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Mechanism of Translesion DNA Synthesis by DNA Polymerase II

COMPARISON TO DNA POLYMERASES I AND III CORE*

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Bypass synthesis by DNA polymerase II was studied using a synthetic 40-nucleotide-long gapped duplex DNA containing a site-specific abasic site analog, as a model system for mutagenesis associated with DNA lesions. Bypass synthesis involved a rapid polymerization step terminating opposite the nucleotide preceding the lesion, followed by a slow bypass step. Bypass was found to be dependent on polymerase and dNTP concentrations, on the DNA sequence context, and on the size of the gap. A side-by-side comparison of DNA polymerases I, II, and III core revealed the following. 1) Each of the three DNA polymerases bypassed the abasic site analog unassisted by other proteins. 2) In the presence of physiological-like salt conditions, only DNA polymerase II bypassed the lesion. 3) Bypass by each of the three DNA polymerases increased dramatically in the absence of proofreading. These results support a model (Tomer, G., Cohen-Fix, O., O'Donnell, M., Goodman, M. and Livneh, Z. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 1376–1380) by which the RecA, UmuD, and UmuC proteins are accessory factors rather than being absolutely required for the core mutagenic bypass reaction in induced mutagenesis in *Escherichia coli*.

The key step in induced mutagenesis is believed to be the insertion of a nucleotide opposite a DNA lesion by a DNA polymerase, a reaction termed bypass or translesion DNA synthesis (reviewed in Refs. 1 and 2). Due to the incorrect coding information of most DNA lesions, such a reaction is potentially mutagenic. In the bacterium *Escherichia coli*, induced mutagenesis by a variety of DNA-damaging agents was found to be dependent on particular gene products in addition to the polymerase, *i.e.* the UmuD, UmuC, and RecA proteins (1, 2). The fact that the expression of these proteins is under the regulation of the SOS stress response suggested that induced mutagenesis is a regulated process, which cannot occur without these SOS proteins (1, 2).

Early experiments clearly demonstrated that DNA lesions arrest DNA synthesis by a variety of DNA polymerases (3, 4), suggesting that bypass synthesis cannot occur without the UmuD, UmuC, and RecA proteins. However, a growing body of literature documents the ability of a variety of purified DNA polymerases to polymerize through DNA lesions unassisted by other proteins (5–16). Using a cell-free assay system for UV mutagenesis, we have demonstrated that crude protein extracts (17, 18) or a reconstituted system consisting of six purified proteins (19) can promote UV mutagenesis *in vitro* in the absence of RecA, UmuD, and UmuC. These observations create an apparent paradox; *in vitro* DNA polymerases can bypass lesions and produce mutations in the absence of UmuD, UmuC, and RecA, but *in vivo* these proteins are required in most mutagenesis assay systems. Notably, there are two exceptions, where pathways of UV mutagenesis were reported to be independent on the Umu proteins: in phage S13 (20) and in the F factor (21).

Very little is known on the ability of DNA polymerase II (pol II)[1] (22, 23) to bypass DNA lesions (11). Polymerase II is UV-inducible, and its gene is SOS-regulated (24, 25). The *in vivo* role of pol II was unknown for many years, and only recently have several studies suggested roles for pol II in adaptive mutagenesis (26, 27), in response to oxidative damage (26), and in bypass of abasic sites *in vivo* (28). Our recent finding that pol III core and pol II can each function to promote UV mutations in an *in vitro* system reconstituted from purified components (19) prompted us to study translesion DNA synthesis by purified DNA polymerase II. Here we describe the analysis of bypass synthesis by DNA polymerase II, and provide a side-by-side comparison of bypass by the three DNA polymerases of *E. coli*: DNA polymerases I, II, and III core.

EXPERIMENTAL PROCEDURES

Materials—Nucleotides were from Boehringer Mannheim. Radiolabeled nucleotides were from The Radiochemical Center, Amersham. Urea was obtained from ICN. EDTA, sodium glutamate, and acrylamide were from BDH. Tris base was from U. S. Biochemical Corp. Potassium glutamate and xylene cyanol were from Sigma. Bromophenol blue and dithiothreitol were from Bio-Rad, glycerol was from Baker, and formamide was from Fluka. DNA polymerase I (6000 units/mg) and its Klenow fragment (5000 units/mg) were obtained usually from Boehringer Mannheim, and occasionally from U. S. Biochemical Corp. A mutant of the Klenow fragment lacking the proofreading exonuclease activity was obtained from U. S. Biochemical Corp. DNA polymerase II and an exonuclease-deficient mutant of DNA polymerase II were purified as described (23). DNA polymerase III core and the α subunit of DNA polymerase III were purified as described (29). Buffer B contained 20 mM Tris·HCl, pH 7.5, 8 μg/ml bovine serum albumin, 5 mM dithiothreitol, 0.1 mM EDTA, 4% glycerol.

DNA Substrates—The 40-nucleotide-long templates AB1 and AB2, each containing a single abasic site analog at a unique position, were synthesized and purified as described (12, 30). The abasic site analog is a modified tetrahydrofuran moiety, which is a stable structural analog of 2′-deoxyribose in the apurinic/apyrimidinic site. It differs from an abasic site by having a hydrogen instead of a hydroxyl residue on 1′

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[1] The abbreviations used are: pol II, polymerase II; PAGE, polyacrylamide gel electrophoresis; ssDNA, single-stranded DNA.

carbon of the deoxyribose ring. It has been shown previously that endonuclease IV and exonuclease III cleave synthetic duplexes containing this analog, and DNA polymerases can bypass it (12). The templates are shown in Fig. 1 along with the complementary oligonucleotides used to form the gapped duplexes. The primers were ^{32}P-end-labeled by T4 polynucleotide kinase and then annealed (2 nmol) along with the phosphorylated downstream complementary oligonucleotide (2 nmol) to the 40-nucleotide-long synthetic DNA template (0.5 nmol) in 35–55 μl of a solution containing 10 mM Tris·HCl, pH 7.5, 1 mM EDTA and 150 mM NaCl. Annealing was carried out for 10 min at 70 °C, followed by slow cooling at room temperature over a period of 2–3 h. The gapped duplex was separated from excess primer and downstream oligonucleotide on a 3-ml Sephadex G-50 superfine column in a buffer containing 10 mM Tris·HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl at a flow of 0.45 ml/min. Fraction were collected and their contents analyzed by 20% PAGE under native conditions, followed by phosphorimaging using a Fuji BAS 1000 instrument. The recovery of the gapped duplex under these conditions was >95%. The preparations contained residual free primer, which did not exceed 5% of the amount of the substrate. This free primer did not interfere with the analysis, but it was degraded by the exonuclease activities of the polymerases, giving rise to radiolabeled fragments that were shorter than the primer (*e.g.* Fig. 2). 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).

Bypass Synthesis—The *in vitro* replication reaction mixture (20–50 μl) contained buffer B, 8 mM or 10 mM $MgCl_2$, 0.5 mM dNTPs, 35–100 nM gapped duplex, and 56–150 nM DNA polymerase. 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 0.05% xylene cyanol, 0.05% bromphenol blue, and 98% formamide. Samples of 10 μl were fractionated by electrophoresis on 20% polyacrylamide gels containing 8 M urea. End-labeled oligonucleotides were used as size standards. Samples were run at 500–600 V for 3–4 h, after which they visualized and quantified using a Fuji BAS 1000 phosphorimager.

Comparison of Bypass Synthesis by DNA Polymerase I, II, and III Core, and Their 3′ → 5′ Exonuclease-deficient Derivatives—The reaction mixture (50 μl), assayed at 30 °C, contained buffer B, 8 mM $MgCl_2$, 0.5 mM dNTPs, and 100 nM primed template AB1m3p7. The reactions were done either without salts or with 0.1 M potassium glutamate. The reactions were started with the addition of 150 nM of the indicated polymerase, carried out for 1–20 min, after which the reaction products were analyzed by 20% PAGE-urea, followed by visualization and quantification by phosphorimaging.

RESULTS

Polymerase II Bypasses an Abasic Site Analog—We have established an assay system for studying bypass synthesis on a defined synthetic gapped duplex DNA, carrying an abasic site analog at a predetermined location. The DNA templates were two 40-nucleotide-long synthetic DNAs, whose sequences were derived from the *lacZ′* portion of M13mp2 (with minor changes), for which *in vivo* data on the specificity of apurinic site mutagenesis existed (31). Template AB1 corresponded to positions 69–108 (when 1 is the beginning of *lacZ′* mRNA), and contained an abasic site analog instead of G^{89}, and oligonucleotide AB2 corresponds to positions 89–128, with an abasic site analog instead of A^{109}. These sites were found to be mutated when depurinated M13mp2 ssDNA was introduced into *E. coli* cells (31)

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

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

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

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

m3                                    T TTGG GACC GCAA TGGG-5'
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                        +9 +7+5 +3+1  -1-3
AB2    5'-GGAA AACC CTGG CGTT ACCC X ACT TAAT CGCC TTGC AGCA-3'

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

m3                                    A ATTA GCGG AACG TCGT-5'
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FIG. 1. **The structures of gapped duplexes containing site-specific abasic site analogs.** Underneath each template (AB1 or AB2) are shown pairs of complementary oligonucleotide that were used to form gapped duplexes. The oligonucleotides on the *right* are the primers, and those on the *left* are the downstream oligonucleotides. The locations of the 3′-end of the primers and the 5′-end of the downstream oligonucleotides are indicated at the *left* side. For example, m3p7 under AB2 represent a gapped duplex formed on oligonucleotide AB2 with the 3′ of the primer located at −3 relative to the abasic site analog (X), and the 5′-end of the downstream oligonucleotide located at the +7 position, forming a gap 9 nucleotides long.

with varying gap lengths (Fig. 1). The names of these gapped substrates describe their structure; For example, AB1m3p7 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 analog (0), and the 5′ end of the downstream oligonucleotide located at plus 7 (p7) relative to the abasic site analog, thus defining a particular 9-nucleotides gap (Fig. 1). Polymerization resulted in extension of the radiolabeled primer, and this was assayed by urea-PAGE, followed by quantitative measurements of the radiolabeled bands by phosphorimaging.

Fig. 2 shows the kinetics of polymerization by pol II with the gapped oligonucleotide AB2m3p7. The initial rapid synthesis stage, terminated opposite the nucleotide preceding the abasic site analog by 1 min. The extension past the lesion was much slower, and led to the accumulation of radiolabeled bypass products varying in length from 23 to 40 nucleotides. The fragments shorter than the primer are mostly due to the degradation by the polymerase of the residual amount of free primer present in the reaction mixture, as well as some of the annealed primer. A cluster of bypass products in the range of 23–28 nucleotides was found to accumulate with time, with a major 27-nucleotide-long product. This cluster represents, most likely, a kinetic pause imposed by the end of the gap. Complete filling-in of the gap should yield a 26-mer polymerization product. While such a product was observed, the major pause product was a 27-mer, and 28- and 29-mer products were observed as well, caused most likely by a partial displacement of the downstream oligonucleotide. In parallel, the accumulation of the full-length 40-mer product was observed (Fig. 2). Unlike pol I, pol II cannot perform nick translation. Therefore, DNA chain elongation beyond the boundary of the gap was accompanied, most likely, by a strand displacement mechanism. As a control we performed the bypass assay in the absence of dATP, where a bypass product 24 nucleotides long was expected. As can be seen in Fig. 2, this was indeed the main bypass product. The accumulation of the 15-mer under these conditions is most likely due to the fact that the two 3′-terminal nucleotides in the 17-mer primer are dAMP residues. Once they are removed by the 3′ → 5′ exonuclease activity of the polymerase, the primer cannot be extended due to the absence of dATP. Quantification of the results (Fig. 2*B*) revealed that bypass increased with reaction time reaching 20% at 30 min.

Bypass Synthesis by pol II Depends on Polymerase, dNTPs,

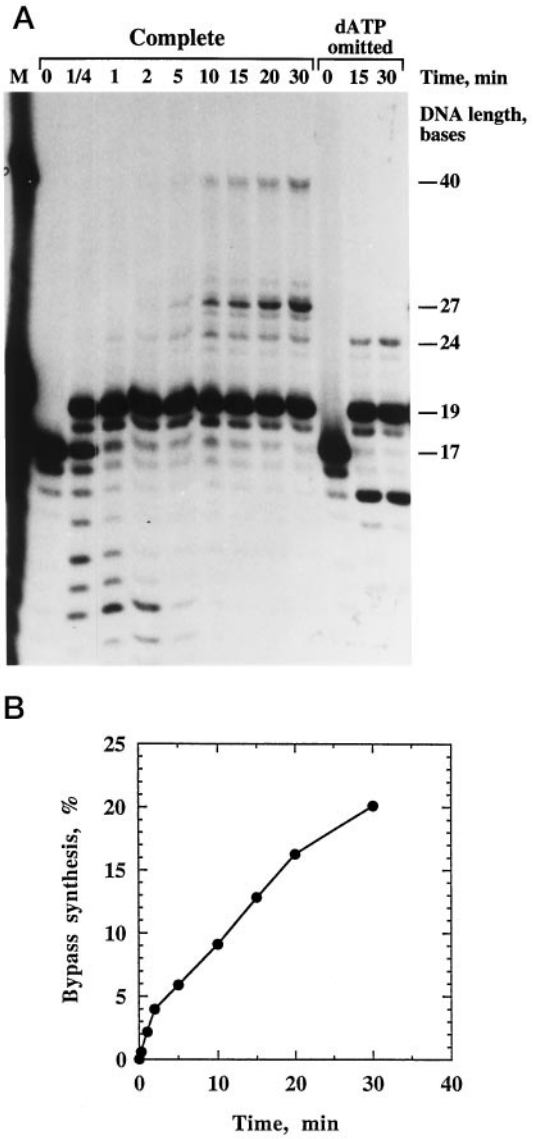

FIG. 2. **Bypass synthesis by pol II.** *A*, the reaction mixture contained buffer B, 8 mM $MgCl_2$, 0.5 mM dNTPs, 35 nM template AB2m3p7, and 56 nM pol II. Reactions were carried out at 30 °C for the indicated time periods. The effect of omission of dATP on bypass synthesis by pol II was assayed under the same reaction conditions in the absence of dATP. Replication products were separated by PAGE on a 20% sequencing gel containing urea and visualized by a phosphorimager. *Lane M* contains a mixture of radiolabeled oligonucleotide markers 17, 19, and 40 bases long. The 40-mer marker was synthesized to have the same sequence as the full bypass product. In this particular preparation the 17-mer primer contained a 5% contamination of the 16-mer (17–1). *B*, quantification of the image showed in *A* was done by scanning with a phosphorimager. Bypass synthesis was calculated by dividing the intensity of the 23–40-nucleotide-long bypass products by the sum of the termination product (the 19-mer) and the bypass products.

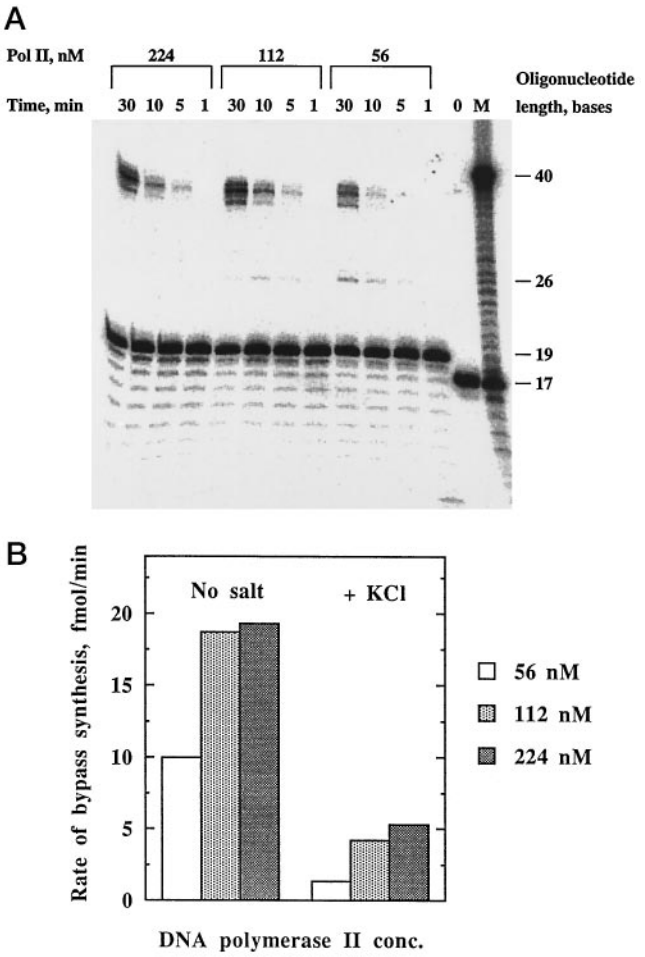

FIG. 3. **Effect of polymerase concentration on bypass synthesis.** *A*, the effect on bypass synthesis of increasing polymerase concentrations was examined in a reaction mixture containing buffer B, 8 mM $MgCl_2$, 0.5 mM dNTP, 35 nM template AB1m3p7, and the indicated polymerase concentrations. Reactions were carried out at 30 °C for the indicated time periods. Replication products were separated by PAGE on a 20% sequencing gel containing urea and visualized by a phosphorimager. *Lane M* contains oligonucleotide markers 17 and 40 bases long. The 40-mer marker was the non-modified template. It migrates slower than the full-length bypass product because of sequence differences. *B*, quantification of the image shown in *A* was done by scanning with a phosphorimager. Results are shown also for the bypass reaction in the presence of 50 mM KCl. The rate of bypass was calculated in the range of 5–30 min, where it was linear.

and Salt Concentrations, and on the DNA Sequence Context—We examined the effect of polymerase concentration on bypass. This was done with the second template, AB1, with the same gap configuration (AB1m3p7). As can be seen in Fig. 3, pol II bypassed the abasic site analog in this template too; however, the product distribution and the rate of bypass were different. With this template, most of the bypass products were full-length or nearly full-length. The slight heterogeneity in the length of the long products might be due to an end effect on this template, since it was also observed with substrate AB1m3, which does not have a downstream oligonucleotide (data not shown). A small fraction of bypass products terminated at the border of the gap, with the major termination site located precisely at the end of the gap (26 nucleotides long compared to 27 nucleotides on AB2m3p7) (Figs. 2 and 3). This suggests that the displacement of the downstream oligonucleotide was easier on AB1m3p7 than on AB2m3p7. The reason may be the stability of the duplex. In AB2m3p7, 7 out of the first 8 base pairs of the downstream duplex region are GC, in contrast to 5 out of 8 in AB1m3p7 (Fig. 1).

The rate of bypass on template AB2m3p7 was 2.5–4-fold faster than on AB1m3p7 (Fig. 4). Since the gap structure was identical, this difference is likely to originate from DNA sequence differences in the vicinity of the lesion. Thus, DNA sequence context may affect the ability of the polymerase to bypass the lesion. The ease of strand displacement past the lesion did not seem to have a significant role in bypass synthesis, since strand displacements appears to be faster with template AB1, on which bypass synthesis was slower (Figs. 3 and 4).

Increasing the concentration of the polymerase led to increased bypass (Fig. 3). This was true also when the reaction

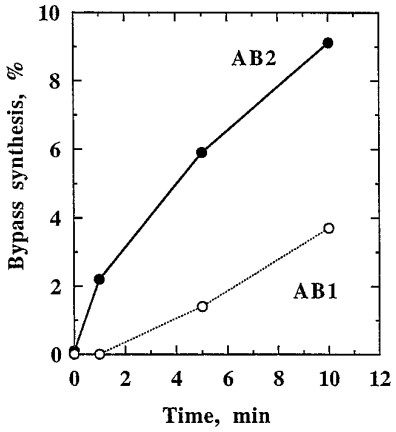

FIG. 4. **Effect of DNA sequence context on bypass synthesis.** Time course of replication by pol II on AB1m3p7 or AB2m3p7 was done under the same reaction conditions described in Fig. 2. Replication products were separated by PAGE on a 20% sequencing gel containing urea and visualized by a phosphorimager. *Solid circles*, bypass synthesis on AB2m3p7; *open circles*, bypass synthesis on AB1m3p7.

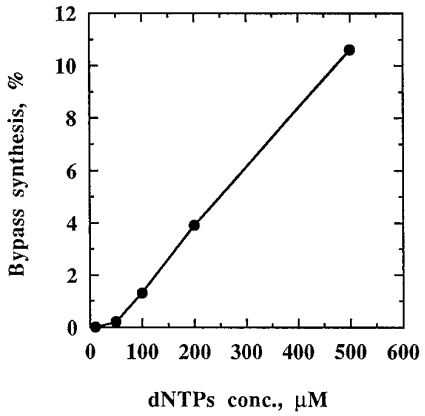

FIG. 5. **Effect of dNTP concentration on bypass synthesis.** The effect on bypass synthesis of dNTP concentration was assayed under the same reaction conditions described in Fig. 2. The reactions were carried out at 30 °C for 15 min.

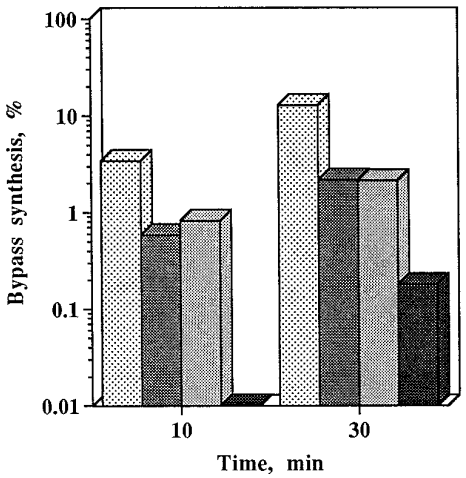

FIG. 6. **Salt sensitivity of bypass synthesis by pol II.** Bypass synthesis by pol II (56 nM) on template AB1m3p7 (35 nM) was assayed as described in Fig. 3 in the absence of salts (*dotted bar*), with potassium chloride (50 mM, *medium gray bar*), potassium glutamate (100 mM, *light gray bar*) or sodium glutamate (100 mM, *dark gray bar*).

was performed in the presence of 50 mM KCl (Fig. 3*B*; see below). This indicates, that the association of the polymerase with the DNA at the lesion site is important for bypass synthesis. We have examined also the effect of dNTP concentration on bypass. As can be seen in Fig. 5, bypass increased linearly with dNTP concentration in the range of 50–500 μM, consistent with a slow polymerization step in the bypass reaction. Examination of the dependence of bypass on Mg^{2+} concentration revealed that 10–12.5 mM $MgCl_2$ were optimal for bypass (data not shown). We have examined bypass synthesis by pol II in the presence of salt. Bypass synthesis was severely inhibited by 100 mM sodium glutamate (up to 65-fold). This inhibition was largely due to the Na^+ ions, since under salt conditions that are considered to be physiological, *i.e.* 0.1 M potassium glutamate (32), inhibition was much smaller (4–6-fold) (Fig. 6).

The Structure of the Gap Affects Bypass Synthesis—In order to examine the effect of structure of the gapped duplex on bypass, we examined bypass with the substrates AB1m3, AB1m3p3, and AB1m3p7 (Fig. 1). As can be seen (Fig. 7), bypass synthesis by pol II showed a dramatic dependence on the size of the ssDNA region. Increasing the ssDNA region from 5 nucleotides (AB1m3p3) to 9 nucleotides (AB1m3p7) increased bypass by up to 16-fold (0.2% *versus* 3.2% bypass, respectively, for the 10-min time point). Bypass on the primed ssDNA (AB1m3), which contained a single-stranded region 23 nucleotides long, was 5.8% under the same conditions, 1.8-fold higher than on AB1m3p7. Similar differences were observed also in the presence of salt (Fig. 7). These differences may be due to stronger binding of the polymerase to the longer ssDNA region, which led to increased bypass.

Bypass Synthesis Increases in the Absence of the Proofreading Exonuclease Activity—In order to examine the role of the 3′ → 5′ proofreading exonuclease activity in bypass synthesis, a mutant pol II deficient in the 3′ → 5′ exonuclease was used. As can be seen (Fig. 8), the rate of bypass synthesis by the mutant polymerase was 15-fold higher than with the wild-type polymerase. A similar result was observed with the second template (AB1), both in the absence and presence of salt (Fig. 9). Notice that the two polymerases gave rise to different distributions of bypass products on template AB1 (Figs. 8 and 9). The mutant, but not the wild-type polymerase exhibited a major pause as it approached the end of the gap on template AB1, with a major pause product of 26 nucleotides, marking the end of the gap. This suggests that the mutant polymerase performs strand displacement slower than the wild-type polymerase. The highly increased bypass capacity of the mutant polymerase was also observed in the presence of 0.1 M sodium or potassium glutamate, and the general patterns of bypass products were similar (Fig. 9*A*). Overall, the percentage of shorter bypass products was 2–3-fold higher with the mutant polymerase as compared to the wild-type enzyme. Interestingly, although there was a significant reduction in the full-length bypass products in the presence of salt, there was only a marginal effect on the overall bypass. This was because a larger proportion of bypass products were terminated at the border of the gap. Thus, salt has little effect on bypass by the mutant polymerase, but it inhibited strand displacement, possibly due to stabilization of the duplex structure under higher ionic strength conditions (Fig. 9).

Comparison of Bypass Synthesis by DNA Polymerases I, II, and III—DNA polymerase III in its holoenzyme form, the major replication machine, is composed of at least 10 subunits, and has very high processivity (33, 34). Since we were studying the filling-in of short ssDNA gaps, we used pol III core, rather than the holoenzyme. Polymerase III core is a tight complex composed of three subunits: α, containing the polymerase catalytic site (35), ε, carrying the proofreading 3′ → 5′ exonuclease (36), and θ, for which there is no assigned function yet (29). pol III core is present in 40 copies/cell, in excess over the holoenzyme, estimated to be present at 8 copies/cell (34). It can be

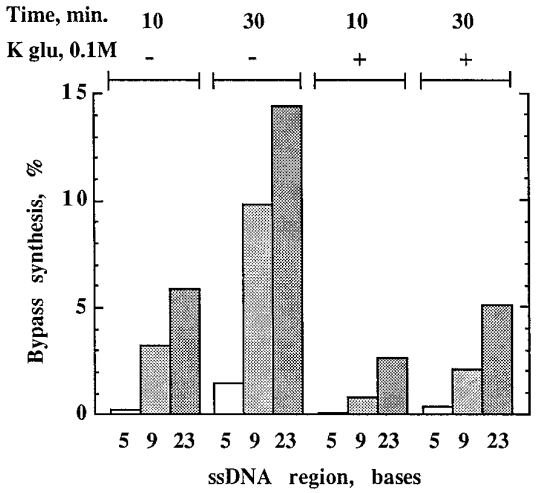

FIG. 7. **The structure of the gap affects bypass synthesis by pol II.** Gapped duplexes (36 nM) with varying location of the 5′-end of the downstream oligonucleotide were assayed in order to determine the effect of gap length on bypass synthesis. AB1m3p3, 5-base gap; AB1m3p7, 9-base gap; AB1m3, 23 bases of ssDNA. The reaction conditions were as described in the legend to Fig. 3. The reactions were carried out in the presence or absence of 100 mM potassium glutamate, at 30 °C for 10 or 30 min.

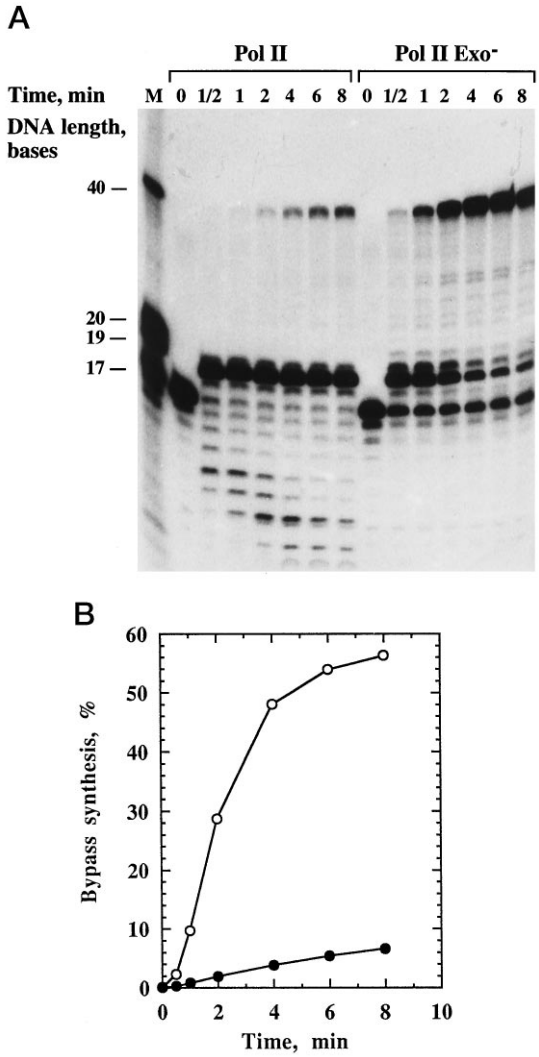

FIG. 8. **Bypass synthesis by pol II greatly increases in the absence of proofreading.** *A*, pol II and a mutant of pol II deficient in the 3′ → 5′ proofreading exonuclease activity were used in replication of template AB2m3 (36 nM) under the same reaction conditions described in Fig. 2 except that 10 mM $MgCl_2$ and 24 nM polymerase were used. Reactions were carried out at 30 °C for the indicated time periods. Replication products were separated by PAGE on a 20% sequencing gel containing urea and visualized by a phosphorimager. *Lane M* contained a mixture of radiolabeled oligonucleotides, which were 17, 19, 20, and 40 bases long. *B*, quantification of the image shown in *A* was done by scanning with a phosphorimager. *Solid circles*, wild-type pol II; *open circles*, mutant of pol II deficient in the 3′ → 5′ exonuclease activity.

purified as an independent entity from extracts of *E. coli* (37), and it may function also in the absence of the other accessory subunits (19).

We have compared bypass synthesis, side by side, by each of the three DNA polymerases of *E. coli*. This was done on template AB1m3p7, both in the absence and presence of 0.1 M potassium glutamate. As can be seen in Fig. 10, pol III core was able to bypass the abasic site analog, albeit at a low rate (0.5% bypass at 20 min). On this particular template, in the absence of salt, bypass by pol I was fastest. It was 2-fold faster than by pol II, and 35-fold faster than by pol III core (Fig. 10*B*). This picture changed dramatically in the presence of 0.1 M potassium glutamate. No bypass was observed with pol III core, bypass by pol I was inhibited 20–30-fold, whereas the rate of bypass by pol II was only slightly affected (Fig. 9*C*). Thus, under physiological salt conditions, only pol II is capable of bypass synthesis. It should be pointed out, however, that the *in vivo* significance of this result is not straightforward, since the intracellular environment, and in particular interactions with other proteins may affect the final activities of the polymerases.

Bypass Synthesis by DNA Polymerases I, II, and III Increases Dramatically in the Absence of Proofreading—We compared the bypass properties of the three DNA polymerases in the absence of proofreading. We used the pol II mutant described above, and an exonuclease-defective mutant of the Klenow fragment of pol I. Instead of pol III core, we used the α subunit of pol III, which contains the polymerase catalytic site, but not the exonuclease active site (35). Each of the exonuclease-deficient polymerases was much more efficient in bypass synthesis than its cognate wild-type enzyme (Fig. 11). This includes the α subunit of pol III, that exhibited a rate of bypass 20–25-fold higher than pol III core (Fig. 11*C*). The α subunit synthesized bypass products that were shorter than those synthesized by the other polymerases (mostly 22–26 nucleotides long; Fig. 11*A*), most likely due to a reduced strand displacement activity. With pol I and pol II, the rates of bypass by the exonuclease-deficient polymerases were 54- and 194-fold higher than with the respective wild-type polymerases (Table I). The addition of salt completely inhibited bypass by the α subunit, whereas the effects on pol I and pol II were smaller (inhibition of 2.7- and 1.4-fold, respectively; Fig. 11, Table I). Under these conditions the rates of bypass by the exonuclease-deficient pol I and pol II were 432- and 147-fold higher that with the respective wild-type enzymes (Table I). The differences here were greater than in Fig. 8, most likely due to the higher polymerase and DNA concentrations. Thus, in the absence of proofreading, the rate of bypass synthesis increased dramatically both in the absence and presence of 0.1 M potassium glutamate.

DISCUSSION

This study establishes the capability of each of the three DNA polymerases of *E. coli* to bypass an abasic site analog, unassisted by other proteins (Table I). Depending on reaction conditions, the extents of bypass vary considerably and can reach substantial extents of up to 80% (*e.g.* Fig. 11), much higher than previously reported (*e.g.* Ref. 11). Several common bypass principles can be drawn based on our experiments. Like any other enzymatic reaction, bypass synthesis was dependent

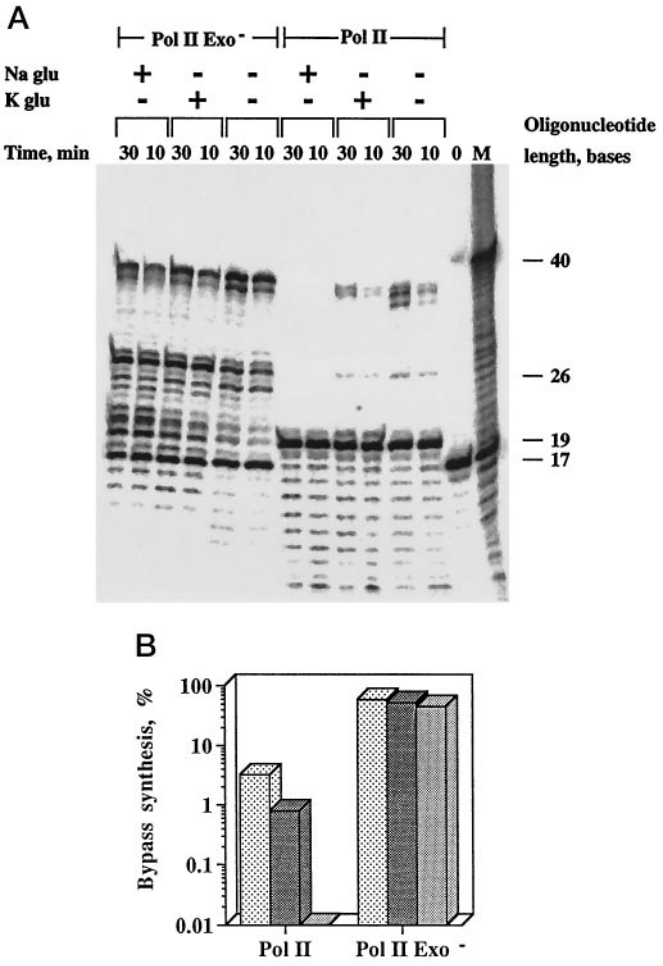

FIG. 9. **Salt inhibits bypass synthesis by pol II but not by an exonuclease-deficient mutant of pol II.** *A*, a mutant of pol II (56 nM) deficient in the 3′ → 5′ proofreading exonuclease activity was used in replication on gapped duplex AB1m3p7 (35 nM) under the same reaction conditions described in Fig. 3 and in the presence of salts. Reactions were carried out at 30 °C for the indicated time periods. Replication products were separated by PAGE on a 20% sequencing gel containing urea and visualized by a phosphorimager. *Lane M* contains oligonucleotide markers 17 and 40 bases long. The 40-mer marker was the non-modified template. It migrates slower than the full-length bypass product because of sequence differences. *B*, quantification of the image shown in *A* was done by scanning with a phosphorimager. *Dotted bar*, no salt; *dark gray bar*, 100 mM potassium glutamate; *light gray bar*, 100 mM sodium glutamate.

on the concentrations of the polymerase, and the two substrates: the DNA[2] and the dNTPs. The involvement of a slow bypass step underscores the importance of these parameters; however, they were frequently not given enough attention in bypass studies for technical reasons. The concentration of the damaged DNA is often kept low in bypass experiments because of quantity limitations, and polymerase concentration is often kept low because of the same reason, or due to interference by associated activities (*e.g.* the 5′ → 3′ exonuclease activity of pol I) or by impurities present in the polymerase preparation. Notably, the intracellular concentration of pol I in *E. coli* was estimated to be 500–700 nM (34), whereas 10–100-fold lower concentrations were usually used in bypass studies. The importance of substrates and enzyme concentrations can be illustrated by the conflicting reports on the ability of the Klenow fragment to bypass a site-specific acetylaminofluorene-guanine adduct. The investigators who reported that bypass was not

[2] T. Paz-Elizur and Z. Livneh, unpublished observation.

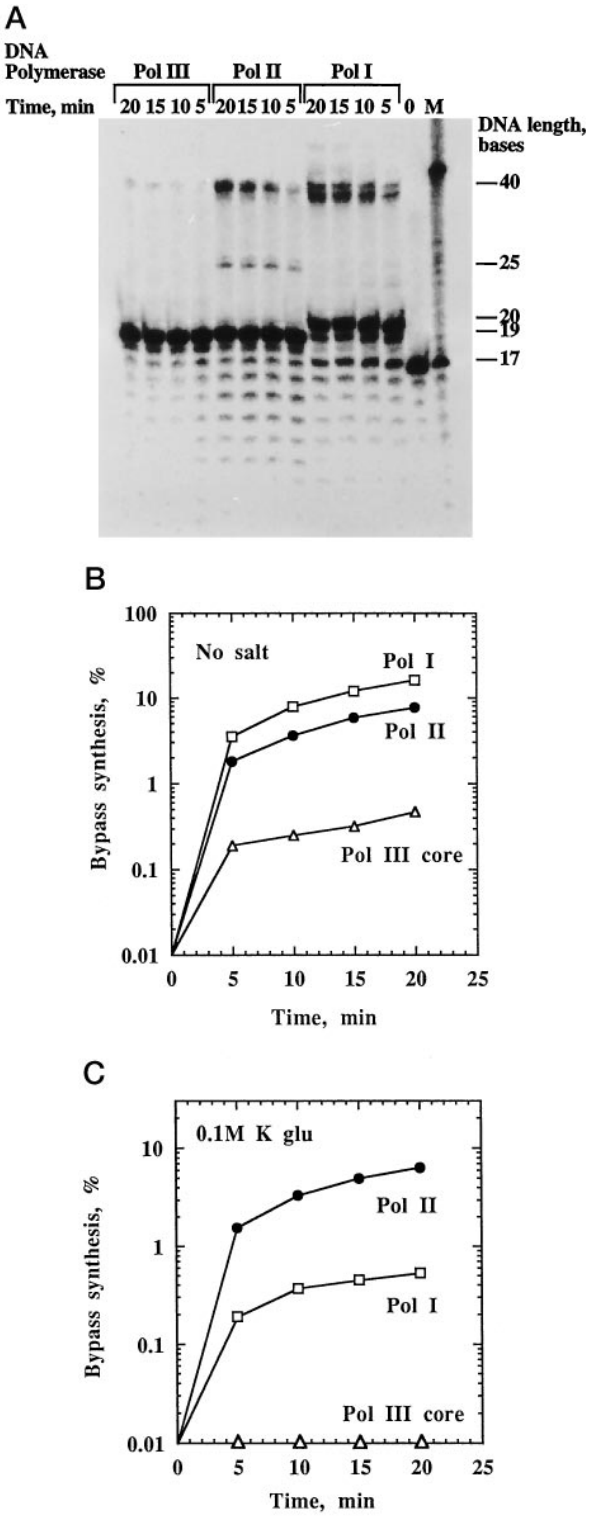

FIG. 10. **Comparison of bypass synthesis by DNA polymerases I, II, and III core.** *A* time course of replication by pol I, pol II, and pol III core on gapped duplex AB1m3p7. The reaction mixture contained buffer B, 8 mM $MgCl_2$, 0.5 mM dNTP, 100 nM gapped duplex AB1m3p7, and 150 nM of the indicated polymerase. Reactions were carried out at 30 °C for the indicated time periods. Replication products were separated by PAGE on a 20% sequencing gel containing urea and visualized by a phosphorimager. *Lane M* contains oligonucleotide markers 17 and 40 bases long. The 40-mer marker was the non-modified template. It migrates slower than the full-length bypass product because of sequence differences. *B*, quantification of the image shown in *A* was done by scanning with a phosphorimager. *C*, comparison of bypass synthesis by DNA polymerases I, II, and III core in the presence of 100 mM potassium glutamate. *Squares*, pol I; *circles*, pol II; *triangles*, pol III core.

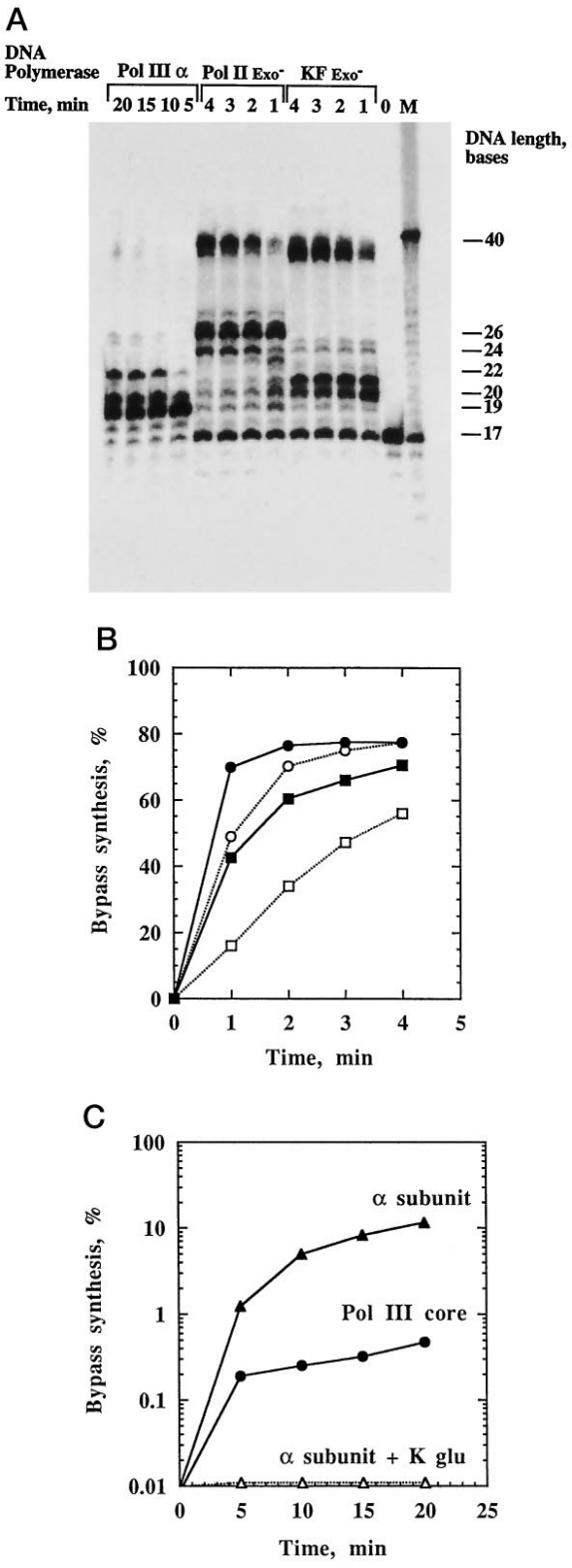

FIG. 11. **Comparison of bypass synthesis by DNA polymerases I, II, and III core in the absence of proofreading.** *A*, time course of replication on gapped duplex AB1m3p7 by the three DNA polymerases in the absence of proofreading. We used exonuclease-deficient mutants of the Klenow fragment of pol I and of pol II. Instead of pol III core, we used the α subunit of pol III, which contains the polymerase catalytic site, but not the exonuclease active site. The reaction conditions were as described in Fig. 11. Replication products were separated by PAGE on a 20% sequencing gel containing urea and visualized by a phosphorimager. *Lane M* contains oligonucleotide markers 17 and 40 bases long. The 40-mer marker was the non-modified template. It migrates slower than the full-length bypass product because of sequence differences. *B*, quantification results of the comparison of bypass synthesis without salts or with 100 mM potassium glutamate by exonuclease-deficient mutants of the Klenow fragment of pol I and of pol II. *Squares*, pol I; *circles*, pol II; *solid symbol*, no salt; *open symbol*, 100 mM potassium glutamate. *C*, comparison of bypass synthesis by DNA polymerase III core and its α subunit. *Solid circles*, pol III core; *solid triangles*, the α subunit of pol III; *open triangles*, the α subunit of pol III with 100 mM potassium glutamate.

TABLE I
Comparison of bypass synthesis of E. coli DNA polymerases through an abasic site analog

The table summarizes the rates of bypass synthesis of the polymerases based on the data presented in Figs. 10 and 11. The reaction mixtures (50 μl) contained 8 mM $MgCl_2$, 0.5 mM dNTPs, 100 nM gapped duplex AB1m3p7, and 150 nM of the indicated polymerase. Reactions were carried out at 30 °C, in the absence or the presence of 100 mM potassium glutamate. The results are presented as fmol of lesions bypassed/min. Under these conditions, a rate of 50 fmol of lesions bypassed/min is equal to 1% of the lesions/min. The α subunit of pol III represents the polymerase without exonuclease activity.

	Rate of bypass synthesis			
	Wild-type polymerase		Exonuclease-deficient polymerase	
	No salt	+ K glu	No salt	+ K glu
	fmol lesions bypassed/min			
pol I	39.5	1.8	2125	800
pol II	18.0	16.5	3490	2440
pol III core	1.3	<0.1	24	<0.1

observed (38) used a 100-fold lower concentration of DNA, a 10-fold lower concentration of enzyme, and a 2.5-fold lower concentration of dNTPs than the investigators who reported a significant extent of bypass (13).

In addition to these factors, which are common to all enzymatic reactions, several other parameters were found to have a substantial effect on bypass synthesis. The structure of DNA affected bypass in two ways. The DNA sequence context of the lesion can have a considerable effect on bypass, as illustrated by the fact that bypass by pol II on template AB2 was 2.5–4-fold faster than on template AB1. The reasons for these differences are not understood. For a gapped duplex substrate, the structure of the gap can greatly affect bypass synthesis, as indicated by the 16-fold variation in bypass by pol II. The critical parameter seems to be the distance of the 5′ terminus of the downstream oligonucleotide from the lesion, which needs to be of a minimal length in order to ensure optimal bypass. Due to the length limitation of the substrates used in our studies, we could not cover a wide range of ssDNA gaps. However, it seems that a ssDNA region of around 10 nucleotides downstream to the lesion is optimal for bypass, at least for pol II. This size, which is similar to the size of a nucleotide excision repair gap (39), may represent an optimal binding site for pol II.

The idea that inhibition of the proofreading of DNA polymerases is needed to enable bypass synthesis was suggested 18 years ago by Radman and colleagues (40). They reasoned, based on the extensive turnover of dNTPs on UV-irradiated DNA, that nucleotides incorporated opposite a damaged nucleotide are partially mismatched and are thus excised by the proofreading exonuclease activity of the polymerase. They proposed that, in the presence of proofreading, the polymerase idles opposite the lesion by performing repeated cycles of addition and excision of the newly added nucleotide, without being able to bypass the lesion. It is clear from our studies, as well as from studies performed by others, that bypass occurs in the presence of normal proofreading and thus inhibition of proofreading is not a prerequisite for bypass. However, the present

study shows clearly that bypass by each of the *E. coli* DNA polymerases was dramatically increased (20–400-fold) in the absence of proofreading (Figs. 8, 9, and 11 and Table I).

Previous studies have demonstrated increased bypass in the absence of proofreading by the Klenow fragment of pol I on templates containing a thymine glycol (14), and an acetylaminofluorene adduct (13), and by the phage T7 DNA polymerase on a template containing acetylaminofluorene modified guanines (41). The absence of bypass by an exonuclease-deficient derivative of the Klenow fragment of pol I, and by the α subunit of pol III on a site-specific acetylaminofluorene-guanine adduct (38) was caused most likely by the use of overly low enzyme, dNTP, and DNA concentrations.

Since extension past the lesion seems to be slow for several DNA polymerases (10, 41, 42), the critical parameter might be the relation between the residence time of the polymerase at the damaged site, and the rate of excision opposite the damaged site. If the residence time is long compared to the rate of excision, inhibition of excision is expected to have a profound effect on bypass synthesis by maintaining the primer terminus opposite the lesion available to repeated extension attempts. These might be the cases presented in the current study. If the residence time is short relative to the excision rate, like was suggested for pol III holoenzyme, inhibition of exonuclease activity might not suffice without additional factors that would stabilize the polymerase-DNA interaction, and thus increase residence time (1, 42–44).

The DNA substrate used in our studies was a synthetic gapped duplex DNA containing a site-specific abasic site analog in the ssDNA region. The examination of bypass synthesis in a duplex containing short ssDNA gaps was motivated by our discovery of a UV mutagenesis pathway associated with nucleotide excision repair gaps (17, 18). The minimal components for this mutagenesis pathway were recently identified in a reaction reconstituted with six purified proteins (19). We found that mutations were promoted in the reconstituted system by pol III core or pol II, but not by the major repair polymerase pol I. One of the reasons maybe that the reconstituted reaction was performed under physiological-like salt conditions that severely inhibit bypass synthesis by pol I. The potency of pol II in promoting mutations in the reconstituted system correlates with its effectiveness in bypass synthesis on the gapped duplex. On the other hand, the ability of pol III to promote mutations is inconsistent with its inability to bypass the abasic site analog in the gapped duplex assay. This may be caused by the difference in templates. The gapped duplex may be too short for stable binding of pol III core, the largest of the three DNA polymerases. Alternatively, one has to consider the difference in the type of lesion (abasic site analog *versus* pyrimidine dimers), or interaction with the other proteins in the reconstituted system (UvrA, UvrB, UvrC, and DNA helicase II).

Bypass synthesis is thought to be the key step in mutagenesis associated with DNA damage. *In vivo* this process depends on the RecA, UmuD, and UmuC proteins in most systems that were examined. This study clearly shows that bypass synthesis can occur unassisted, at effective rates. This is consistent with our results in the reconstituted repair-gap mutagenesis system, where UV mutations were generated in the absence of RecA, UmuD, and UmuC (19). This suggests that the core mutagenic bypass reaction in induced mutagenesis does not require RecA, UmuD, and UmuC. These proteins may function indirectly to inhibit competing reactions, *e.g.* recombinational repair (45). Alternatively, the UmuD/C and RecA proteins may serve as accessory loading factors that stabilize the binding of the polymerase to DNA, thus enabling bypass under unfavorable conditions (*e.g.* low polymerase concentration). The bypass system developed in this study is currently used to test these ideas.

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Nucleic Acids, Protein Synthesis, and Molecular Genetics:

Mechanism of Translesion DNA Synthesis by DNA Polymerase II: COMPARISON TO DNA POLYMERASES I AND III CORE

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