymerases that rapidly dissociate under such conditions.

DNA polymerases can face elongation bypass or initiation bypass, situations that may result in a different behavior at a lesion. In both cases, the residence time on DNA is crucial. For elongation bypass, the processivity of the polymerase may be a good measure for bypass, predicting that highly processive polymerases might have a better chance for elongation bypass. In initiation bypass, the processivity may be irrelevant because there may be no way to assemble the polymerase in a stable "processive" mode at a lesion. In such cases, bypass will be much lower in the initiation mode.

An additional factor that can influence bypass synthesis is the molecular-sensing mechanism that some DNA polymerases may possess. When operative, such molecular sensor may be the dominant factor and cause termination of polymerization at DNA lesions.

The fidelity of DNA polymerases and their bypass capabilities are interrelated. Some factors that decrease the fidelity of DNA polymerases such as manganese ions were found to increase bypass synthesis. However, the exact relationship between fidelity of a DNA polymerase and its bypass properties is not fully understood.

Can our current knowledge on bypass synthesis by purified DNA polymerases be used to predict what is required of SOS-inducible factors to allow bypass synthesis *in vitro*? The two major factors that limit bypass synthesis by pol III are the slow past-lesion extension step and the rapid dissociation from DNA at a lesion. Thus, the role of the SOS-inducible factors may be to stimulate the extension step and/or stabilize the polymerase-DNA complex at a lesion to decrease the dissociation rate. In addition, inhibition of the $3' \rightarrow 5'$ exonuclease activity of the polymerase may be necessary. The recent report that bypass of an abasic site by pol III holoenzyme was stimulated by adding RecA, UmuD′, and UmuC[294] provides an experimental system to test these predictions.

4. The Two-Step Model of UV Mutagenesis

When cells were subjected to photoreactivation immediately after UV irradiation, most of the UV mutations were eliminated. However, UV mutations were observed if a post-UV incubation period was added before photoreactivation (delayed photoreactivation). Under these conditions, UV mutagenesis did not require the RecA or Umu proteins. These findings led to the two-step model of UV mutagenesis, according to which bypass synthesis, assumed to be responsible for UV mutagenesis, can be separated into two steps: (1) a misincorporation or addition step in which a nucleotide is polymerized opposite the lesion and (2) an extension step in which the added nucleotide is extended. According to this model, the misincorporation step does not require RecA and UmuC and is presumably carried out by pol III. The subsequent extension step requires both RecA and UmuC. It was further suggested that the delayed photoreactivation converted the cyclobutane dimers into the normal bases *after* the misincorporation step has occurred but before completion of bypass. This enabled the extension step to proceed freely without the need for UmuD/C or RecA because there was no lesion to bypass.[34,36,43]

Assuming that UV mutagenesis involves bypass synthesis, then, in a sense, this model may be correct regardless of whether it explains the delayed photoreactivation phenomenon. The kinetic analysis of bypass synthesis distinguishes between the addition (misincorporation) and the extension steps. The *in vitro* data on the idling-turnover of pol I and pol III argue strongly that the addition step occurs without RecA or UmuD/C and that the problematic step is the extension step. Thus, it is likely that the activity of SOS proteins (UmuD/C? RecA?) is required for this step by either stimulating it directly and/or by increasing the residence time of the polymerase on the DNA.[216,339] An intriguing point in the two-stage model is the stability of the nucleotide added both opposite the putative photodimer and opposite a template pyrimidine after the photoreactivation treatment. Based on *in vitro* studies, such a nucleotide is likely to be rapidly excised by the $3' \rightarrow 5'$ exonu-

clease activity of polymerases or by other exonucleases present in the cell. Therefore, if it is stable *in vivo* long enough to survive the delayed photoreactivation experimental protocol, it must be protected in some way from the activity of nucleases. As discussed previously, the two-stage model was recently challenged by the suggestion that deamination of cytosine in photodimers and 6–4 adducts can explain delayed photoreactivation.[370,371] The importance of deamination in UV mutagenesis is not clear at this point; however, it is unlikely to account for all UV mutagenesis.[36]

5. An In Vitro System for UV Mutagenesis

The development of *in vitro* systems that mimic *in vivo* processes is invaluable for the elucidation of the molecular mechanisms of many processes, including DNA replication, repair, transcription, and translation. It paves the way to partial or full reconstitution of the reaction with purified components, thus enabling a detailed biochemical analysis of the process. Only recently was such a system developed for UV mutagenesis.[71] It consists of two stages: (1) an *in vitro* stage that involves incubation of a UV-irradiated plasmid carrying the *cro* gene with a soluble protein extract prepared from *E. coli* cells and (2) detection of mutations produced in the *in vitro* stage in the *cro* gene by a subsequent bioassay step,[71] based on the *cro* mutagenesis assay system.[344] This stage involves transformation of an indicator strain with plasmid DNA that was isolated from the reaction mixture, followed by scoring mutant *cro* colonies on indicator plates. The indicator strain is unable to carry out UV mutagenesis by itself because of a Δ*recA* mutation.

This assay system enabled the identification of two UV mutagenesis pathways: (1) a pathway termed "type I UV mutagenesis" that depends on DNA replication and requires the *recA* and *umuC* gene products and (2) a second pathway termed "type II UV mutagenesis" that depends on DNA excision-repair genes but not on DNA replication. DNA sequence analysis of the mutations that were produced *in vitro* by type II UV mutagenesis revealed a spectrum similar to that of *in vivo* UV mutagenesis.[71] The identification of these two pathways of mutagenesis *in vitro* supports previous suggestions for their existence based on *in vivo* studies. Moreover, it provides direct evidence for the dependence of one of these pathways, on the UvrA, UvrB, and UvrC proteins. The Uvr-dependent UV mutagenesis may occur at sites of two closely opposed UV lesions, as discussed earlier. The excision of one of these lesions by the UvrABC nuclease, possibly enlarged by exonuclease and/or helicase activities, will leave a ssDNA gap in the DNA with a nonrepairable lesion on it. The mutation may occur on the filling in of this gap by a specialized form of pol III, an event that may be appropriately termed "error-prone repair". The advantage of such an *in vitro* system is that it assays mutations directly, without a need to assume any particular mechanism. This system as well as other systems developed in the future along similar lines are an important and powerful research direction in the efforts to elucidate the mechanism of UV mutagenesis.

D. Strand Specificity of UV Mutagenesis

In recent years, it has become clear that in many cases, DNA repair occurs preferentially in transcribed genes.[26,130] Moreover, the repair in the transcribed gene occurs preferentially on the transcribed strand, leaving the UV lesions on the nontranscribed strand. This was first discovered in mammalian cells, but was shown to occur also in *E. coli*.[253] The targeting of DNA repair to the transcribed strand is mediated via a specific protein, the transcription-repair coupling factor (TRCF;[325]). *In vitro*, in the absence of TRCF, RNA polymerase is stuck at UV lesions and interferes with excision repair, leading to preferential repair of the nontranscribed strand. Based on genetic experiments, it was suggested that the phenomenon of mutation-frequency decline described earlier, involves the preferential excision repair of premutational lesions on the transcribed strand of DNA.[25] This prediction seems rather accurate: extracts prepared from an *mfd* strain were found to be deficient in strand-specific excision repair, and this activity was restored on addition of puri-

fied TRCF, indicating the *mfd* gene encodes TRCF.[326] A direct correlation between the activity of the *mfd* gene product and the strandness of excision repair was recently demonstrated *in vivo* in the *lacI* gene.[184] The asymmetry in repair is readily manifested in a similar asymmetry in UV mutability of the two DNA strands. Indeed, most mutations in the *lacI* gene could be ascribed to UV lesions present on the nontranscribed strand.[170,184,279] In contrast, in an *mfd* strain, mutations arose predominantly from lesions on the transcribed strand.[279] In *uvr* strains where there is no excision repair, mutations were distributed over both strands.[170]

E. Untargeted Mutagenesis

Untargeted mutagenesis is defined as the appearance of mutations at undamaged sites because of a mutagenic treatment such as UV irradiation.[404] It is most clearly demonstrated by the elevated mutation frequency of unirradiated phage DNA observed on infection of UV-irradiated *E. coli* cells.[148] It was estimated that untargeted mutagenesis accounts for up to 10% of the total number of UV mutations in *E. coli*.[66,187] In *mutH, mutL,* or *mutS* mutants, which are deficient in mismatch repair, untargeted mutagenesis was higher than in wild-type cells.[59] This raised the possibility that untargeted mutagenesis initially produces a large number of mismatches, most of which are corrected by the methylation-directed generalized mismatch repair system.[69,262]

The most commonly used experimental systems for studying untargeted mutagenesis are the infection of irradiated host cells with nonirradiated phage and the transfer by conjugation of an F′ episome from a nonirradiated donor to an irradiated acceptor. Using such systems, some significant differences between targeted and untargeted mutagenesis were found. Unlike targeted mutagenesis, untargeted mutagenesis of dsDNA is independent of the *umuC* gene product.[225,406] This finding is consistent with the notion that the Umu proteins are involved in bypass of DNA lesions, which are presumably not involved in untargeted mutagenesis. Surprisingly, however, UmuC is required for untargeted mutagenesis of ssDNA.[226]

Both targeted and untargeted mutagenesis share the requirement for the *recA* gene product. Unlike targeted mutagenesis, however, in untargeted mutagenesis the role of RecA is limited to the derepression of the SOS regulon. Irradiation of *lexA*(Def)Δ*recA* cells in which the SOS genes were constitutively expressed but that lacked the *recA* gene, yielded similar levels of untargeted mutagenesis as in wild-type cells.[45,406] It is interesting to note that although untargeted mutagenesis is an SOS function, derepression of the SOS regulon is not sufficient for generating untargeted mutations and UV irradiation is still required. This may indicate the requirement for the induction of a factor not solely controlled by LexA and RecA.[406] Another possibility is that irradiation is required for the activation of an SOS controlled factor.

Untargeted, but not targeted, mutagenesis of dsDNA requires pol I[148,226] and the Uvr excision-repair proteins (but not for ssDNA;[226]). It is not clear whether the involvement of pol I is direct or a result of the inability of irradiated *polA* mutants to support phage growth used to assay untargeted mutagenesis.[148] Interestingly, untargeted mutagenesis does not occur in cells carrying a *dinB* mutation.[45] The *dinB* gene encodes a DNA damage-inducible protein whose function is still unknown.[166]

The mutational spectrum of untargeted mutagenesis differs from that of targeted mutagenesis. In a study assaying mutations in the phage λ *cI* gene, 75% of the untargeted mutations were found to be frameshifts,[406] whereas base substitutions in the same system dominated the spectrum of UV-targeted mutations.[408] The frameshift mutations were concentrated in runs of identical bases, suggesting that they were generated by a "slippage" of the replication apparatus.[355]

The increase in untargeted mutations following UV irradiation has lead to the suggestion that they are produced by a DNA polymerase with reduced fidelity. This is consistent with the involvement in untargeted mutagenesis of pol III, suggested by genetic studies using various *polC*-mutator mutants.[46] According to one model, the polymerase complex that bypasses DNA lesions has a reduced fidelity on undamaged DNA. This model depicts untargeted mutagenesis as a

byproduct of targeted mutagenesis. An alternative explanation is the induction of a specialized low-fidelity polymerase that operates under SOS conditions. It may be related to pol I*, a low-fidelity form of pol I isolated from SOS-induced cells.[188,189]

One cannot exclude the possibility that untargeted mutagenesis occurs at cryptic lesions that are formed in DNA under normal physiological conditions. These lesions may not be effective as inducers of the SOS response but may be mutagenic once the SOS response is induced by external agents. If such is the case, the term "untargeted" is inadequate.

IV. FUTURE DIRECTIONS

The data discussed earlier provide substantial support for the notion that bypass synthesis of UV lesions is the major mechanism of UV mutagenesis. However, significant parts of the overall picture are still missing.

UV light produces a multiplicity of products in DNA, of which only a few have been chemically identified. This is particularly true for long-wavelength UV radiation. This UV light is more relevant to the biological effects of sunlight than short-wavelength UV radiation, widely used in past and current studies on UV mutagenesis. It is important to determine the chemical structure of new DNA photoproducts, including those arising by photosensitization of UV-absorbing metabolites in the cell. This, combined with the powerful methodology of engineering of specific lesions at specific sites in DNA, will enable a better assignment in the future of the mutational specificity of individual DNA lesions. The full understanding and prediction of mutational spectra produced by UV radiation in a given gene is a difficult task. Besides a comprehensive knowledge on the types of lesions produced in DNA, it will require a detailed understanding of the factors that determine their distribution in DNA and the efficiency of their removal by DNA repair mechanisms.

The effects of DNA damage on DNA replication and transcription are poorly understood. This calls for extensive genetic, biochemical, and enzymological studies of these mechanisms in *E. coli* to serve as convenient model systems for higher organisms. Similarly to *E. coli,* in eukaryotes, the inhibitory effects of DNA-damaging agents on DNA replication and cell division were suggested to allow optimal repair of damage before the cell reinitiates replicative DNA synthesis in the S phase (G1 arrest) and/or begins mitosis (G2 arrest). In the yeast *Saccharomyces cerevisiae,* a specific gene *RAD9* was shown to control the cell-cycle response to DNA damage. It seems that the RAD9-dependent response detects potentially lethal DNA damage and causes arrest of cells in G2 until such damage is repaired.[131,385] It was recently suggested that the p53 tumor suppressor, the most commonly mutated gene in human cancers, may play a role in the G1 arrest that occurs after treatments with DNA-damaging agents.[161,183] These studies illustrate the importance in carcinogenesis of the responses of DNA replication to DNA damage and will hopefully stimulate the study of these topics in *E. coli.*

Whereas genetic and biological studies are the scouts of mechanistic research and provide initial information, the elucidation or the confirmation of molecular mechanisms cannot proceed without a detailed biochemical analysis. The field of UV mutagenesis has reached a stage where many more biochemical studies are essential to reach an insight into molecular mechanisms. The recent development of the cell-free system for UV mutagenesis is a step in this direction. In parallel, experiments with purified proteins that are related to the mutagenic reaction are equally important. Induced mutagenesis is an interdisciplinary research field, intimately connected to DNA structure, DNA replication, repair, recombination, and transcription. The understanding of mutagenic mechanisms in their true cellular context requires the use of complex multiprotein systems. From this standpoint, the advanced state of the enzymology of replication, repair and recombination is likely to ease the establishment of such multiprotein assay systems.

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