Analysis of Unassisted Translesion Replication by the DNA Polymerase III Holoenzyme†

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ABSTRACT: DNA damage-induced mutations are formed when damaged nucleotides present in single-stranded DNA are replicated. We have developed a new method for the preparation of gapped plasmids containing site-specific damaged nucleotides, as model DNA substrates for translesion replication. Using these substrates, we show that the DNA polymerase III holoenzyme from *Escherichia coli* can bypass a synthetic abasic site analogue with high efficiency (30% bypass in 16 min), unassisted by other proteins. The θ and τ subunits of the polymerase were not essential for bypass. No bypass was observed when the enzyme was assayed on a synthetic 60-mer oligonucleotide carrying the same lesion, and bypass on a linear gapped plasmid was 3−4-fold slower than on a circular gapped plasmid. There was no difference in the bypass when standing-start and running-start replication were compared. A comparison of translesion replication by DNA polymerase I, DNA polymerase II, the DNA polymerase III core, and the DNA polymerase III holoenzyme clearly showed that the DNA polymerase III holoenzyme was by far the most effective in performing translesion replication. This was not only due to the high processivity of the pol III holoenzyme, because increasing the processivity of pol II by adding the γ complex and β subunit, did not increase bypass. These results support the model that SOS regulation was imposed on a fundamentally constitutive translesion replication reaction to achieve tight control of mutagenesis.

Genomic DNA in each cell suffers from continuous damage by both intracellular and external agents. Most of these DNA lesions are eliminated by error-free DNA repair mechanisms (*1*). When lesions that have escaped repair are replicated, they give rise to mutations, due to misinsertion opposite the damaged site by DNA polymerases (reviewed in refs *2* and *3*). Bypass of lesions that are highly blocking with respect to replication is regulated by the SOS stress response in *Escherichia coli*, and is the classical paradigm for genetically regulated DNA damage-dependent mutagenesis (*3*−*5*). The mechanism of this pathway is not fully understood, but the recent reconstitution of the reaction with purified components by two groups (*6*, *7*) has shown that it proceeds via RecA-, UmuD′-, and UmuC-stimulated translesion replication by the DNA polymerase III holoenzyme, thus confirming the previous results of Rajagopalan et al. (*8*).

It is well established that DNA polymerase I (*9*−*14*) and DNA polymerase II (*15*, *16*) can bypass blocking DNA lesions unassisted by SOS-induced proteins, with efficiencies that vary considerably depending on the reaction conditions (*16*). However, there was a controversy about the bypass ability of the DNA polymerase III holoenzyme. While our laboratory determined that the pol III[1] holoenzyme can bypass both UV lesions (*17*−*19*) and abasic sites (*20*) during replication of ssDNA, others have reported essentially no bypass (*8*, *15*, *21*). This is an important point, because the available genetic data point toward pol III being the only DNA polymerase required for SOS mutagenesis (*22*−*24*). The high complexity of the DNA polymerase III holoenzyme (*25*−*27*) led us to reason that the disagreement in the in vitro results may stem from the usage of different types of DNA substrates. Whereas we used circular ssDNA with randomly distributed lesions (*17*−*20*), others used either synthetic oligonucleotides (*21*) or linear ssDNA with a site-specific lesion situated close to the DNA end (*8*, *15*). To resolve these differences, and to provide native-like DNA substrates for translesion replication studies, we developed a new method for the quantitative preparation of gapped plasmid DNA, containing a site-specific lesion in the ssDNA region (lesion/gap plasmids). Using this type of substrate, we show that the DNA polymerase III holoenzyme can bypass an abasic site analogue, unassisted by other proteins, and we analyze the characteristics of this reaction.

MATERIALS AND METHODS

Materials. The sources of materials used were as follows: nucleotides, dithiothreitol, bovine serum albumin, and

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[1] Abbreviations: pol I, DNA polymerase I; pol II, DNA polymerase II; pol III, DNA polymerase III; PAGE, polyacrylamide gel electrophoresis; ssDNA, single-stranded DNA; SSB, single strand-binding protein.

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proteinase K from Boehringer-Mannheim; [γ-^{32}P]ATP from NEN Research Products; urea from ICN; EDTA and $MgCl_2$ from Merck; acrylamide from BDH; Tris base, potassium glutamate, and xylene cyanol from Sigma; bromophenol blue from Bio-Rad; glycerol from Baker; formamide from Fluka; NA45 DEAE membranes from Schleicher & Schuell; and dSpacer CE phosphoramidite, an abasic site analogue building block used in oligonucleotides synthesis, from Glen Research.

Proteins. The DNA polymerase III holoenzyme was purified as described previously (*28*). DNA polymerase I (6000 units/mg) and its Klenow fragment (5000 units/mg) were from Boehringer-Mannheim. DNA polymerase II, purified according to the method described in ref *29*, was a gift from M. Goodman (University of Southern California, Los Angeles, CA). The DNA polymerase III core, the γ complex, and the α, ϵ, θ, β, and τ subunits of pol III were gifts from M. O'Donnell (Rockefeller University, New York, NY). Restriction nucleases *Bst*XI, *Bsa*I, *Xmn*I, and *Pvu*II, T4 DNA ligase, and T4 polynucleotide kinase were from New England Biolabs. Restriction nuclease *Dra*III was from Boehringer-Mannheim, and S1 nuclease was from Promega.

DNA. All oligonucleotides were synthesized and purified by the Synthesis Unit of the Biological Services Department of the Weizmann Institute of Science. Oligonucleotides containing a synthetic abasic site were synthesized similarly using dSpacer CE phosphoramidite as a building block. The abasic site analogue is a modified tetrahydrofuran moiety which is a stable analogue of 2′-deoxyribose in the abasic site. It has a hydrogen instead of a hydroxyl residue at C-1′ of the deoxyribose ring (*12*). Plasmid pSKSL was constructed as follows. The 1091 bp *Aat*II−*Hin*dIII fragment from pACYC177 was ligated to a 3800 bp *Aat*II−*Hin*dIII fragment from pOC2 (*30*) to generate plasmid pSL1 (4891 bp). The 1371 bp *Bst*EII fragment of phage λ DNA, containing a *Bst*XI site, was inserted into the single *Pst*I site of pSL1, forming plasmid pSL2 (6262 bp). Plasmid pSL3 (4308 bp) was constructed by deletion of the 1954 bp *Eco*RV−*Bst*EII fragment from pSL2. Plasmid pSL3 carries the medium-copy number origin of replication from pBR322. To render the plasmid more suitable for quantitative preparations, the 1291 bp *Fsp*I−*Pvu*II fragment from pBluescript II SK(+) (Stratagene), which carries a high-copy number origin of replication, was ligated to the 2070 bp *Fsp*I−*Fsp*I fragment from plasmid pSL3, containing the *Bst*XI site and the gene for kanamycin resistance, to form plasmid pSKSL (3361 bp).

Construction of Lesion/Gap Plasmids. The gap/lesion plasmids were constructed by ligating a gapped duplex oligonucleotide carrying a site-specific synthetic abasic site to a restriction nuclease-cleaved plasmid via nonpalindromic cohesive termini (Figure 1). The gapped duplex oligonucleotide was formed by annealing a 50- or 60-mer template with or without a synthetic abasic site, a 5′ ^{32}P-labeled primer, and an unlabeled 15-mer oligonucleotide. The DNA sequences of the oligonucleotides and the structures of the gapped duplexes are shown in Figure 2. The primer was 5′ end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP, and then annealed (4 nmol) together with the phosphorylated downstream 15-mer oligonucleotide (4 nmol) to the template (1 nmol) in 100 μL of a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 150 mM NaCl by heating to 70 °C for 10 min, and then cooling to room temperature

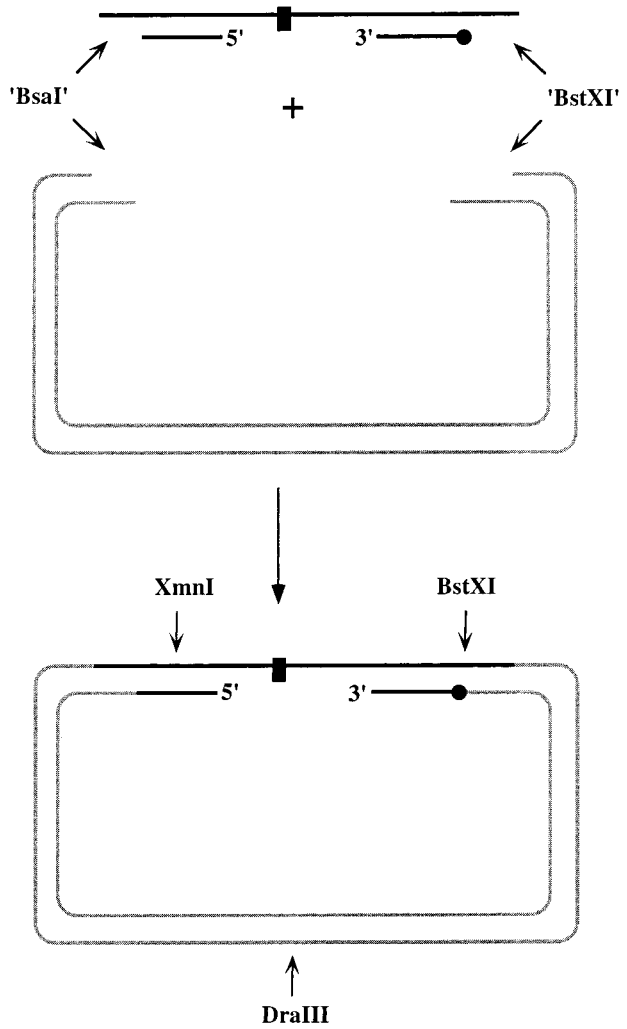

FIGURE 1: Schematic description of the construction of gap/lesion plasmids. The final substrate was prepared by ligating two components: a plasmid cleaved with restriction nucleases *Bst*XI and *Bsa*I, which generated nonpalindromic termini, and a gapped duplex oligonucleotide, containing a site-specific abasic site analogue opposite the gap. The termini of the gapped duplex were complementary to the termini of the cut plasmid, such that ligation created a circular gapped DNA containing a gap/lesion structure. The ligation restored the *Bst*XI site, but not the *Bsa*I site. The primer in the gapped oligonucleotide contained a ^{32}P-labeled 5′ end (marked by a black circle) that becomes internal upon ligation. Translesion replication on this substrate caused gap filling. The newly synthesized DNA was released from the substrate by cleavage with restriction nucleases *Bst*XI (which cleaves just upstream of the radiolabel) and *Xmn*I (which cleaves in the plasmid downstream of the gap) and analyzed by urea−PAGE.

over a period of 2−3 h. The gapped duplex oligonucleotide was purified by PAGE (8%), and the band containing the gapped duplex was excised, crushed, and soaked in a buffer containing 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS at room temperature for 8−12 h. The gapped duplex was concentrated by ethanol precipitation, and dissolved in 10 mM Tris-HCl and 1 mM EDTA (pH 7.5). The vector was the 2945 bp *Bst*XI−*Bsa*I fragment of plasmid pSKSL. It was obtained by cleavage of the plasmid, followed by fractionation on a 0.8% agarose gel, and purification by electroelution onto an NA45 DEAE membrane. The fragment was eluted from the membrane with a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1.5 M NaCl, at 65 °C for 2 h, with a yield of

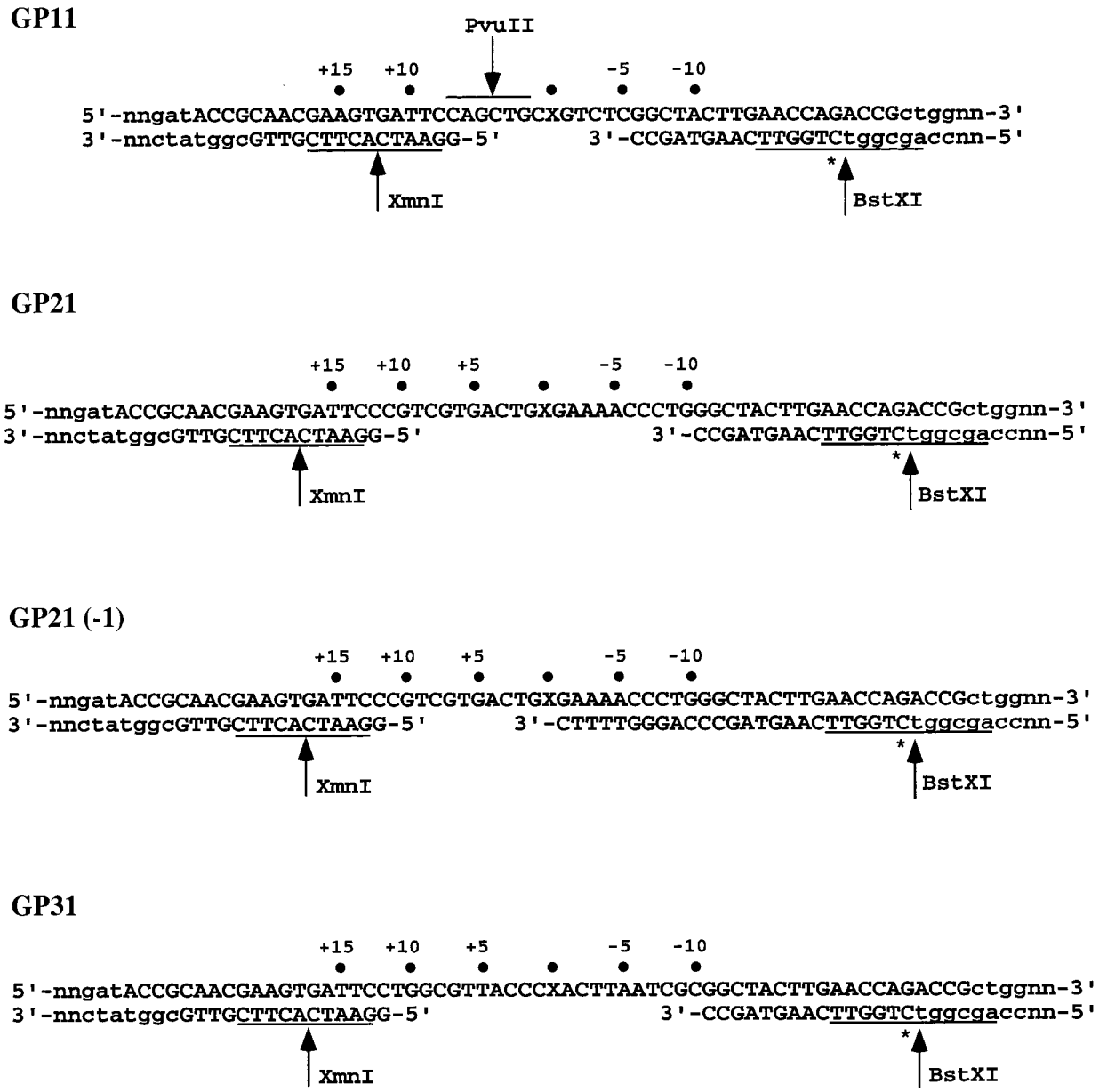

FIGURE 2: DNA sequence of the gap/lesion region in substrates used in this study. The gapped plasmid GP11 contains the 3′-OH at position −6; GP21 and GP31 each contain the 3′-OH terminus located at position −11, and GP21(−1) contains the 3′-OH terminus at position −1 (the 0 position refers to the location of the abasic site analogue, marked with an X). They differ in the DNA sequence at the vicinity of the lesion. Each of the gapped plasmids contains *Xmn*I and *Bst*XI restriction nuclease sites. The asterisks denote the radiolabel. Capital letters refer to the sequence of the gapped duplex oligonucleotide, whereas small letters refer to plasmid sequences.

35%. Preperative ligation of the insert to the vector was carried out in a 1:1 molar ratio, with 12.5 ng/μL vector DNA, and 0.025 Weiss unit/μL T4 ligase at 16 °C for 8−12 h. After ligation, the enzyme was heat inactivated at 65 °C for 10 min, and the DNA was then ethanol precipitated and redissolved in 500 μL of 10 mM Tris-HCl and 1 mM EDTA (pH 7.5). The reaction products were fractionated by electrophoresis on 0.8% agarose gels in Tris-borate buffer, and the gapped circular plasmid was extracted from the gel by electroelution onto an NA45 membrane, as described above. The concentration of the gapped plasmid was determined on the basis of the specific activity of the radiolabeled oligonucleotide. Typically, from 1 mg of plasmid pSKSL we obtained 300 μg of gel-purified vector. The gapped plasmid comprised 20% of the ligation products, i.e., 60 μg, 20 μg of which was recovered from the gel. This was sufficient for 200−400 translesion replication assays. To extend the gap to an average of 350 nucleotides, the gapped plasmid was treated with T7 gp6 5′ → 3′ exonuclease, as recently described (*31*).

Translesion Replication Assay. The translesion replication reaction mixture (25 μL) contained buffer B [20 mM Tris-HCl (pH 7.5), 8 μg/mL bovine serum albumin, 5 mM dithiothreitol, 0.1 mM EDTA, and 4% glycerol], 1 mM ATP, 10 mM $MgCl_2$, dATP, dGTP, dTTP, and dCTP (0.5 mM each), 0.1 μg (2 nM) of the gapped plasmid, and 1−90 nM DNA polymerase. Reactions were carried out at 37 °C for up to 16 min, after which they were terminated by adding SDS to a final concentration of 0.2% and EDTA to a final concentration of 20 mM and the mixtures heat inactivated at 65 °C for 10 min. The proteins were digested with 0.4 mg/mL proteinase K at 37 °C for 30 min, after which the DNA was extracted with phenol/chloroform and ethanol precipitated. The DNA was digested with *Xmn*I (substrate

GP11, GP21, or GP31) or with *Pvu*II (substrate GP11, when indicated) at 37 °C for 1 h (3 units/tube). Then, 5 units of *Bst*XI was added and incubation continued at 55 °C for an additional 1 h. The DNA was fractionated by electrophoresis on 15% polyacrylamide gels containing 8 M urea. Gels were run at 1500−2000 V for 2−3 h, after which they were dried, and visualized and quantified using a Fuji BAS 2000 phosphorimager. The extent of translesion replication was calculated by dividing the amount of bypass products by the amount of the extended primers.

RESULTS

Construction of Gapped Plasmids Carrying a Site-Specific Synthetic Abasic Site in the Single-Stranded Region. The construction of native-like long DNA substrates carrying site-specific lesions had led to significant progress in our understanding of translesion replication in vivo (reviewed in ref *32*). However, the utilization of such substrates for in vitro mechanistic studies proved to be slow, primarily due to the difficulties in constructing quantitative amounts of high-quality substrates (*33*−*37*). Using synthetic oligonucleotides carrying site-specific lesions provided a convenient solution in some cases (*12*, *13*, *16*, *21*, *38*, *39*). However, long DNAs with site-specific gap/lesion structures are much needed, especially for studies with complex multisubunit DNA polymerases, such as the DNA polymerase III holoenzyme (*25*, *27*), and for studying the effects on translesion replication of proteins such as RecA or SSB, which bind in a stoichiometric and cooperative manner to long DNA (*40*, *41*). For these types of studies, we developed a new method for the preparation of gap/lesion plasmids.

The method involved cutting of a plasmid with restriction nucleases *Bsa*I and *Bst*XI and ligating it to a synthetic gapped duplex oligonucleotide, whose ends were complementary to those of the plasmid. The gapped duplex oligonucleotide (termed GD) contained a site-specific synthetic abasic site opposite the gap, and the 5′ end of the primer oligonucleotide was ^{32}P labeled (Figure 1). The gapped circular plasmid (termed GP) obtained after ligation was gel purified, and used for replication assays. Since the strand that was extended by the polymerase contained an internal radiolabeled phosphate group, replication was assayed as the extension of the radiolabeled strand. The analysis was carried out by cleaving the plasmid with *Bst*XI, which cuts just upstream of the radiolabel, and with *Xmn*I, which cuts downstream of the gap (Figure 1). The radiolabeled products were then analyzed by urea−PAGE. A critical parameter in this method was the choice of nonpalindromic restriction nuclease cleavage sites designed to form the ligation junctions. This prevented extensive multimerization of the cut plasmid or of the gapped duplex oligonucleotide, thus increasing the yield of the desired ligation product.

Figure 2 shows the gap/lesion configurations that were used in this study. The only elements required to be present in the gapped oligonucleotide are termini complementary to the plasmid ends, and an *Xmn*I site downstream of the lesion. This allows flexibility in the choice of the type of lesion, and in the configuration of the gap, i.e., its length, its DNA sequence context, and the location of the gap boundaries relative to the lesion. Figure 3 shows the products of the ligation of the synthetic gapped duplex oligonucleotide to

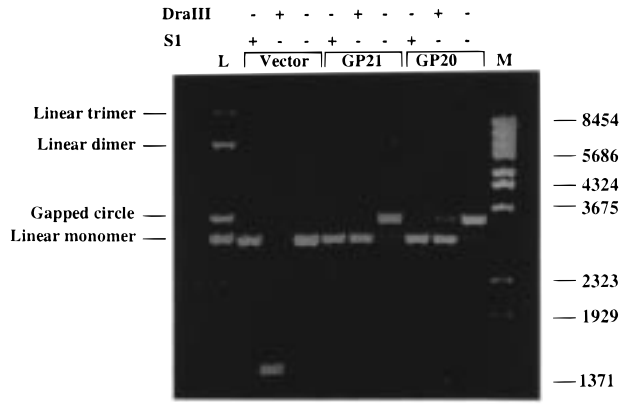

FIGURE 3: Agarose gel electrophoresis analysis of gap/lesion plasmids. The gap/lesion circular plasmid was gel purified from the ligation mixture, a sample of which is shown in lane L. Lanes marked GP21 show gapped plasmid GP21 before and after treatments with S1 nuclease or restriction nuclease *Dra*III. Substrate GP20 is the same as GP21, except that it has no lesion. The lanes marked Vector show a marker of the cut plasmid, without the ligated gapped duplex. S1 nuclease converts the gapped circular substrates into a linear form, but has no effect on the cut vector. *Dra*III, which cuts once in the plasmid, converts substrates GP20 and GP21 into the linear form, confirming that they are circular. Treatment of the linear vector generates two shorter fragments of 1492 and 1453 bp that comigrate in the gel. Lane M shows the migration of DNA size markers.

the linearized plasmid. As can be seen, multiple ligation products existed, as expected. The yield of the desired gapped plasmid depended strongly on the concentration of the DNA (not shown), with a concentration of 12.5 ng/μL yielding reproducibly the best yield. The band suspected to be the gapped plasmid was isolated and treated with S1 nuclease, which is specific to ssDNA. As can be seen, S1 nuclease treatment converted the DNA to a form that comigrated with the linear form of the plasmid. To prove that the DNA was circular, we cleaved the gapped plasmid with *Dra*III, which cleaves at a unique site away from the gap. As can be seen in Figure 3, this treatment converted the plasmid to a form that comigrated with the linear plasmid, confirming that the substrate was circular (a linear DNA would have resulted in two shorter fragments; see the vector control lanes in Figure 3). Finally, we introduced the gapped plasmid into *E. coli* cells, propagated the transformed cells under kanamycin selection, and extracted the plasmid from the cells. DNA sequence analysis of the gap region revealed that the plasmid contains the expected insert.

DNA Polymerase I and II, but Not the DNA Polymerase III Core, Bypass the Synthetic Abasic Site. We examined the ability of purified DNA polymerases from *E. coli* to bypass the synthetic abasic site in the gapped plasmid. We first used substrate GP11, which has a gap of 12 nucleotides, and the primer terminus is placed at the −6 position relative to the synthetic abasic site (Figure 2). This substrate also contains a *Pvu*II restriction nuclease site in the ssDNA region downstream of the lesion (Figure 2). Translesion replication was expected to convert the ssDNA region into a duplex form, thus rendering the substrate sensitive to *Pvu*II. Following replication, the reaction products were cleaved with restriction nucleases *Bst*XI and *Pvu*II, and fractionated by urea−PAGE. As can be seen in Figure 4A, a strong replication block was observed opposite the synthetic abasic site (for pol I) or at the nucleotide preceding the lesion (for

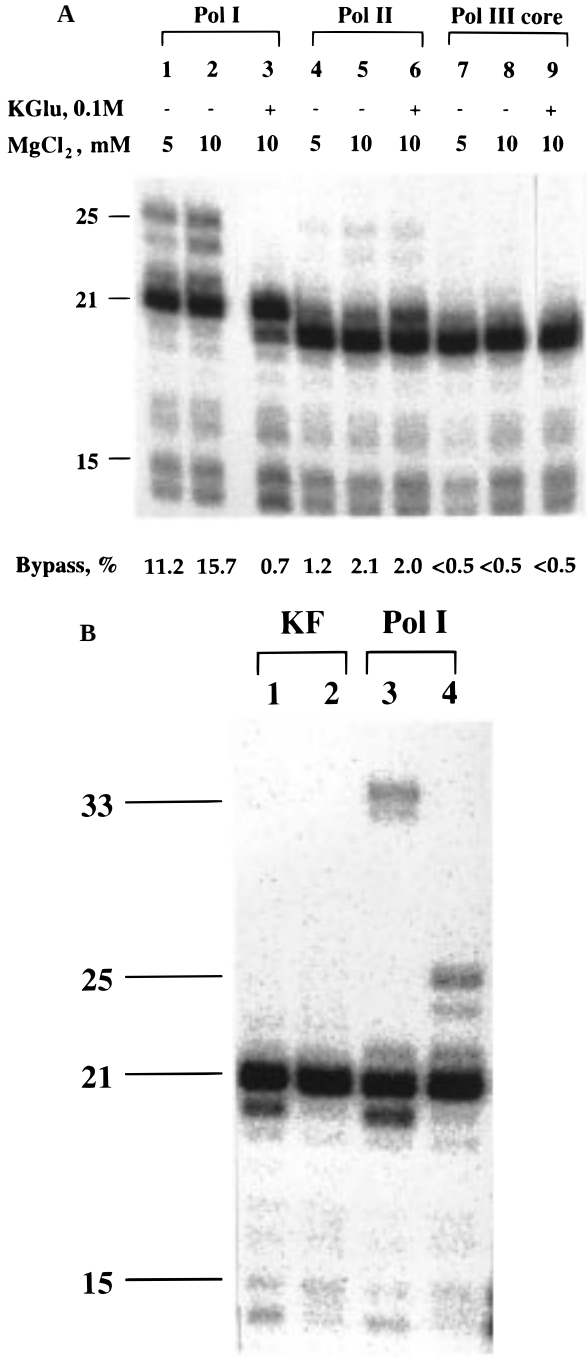

FIGURE 4: Pol I and pol II, but not the pol III core, bypass the abasic site analogue in substrate GP11. (A) Translesion replication assays were performed with each of the indicated DNA polymerases (at 90 nM) on substrate GP11 for 15 min, as described in Materials and Methods, except that $MgCl_2$ and potassium glutamate (KGlu) were present as indicated. The DNA was purified, cleaved with restriction nucleases *Bst*XI and *Pvu*II, and fractionated by urea−PAGE. Analysis of the radiolabeled products was carried out by phosphorimaging, and the extents of bypass synthesis are given at the bottom of each lane. (B) The reactions were performed and analyzed as described for panel A, except that the newly synthesized DNA strand was released prior to urea−PAGE analysis by cleavage with *Bst*XI and *Xmn*I (lanes 1 and 3) or with *Bst*XI and *Pvu*II (lanes 2 and 4). KF represents the Klenow fragment of pol I. In these gels, the unextended primer is represented by the 15-mer; the 20-mer and 21-mer represent replication stops preceding and opposite the lesion, respectively, and the 25-mer and 33-mer represent bypass products for analysis with *Bst*XI and *Pvu*II, and *Bst*XI and *Xmn*I, respectively.

pol II and the pol III core). Still, both pol I and pol II were able to replicate through the lesion. Pol I was most effective in translesion replication, showing a 15.7% bypass level at 15 min. Pol II was considerably less effective, with a 2.1% bypass level, and the pol III core exhibited essentially no bypass (<0.5%). Addition of potassium glutamate [the intracellular salt in *E. coli* (*42*)] caused a drastic 22-fold reduction in the bypass level by DNA polymerase I, but not by DNA polymerase II, consistent with previous results obtained with these polymerases on synthetic gapped oligonucleotide templates (*14*, *16*). Cutting the reaction product with *Xmn*I instead of with *Pvu*II was expected to yield a 33-nucleotide product, since *Xmn*I cuts downstream of *Pvu*II (Figure 2). Indeed, such a product was formed (Figure 4B). A reaction with the Klenow fragment of pol I showed no bypass under these conditions (Figure 4B). This was not investigated further, but served to show that essentially all the substrate molecules (>99.5%) contained the lesion. Notice that to be cleaved by *Xmn*I, the nascent DNA strand must have been extended past the *Xmn*I cleavage site, which is located in the double-stranded region downstream of the gap (Figure 2). In the case of pol I, this was achieved, most likely, by nick translation, which depends on its 5′ → 3′ exonuclease activity. For pol II and pol III, which do not have a 5′ → 3′ exonuclease activity, this occurred most likely via a strand displacement mechanism.

The DNA Polymerase III Holoenzyme Bypasses the Synthetic Abasic Site in a Gapped Plasmid. We prepared two gapped plasmids, GP21 and GP31, each containing a gap of 22 nucleotides, and a primer terminus located at the −11 position relative to the synthetic abasic site (Figure 2). The DNA sequences surrounding the lesions have been used by us in the past to study translesion replication on synthetic gapped duplex oligonucleotides; in substrate GP21, the sequence was taken from oligonucleotide AB1, whereas in substrate GP31, it was the same as in oligonucleotide AB2 (*14*, *16*). Figure 5 shows replication of these substrates with pol I, pol II, the pol III core, and the pol III holoenzyme. The synthetic abasic site caused a strong inhibition of replication by each of the polymerases. Pol I bypassed the abasic site in these two substrates with similar efficiencies (Figure 5). Pol II showed a strong DNA sequence context effect; it performed effective bypass synthesis on substrate GP31 (30.3% bypass level in 15 min, at a concentration of 90 nM), whereas on substrate GP21, the bypass level was 9-fold lower (3.3% bypass under the same conditions). The pol III core showed essentially no bypass on either of these substrates (<0.5%; Figure 5). In contrast, the pol III holoenzyme showed effective bypass on each of the two substrates. It reached a 24% bypass level on GP31, and 45.3% on GP21 within 15 min (Figure 5). Notice that the concentration of the pol III holoenzyme was 1 nM, 5-fold lower that the lowest of the concentrations of the other polymerases (Figure 5).

Preparations of the pol III holoenzyme may contain impurities which might affect its translesion replication ability. An effective way to ascertain that translesion replication by the pol III holoenzyme was indeed unassisted by other proteins is to assay the polymerase reconstituted from its purified subunits (reviewed in refs *25* and *27*). When the pol III holoenzyme reconstituted from purified subunits was examined, it exhibited effective translesion replication

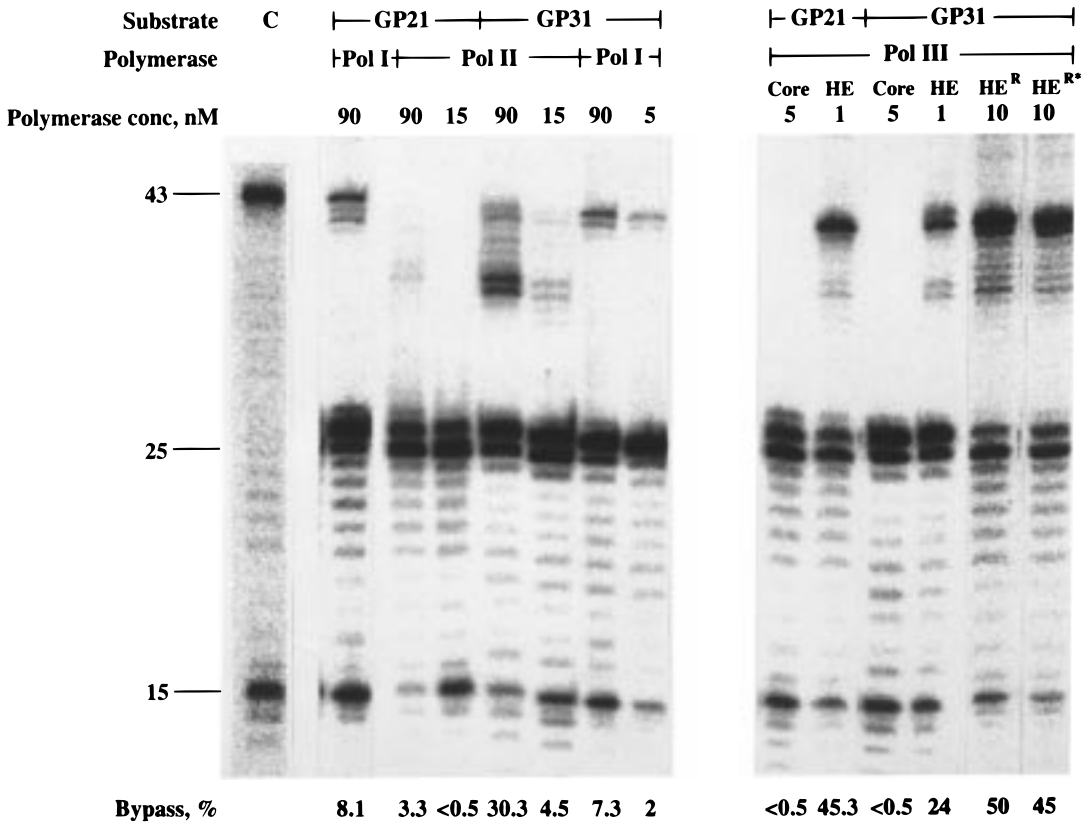

FIGURE 5: Translesion replication by purified DNA polymerases on templates GP21 and GP31. Reactions were performed for 15 min under the conditions described in Materials and Methods with pol I (90 or 5 nM), pol II (90 or 15 nM), the pol III core (5 nM), the pol III holoenzyme (1 nM, marked HE), or the reconstituted pol III holoenzyme (10 nM, marked HE^R; HE^{R*} is holoenzyme reconstituted without the θ subunit). Lane C shows the result of a control reaction with the pol III holoenzyme on substrate GP20, which had no lesion. The numbers to the left of the gel mark the unextended primer (15-mer), the products terminated one nucleotide before the lesion (25-mer), and the full bypass products (43-mer). The extents of translesion replication are indicated at the bottom of each lane.

(Figure 5, the lane marked HE^R; 50% bypass level). A similar result was obtained with a nine-subunit subassembly of the polymerase, which did not contain the θ subunit (Figure 5, the lane marked HE^{R*}; 45% bypass level). These results argue that translesion replication by the pol III holoenzyme was indeed unassisted by other proteins. In addition, these results show that the θ subunit is not essential for translesion replication, consistent with a lack of phenotype in mutants lacking the *holE* gene, encoding the θ subunit (*43*).

To examine the progress of the bypass reaction, we examined a time course of the reaction on template GP21 with the pol III holoenzyme or with pol I. As can be seen in Figure 6, bypass by pol III holoenzyme proceeded in a nearly linear rate, reaching 30% at 16 min. Bypass by a 20-fold higher concentration of pol I was 3-fold slower (Figure 6). Notice that bypass products were synthesized by the pol III holoenzyme by 2 min, when most of the primers were not extended yet. This suggests a processive mechanism of bypass. Indeed, we have shown that this bypass depends on the β subunit of pol III, the sliding DNA clamp responsible for the high processivity of the polymerase (*31*). The major replication pause of the pol III holoenzyme in the vicinity of the lesion was at the "−1" position with some products extending to the position opposite the lesion. This is in contrast to pol I, where most of the pauses were opposite the lesion (Figure 6A). There is also a difference in the bypass products. The pol III holoenzyme generated products that were 42 nucleotides long, one nucleotide shorter than the expected length, as a result of skipping over the abasic site. This was recently confirmed by DNA sequence analysis of replication products (*31*). In contrast, pol I generated the expected full replication product, 43 nucleotides long (Figure 6A). In addition, pol I generated a −2 deletion product (41 nucleotides long), consistent with the results of Shibutani et al. (*44*).

Processivity Accessory Proteins of Pol III Greatly Increase the Extent of Translesion Replication by the Pol III Core, but Not by Pol II. One of the major differences in activity between the pol III holoenzyme and the other two DNA polymerases of *E. coli* is the great processivity of the former (*26*). When the pol III core, the low-processivity catalytic core of the holoenzyme, was assayed for translesion replication, no bypass could be observed (Figure 7A). Addition of the γ complex clamp loader and the β subunit processivity clamp caused a dramatic 20-fold increase in the extent of translesion replication (from <0.5 to 10.6%; Figure 7A). Notice the bypass products show a length distribution of 37−43 nucleotides. The 37-nucleotide product is formed by pausing at the end of the gap, whereas the longer products are due to strand displacement. Strand displacement in this experiment was less effective than in the experiments whose results are presented in Figure 6, possibly due to the lack of the τ subunit. This experiment also shows that the τ subunit of the pol III holoenzyme is not essential for translesion replication.

The γ complex and the β subunit of pol III were also shown to endow pol II with high processivity (*15*, *45*). Do these proteins stimulate translesion replication by pol II?

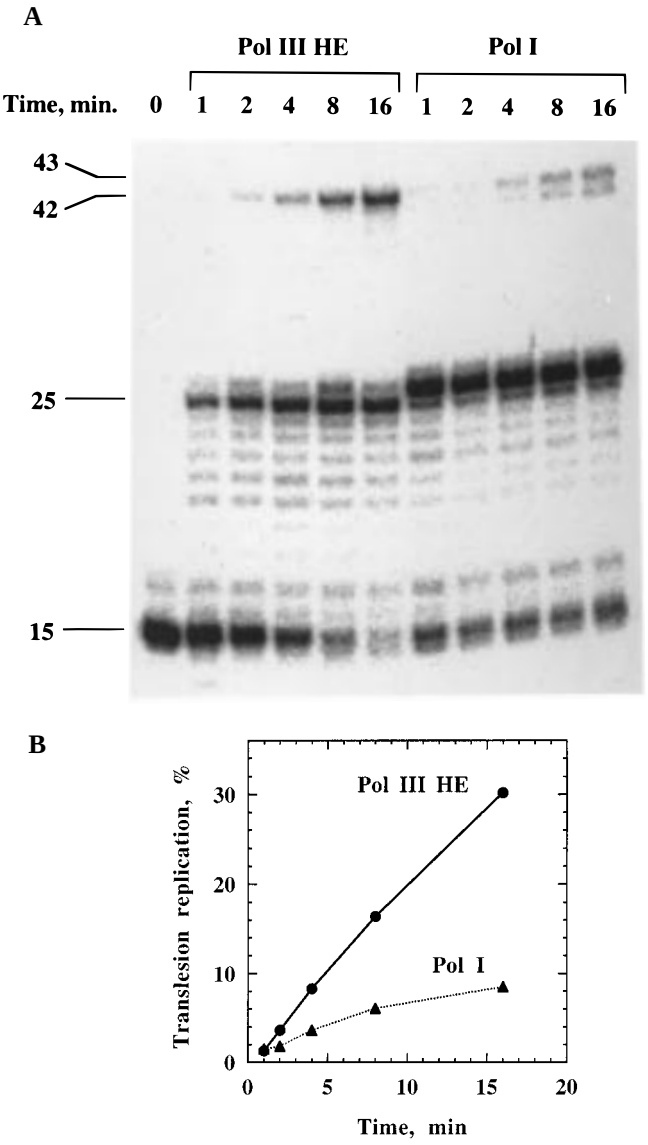

FIGURE 6: Time course of translesion replication by the pol III holoenzyme and pol I. Reaction conditions and analysis were as described in Materials and Methods, except that the pol III holoenzyme concentration was 1 nM, whereas the pol I concentration was 20 nM: (A) phosphorimage of the radiolabeled reaction products and (B) quantification of the results shown in panel A. The unextended primer is a 15-mer; termination products preceding or opposite the lesion are 25 and 26 nucleotides long, respectively, and full bypass products were 43 nucleotides long.

Control experiments have confirmed that the γ complex and the β subunit confer high processivity on pol II (not shown). However, as can be seen in Figure 7A, in contrast to their effect on the pol III core, the γ complex and β subunit did not stimulate translesion replication by pol II. In fact, a slight inhibition was observed (Figure 7A). A similar result was obtained with a gapped plasmid with a large gap, where SSB was also added (Figure 7B). Thus, the stimulation of bypass by the γ complex and the β subunit is specific to pol III. Notice that although the overall level of bypass by pol II was not stimulated by the γ complex and the β subunit, its specificity was changed. Pol II alone produced primarily 42-mers, whereas in the presence of the accessory proteins, it produced mainly the full-length 43-mers (Figure 7B). The basis of this behavior is currently under investigation.

The Pol III Holoenzyme Does Not Bypass the Synthetic Abasic Site on a 60-mer Oligonucleotide. We examined the importance of using native-like long DNA substrates by performing side-by-side bypass experiments using a gapped plasmid and a synthetic gapped duplex oligonucleotide, with a 60-nucleotide template. We used the same oligonucleotide that was used for the preparation of the gap/lesion plasmids, thus ensuring identical DNA sequence context. Figure 8 shows the results of replicating these substrates with the pol III holoenzyme. Whereas the level of bypass on the gapped plasmid GP21 reached 14.2% at 8 min, no bypass was observed on the gapped oligonucleotide (<0.5%). These results clearly demonstrate that the pol III holoenzyme replicates through the synthetic abasic site when assayed on native-like long DNA, but not on a 60-nucleotide template. The pol III holoenzyme was shown to perform processive synthesis on longer 80-mer and 100-mer synthetic oligonucleotides (*46*, *47*); however, it was not shown to be able to bypass blocking lesions on such substrates.

Is the circular structure of the DNA important for bypass? This was examined by assaying side-by-side circular gapped plasmid, and linear gapped plasmid, generated by restriction. As can be seen in Figure 8, the pol III holoenzyme did perform translesion replication on the linear gapped plasmid. However, bypass on the circular form was 3−4-fold faster than on the linear form.

Running-Start and Standing-Start Translesion Synthesis Yield Similar Rates. The distinction was made in the past between standing-start (or initiation mode) bypass and running-start (elongation mode) bypass. In the former, the primer terminus is located opposite the nucleotide preceding the lesion, and thus, synthesis begins with insertion opposite the lesion. In the latter mode, the primer terminus is located upstream of the lesion, and thus, replication is initiated at an unperturbed primer−template configuration; the polymerase encounters the lesion during the elongation mode of polymerization. We prepared two gap/lesion plasmids with the primer terminus located at the −1 and −11 positions, and used them to compare initiation and elongation modes of bypass by the pol III holoenzyme. Bypass synthesis with the "−11" substrate was similar to that with the "−1" substrate (Figure 9). Thus, within the range tested, there was no major difference between the two modes of bypass. At this point, we do not know what the effect of placing the primer terminus more than 11 nucleotides away from the lesion is.

DISCUSSION

The methodology developed in this study provides a quantitative, reproducible, and flexible method for the preparation of gapped duplex plasmids, with a site-specific lesion opposite the gap. The following are advantages of the method. (1) It produces a native-like 3 kb gapped plasmid with a site-specific lesion opposite the gap. (2) It can be applied to any lesion that can be incorporated in a synthetic oligonucleotide. (3) Any DNA sequence context in the vicinity of the DNA lesion can be chosen. (4) The gap parameters (length and location of boundaries) can be varied. (5) The substrate can be used for in vivo studies, after being introduced into *E. coli* cells by transformation. This enables the performance of in vivo and in vitro experiments using

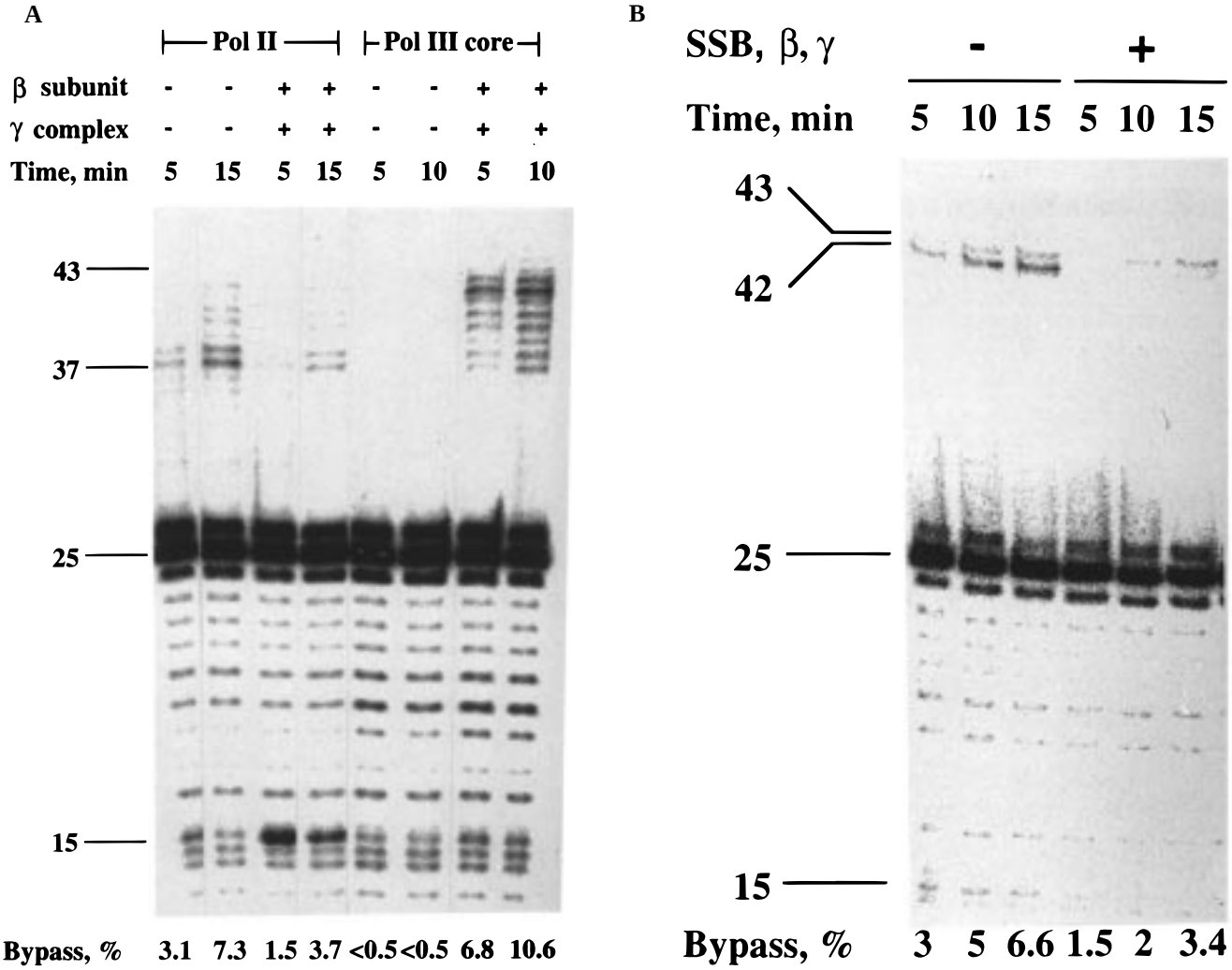

FIGURE 7: Effect of the γ complex clamp loader and the β subunit of pol III on translesion replication by pol II and the pol III core. Reactions were carried out for the indicated time periods as described in Materials and Methods, using gapped plasmid GP31. (A) Reactions with a 22-nucleotide gap. The pol II, pol III core, and β subunit concentrations were each 20 nM, and the γ complex concentration was 0.7 nM. (B) Translesion replication by pol II on a gapped plasmid containing a large gap of 350 nucleotides. The location of the 3′-OH is the same as in GP31. The concentrations of the proteins were as follows: 30 nM pol II, 600 nM SSB (as a tetramer), 0.7 nM γ complex, and 30 nM β subunit. The extents of translesion replication are indicated at the bottom of each lane.

the same DNA substrate. The following are disadvantages of the method. (1) It is complex, involving many steps. (2) The yield is not high, with 20 μg typically obtained from 1 mg of plasmid. Still, 20 μg provides a substrate sufficient for 200−400 assays. (3) The gap size is limited by the length of the synthetic oligonucleotide template. To overcome this disadvantage, we have developed a modification of the methodology, in which the gap is extended (*31*). These substrates should be useful in a wide variety of studies in the DNA repair and mutagenesis field.

As far as translesion replication is concerned, the two important features of the gap/lesion plasmids are its large size and the fact that the lesion is not close to the DNA end. This is important for studying complex multisubunit DNA polymerases such as the pol III holoenzyme. Indeed, we saw no bypass by the pol III holoenzyme on a synthetic gapped 60-mer oligonucleotide (<0.5% in 8 min), nor was bypass by the pol III holoenzyme on a synthetic oligonucleotide reported by others. In contrast, an impressive extent of bypass was observed using a gap/lesion plasmid (15% bypass in 8 min). These results are consistent with those of our earlier studies on the ability of the pol III holoenzyme to bypass UV lesions (*17*−*19*) and abasic sites (*20*) during replication of circular ssDNA containing multiple random lesions. Two studies have previously reported the inability of the pol III holoenzyme to bypass a site-specific synthetic abasic site in a long DNA (*8*, *15*). The lesion in those substrates was located 30 bases away from the 5′ DNA terminus, and that proximity of the lesion to the DNA end might have limited bypass by the large pol III holoenzyme complex due to DNA end effects. In addition, as shown in this study, bypass on a linear DNA was slower than on circular DNA. This observation may be related to our recent observation that bypass by the pol III holoenzyme depends on its β subunit, the sliding DNA clamp (*31*). Since the β subunit can slide off linear double-stranded DNA, but not circular DNA (*48*), the lower bypass observed on linear as compared to circular plasmid (Figure 8) may be explained by the dissociation of the β subunit, although this remains to be proven.

Several studies have previously reported that *E. coli* DNA polymerases can bypass blocking lesion in an unassisted manner (*9*, *11*−*14*, *16*−*18*, *20*, *49*). We have shown that by adjustment of reaction conditions bypass synthesis by pol I and pol II can reach very high extents (*14*, *16*). In all these

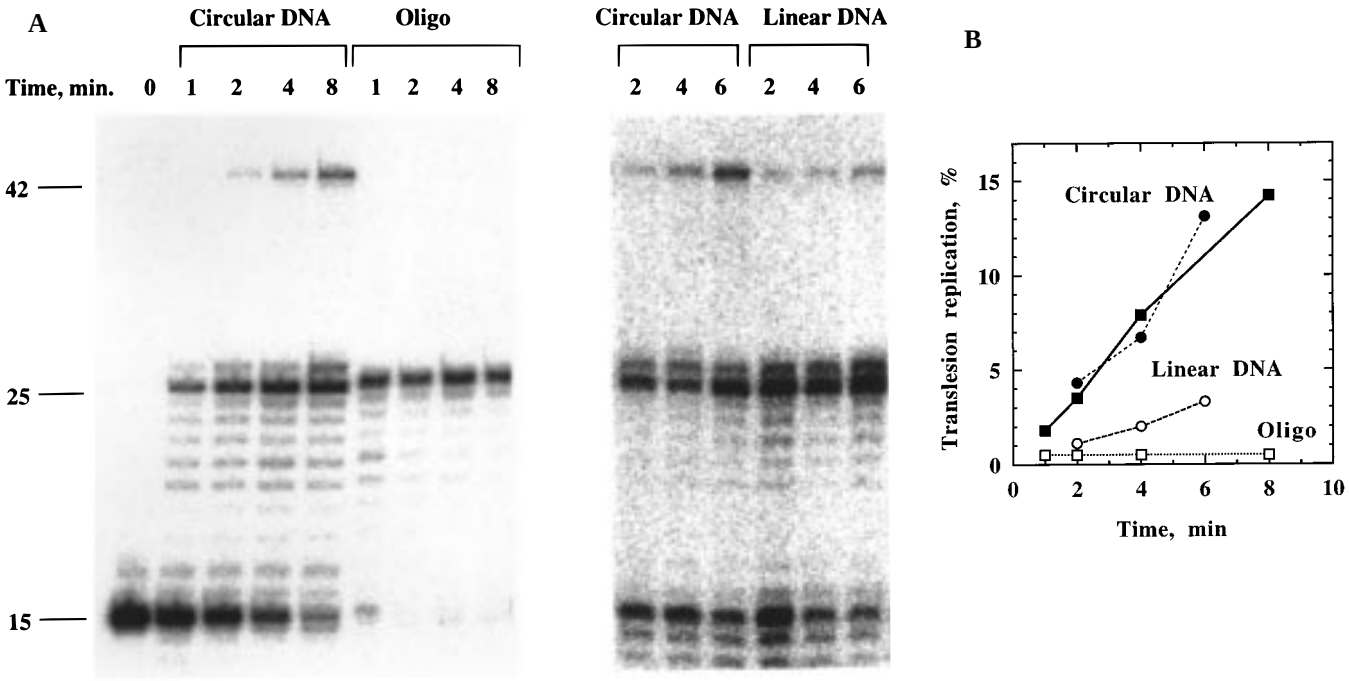

FIGURE 8: Comparison of translesion replication by pol III holoenzyme on a gap/lesion plasmid and a gapped duplex oligonucleotide. Reaction conditions and analysis were as described in the legend of Figure 6. The numbers to the left of the gel mark the unextended primer (15-mer), the products terminated before the lesion (25-mer), and the bypass product (42-mer). (A) A time course of translesion replication by the pol III holoenzyme on the circular gap/lesion plasmid GP21 and the corresponding gap/lesion oligonucleotide GD21 (left panel) or the *Dra*III-cleaved linear form of the gap/lesion plasmid (right panel). (B) Quantification of the results shown in panel A: (■) circular gapped plasmid, (□) synthetic 60-mer gapped duplex oligonucleotide, (●) circular gapped plasmid, and (○) linear gapped plasmid.

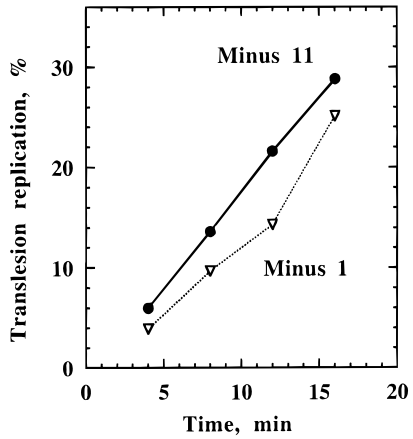

FIGURE 9: Comparison of running-start and standing-start translesion replication by the pol III holoenzyme. Reactions were performed with substrate GP21, or with a similar substrate in which the primer terminus was located at position −1 as described in the legend of Figure 2. The figure shows the quantification of the phosphorimage of the gel-fractionated reaction products: (●) primer terminus located at position −11 relative to the lesion and (▽) primer terminus located at position −1.

experiments, the concentration of the DNA polymerase was critical, and it was in excess over the substrate. Under those conditions, bypass synthesis occurred by allowing multiple bypass attempts. This may represent a real in vivo situation, because the concentration of pol I in the cell is relatively high (750 nM; *26*). Compared to that of pol I, the concentration of the pol III holoenzyme is much lower (estimated to be 10−15 nM). Under such concentrations, pol I exhibited a low level of translesion replication. In contrast, as shown in this study, the pol III holoenzyme performed effective bypass synthesis when present at a low concentration of 1 nM. In fact, the polymerase seems to be able to bypass the lesion under single-cycle replication conditions, as suggested by the bypass observed when a substantial portion of the primer remained unextended (Figure 6A). Thus, compared per single-cycle bypass attempt, the pol III holoenzyme is much more effective than any other *E. coli* DNA polymerase.

Bypass by the pol III holoenzyme was recently shown to depend on the β subunit sliding DNA clamp (*31*), suggesting a major role for processivity in bypass. However, processivity alone does not seem to be sufficient, since increasing the processivity of pol II by adding SSB, the γ complex, and the β subunit did not lead to an increased bypass level (Figure 7). These results disagree with those of Bonner et al. (*15*), who reported that the accessory proteins increase the extent of translesion replication by pol II. The reason for this difference is not clear. The fact that increased processivity stimulates bypass by pol III, but not by pol II, may stem from the difference in their rates of polymerization. The rate of polymerization by processive pol II (20−30 nucleotides/s) (*15*) is much slower than that of the pol III holoenzyme (500 nucleotides/s) (*27*). This high level of effectiveness of the pol III holoenzyme in bypass is a significant finding because according to genetic analysis pol III is the only DNA polymerase required for SOS-regulated mutagenesis (*22*, *23*, *50*−*52*).

Translesion replication by the pol III holoenzyme is the basic reaction on which RecA, UmuD′, and UmuC exert their effects during in vivo mutagenesis. Why are these proteins required in vivo, if pol III holoenzyme can bypass lesions in an unassisted manner in vitro? Recently, we and the Goodman group have independently reconstituted SOS translesion replication with purified components (*6*, *7*). These studies confirmed a previous study (*8*) which showed that RecA, UmuD′, and UmuC stimulated translesion replication

by the pol III holoenzyme through a synthetic abasic site. Notice that although substantial bypass rates can be obtained with the pol III holoenzyme alone, they vary considerably depending on the DNA structure (Figures 5−8), and on the size of the gap. The level of bypass of a synthetic abasic site located in a ssDNA region of 350 nucleotides was found to be 5-fold lower than in a ssDNA region of 22 nucleotides (*31*). Therefore, RecA, UmuD′, and UmuC are required to increase the rate of translesion replication.

Analysis of the types of mutations formed during unassisted translesion replication by the pol III holoenzyme revealed exclusively small deletions, primarily −1 frameshifts, as a result of polymerase skipping over the lesion, rather than replicating it (*7*, *31*). This high prevalence of minus frameshifts resembles a previous result from our laboratory with an in vitro UV mutagenesis reaction promoted by six purified proteins, including pol III, but without RecA, UmuD′, and UmuC (*53*). In that system, an unusually high proportion of 40% of the UV mutations were frameshifts. Therefore, unassisted translesion replication seems to produce a very high proportion of frameshifts, a lethal type of mutation. Analysis of the mutations produced during translesion replication by the pol III holoenzyme in the presence of SSB, RecA, UmuD′, and UmuC revealed primarily base substitution (*7*). Consistent with in vivo results (*54*), an A was most frequently inserted opposite the abasic site under these conditions (*7*). Thus, SOS proteins not only increase translesion replication but also change its specificity from lethal frameshifts to the milder base substitutions (*7*).

Other relevant aspects of translesion replication are its inhibition by intracellular inhibitors and the competition with recombinational repair. We have recently shown that DNA damage-binding proteins are direct inhibitors of translesion replication (*55*). Thus, in vivo all or some of the proteins (RecA, SSB, UmuD′, and UmuC) may be required to counteract the inhibition by these or other intracellular factors. The UmuD and -C proteins may be required also to inhibit recombinational repair, and switch from an error-free repair mode (recombinational repair) into an error-prone repair mode (translesion replication) as suggested by Devoret and co-workers (*56*, *57*). Overall, these reactions are regulated by SOS stress response, to allow tight control of mutagenesis.

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REFERENCES

1. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) *DNA repair and mutagenesis*, American Society for Microbiology, Washington, DC.
2. Strauss, B. S. (1985) *Cancer Surv. 4*, 493−516.
3. Livneh, Z., Cohen-Fix, O., Skaliter, R., and Elizur, T. (1993) *CRC Crit. Rev. Biochem. Mol. Biol. 28*, 465−513.
4. Walker, G. C. (1995) *Trends Biochem. Sci. 20*, 416−420.
5. Woodgate, R., and Levine, A. S. (1996) *Cancer Surv. 28*, 117−140.
6. Tang, M., Bruck, I., Eritja, R., Turner, J., Frank, E. G., Woodgate, R., O'Donnell, M., and Goodman, M. F. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 9755−9760.
7. Reuven, N. B., Tomer, G., and Livneh, Z. (1998) *Mol. Cell 2*, 191−199.
8. Rajagopalan, M., Lu, C., Woodgate, R., O'Donnell, M., Goodman, M., and Echols, M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10777−10781.
9. Kunkel, T. A., Schaaper, R. M., and Loeb, L. A. (1983) *Biochemistry 22*, 2378−2384.
10. Schaaper, R. M., Kunkel, T. A., and Loeb, L. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 487−491.
11. Sagher, D., and Strauss, B. (1983) *Biochemistry 22*, 4518−4526.
12. Takeshita, M., Chang, C., Johnson, F., Will, S., and Grollman, A. P. (1987) *J. Biol. Chem. 262*, 10171−10179.
13. Taylor, J. S., and O'Day, C. L. (1990) *Biochemistry 29*, 1624−1632.
14. Paz-Elizur, T., Takeshita, M., and Livneh, Z. (1997) *Biochemistry 36*, 1766−1773.
15. Bonner, C. A., Stukenberg, P. T., Rajagopalan, M., Eritja, R., O'Donnell, M., McEntee, K., Echols, H., and Goodman, M. F. (1992) *J. Biol. Chem. 267*, 11431−11438.
16. Paz-Elizur, T., Takeshita, M., Goodman, M., O'Donnell, M., and Livneh, Z. (1996) *J. Biol. Chem. 271*, 24662−24669.
17. Livneh, Z. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4599−4603.
18. Livneh, Z. (1986) *J. Biol. Chem. 261*, 9526−9533.
19. Shwartz, H., Shavitt, O., and Livneh, Z. (1988) *J. Biol. Chem. 263*, 18277−18285.
20. Hevroni, D., and Livneh, Z. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5046−5050.
21. Belguise-Valladier, P., Maki, H., Sekiguchi, M., and Fuchs, R. P. P. (1994) *J. Mol. Biol. 236*, 151−164.
22. Bridges, B. A., Motershead, R. P., and Sedgwick, S. G. (1976) *Mol. Gen. Genet. 144*, 53−58.
23. Brotcorne-Lannoye, A., Maenhaut-Michel, G., and Radman, M. (1985) *Mol. Gen. Genet. 199*, 64−69.
24. Sharif, F., and Bridges, B. A. (1990) *Mutagenesis 5*, 31−34.
25. McHenry, C. S. (1991) *J. Biol. Chem. 266*, 19127−19130.
26. Kornberg, A., and Baker, T. (1991) *DNA Replication*, W. H. Freeman and Co., New York.
27. Kelman, Z., and O'Donnell, M. (1995) *Annu. Rev. Biochem. 64*, 171−200.
28. Cull, M. G., and McHenry, C. S. (1995) *Methods Enzymol. 262*, 22−35.
29. Cai, H., Yu, H., McEntee, K., Kunkel, T. A., and Goodman, M. F. (1995) *J. Biol. Chem. 270*, 15327−15335.
30. Cohen-Fix, O., and Livneh, Z. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3300−3304.
31. Tomer, G., Reuven, N. B., and Livneh, Z. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 14106−14111.
32. Singer, B., and Essigmann, J. M. (1991) *Carcinogenesis 12*, 949−955.
33. Moriya, M., Ou, C., Bodepudi, V., Johnson, F., Takeshita, M., and Grollman, A. P. (1991) *Mutat. Res. 254*, 281−288.
34. Thomas, D. C., Veaute, X., Kunkel, T. A., and Fuchs, R. P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 7752−7756.
35. Carty, M. P., Lawrence, C. W., and Dixon, K. (1996) *J. Biol. Chem. 271*, 9637−9647.
36. Koffel-Schwartz, N., Coin, F., Veaute, X., and Fuchs, R. P. P. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 7805−7810.
37. Wang, G., Rahman, M. S., and Humayun, M. Z. (1997) *Biochemistry 36*, 9486−9492.
38. Randall, S. K., Eritja, R., Kaplan, B. E., Petruska, J., and Goodman, M. (1987) *J. Biol. Chem. 262*, 6864−6870.
39. Latham, G. J., Zhou, L., Harris, C. M., Harris, T. M., and Lloyd, R. S. (1993) *J. Biol. Chem. 268*, 23427−23434.
40. Roca, A. I., and Cox, M. M. (1990) *CRC Crit. Rev. Biochem. Mol. Biol. 25*, 415−456.
41. Lohman, T. M., and Ferrari, M. E. (1994) *Annu. Rev. Biochem. 63*, 527−570.
42. Richey, B., Cayley, D. S., Mossing, M. C., Kolka, C., Anderson, C. F., Farrar, T. C., and Record, M. T., Jr. (1987) *J. Biol. Chem. 262*, 7157−7164.

43. Slater, S. C., Lifsics, M. R., O'Donnell, M., and Maurer, R. (1994) *J. Bacteriol. 176*, 815−821.
44. Shibutani, S., Takeshita, M., and Grollman, A. P. (1997) *J. Biol. Chem. 272*, 13916−13922.
45. Hughes, A. J., Bryan, S. K., Chen, H., Moses, R. E., and McHenry, C. S. (1991) *J. Biol. Chem. 266*, 4568−4573.
46. Bloom, L. B., Turner, J., Kelman, Z., Beechem, J. M., O'Donnell, M., and Goodman, M. F. (1996) *J. Biol. Chem. 271*, 30699−30708.
47. Bloom, L. B., Chen, X., Fygenson, D. K., Turner, J., O'Donnell, M., and Goodman, M. F. (1997) *J. Biol. Chem. 272*, 27919−27930.
48. Stukenberg, P. T., Studwell-Vaughan, P. S., and O'Donnell, M. (1991) *J. Biol. Chem. 266*, 11328−11334.
49. Schaaper, R. M., Glickman, B. W., and Loeb, L. A. (1982) *Mutat. Res. 106*, 1−9.
50. Witkin, E. M. (1976) *Bacteriol. Rev. 40*, 869−907.
51. Bates, H., Randall, S. K., Rayssiguier, C., Bridges, B. A., Goodman, M. F., and Radman, M. (1989) *J. Bacteriol. 171*, 2480−2484.
52. Kow, Y. W., Faundez, G., Hays, S., Bonner, C. A., Goodman, M. F., and Wallace, S. S. (1993) *J. Bacteriol. 175*, 561−564.
53. Tomer, G., Cohen-Fix, O., O'Donnell, M., Goodman, M., and Livneh, Z. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1376−1380.
54. Lawrence, C. W., Borden, A., Banerjee, S. K., and LeClerc, J. E. (1990) *Nucleic Acids Res. 18*, 2153−2157.
55. Paz-Elizur, T., Barak, Y., and Livneh, Z. (1997) *J. Biol. Chem. 272*, 28906−28911.
56. Sommer, S., Bailone, A., and Devoret, R. (1993) *Mol. Microbiol. 10*, 963−971.
57. Boudsocq, F., Campbell, M., Devoret, R., and Bailone, A. (1997) *J. Mol. Biol. 270*, 201−211.

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