Mechanism of Bypass Synthesis through an Abasic Site Analog by DNA Polymerase I†

Tamar Paz-Elizur,‡ Masaru Takeshita,§ and Zvi Livneh*,‡

Department of Biochemistry, Weizmann Institute of Science, Rehovot 76100, Israel, and Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, New York 11794

Received August 26, 1996; Revised Manuscript Received December 10, 1996⊗

ABSTRACT: Bypass synthesis by DNA polymerase I was studied using synthetic 40-nucleotide-long gapped duplex DNAs each containing a site-specific abasic site analog, as a model system for mutagenesis associated with DNA lesions. Bypass synthesis proceeded in two general stages: a fast polymerization stage that terminated opposite the abasic site analog, followed by a slow bypass stage and polymerization down to the end of the template. The position of the 3′-terminus of the primer relative to the abasic site analog did not affect bypass synthesis in the range of −1 to −5. In contrast, bypass synthesis increased with the distance of the 5′-boundary of the gap from the lesion for up to 3-fold in the range of +1 to +9. Bypass synthesis was severely inhibited by moderate concentrations of salts, and under conditions that were optimal for the synthetic activity of DNA polymerase I (100 mM K^+), bypass synthesis was completely inhibited (<0.02% bypass). Elimination of the 3′→5′ proofreading exonuclease activity of the polymerase, by using a mutant DNA polymerase, caused a dramatic 10−60-fold increase in bypass synthesis. Determination of the kinetic parameters for insertion opposite the abasic site analog revealed a strong preference for the insertion of dAMP, dictated by a lower K_m and a higher k_{cat} as compared to the other nucleotides. The rate of bypass was increased by omitting one or two dNTPs, most likely due to the facilitation of the polymerization past the lesion.

The abasic site is a major type of lesion produced in DNA. It is generated by spontaneous hydrolysis of the glycosylic bond of purine nucleotides, and as an intermediate in base excision repair, by the action of DNA *N*-glycosylases on damaged or foreign bases in DNA. In addition, chemical modification of purines by a variety of chemicals and carcinogens greatly labilizes the glycosylic bond, leading to enhanced spontaneous base loss (Loeb, 1985; Loeb & Preston, 1986). Mutagenesis caused by abasic sites is believed to result from the insertion of nucleotides opposite the abasic site by DNA polymerases. In the bacterium *Escherichia coli*, abasic site mutagenesis depends on induction of the SOS stress response (Schaaper & Loeb, 1981; Lawrence et al., 1990) and on the RecA, UmuC, and RecF proteins (Schaaper et al., 1982). However, abasic sites positioned as a bistrand lesion in a plasmid caused mutagenesis in the absence of SOS induction (Takeshita & Eisenberg, 1994). Genetic support for the involvement of abasic sites in chemical mutagenesis came from the observation that *xth* mutants, that lack the major AP endonuclease, displayed increased mutability by MNNG in an SOS-dependent fashion. This mutagenesis was suppressed by introducing an additional *alkA* mutation which eliminated the major 3-methyladenine−DNA glycosylase activity. This suggested that abasic sites generated by the glycosylase were the cause of increased mutagenesis (Foster & Davis, 1987).

It is well established that abasic sites are obstacles to DNA polymerases (Kunkel et al., 1983; Sagher & Strauss, 1983; Hevroni & Livneh, 1988). Still, most DNA polymerases exhibit the capacity to replicate through abasic sites to some extent. This is the case for DNA polymerase I (Pol I)[1] (Kunkel et al., 1981; Sagher & Strauss, 1983; Takeshita et al., 1987; Bonner et al., 1992), Pol II (Bonner et al., 1992), and Pol III holoenzyme of *E. coli* (Hevroni & Livneh, 1988). The mechanism of this reaction, termed bypass synthesis or translesion replication, is only partially understood (Strauss, 1985; Livneh et al., 1993). We have previously studied the mechanism of bypass by DNA polymerase II (Paz-Elizur et al., 1996). The current study addresses the mechanism of bypass by DNA polymerase I through a site-specific abasic site analog (tetrahydrofuran) in a synthetic gapped duplex DNA.

MATERIALS AND METHODS

Materials. Nucleotides were obtained from Boehringer-Mannheim, and radiolabeled nucleotides were purchased from The Radiochemical Center, Amersham. Sodium glutamate and acrylamide were from BDH, and potassium glutamate was from Sigma. DNA polymerase I (6000 units/mg) and its Klenow fragment (5000 units/mg) were obtained usually from Boehringer-Mannheim, and occasionally from US Biochemicals. A mutant of the Klenow fragment lacking the proofreading exonuclease activity was obtained from US

† This work was supported by grants from the Scheuer Research Foundation of the Israel Academy of Sciences, and from The Forchheimer Center for Molecular Genetics.

* To whom correspondence should be addressed at the Department of Biochemistry, Weizmann Institute of Science, Rehovot 76100, Israel. Telephone: 972-8-934-3203. Fax: 972-8-934-4169. Email: bclivneh@weizmann.weizmann.ac.il.

‡ Weizmann Institute of Science.

§ State University of New York at Stony Brook.

⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1997.

[1] Abbreviations: AP sites, apurinic/apyrimidinic sites, abasic sites; Pol I, DNA polymerase I; Pol II, DNA polymerase II; Pol III, DNA polymerase III; PAGE, polyacrylamide gel electrophoresis; ssDNA, single-stranded DNA.

S0006-2960(96)02132-0 CCC: $14.00

Biochemicals. Buffer B contained 20 mM Tris·HCl, pH 7.5, 8 μg/mL BSA, 5 mM DTT, 0.1 mM EDTA, pH 7.5, 4% glycerol, and 8 mM $MgCl_2$.

DNA Substrates. The DNA substrates were described before (Paz-Elizur et al., 1996). Templates AB1 and AB2 are 40-nucleotide-long synthetic DNAs, each containing a single abasic site analog at a unique position (Paz-Elizur et al., 1996). They were synthesized and purified as described (Takeshita et al., 1987; Takeshita & Eisenberg, 1994). The abasic site analog is a modified tetrahydrofuran moiety which is a stable analog of 2′-deoxyribose in the abasic site (Takeshita et al., 1987). It has a hydrogen instead of a hydroxyl residue on the 1′ carbon of the deoxyribose ring (Takeshita et al., 1987). The templates are shown in Figure 1 along with the complementary oligonucleotides used to form the gapped duplexes. The preparation of the gapped duplexes was described before (Paz-Elizur et al., 1996). Briefly, the primers were ^{32}P-end-labeled by T4 polynucleotide kinase and then annealed (2 nmol) along with the phophorylated downstream complementary oligonucleotide (2 nmol) to the 40-nucleotide-long synthetic DNA template (0.5 nmol) in 45 μL of a solution containing 10 mM Tris·HCl, pH 7.5, 1 mM EDTA, and 150 mM NaCl. The gapped duplex was separated from excess primer and downstream oligonucleotide by size exclusion on a Sephadex G-50 superfine column. The recovery of the gapped duplex under these conditions was >95%. The specific activity of the gapped duplex was calculated by dividing the total amount of radioactivity that was annealed to the template, as assayed on the gel, by the total amount of the template in the annealing reaction (under these conditions, essentially all the template was annealed). The concentration of the purified template in each fraction was determined based on this specific activity, and was verified by measuring its absorbance at 260 nm in a Pharmacia GeneQuant spectrophotometer using a microcapillary cuvette. Essentially all template molecules contain the abasic site analog (>99.98%). This was deduced from the fact that when bypass was assayed with Pol I in the presence of 0.1 M KCl, all the primers were extended up to the lesion, but no bypass was observed (<0.02%, representing the limit of our detection; Paz-Elizur et al., 1996). Single-stranded DNA (ssDNA) from phage M13mp8 was prepared as previously described (Livneh, 1986b).

Bypass Synthesis. The *in vitro* replication reaction mixture (20−50 μL) contained buffer B, 0.5 mM dNTPs, 60−90 nM gapped duplex, and DNA polymerase I or one of its derivatives. Reactions were carried out at 30 °C for up to 30 min, after which they were terminated by adding an equal volume of loading buffer containing 0.02% xylene cyanol, 0.02% bromophenol blue, and 98% formamide. Samples of 10 μL were fractionated by electrophoresis on 15% or 20% polyacrylamide gels containing 8 M urea at 1500−1600 V for 3−4 h, after which they were visualized and quantified using a Fuji BAS 1000 phosphorimager.

DNA Synthesis. M13mp8 ssDNA was annealed at a molar ratio of 1:30 to the oligonucleotide primer 5′-GAAAC-CATCGATAGC-3′, complementary to positions 2538−2524. Annealing was carried out for 10 min at 70 °C in 10 mM Tris·HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl, followed by cooling to room temperature over a period of 2−3 h. The replication reaction mixture (25 μL) contained buffer B, 100 μM each of dGTP, dTTP, and dCTP, 20 μM [α-^{32}P]dATP, 4 nM primed M13mp8 ssDNA, and 40 nM Pol I or 80 nM of its Klenow fragment. The reaction was carried out at 30 °C for 5 min (Pol I), or 10 min (Klenow fragment), after which it was stopped by adding 150 μL of a solution containing 10 mM Tris·HCl, pH 7.5, 25 mM EDTA, and 0.1 M sodium pyrophosphate. The amount of acid-insoluble radiolabeled material was determined by trichloroacetic acid precipitation as described (Livneh, 1986a).

Determination of Kinetic Parameters for Polymerization Opposite the Abasic Site Analog. The reaction mixture (20 μL) contained buffer B, 70 nM of template AB1 primed with an oligonucleotide whose 3′-terminus was located at the −1 position (Figure 1), and a single dNTP. The concentration ranges and the reaction times used for each dNTP, determined empirically, were as follows: dATP, 10−50 μM, 0.5−1 min; dGTP, 10−50 μM, 1.5−3 min; dCTP, 60−2000 μM, 3.5−7 min; dTTP, 30−300 μM, 8 min. The reaction was started by adding 60 fmol of Pol I to a reaction preheated to 30 °C. The reactions were terminated by adding 20 μL of the loading buffer containing 0.02% xylene cyanol, 0.02% bromophenol blue, and 98% formamide. Samples of 5 μL were fractionated by electrophoresis on 20% polyacrylamide sequencing gels containing 7 M urea, at 1700−2000 V, 45−50 °C for 4 h. The gels were visualized and quantified by phosphorimaging. The rates of addition opposite the abasic site were calculated from the intensities of the unextended and extended primers as previously described (Randall et al., 1987).

RESULTS

DNA Polymerase I Bypasses an Abasic Site Analog in a Synthetic Gapped Duplex DNA. We have previously described an assay system for studying bypass synthesis on a defined synthetic gapped duplex DNA, carrying an abasic site analog at a predetermined location (Paz-Elizur et al., 1996). The DNA templates were two 40-nucleotide-long synthetic DNAs, whose sequences were derived (with minor changes) from the *lacZ*′ portion of M13mp2, for which *in vivo* data on the specificity of apurinic site mutagenesis existed (Kunkel, 1984). Template AB1 contains the abasic site analog instead of G89, and oligonucleotide AB2 has an abasic site analog instead of A109, sites that were found to be mutated when depurinated M13mp2 ssDNA was introduced into *E. coli* cells (Kunkel, 1984).

The gapped DNAs were prepared by annealing two short oligonucleotides to the 40-mer template: a ^{32}P-labeled primer, and an unlabeled oligonucleotide, complementary to the 5′ end of the template (the downstream oligonucleotide). A series of short oligonucleotides was used to create gapped substrates with varying gap length, and varying locations of the 3′-terminus of the primer and the 5′-terminus of the downstream oligonucleotide relative to the location of the abasic site (Figure 1). The names of these gapped substrates describe their structure; for example, AB1m3p5 stands for a gapped duplex prepared from template AB1, with the 3′-terminus of the primer located at the minus 3 (m3) position relative to the abasic site (0), and the 5′-terminus of the downstream oligonucleotide located at plus 5 (p5) relative to the abasic site, thus defining a particular 7-nucleotide gap (Figure 1). Polymerization resulted in extension of the radiolabeled primer, and this was assayed by urea−PAGE,

```
                          +9 +7+5 +3+1  -1-3
AB1    5'-GCTG TACA ACGT CGTG ACTG X GAA AACC CTGG CGTT ACCC-3'

m3p1   3'-CGAC ATGT TGCA GCAC TGAC    T TTGG GACC GCAA TGGG-5'

m3p3   3'-CGAC ATGT TGCA GCAC TG      T TTGG GACC GCAA TGGG-5'

m3p5   3'-CGAC ATGT TGCA GCAC         T TTGG GACC GCAA TGGG-5'

m3p7   3'-CGAC ATGT TGCA GC           T TTGG GACC GCAA TGGG-5'

m3p9   3'-CGAC ATGT TGCA              T TTGG GACC GCAA TGGG-5'

                       +9 +7+5 +3+1  -1-3

                          +9 +7+5 +3+1  -1-3
AB2    5'-GGAA AACC CTGG CGTT ACCC X ACT TAAT CGCC TTGC AGCA-3'

m1p1   3'-CCTT TTGG GACC GCAA TGGG  TGA ATTA GCGG AACG TCGT-5'

m1p3   3'-CCTT TTGG GACC GCAA TG    TGA ATTA GCGG AACG TCGT-5'

m3p1   3'-CCTT TTGG GACC GCAA TGGG    A ATTA GCGG AACG TCGT-5'

m3p3   3'-CCTT TTGG GACC GCAA TG      A ATTA GCGG AACG TCGT-5'

m3p5   3'-CCTT TTGG GACC GCAA         A ATTA GCGG AACG TCGT-5'

m3p7   3'-CCTT TTGG GACC GC           A ATTA GCGG AACG TCGT-5'

m5p1   3'-CCTT TTGG GACC GCAA TGGG       TTA GCGG AACG TCGT-5'

m5p3   3'-CCTT TTGG GACC GCAA TG         TTA GCGG AACG TCGT-5'

                       +9 +7+5 +3+1  -1-3-5
```

FIGURE 1: Structures of gapped duplexes each containing a site-specific abasic site analog. Underneath each template (AB1 or AB2) are shown the pairs of complementary oligonucleotide that were used to form the gapped duplexes. The oligonucleotides on the right are the primers, and those on the left are the downstream oligonucleotides. The locations of the 3′-ends of the primers and the 5′-ends of the downstream oligonucleotides are indicated at the left side. For example, m3p5 under AB1 represents a gapped duplex formed on oligonucleotide AB1 with the 3′ of the primer located at the −3 (**m**inus 3) relative to the abasic site (X), and the 5′-end of the downstream oligonucleotide located at the +5 (**p**lus 5) position, forming a gap 7 nucleotides long.

followed by quantitative measurements of the radiolabeled bands by phosphorimaging.

Figure 2 shows the reaction products of Pol I with a gapped DNA (AB1m3p9; 11-nucleotides gap). The reaction can generally be divided into two stages: a rapid synthesis stage up to the lesion, followed by a slow bypass step. Interestingly, DNA synthesis terminated opposite the lesion, suggesting that addition opposite the abasic site analog was faster than excision of the added nucleotide or the past-lesion polymerization step. The extension past the lesion was much slower, and led to the accumulation of a radiolabeled 40-mer, indicating that the polymerase did bypass the abasic site analog (Figure 2A). Following the completion of gap-filling, the polymerase continued synthesis while replacing the downstream oligonucleotide, most likely by its nick translation activity (Kornberg & Baker, 1991). The extent of bypass was significant, reaching nearly 8% bypass in 20 min (Figure 2B). The 5′→3′ exonuclease activity of the polymerase can excise the radiolabeled primer from the 5′-end, leading to an overall loss of radiolabel from the synthetic DNA. We found that less than 10% of the radiolabel was lost after 20 min of reaction. No such loss was observed with the Klenow fragment, as expected. We have also observed a residual amount of approximately 5% of the unextended primer (Figure 2). This is likely to be residual

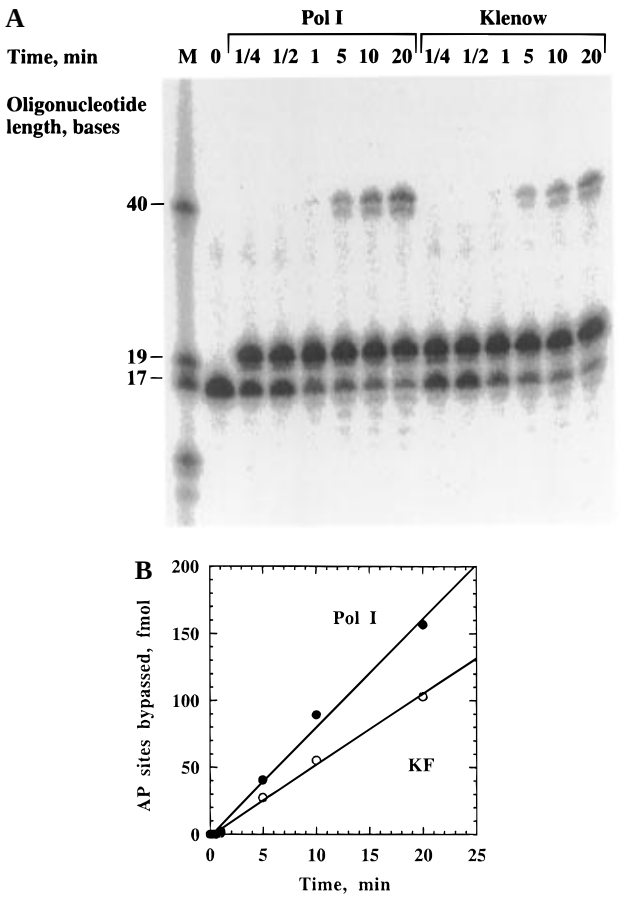

FIGURE 2: Bypass synthesis by Pol I and its Klenow fragment. (A) Time course of replication by Pol I or its Klenow fragment on AB1m3p9, a gapped duplex carrying a single abasic site analog. The reaction mixture contained buffer B, 0.5 mM dNTP, 83 nM gapped duplex, and 90 nM polymerase. Reactions were carried out at 30 °C for the indicated time periods. Replication products were separated by PAGE on a 20% gel containing urea and visualized by phosphorimaging. (B) Quantification of the image shown in panel A was done by phosphorimaging. Bypass synthesis was calculated by dividing the intensity of the bypass products by the sum of the termination and bypass products. Bypass of 20.8 fmol of AP sites represents 1% of the substrate.

nonhybridized primer, that did not separate completely from the duplex template during its purification.

The ability of the Klenow fragment of Pol I to bypass the abasic site analog was assayed under conditions identical to those used to assay Pol I. Similarly to Pol I, the Klenow fragment was capable of bypass synthesis (Figure 2). Interestingly, the polymerase did not stop at the gap boundary, and the bypass product was the fully replicated 40-mer oligomer. The rate of bypass of the abasic site analog was 50% higher for Pol I, as compared to the Klenow fragment under the same conditions (Figure 2B). Since the Klenow fragment cannot perform nick translation, the downstream oligonucleotide was removed most likely by a strand displacement mechanism, resulting in extension down to the end of the template. Omitting a single dNTP from the reaction mixture is expected to lead to the formation of a shorter bypass product. This was examined with the DNA substrate AB2m3p7, that contains the second template. As can be seen in Figure 3, the Klenow fragment did bypass the abasic site analog in this template too, and the major bypass product was 40 nucleotides long. Omission of dCTP led to the formation of a bypass product 26 nucleotides long (Figure 3), as expected from the location of the first G in

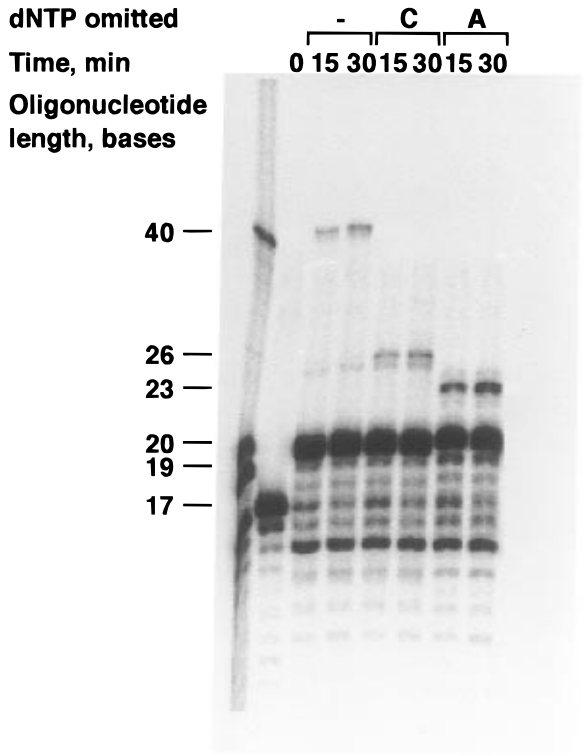

FIGURE 3: Effect of omission of a single dNTP on bypass synthesis by the Klenow fragment of Pol I. The reaction conditions were as described in the legend to Figure 2 except that template AB2m3p7 was used, and parallel reactions were carried out with the four dNTPs, in the absence of dCTP, and in the absence of dATP. Replication products were separated by PAGE on a 20% sequencing gel containing urea and visualized by phosphorimaging.

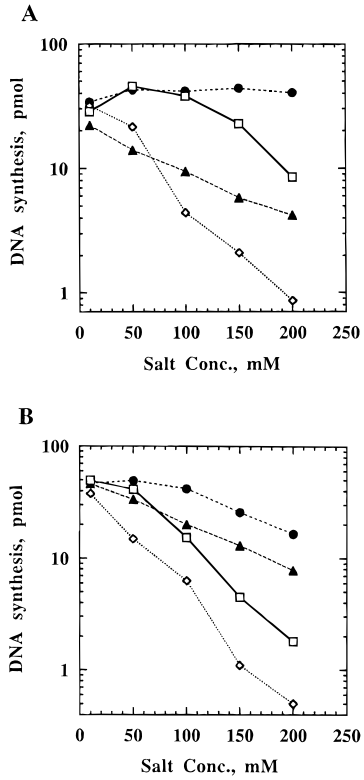

FIGURE 4: Salt sensitivity of polymerization by Pol I or its Klenow fragment. The reaction mixture (25 mL) was as described in the legend to Figure 2 except that it contained 4 nM primed M13mp8 ssDNA, 40 nM Pol I or 80 nM Klenow fragment, and the indicated salt. After 5 min (Pol I), or 10 min (Klenow fragment), the reaction was stopped, and the amount of acid-insoluble radiolabeled material was measured. (A) Pol I; (B) the Klenow fragment. White squares, KCl; white diamonds, NaCl; black circles, potassium glutamate; black triangles, sodium glutamate.

the template (Figure 1). In addition, a fainter band, one nucleotide shorter, was observed (Figure 3). Omission of dATP yielded a major bypass product 23 nucleotides long, 1 nucleotide shorter than the expected product (Figure 1), although the 24-nucleotide product was observed too (Figure 3).

The effect on bypass synthesis of increasing the polymerase concentrations was measured with a gapped duplex containing a 9-nucleotide gap (AB2m3p7). It was found that bypass synthesis increased with polymerase concentration both for Pol I and for the Klenow fragment (data not shown), indicating that the association of the polymerase with the DNA at the lesion site is important in determining bypass synthesis. Bypass synthesis depended on the concentration of dNTPs, consistent with the involvement of a slow polymerization step (not shown). Maximal bypass required dNTP concentrations higher than 100 μM.

Bypass Synthesis Is Severely Inhibited under Salt Conditions That Are Optimal for Polymerization. We have examined the sensitivity of polymerization by either Pol I or the Klenow fragment to NaCl, KCl, sodium glutamate, and potassium glutamate, the latter being the major intracellular salt in *E. coli* (Richey et al., 1987). As can be seen (Figure 4A), the combination of the Na^+ cation and the Cl^- anion had the greatest inhibitory effect on polymerization by Pol I on undamaged primed M13mp8 ssDNA, reaching a 40-fold inhibition at 200 mM NaCl. With potassium glutamate, polymerization activity remained remarkably constant up to 200 mM. The Klenow fragment was generally more salt sensitive than the intact polymerase, but still the best salt resistance was found with potassium glutamate (Figure 4B).

Bypass synthesis by Pol I was highly sensitive to KCl that caused a 100-fold inhibition at a concentration of 25 mM (Figure 5A). Examining several salts revealed a similar picture, with inhibition decreasing in the order KCl > potassium glutamate > NaCl > sodium glutamate. Thus, potassium ions were more inhibitory than sodium ions, and chloride ions were more inhibitory than glutamate ions. Bypass synthesis by the Klenow fragment was similarly highly sensitive to K^+ ions (Figure 5B). However, in contrast to Pol I, the identity of the anion (Cl^- or glutamate) did not affect bypass synthesis (Figure 5B). Most remarkably, under conditions that are optimal for the polymerization activity of Pol I, and resemble the intracellular salt concentration, i.e., 0.1−0.2 M potassium glutamate (Richey et al., 1987), bypass synthesis was completely inhibited (Figure 5C). The inhibition was caused primarily at the past-lesion polymerization step, since the extended primer terminated opposite the lesion, similar to termination in the absence of salt (not shown).

The Structure of the Gap Affects Bypass Synthesis. At least three gap parameters can in theory affect the bypass reaction: the distance of the primer 3′-OH terminus from the abasic site, the distance of the 5′ boundary of the gap from the abasic site, and the size of the gap. Determination of bypass synthesis with a series of gapped duplexes showed similar bypass kinetics for primer termini located at the minus 1, minus 3, or minus 5 positions relative to the abasic site

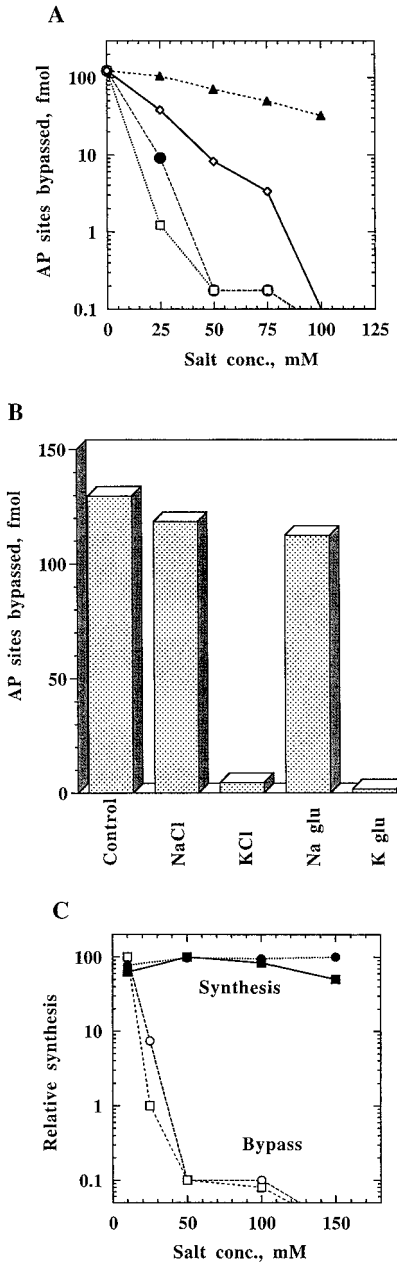

FIGURE 5: Salt sensitivity of bypass synthesis by Pol I or its Klenow fragment. Bypass synthesis was assayed for 15 min in buffer B containing 0.5 mM dNTPs, and either 70 nM template AB2m3 and 70 nM Pol I (A), or 70 nM template AB2m3p3 and 140 nM Klenow fragment (B). White squares, KCl; white diamonds, NaCl; black circles, potassium glutamate; black triangles, sodium glutamate. The concentration of each of the salts in panel B was 25 mM. In the absence of salt, 123 fmol (7%) and 130 fmol (7.4%) of abasic sites were bypassed in 15 min by Pol I and the Klenow fragment, respectively. (C) Comparison of the effects of potassium salts on polymerization and bypass by Pol I. Squares, KCl; circles, potassium glutamate; black symbols, DNA synthesis on undamaged ssDNA; white symbols, bypass synthesis. The data were taken from panels A and B, and from Figure 4A.

analog (Figure 6). This is consistent with the two-stage course of the reaction: The rapid stage causes extension up to the lesion. Since the following bypass stage is much slower (Figure 2), essentially all bypass events initiate from the primer terminus located opposite the lesion.

In order to examine the effect of the location of the 5′ boundary of the gap, we examined bypass with the substrates AB1m3p1, AB1m3p3, AB1m3p5, AB1m3p7, and AB1m3p9 (Figure 1). As can be seen in Figure 7, bypass synthesis by

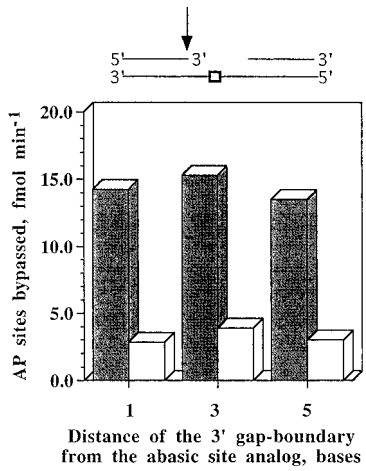

FIGURE 6: Distance of the 3′-terminus of the primer from the abasic site does not affect bypass synthesis. Gapped duplexes (60 nM) which have primer termini located at the minus 1 position (AB2m1p1 or AB2m1p3), at the minus 3 position (AB2m3p1 or AB2m3p3), or at the minus 5 position (AB2m5p1 or ABm5p3) were used as substrates for bypass synthesis by the Klenow fragment (330 nM) in the presence of 1 mM dNTPs. Replication products were separated by PAGE on a 15% gel containing urea and then visualized and quantified by a phosphorimager. Full and empty columns refer to substrates with the 5′ boundary of the gap located at the plus 3 and plus 1 positions, respectively.

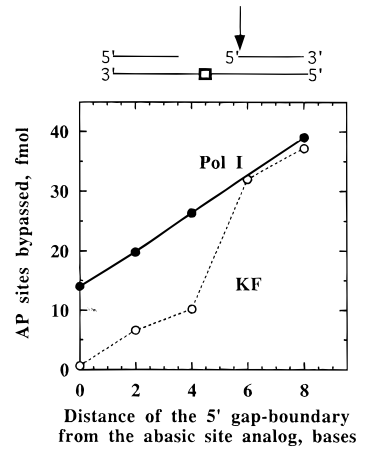

FIGURE 7: Distance of the 5′ boundary of the gap from the abasic site affects bypass synthesis. Gapped duplexes (80 nM) with varying locations of the 5′-end of the downstream oligonucleotide were assayed under conditions described in the legend to Figure 2 in order to determine the effect of gap length on bypass synthesis. The distances of the 5′ boundary of the gap from the abasic site analog were as follows: AB1m3p1, 0 bases; AB1m3p3, 2 bases; AB1m3p5, 4 bases; AB1m3p7, 6 bases; and AB1m3p9, 8 bases. Replication products were separated by PAGE on a 15% gel containing urea, then visualized and quantified by a phosphorimager. Black circles, bypass synthesis by Pol I (80 nM) for 5 min; white circles, bypass synthesis by the Klenow fragment (80 nM) for 10 min. Bypass of 20 fmol of AP sites represents 1% of the template.

Pol I increased with the size of the gap, from 14 fmol of AP sites bypassed in 5 min for AB1m3p1 up to 40 fmol for AB1m3p9, which contains a gap of 11 nucleotides. In fact, bypass on this gapped duplex was similar to that on the primed ssDNA template (46 fmol of AP bypassed in 5 min). In general, the behavior of the Klenow fragment was similar, except that it showed lower extents of bypass (the curves in Figure 7 correspond to reaction times of 5 and 10 min for Pol I and the Klenow fragment, respectively).

Interestingly, when the 5′ end of the downstream oligonucleotide started at the plus 1 position, i.e., next to the abasic

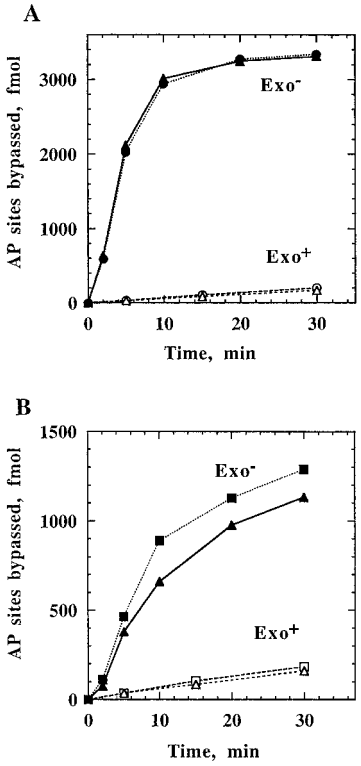

FIGURE 8: Bypass synthesis greatly increases in the absence of proofreading. The Klenow fragment or a mutant of the Klenow fragment deficient in the 3′→5′ proofreading exonuclease activity was used (330 nM) in replication of gapped duplexes (160 nM). Replication products were separated by PAGE on a 15% gel containing urea, and then visualized and quantified by a phosphorimager. (A) Triangles, AB2m3p3; circles, AB2m1p3. (B) Squares, AB2m3p1; triangles, AB2m1p1; black symbols, reactions with an exonuclease-deficient mutant of the Klenow fragment; white symbols, reactions with the Klenow fragment.

site analog, the rate of bypass synthesis by the Klenow fragment was reduced 4−5-fold, whether the primer was located at the minus 1, minus 3, or minus 5 positions (Figure 6). Since the Klenow fragment of Pol I lacks the 5′→3′ exonuclease activity, bypass synthesis on such templates requires strand displacement prior to the first past-lesion polymerization reaction, a process that is likely to further slow down the extension step. Indeed, Pol I that can perform nick translation, thus excising the 5′ end of the downstream oligonucleotide, was able to perform bypass synthesis on the m3p1 template, on which the Klenow fragment did not show any bypass (14 and <0.4 fmol of AP sites bypassed in 5 min, respectively; Figure 7).

Inhibition of the 3′→5′ Exonuclease Activity of the Polymerase Greatly Increases Bypass Synthesis. We have previously shown that bypass synthesis on template AB1 was increased in the absence of proofreading by each of the *E. coli* DNA polymerases (Paz-Elizur et al., 1996). Using a mutant Klenow fragment deficient in the 3′→5′ exonuclease, we have extended these experiments to template AB2 with several gap configurations. As can be seen (Figure 8), the rate of bypass synthesis was dramatically increased up to 60-fold higher than that of the Klenow fragment. The same effect was obtained with templates AB2m1p3 and AB2m3p3 (Figure 8A). By 5 min, as much as 50% (2116 fmol) of the abasic sites were replicated, whereas less than 0.8% (32 fmol) were bypassed by the Klenow fragment under similar conditions (Figure 8A). For two other templates, in which the 5′-termini of the downstream oligonucleotides were located next to the abasic site analog (AB2m1p1 and AB2m3p1), bypass synthesis was generally slower, but still 10-fold faster than with the Klenow fragment (Figure 8B).

Table 1: Kinetic Parameters for Addition of dNMP Residues Opposite the Abasic Site Analog by DNA Polymerase I[a]

substrate	K_m, mM	k_{cat}, s^{-1}	k_{cat}/K_m, $s^{-1}M^{-1}$
dATP	32 ± 5 (1)	0.059 (8.8)	1844 (110)
dGTP	56 ± 9 (1.7)	0.016 (2.4)	287 (17.1)
dTTP	228 ± 50 (7.1)	0.014 (2.1)	61.4 (3.7)
dCTP	400 ± 85 (12.5)	0.0067 (1)	16.8 (1)

[a] Reactions were carried out with the template AB1m1 and with a single dNTP as described under Materials and Methods. The products were fractionated by PAGE on a 20% gel containing urea, after which the bands were visualized and quantified using a phosphorimager. Each experiment was performed 3−4 times, and the results were averaged and presented as double-reciprocal plots. The K_m and k_{cat} values were calculated from these plots. The numbers in parentheses represent relative values normalized to a particular dNTP that was set to 1.

Kinetic Parameters for Polymerization Opposite the Abasic Site Analog. The specificity of insertion opposite the abasic site analog was examined by determining its K_m and k_{cat} values for each of the four dNTPs. This was done with oligonucleotide AB1 primed with a 19-mer primer whose 3′ was located opposite the template base preceding the abasic site analog. The extent of addition of a single dNMP residue to the primer (i.e., addition opposite the abasic site analog) was assayed as a function of dNTP concentration in a reaction with a single dNTP. This was repeated for each dNTP. The results are summarized in Table 1, where the values of the kinetic parameters are presented. As can be seen, dATP is clearly the best substrate for incorporation opposite the abasic site analog, exhibiting a K_m which is an order of magnitude lower than for the pyrimidine dNTPs, and nearly 2-fold lower than for dGTP, and a k_{cat} which is 4−9-fold higher than for the other dNTPs (Table 1). This leads to an overall insertion specificity factor (k_{cat}/K_m) which is 110-, 33-, and 6.6-fold higher than that of dTTP, dCTP, and dGTP, respectively.

Bypass Synthesis Increases When Only Three or Two dNTPs Are Present. Careful examination of Figure 3 reveals that the extent of bypass increased when dATP was omitted from the reaction mixture. This was somewhat surprising in light of the clear preference for insertion of dAMP opposite the abasic site analog. We examined the effect of the composition of dNTPs on bypass synthesis by Pol I on template AB2m3p7. The sequence following the abasic site analog is 3′...XCCCATTG...5′ (Figure 1). Thus, bypass synthesis could be assayed in the absence of dCTP, dATP, or both (Figure 3). As can be seen in Table 2, bypass synthesis was 2.4-fold faster when dCTP was omitted, 3.2-fold faster when dATP was omitted, and 4.5-fold faster when both dCTP and dATP were absent. Similar effects were obtained with the Klenow fragment, and with the exonuclease-deficient mutant of the Klenow fragment (Table 2), implying that the effect was not dependent on either one of the two exonucleolytic activities of the polymerase. These results are consistent with the notion that extension past the lesion is slower than addition opposite the lesion, and they suggest that binding of the dNTP to the polymerase may affect the kinetics of bypass polymerization (see below).

Table 2: Bypass Synthesis Increases When Fewer dNTPs Are Present[a]

	bypass synthesis, % min^{-1}		
	Pol I[b]	Klenow fr.[c]	exo$^-$ Klenow fr.[d]
all dNTPs	0.15 (1.0)	0.50 (1.0)	4.1 (1.0)
no dCTP	0.36 (2.4)	0.73 (1.5)	13.2 (3.2)
no dATP	0.48 (3.2)	1.03 (2.1)	nd
no dATP, dCTP	0.67 (4.5)	1.33 (2.7)	nd

[a] Bypass reactions were carried out in the presence of 1 mM of each of the indicated dNTPs, at 30 °C for 5−15 min. Replication products were fractionated by urea−PAGE and analyzed and quantified by phosphorimaging. The values in parentheses are relative bypass synthesis rates normalized to the rates in the presence of all four dNTPs. [b] Reaction mixtures contained 70 nM substrate AB2m3p7, and 70 nM Pol I. [c] Reaction mixtures contained 160 nM substrate AB2m3p5 and 330 nM Klenow fragment of Pol I. [d] Reaction mixtures contained 160 nM substrate AB2m3p5 and 330 nM of an exonuclease-deficient mutant of the Klenow fragment of Pol I.

DISCUSSION

Induced mutations are caused by DNA lesions present in segments of ssDNA, i.e., in a structure of a gapped duplex (Walker, 1984; Livneh et al., 1993). This can be produced by termination of replication at the lesion, followed by replication restart past the lesion (Rupp & Howard-Flanders, 1968), or by the activity of nucleotide excision repair (Cohen-Fix & Livneh, 1992, 1994; Tomer et al., 1996). In addition, several drugs and carcinogens such as mitomycin C produce interstrand cross-links that are converted to single-chain lesions in a ssDNA gap after the action of the UvrABC excision repair system (Van Houten, 1990). Although abasic sites are repaired by a very short repair patch, they may be occasionally found in ssDNA regions. For example, the drugs bleomycin and neocarzinostatin form bistrand lesions in DNA, with an abasic site on one strand (Steighner & Povirk, 1990), that are refractory to repair endonucleases (Povirk & Goldberg, 1985). We have utilized defined synthetic gapped duplexes each containing a site-specific abasic site analog, and used them as model substrates for the bypass synthesis step of mutagenesis associated with DNA damage in general, and with abasic sites in particular.

Consistent with previous reports (Kunkel et al., 1981; Sagher & Strauss, 1983; Takeshita et al., 1987; Bonner et al., 1992), Pol I was capable of polymerizing through the abasic site analog on two different synthetic DNAs. Generally the reaction can be divided into two stages: a rapid polymerization step that extends the primer to the position opposite the lesion, followed by a slower step that involves extension past the lesion, and elongation down to the end of the template. The rapid accumulation of intermediates that terminated opposite the abasic site analog implies that (a) addition opposite the lesion is faster than excision and (b) the bypass reaction is limited by a kinetic step that occurs after the addition. This is supported by our finding that omission of dATP, the nucleotide which is the best substrate for incorporation opposite the lesion, causes an increase, rather than a decrease, in the rate of bypass synthesis.

At least three parameters may be responsible for limiting the bypass reaction past the addition step: a slow extension step, the action of the 3′→5′ proofreading exonuclease activity of the polymerase that aborts extension attempts (Villani et al., 1978), and rapid dissociation of the polymerase from the template (Shwartz & Livneh, 1987; Shwartz et al., 1988). The dramatic effect on bypass synthesis of eliminating the 3′→5′ proofreading activity suggests that excision of the nucleotide inserted *past* the lesion is faster than polymerization at that site. Moreover, it suggests that the residence time of the polymerase at the primer/lesion junction, after the extension step, is long enough to make excision at this site a significant parameter in inhibiting bypass. When the exonuclease activity is eliminated, the nucleotides inserted opposite and past the lesion remain in place until the nascent chain is further extended by the polymerase. Increased bypass in the absence of proofreading was reported for the Klenow fragment on DNA containing a site-specific thymine glycol (Clark & Birdsley, 1989) or an acetylaminofluorene-guanine adduct (Shibutani & Grollman, 1993), and for phage T7 DNA polymerase on a template containing acetylaminofluorene-modified guanines (Strauss & Wang, 1990).

It was previously shown that Pol I inserts preferentially a dAMP residue opposite abasic sites (Sagher & Strauss, 1983); however, the precise kinetic parameters were not determined. Using a template containing a site-specific abasic site analog enabled us to measure the K_m and k_{cat} of insertion of each of the dNMPs opposite the lesion. We found that dATP is indeed the best substrate for addition opposite the abasic site analog, exhibiting the lowest K_m and highest k_{cat} (Table 1). Purine nucleotides had an insertion specificity factor (k_{cat}/K_m) higher than pyrimidine nucleotides, primarily due to a much lower K_m (Table 1). Between the purine nucleotides, the difference is primarily due to a higher k_{cat} for dATP compared to dGTP, whereas between the two pyrimidine nucleotides there are nearly equal contributions of K_m and k_{cat} that lead to a higher insertion specificity factor for dCTP. This specificity is likely to be dictated primarily by the polymerization step since proofreading by the 3′→5′ exonuclease activity does not discriminate among the four possible combinations of a misinserted nucleotide opposite an abasic site analog (Ide et al., 1995).

The finding that the rate of bypass synthesis increased when dCTP, dATP, or both were omitted was somewhat surprising, primarily in light of the great preference for inserting dAMP opposite the abasic site analog. In template AB2, there is a stretch of three C's 3′ to the lesion. Thus, the first three nucleotides to be incorporated are dGMPs. The fact that omitting dATP caused an increase in the rate of bypass is consistent with the notion that extension past the lesion rather than addition opposite the lesion is rate-limiting under our conditions. The decrease in the rate of bypass synthesis with the increase in the number of dNTPs can be explained by competition for the dNTP binding site. Only dGTP is needed for past-lesion extension, and thus any other dNTP is, in a sense, a competitive inhibitor. It was previously shown that upon binding of a dNTP to the polymerase−DNA complex, the polymerase undergoes a conformational change in a process that is rate-limiting (Kornberg & Baker, 1991; Kuchta et al., 1987). When all four dNTPs are present, but only dGTP is needed, the polymerase 'waists' time in binding−dissociation cycles with incorrect dNTPs. Eliminating these dNTPs reduces the time spent by the polymerase in the base selection process, leading to an increase in the rate of bypass.

The size of the premutagenic ssDNA gap is unknown, although for repair-dependent mutagenesis it is likely to be in the range of an excision−repair gap (12−13 nucleotides;

Sancar & Rupp, 1983). We found that the structure of the gap affected bypass synthesis up to 3-fold, depending on the location of the 5′ boundary of the gap relative to the abasic site analog. The location of the primer terminus was less important, since extension up to the abasic site analog was fast, such that essentially most bypass events start from the primer terminus located opposite the lesion, regardless of its initial position. Within the limits dictated by our synthetic DNA substrates, bypass increased with gap size up to 11 bases, similar to the gap generated by the UvrABC repair nuclease (12−13 nucleotides; Sancar & Rupp, 1983). Pol I is known to bind strongly ssDNA (Kornberg & Baker, 1991), and the higher rate of bypass with the larger gap may reflect the higher affinity of Pol I to this substrate.

The extreme salt sensitivity of bypass synthesis by Pol I is intriguing. Under 100−200 mM potassium glutamate, which represents the intracellular ionic environment (Richey et al., 1987), and under which the polymerizing activity of Pol I is optimal, bypass synthesis was essentially eliminated. At this point, it is not clear whether this salt sensitivity implies that *in vivo* Pol I is not involved in bypass of abasic sites, since the *in vivo* microenvironment at the damaged site might be different, and interaction with other proteins may affect the properties of the polymerase.

REFERENCES

Bonner, C. A., Stukenberg, P. T., Rajagopalan, M., Eritja, R., O'Donnell, M., McEntee, K., Echols, H., & Goodman, M. F. (1992) *J. Biol. Chem. 267*, 11431−11438.

Clark, J. M., Beardsley, G. P. (1989) *Biochemistry 28*, 775−779.

Cohen-Fix, O., Livneh, Z. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3300−3304.

Cohen-Fix, O., & Livneh, Z. (1994) *J. Biol. Chem. 269*, 4953−4958.

Foster, P. L., & Davis, E. F. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2891−2895.

Hevroni, D., & Livneh, Z. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5046−5050.

Ide, H., Murayama, H., Sakamoto, S., Makino, K., Honda, K., Nakamuta, H., Sasaki, M., & Sugimoto, N. (1995) *Nucleic Acids Res. 23*, 123−129.

Kornberg, A., & Baker, T. (1991) *DNA Replication*, W. H. Freeman and Co., New York.

Kuchta, R. D., Mizrahi, V., Benkovic, P. A., Johnson, K. A., & Benkovic, S. J. (1987) *Biochemistry 26*, 8410−8417.

Kunkel, T. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1494−1498.

Kunkel, T. A., Shearman, C. W., & Loeb, L. A. (1981) *Nature 291*, 349−351.

Kunkel, T. A., Schaaper, R. M., & Loeb, L. A. (1983) *Biochemistry 22*, 2378−2384.

Lawrence, C. W., Borden, A., Banerjee, S. K., & LeClerc, J. E. (1990) *Nucleic Acids Res. 18*, 2153−2157.

Livneh, Z. (1986a) *J. Biol. Chem. 261*, 9526−9533.

Livneh, Z. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4599−4603.

Livneh, Z., Cohen-Fix, O., Skaliter, R., & Elizur, T. (1993) *CRC Crit. Rev. Biochem. Mol. Biol. 28*, 465−513.

Loeb, L. A. (1985) *Cell 40*, 483−484.

Loeb, L. A., & Preston, B. D. (1986) *Annu. Rev. Genet. 20*, 201−230.

Paz-Elizur, T., Takeshita, M., Goodman, M., O'Donnell, M., & Livneh, Z. (1996) *J. Biol. Chem. 271*, 24662−24669.

Povirk, L. F., & Goldberg, I. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3182−3186.

Randall, S. K., Eritja, R., Kaplan, B. E., Petruska, J., & Goodman, M. (1987) *J. Biol. Chem. 262*, 6864−6870.

Richey, B., Cayley, D. S., Mossing, M. C., Kolka, C., Anderson, C. F., Farrar, T. C., & Record, M. T., Jr. (1987) *J. Biol. Chem. 262*, 7157−7164.

Rupp, W. D., & Howard-Flanders, P. (1968) *J. Mol. Biol. 31*, 291−304.

Sagher, D., & Strauss, B. (1983) *Biochemistry 22*, 4518−4526.

Sancar, A., & Rupp, W. D. (1983) *Cell 33*, 249−260.

Schaaper, R. M., & Loeb, L. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1773−1777.

Schaaper, R. M., Glickman, B. W., & Loeb, L. A. (1982) *Mutat. Res. 106*, 1−9.

Shibutani, S., & Grollman, A. P. (1993) *J. Biol. Chem. 268*, 11703−11710.

Shwartz, H., & Livneh, Z. (1987) *J. Biol. Chem. 262*, 10518−10523.

Shwartz, H., Shavitt, O., & Livneh, Z. (1988) *J. Biol. Chem. 263*, 18277−18285.

Steighner, R. J., & Povirk, L. F. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8350−8354.

Strauss, B. S. (1985) *Cancer Surv. 4*, 493−516.

Strauss, B. S., & Wang, J. (1990) *Carcinogenesis 11*, 2103−2109.

Takeshita, M., & Eisenberg, W. (1994) *Nucleic Acids Res. 22*, 1897−1902.

Takeshita, M., Chang, C., Johnson, F., Will, S., & Grollman, A. P. (1987) *J. Biol. Chem. 262*, 10171−10179.

Tomer, G., Cohen-Fix, O., O'Donnell, M., Goodman, M., & Livneh, Z. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1376−1380.

Van Houten, B. (1990) *Microbiol. Rev. 54*, 18−51.

Villani, G., Boiteux, S., & Radman, M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3037−3041.

Walker, G. C. (1984) *Microbiol. Rev. 48*, 60−93.

BI9621324