Mechanism of Replication of Ultraviolet-irradiated Single-stranded DNA by DNA Polymerase III Holoenzyme of Escherichia coli

IMPLICATIONS FOR SOS MUTAGENESIS*

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Replication of UV-irradiated oligodeoxynucleotide-primed single-stranded φX174 DNA with Escherichia coli DNA polymerase III holoenzyme in the presence of single-stranded DNA-binding protein was investigated. The extent of initiation of replication on the primed single-stranded DNA was not altered by the presence of UV-induced lesions in the DNA. The elongation step exhibited similar kinetics when either unirradiated or UV-irradiated templates were used. Inhibition of the 3'→5' proofreading exonuclease activity of the polymerase (case by dGMP or by a mutD mutant) did not increase bypass of pyrimidine photodimers, and neither did purified RecA protein influence the extent of photodimer bypass as judged by the fraction of full length DNA synthesized. Single-stranded DNA-binding protein stimulated bypass since in its absence the fraction of full length DNA decreased 5-fold. Termination of replication at putative pyrimidine dimers involved dissociation of the polymerase from the DNA, which could then reinitiate replication at other available primer templates. Based on these observations a model for SOS-induced UV mutagenesis is proposed.

UV mutagenesis, being part of the SOS response in Escherichia coli, is an induced regulated process under the control of the recA (activator) and lexA (repressor) gene products (1–3). Genetic studies have demonstrated that the umuC and umuD gene products are required for UV mutagenesis, as well as mutagenesis induced by a variety of chemical mutagens and carcinogens (4–5). RecA protein, in addition to its regulatory role, is directly involved in the process too (6–8).

The current view is that mutagenesis occurs by error-prone replication through UV-induced lesions such as pyrimidine photodimers, possibly by an altered error-prone polymerase (2, 9). This polymerase is likely to be DNA polymerase III (10, 11), the multisubunit enzyme, principally responsible for replicating the E. coli chromosome. Thus, it was proposed that SOS-induced proteins (possibly umuC and/or umuD) interact with the polymerase and reduce its fidelity to allow bypass of pyrimidine photodimers.

Most attempts to approach the problem biochemically were based on model systems utilizing DNA polymerase I, the major repair polymerase in E. coli (12–14). This polymerase, however, does not seem to play a major role in UV mutagenesis, since polA mutants exhibit normal UV mutagenesis (Ref. 2, but see Refs. 15 and 16). The recent advance in in vitro replication systems and the purification and characterization of DNA polymerase III holoenzyme, the major polymerase responsible for replicating the E. coli chromosome, made it possible for us to investigate some of the fundamental characteristics of replication of UV-irradiated DNA, using complete in vitro replication systems.

In undertaking a biochemical analysis of UV-induced mutagenesis we have studied the replication of UV-irradiated ssDNA1 by purified enzymes isolated from wild-type cells (single-stranded → replicative form reaction; Ref. 17). We have previously presented evidence that DNA polymerase III holoenzyme bypasses pyrimidine photodimers to a significant extent during replication of UV-irradiated ssDNA, even in the absence of SOS-induced proteins (18). The present study provides a biochemical characterization of the in vitro replication of UV-irradiated ssDNA with DNA polymerase III holoenzyme. Based on our findings we propose a model for the mechanism of UV mutagenesis in E. coli.

EXPERIMENTAL PROCEDURES

Materials—E. coli DNA polymerase III holoenzyme and single-stranded DNA-binding protein were purified as described (Refs. 19, 20, and 21, respectively). RecA protein was purified as described (22). mutD Pol III HE partially purified from mutD mutants was a gift from H. Echols. ssDNAs from phages φX174 and M13mp8 were prepared as described (Refs. 23 and 24, respectively). Bovine serum albumin (Penetex) was obtained from Miles Laboratories, and dGMP was purchased from Sigma. The oligodeoxyribonucleotide primer 5'-GTTTTCCAGTCGACACG-3' (for M13mp8 ssDNA) was purchased from New England Biolabs. Buffer R contained 20 mM Tris-HCl (pH 7.5), 80 µg/ml bovine serum albumin, 8 mM dithiothreitol, 4% glycerol, 8 mM MgCl2, and 40 mM NaCl.

Synthesis of Primers—Oligodeoxyribonucleotide primers (15 nucleotides long) complementary to φX174 ssDNA were synthesized either by the solid phase triester method (25) or a gift from M. O'Donnell, Stanford University) or by phosphoramidite coupling (26). The primers' sequences were as follows (map position of the 5' end on the φX174 sequence is given in parentheses): primer1 5'-ATTCGCCCAGCC TAGCC-3' (2808); primer2 5'-GACCAAGGCGGACGGC-3' (887); primer3 5'-CGCATATAATGTCGAC (4047); primer4 5'-AGCCTGCTACG CTCA-3' (4793). The sums of the extinction coefficients of the individual bases were used to determine the oligonucleotides' concentrations. A260 values of 1 µmol of the primers were 152, 152, 165, and 166 for primers 1–4, respectively.

UV-irradiated φX174 ssDNA—DNA (72 µg/ml) in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA was spread on Parafilm as droplets (3 µl each) and UV irradiated (254 nm) on ice, using a low pressure mercury germicidal lamp. The dose rate was 0.8 J/m2 s−1 as determined by a UV products radiometer using a UV-X 25 sensor. The average number of pyrimidine dimers/DNA molecule was determined by acid hydroly-

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1 The abbreviations used are: ssDNA, single-stranded DNA; Pol III HE, DNA polymerase III holoenzyme; SSB, single-stranded DNA-binding protein; EDTA, ethylenediaminetetraacetic acid, sodium salt; RF, replicative form.
ysis of \(^{3}H\) thymidine-labeled ssDNA as described (27) and was found to be 1.4 \(\times 10^{-5}\) dimers/nucleotide/Jm\(^{-2}\). This value is slightly higher than the value described previously (18) and is likely to be the result of different radiomarkers used in these determinations.

**Replication of Oligonucleotide-primed \(\phiX174\) ssDNA**—The synthetic oligonucleotides (at 2 \(\mu\)M) were annealed at a molar ratio of 1:8 to SSBr-conjugated ssDNA (0.64 \(\mu\)M of SSBr and 220 pmol, as nucleotide, of unirradiated or UV-irradiated \(\phiX174\) DNA). Annealing was carried out in a buffer containing (in 25 \(\mu\)L) 20 mM Tris-HCl (pH 7.5), 80 \(\mu\)g/ml bovine serum albumin, 8 mM dithiothreitol, 4% glycerol, 8 mM MgCl\(_2\), 40 mM NaCl for 10 min at 32°C. Alternatively, the primers could be annealed to the DNA in the absence of SSBr. In this case the primers at a concentration of 8 \(\mu\)M were annealed at a molar ratio of 250:1 to \(\phiX174\) ssDNA in a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA for 30 min at 32°C. Each primer hybridized primarily at a unique site in \(\phiX174\) ssDNA as evident from the production of a single full length DNA product following replication with Pol III HE.

The standard reaction mixture included (in 25 \(\mu\)L) 20 mM Tris-HCl (pH 7.5), 80 \(\mu\)g/ml bovine serum albumin, 8 mM dithiothreitol, 4% glycerol, 8 mM MgCl\(_2\), 40 mM NaCl (buffer R), 0.5 mM ATP, 50 \(\mu\)M each of dATP, dGTP, dCTP, and dTTP, 2-10 \(\mu\)Ci of \([\alpha^{-32}P]dTTP\) (1 Ci/mM). The reaction mixture was incubated to a molar ratio of unirradiated or UV-irradiated oligonucleotide-primed \(\phiX174\) ssDNA, 0.64 \(\mu\)g of SSBr, and 0.3 \(\mu\)g of DNA polymerase III holoenzyme (Fraction V, 7 \(\times\) 10\(^{6}\) units/ml). Incubations were for 5 min at 32°C. The reactions were stopped by adding EDTA to 20 mM and sodium dodecyl sulfate to 0.5% and kept on ice until assayed.

**Analysis of Products by Alkaline Agarose Gel Electrophoresis**—The radiolabeled replication products were separated by electrophoresis in alkaline agarose gels as described (18). Briefly, the extent of incorporation was determined by acid precipitation, and similar amounts of radiolabeled products were electrophoresed in alkaline agarose gels. Following electrophoresis the gels were dried and autoradiographed using Kodak XAR-5 x-ray film. The gel was dried onto a sheet of DEAE-cellulose paper which binds strongly single-stranded DNA and thus avoids loss of short DNA fragments.

We compared the distribution of product sizes as obtained by densitometry to that obtained by cutting the dried gel into 0.5-cm pieces and directly counting the pieces in toluene-based scintillation liquid, using a liquid scintillation counter. A representative result is presented in Fig. 1. It can be seen that both methods gave essentially an identical picture, implying that whatever densitometric measurements are made within the linear range of the densitometer, the tracing does faithfully represent the distribution of replication products.

**Initiation of Replication on Oligonucleotide-primed \(\phiX174\) ssDNA**—Reaction mixtures containing (in 25 \(\mu\)L) 20 mM Tris-HCl (pH 7.5), 80 \(\mu\)g/ml bovine serum albumin, 8 mM dithiothreitol, 4% glycerol, 8 mM MgCl\(_2\), 40 mM NaCl (buffer R), 0.5 mM ATP, 50 \(\mu\)M each of dATP, dGTP, dCTP, and dTTP (dTTP was omitted), 0.5 \(\mu\)Ci of \([\alpha^{-32}P]dTTP\), 60 \(\mu\)M of unirradiated or UV-irradiated oligonucleotide-primed \(\phiX174\) ssDNA, 0.64 \(\mu\)g of SSBr, and 0.3 \(\mu\)g of DNA polymerase III holoenzyme (Fraction V, 7 \(\times\) 10\(^{6}\) units/ml). Incubations were for 5 min at 32°C. The reactions were stopped by adding EDTA to 20 mM and SDS to 0.5%, and products were electrophoresed in 1.2% alkaline agarose gels.

**Turnover Assay**—The assay for turnover of dTTP into dTMP by Pol III HE was carried out as follows: Reaction mixtures (in 25 \(\mu\)L of buffer R) included 50 \(\mu\)M each of dATP, dGTP, dCTP, and dTTP, 10 \(\mu\)Ci of [\(\alpha^{-32}P\]dTTP (400 Ci/mmol), 220 pmol (as nucleotide) of oligonucleotide-primed \(\phiX174\) ssDNA (primer 4) UV-irradiated at a dose of 90 J/m\(^2\), 0.64 \(\mu\)g of SSBr, and 0.3 \(\mu\)g of Pol III HE. Incubation was for 5 min at 32°C after which the reaction was stopped by adding EDTA to 20 mM and SDS to 5%, and products were electrophoresed in 1.2% alkaline agarose gels and analyzed as described under "Experimental Procedures." Lower panel, densitometric trace of an autoradiogram obtained from the dry gel. The fraction of full length DNA among products was determined by cutting out the peaks and weighing these on an analytical balance and was found to be 28%. Upper panel, radioactivity distribution along the dry gel. The dry gel was cut into 5-mm slices, and each of these was counted in a scintillation counter using a toluene-based scintillation liquid. The fraction of full length DNA using this method was 30%. Notice that the output recording by the densitometer stretches out the abscissa compared to the actual dimensions of the dry gel, which are represented in the upper panel.

**Turnover Assay**—The assay for turnover of dTTP into dTMP by Pol III HE was carried out as follows: Reaction mixtures (in 25 \(\mu\)L of buffer R) included 50 \(\mu\)M each of dATP, dGTP, dCTP, and dTTP, 10 \(\mu\)Ci of [\(\alpha^{-32}P\]dTTP (400 Ci/mmol), 220 pmol (as nucleotide) of oligonucleotide-primed \(\phiX174\) ssDNA (primer 4). Either dGMP (final concentration 3.66 \(\mu\)M) or RecA protein (up to 8 \(\mu\)g), or both, were included in the reaction mixture. Replication was initiated by the addition of 0.3 \(\mu\)g of Pol III HE and incubated at 32°C. Four \(\mu\)l samples were quenching by addition of a solution (4 \(\mu\)l) containing 0.1 mM EDTA, 1.7 mM dTTP, and 2.3 mM dGTP. Free dTTP and DNA were fractionated by PEI-cellulose thin-layer chromatography of a 2-\(\mu\)l aliquot, after which the PEI-cellulose plate was sliced and radioactivity was determined by scintillation counting (30). For free dTTP the chromatography solvent was 0.5 M LiCl, 1 M formic acid, whereas for dTTP in DNA the chromatography solvent was 1 M LiCl, 1 M formic acid (30).

**Effect of Inhibitors of the 3'-5' Exonuclease Activity of Pol III HE**—Replication mixtures (in 25 \(\mu\)L of buffer R) included 50 \(\mu\)M each of dATP, dGTP, dCTP, and dTTP, 10 \(\mu\)Ci of [\(\alpha^{-32}P\]dTTP, 1.25 \(\mu\)g of SSBr, 440 pmol (nucleotide) of \(\phiX174\) ssDNA, and a 2 \(\mu\)M primer 1. Reactions were started by the addition of 0.3 \(\mu\)g of Pol III HE and incubated at 32°C. Four \(\mu\)l samples were quenching by addition of a solution (4 \(\mu\)l) containing 0.1 mM EDTA, 1.7 mM dTTP, and 2.3 mM dGTP. Free dTTP and DNA were fractionated by PEI-cellulose thin-layer chromatography of a 2-\(\mu\)l aliquot, after which the PEI-cellulose plate was sliced and radioactivity was determined by scintillation counting (30). For free dTTP the chromatography solvent was 0.5 M LiCl, 1 M formic acid, whereas for dTTP in DNA the chromatography solvent was 1 M LiCl, 1 M formic acid (30).
mmol), and 220 pmol (nucleotide) of unirradiated or UV-irradiated oligonucleotide-primed ϕX174 ssDNA (primer 1). Reactions were started by the addition of 0.3 μg of Pol III HE and incubated at 32°C for the indicated periods of time. Products were analyzed by electrophoresis in alkaline agarose gels as described above.

Effect of Polymerase Concentration—The reaction was performed under standard conditions with Pol III HE at amounts ranging from 0.3–2.4 μg (up to 16-fold molar concentration). When multiple aliquots of polymerase were added, reactions were incubated with 0.3 μg of Pol III HE at 32°C for 10 min, after which 0.3 μg of Pol III HE was added and the mixture incubated for 10 additional minutes. Experiments were also performed in which additional 0.3–μg aliquots of Pol III HE were added 7 and 14 min after the beginning of reaction and incubated at 32°C for a total of 20 min. Products were analyzed by electrophoresis in alkaline agarose gels as described above.

Recycling of Pol III HE—Reaction mixture (in 200 μl of buffer R) contained 0.5 mM ATP, 50 μM each of dATP, dGTP, and dCTP, dTTP, 100 μCi of [α-32P]dCTP, 8.3 μg of SSB, and 3.52 nmol (as nucleotide) of oligonucleotide-primed ϕX174 ssDNA (primer 1). The mixture was incubated at 32°C for 90 s after which the reaction was started by the addition of 0.15 μg of Pol III HE. At the appropriate time points, 20-μl samples were withdrawn, added into 100 μl of ice-cold 0.1 M sodium pyrophosphate to stop the reaction, and the extent of DNA synthesis was determined by acid precipitation as described (18). Under these conditions, DNA is present at a 8-fold molar excess over Pol III HE.

Transfer of Pol III HE from Replicated ϕX174 DNA to Primed M13mp8 ssDNA—An initiation complex for M13mp8 ssDNA was formed by 2-min incubation of 32°C of 60 μg of Pol III HE with ϕX174 ssDNA primed with primer 1 (1320 pmol as nucleotide) in 75 μl of buffer R containing 0.5 mM ATP, 50 μM each of dGTP and dCTP, and 3.9 μg of SSB. Replication was initiated at 32°C upon adding 25 μl of buffer R containing oligonucleotide-primed M13mp8 ssDNA (300 pmol as nucleotide), 0.5 mM ATP, 50 μM dATP, 50 μM [α-32P]dTTP, and 0.9 μg of SSB. At various times, 15-μl aliquots were quenched by addition to a solution (15 μl) containing 70 mM EDTA, 1.5% sodium dodecyl sulfate, and fractionated by electrophoresis in neutral 0.7% agarose gels. The gels were dried and autoradiographed.

RESULTS

Replication of Oligonucleotide-primed UV-irradiated ϕX174 ssDNA—Oligonucleotide-primed UV-irradiated ϕX174 ssDNA was replicated in vitro with DNA polymerase III holoenzyme in the presence of SSB, and the radiolabeled products were analyzed by electrophoresis in alkaline agarose gels. As described for the replication of UV-irradiated M13 ssDNA or G4 ssDNA (18), densitometric scans of autoradiograms obtained from the dried agarose gels revealed full length as well as shorter newly replicated DNA chains (Fig. 2). We interpreted the full length DNA chains as resulting from bypass of pyrimidine dimers during replication (18). The shorter DNA chains were interpreted as termination/pause products. Thus, the fraction of full length DNA products out of all DNA products synthesized is a measure for bypass; the greater the fraction, the higher is the extent of bypass (Table I). We have used this assay system to study the bypass/termination characteristics of DNA polymerase III during replication of UV-irradiated ssDNA under a variety of conditions.

UV Irradiation of the Template DNA Does Not Alter the Extent of Initiation by Pol III HE—In order to determine whether the extent of initiation by Pol III HE is altered by UV irradiation of the DNA template the following experiment was performed. Primer 1 was annealed to various samples of UV-irradiated ssDNA, and replication was initiated by the addition of Pol III HE, SSB, dATP, dGTP, and dCTP (dTTP was omitted). This allows Pol III HE to initiate at the primer and polymerize the first 10 nucleotides until the first dTTP residue is needed for replication (Fig. 3). Six out of the first 10 nucleotides are G residues; thus, inclusion of [α-32P]dCTP in the reaction mixture is expected to label well the extended primer. When such a reaction mixture is analyzed by electrophoresis on neutral agarose gels and visualized by autoradiography (Fig. 4) radioactive bands appear at the position of ssDNA. This is the primer-template extended with the additional 10 nucleotides. This band did not appear if Pol III HE was omitted from the reaction mixture, whereas when the missing dTTP was added, the fully replicated RFII molecule was observed (Fig. 4). The intensity of the primed ssDNA bands reflects the extent of initiation by Pol III HE. As seen in Fig. 4, ssDNAs UV-irradiated with various UV doses gave similar extents of primer extension. Thus, differences in the fraction of full length DNA found in replication experiments

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**TABLE I**

Extent of full-length DNA synthesized by DNA polymerase III holoenzyme on UV-irradiated ϕX174 ssDNA

UV-irradiated oligonucleotide-primed ϕX174 ssDNA (primer 1) was replicated with DNA polymerase III holoenzyme in the presence of SSB as described in the legend to Fig. 2, and radiolabeled products were analyzed by alkaline gel electrophoresis followed by autoradiography. Data was obtained from the densitometric trace in Fig. 2 by cutting and weighing the peaks. Expected radioactivity fraction in the full length products was calculated from the Poisson distribution and is equal to λe−λ, where λ is the average number of pyrimidine dimers/molecule. The values in parentheses give the percent of molecules which were fully replicated. These are obtained by dividing the measured fraction of full length DNA by λ, the average number of photodimers per nucleotide to compensate for the fact that the longer products have more incorporated radioactivity (18). The expected fraction of fully replicated molecules is λ−1 (Poisson distribution (18)).

<table>
<thead>
<tr>
<th>UV dose Jm⁻²</th>
<th>Average number of photodimers/circle</th>
<th>Full length DNA in products</th>
<th>Found</th>
<th>Expected</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>100 (100)</td>
<td>100 (100)</td>
<td></td>
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<tr>
<td>45</td>
<td>3.4</td>
<td>34 (10)</td>
<td>11.4 (3.3)</td>
<td></td>
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<tr>
<td>90</td>
<td>6.8</td>
<td>29 (4.3)</td>
<td>0.76 (0.11)</td>
<td></td>
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<tr>
<td>150</td>
<td>11.3</td>
<td>23 (2.0)</td>
<td>0.014 (0.0012)</td>
<td></td>
</tr>
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</table>
which are 0.64 mixture (in of replication was similar, and replication was completed by the level of seconds time scale. Since 1 nucleotide is added to elongation step with no need to involve the initiation step. can be interpreted by the bypass/termination balance in the unirradiated ssDNA with the addition of dTTP.

Primer DNA; a growing chain in 2.5-3 s reduce the rate of elongation 10-fold will cause a delay of 0.06 s/dimer or 0.6 s for a molecule carrying an average of 10 dimers, a pause which wouldn't be detected in our assay system. However, a pause of 100-fold should yield a delay of a few seconds and should be detected. Thus we conclude that if the polymerase pauses at a pyrimidine photodimer before bypassing it, the inhibition in elongation is 50-fold or less.

FIG. 3. Nucleotide sequence of primer 1 and the 4x174 sequence to which it hybridizes. Omitting dTTP from the replication mixture allows extending the primer with 10 nucleotides, 6 of which are G residues.

FIG. 4. Limited primer extension by Pol III HE on UV-irradiated oligonucleotide-primed 4x174 ssDNA. Reaction mixture (in 25 μl of buffer R) contained 0.5 mM ATP, 50 μM each of dATP, dGTP, and dCTP (dTTP was omitted), 5 μCi of [α-32P]dGTP, 0.64 μg of SSB, and 220 pmol of unirradiated or UV-irradiated DNA. Primer 1 was added at a final concentration of 2 μM, and the mixture was incubated at 32 °C for 10 min to hybridize the primer to the DNA. Pol III HE (0.3 μg) was added, and the mixture was incubated at 32 °C for 5 min. Samples were analyzed by neutral agarose gel electrophoresis as described under “Experimental Procedures.” Lanes 1 and 2, unirradiated DNA; other lanes represent 4x174 ssDNA irradiated with 45 Jm^-2 (lanes 3 and 4), 90 Jm^-2 (lanes 5 and 6), and 150 Jm^-2 (lanes 7 and 8). Lanes 9, no Pol III HE added. Lane 10, unirradiated ssDNA with the addition of dTTP. SS, single-stranded DNA; RF2, circular open duplex DNA.

Kinetics of Elongation—A time course analysis of replication of unirradiated 4x174 ssDNA shows that by 15 s the DNA is fully replicated (Fig. 5). Since 4x174 DNA is 5400 bases long, it means that Pol III HE polymerizes at a rate of nearly 400 nucleotides/s, a rate not far away from the in vivo rate of DNA chain growth of about 1000 nucleotides/s (28, 29, 31).

When UV-irradiated templates were used the time course of replication was similar, and replication was completed by 15 s (Fig. 5). Specifically, the appearance of the full length DNA products was not delayed, implying no kinetic pause on the level of seconds time scale. Since 1 nucleotide is added to a growing chain in 2.5-3 × 10^-3 s, a kinetic pause which will reduce the rate of elongation 10-fold will cause a delay of 0.06 s/dimer or 0.6 s for a molecule carrying an average of 10 dimers, a pause which wouldn't be detected in our assay system. However, a pause of 100-fold should yield a delay of a few seconds and should be detected. Thus we conclude that if the polymerase pauses at a pyrimidine photodimer before bypassing it, the inhibition in elongation is 50-fold or less.

Inhibition of 3′→5′ Proofreading Exonucleolytic Activity Does Not Increase Bypass of Pyrimidine Photodimer—The finding that inhibition of the 3′→5′ proofreading exonucleolytic activity of polymerases reduces the fidelity of polymerization and increases misincorporation (32-34) led to the suggestion that it is the inhibition of the 3′→5′ exonucleolytic activity of Pol III HE by SOS-induced proteins, which relaces its fidelity and enables bypass of pyrimidine dimers (35, 36). We tested this hypothesis using our assay system by monitoring the fraction of full length DNA under conditions in which the 3′→5′ exonucleolytic activity is inhibited. It has been shown that dGMP inhibits the 3′→5′ exonucleolytic activity of Pol III HE and that RecA protein has the same effect on Pol III HE when assayed on synthetic polynucleotides (34). Under our replication conditions we observed a 5-fold inhibition of the 3′→5′ exonucleolytic activity of Pol III HE by

FIG. 5. Time course of elongation by Pol III HE. The reaction mixture (in 25 μl of buffer R) included: 0.5 mM ATP, 50 μM each of dGTP and dCTP, 0.64 μg of SSB and 220 pmol, as nucleotide, of 4x174 ssDNA primed with primer 1. Pol III HE (0.3 μg) was added, and the mixture was incubated at 32 °C for 2 min to form an initiation complex (see “Experimental Procedures”). Elongation was started by the addition of a mixture of dTTP and dATP (final concentration in the reaction mixture was 50 μM each) and 10 μCi of [α-32P]dTTP. Samples taken at various time points were analyzed by alkaline agarose gel electrophoresis as described under “Experimental Procedures.” A, autoradiogram of replication products. B, densitometric trace of the autoradiogram. Scanning was done as described in the legend to Fig. 2.
Replication of UV-irradiated DNA

dGMP as judged by the turnover assay of dTTP into dTMP ((30) Fig. 6, Table II). This was true whether unirradiated or UV-irradiated ssDNA (45 Jm⁻² and 90 Jm⁻²) were used as templates (Fig. 6, Table II). Addition of dGMP, RecA protein, or both, to the reaction mixture did not seem, however, to increase bypass of pyrimidine photodimers since it did not increase the extent of full length DNA synthesized on UV-irradiated φX174 ssDNA. Thus, the fraction of full length DNA was 28% for ssDNA irradiated at 90 Jm⁻² and 23% for ssDNA irradiated at 150 Jm⁻² and did not change upon addition of either dGMP or RecA protein, or both. The same result was obtained when the DNA was replicated with Pol III HE isolated from an E. coli mutD mutant. This mutant polymerase was shown to be deficient in the 3'→5' exonucleolytic activity in vitro (37).

Role of dNTPs and Pol III HE Concentrations—Variations in the relative concentrations of the dNTPs have been shown to cause misincorporation during replication (38). However, increasing the concentration of each of the dNTPs or all of them up to 1 mM, a 20-fold increase over the standard concentration of dNTPs in our replication assay, did not increase bypass of pyrimidine dimers.

Increasing Pol III HE concentration up to a 16-fold molar concentration did not increase bypass either. Neither did prolonged incubation periods (up to 30 min) or several additions of Pol III HE during the reaction. This implies that a terminated DNA chain (presumably at a pyrimidine dimer) cannot be extended by DNA Pol III HE under the experimental conditions tested.

SSB Facilitates Pyrimidine Dimers Bypass—E. coli single-stranded DNA-binding protein stimulates the activity of Pol III HE on single-stranded DNA (31). Accordingly when we omitted SSB from the reaction mixture, replication was slowed down (Fig. 7); still by 4 min 85% of the products were full length DNA. The products synthesized on UV-irradiated DNA reached their final distribution within 3 min, and it remained unaltered upon further incubation (Fig. 7). The fraction of full length DNA was reduced 5-fold with a concomitant increase in the fraction of termination products (Fig. 7, Table III). A large number of discrete termination/pause products was observed, presumably at sites where pyrimidine dimers impede elongation.

DNA Polymerase III Holoenzyme Recycles after Replicating UV-irradiated DNA—Termination of replication at a pyrimidine dimer may involve dissociation of Pol III HE from the DNA or else the polymerase may remain bound to the primer template but unable to continue elongation. If dissociation takes place, Pol III HE may lose its activity (e.g. by losing one of its subunits) or else it may dissociate fully active and be available for reinitiation and elongation. In order to find out which of the possibilities holds for Pol III HE, we used our replication assay with a limiting amount of polymerase (i.e. excess DNA). Elongation can be synchronized by forming an initiation complex between the polymerase and the primer-template. This is done by adding Pol III HE in the presence of two dNTPs only. Pol III HE will bind the primer-template under these conditions and will remain bound for at least 10 min (29). Upon addition of the remaining two dNTPs elongation commences. As can be seen from Fig. 8, when unirradiated DNA was used as the template there was a fast phase of DNA synthesis, which corresponded to the completion of the first cycle of replication, followed by a slower rate of DNA synthesis. This slower phase involved dissociation of Pol III HE from the replicated DNA molecules and reinitiation at the unreplicated ones (39). This recycling of Pol III HE took 1–2 min (29, 39). The same behavior was observed for UV-irradiated DNA. In each case there was a rapid phase of replication of the initiated DNA molecules followed by a slower rate of DNA synthesis which involved dissociation, reinitiation, and elongation. The recycling time (the period it takes for successive increases in DNA synthesis equal to the amount of synthesis in the initial rapid phase of replication) was the same whether unirradiated or UV-irradiated DNAs were used and was 1–2 min (Fig. 8).

A direct demonstration of the transfer of Pol III HE from replicated UV-irradiated φX174 ssDNA to oligonucleotide-primed M13mp8 ssDNA is presented in Fig. 9. An initiation complex of Pol III HE with a 16-fold molar excess of oligonucleotide-primed UV-irradiated φX174 ssDNA could not initiate DNA synthesis (data not shown). In the absence of an initiation primer, DNA synthesis was also not initiated (data not shown). When an initiation complex was prepared, however, it could synthesize DNA in the presence of dNTPs and Pol III HE. A linear rate of DNA synthesis was observed, presumably at sites where pyrimidine dimers impede elongation.
Replication of UV-irradiated DNA

**Table III**

*Extents of full length DNA synthesized by DNA polymerase III holoenzyme on UV-irradiated φX174 ssDNA in the presence or the absence of SSB*

UV-irradiated oligonucleotide-primed φX174 ssDNA (primer 1) was replicated with DNA polymerase III holoenzyme in the presence or the absence of SSB as described in the legends to Figs. 2 and 7, and radiolabeled products were analyzed by electrophoresis in alkaline agarose gels. Data for the +SSB reaction was obtained from Fig. 2 and for the −SSB reaction from Fig. 7. The values of per cent full length DNA were taken from the 3-, 4-, and 5-min time points, all of which gave identical results.

<table>
<thead>
<tr>
<th>UV dose (Jm⁻²)</th>
<th>Average number of photodimers/circle</th>
<th>Full length DNA in products (%)</th>
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</tr>
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**Fig. 8. Recycling of DNA polymerase III holoenzyme during replication of UV-irradiated DNA.** Reaction mixture (in 200 μl of buffer R) contained 0.5 mM ATP, 50 μM each of dATP, dGTP, dCTP, and dTTP, 100 μCi of [α-³²P]dGTP, 8.3 μg of SSB, and 3.52 nmol (as nucleotide) of oligonucleotide-primed φX174 ssDNA (primer 1). The mixture was incubated at 32°C for 90 s after which the reaction was started by the addition of 0.15 μg of Pol III HE. Incubation time points were as indicated. The extent of DNA synthesis was determined by acid precipitation (see "Experimental Procedures").

**DISCUSSION**

Current models propose that UV mutagenesis, also termed error-prone repair, occurs by error-prone bypass of pyrimidine photodimers and other lesions (1, 2, 40). Moreover, it has been suggested that this bypass occurs via inhibition of the 3′→5′ proofreading exonuclease activity of the polymerase, which under normal conditions removes mismatched nucleotides misincorporated at the replication fork (35, 36). We have used the *in vitro* replication of ssDNA developed by Kornberg and his colleagues (17) as an assay system to elucidate the mechanism of replication of UV-irradiated DNA and to probe some of the hypotheses concerning UV mutagenesis. In this analysis we have used primarily DNA polymerase III holoenzyme, the multisubunit enzyme, principally responsible for replicating the *E. coli* chromosome. This is the enzyme which encounters the pyrimidine photodimers upon replication, and in *in vivo* studies have implicated its involvement in UV mutagenesis (10, 11).

Our main results, presented here and previously (18), can be summarized as follows. 1) Under normal *in vitro* condi-
or UV-irradiated &X174 DNA to primed M13mp8 ssDNA. The small fraction and 0.9 pg of dTTP and 0.9 pg of SSB. Replication was initiated at 32 °C upon addition of buffer R containing 0.5 mM ATP, 50 μM dATP, and 50 μM [α-³²P]dTTP and 0.9 μg of SSB. Replication was initiated at 32 °C upon adding 25 μl of buffer R containing 300 pmol (as nucleotide) of primed M13mp8 ssDNA, 0.5 mM ATP, 50 μM dATP, and 50 μM [α-³²P]dTTP and 0.9 μg of SSB. At the times indicated 15-μl samples were quenched by addition to 15 μl of a stop solution containing 70 mM EDTA, 1.5% sodium dodecyl sulfate, and fractionated a native 0.7% agarose gel. The small fraction of short DNA products in the lanes of unirradiated DNA arose in part from secondary hybridization sites of the two primers present in solution and in part from interruptions of chain elongation caused by stopping the reaction.

Our results indicate that DNA polymerase III holoenzyme has the inherent ability of copying pyrimidine dimers in the presence of SSB. This may be less unexpected than one might think in light of recent in vitro studies demonstrating the ability of DNA polymerase I to bypass lesions such as apurinic and apyrimidinic sites (41, 42), psoralen monoadducts (43), guanine 8-aminofluorene adducts (44, 45), and even pyrimidine dimers, although the latter required Mn²⁺ ions (known to relax the fidelity of DNA polymerase I) and high concentrations of dNTPs (46). If true in vivo, the bypass of pyrimidine photodimers by Pol III HE is expected to yield some UV-induced mutations which are independent on the SOS pathway, i.e. recA and umuC independent. Indeed, Tessman (47) has recently reported that mutagenesis in UV-irradiated phage S13 can occur in the absence of the recA and umuC gene product. In a previous study Kato et al. (48) found that 10–15% of the mixed burst mutations in the cI gene of bacteriophage λ were recA independent. Our results, however, although indicating bypass of pyrimidine dimers in vitro, do not necessarily imply the same extent of bypass in vivo. It is possible that proteins, present in the cell, inhibit bypass, and

SOS-induced proteins are then required to allow reinitiation and transdimer synthesis. In contrast to several hypotheses raised, our results argue against inhibition of the 3’→5’ exonucleolytic activity as a sufficient condition for bypass of pyrimidine dimers. In fact, we found no effect on bypass when this activity was inhibited 5-fold. Inhibition of the 3’→5’ exonucleolytic activity may still be necessary (but not sufficient), and it may also play an important role in determining the specificity of nucleotides inserted opposite the dimers once SOS-induced proteins enable bypass. The 3’→5’ exonucleolytic activity may also play an important role in untargeted mutagenesis, present in SOS-induced cells and responsible for an increased level of mutagenesis in unirradiated replicons (e.g. phage λ) after they enter the cell (49). An SOS-induced protein (RecA protein itself?) which inhibits the 3’→5’ exonucleolytic activity may be responsible for this mutagenic process. Interestingly, it has been observed that although the recA gene product is required for untargeted mutagenesis the umuC and umuD gene products, which are required for targeted UV mutagenesis, are dispensable for untargeted mutagenesis (50, 51).

What are then the factors which are important in photodimers bypass? We would like to propose that the processivity of the polymerase, i.e. the number of nucleotides polymerized by a molecule of polymerase/a single initiation event and before it dissociated from the template, is the factor which determines the ability of the polymerase to bypass lesions such as pyrimidine dimers. The processivity of Pol III HE is
very high (>5400), and in fact once initiating replication it does not leave a single-stranded DNA template until the DNA is fully replicated (31, 52–54). The processivity of most other purified polymerases is much lower, e.g. 20 for E. coli DNA polymerase I and around 10 for DNA polymerase α from calf thymus (52). Thus, a single initiating event of a single Pol III HE molecule is sufficient to promote full replication of 4×174 ssDNA, while many initiating events (200 or more) are needed for DNA polymerase I to complete the same DNA synthesis.

We imagine that when Pol III HE encounters a lesion such as a pyrimidine dimer, it pauses and may either bypass the lesion or else dissociate from the DNA. We argue that the higher the processivity of the enzyme, the longer it stays bound to the DNA during the pause, and thus the bigger are its chances to bypass the lesion. One would then expect that factors which reduce processivity substantially will reduce bypass of pyrimidine dimers. SSB was found to increase the processivity of Pol III HE (54). Indeed, in the absence of SSB we found a 5-fold decrease in the fraction of full length DNA (our measure for the extent of bypass). However, the contribution of SSB may be not only due to the increase in processivity of Pol III HE, but also due to a steric effect via interaction with the DNA template. The latter may lead to reorientation of the distorted region in the vicinity of pyrimidine dimers, making it easier to replicate. A more direct test of this suggestion would be to examine bypass of pyrimidine dimers by DNA polymerase III*, a subassembly of Pol III HE lacking the β subunit, which has a processivity of only 190 (at least 30-fold lower than Pol III HE). The bypass properties of Pol III HE are under investigation.

Based on the finding presented here and previously (18) we propose the following scheme for SOS-induced UV mutagenesis (Fig. 10). When the polymerase encounters a lesion such as a pyrimidine photodimer it pauses and can take one of two pathways: bypass, which allows uninterrupted chain elongation, or termination, in which the polymerase dissociates from the DNA, leaving a DNA structure where the daughter strand is terminated at the pyrimidine dimer. We propose that Pol III HE cannot elongate such a primer-template. DNA synthesis can, however, resume at a new site downstream to the blocking dimer, thus generating a persisting single-stranded gap in the DNA which can trigger the induction of the SOS response (3). SOS-induced proteins (umuC? umuD? recA?) interact with the polymerase and/or with the primer-template to allow reinitiation and elongation of the terminated DNA chain. According to this model the polymerase has the inherent capability of copying pyrimidine dimers, but cannot do so, once dissociated from the DNA. The major function thus suggested for the SOS-induced proteins is to allow a successful reinitiation event leading to chain elongation. Inhibition of the 3'→5' exonuclease activity may be involved in determining the specificity of the mutation created opposite the pyrimidine dimer and may also be involved in mutating undamaged portions of the DNA (undamaged mutagenesis). There is a possibility that under normal in vivo conditions an inhibitor(s) reduces bypass of pyrimidine dimers. If this is the case, one of the SOS-induced proteins may be a factor which will neutralize this inhibition.

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