Replication of UV-irradiated single-stranded DNA by DNA polymerase III holoenzyme of Escherichia coli: Evidence for bypass of pyrimidine photodimers

(SOS response/UV mutagenesis)

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ABSTRACT Replication of UV-irradiated circular single-stranded phage M13 DNA by Escherichia coli DNA polymerase (EC 2.7.7.6) and DNA polymerase III holoenzyme (EC 2.7.7.7) in the presence of single-stranded DNA binding protein yielded full-length as well as partially replicated products. A similar result was obtained with phage G4 DNA primed with E. coli DNA primase, and phage φX174 DNA primed with a synthetic oligonucleotide. The fraction of full-length DNA was several orders of magnitude higher than predicted if pyrimidine photodimers were to constitute absolute blocks to DNA replication. Recent models have suggested that pyrimidine photodimers are absolute blocks to DNA replication and that SOS-induced proteins are required to allow their bypass. Our results demonstrate that, under in vitro replication conditions, E. coli DNA polymerase III holoenzyme can insert nucleotides opposite pyrimidine dimers to a significant extent, even in the absence of SOS-induced proteins.

Pyrimidine photodimers, the major UV-induced lesions in DNA, are believed to constitute an absolute block to DNA replication, which then resumes at a site distal to the dimer, generating a single-stranded gap within the DNA duplex (refs. 1-3; but see ref. 4). Current models suggest that recA protein binds to the single-stranded region, resulting in its cleavage as a specific protease, which can then lead to the cleavage of lexA repressor. As a consequence, at least 16 cellular genes are induced, generating a diversity of phenomena including inhibition of cell division, enhanced postreplicative repair, and mutagenesis. The process of mutagenesis, which requires the recA (1), umuC, and umuD (5, 6) gene products, is of particular interest. It has been suggested that mutagenesis occurs by error-prone replication through the pyrimidine dimers, possibly by an altered, error-prone, DNA polymerase (1). In vitro studies have suggested that DNA polymerase III (EC 2.7.7.7) is involved in UV mutagenesis (7, 8). Lackey et al. (9) have isolated an altered DNA polymerase I from SOS-induced cells that shows a high frequency of miscoding in vitro. However, the significance of this activity in vivo is unclear in view of the normal UV-mutability observed with polA mutants (1).

In undertaking a biochemical analysis of UV-induced mutagenesis, we began by examining the replication of UV-irradiated circular single-stranded DNAs from phages M13, G4, and φX174 with purified enzymes isolated from wild-type cells ("SS → RF reaction") (10). We have found that, contrary to expectations, pyrimidine photodimers do not constitute an absolute block to DNA replication and can be bypassed to a significant extent, possibly with formation of mutations, even in the absence of SOS-induced proteins.

MATERIALS AND METHODS

DNA Preparations. M13mp8 single-stranded (ss) DNA (11) and φX174 ss DNA were prepared as described (12, 13). M13Gori ss DNA was a gift from D. Soltis and G4 ss DNA was a gift from M. Stayton.

Enzymes and Proteins. Escherichia coli DNA polymerase III holoenzyme and ss DNA-binding protein (SSB) were purified as described (14-16). Other E. coli replication proteins were gifts from the groups of A. Kornberg and I. R. Lehman as follows: RNA polymerase (EC 2.7.7.6), J. Kaguni; DNA primase, J. Flynn; DNA polymerase I, J. Kelly; DNA ligase (EC 6.5.1.2), I. R. Lehman. Phage T4 endonuclease V (EC 3.1.25.1) was a gift from P. Seawell and A. Ganesan. Bovine serum albumin (Pentex) was purchased from Miles.

Biochemicals. Deoxyribonucleoside triphosphates and ribonucleoside triphosphates were obtained from P-L Biochemicals. [α-32P]dTTP (800 Ci/mmole; 1 Ci = 37 GBq) was obtained from Amersham and New England Nuclear.

UV-Irradiated ss DNA. 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) using a low-pressure mercury germicidal lamp. The dose rate was 1 J·m⁻²·sec⁻¹ as determined by a Latarjet UV meter. The average number of pyrimidine dimers per DNA molecule was determined by acid hydrolysis of [3H]thymidine-labeled ss DNA as described (17) and was found to be 1.2 × 10⁻⁵ pyrimidine dimers per nucleotide per J·m⁻² at doses up to 300 J·m⁻².

Replication of ss DNA (SS → RF Reaction). The standard reaction mixture included in (25 μl): 20 mM Tris-HCl at pH 7.5, bovine serum albumin at 80 μg/ml, 8 mM dithiothreitol, 4% (vol/vol) glycerol, 8 mM MgCl₂, 2 mM ATP, GTP, CTP, and dTTP at 0.1 mM each, dATP, dGTP, dCTP, and dTTP at 50 μM each, 2–10 μCi of [α-32P]dTTP (800 Ci/mmole), 220 pmol (as nucleotide) of unirradiated or UV-irradiated ss DNA, 0.6 μg of SSB, and 0.3 μg of DNA polymerase III holoenzyme (fraction V, 7 × 10⁵ units/mg) (14).

For priming at the M13 origin, 0.03 μg of RNA polymerase was added; for priming at the G4 origin, 0.1 μg of DNA primase was added. φX174 ss DNA was primed with the synthetic oligodeoxynucleotide 5'-ATGTCCCGGCCTACG-3' (a gift from M. O'Donnell), complementary to the φX174 sequence at positions 2794–2808 (18). Replication of the primed φX174 ss DNA was performed in the absence of ribonucleoside triphosphates. To obtain closed circular duplex DNA, reaction mixtures were supplemented with 0.2 μg of DNA polymerase I, 0.5 μg of E. coli DNA ligase, and 40

Abbreviations: ss DNA, single-stranded DNA; SSB, ss DNA-binding protein; kb, kilobase(s); RF, replicative form.

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Analysis of Products by Agarose Gel Electrophoresis. The incorporation of $[\alpha^{32}P]dTTP$ into acid-insoluble material was determined by assaying 5-µl samples from each reaction mixture prior to analysis on agarose gels. All samples that were loaded onto the gels contained similar amounts of incorporated $[\alpha^{32}P]dTTP$, 20 mM EDTA, 0.1 µg of carrier plasmid DNA (plasmid pMOB45), 5% glycerol, 0.02% bromoresol green dye, and 0.2 M NaOH in 1% NaDodSO4. Horizontal 1% agarose gels made with 30 mM NaOH/1 mM EDTA (20) were used for electrophoresis in 30 mM NaOH/1 mM EDTA at 45 V for 16–20 hr. Care was taken to ensure that the running solution formed a contact with the gel edges but did not overflow the gel. This eliminates contamination of the gel by unreacted $[\alpha^{32}P]dTTP$.

For analysis on neutral agarose gels, the NaOH was omitted from the loading mixture. Electrophoresis was performed in 0.8% agarose gels in 90 mM Tris/borate, pH 8.1/2.5 mM EDTA containing ethidium bromide at 0.5 µg/ml, at 35 V for 14–16 hr.

After electrophoresis, the gels were dried and autoradiographed with Kodak XAR-5 x-ray film. The autoradiograms were scanned with a Helena Laboratories (Beaumont, TX) Quick Scan, Jr. densitometer. The scans were quantitated by cutting out the peaks on the tracing paper and weighing them on an analytical balance.

**T4 Endonuclease V Digestion.** The DNA was incubated in a buffer containing 10 mM Tris-HCl at pH 8, 10 mM EDTA, 0.1 M NaCl, bovine serum albumin at 0.1 mg/ml, and $2 \times 10^5$ units of T4 endonuclease V, at 37°C for 60 min.

**RESULTS**

When UV-irradiated circular ss M13 DNA was replicated with RNA polymerase, DNA polymerase III holoenzyme, and SSB, there was a decrease in nucleotide incorporation as a function of UV dose (Fig. 1). The incorporation was inversely proportional to UV dose, hence, directly proportional to the distance between pyrimidine dimers. It was, however, 2- to 3-fold higher than that predicted from the Poisson distribution, assuming that each dimer represents a complete block to replication (Fig. 1; Table 1).

When analyzed by electrophoresis in alkaline agarose gels (Fig. 2) the length distribution of products revealed a large proportion of polynucleotides longer than the average interdimer distances, including a high proportion of full-length molecules. Similar results were obtained with G4 or M13Goril ss DNA (21) primed with DNA primase, with M13mp8 ss DNA (11) primed with RNA polymerase, and with φX174 ss DNA primed with a synthetic 15-residue oligodeoxynucleotide. The synthesis of full-length molecules is therefore independent of the source of ss DNA and the mode of priming and is primarily a function of the DNA

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**Table 1. Product analysis of replicated UV-irradiated M13Goril ss DNA**

<table>
<thead>
<tr>
<th>UV dose, J/m$^2$</th>
<th>Average no. of dimers per circle</th>
<th>% incorporation</th>
<th>% full-length DNA in products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found$^a$</td>
<td>Expected$^b$</td>
<td>Ratio</td>
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<tr>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
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<tr>
<td>45</td>
<td>5</td>
<td>40</td>
<td>24</td>
</tr>
<tr>
<td>90</td>
<td>10</td>
<td>24</td>
<td>9</td>
</tr>
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</table>

UV-irradiated M13Goril ss DNA was replicated as described in the legend to Fig. 1, using priming with DNA primase, and the products were analyzed by gel electrophoresis.

$^a$Radiolabeled dTTP incorporated into acid-insoluble material.

$^b$Calculated from the Poisson distribution. Expected incorporation = 1/($\lambda + 1$); expected full-length = $he^{-\lambda}$, in which $\lambda$ is the average number of dimers per molecule. See text.

$^c$The length distribution was obtained by densitometric tracing of the autoradiogram in Fig. 2. The numbers in parentheses give the percentage of molecules that were fully replicated. These values are obtained by dividing the observed fraction of full-length DNA by the average number of photodimers per molecule.
replication, the G4 origin on a 2.2-kilobase-pair DNA fragment inserted into the intergenic region (21). Priming at either origin yielded a similar fraction of full-length DNA products after replication by DNA polymerase III holoenzyme, although the distributions of the shorter DNA products were not identical. The differences in distributions are probably due to differences in termination (Fig. 2).

When replication mixtures were supplemented with DNA polymerase I (to excise the RNA primer) and DNA ligase (to seal the remaining nick) and the products were analyzed by electrophoresis in neutral agarose gels containing ethidium bromide, a high proportion of supercoiled products was observed. Denaturation of the products with alkali followed by renaturation did not alter the migration of the replicative form (RF) I product, verifying that it is covalently closed circular duplex DNA (Fig. 3).

The assumption that a pyrimidine dimer is a complete block to DNA replication implies that any full-length product is the result of replication of that fraction of the UV-irradiated DNA molecules lacking dimers. Assuming a random distribution of pyrimidine dimers among DNA molecules, this fraction \( n_0 \) can be predicted from the Poisson equation, \( N_0 = e^{-\lambda} \), in which \( \lambda \) is the average number of dimers per molecule (22). When a number rather than a length distribution is used the equation becomes \( N_0 = e^{-\lambda} \).

The distribution we measure is the length distribution of products. To translate the measured fraction of full-length DNA to the fraction of molecules that were fully replicated, one has to divide the measured values by the average number of photodimers per molecule. For M13Goril ss DNA carrying averages of 5, 10, and 16.4 photodimers per molecule, the measured fractions of full-length products were 25%, 17%, and 11%, respectively (Table 1). These represent 5%, 1.7%, and 0.7% of the molecules, respectively, being fully replicated. Thus the assay amplifies the signal generated by fully replicated molecules and enables their accurate determination. Our results show that the observed fraction of full-length products was up to several orders of magnitude higher than expected (Table 1). Furthermore, the ratio of found to expected increased with UV dose from a value of 7 at a dose of 45 J\cdot m^{-2}, through 400 at 90 J\cdot m^{-2}, up to 10^5 at 150 J\cdot m^{-2}. These results suggest strongly that DNA molecules that

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**Fig. 2.** (A) Autoradiogram of replicated UV-irradiated M13Goril ss DNA analyzed by alkaline gel electrophoresis. Replication mixtures were as described in the legend to Fig. 1. Mixtures were incubated at 32°C for 20 min, after which reactions were stopped by the addition of EDTA to a final concentration of 20 mM and NaDodSO4 to a final concentration of 1%. Samples containing similar amounts of incorporated \( [\text{a-32P}]d\text{TMP} \), 20 mM EDTA, 0.1 \( \mu \)g of carrier DNA (plasmid pMOB45), 5% glycerol, 0.02% bromoresol green dye, 0.2 M NaOH, and 1% NaDodSO4 were loaded onto horizontal 1% agarose gels and electrophoresed in 30 mM NaOH/1 mM EDTA at 45 V for 16–20 hr. After electrophoretic separation the gels were dried and autoradiographed. kb, Kilobases. (B) Densitometry trace of the autoradiogram (replication primed with DNA primase). Densitometry sensitivity was adjusted for each lane to yield the largest signal within the linear range of the instrument to allow an accurate determination of the fraction of fully replicated molecules. Thus the peaks of full-length DNAs from different lanes cannot be compared directly. The comparison is made by determining the fraction of full-length DNA among replication products in each lane (see Table 1). Usually different exposures were required to allow scanning of different lanes.

polymerase III holoenzyme. The lack of dependence on the priming system is illustrated in the replication of M13Goril ss DNA (Fig. 2), which has, in addition to the M13 origin of
contain pyrimidine dimers have been fully replicated—i.e., pyrimidine dimers have been bypassed.

This conclusion relies on the applicability of the Poisson distribution to our experimental situation. Since it assumes a random distribution of pyrimidine dimers, any deviation from randomness—e.g., clustering of pyrimidine dimers—would alter the predicted fraction of dimer-free molecules; however, no clustering of pyrimidine dimers has been observed in UV-irradiated ss M13 DNA (23). Moreover, if pyrimidine dimers were formed preferentially at a limited number of hot spots, rather than distributed equally among all pyrimidine-pyrimidine sequences, then the value of \( n_0 \) should be calculated from the binomial distribution, which gives values that are even lower than the Poisson distribution (22).

To obtain a direct measure of the fraction of pyrimidine dimer-free ss DNA molecules within a population of UV-irradiated ss DNA, the following experiment was performed. 32P-labeled M13mp8 and \( \phi X174 \) ss DNAs were irradiated and treated with T4 endonuclease V, an enzyme that acts specifically on UV-irradiated double-stranded DNA, cleaving the DNA at pyrimidine dimers (24–27). The products were then analyzed in alkaline agarose gels. Since UV-irradiated ss DNA is a poor substrate for T4 endonuclease V (24–27), a large excess of enzyme was required, resulting in some cleavage of the control unirradiated ss DNA. As shown in Fig. 4, extensive degradation of the UV-irradiated ss DNA did occur. The fraction of dimer-free ss DNA as determined by this method was much less than the fraction of full-length products obtained after replication of the UV-irradiated ss DNA (compare Fig. 4A and Fig. 2B). The percentage of full-length molecules at each UV dose is shown in Fig. 4B. At a dose of 45 J m\(^{-2}\) the results obtained after T4 endonuclease V cleavage were as predicted by the Poisson distribution. At higher doses the experimental values were considerably higher than predicted possibly as a result of incomplete cleavage by the T4 endonuclease V. Nevertheless, it is clear that at all doses tested, the fraction of full-length molecules obtained after replication is much higher than that expected from T4 endonuclease V cleavage.

**DISCUSSION**

There is at present no direct evidence in vivo that pyrimidine dimers constitute absolute blocks to DNA replication in *E. coli* (4).

Studies in vitro (3, 28) have, however, demonstrated that pyrimidine dimers reduce the extent of DNA synthesis and that replication frequently terminates at the site of a dimer (29, 30). Our results confirm these observations; however, our experiments, which were designed to examine all of the products of replication rather than interruptions in synthesis, have shown that DNA polymerase III holoenzyme in the presence of SSB can replicate through pyrimidine dimers. It is noteworthy that T4 DNA polymerase, in the presence of T4 gene 32 protein, is deficient in its ability to replicate past a dimer (unpublished results).

The duplex DNA product obtained by replication of UV-irradiated ss DNA was resistant to T4 endonuclease V and to *Micrococcus luteus* "UV" endonuclease unless a high excess of enzyme was used or the product DNA was denatured prior to digestion (unpublished results). This would indicate that there is some alteration in the structure of the duplex opposite the replicated pyrimidine dimer.

A related observation was reported by two groups (31, 32), who found that after replication of UV-irradiated DNA in vivo, in *E. coli* (31) or in mouse cells infected with the minute virus of mice (32), pyrimidine dimers, while still present in the DNA, become resistant to *M. luteus* UV endonuclease or T4 endonuclease V, respectively. The in vivo replication products were also insensitive to nuclease S1 (unpublished results): thus if looped-out structures are formed during the replication they are unlikely to be very long, probably three nucleotides or less (33).

The simplest interpretation of the structural alteration...
opposite the pyrimidine dimer is that a mutation has been generated—e.g., only one nucleotide has been inserted opposite the photodimers. If applicable in vivo, this implies the existence of a branch of UV mutagenesis that is constitutive and independent of SOS-induced functions. Indeed, Kato et al. (34), investigating forward mutation in the cI gene of phage λ, found that 10–15% of the mixed-burst mutations scored were recA independent. Recently, Tessman (35) has demonstrated umuc- and recA-independent mutagenesis in UV-irradiated phage S13.

It has recently been suggested that pyrimidine-photodimer (4–6) photoproducts may be involved in UV mutagenesis in addition to pyrimidine dimers (36). In UV-irradiated double-stranded DNA, pyrimidine adducts amount to approximately 10% of the number of pyrimidine dimers; however, in ss DNA this value is reduced to 2%–37%. Thus the substrates used in our experiments contained between 0.1 and 0.3 adduct per molecule, a number too small to allow measurement of bypass.

Interestingly, the large fragment of DNA polymerase I has recently been shown to catalyze polymerization opposite pyrimidine dimers (38). However, the bypass required Mn²⁺ and a high concentration of deoxyribonucleoside triphosphates.

Recent studies reveal that DNA polymerase I can bypass DNA lesions such as psoralen monoaadducts (39), apurinic sites (40, 41), and guanine-8-aminofluorene adducts (42). Thus it would appear that even bulky modifications of bases in DNA are not a great barrier to the major repair polymerase in E. coli, DNA polymerase I. The same may be true for the major replicative polymerase in E. coli, DNA polymerase III holoenzyme, as shown in this paper for the case of pyrimidine photodimers.

Our findings suggest that when DNA polymerase III holoenzyme encounters a pyrimidine photodimer, it pauses, and may then take one of two possible pathways: The major route is termination, which produces a persistent single-stranded region downstream from the pyrimidine dimer. This, as the current model suggests, would activate recA protein and generate the SOS response (43). The minor pathway is bypass, in which replication proceeds through the pyrimidine photodimer. We estimate that the probability of bypass of a pyrimidine dimer is at least 30% (unpublished results).

The connection between targeted SOS-induced UV mutagenesis and the constitutive bypass of pyrimidine dimers is presently unclear; however, our findings do raise the possibility that proteins induced by the SOS response may alter the efficiency and/or the specificity of the replication through pyrimidine dimers by DNA polymerase III holoenzyme, thereby leading to increased levels of mutagenesis. Alternatively, a completely different mechanism for SOS-induced UV mutagenesis may exist.

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