Replication of M13 Single-Stranded Viral DNA Bearing Single Site-Specific Adducts by *Escherichia coli* Cell Extracts: Differential Efficiency of Translesion DNA Synthesis for SOS-Dependent and SOS-Independent Lesions†

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ABSTRACT: In order to characterize mutagenic translesion DNA synthesis in UVM-induced *Escherichia coli*, we have developed a high-resolution DNA replication system based on *E. coli* cell extracts and M13 genomic DNA templates bearing mutagenic lesions. The assay is based on the conversion of M13 viral single-stranded DNA (ssDNA) bearing a single site-specific DNA lesion to the double-stranded replicative form (RF) DNA, and permits one to quantitatively measure the efficiency of translesion synthesis. Our data indicate that DNA replication is most strongly inhibited by an abasic site, a classic SOS-dependent noninstructive lesion. In contrast, the efficiency of translesion synthesis across SOS-independent lesions such as O^6-methylguanine and DNA uracil is around 90%, very close to the values obtained for control DNA templates. The efficiency of translesion synthesis across 3,N^4-ethenocytosine and 1,N^6-ethenoadenine is around 20%, a value that is similar to the *in vivo* efficiency deduced from the effect of the lesions on the survival of transfected M13 ssDNA. Neither DNA polymerase I nor polymerase II appears to be required for the observed translesion DNA synthesis because essentially similar results are obtained with extracts from *polA*- or *polB*-defective cells. The close parallels in the efficiency of translesion DNA synthesis *in vitro* and *in vivo* for the five site-specific lesions included in this study suggest that the assay may be suitable for modeling mutagenesis in an accessible *in vitro* environment.

Translesion DNA synthesis, the key step in mutagenesis induced by DNA damaging agents, is believed to have differing requirements depending on the characteristics of the lesion. Translesion synthesis across the so-called *mispairing* lesions appears not to require special factors, presumably because the normal replication apparatus is able to extract coding information from such lesions, even though the information is wrong in a biological sense. Thus, mispairing lesions such as O^6-methylguanine (O^6mG)[1] and DNA uracil (dU) produced by deamination of DNA cytosine are known to neither block DNA replication nor depend on inducible factors for translesion synthesis.

However, a majority of known mutagenic DNA lesions fall into the category of *noninstructive* DNA lesions, best exemplified by abasic (AP) sites, that tend to block DNA synthesis. Translesion synthesis across this class of lesions is poorly understood, and in *Escherichia coli*, it is hypothesized to depend on additional induced functions such as those provided by RecA, UmuD, and UmuC proteins of the SOS system (*1*−*3*). In SOS-induced cells, translesion DNA synthesis at noninstructive lesions appears to be facilitated at the cost of increased mutagenesis; i.e., a transient increase in replication efficiency is coupled to a decrease in fidelity in such cells. However, the biochemical mechanism mediating such transient "error-prone" replication remains unknown.

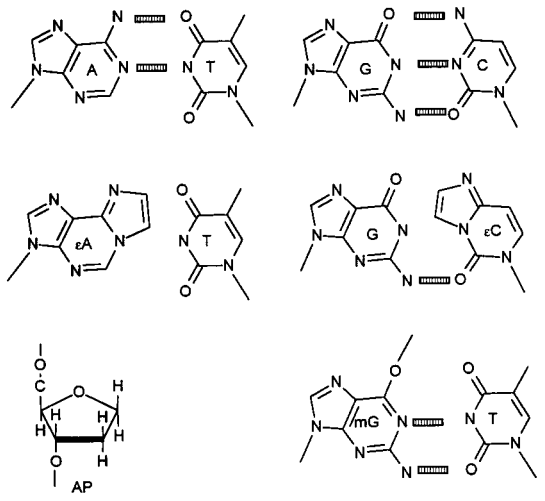

FIGURE 1: Chemical structures of mutagenic DNA lesions used in this study. Below the normal Watson−Crick base pairs (A·T, G·C), ϵA·T and ϵC·G "pairs" are shown to highlight the interference to Watson−Crick base pairing expected from the etheno rings in these lesions. Also shown is a potential base pairing of O^6mG·T and the structure of the artificial AP site in this study.

Recent results from this laboratory have suggested the existence of a third class of mutagenic lesions (termed class 2 noninstructive lesions; *4*−*6*) exemplified by the exocyclic lesion 3,N^4-ethenocytosine (ϵC; Figure 1), known to be

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[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; RF-DNA, replicative form DNA; ϵC, ethenocytosine; ϵA, ethenoadenine; AP, abasic site; dU, DNA uracil; O^6mG, O^6-methylguanine; ϵC-, ϵA-, O^6mG-, or U-DNA, M13 ssDNA constructs bearing the corresponding lesions; C-DNA, control M13 ssDNA construct bearing a normal cytosine at the lesion site; pol-I, pol-II, and pol-III, *Escherichia coli* DNA polymerases I, II, and III, respectively.

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induced by industrial carcinogens such as vinyl chloride (*7*) and ethyl carbamate (*8*), as well as by certain endogenous agents (*9*). ϵC resembles mispairing lesions in two key characteristics: mutagenesis at ϵC is independent of the *recA* gene; and although ϵC appears to impede replication, it does so to a lesser degree than that observed for the classical SOS-dependent lesions such as abasic sites. Interestingly, ϵC resembles noninstructive lesions in two key features: it appears to be incapable of Watson−Crick template instruction as evidenced by its *in vitro* template characteristics (*10*), and by its *in vivo* mutational signature (*11*, *12*); second, mutagenesis at ϵC is strongly affected by *inducible cellular functions*. Thus, mutagenesis at ϵC is significantly elevated in cells pretreated with DNA damaging agents, an effect that we have termed UVM (for UV modulation of mutagenesis) because it is independent of all known *E. coli* cellular responses to DNA damage, including the SOS response (*4*, *5*, *13*, *14*). Recent evidence suggests that UVM may also modulate mutagenesis at 1,N^6-ethenoadenine (ϵA), another class 2 noninstructive lesion, but apparently not at the mispairing lesion O^6mG (*6*).

Translesion DNA synthesis has been studied at a high resolution indirectly *in vivo*, and more directly *in vitro* (for a review, see ref 15). Because blocking DNA lesions can be overcome *in vivo* through recombinational repair, their physical removal (i.e., damage repair) can occur *before* or *after* DNA replication is completed. Therefore, *in vivo* mutagenesis data normally represent the results of multiple cellular processes that include DNA replication, recombination, and repair. On the other hand, results from *in vitro* studies based on oligonucleotide templates and purified DNA polymerases are highly variable depending on the polymerase used (*16*−*18*).

SOS replication is thought to require DNA polymerase III (pol-III; *3*, *19*). Because UVM appears not to require DNA polymerases I (pol-I) or II (pol-II; *4*), by default pol-III (or a subassembly of pol-III) is assumed to mediate UVM. Because the form of pol-III involved in translesion DNA synthesis is not known, we have explored cell-free extracts as a first step toward modeling mutagenic translesion synthesis during the UVM response. We describe here an *in vitro* DNA replication system using *E. coli* cell extracts and unprimed M13 ssDNA templates bearing single, site-specific mutagenic lesions.

MATERIALS AND METHODS

Materials. Deoxyribonucleoside and ribonucleoside triphosphates were purchased from Pharmacia, and [α-^{32}P]dCTP (10 mCi/mL, 6000 Ci/mmol) was from Amersham. Restriction endonucleases, T4 DNA ligase, and proteinase K were obtained from New England Biolabs. The DNA purification kit used (tip-20) was supplied by Qiagen. Oligonucleotides (17-mers) containing single site-specific lesions (ϵC, ϵA, O^6-mG, AP, and dU) were synthesized and HPLC-purified by the Midland Certified Reagent Co. The synthetic AP lesion is a modified tetrahydrofuran moiety which has two hydrogens on carbon 1′ of the deoxyribose ring (Figure 1). Other oligonucleotides were synthesized by the New Jersey Medical School Molecular Resource Facility. All synthetic oligonucleotides were subsequently purified by high-resolution gel electrophoresis before use.

Bacterial and Phage Strains. The *E. coli* strains KH2 Δ(*lac-pro*) *trpE4777* (F′ *lacI*^{q}*Z* ΔM15 *pro*$^+$), KH2R Δ(*srlR-recA*)306::Tn*10*(Tet^r) in KH2 (*20*), CJ231 Δ*polA* Km^r/pCJ102 (pCJ102 = F′ 5′ Exo Cm^r (*21*), and VP100 Δ*polB* F′ (pOX38::Cm^r) (*4*) were used for making cell-free extracts. M13 mp7L2 is a derivative of phage M13mp7 (*22*).

Construction of M13 Single-Stranded DNA (ssDNA) Bearing a Site-Specific Lesion. Procedures used for the construction of M13 ssDNA bearing single site-specific lesions have been described previously (*13*) and can be summarized as follows: (1) linearization of M13 ssDNA by *Eco*RI digestion; (2) annealing with the 57-mer scaffold and a 17-mer containing a single site-specific lesion; and (3) DNA ligation. The ligation mix was deproteinized by a phenol extraction, and the DNA was recovered by ethanol precipitation and resuspension in a small volume of water. To completely remove the 57-mer scaffold, the DNA construct was heat-denatured in the presence of an excess of anti-57-mer oligonucleotide and immediately loaded on a Qiagen (tip-20) resin column. The oligonucleotides were washed out of the column with 1.0 M NaCl at pH 7.0 (buffer QC provided by Qiagen), and the ssDNA molecules were eluted with 1.25 M NaCl at pH 8.5 (buffer QF; Qiagen). Figure 2 is a diagrammatic representation of constructed lesion-containing M13 ssDNA.

Preparation of E. coli Cell Extracts. E. coli cells were grown in 1300 mL of LB medium at 37 °C to late-log phase (OD_{600} = 1.0) and harvested by centrifugation. Cell pellets were rinsed with a small volume of buffer A (25 mM Hepes, pH 7.6/1 mM EDTA) and resuspended in 6 mL of buffer A, followed by freezing in a dry ice/ethanol bath and storage at −70 °C. Frozen cell suspensions were thawed at 15 °C and adjusted to 80 mM KCl, 2 mM dithiothreitol, 200 μg/mL egg lysozyme. The cell suspension was incubated at 0 °C for 20 min, and the cells were lysed by two freeze−thaw cycles in which cells were frozen at −70 °C for 2 min, followed by thawing at 15 °C for 20 min. The freeze−thaw lysates were clarified by centrifugation at 100000*g* for 30 min at 4 °C (fraction I). To remove chromosomal DNA, 0.1 volume of a 30% (w/v) solution of streptomycin sulfate was added to fraction I followed by stirring at 0 °C for 30 min. DNA precipitates were removed by centrifugation at 20000*g* for 10 min at 4 °C. To the suspension was slowly added solid ammonium sulfate at 0 °C with stirring over a 15 min period to a final concentration of 0.28 g/mL (for 45% saturation) or 0.47 g/mL (for 70% saturation), and the suspension was stirred for an additional 20 min at 0 °C. Precipitating proteins were collected by centrifugation at 20000*g* for 10 min at 4 °C. The pellets were resuspended in 0.5 mL of buffer B (25 mM Hepes, pH 8.0, 0.1 mM EDTA, 2 mM dithiothreitol), and the suspension was dialyzed against 100 volumes of precooled buffer B at 0 °C for 2 h. The dialysate (fraction II) was frozen in 0.05 mL aliquots in a dry ice/ethanol bath, and stored at −70 °C. Protein concentration of the cell extract (fraction II) ranged from 40 to 80 mg/mL as determined by Biorad protein assay using bovine serum albumin as a standard.

In Vitro Replication Assay. The reaction mix (25 μL) contained 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 12 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 5% ethylene glycol (v/v), 2 mM ATP, 0.5 mM each of UTP, GTP, and CTP, 50 μM NAD, 50 μM cAMP, 50 μM each of the four dNTPs, 7 μCi of [α-^{32}P]dCTP (10 mCi/mL, 6000 Ci/mmol), 70 ng of ssDNA, and 350 μg of *E. coli* cell extract protein. The reaction was allowed to proceed at 37 °C for 90 min and

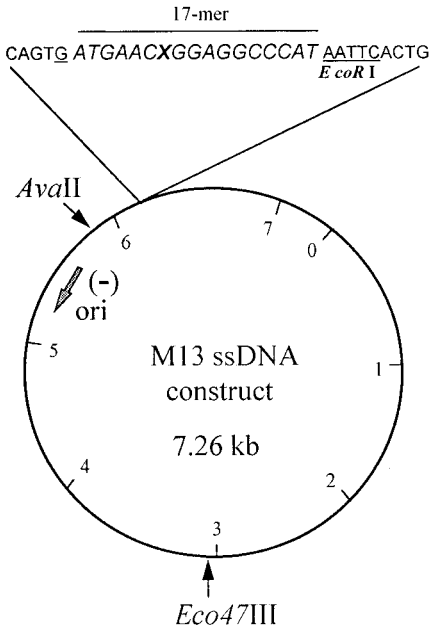

FIGURE 2: Diagrammatic representation of M13 ssDNA constructs bearing site-specific DNA lesions. M13 ssDNA bearing a site-specific lesion was constructed by replacing the polylinker sequence in M13 mp7L2 ssDNA with a lesion-bearing 17-mer oligonucleotide (*13*). The *X* within the 17-mer sequence shown represents the lesion. The gray arrow indicates the location of the minus-strand origin of replication and the direction of primer synthesis. Also shown are the recognition sites for the restriction enzymes *Ava*II and *Eco*47III that would exist if the DNA were double-stranded as in RF DNA. Double-digestion of the RF DNA (7.26 kb) with *Ava*II and *Eco*47III should yield two restriction fragments (4.38 kb and 2.88 kb).

was terminated by incubation with proteinase K at a concentration of 0.4 mg/mL for 30 min at 37 °C followed by phenol extraction. Aliquots of the replication products either were directly analyzed by electrophoresis on 1% agarose gels in TAE buffer (40 mM Tris−acetate, 1 mM EDTA, pH 8) containing 0.5 μg/mL ethidium bromide or were subjected to ethanol precipitation and subsequent restriction enzyme digestion before gel electrophoresis as above. The positions of the RF-I (supercoiled circular) and RF-II (nicked circular) DNA were identified by coelectrophoresing purified M13 RF DNA markers. DNA synthesis was visualized by autoradiography of the dried gel, and quantitated by computing densitometry as described (*10*).

RESULTS

Characteristics of in Vitro M13 Viral ssDNA-to-dsDNA Replication. Our system is based on the conversion of the single-stranded viral genome of the phage M13 to the double-stranded DNA (dsDNA or RF DNA) form. No phage proteins are required for this so-called stage I replication (*23*, *24*). DNA synthesis is initiated at a unique site called the minus-strand origin (Figure 2), where a 20-nucleotide RNA primer is synthesized by the host RNA polymerase (*25*). The primer is subsequently elongated by DNA polymerase III holoenzyme around the ssDNA circle. Finally, DNA polymerase I excises the primer RNA and refills the gap with deoxyribonucleotides. Subsequent actions of DNA ligase and DNA gyrase generate a covalently closed supercoiled dsDNA termed RF-I DNA.

To prepare cell-free protein extracts that support the replication of M13 ssDNA efficiently, we employed modifications of available methods (*26*, *27*) as described under Materials and Methods. In *E. coli* KH2 and KH2R cell extracts, we find that M13 replicating activities are precipitated at an ammonium sulfate concentration representing 45% saturation (i.e., 0−45% ammonium sulfate fraction), whereas almost no activity is detected in the 45−70% fraction. The 0−70% fraction had about 2-fold higher levels of protein, but approximately the same level of replication activity as the 0−45% fraction (Figure 3). However, the 0−70% fraction had no significant loss of replicating activity after storing at −70 °C for 4 months, while the 0−45% fraction retained only 10−20% of the original activity, suggesting that factors stabilizing the replication activities were present in the 0−70%, but apparently not in the 0−45%, fraction. In order to ensure that all proteins required for supporting DNA replication, as well as any possible accessory proteins necessary for translesion synthesis, we used the 0−70% ammonium sulfate protein fraction for the experiments described below.

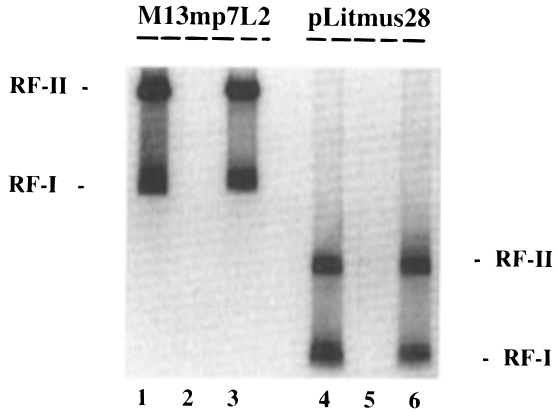

FIGURE 3: Characteristics of the *in vitro* ssDNA-to-dsDNA replication system using *E. coli* KH2 cell extracts. Shown is an autoradiograph of a 1% agarose electrophoretic gel on which *in vitro* replication products have been analyzed. Since [α-^{32}P]dCTP was used in the assay, only newly synthesized strands can be seen in the autoradiograph. Reactions were carried out at 37 °C for 90 min. The ssDNA templates used were M13 mp7L2 (lanes 1−3) and pLitmus28 (lanes 4−6). Cell extracts used (350 μg of protein for a 25 μL reaction) were the protein fractions collected by precipitation with 0−45% (lanes 1 and 4), 45−70% (lanes 2 and 5), and 0−70% (lanes 3 and 6) saturated ammonium sulfate. The positions of covalently closed circular DNA (RF-I) and nicked or gapped circular DNA (RF-II) are marked on the side.

To optimize the replication conditions for our cell extracts and DNA substrates, we tested different parameters including temperature, time, and the amounts of DNA template and protein extract. We determined that M13 ssDNA-to-dsDNA replication is optimal when carried out at 37 °C for 90 min as described under Materials and Methods. Reaction times of 120 min or longer resulted in a decrease in the amount of DNA synthesis (data not shown), presumably due to degradation by nuclease activities present in the extract. The efficiency of replication in our system appears to be appreciable. In a typical 25 μL reaction with 70 ng of ssDNA template, we obtain about 100 ng of DNA replication products as judged by ethidium bromide staining of agarose electrophoretic gels (data not shown), suggesting that approximately 70% of the input ssDNA molecules were replicated to the RF-II and RF-I stages. Under optimal conditions, the supercoiled circular DNA (RF-I) constituted about 40% of the replication products using unmodified

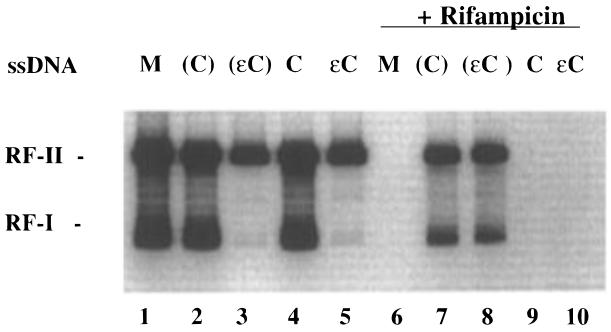

FIGURE 4: *In vitro* replication of ssDNA templates in the absence and presence of rifampicin. *In vitro* replication reactions were carried out at 37 °C for 90 min using *E. coli* KH2 (*recA*+) cell extracts prepared by precipitation with 0−70% saturated ammonium sulfate. DNA templates used here are as follows: M, M13mp7L2 ssDNA; (C) and (ϵC), unpurified M13 ssDNA construct containing a site-specific C and ϵC, respectively, at position X (Figure 2); C and ϵC, M13 ssDNA constructs containing a site-specific C and ϵC, respectively, after purification through a Qiagen tip-20 column. Rifampicin was added to 100 μg/mL for the *in vitro* replication reactions analyzed in lanes 6 through 10. Positions of the two forms of replication products (RF-I and RF-II) are indicated at the left.

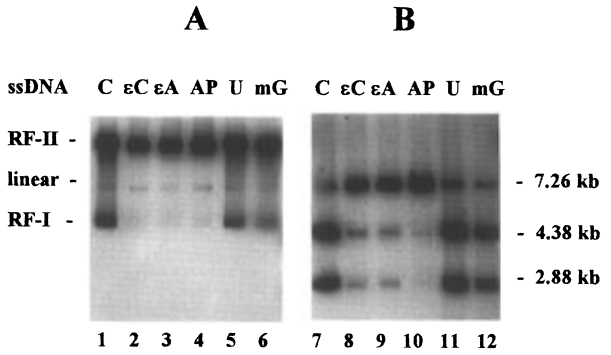

FIGURE 5: Effects of lesions on the replication of M13 ssDNA by *E. coli* KH2R (Δ*recA*) cell extract. (A) Autoradiograph of an agarose electrophoretic gel on which replication products with different ssDNA templates were analyzed. C, control DNA construct containing normal cytosine in place of the lesion; ϵC, ϵA, AP, U, and mG, DNA constructs containing the corresponding site-specific lesions. The conditions for *in vitro* replication were the same as those given in the legend for Figure 4. The positions of RF-I, RF-II, and RF-III (linear) DNAs are indicated at left. (B) The replication products shown in panel A were digested by *Ava*II and *Eco*R47III (37 °C for 1 h with a 10-fold enzyme excess) before analysis on agarose gel electrophoresis. The length of each band is extrapolated from a "1 kb marker DNA ladder" coelectrophoresed on the same gel (not shown).

(control) M13 ssDNA as a template (see Figure 3). When the smaller pLitmus28 ssDNA genome (a phagemid containing M13 replication origin; 2.6 kb) was used as the template, about 60% the replication products were RF-I (Figure 3).

Replication of Lesion-Containing ssDNA Templates. The strategy used for constructing lesion-containing M13 ssDNA (*13*) uses a 57-mer oligonucleotide as a scaffold at the DNA ligation step. The scaffold is later removed by denaturation and dilution before transfection into competent *E. coli* cells. Because the 57-mer scaffold is complementary to the lesion-containing region of the ssDNA construct, any residual scaffold may serve as a primer for DNA synthesis *in vitro* and confound the analysis of *de novo* translesion synthesis. To ensure that replication initiation on the template DNA occurred predominantly at the minus-strand origin by mechanisms similar to those prevailing *in vivo*, it proved necessary to completely remove the scaffold from the constructed ssDNA template. We achieved this result by heat-denaturing the ssDNA construct in the presence of an anti-57-mer oligonucleotide immediately followed by passage through a Qiagen tip-20 resin column. Oligonucleotides were washed out from the column with a buffer containing 1.0 M NaCl at pH 7.0, and the ssDNA construct (approximately 7300 nt long) was eluted with a buffer containing 1.25 M NaCl at pH 8.5. The efficiency of DNA recovery in this procedure ranged from 30% to 50%.

Because initiation of replication at the M13 minus-strand origin requires RNA polymerase to synthesize an RNA primer, initiation of replication of M13 ssDNA is sensitive to rifampicin, an inhibitor of RNA polymerase. The data in Figure 4 show that DNA synthesis on M13 reference ssDNA (M13 mp7L2, lane 6), and on DNA constructs purified as above (ϵC-DNA, lane 10; and the control, or C-DNA, lane 9), was undetectable in the presence of rifampicin (100 μg/mL). In contrast, only about half the amount of DNA synthesis was suppressed by rifampicin on unpurified C-DNA templates (lane 7, with rifampicin, vs lane 2, no rifampicin). Rifampicin failed to inhibit DNA synthesis on unpurified ϵC-DNA (lane 8, with rifampicin, vs lane 3, no rifampicin). These data suggest that scaffold-removal techniques used in transfection experiments (*4*−*6*, *13*, *14*) although adequate for *in vivo* experiments, are inadequate for an *in vitro* system. Adventitious replication initiation could be a potentially serious confounding factor in experiments where similar technologies are used for introducing site-specific lesions.

Efficiency of Translesion Synthesis. By using the system optimized as above and described under Materials and Methods, we tested *in vitro* replication of M13 ssDNA containing the following single, site-specifically located DNA lesions: O^6mG, dU, AP site, ϵA, and ϵC. In addition, an M13 ssDNA construct containing normal cytosine in place of the lesion (C-DNA) was prepared as a control. Figure 5A shows an analysis of the replication products from lesion-containing ssDNAs by *E. coli* KH2R (Δ*recA*) cell extracts. Replication of the control C-DNA (lane 1) produced maximal amounts of DNA synthesis products with approximately one-third of the products being RF-I. For the ssDNA construct bearing uracil (U-DNA) or O^6mG (O^6mG-DNA), a similar level of DNA synthesis was observed (lanes 5 and 6). For ϵC, ϵA, and AP lesion-containing DNAs (ϵC-DNA, ϵA-DNA, and AP-DNA, respectively), somewhat lower amounts of DNA were synthesized, but more significantly, most of the replication products were RF-II (lanes 2, 3, 4), indicating that these lesions might be blocking replication at the lesion sites.

Because in our ssDNA constructs the lesion is located near the terminus of replication (Figure 2), it is hard to distinguish (much less quantitate) gapped duplex DNA in which DNA synthesis has been arrested at the lesion site from RF-II DNA in which translesion synthesis has occurred. In both cases, the DNA is expected to have the mobility of form II DNA under commonly used conditions of gel electrophoresis, although a small difference can be seen in the gel represented by Figure 5A (*e.g.*, compare the RF-II bands in lanes 2−4 to that in lane 1). To measure translesion synthesis, we subjected the replication products to double digestion with the restriction endonucleases *Ava*II and *Eco*47III. As shown on the M13 DNA map in Figure 2, the size of RF DNA

Table 1: Efficiencies of Translesion Synthesis *in Vitro*[a]

E. coli strain	translesion synthesis (%) across					
	C	ϵC	ϵA	AP	dU	O^{6}mG
KH2R Δ*recA*	95 ± 4	18 ± 3	23 ± 5	9 ± 3	92 ± 5	89 ± 6
KH2 *recA*+	97.00	21.00	22.00	11.00	89.00	91.00
CJ231 Δ*polA*	92.00	19.00	ND	ND	ND	80.00
VP100 Δ*polB*	94.00	17.00	ND	ND	ND	87.00

[a] *In vitro* replication assays were carried out as described in the text using cell extracts from the strains shown. Replication products were subjected to the *Ava*II and *Eco*47III digestion assay as described. Each lane in the autoradiographs was scanned with a computing densitometer, and the percentage of translesion synthesis was calculated as described in the text. The data (±standard deviation) for the KH2R strain were averaged from three independent experiments. ND, not determined.

linearized (to RF-III) by a single cut is 7.26 kb, whereas a double cut will give rise to two fragments (4.38 kb and 2.88 kb) that are well-separated from each other in 1% agarose gels. The *Ava*II site can only be generated by DNA synthesis past the lesion site. A replication block at the lesion site will give rise to products that are uncleavable by *Ava*II. Therefore, translesion synthesis can be measured as follows: % translesion synthesis = [(4.38 kb + 2.88 kb)/(4.38 kb + 2.88 kb + 7.26 kb)] × 100.

An example of the endonuclease digestion analysis for translesion synthesis past different lesions is shown in Figure 5B. For C-DNA (control; lane 7), the fraction of replication products that passed the *Ava*II site is about 95%, suggesting that only a minor fraction of DNA molecules is incompletely replicated (or, less likely, selectively degraded). It is interesting that translesion synthesis for dU-DNA (lane 11) and for O^{6}mG-DNA (lane 12) is a similarly high 90%, suggesting that these lesions do not significantly impede replication. In contrast, the efficiencies of translesion synthesis across ϵC (lane 8) and ϵA (lane 9) are about 20%, indicating a significant blockage of replication by these lesions. The strongest inhibition was observed with AP-DNA (abasic site; lane 10), although we did find a minor fraction of the replication proceeding past this lesion. Table 1 gives a quantitative summary of data for the efficiency of translesion synthesis across different lesions averaged from three independent experiments. In addition, a similar experiment using extracts prepared from *E. coli* KH2 (*recA*+) cells was conducted, and the data showed that the efficiencies of translesion synthesis across the lesions examined here were very similar to those observed using the Δ*recA* cell extracts.

It is possible that in "blocked RF-II DNA" (*i.e.*, RF DNA bearing a gap due to blockage of DNA synthesis at the lesion), the single-stranded gap becomes vulnerable to endonucleases present in the cell-free extracts, resulting in an overestimation of translesion synthesis. However, gel analysis of "blocked DNAs" singly digested with *Eco*47III (but not with *Ava*II) did not reveal the formation of the two smaller fragments to a significant extent (data not shown).

Depletion of Pol-I or Pol-II Does Not Affect the Efficiency of Translesion Synthesis. In addition to pol-III, *E. coli* has two other DNA polymerases, pol-I and pol-II, encoded by the genes *polA* and *polB*, respectively. To test for a possible indispensable role for pol-I or pol-II in translesion synthesis observed in our system, we replicated lesion-containing ssDNAs using cell extracts prepared from *E. coli* strains defective for *polA* or *polB* genes. Figure 6 shows the results from *in vitro* replication of ϵC- and O^{6}mG-containing M13

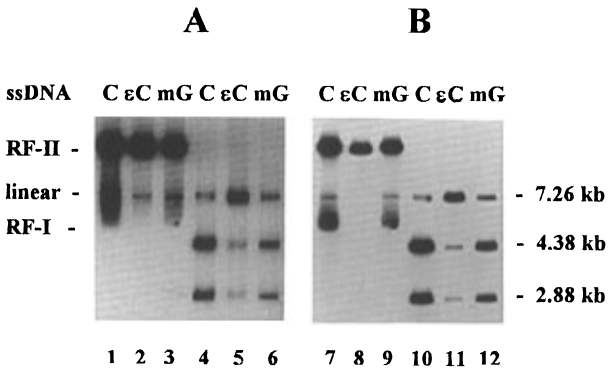

FIGURE 6: Effects of depletion of pol-I or pol-II on translesion synthesis. C-, ϵC-, and O^{6}mG-containing M13 ssDNA were replicated *in vitro* using the cell extracts prepared from *E. coli* strains CJ231 Δ*polA* (defective for pol-I; panel A) and VP100 Δ*polB* (defective for pol-II; panel B). Half of the replication products was analyzed directly on an agarose electrophoretic gel (lanes 1−3 and 7−9), and the other half was digested by *Ava*II and *Eco*47III before agarose gel electrophoresis (lanes 4−6 and 10−12). The positions of RF-I, RF-II, and RF-III (linear) DNAs are indicated at the left. The length of each band from endonuclease digestion assay is indicated at the right.

ssDNA by the cell extracts from *E. coli* CJ231 Δ*polA* (panel A) and VP100 Δ*polB* (panel B), and the quantitative data for the efficiency of translesion synthesis are given in Table 1. It is interesting that depletion of pol-I in *polA* cells results in RF-I molecules with apparently altered topology, as indicated by the reduced mobility of the "RF-I" fraction (see smears at the position between the bands for RF-III and RF-I DNAs; Figure 6A, lanes 1−3). However, the restriction endonuclease digestion assay (lanes 4−6) showed that the efficiency of translesion DNA synthesis was similar to that observed with *polA*+ cell extract (cf. Figure 5), suggesting that pol-I is not required for translesion synthesis. Similarly, translesion synthesis by Δ*polB* cell extracts (Figure 6B) was similar to that observed for *polB*+ cell extracts (cf. Figure 5), indicating that pol-II does not play an indispensable role in translesion synthesis in this system.

DISCUSSION

The aim of this work has been to develop an *in vitro* system in which M13 ssDNA containing single site-specific lesions is replicated by *E. coli* cell extracts with the ultimate goal of using such a system to identify and purify factors required for translesion synthesis. Building on other available systems for *in vitro* DNA replication, we have optimized conditions suitable for *E. coli* strains and DNA substrates that we have previously used for analyzing inducible mutagenic phenomena *in vivo*. The rifampicin sensitivity of DNA replication observed in our experiments with purified ssDNA constructs suggests that replication initiation *in vitro* occurs by a mechanism similar to that known to occur *in vivo*. These experiments also indicate an absolute requirement for a rigorous purification of template DNA to eliminate adventitious initiation events likely to be associated with DNA constructs bearing single lesions. The efficiency of DNA synthesis in our system is appreciable. The lack of complete conversion of RF-II DNA to RF-I may indicate limited access to a required enzyme such as DNA polymerase I, DNA ligase, or gyrase in the extract, or it may represent an equilibrium between synthetic and degradative activities present in the extract. Livneh and co-workers have also

developed *in vitro* translesion DNA synthesis systems based on cell extracts as well as purified proteins (*18*, *28*, *29*). The use of site-specific adduction technology, as well as the incorporation of a simple quantitative assay as described here, permits one to use the present system as a routine high-resolution assay for characterizing translesion DNA synthesis.

Using this system, we have determined the relative efficiencies of translesion synthesis across a collection of DNA lesions that includes examples of SOS-independent mispairing lesions (O^6mG and dU), SOS-dependent noninstructive lesions (AP site), and also class 2 noninstructive lesions (ϵC and ϵA). O^6mG, the archetypal mispairing lesion, is believed to be the most mutagenic of the modified bases formed by mutagenic alkylating agents. O^6mG can pair with thymine in addition to cytosine during DNA replication. The mispairing of O^6mG with T results in the almost exclusive induction of G-to-A transition mutations *in vivo* and *in vitro* (*30*, *31*). The template characteristics of O^6mG, as well as the known SOS-independence of O^6mG mutagenesis, suggest that O^6mG does not cause a strong block on DNA synthesis. Our data here confirm this expectation in that translesion synthesis occurs in 90% of the replication products. DNA uracil, arising from the deamination of DNA cytosine, is an example of a monospecific miscoding lesion in which the template instruction is invariably mutagenic, as contrasted to the dual coding ability of O^6mG. Our observation that the efficiency of DNA synthesis past dU is similar to that past a normal base is in good agreement with the expected efficient coding ability of uracil during DNA synthesis.

AP sites are believed to be among the most common of the spontaneous DNA lesions in all organisms. In addition, AP sites are also among the most common forms of DNA damage induced directly or indirectly by physical and chemical mutagens. AP sites exemplify the noninstructive class of lesions that by definition do not have *accessible* coding information, and are hypothesized to block the progression of replicative DNA polymerases. Translesion synthesis across such noninstructive lesions is hypothesized to require three proteins under SOS regulation, namely, RecA*, UmuD', and UmuC. The data presented here show a strong block to DNA replication by the single AP site such that <10% of the replication products appeared to have undergone translesion synthesis. If one applies a correction factor to compensate for the small fraction (about 5%) of control ssDNA molecules that is not fully replicated (*i.e.*, not fully cut by the restriction enzymes), the actual bypass efficiency may be slightly higher. However, the known *in vivo* effects of AP sites predict a translesion synthesis efficiency across AP sites of 1% or less. It is interesting that the efficiency measured here is comparable to the 10−15% efficiency estimated for translesion synthesis across AP sites by pol-III (*29*) *in vitro*. Whether the increased efficiency is an artifact of the *in vitro* assay or indicates the absence of a required but unidentified fidelity factor remains to be determined.

The exocyclic DNA lesions ϵC and ϵA cause a significant blockage to translesion synthesis compared to mispairing lesions (O^6mG and dU) or a normal base. Our observations indicate that bypass of these lesions occurs in about 20% of molecules replicated by cell extracts from *E. coli recA*+ as well as Δ*recA* strains. DNA excision repair cannot account for this level of bypass because the lesions are contained on ssDNA. However, we cannot exclude the possibility that the ϵC and ϵA lesions were repaired by an unknown (hypothetical) *nonexcisive* repair activity before replication occurs. If so, such a repair enzyme must be contained within the cell extracts used in our study. The data presented here are in reasonable agreement with *in vivo* observations in that transfection efficiencies of ϵC- and ϵA-ssDNA were in a range of 10−40% of that for control ssDNA in *recA*+ and Δ*recA* cells (*6*, *13*). Although we have not yet characterized the mutagenic effects of *in vitro* translesion synthesis at ϵC and ϵA, our results so far are consistent with the notion that ϵC and ϵA are class 2 noninstructive lesions. Because the *in vitro* replicative behavior of the model lesions used here parallels that deduced from *in vivo* experiments, the experimental system described in this paper appears to be a reasonable starting point for the investigation of inducible mutagenic phenomena such as UVM.

The *E. coli* pol-III complex is known to be required for replication of the genome, as well as for SOS mutagenesis (*32*). Normally, pol-I is thought to be responsible for excising the RNA primers used to initiate Okazaki fragments and for gap-filling DNA synthesis during DNA excision repair (*33*). Although *polB* is now known to be a DNA damage-inducible SOS gene, an indispensable cellular function is not established for pol-II. Recent studies suggest that pol-II might have a back-up function in that it is able to catalyze both replicative and repair DNA synthesis (*18*, *34*, *35*). The *in vitro* data presented here do not support a possible essential role for pol-I or pol-II in translesion synthesis. Therefore, pol-III appears to be the enzyme responsible for the translesion DNA synthesis observed in this system, but a possible contribution to translesion synthesis by other pol-I and pol-II cannot be ruled out. Whether pol-III is in fact the enzyme responsible for the translesion synthesis observed in this system and, if so, what form of the enzyme is involved are questions that we hope to address in the future.

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