Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 1376–1380, February 1996
Biochemistry

Reconstitution of repair-gap UV mutagenesis with purified proteins from *Escherichia coli*: A role for DNA polymerases III and II

(DNA repair/excision repair/error-prone repair/carcinogenesis)

GUY TOMER*, ORNA COHEN-FIX*, MICHAEL O'DONNELL†, MYRON GOODMAN‡, AND ZVI LIVNEH*§

*Department of Biochemistry, Weizmann Institute of Science, Rehovot 76100, Israel; †Department of Microbiology, Cornell University Medical College, New York, NY 10021; and ‡Department of Molecular Biology, University of Southern California, Los Angeles, CA 90089

Communicated by I. Robert Lehman, Stanford University Medical Center, Stanford, CA, October 26, 1995

ABSTRACT **Using a cell-free system for UV mutagenesis, we have previously demonstrated the existence of a mutagenic pathway associated with nucleotide-excision repair gaps. Here, we report that this pathway can be reconstituted by using six purified proteins: UvrA, UvrB, UvrC, DNA helicase II, DNA polymerase III core, and DNA ligase. This establishes the minimal requirements for repair-gap UV mutagenesis. DNA polymerase II could replace DNA polymerase III, although less effectively, whereas DNA polymerase I, the major repair polymerase, could not. DNA sequence analysis of mutations generated in the *in vitro* reaction revealed a spectrum typical of mutations targeted to UV lesions. These observations suggest that repair-gap UV mutagenesis is performed by DNA polymerase III, and to a lesser extent by DNA polymerase II, by filling-in of a rare class of excision gaps that contain UV lesions.**

DNA repair is the major defense mechanism of cells against DNA damage and its deleterious effects, primarily cancer (1, 2). A major repair mechanism both in prokaryotes and in eukaryotes is nucleotide excision repair (3). In the bacterium *Escherichia coli*, this process is initiated by the UvrABC repair nuclease that incises the DNA at both sides of the lesion, at a distance of 12 or 13 nucleotides, followed by removal of the damaged oligonucleotide by DNA helicase II (4, 5). The excision gap is filled-in by DNA polymerase I (Pol I), followed by ligation by DNA ligase. This process is error free and leads to restoration of the integrity of the genetic information.

Unrepaired DNA lesions are frequently processed into mutations by genetically regulated pathways. The classical example of this type of active mutagenesis is UV mutagenesis in *E. coli* (also termed error-prone repair, or SOS mutagenesis), which depends on the UV-inducible genes *recA*, *umuD*, and *umuC* that belong to the SOS stress regulon (reviewed in refs. 6 and 7). Similar mechanisms are found in eukaryotes (1, 8, 9). UV mutagenesis is believed to occur opposite a UV lesion located in a segment of single-stranded DNA (ssDNA). Such premutagenic sites can be generated by an interruption of DNA replication at the lesion (7, 10) or by nucleotide excision repair of closely opposed UV lesions (11, 12), where the removal of a UV lesion from one strand results in a ssDNA excision gap containing the second UV lesion. The key step in UV mutagenesis is thought to be a translesion DNA synthesis reaction (also termed bypass synthesis), in which an incorrect nucleotide is incorporated opposite the lesion (6, 7, 13, 14).

With the goal of elucidating the molecular mechanism of UV mutagenesis, we have recently established a cell-free assay system for UV mutagenesis using protein extracts prepared from *E. coli* cells (15). Using this assay system, we have identified two pathways: type I, or replicative UV mutagenesis, that depended on DNA replication, and type II, or repair-gap UV mutagenesis, that depended on nucleotide excision repair (15, 16). Here, we report the reconstitution and characterization of repair-gap UV mutagenesis using purified components.

MATERIALS AND METHODS

Materials. The sources of materials were as follows: unlabeled dNTPs and creatine phosphate, Boehringer Mannheim; [α-^{32}P]dNTPs (400 Ci/mmol; 1 Ci = 37 GBq), Amersham; eosin yellow and methylene blue, Riedel de Haen; and bacteriological media, Difco. Plasmid pOC2 is a 5.0-kb pBR322 derivative carrying the *cro*, *kan*, and *bla* genes (15). It was UV irradiated (254 nm) as described (17) at doses of 100, 200, or 400 J·m^{-2} to produce 5, 10, or 20 photodimers per molecule, respectively. UvrA (fraction IV, 0.33 mg/ml), UvrB (fraction V, 0.21 mg/ml), and UvrC (fraction IV, 0.02 mg/ml) were purified as described (18, 19). UvrD (1.5 mg/ml) was kindly given to us by R. Bryant (Johns Hopkins University, Baltimore). DNA polymerase II (Pol II; fraction IV; 0.5 mg/ml) was purified as described (20). DNA polymerase III (Pol III) core (1 mg/ml) was prepared by reconstitution from the individual purified subunits (21). *E. coli* DNA photolyase (fraction IV, 1.0 mg/ml) was a gift from A. Sancar (University of North Carolina, Chapel Hill). Creatine kinase, RNase I, *E. coli* DNA ligase, T4 DNA ligase, and DNA polymerase I (Pol I) were from Boehringer Mannheim.

Excision Repair. The reaction mixture (25 μl) contained 40 mM Tris·HCl (pH 7.6); 85 mM KCl; 15 mM $MgCl_2$; 1 mM dithiothreitol; 1 mM EDTA; 50 μg of bovine serum albumin per ml; 2 mM ATP; 40 mM creatine phosphate; 0.4 mg of creatine kinase per ml; 100 μM each of dATP, dCTP, and dTTP; 10 μM [α-^{32}P]dGTP; 0.3 μg (90 fmol circles) of UV-irradiated plasmid pOC2; 12 nM each of UvrA, UvrB, and UvrC; 20 nM DNA helicase II (UvrD); 40 nM DNA polymerase; and 1 unit of *E. coli* or phage T4 DNA ligase. When the bacterial ligase was used, 50 μM NAD was added. The reaction components were mixed on ice and then incubated at 37°C for up to 1 h. The DNA was then deproteinized with proteinase K and fractionated on a neutral 0.8% agarose gel. The gel was then dried and scanned by using a Fuji BAS 1000 Bioimaging Analyzer.

***In Vitro* UV Mutagenesis Reaction.** The reaction mixture (75 μl) contained 40 mM Tris·HCl (pH 7.6); 85 mM KCl; 15 mM $MgCl_2$; 1 mM dithiothreitol; 1 mM EDTA; 50 μg of bovine serum albumin per ml; 2 mM ATP; 40 mM creatine phosphate; 0.4 mg of creatine kinase per ml; 100 μM each of dATP, dCTP, dGTP, and dTTP; 0.9 μg (270 fmol circles) of UV-irradiated plasmid pOC2; 12 nM each of UvrA, UvrB, and UvrC; 20 nM DNA helicase II (UvrD); 40 nM DNA polymerase; and 1 unit

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§To whom reprint requests should be addressed.

of *E. coli* or phage T4 DNA ligase. When the bacterial ligase was used, 50 μM NAD was added. The reaction components were mixed on ice and then incubated at 37°C for up to 1 h. The reaction was stopped by adding EDTA and SDS to final concentrations of 20 mM and 1%, respectively. Under these conditions deamination of cytosine-containing cyclobutyl dimers occurs with a half-life of nearly 10 h (22) and thus does not affect the mutagenesis results. Cro$^-$ mutations generated during the *in vitro* reaction were detected as previously described (15, 16) by transforming an indicator strain which gives rise to dark red colonies when transformed with a Cro$^-$ plasmid and white colonies when transformed with a wild-type *cro* plasmid. A new indicator strain, *E. coli* WBY11T (22), was used that carried a Δ*umuDC595*::*cat* mutation (23) in addition to the Δ*recA* mutation. Each experiment was performed three times, and a typical experiment contained 1.5–3 × 10^5 transformants. Transformation frequency was calculated by dividing the number of mutants by the total number of transformants. Standard deviations of the mutation frequencies were ±15–20%.

DNA Sequence Analysis of Mutants. *In vitro* UV mutagenesis reactions were carried out with UV-irradiated plasmid pOC2 (400 J·m^{-2}), and the reaction products were bioassayed for Cro$^-$ mutations as described above. Plasmid DNA was extracted from mutant (dark red) colonies obtained from independent transformation reactions by using the Promega Wizard Miniprep DNA purification system. The sequence of the *cro* gene carried by these plasmids was determined in the Biological Services Unit in our institute by automated DNA sequence analysis using *Taq* DyeDeoxy Terminator Cycle Sequencing in an Applied Biosystems model 373 DNA sequencer.

RESULTS

Repair-Gap UV Mutagenesis Can Be Reconstituted with Six Purified Proteins. Using a cell-free assay system for UV mutagenesis, we have previously demonstrated the existence of a mutagenic pathway that depended on the excision repair proteins UvrA, UvrB, and UvrC. In an attempt to reconstitute this mutagenic pathway with purified components, we performed a series of experiments designed to establish its minimal requirements. The experimental methodology was essentially as described previously (15, 16), except that purified proteins were used. It involved an *in vitro* reaction of UV-irradiated plasmid pOC2 with purified proteins that acted to produce mutations in the *cro* reporter gene present in the plasmid. Following the *in vitro* reaction, the DNA was purified and assayed for the presence of Cro$^-$ mutations by transforming an *E. coli* Δ*recA* Δ*umuDC* indicator strain. This strain is defective in UV mutagenesis, but it detects preexisting mutations in *cro*. Prior to transformation, the plasmid was subjected to enzymatic photoreactivation with purified DNA photolyase to eliminate remaining UV dimers (24) and thus increase transformation efficiency (16).

The mutagenesis reaction mixture included UV-irradiated plasmid pOC2 carrying the *cro* reporter gene, purified UvrA, UvrB, and UvrC proteins that constitute the repair nuclease, DNA helicase II (the UvrD protein), a DNA polymerase, and DNA ligase. *E. coli* contains three DNA polymerases (25): Pol I is the major repair polymerase, Pol III in its holoenzyme form is the major replicative polymerase, whereas the role of Pol II is not clear, although it is likely to be involved in DNA repair. When Pol III core [the three-subunit catalytic core of Pol III holoenzyme (26)] was used in the reaction, Cro$^-$ mutations were produced in a time-dependent manner increasing by up to 40-fold, from a background of 2 × 10^{-5} up to 84 × 10^{-5} (Fig. 1). A similar result was obtained with Pol II, where mutation frequency increased 50-fold. In contrast, a very low mutation frequency was obtained with Pol I, the major repair polymerase (Fig. 1). The mutagenic reaction was dependent on the UvrA, UvrB, and UvrC proteins, on DNA polymerase, on UV-irradiation, on ATP, and on Mg^{2+} (Table 1). A modest mutagenic effect was observed in the absence of DNA helicase II (UvrD), suggesting that the DNA polymerases can displace the incised oligonucleotide. DNA ligase was not required (data not shown); presumably ligation can occur in the cell. The same requirements were observed for reactions with either Pol II or Pol III core (Table 1).

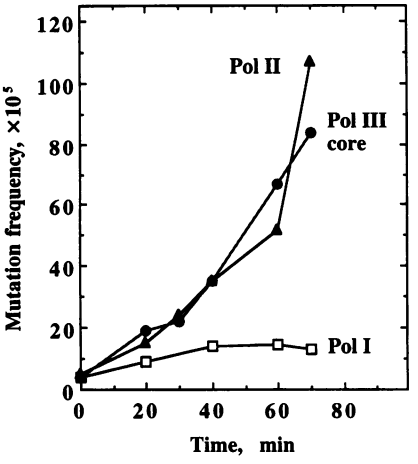

FIG. 1. Kinetics of *in vitro* reconstituted repair-gap UV mutagenesis. The *in vitro* reactions contained UV-irradiated plasmid pOC2 (400 J·m^{-2}), UvrA, UvrB, UvrC, DNA helicase II, DNA ligase, and Pol III core (closed circles), Pol II (closed triangles), or Pol I (open squares). Cro$^-$ mutations were assayed by transforming the indicator strain with the reaction products, as described in *Materials and Methods*.

The production of Cro$^-$ mutations in the reconstituted system was dependent on the UV dose applied to the plasmid (Fig. 2). Enzymatic photoreactivation of the UV-irradiated plasmid prior to incubation led to a 50% reduction in mutation frequency (Fig. 2). This was not due to incomplete photoreactivation since the photolyase removed >90% of the cyclobutyl dimers under our assay conditions (data not shown). This suggests that both cyclobutyl pyrimidine dimers and 6–4 pyrimidine–pyrimidone adducts give rise to mutations in the reconstituted system.

Activity of Pol I, II, and III Core in Repair Synthesis. The results presented above suggest that the mutagenic reaction occurs at excision repair gaps. To demonstrate that excision repair occurs under our reaction conditions, we assayed repair synthesis directly by including a radiolabeled dNTP. After incubation, the reaction mixture was deproteinized, analyzed

Table 1. Requirements of reconstituted repair gap UV mutagenesis

Reaction conditions	Mutation frequency, ×10^5	
	Pol II	Pol III core
Complete	64.6	76.7
Without UvrA, UvrB, and UvrC	4.6	5.2
Without DNA helicase II	15.9	20.7
Without DNA polymerase	12.6	12.6
Without ATP	6.2	8.4
Without Mg^{2+}	4.1	3.4
No UV lesions in DNA	2.9	1.9

UV-irradiated plasmid pOC2 (400 J·m^{-2}) was incubated with purified proteins at 37°C for 60 min as described in *Materials and Methods*. The requirements of the reaction were assayed by omitting the indicated components. The production of Cro$^-$ mutations was assayed in the indicator strain as described in *Materials and Methods*.

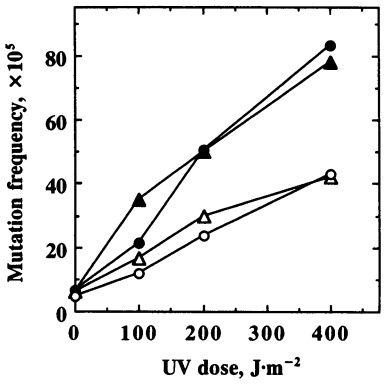

FIG. 2. UV dose dependence of repair-gap mutagenesis. The *in vitro* reaction was performed as described in the legend to Fig. 1, except that the plasmid was UV irradiated at the indicated doses (closed symbols). In parallel, the UV-irradiated plasmids were photoreactivated prior to the *in vitro* reaction (open symbols). Cro^- mutations were assayed by transforming the indicator strain, as described in *Materials and Methods*. Circles, reactions with Pol III core; triangles, reactions with Pol II.

by electrophoresis on nondenaturing agarose gels, and visualized in a PhosphorImager and by autoradiography. As can be seen in Fig. 3, all three DNA polymerases supported the incorporation of radiolabeled dNTPs into DNA. This incorporation was dependent on the presence of UV lesions in the plasmid and on the presence of UvrA in the reaction mixture (Fig. 3), implying that it resulted from nucleotide excision repair. When total incorporation into excision gaps was compared, Pol I and Pol II showed comparable efficiencies (Fig. 4*B*). Pol III core, on the other hand, showed an 80–90% slower synthesis rate (Fig. 4*B*). There was also a difference in the types of radiolabeled products obtained. Thus, with Pol I, most of the products were covalently closed (form IV), indicating a complete repair reaction including ligation (Fig. 4*A*). In contrast, with either Pol II or Pol III core, most of the radiolabeled products were nicked circles (Fig. 4*A*), suggesting that Pol II and Pol III do not complete the filling-in of excision gaps.

As expected, there was no correlation between repair synthesis, as assayed biochemically, and mutagenesis. This is because these two assays monitor different events; repair synthesis reflects primarily error-free excision repair, whereas the bioassay monitors mutagenic gap-filling, most likely in

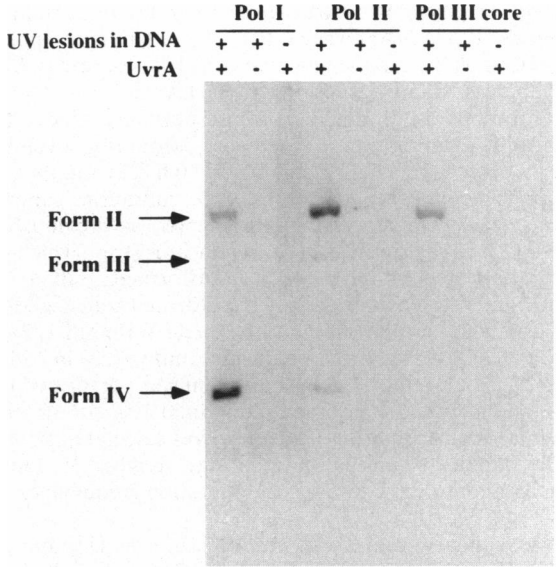

FIG. 3. Nucleotide excision repair in the reconstituted system. Reactions were carried out as described in the legend to Fig. 1, with plasmid pOC2 irradiated at 400 $J \cdot m^{-2}$, except that radiolabeled [α-^{32}P]dTTP was included. Following the reaction, the DNA was fractionated by electrophoresis on a nondenaturing 0.8% agarose gel, which was then dried, and the bands were visualized by autoradiography. Form II, nicked circular DNA; form III, linear DNA; form IV, covalently closed circular DNA.

scarce ssDNA gaps that contain UV lesions (see below). A better comparison of the mutagenic efficiencies of the polymerases is obtained by normalizing for the differences in their synthetic activities. When this is done (Fig. 5), Pol III core is clearly the most effective polymerase, being 26-fold more effective than Pol I, and 4-fold more effective than Pol II. Pol I is essentially inactive in promoting repair-gap UV mutagenesis.

Spectrum of UV Light Mutations in the Reconstituted System. The spectrum of mutations produced in DNA is characteristic to the damaging agent that induced them (27). We have examined the types of mutations produced in the reconstituted system by DNA sequence analysis of plasmids isolated from mutant colonies (Table 2). The major types of mutations produced in the Pol II-dependent reaction were G·C → A·T transitions (10/19; 53%) and −1 or +1 nt frameshifts

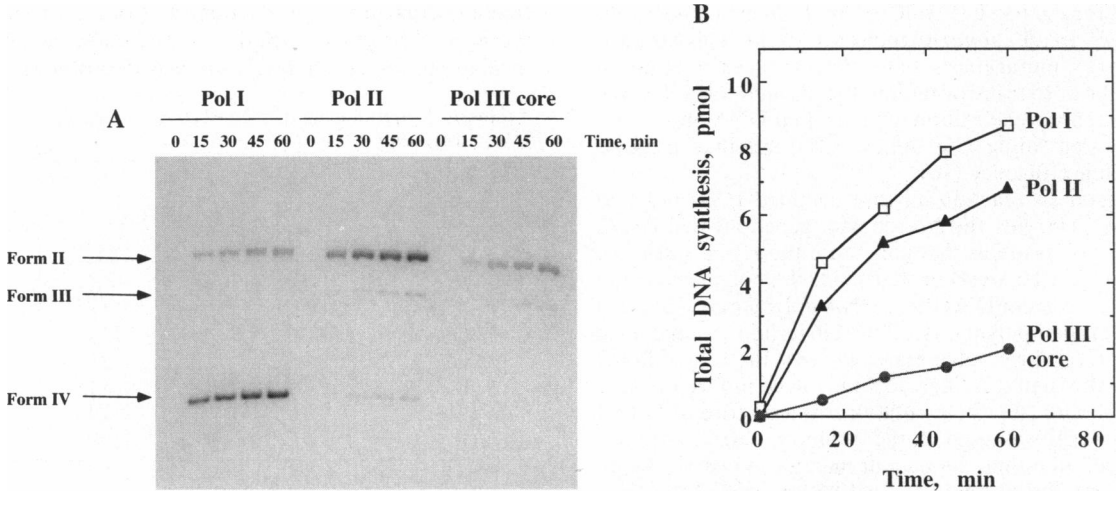

FIG. 4. Kinetics of excision repair synthesis in the reconstituted system. Reactions were carried out as described in the legend to Fig. 1, with plasmid pOC2 irradiated at 400 $J \cdot m^{-2}$, except that radiolabeled [α-^{32}P]dTTP was included. Reaction products were fractionated by electrophoresis on a nondenaturing 0.8% agarose gel, and the bands were visualized by autoradiography (*A*), and quantified in a PhosphorImager (*B*).

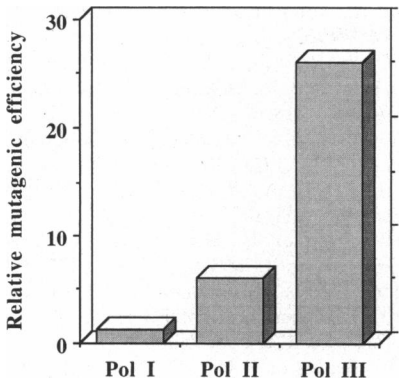

FIG. 5. Relative mutagenic efficiency of purified DNA polymerases in repair-gap UV mutagenesis. The mutagenic efficiency was calculated by dividing the frequency of UV mutations produced by a particular DNA polymerase by its repair synthesis activity. The values were taken from the 60-min time points in Figs. 1 and 4. The mutagenic efficiencies are given relative to that of Pol I, which was set to 1.

(5/19; 26%). Two of the 19 mutants (11%) were tandem double mutations at adjacent pyrimidines, a hallmark of *in vivo* UV light mutagenesis (28, 29). The data for Pol III core were generally similar and characteristic of the specificity of UV mutagenesis *in vivo*. Thus, under our assay conditions, there is no apparent difference in the specificity of mutations produced by Pol II or Pol III core.

DISCUSSION

Using a UV mutagenesis assay system consisting of a cell-free reaction coupled to bioassay, we previously demonstrated the existence of a UV mutagenesis pathway that was associated with nucleotide excision repair (15, 16). Here, we report that the full mutagenic reaction could be reconstituted by using UV-irradiated double-stranded DNA, the UvrA, UvrB, and UvrC proteins, DNA helicase II, DNA ligase, and DNA polymerase III core or, to a lesser degree, Pol II.

Pol I was ineffective in the mutagenic reaction, consistent with our previous results with the crude system (16) and with *in vivo* data showing that UV mutagenesis occurs in the absence of Pol I (30). The activity of Pol III core in the mutagenic reaction suggests that *in vivo* this three-subunit minimal subassembly of Pol III holoenzyme (26) may function also independently of the other seven subunits of the holoenzyme. It is consistent with the results from the crude system, where the α subunit of Pol III but not the processivity β subunit was required (16), and with *in vivo* data suggesting that Pol III is involved in UV mutagenesis (31). DNA polymerase II is SOS inducible (32–34). However, Δ*polB* mutants lacking the polymerase show no defect in UV repair or mutagenesis, and most other cellular functions seem to be unaffected (25, 35, 36). Thus, Pol II is not essential for UV repair or mutagenesis; however, this does not exclude the possibility that when present, it takes part in these processes. We have previously shown that Pol II was not required for repair-gap UV mutagenesis in a crude extract (16). This is consistent with the higher effectiveness of Pol III in promoting UV mutagenesis in the reconstituted system and suggests that Pol III is the major polymerase responsible for generating UV mutations, similar to the *in vivo* situation (31).

Table 2. Specificity of mutations in the reconstituted UV mutagenesis reaction

Mutation type	Pol II	Pol III core
Transition		
G·C → A·T	10 (53%)	9 (45%)
Transversion		
G·C → C·G	1 (5%)	
Double mutations		
GG → AA CC → TT	2 (11%)	1 (5%)
Frameshift	5 (26%)	8 (40%)
Deletion	1 (5%)	2 (10%)
Total	19 (100%)	20 (100%)

Repair-gap UV mutagenesis reactions were carried out and bioassayed as described in *Materials and Methods* using DNA irradiated at 400 J·m^{-2}. Cro$^-$ mutant colonies (dark red) were isolated, and their plasmid content was extracted and analyzed for mutations in *cro* by automated DNA sequence analysis.

Our data on repair synthesis demonstrate that although Pol I is most effective, Pol II and, to a lesser extent, Pol III core can participate in excision repair of UV damage, consistent with *in vivo* results (37, 38). Pol II and Pol III core were inefficient in completing the filling-in of the gap, such that the remaining nick could not be ligated. This process may require the β subunit of Pol III which endows Pol III (39–41) and also Pol II (20, 42) with high processivity, once loaded on the DNA by the γ complex.

The fact that enzymatic photoreactivation of the plasmid prior to incubation in the mutagenesis reaction mixture reduced mutagenesis by only 50% suggests that part of the mutations are targeted to 6–4 pyrimidine–pyrimidone adducts. These adducts were estimated to comprise 20–25% of the UV lesions in UV-irradiated DNA (1). Their higher contribution to UV mutagenesis in the reconstituted system is likely to be due to their higher mutagenicity, as compared with cyclobutyl dimers (7, 43).

UV mutagenesis requires *in vivo* the RecA (44, 45), UmuD, and UmuC proteins (46, 47), whereas the minimal reconstituted reaction does not contain any of these proteins. Thus, at least under *in vitro* conditions, the core mutagenic reaction occurs in the absence of RecA, UmuD, and UmuC. It is possible that the minimal reconstituted reaction is not optimal and requires additional factors for full activity, including RecA, UmuD, and UmuC. It is noteworthy that it has been previously suggested that the Umu proteins are not essential but rather are stimulatory to the mutagenic reaction (48, 49).

The findings presented in this study support a model for repair-gap UV mutagenesis that we have presented previously (16). In the first step the UvrA, UvrB, and UvrC proteins and DNA helicase II form excision gaps in the UV-damaged DNA. The majority of the gaps do not contain UV lesions, and they are filled in by Pol I in an error-free reaction. A minority of the gaps contain UV lesions, and those are the premutagenic gaps. They are filled in by Pol III core or by Pol II and involve bypass of the UV lesion in the gap, leading to an increased level of mutations. One possibility is that the premutagenic gaps and the mutations occur at sites of closely opposed UV lesions. These are formed at frequencies much higher than expected on the basis of pure statistics (12) and can account for most of the UV mutations in our *in vitro* system (15).

This work was supported by grants from the United States–Israel Binational Science Foundation (91-00281), Minerva, the Committee for Scientific Cooperation Between Germany and Israel, the Israel Science Foundation (505/94), and the Forchheimer Center for Molecular Genetics.

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