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Influence of Template Strandedness on in Vitro Replication of Mutagen-Damaged DNA†

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ABSTRACT: We analyzed the ability of DNA polymerases to bypass damage on single- and double-stranded templates. In vitro DNA synthesis was studied on UV-irradiated and polyaromatic hydrocarbon reacted (benzo[*a*]pyrenediol epoxide and oxiranylpyrene) double-stranded templates by a protocol involving initiation on a uniquely nicked circular double-stranded template. The template was prepared by treating single-stranded (+)M13mp2 circular strands with mutagen and then hybridizing with restricted M13 RFmp2, followed by isolation of the nicked RFII forms. The protocol permits either (+), (−), or both strands to carry lesions. We found that the rules for termination and bypass of lesions previously observed with single-stranded DNA templates also hold for double-stranded templates. Termination of synthesis occurs primarily one nucleotide 3′ to the lesion in the template strand. Bypass of UV-induced lesions can be followed in a series of three partial reactions in the presence of Mn^{2+} and dGMP, which relax the specificity of nucleotide insertion and 3′ → 5′ exonuclease activity, respectively. There is no evidence for greater permissivity of bypass in double- as opposed to single-stranded templates. As with single-stranded templates, purines and preferentially deoxyadenosine (dA) are inserted opposite lesions. Lesions in the nontemplate strand elicit neither termination nor pausing. The addition of Rec A protein resulted in a measurable increase of bypass in this system.

Termination of DNA synthesis as a result of lesions introduced into the template by mutagenic and/or carcinogenic agents has now been studied in a variety of systems [see Strauss (1985) for review]. For almost all of these cases a set of rules can be formulated, indicating just where the majority of terminations will occur. Most of the lesions previously studied result in termination exactly one nucleotide prior to (3′ to) the lesion on the template strand, especially when polymerases with active 3′ → 5′ exonuclease proofreading functions are utilized. Exceptions do occur but these often are related to the special stereochemical properties of the altered nucleotide [e.g., Moore et al. (1981), Clark and

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Beardsley (1986), Hayes and LeClerc (1986), and Ide et al. (1985)].

The majority of published studies utilize single-stranded templates primed with either a restriction fragment or a relatively short oligonucleotide (Strauss, 1985). The relationship between this system and in vivo DNA replication is not clear since most DNA structures are double stranded and the structure of the growing point still defies precise description. It might therefore be supposed that different rules govern both synthesis and termination on double-stranded templates. In fact, there have been reports of differences between synthesis on double- and single-stranded DNA. O'Connor and Stohrer (1985) used nicked double-stranded oligonucleotides and primed single-stranded oligonucleotides to replicate a specifically placed guanyl 8-aminofluorene residue. They report bypass of the lesion by *Escherichia coli* Pol I[1] (large fragment) with more bypass occurring in a strand displacement mode of synthesis. Similarly, Piette and Hearst (1983) concluded that *E. coli* Pol I "is able to bypass psoralen monoadducts when nick-translating double-stranded DNA. When situated on the template strand, the monoadducts act only as attenuators of the rate of synthesis". Since much evidence indicates that aminofluorene [as opposed to (acetylamino)fluorene] adducts are not absolute blocks to DNA synthesis (Lutgerink et al., 1985) and since Piette and Hearst (1983) also report that non-cross-linked psoralen adducts are not absolute blocks to DNA synthesis, we decided to test the termination behavior of DNA containing UV-induced lesions. These lesions block DNA chain progression in vivo (Berger & Edenberg, 1986) and are absolute blocks to DNA synthesis in vitro (Moore & Strauss, 1979; Chan et al., 1985). In this study we constructed templates with UV and polyaromatic hydrocarbon induced lesions and studied their behavior as templates in both single- and double-stranded configurations. For this purpose, double-stranded templates were constructed with a unique nick at the *Eco*RI, *Bgl*I, or *Pvu*I sites of bacteriophage M13mp2. Lesions were selectively placed in either the template or complementary strand. Five questions have been addressed: (1) Are the basic "rules" for termination on damaged, double-stranded templates the same as those for synthesis on damaged, single-stranded templates? (2) Are the bands seen at the site of DNA damage pause sites or are they permanent arrest sites? (3) What is the effect of a lesion in the nontemplate strand on DNA synthesis? (4) What nucleotides are added opposite lesions when the polymerase is induced to make an insertion? (5) If DNA polymerase can be induced to bypass a template lesion, will this bypass synthesis be influenced by the structure of the template? We have been able to induce bypass of lesions in double-stranded DNA with *E. coli* DNA polymerase I, but only by following the same rigorous protocol required for bypass on single-stranded templates (Rabkin et al., 1983).

Materials and Methods

Reagents. Deoxynucleoside triphosphates (HPLC purified) were obtained from Pharmacia; [α-^{32}P]dNTPs were from Amersham. Oligonucleotide sequencing primers were purchased from New England Biolabs. Matrices for DNA separation and purification were purchased as follows: Ultrapure (DNA grade) agarose, Bio-Gel A1.5 agarose (100–200 mesh), and P6 acrylamide (200–400 mesh) were from Bio-Rad; Sepharose 4B was from Pharmacia. [^{3}H]-*anti*-(+)-*r*-7,*t*-8-Dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (benzo[*a*]pyrenediol epoxide, BPDE) and [^{3}H]-1-pyrenyloxirane (oxiranylpyrene, ORP) were generously supplied by the NCI Radiochemical Repository/Midwest Research Institute.

Enzymes. Restriction endonucleases were supplied by New England Biolabs (*Eco*RI, *Pvu*I, and *Bgl*I) and Boehringer Mannheim (*Eco*RI). *E. coli* DNA polymerase I (endonuclease free) was purchased from Boehringer Mannheim, polymerase I Klenow fragment (Kf) from Boehringer Mannheim and Pharmacia, and avian myeloblastosis virus reverse transcriptase from Life Sciences. Rec A protein was supplied by K. McEntee. A recent assay of the ATPase activity of this preparation gave a value of 0.56 unit/mg.

Isolation of M13mp2 DNA. The phage strain used was an M13mp2 strain without amber mutations. *E. coli* K12 hosts were JM103 or CSH50F'*traD36 proAB lacIqZcM15* constructed by E. LeClerc. Phage and bacterial stocks were generously provided by E. LeClerc. Viral strand and replicative form I M13mp2 DNA were prepared by a modification of the procedure suggested by Messing (Sagher & Strauss, 1983).

Construction of Uniquely Nicked, Double-Stranded Templates. The priming strand was created by treating replicative form I M13mp2 DNA with *Eco*RI or *Bgl*I (New England Biolabs), which cleaves the molecule only once. Cleavage was carried out under the conditions recommended by the supplier. Template DNA was purified from contaminating circular viral strands and from linear molecules that had reannealed to form RFIII as described by Larson et al. (1985).

Preparation of Oligonucleotide Primed Single-Stranded Templates. A 17-mer sequencing primer, 5'-GTAAAACGACGGCCAGT-3', was purchased from New England Biolabs. The primer is complementary to M13mp2 viral-strand DNA adjacent to the unique *Eco*RI site. Sequencing primer and viral-strand DNA were incubated with 100 mM KCl at 75 °C and then at room temperature for 20 min. The molar ratio of primer to viral strand was 15 to 1. Hybridizations were carried out at a viral-strand concentration of 83 μg/mL.

Construction of Templates for Runoff Assays. Circular, double-stranded M13mp2 RFI molecules were cleaved with *Bgl*I (New England Biolabs) under conditions suggested by the supplier. The full-length linear (RFIII) product was phenol/chloroform and ether extracted and then precipitated with sodium acetate/ethanol. DNA was resuspended in 10 mM Hepes/1 mM EDTA, pH 8.0 (HE buffer). *Bgl*I-linearized DNA was denatured at 100 °C for 5 min and quenched on ice. Viral-strand DNA was added and the salt concentration adjusted to 100 mM KCl before hybridization at 65 °C for 30 min with subsequent slow cooling. The product of the hybridization was cut with *Pvu*I (New England Biolabs) by the supplier's recommended procedure to yield a full-length linear molecule with a unique nick at the *Bgl*I site of M13mp2. DNA was chloroform/phenol extracted, ether extracted, and sodium acetate/ethanol precipitated. Recovered DNA was suspended in 10 mM Hepes/1 mM EDTA, pH 8.0.

Reaction of DNA with Benzo[a]pyrenediol Epoxide and Oxiranylpyrene. M13mp2 viral-strand DNA was reacted at

[1] Abbreviations: *E. coli* Pol I (Kf), *Escherichia coli* polymerase I Klenow (large) fragment; AMV rt, avian myeloblastosis virus reverse transcriptase; BPDE, *anti*-(+)-*r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (benzo[*a*]pyrenediol epoxide); ORP, 1-pyrenyloxirane (oxiranylpyrene); dGMP, 2'-deoxyguanosine 5'-phosphate; dA, deoxyadenosine; HPLC, high-performance liquid chromatography; dNTP, unspecified deoxynucleoside 5'-triphosphate; RF, replicative form; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

a concentration of 500 μg/mL in 10 mM Hepes at pH 8.0. Reaction was for 1 h at 37 °C with 421 mCi/mmol oxiranylpyrene (ORP) or 398 mCi/mmol benzo[*a*]pyrenediol epoxide (BPDE). Unreacted ORP and BPDE were removed from samples by extraction 6 times with water-saturated butanol. DNA was precipitated once with sodium acetate/ethanol and resuspended in 10 mM Hepes/1 mM EDTA. Quantitation of radioactivity fixed in DNA indicated the presence of between 60 and 130 adducts per molecule depending on the particular preparation.

Treatment of DNA with Ultraviolet Light. Viral strand or linearized, full-length double strands (RFIII) were irradiated with ultraviolet light from a GE germicidal lamp at a dose rate of 2 J m^{-2} s^{-1} to a total dose of 1 kJ. DNA was irradiated at a concentration of 100 μg/mL in HE buffer.

DNA Polymerase Reactions. For time course studies, reactions contained 1.0 μg of nicked, double-stranded DNA, 0.6 μM each of [^{32}P]dATP, -dGTP, -dCTP, and -dTTP (sp act. 400 μCi/mmol each), and 1.4 μM each of dATP, dGTP, dCTP, and dTTP in standard magnesium reaction buffer (50 mM Hepes, pH 8.0, 8 mM $MnCl_2$, 5 mM dithiothreitol), 100 mM KCl, and polymerase in a total volume of 52.5 μL. Two units of *E. coli* polymerase I or polymerase I (Kf) or 60 units of AMV reverse transcriptase was used. Reactions were incubated at 37 °C (or 42 °C for AMV rt) and 7.5-μL aliquots removed after 2, 5, and 10 min. dNTP concentrations were adjusted to 100 μM each, and incubation was continued. Aliquots were removed after 5, 15, 30, and 45 min. Reactions were stopped by heat inactivation of the polymerase at 65 °C for 10 min. *Eco*RI was added, and samples were incubated for 45 min at 37 °C. For runoff assays, reactions contained 0.28 μg of DNA, 0.33 units of endonuclease-free polymerase I, 50 mM Hepes, pH 8.0, 100 mM KCl, 8 mM $MnCl_2$, 5 mM DTT, and 1.2 μM each of [α-^{32}P]dNTP. DNA was incubated for 15 min at 37 °C, then all four unlabeled dNTPs were aded to 100 μM, and incubation was continued for 15 min. Polymerase was inactivated at 65 °C for 10 min. Additions were present at the following concentrations: dGMP, 10 mM; dAMP, 10 mM; ATP, 3 mM; Rec A protein, 0.28 mg/mL; ssB, 4 μM.

Two- and Three-Stage Reactions. Stage 1 reactions contained 1 pmol of uniquely nicked, double-stranded DNA or primed, single-stranded DNA, 4 units of *E. coli* polymerase I large fragment (Kf), standard magnesium reaction buffer, 28 mM KCl, and 40 μCi each of [α-^{32}P]dNTP (400 Ci/mmol) in a volume of 70 μL. Labeling was for 30 min at 30 °C; all four dNTPs were then added to 100 mM, and incubation was continued for 10 min before reactions were stopped with the addition of $^1/_{16}$ volume of 250 mM EDTA/saturated sodium pyrophosphate stop solution. tRNA carrier was added, and nucleic acids were precipitated with sodium acetate/ethanol. After suspension in 10 mM Hepes/1 mM EDTA, pH 8.0, high molecular weight DNA was separated from nucleotides on a 1-mL P6 column. DNA was precipitated with sodium acetate/ethanol and resuspended in 10 mM Hepes, pH 8.0. Stage 2 reactons were run in standard manganese buffer (50 mM Hepes, 0.5 mM $MnCl_2$, 5 mM DTT, pH 8.0) with 28 mM KCl and 500 μM dATP, dGTP, dCTP, or dTTP added individually or 500 μM dATP if a stage 3 reaction was to be performed. Synthesis was for 10 min at 30 °C after which time the polymerase was heat inactivated at 65 °C for 10 min and DNA restricted with *Eco*RI for 45 min at 37 °C. If a third-stage reaction was to be carried out, reactions were terminated with EDTA/sodium pyrophosphate and DNA was precipitated with sodium acetate/ethanol. DNA suspended in 10 mM Hepes was purified from nucleotide (dATP) on a P6 column. Additions to individual two-stage reactions were as described above for runoff assays. Individual third-stage reactions were run in standard magnesium or manganese buffer with 28 mM KCl, 10 mM dGMP, 500 μM dATP, dGTP, dCTP, or dTTP, added individually or in combination, and 0.3 unit of polymerase I (Kf). Synthesis was for 10 min at 30 °C. The polymerase was heat inactivated as above. Final DNA products were restricted with *Eco*RI at 37 °C for 45 min after the KCl concentration was increased to 100 mM.

Polyacrylamide Gel Electrophoresis and Quantification. Electrophoresis was carried out as previously described (Sagher & Strauss, 1983). Autoradiograms were scanned with a Gilford 250 spectrophotometer with a linear transport attachment. Integration of peaks was accomplished by a Varian 401 computer. The linearity of the film response was determined by scanning several timed exposures. Alternatively, autoradiograms were used to locate bands on gels that were excised, and the radioactivity was quantified in a scintillation counter by the Cherenkov method (Kobayashi & Maudsley, 1974).

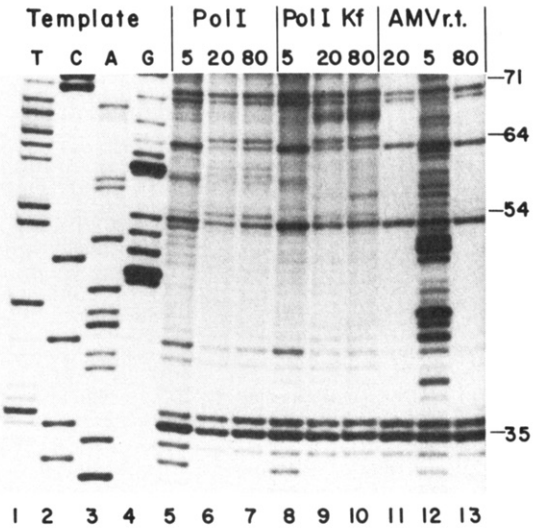

FIGURE 1: DNA synthesis on UV-treated double-stranded template catalyzed by *E. coli* polymerase I (holoenzyme), polymerase I (Kf), or AMV reverse transcriptase. DNA templates were irradiated to a total dose of 1 kJ. Polymerase reactions contained 0.1 μg of DNA, 0.4 unit of Pol I (holoenzyme), 0.4 unit of Pol I (Kf), or 8 units of AMV reverse transcriptase in 50 mM Hepes, pH 8.0, 8 mM $MgCl_2$, and 5 mM DDT. KCl concentrations were 20 mM for Pol I reactions and 100 mM for reactions containing AMV reverse transcriptase. Reactions initially contained 0.6 μM each of [α-^{32}P]dATP, dGTP, dCTP, dTTP and were incubated for 10 min before nucleotide concentrations were adjusted to 100 mM. Incubation was at 30 °C for Pol I or 35 °C for AMV reverse transcriptase. Reactions were terminated by incubation at 65 °C for 10 min, 5, 20, or 80 min after nucleotide concentrations were raised. The reaction products were digested with *Eco*RI and analyzed by electrophoresis on 14% polyacrylamide/8 M urea gels. The sequence studied was

$^{3}_{1}5$ $^{5}_{1}4$ $^{6}_{1}4$ $^{7}_{1}1$
3'-ACTTTAACAATAGGCGAGTTAAGGTGTGTTGTATGCTCGGCCTTCG-5'

RESULTS

The Basic Rules for Synthesis on Damaged, Double-Stranded Templates Are the Same as for Single-Stranded Templates. When DNA synthesis was catalyzed by *E. coli* Pol I on a UV-treated, double-stranded template, termination bands were observed one nucleotide prior to (3′ to) a presumptive pyrimidine dimer (Figure 1, lane 7, positions 34–36, 53, 63, 70 and 71). This is the common finding for three enzymes, Pol I holoenzyme (Figure 1, lane 7), Pol I (Kf)

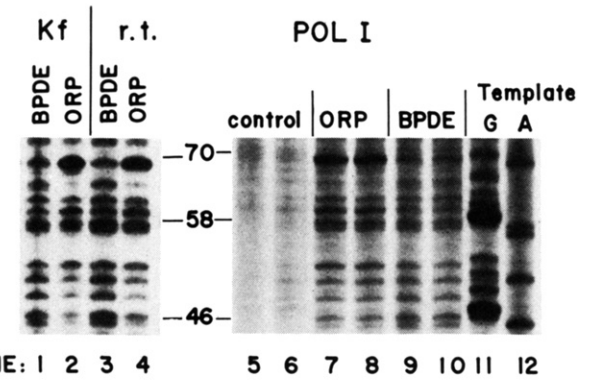

FIGURE 2: DNA synthesis on benzo[*a*]pyrenediol epoxide (BPDE) or oxiranylpyrene (ORP) treated double-stranded templates catalyzed by *E. coli* polymerase I (holoenzyme), polymerase I (Kf), or AMV reverse transcriptase. Synthesis was from the unique *Eco*RI site on M13mp2. Templates were reacted with BPDE or ORP as described under Materials and Methods. Polymerase reactions contained 0.1 μg of DNA, 0.2 unit of Pol I (holoenzyme), 0.2 unit of Pol I (Kf), or 25 units of AMV reverse transcriptase in 5 mM Hepes, pH 8.0, 8 mM $MgCl_2$, 5 mM DDT, and 20 mM KCl. Reactions initially contained 0.6 μM [α-^{32}P]dTTP and 50 μM each of dATP, dGTP, and dCTP and were incubated at 30 °C for 15 min before unlabeled dTTP was added to 50 μM. Reactions were terminated after an additional 15-min incubation by heating at 65 °C for 10 min. The reaction products were digested with *Eco*RI and analyzed by electrophoresis on 15% polyacrylamide/8 M urea gels. The sequence studied was

$$\overset{46}{\underset{}{}}\qquad\overset{58}{\underset{}{}}\qquad\overset{70}{\underset{}{}}$$
3'-AGGCGAGTGTTAAGGTGTGTTGTATG-5'

(Figure 1, lane 10), and AMV reverse transcriptase (Figure 1, lane 13). Pol I and Pol I (Kf) have a tendency to insert a nucleotide opposite the first (3′) nucleotide in a presumptive dimer (positions 54 and 64, lanes 7 and 10). Band densities at these two positions are approximately equal to that seen in the band just prior to the dimer. Termination of synthesis by Pol I prior to (3′ to) a reacted nucleotide is also observed on double-stranded templates treated with oxiranylpyrene (ORP) and benzo[*a*]pyrenediol epoxide (BPDE), respectively (Figure 2, lanes 7 and 9). For BPDE-reacted templates, bands appear at positions 45–48, 52, 57–59, 62, 65, and 69, one nucleotide prior to each guanine in the template strand (Figure 2). The band at position 69 consistently contains more radioactivity when the template lesion is a result of reaction with ORP whereas a band at position 65 is often missing on such templates. Similar results are obtained with Pol I (Kf) (Figure 2, lanes 1 and 2) and AMV reverse transcriptase (Figure 2, lanes 3 and 4). To examine further the discrepancies between BPDE and ORP treatment of DNA, a second set of templates was constructed with the start for DNA synthesis at a new point along the M13 template (Figure 3). AMV reverse transcriptase produces termination bands 3′ to guanine in the template at positions 48, 51, and 56 on ORP-treated double- or single-stranded templates (Figure 3). When DNA synthesis occurs on templates that have been treated with BPDE, whether double- or single-stranded, a fourth band at position 53 appears. The new band is either one nucleotide 3′ to or opposite a thymidine in the template. Synthesis by Pol I (Kf) also results in the same pattern of bands whether templates are double- or single-stranded (gapped) molecules. In summary, for both UV irradiation induced pyrimidine dimers and at large bulky adducts resulting from reaction of guanine with BPDE or ORP, termination of synthesis at lesions shows the same pattern on double- and single-stranded templates.

Are Blocks Permanent Arrest Sites? The bands resulting from synthesis by DNA polymerases on damaged templates could represent pausing at lesions or complete termination of

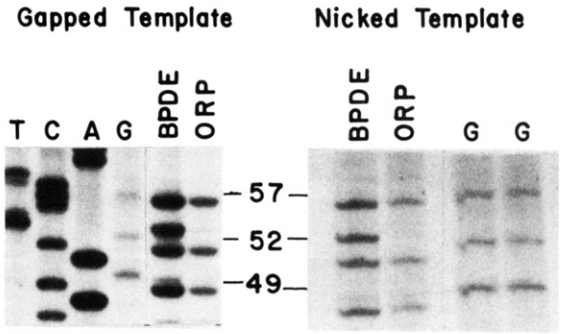

FIGURE 3: DNA synthesis on benzo[*a*]pyrenediol epoxide (BPDE) or oxiranylpyrene (ORP) treated nicked, double-stranded templates or gapped templates. Synthesis by AMV reverse transcriptase was from the unique *Pvu*I site on M13mp2. The gapped region extended 123 bases from the *Pvu*I site to the *Eco*RI site. Reaction conditions were as described in Figure 2 except digestion of products was with *Pvu*I. The sequence shown is

$$\overset{49}{\underset{}{}}\qquad\overset{52}{\underset{}{}}\qquad\overset{57}{\underset{}{}}$$
3'-CACGACGTTCCGC-5'

synthesis. If the blocks observed are pauses and not arrest sites, radioactivity should be lost from bands on the gel over time or under other conditions favorable for bypass. We allowed *E. coli* Pol I and AMV reverse transcriptase catalyzed synthesis to occur on UV-treated double-stranded templates for various time periods and with different nucleotide concentrations (Figure 4). Lanes 3–5 of Figure 4A show synthesis after 2, 5, or 10 min at 2 μM dNTP concentration. After 2 min of synthesis, bands appear before the expected pyrimidine dimer sites close to the primer (positions 35–38, 54, 64, 71, and 72). A light band also appears at position 33, two nucleotides prior to a dimer site. Further from the primer, termination bands are observed on both UV-treated and undamaged templates and do not correlate with the sites of predicted dimers. After 5 or 10 min of synthesis (Figure 4A, lanes 4 and 5) bands appear one nucleotide 3′ to each position at which a presumptive lesion occurs (positions 34–36, 53, 63, 70, 71, 75–78, 83–85, 90–92). The band at position 33, two nucleotides 3′ to a dimer, also remains in these lanes. Synthesis was allowed to continue for 10 min with 2 μM nucleotides, before the nucleotide concentration was adjusted to 100 μM. Lane 6 represents synthesis for 5 min at the higher nucleotide concentration. The bands now assume the pattern described earlier for UV-treated templates. Bands appear one nucleotide 3′ to each presumptive dimer (positions 34–36, 53, 63, 70, 71, 75–78, 83–85, and 90–92), at positions opposite the 3′ nucleotide in a dimer (positions 54 and 64), and the band two nucleotides prior to a dimer disappears (position 33). The pattern is maintained through 45 min of synthesis at the higher nucleotide concentration. Time course experiments repeated with AMV reverse transcriptase give results that differ only in detail from experiments performed with Pol I (Figure 4B). Synthesis through the region examined was slower in the case of the reverse transcriptase (compare lanes 3 and 4 of Figure 4A with lanes 5 and 6 of Figure 4B). Bands were never present at position 33, two nucleotides 3′ to a dimer, nor did the polymerase insert a nucleotide opposite the 3′ member of a dimer (positions 54 and 64).

Quantification of the time course experiments described above was achieved by densitometric scanning (Table I). Three experiments were averaged for each polymerase; the lanes for the 5- and 45-min reactions at 100 μM dNTP concentration were scanned in every case. There was no consistent change in the distribution of radioactivity when synthesis at the higher nucleotide concentration was compared after 5 and

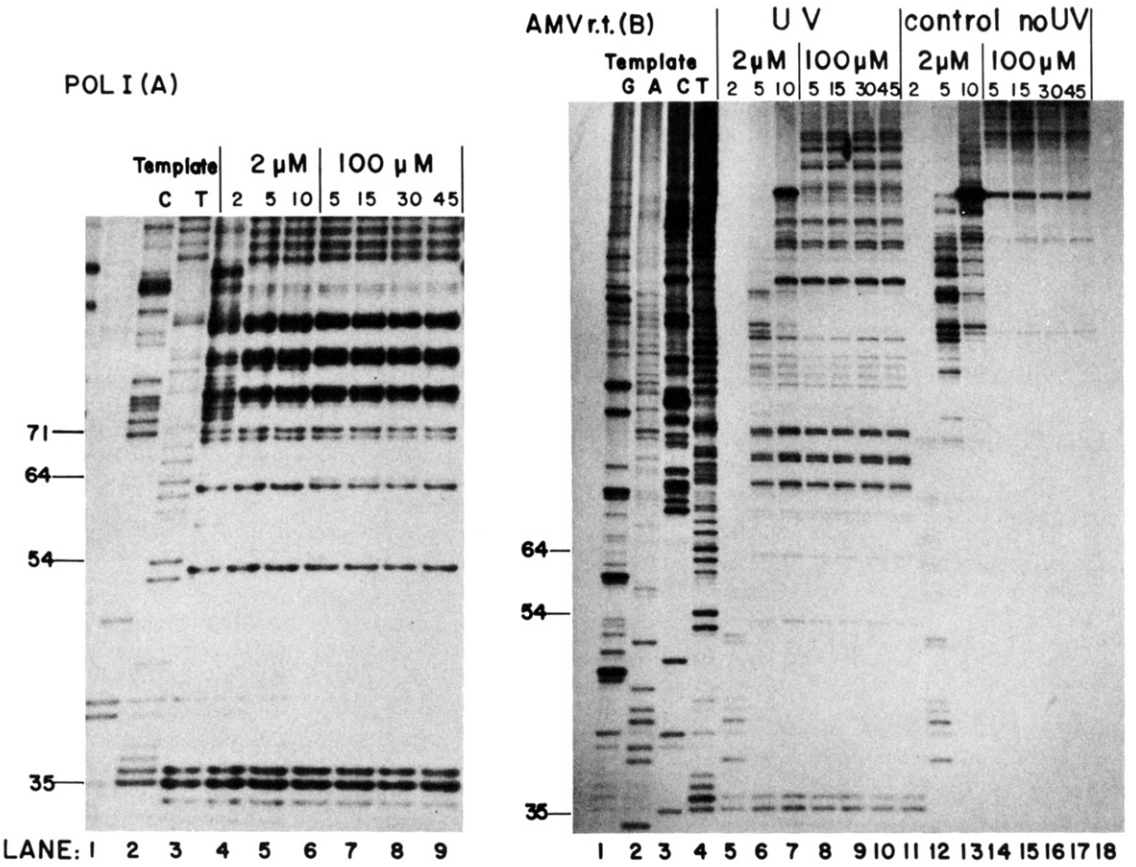

FIGURE 4: Effect of incubation time and nucleotide concentration on arrest of DNA synthesis at UV-induced lesions on double-stranded templates by *E. coli* polymerase I (holoenzyme). Polymerase reactions contained 1.2 μg of DNA, 2 units of Pol I (holoenzyme), 0.6 μM [α-^{32}P]dATP, dGTP, dCTP, and dTTP, and 1.4 μM unlabeled nucleotides in 50 mM Hepes, pH 8.0, 8 mM $MgCl_2$, 5 mM DDT, and 100 mM KCl in a volume of 52 μL. Aliquots were removed 2, 5, and 10 min after incubation at 37 °C. Nucleotide concentrations were raised to 100 μM and aliquots removed after an additional 5, 15, 30, and 45 min. All reactions were terminated by heating at 65 °C for 10 min. After digestion with *Eco*RI, products were analyzed on 14% polyacrylamide/8 M urea gels. The sequence used is given in the legend to Figure 1. (A) *E. coli* DNA polymerase I; (B) AMV reverse transcriptase.

Table I: Quantitation of the Effect of Incubation Time on Arrest of DNA Synthesis at UV-Induced Lesions on Double-Stranded Templates[a]

band	a	b	c	d
34	5.3 ● 0.9	5.0 ± 0.8	4.0 ± 0.8	5.0 ± 0.8
35	31.7 ± 2.1	32.3 ± 2.5	33.0 ± 1.4	36.3 ± 0.5
36	20.7 ± 0.5	22.0 ● 2.2	20.0 ● 0.8	20.6 ± 1.7
54	14.7 ± 0.5	14.6 ± 1.2	13.7 ± 1.2	13.6 ± 1.2
64	12.0 ± 0	11.6 ± 0.9	13.0 ± 0.8	11.0 ± 1.4
71	15.0 ± 0.8	14.0 ± 2.2	15.3 ± 1.2	14.6 ± 1.7

[a] Densitometric scans were made of the gels represented in Figure 4. a is the scan of products of synthesis after 5 min at 100 μM nucleotide concentration with Pol I (holoenzyme); b is the scan of reaction products after 45 min; c is the scan of products of synthesis after 5 min at 100 μM nucleotide concentration with AMV reverse transcriptase; d is the scan of reaction product after 45 min. The area under the peaks listed was totaled and set at 100%. The percent of the total radioactivity contained in the individual peaks were calculated, and the results are listed above. Three experiments were scanned for each polymerase.

45 min. To the extent that analysis is possible in this system, pyrimidine dimers appear to be permanent arrest sites for DNA synthesis rather than sites for polymerase pausing.

Effects of Lesions in the Displaced Strand. Pyrimidine dimers are known to block the activity of exonuclease III (Royer-Pokora et al., 1981) but do not seem to affect the activity of the 5′ → 3′ exonuclease activity of *E. coli* Pol I as measured by release of labeled nucleotides from UV-irradiated double-stranded DNA (Kelly et al., 1969). Templates were constructed that contained UV lesions in either the template or nontemplate strand. Synthesis by Pol I on uniquely nicked templates generates the expected pattern of termination bands when the template strand contains UV lesions (Figure 5, lanes 5–7). Synthesis on nontreated DNA generates a few light bands, generally toward the top of the gel (lanes 11–13), which are at positions different from those where UV damage is expected. Light bands also appear in lanes 8–10, which represent synthesis on templates containing UV damage in the noncomplementary strand. These bands, when present, are always at positions where bands appear in the untreated control lanes. Pol I (Kf), like Pol I (holoenzyme), is not affected by lesions in the complementary strand; the results obtained for synthesis with AMV reverse transcriptase on these three templates are similar to those obtained for Pol I (data not shown). As might be expected, no blocks or pauses to DNA synthesis by Pol I or AMV reverse transcriptase are generated by lesions in the complementary strand.

Two-Stage Reactions. The data above indicate that the general rules governing termination of synthesis at template lesions in single-stranded DNA hold also for double-stranded DNA. Termination generally occurs one nucleotide 3′ to a lesion, although termination occurs opposite some lesions in a site-specific manner. It is possible to determine which nucleotide is added opposite a lesion at sites where this behavior is noted (Moore et al., 1982). Polymerase reactions are run in the presence of all four nucleotides. The reaction products are then isolated by passage through a Bio-Rad A1.5 column and used as substrate for a second series of reactions. This time the reactions are run in the presence of the individual nucleotides. The specific nucleotides that promote elongation

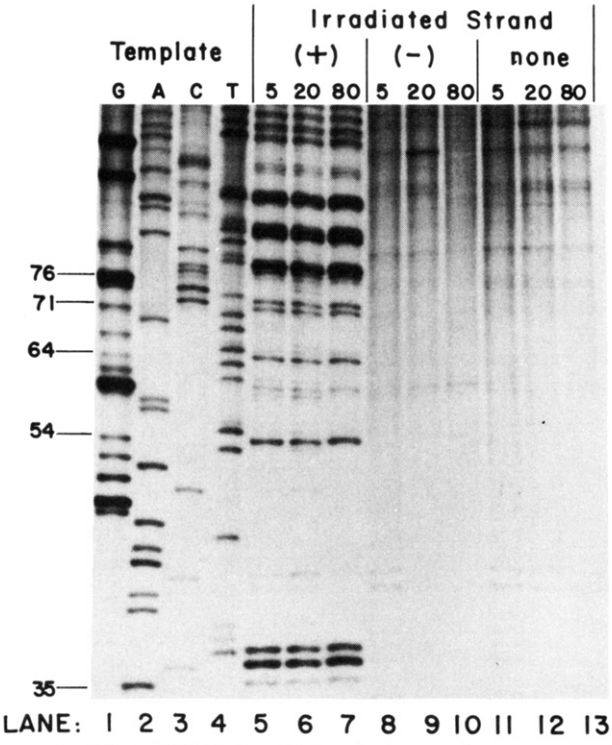

FIGURE 5: Effect of UV-induced lesions in the complementary strand on *E. coli* polymerase I (holoenzyme) catalyzed DNA synthesis. Double-stranded molecules contained lesions in the template strand only or in the complementary strand only or were not treated. Polymerase reactions contained 0.1 μg of DNA, 0.3 unit of Pol I (holoenzyme), 50 mM Hepes, pH 8.0, 8 mM $MgCl_2$, 5 mM DDT, 100 mM KCl, and 0.6 μM each of [α-^{32}P]dATP, dGTP, dCTP, and dTTP. Reactions were incubated at 30 °C for 10 min, nucleotide concentrations adjusted to 100 μM and incubations continued for an additional 5, 20, or 80 min before being terminated by heating at 65 °C for 10 min. The template strand used is shown in the legend to Figure 1. (+), template strand UV irradiated; (-), (-) strand UV irradiated; none, unirradiated control.

of the template to a position opposite a lesion can then be identified. With single-stranded DNA, dATP promotes most elongation on damaged templates (Rabkin et al., 1983). However, the results depend on the particular cation present and on the 3′ → 5′ proofreading exonuclease activity as affected by the presence of dAMP. Double-stranded, nicked, irradiated DNA, used as a substrate for DNA synthesis, with termination bands just prior to putative dimers (Figure 6, lanes 5 and 18, positions 34–36, 53, 63, 70, and 71) was used as a substrate for a second-stage reaction. This substrate also produced a termination site at position 44 opposite a T in an ATA sequence also seen in control (nonirradiated) reactions (see Figure 5). With Mg^{2+} as a cation and no dAMP, Pol I (Kf) was able to elongate the substrate at only one site and to a very limited extent where a faint band can be seen at position 64, opposite the 3′ member of the dimer at positions 64–65 (lane 11). There was no elongation to position 54, opposite the 3′ member of the dimer at positions 54–55, although the sequences immediately 3′ to these two T-T dimers are identical [3′-GTG(T54)TAAG vs. 3′-GTG(T64)TGTA]. The substitution of Mn^{2+} for Mg^{2+} and the addition of dAMP resulted in complete elongation of substrate to a position opposite the 3′ member of the dimer at five sites (lane 12). The termination band at position 34 disappeared almost completely, elongation from positions 53 to 54, 63 to 64, and 70 to 71 was complete. In addition, band 35 lost radioactivity. Partial elongation was obtained either when Mn^{2+} was substituted for Mg^{2+} (lane 10) or when dAMP was added in the presence of Mg^{2+} (lane 17). Radioactivity disappeared from band 34,

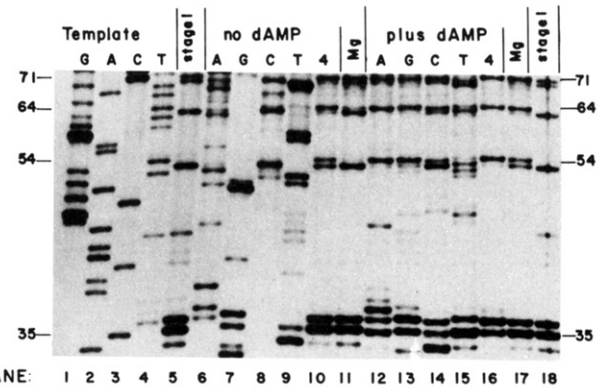

FIGURE 6: Identity of nucleotides added opposite UV-induced lesions. Two-stage polymerase reactions are described in the text. All reactions were carried out in the presence of manganese except where labeled Mg. Magnesium was substituted for manganese in these lanes. The dAMP (lanes 12–18) concentration was 10 mM. A, T, G, or C denotes the nucleoside triphosphates added to reactions individually; 4 denotes the presence of all four dNTPs. The DNA sequence is as given in the legend to Figure 1.

about half the radioactivity in the termination band at position 53 appeared at position 54, virtually all the substrate that terminated at position 63 elongated to position 64, and the termination band at position 70 disappeared.

To determine whether all nucleotides were equally effective in mediating elongation, each nucleotide was added individually in a second round of synthesis in the presence of dAMP and with Mn^{2+} as the cation. When TTP was added as the sole dNTP, there was limited elongation at some sites (e.g., Figure 6, lane 15, position 64), degradation at others (from site 53 to site 52 or 51), and no change at others (positions 34–36). Addition of dCTP (Figure 6, lane 14) supported partial elongation at three sites. At one site, only degradation was observed (site 34 to site 33). dGTP (lane 13) induced complete elongation at those sites at which partial elongation had been obtained in the presence of dCTP. In addition, there was elongation from band 34 to band 35, from band 35 to band 36, and from 36 to a new termination band at position 37. dATP (Figure 6, lane 12) was even more effective than dGTP in supporting elongation. The radioactivity in bands 34–36 moved further up the gel into new bands at positions 38 and 39. Similar results were obtained when single-stranded DNA was used as template (data not shown), consistent with previous results obtained for UV-irradiated single-stranded ϕX174 templates (Rabkin et al., 1983). The reactions shown in lanes 12–15 (Figure 6) were repeated in the absence of monophosphate (lanes 6–11). In all cases there was degradation of substrate at most sites, presumably due to 3′ → 5′ exonuclease activity.

Comparison of Bypass of UV Dimers on Double-Stranded and Single-Stranded Templates. Polymerase I (Kf) can be induced to synthesize DNA past lesions in vitro in a series of three partial reactions (Rabkin et al., 1983). The amount of bypass rendered possible in this in vitro system can be followed at each step, examined visually on polyacrylamide gels, and quantitated by densitometric scanning for both double- and single-stranded templates [see Figure 1 in Rabkin et al. (1983)]. Lane 1 (Figure 7) shows the products of a first-stage reaction on a double-stranded template. There is termination at positions 34–36, one nucleotide before presumptive dimers. A second round of synthesis results in elongation to positions 37–39 (lane 2). A third round of synthesis in the presence of dGMP and dTTP (lane 6) allowed the demonstration of complete bypass of the dimer with elongation continuing to position 39 in the template. When both dTTP and dGTP were

Table II: Quantitation of Trans Dimer Synthesis on UV-Treated Double- and Single-Stranded Templates[a]

DNA	base added	position 34	35	36	40	43
ss	stage 1	15.3 ± 2	49.0 ± 2.9	33.6 ± 7.6	0.3 ± 0.5	1.3 ± 1.9
ds	stage 1	14.0 ● 0	49.3 ± 1.9	34.6 ± 0.9		1.3 ± 1.2
ss	T	35.0 ± 9	29.0 ± 2.9	16.7 ± 4.2	14.7 ± 4.5	5.7 ± 0.5
ds	T	29.7 ± 4	26.3 ± 3.7	16.3 ± 3.7	18.7 ± 3.3	8.0 ± 3.6
ss	T + G	6.0 ± 0	45.0 ± 4.2	18.0 ± 2.9	0.7 ± 0.9	29.3 ± 2.6
ds	T + G	8.7 ± 4.5	44.0 ± 5.0	21.7 ± 5.4	1.3 ± 1	24.3 ± 3.3
ss	T + G + C + A	4.0 ± 2.8	39.0 ± 2.9	54.6 ± 6.8		2.3 ± 1.7
ds	T + G + C + A	10.3 ± 4.8	40.6 ± 2.0	47.0 ± 5.0	2.7 ± 1.8	

[a] Densitomeric scans were made of gels from experiments similar to that described in Figure 7. Gels of (a) stage 1 products, (b) the products of a third-stage reaction synthesized in the presence of magnesium, dGMP, and dTTP, (c) the products of a third-stage reaction synthesized in the presence of magnesium, dGMP, and both dTTP and dGTP, or (d) the products of a third-stage reaction with magnesium, dMP, and all four dNTPs were scanned. The area under the peaks corresponding to sequence positions 34–36, 40, and 43 (see Figure 7) was totaled and set at 100%. The percent of the total radioactivity contained in the individual peaks was calculated, and results are listed. ds, double-stranded template; ss, single-stranded template. Three replicate experiments were performed for both double-stranded and single-stranded templates.

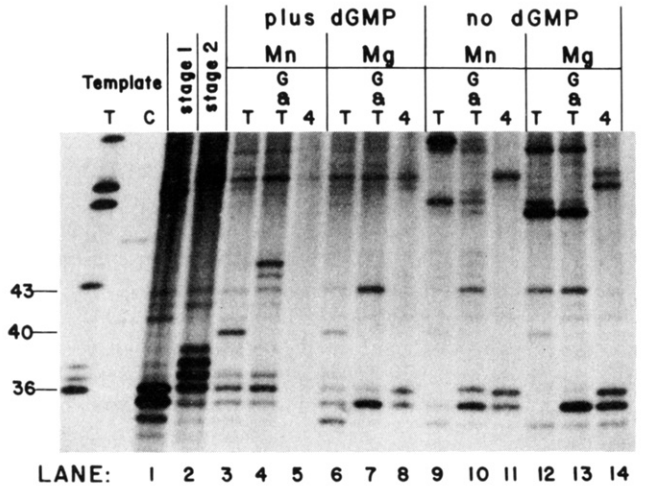

FIGURE 7: Trans dimer synthesis catalyzed by *E. coli* polymerase I (Kf) on UV-treated double-stranded DNA templates. Lane 1, first-stage reaction products; lane 2, second-stage reaction products; lane 3, third-stage reaction products synthesized in the presence of dTTP only; lane 4, third-stage products synthesized in the presence of all four dNTPs. Reactions in lanes 3–5 contained dGMP and manganese as the divalent cation. Lanes 6–8 correspond to lanes 3–5, respectively, except magnesium was substituted for manganese. Lanes 9–14 correspond to lanes 3–8, respectively, but with the omission of dGMP. Conditions for polymerase reactions are given in detail under Materials and Methods. The template sequence illustrated is

$$\overset{3|5}{}\qquad\overset{4|4}{}$$
3'- CACTTTAACAATAG -5'

provided (lane 7), elongation continued to position 43. With the addition of all four nucleotides (lane 8), the bypass products were lost from this section of the gel. In lanes 3–5, repetition of these three reactions in the presence of both dGMP and manganese resulted in manganese-induced extension of the third-stage products to positions one or two nucleotides past the expected site. Parallel reactions to those that appear in lanes 3–8, but in which dGMP is omitted, resulted only in degradation of the stage 3 products, especially in the presence of magnesium and dTTP only (see lane 9). Bypass can be followed quantitatively by densitometric scans of individual lanes (Table II). Total radioactivity in bands 34–43 was set at 100%, and the percent radioactivity in each band was determined. We observed no significant differences in the bypass synthesis obtained on single- and double-stranded templates.

Effect of Rec A and Single-Stranded Binding Protein on Bypass of Lesions. Lu et al. (1986) showed that Rec A protein binds to the subunit of *E. coli* polymerase III and reduces the

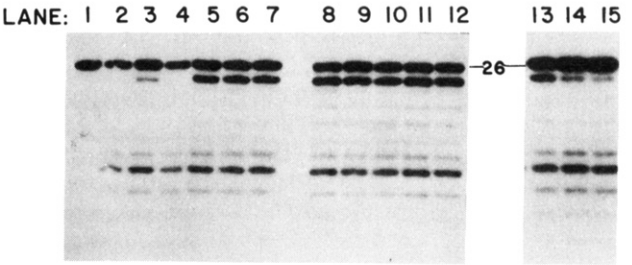

FIGURE 8: Effect of Rec A and single-stranded binding protein (ssB) on synthesis past UV-induced lesions by *E. coli* polymerase I (holoenzyme). Standard reactions included 0.1 μg of DNA, 0.3 unit of Pol I, 50 mM Hepes, pH 7.5, 8 mM $MgCl_2$, 5 mM DDT, 20 mM KCl, and [α-^{32}P]dATP, dGTP, dCTP, and dTTP. Reactions were incubated at 37 °C for 15 min before nucleotide concentrations were adjusted to 100 μM with unlabeled nucleotides. Additions were as follows: ATP, 0.28 μM; Rec A, (a) 0.28 mg/mL, (b) 0.56 mg/mL, and (c) 1.12 mg/mL; ssB, 4 μM; dGMP, 10 mM. The template sequence immediately preceding the runoff nucleotide was

$$\overset{1|7}{}\qquad\overset{2|6}{}$$
3'- AACCTTCCCGC-5'

Lane 1, nonirradiated, no addition; lane 2, no addition; lane 3, ATP; lane 4, ssB; lane 5, Rec A (a); lane 6, Rec A (b); lane 7, Rec A (c); lane 8, Rec A (a), ATP; lane 9, Rec A (c), ATP; lane 10, Rec A (a), ATP, ssB; lane 11, Rec A (b), ATP, ssB; lane 12, Rec A (c), ATP, ssB; lane 13, Rec A (a), ATP; lane 14, dGMP, Rec A (a), ATP; lane 15, dGMP, ssB.

fidelity of the polymerase. They find that Rec A binds specifically to UV dimers in damaged, double-stranded DNA. *E. coli* single-stranded binding protein (ssB) has also been reported to have effects on fidelity (Kunkel et al., 1979). In order to study the effects of both Rec A and ssB proteins on the bypass of lesions, a runoff assay was developed that would allow bypass to be quantitated more easily. The effects of ssB and Rec A on termination of synthesis at UV dimers by *E. coli* Pol I were examined in this system. A linear, double-stranded molecule with a unique nick was constructed so that synthesis on the molecule would proceed from the unique *Bgl*I site of M13mp2 to the end of the molecule at the unique *Pvu*I site. Within the 26-base region over which synthesis occurs, there are 10 potential sites for UV dimer formation. At 1 kJ/m^2 UV irradiation, one in 40 T's, at most, will be involved in dimer formation (Reynolds et al., 1981). If a random distribution of dimers is assumed, each molecule should contain one or no dimers within the 26-base region examined. If termination did occur, any subsequent bypass of the lesion would result in synthesis to the end of the molecule. Digestion with *Bgl*I and visualization of the products on denaturing acrylamide gels should result in the production of a 26-base

Table III: Ratio of Radioactivity in the Runoff to That in the Termination Bands[a]

condition	ratio
untreated control	2.4 ± 0.9
UV, no addition	0.8 ± 0.2
UV, ATP	1.3 ± 0.1
UV, Rec A	1.6 ± 0.1
UV, Rec A (0.28 mg/mL), ATP	1.2 ± 0.2
UV, Rec A (0.56 mg/mL), ATP	1.3 ± 0.3
UV, Rec A (1.12 mg/mL), ATP	1.3 ± 0.9

[a] Radioactivity corresponding to bands 25 and 26 (runoff bands, Figure 8) was determined by cutting and counting the gel, and this radioactivity was compared to that obtained by cutting out and determining the radioactivity in the termination bands (Figure 8). This result is expressed as a ratio. The figures represent the average of three experiments.

runoff fragment. We examined the effect of various additions on runoff synthesis on UV-treated DNA templates (Figure 8). The runoff band is present at position 26. Synthesis under standard conditions on control DNA is shown in lane 1 and on UV-treated DNA in lane 2. The runoff band and faint termination bands are seen. Addition of ATP (lane 3) or Rec A (lanes 5–7) stimulated synthesis. In addition, a new band appeared at position 25, one nucleotide prior to the end of the molecule. *E. coli* single-stranded binding protein (lane 4) has no apparent effect on the reaction. The results for Rec A added in combination with ATP (lanes 8–9) or with ATP and SSB (lanes 10–11) resemble those for Rec A added alone. Lanes 13–15 indicate that the penultimate termination band is due to the 3′ → 5′ exonuclease activity of the polymerase since it disappears on addition of dGMP to the reaction mixture. In combination with Rec A and ATP, the addition of dGMP to inhibit the 3′ → 5′ exonuclease activity suppresses the amount of termination seen at position 25 (compare lane 13 to lanes 14 and 15). Gels were quantified by cutting out individual bands and counting Cherenkov radiation. The total radioactivity contained in bands 25 and 26 (runoff band) was compared to the total radioactivity contained in the region of the termination bands (Table III). The ratio of radioactivity in the runoff to that in the termination bands doubled on addition of Rec A protein and also increased on addition of ATP. However, since a doubling in ratio by transfer of radioactivity from one band to another represents less than a twofold difference, we suppose the Rec A effect in this in vitro system to be minimal. The observation does not, of course, preclude a more important in vivo role for the Rec A protein in bypass reactions.

Discussion

Our major goal in these studies was to determine whether the rules for termination of DNA synthesis on damaged templates as established for in vitro synthesis on primed single-stranded templates are applicable to double-stranded templates. We used specifically nicked double-stranded DNA as a model substrate to mimic replication on double-stranded DNA as a model substrate to mimic replication on double-stranded templates using either the strand-displacement or nick-translation properties of the polymerases. It has been established that, during nick translation, *E. coli* Pol I does not behave in a strictly exonucleolytic fashion cleaving individual bases but rather produces an overhang up to 10 nucleotides in length, which on cleavage produces a distribution of short oligonucleotides (Lundquist & Olvera, 1982). This mechanism may account for the inability of lesions in the nontemplate strand to block synthesis (Figure 5; Kelly et al., 1969) since exonuclease action *at* the site of the lesion is not required. It had been pointed out by Piette and Hearst (1983) that the structure of the growing points in single-stranded and nick-translated double-stranded templates differed because of the presence in the latter case of these displaced overhangs (Lundquist & Olvera, 1982). In addition, the use of the eukaryotic enzyme AMV reverse transcriptase permits only limited strand displacement (Larson et al., 1985), and nick translation is not possible. Our experiments were designed to determine if the presence of a second strand during synthesis influences termination at, or bypass of, lesions.

The first approach taken was to determine the basic rules for termination at lesions on double-stranded DNA molecules. For templates containing UV dimers, termination occurred primarily one nucleotide prior (3′) to the 3′ member of each presumptive template dimer. This behavior is not polymerase specific since it is observed with Pol I, Pol I (Kf), and AMV reverse transcriptase. At two sites with similar flanking sequences (3′-GTGTTA-5′; 3′-GTGTTG-5′), a nucleotide may be inserted opposite the first (3′) member of a UV dimer by Pol I or Pol I (Kf) (Figure 1). This tendency is not seen with AMV reverse transcriptase.

Reaction of BPDE and ORP with DNA produces adducts at guanines. As might be predicted from the results with single-stranded templates (Moore & Strauss, 1979), termination on double-stranded templates is observed one nucleotide 3′ to template guanines. At three sites, quantitative differences were observed between templates reacted with ORP as opposed to the closely related BPDE. The differences could be due to differing reaction patterns or to polymerase-specific differences in termination. However, termination patterns were the same when synthesis was through either a double- or a single-stranded region and termination was not polymerase specific.

We have previously reported (Larson et al., 1985) that the termination of synthesis resulting from interaction of polymerase with DNA reacted with dimethyl sulfate occurred 3′ to adenosine on the template strand for double-stranded and single-stranded templates, presumably as the result of the formation of either 1- or 3-methyladenine. Although the major reaction product is with guanine, termination prior to guanines did not, in general, occur until the DNA had been heat treated to induce abasic sites. Therefore, in the three cases examined, UV-induced lesions, reactions with polyaromatic hydrocarbon derivatives, and methylation damage, the termination patterns generated are the same for both single- and double-stranded templates and for both small and bulky lesions.

The observation by Piette and Hearst (1983) that termination bands at psoralen-induced monoadducts seemed to disappear with time prompted us to reexamine the question of whether the termination bands seen in experiments of the type we report are "pauses" or permanent blocks to synthesis. Earlier quantitative work (Moore et al., 1980) indicated that (acetylamino)fluorene lesions constituted permanent blocks to synthesis. In our experiments with AMV reverse transcriptase at early times in synthesis at low nucleotide concentrations, termination bands are seen, which then disappear at later times when the nucleotide concentration is increased (Figure 4B). Our experiments were done at dimer levels that would not result in multiple lesions being present in the region studied so that bypass of one lesion would not result in a termination at a second lesion in the region studied. Overall diminution of bands lower in the gel or at specific sites might be expected if the bands visualized were merely pauses. However, no changes were observed after the earliest times at low dNTP concentration, and we therefore suppose that for

UV-induced lesions and for methylation-induced stops (Larson et al., 1985) the termination bands represent permanent blocks to synthesis with the enzymes and accesory proteins used.

Our experiments confirm the observations of Rabkin et al. (1983) on the specificity of insertion opposite lesions. A variety of conditions can be found under which a polymerase will insert a nucleotide opposite a lesion. In the presence of Mn^{2+} or dGMP and all four nucleoside triphosphates, *E. coli* Pol I will elongate terminated substrates to a position opposite the 3′ member of a UV dimer. When *both* Mn^{2+} and dGMP are present, elongation to this position is complete in some cases. In contrast to the results previously obtained in this laboratory [e.g., Rabkin et al. (1983)] we find it necessary to add inhibitors of 3′ → 5′ nuclease action in order to obtain elongation. On the basis of the 3′ → 5′ degradation of template seen in the absence of dGMP, we presume that changes in the nuclease/polymerase ratios of the different enzyme preparations account for this difference. In elongation reactions in which Mn^{2+} and dGMP were present with individual nucleotides, the four nucleoside triphosphates were not equally effective in template extension. Preference for nucleotide insertion was as follows: dATP > dGTP > dCTP > dTTP. This result is not significantly different from that obtained for single-stranded templates (Rabkin et al., 1983). Insofar as model systems can predict in vivo events, these findings provide additional support for the "A rule" in mutation (Howard & Tessman, 1964; Tessman, 1976, 1985; Strauss et al., 1982; Sagher & Strauss, 1984): when cells are confronted with noninstructional lesions, they tend to insert adenine nucleotides.

The data above seem to indicate that the "quality" of termination at lesions is the same for double-stranded and single-stranded templates. Bypass can be induced in a series of three partial reactions and was compared for synthesis on double- and single-stranded templates with Pol I (Kf). Synthesis terminated one nucleotide before a lesion in a first-stage reaction and was followed as translesion synthesis was permitted in the presence of Mg^{2+} and dGMP (Table II). Translesion synthesis of double-stranded DNA does not seem to be facilitated by the presence of a complementary strand but can readily be induced at some sites by manipulating the catalytic reactions of the enzyme.

How then can one account for the observation of Piette and Hearst (1983) and of O'Connor and Stohrer (1985) that bypass occurs more readily in nick-translation reactions? We suppose that these observations may be accounted for by the unique nature of adducts and their interaction with polymerases. O'Connor and Stohrer (1985) used a guanyl–aminofluorene adduct in their experiments, and there is both biological (Lutgerink et al., 1985) and biochemical (Johnson et al., 1986) evidence that such adducts are not absolute blocks to DNA synthesis as are the (acetylamino)fluorene adducts. The termination site depends on the conformation of the DNA–adduct complex. For example, a guanyl–(acetylamino)fluorene adduct in the syn conformation presents an informational face to a polymerase so that termination with AMV reverse transcriptase is opposite the lesion, whereas the aminofluorene derivative is in the anti conformation so that termination (or pausing) occurs 3′ to the lesion (Moore et al., 1981, 1982). It is possible that changes in the conformation of psoralen monoadducts could occur in going from single- to double-stranded DNA at particular sites, but it is probable that only additional work will resolve the differences between the observations. However, our experiments indicate that UV-induced lesions exist in a stable conformation that blocks DNA synthesis on either double- or single-stranded templates.

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Registry No. BPDE, 63323-31-9; ORP, 61695-74-7.

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