

# Processive DNA Synthesis by DNA Polymerase II Mediated by DNA Polymerase III Accessory Proteins\*

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An interesting property of the *Escherichia coli* DNA polymerase II is the stimulation in DNA synthesis mediated by the DNA polymerase III accessory proteins  $\beta$ ,  $\gamma$  complex. In this paper we have studied the basis for the stimulation in pol II activity and have concluded that these accessory proteins stimulate pol II activity by increasing the processivity of the enzyme between 150- and 600-fold. As is the case with pol III, processive synthesis by pol II requires both  $\beta$ ,  $\gamma$  complex and SSB protein. Whereas the intrinsic velocity of synthesis by pol II is 20–30 nucleotides per s with or without the accessory proteins, the processivity of pol II is increased from approximately five nucleotides to greater than 1600 nucleotides incorporated per template binding event. The effect of the accessory proteins on the rate of replication is far greater on pol III than on pol II; pol III holoenzyme is able to complete replication of circular single-stranded M13 DNA in less than 20 s, whereas pol II in the presence of the  $\gamma$  complex and  $\beta$  requires approximately 5 min. We have investigated the effect of  $\beta$ ,  $\gamma$  complex proteins on bypass of a site-specific abasic lesion by *E. coli* DNA polymerases I, II, and III. All three polymerases are extremely inefficient at bypass of the abasic lesion. We find limited bypass by pol I with no change upon addition of accessory proteins. pol II also shows limited bypass of the abasic site, dependent on the presence of  $\beta$ ,  $\gamma$  complex and SSB. pol III shows no significant bypass of the abasic site with or without  $\beta$ ,  $\gamma$  complex.

*Escherichia coli* contains three DNA polymerases: pol I,<sup>1</sup> pol II, and pol III (for a review, see Ref. 1). pol III is the main replicative polymerase (2, 3), and the functional holoenzyme contains at least 10 subunits (4). The polymerase function

resides in the  $\alpha$  subunit (5) and the 3' to 5' proofreading exonuclease is localized in the  $\epsilon$  subunit (6). These two proteins, along with a third subunit,  $\theta$ , comprise the pol III core (7), an enzyme with relatively low synthetic processivity, synthesizing approximately 11 nucleotides per template binding event (8). The accessory proteins,  $\beta$  and the five-protein  $\gamma$  complex subassembly, form a  $\beta$ -sliding clamp on duplex DNA (9) which tethers the pol III core to the template thus converting pol III core into a highly processive enzyme capable of replicating long (>5000 nucleotides) SSB coated single-stranded circular DNA viral templates in less than 20 s (4, 8, 10, 11). pol I is a single-subunit enzyme (103 kDa) possessing polymerase as well as 3'→5'- and 5'→3'-exonuclease activities (1). Since cells containing *polA* deletions are viable, pol I is not required for chromosome replication in *E. coli* (12). However, under normal growth conditions, the 5'→3'-exonuclease of pol I acts on the lagging strand during replication to excise RNA primers from Okazaki fragments (1). pol I has also been shown to play an important role in repair of UV-damaged DNA (1). pol II is comprised of a single 89.9-kDa subunit (13, 14) which contains both polymerase and 3'→5'-exonuclease activities (1). The function of pol II in DNA replication or repair in *E. coli* remains unknown.

There is genetic evidence suggesting possible roles for both pol II and pol III in the pleiotropic SOS response to DNA damage which enhances capacity for excision repair and recovery of DNA replication by error free (replication restart) and mutagenic pathways (15, 16). Bridges *et al.* (17) showed that strains with a temperature-sensitive *dnaE* gene (which encodes the  $\alpha$  subunit) exhibit little UV-induced mutation at nonpermissive temperatures. Hagansee *et al.* (18) demonstrated an absence of UV-induced mutation in a *dnaEts* strain at nonpermissive temperatures when viability was conferred by a suppressor mutation, *pcbA*, that allows replication to occur at the nonpermissive temperature (perhaps through substitution of either pol I or pol II for the  $\alpha$  subunit). *E. coli polA* mutants (19) exhibit normal UV mutability, demonstrating that the role of pol I is not essential for this process (20, 21).

Bonner *et al.* (22) showed that DNA pol II activity is increased 7-fold in cells induced for the SOS response and that the polymerase is capable of limited insertion and bypass of abasic lesions *in vitro*. The structural gene for pol II has recently been shown to be the same as the *dinA* gene (13, 23), which is a member of the SOS regulon controlled directly by Lex A repressor (24). These results clearly demonstrate that pol II is an SOS-induced polymerase and likely participates

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<sup>1</sup> The abbreviations used are: pol I, DNA polymerase I; pol II, DNA polymerase II; pol III, DNA polymerase III;  $\gamma$  complex, complex consisting of stoichiometric concentrations of pol III accessory proteins  $\gamma$ ,  $\delta$ ,  $\delta'$ ,  $\chi$ , and  $\psi$ ; SSB, single-stranded binding protein from *E. coli*; ssM13 DNA, bacteriophage single-stranded M13 DNA; TdT, terminal deoxynucleotidyltransferase; kb, kilobase(s); dNTP, deoxynucleoside triphosphate.

in some aspect of error free or error prone DNA synthesis.

Evidence from earlier studies has suggested that the  $\beta$  and  $\gamma$  subunits of the pol III holoenzyme can stimulate DNA synthesis by pol II (25). Recently, Hughes *et al.* (26) have shown with highly purified proteins that pol II-dependent DNA synthesis is stimulated by the  $\beta, \gamma$  complex accessory proteins of pol III holoenzyme. Since these accessory proteins are known to stimulate pol III activity by increasing the processivity of the enzyme (4, 27, 28), they might affect pol II activity by a similar mechanism. If so, addition of  $\beta, \gamma$  complex, perhaps in the presence of other SOS-induced proteins, such as UmuC and UmuD (15, 16) may stimulate bypass of blocking lesions in DNA. In the work reported here, we have characterized the effect of  $\beta, \gamma$  complex accessory proteins on the processivity of pol II. We have also compared the bypass of abasic lesions by all three *E. coli* DNA polymerases.

#### EXPERIMENTAL PROCEDURES

**Materials**—Unlabeled and labeled nucleotides were purchased from ICN Biochemicals. DNA oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. The abasic site containing oligonucleotides were synthesized as described (29). M13 mp18 ssDNA uniquely primed with a synthetic DNA 30-mer was prepared as described (9). *E. coli* DNA polymerase II was prepared by a modification of a published procedure (22) from an overproducing strain<sup>2</sup> where phosphocellulose peak fractions were applied to a diethylaminoethyl cellulose (Whatman DE52) column followed by chromatography on an AGATP type 2 column (Pharmacia LKB Biotechnology Inc.). Other replication proteins were purified as described:  $\alpha$  (5),  $\epsilon$  (6),  $\alpha\epsilon$  complex (30),  $\gamma$  complex (31), pol III holoenzyme (32), and SSB (33). Concentration of proteins was determined by the method of Bradford (34) using bovine serum albumin as a standard. Terminal deoxynucleotidyltransferase (TdT) was purchased from Boehringer Mannheim, and  $T_4$  polynucleotide kinase was purchased from United States Biochemical.

**Buffers**—TdT buffer is 200 mM potassium cacodylate, 25 mM Tris-HCl, 250  $\mu$ g/ml bovine serum albumin, 1 mM  $\text{CoCl}_2$ , pH 6.6. Annealing buffer is 50 mM Tris-HCl, pH 8.0, 5 mM  $\text{MgCl}_2$ , 2 mM  $\beta$ -mercaptoethanol, and 100  $\mu$ g/ml bovine serum albumin. Buffer A is 20 mM Tris-HCl, pH 7.5, 8 mM  $\text{MgCl}_2$ , 5 mM dithiothreitol, 0.1 mM EDTA, 4% glycerol, and 40  $\mu$ g/ml bovine serum albumin.

**Terminal Deoxynucleotidyltransferase Reactions**—Oligonucleotides were extended in TdT buffer (40  $\mu$ l volume) containing 2.5  $\mu$ M oligonucleotide, 2.5 mM dNTP (0.6 mM each dATP, dCTP, dGTP, dTTP), and 100 units of TdT. Reactions were incubated at 37 °C for 2 h, followed by addition of another 100 units of TdT and an additional 1-h incubation. Reactions were then heated 10 min at 70 °C and cooled on ice. Extended oligonucleotides were phenol/chloroform-extracted and ethanol-precipitated in the presence of sodium acetate as described (35). DNA concentrations were checked by absorbance at 260 nm. Extended oligonucleotides were 5' end-labeled using [ $\gamma$ -<sup>32</sup>P]ATP and  $T_4$  polynucleotide kinase (35) and electrophoresed on 8% denaturing polyacrylamide gels. Oligonucleotides were extended from 60 or 71 nucleotides to an average length of 150 nucleotides.

**Polymerase Assays on Synthetic Oligonucleotides**—15-mer DNA primers were 5' end-labeled using [ $\gamma$ -<sup>32</sup>P]ATP and  $T_4$  polynucleotide kinase (35) and annealed to DNA oligonucleotides in annealing buffer by heating to 100 °C, followed by slow cooling (at least 2 h) to room temperature. Primers (15-mers) were annealed to templates 56 nucleotides from the template 5' end. Thus, extension of the primer to the end of the template yields products 71 nucleotides long. Polymerase reactions were in buffer A. Preinitiation complexes were formed in 10- $\mu$ l volumes using 14 nM primed oligonucleotide (as 150-mer), 130 nM SSB (as tetramer), 0.5 mM ATP, 60  $\mu$ M dCTP, 60  $\mu$ M dGTP, 11 nM  $\beta$  (as dimer), 7 nM  $\gamma$  complex, and either 38 nM  $\alpha\epsilon$  complex or pol II and incubated 5 min at 37 °C. Reactions were initiated by addition of dATP and dTTP to final concentrations of 60  $\mu$ M each and terminated with the addition of 10  $\mu$ l of 20 mM EDTA in 95% formamide. Samples of the polymerase reaction mixtures were heat-denatured at 100 °C for 5 min, cooled on ice, and loaded onto 10%

polyacrylamide gels containing 8 M urea. Electrophoresis was performed at 2000 V for 2 h to resolve extended primers. Autoradiograms of gels were made by overlaying medical x-ray film (Kodak GPB-1) with an intensifying screen and exposing overnight at -70 °C. Alternatively, gels were exposed to phosphorimager cassettes overnight and scanned on a phosphorimager (Molecular Dynamics). Reactions on synthetic oligonucleotides containing a single abasic site were performed as described above except that 50 nM  $\alpha\epsilon$  complex or pol II were used on 50 nM SSB-coated primed oligonucleotide.

**Polymerase Reactions on M13 mp18**—Reconstitution reactions were performed in 25- $\mu$ l reactions by incubating 5 nM  $\beta$  (as dimer), 0.9 nM  $\gamma$  complex, and either 11.2 nM  $\alpha\epsilon$  complex or pol II with 1.2 nM primed M13 mp18 ssDNA, 0.4  $\mu$ M SSB (as tetramer), 0.5 mM ATP, 60  $\mu$ M each dCTP and dGTP in buffer A for 5 min at 37 °C. Synchronous replication was initiated upon addition of dATP and [ $\alpha$ -<sup>32</sup>P]dTTP to final concentrations of 60 and 20  $\mu$ M, respectively. Replication reactions were quenched after 5 min with 25  $\mu$ l of 1% SDS and 40 mM EDTA. Replication time courses containing 1, 10, or 140 nM pol II were performed as described above except 1.2 nM primed ssDNA, 0.4  $\mu$ M SSB, and where indicated 5 nM  $\beta$  (as dimer), and 0.9 nM  $\gamma$  complex were present in a 175- $\mu$ l final volume of buffer A. After preincubation for 5 min at 37 °C, replication was initiated upon addition of dATP and [ $\alpha$ -<sup>32</sup>P]dTTP to 60 and 20 mM, respectively, and 20  $\mu$ l was removed and quenched as above at the indicated times. Reaction products were visualized by autoradiography following electrophoresis of reaction products on 0.8% agarose gels.

**Polymerase Reactions on  $\phi$ X174 Containing a Single Abasic Site**—The 5.4-kb linear single-stranded DNA substrate with an abasic site at a defined position (30 nucleotides from the 5' end) was constructed by ligating a 60-mer oligonucleotide with an abasic site to a linear  $\phi$ X174 ssDNA.<sup>3</sup> The resulting template was then primed with two 20-mer oligonucleotides. The one closer to the abasic site was <sup>32</sup>P end-labeled using  $T_4$  polynucleotide kinase. Replication from the second primer (closer to the 3' end) helps to overcome technical complications from long regions of single-stranded DNA. Replication reactions were performed in 10  $\mu$ l of buffer A (plus 0.1 mM ATP). The primed substrate (2.5 nM) was preincubated with SSB (3.1  $\mu$ M),  $\beta$  (100 nM), and  $\gamma$  complex (3.6 nM) for 5 min at 30 °C (protein concentrations in monomer units). Respective polymerases (20 nM) were added on ice, and replication was initiated upon adding dNTPs to a final concentration of 60  $\mu$ M each. Reactions were carried out at 37 °C for 10 min and quenched with formamide/EDTA. Products were separated on 10% acrylamide-urea gels and visualized by autoradiography of dried gels. Only the products of replication from the <sup>32</sup>P end-labeled primer can be visualized on autoradiograms. The major replication products detectable by this experimental system are: (i) replication block, replication products that stop opposite the base before the abasic site (85 nucleotides); (ii) misincorporation products, replication products that terminate opposite the abasic site (86 nucleotides); (iii) bypass products, replication products that bypass the abasic site and terminate at the end of the template (116 nucleotides).

#### RESULTS

**Stimulation of pol II by pol III Accessory Proteins Using Synthetic Template-Primer Molecules**—To examine the effect of the  $\beta, \gamma$  complex accessory proteins on the processivity of pol II, we have used both a synthetic primer-template system and uniquely primed single-stranded M13 DNA. The primer-template region required to bind and hold onto the  $\beta, \gamma$  complex is quite large; approximately 36 bases upstream and downstream of the primer 3' terminus are required.<sup>4</sup> To satisfy these length requirements, we synthesized a 71-mer oligonucleotide and extended it with terminal deoxynucleotidyltransferase to produce average template lengths of 150 nucleotides. Extended templates were then annealed to a specific 5'-<sup>32</sup>P-end-labeled primer (15 nucleotides long), preincubated with SSB protein,  $\beta$  and/or  $\gamma$  complex accessory proteins, and pol II. DNA synthesis was initiated upon addition of dNTP and terminated after 10 s by addition of EDTA/formamide. Heat-

<sup>3</sup> M. Rajagopalan and H. Echols, unpublished data.

<sup>2</sup> C. A. Bonner, S. Hays, K. McEntee, and M. F. Goodman, unpublished data.

<sup>4</sup> P. T. Stukenberg, S. Rahman, S.-Y. Chang, and M. O'Donnell, unpublished data.

denatured reaction products were resolved on 10% polyacrylamide gels, and primer extension was visualized either by autoradiography or phosphorimaging.

The effect of the pol III accessory proteins on synthesis by pol II is shown in Fig. 1. There were no full length DNA chains synthesized by pol II alone or pol II in the presence of either the  $\beta$  or  $\gamma$  complex accessory proteins added separately (Fig. 1, lanes 1–3). In these reactions there is a pattern of products ranging from one to 15 nucleotides added to the primer. However, addition of both  $\beta$  and the  $\gamma$  complex resulted in full length synthesis of the oligonucleotide within the shortest incubation period examined (10 s; Fig. 1, lane 4). A primer-template reaction without added polymerase is shown to verify the lack of polymerase contamination in the accessory proteins (Fig. 1, lane 5). The effect of  $\beta, \gamma$  complex on the  $\alpha\epsilon$  subunits of pol III is shown for comparison (Fig. 1, lanes 6 and 7). Only low molecular weight products are produced by pol II alone suggesting that the polymerase acts distributively or is very slow in elongation. The addition of both  $\gamma$  complex and  $\beta$ , which forms a sliding clamp on the primed DNA, conferred onto pol II a rate greater than five nucleotides per s. A possible explanation of the data of Fig. 1 indicates that, as with pol III, the effect of added  $\beta, \gamma$  complex is to increase the affinity of pol II for the DNA primer-template. No stimulation of pol II was observed using 71-mer

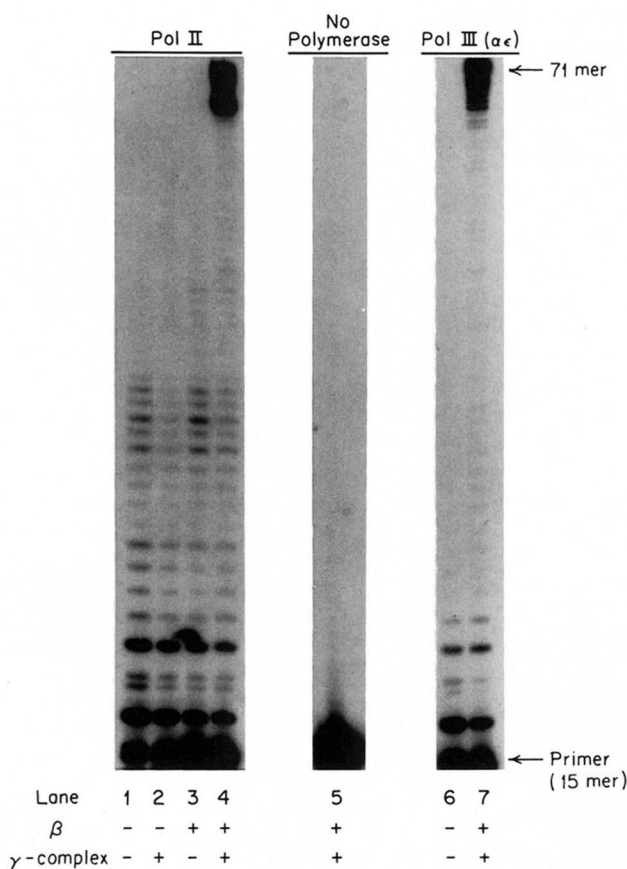
primer-templates that were not extended by terminal transferase (data not shown), consistent with the stimulation being conferred by the accessory proteins since there is a minimum length of DNA required for the assembly of the accessory protein clamp.<sup>4</sup>

**Effect of  $\beta, \gamma$  Complex on pol II Using M13 ssDNA Templates**—A study of the  $\beta$  and  $\gamma$  complex accessory proteins on either pol II or the  $\alpha\epsilon$  subunits of pol III on short synthetic templates (Fig. 1) demonstrated that the pol III accessory proteins stimulate both polymerases (Fig. 1, lanes 4 and 7). To investigate the differences in the degree to which  $\beta$  and the  $\gamma$  complex can stimulate pol II compared to pol III, we used the longer template molecule, M13 mp18 single-stranded (ss) DNA primed with a synthetic DNA 30-mer in either the presence or absence of SSB. Products of DNA synthesis were analyzed in native agarose gels (Fig. 2A) and alkaline agarose gels (Fig. 2B), and the total amount of nucleotide incorporated was also quantitated (Fig. 2C). In the absence of  $\beta, \gamma$  complex, or in the presence of either  $\beta$  or the  $\gamma$  complex, the  $\alpha\epsilon$  polymerase showed no detectable product (Fig. 2, lanes 1, 2, and 3). In the presence of  $\beta$  and  $\gamma$  complex the  $\alpha\epsilon$  polymerase completed synthesis of some of the 7.2-kb M13 mp18 templates within 5 min (Fig. 2, A and B, lane 4). pol II alone extended the primer on M13 mp18 slightly (Fig. 2A, lane 9); the product length was below the 500-base resolution limit of the alkaline gel (Fig. 2B, lane 9). The extent of synthesis by pol II was unaffected by the presence of either  $\beta$  or the  $\gamma$  complex, or both  $\beta$  and the  $\gamma$  complex (Fig. 2, A–C, lanes 10–12).

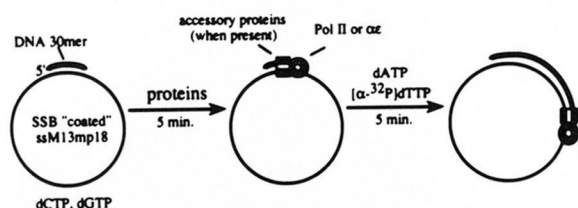
**Effect of SSB Protein on the  $\beta, \gamma$  Complex Stimulation of pol II**—The presence of SSB protein is required to achieve highly processive synthesis by the pol III holoenzyme on ssDNA (8). The  $\alpha\epsilon$  polymerase was much more efficient at synthesis of the M13 mp18 template in the presence of SSB and its accessory proteins than in the absence of SSB (compare lanes 8 and 4 in Fig. 2). In order to determine the effect of SSB on pol II synthesis stimulated by  $\beta, \gamma$  complex, we repeated the assay described for Fig. 2 in the presence of SSB. pol II alone was stimulated approximately 8-fold by SSB (compare lanes 9 and 13, Fig. 2C). Synthesis by pol II in the presence of SSB was unaffected by either  $\beta$  or  $\gamma$  complex, wherein the primer was extended approximately 1.5–2 kb (Fig. 2, A and B, lanes 13–15). However, in the combined presence of  $\beta, \gamma$  complex, and SSB (Fig. 2, lane 16), pol II completely replicated the M13 mp18 template. Hence, in the presence of SSB the accessory proteins had a profound effect on pol II, but in the absence of SSB the accessory proteins had little effect on pol II.

**Accessory Proteins Do Not Increase the Intrinsic Velocity of pol II**—To determine the rate of pol II replication in the presence and absence of accessory proteins, kinetic experiments were performed on uniquely primed M13 mp18 ssDNA “coated” with SSB. An autoradiogram of a 0.8% agarose gel of the replication time courses is shown in Fig. 3. Three levels of pol II enzyme were used in these assays: 27 fmol (1 nM, 0.8:1 polymerase to primed circle), 270 fmol (10 nM, 8:1 polymerase to primed circle), 3500 fmol (140 nM, 100:1 polymerase to primed circle).

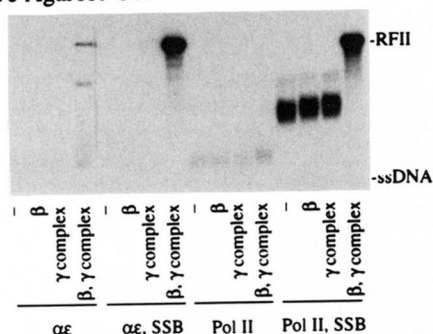
With approximately stoichiometric levels of pol II alone, little synthesis was detected before 6 min, and no full length product was observed (Fig. 3A). Addition of  $\beta, \gamma$  complex, however, resulted in accumulation of full length replicative form II between 4 and 6 min (Fig. 3A). A somewhat different response was observed with the 8-fold molar excess of pol II over template (Fig. 3B). Without the accessory proteins, full length replicative form II was produced by 12 min. The



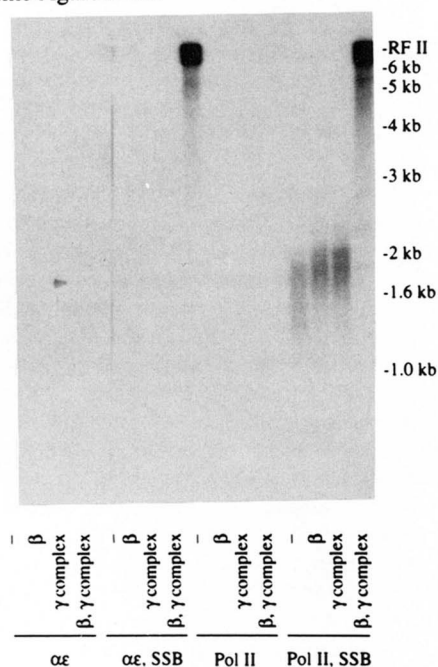
**FIG. 1. pol II is stimulated by pol III accessory proteins on synthetic oligonucleotide templates.** Autoradiogram showing stimulation of both pol II and III ( $\alpha\epsilon$  complex) by  $\beta$  and the  $\gamma$  complex. Reconstitution assays were performed as described under “Experimental Procedures” using either 38 nM pol II or  $\alpha\epsilon$  complex on 14 nM SSB-coated extended oligonucleotides. Reaction times were 10 s following a 5-min preincubation of the polymerase with accessory proteins. Lanes 1–4, pol II alone (lane 1), + $\gamma$  complex (lane 2), + $\beta$  (lane 3), + both  $\beta$  and  $\gamma$  complex (lane 4). Lane 5, reconstitution assay without added polymerase. Lanes 6 and 7,  $\alpha\epsilon$ , alone (lane 6) or with  $\beta, \gamma$  complex (lane 7).



## A. Native Agarose Gel



## B. Alkaline Agarose Gel



## C. Replication Activity

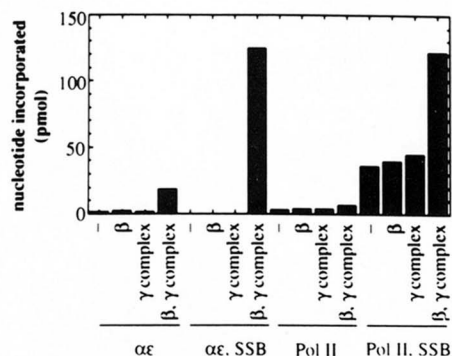


FIG. 2. **pol II is stimulated by pol III accessory proteins on natural single-stranded M13 mp18 templates.** Reconstitution reactions were performed as summarized in the scheme at the top in 25- $\mu$ l reactions upon incubating 5 nM  $\beta$  (as dimer), 0.9 nM  $\gamma$  complex, and either 11.2 nM  $\alpha\epsilon$  complex or pol II with 1.2 nM primed M13

addition of the accessory proteins resulted in the appearance of full length replicative form II between 2 and 4 min. Yet another result was obtained with the huge molar excess of pol II (Fig. 3C). Now the pol II replicated the template to full length between 2 and 4 min whether the accessory proteins were present or not, indicating that the accessory proteins did not increase the intrinsic velocity of synthesis by pol II. The accessory protein stimulation observed at low levels of pol II is likely due to an increase in processivity but may be explained by an increased rate of association with the primer template. In the next experiment (Fig. 4), it is shown that the  $\beta, \gamma$  complex do increase the processivity of pol II. However, the  $\beta, \gamma$  complex must not provide pol II with sufficient processivity to allow it to completely replicate the M13 mp18 template in a single binding event. If pol II would not have dissociated from the accessory proteins for a full round of M13 mp18 synthesis, then 27-fmol (1 nM) pol II would have formed full length M13 mp18 DNA in the same amount of time as seen for 270-fmol (10 nM) pol II.

The stimulation by  $\beta, \gamma$  complex on the rates of DNA synthesis by pol II was quantified by measuring the kinetics of deoxyribonucleotide incorporation (Fig. 3). The rate of incorporation depended on the concentration of pol II when measured in the presence or absence of  $\beta, \gamma$  complex. In the absence of the accessory proteins, the template was replicated at a rate of 10 nucleotides per s with 270 fmol (10 nM) of pol II; with 27 fmol (1 nM) of pol II, replication was about 10-fold slower. Addition of accessory proteins resulted in at least a 3-fold stimulation of the rate of incorporation with 10 nM pol II, to a value of 30 nucleotides per s. At 1 nM pol II, inclusion of accessory proteins produced a 20-fold stimulation, from 1 to 20 nucleotides/s.

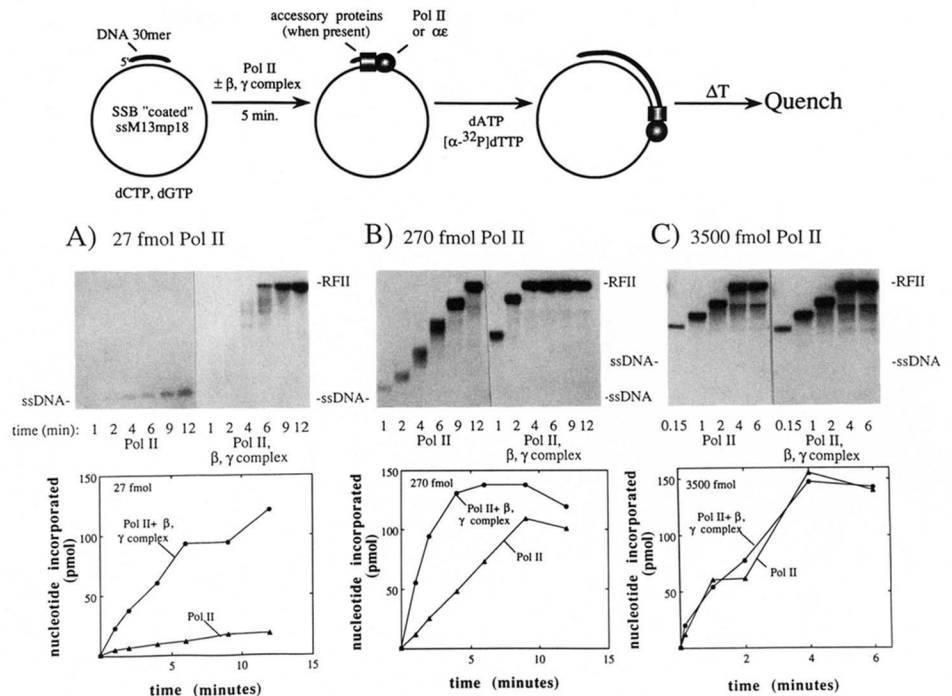
**Processivity of pol II in the Presence and Absence of Accessory Proteins**—To measure the processivity of pol II in the absence of accessory proteins and SSB, an experiment using excess challenge DNA was performed using stoichiometric pol II with the extended oligonucleotide (~150-mer) primed with a  $^{32}$ P end-labeled DNA 15-mer (Fig. 4A). pol II was preincubated with the primed template and replication initiated by the addition of 10-fold excess unlabeled challenge DNA and unlabeled nucleotides. For reaction times between 30 s and 3 min following addition of challenge DNA, product distribution ranged from one to 20 nucleotides. The processivity of pol II alone is about five nucleotides as determined by computing the weighted average of integrated band intensities (Fig. 4A, 0.5- and 1-min reactions).

The processivity of pol II on SSB "coated" primed M13 mp18 ssDNA containing the accessory protein clamp was measured directly in an excess challenge DNA processivity assay (Fig. 4B). In this processivity experiment, stoichiometric pol II along with  $\beta, \gamma$  complex were preincubated with M13 mp18 ssDNA primed with a  $^{32}$ P end-labeled DNA 30-mer and "coated" with SSB. Replication was initiated upon addition of a 4-fold excess of challenge primed (unlabeled) M13 mp18 ssDNA which also contained a preinitiation complex formed using the  $\beta, \gamma$  complex accessory proteins. Hence, as pol II dissociates from the  $^{32}$ P end-labeled primer it will become trapped on the excess unlabeled challenge primed template. At various times the extension reaction was sampled and the

mp18 ssDNA, 0.4  $\mu$ M SSB (when present), 0.5 mM ATP, 60  $\mu$ M each dCTP and dGTP, in buffer A for 5 min at 37  $^{\circ}$ C. Synchronous replication was initiated upon addition of dATP and [ $\alpha$ - $^{32}$ P]dTTP to final concentrations of 60 and 20  $\mu$ M, respectively. Replication reactions were quenched after 5 min with 25  $\mu$ l of 1% sodium dodecyl sulfate and 40 mM EDTA. Quenched reactions were analyzed by a 0.8% agarose gel (A), 1% alkaline agarose gel (B), and total nucleotide incorporation (C).



**FIG. 3. The level of accessory protein stimulation depends on the concentration of pol II.** Replication time courses containing 1 nM (27 fmol) (A), 10 nM (270 fmol) (B), or 140 nM (3500 fmol) (C) pol II were performed as in Fig. 2 (summarized in the scheme at the top), except 1.2 nM primed ss DNA, 0.4  $\mu$ M SSB, and, where indicated, 5 nM  $\beta$  and 0.9 nM  $\gamma$  complex were present in a 175- $\mu$ l final volume of buffer A. After preincubation for 5 min at 37  $^{\circ}$ C, replication was initiated upon addition of dATP and [ $\alpha$ - $^{32}$ P]dTTP to 60 and 20  $\mu$ M, respectively, and 20  $\mu$ l was removed and quenched as in Fig. 2 at the indicated times. Autoradiograms of 0.8% agarose gels of the time course reactions are shown above graphs of deoxynucleotide incorporation per 25  $\mu$ l of reaction.



length of primer extension was analyzed in an alkaline agarose gel. The analysis shows at least some pol II remains associated with the initial  $^{32}$ P primed template for over 2 min. The length distribution of products finally stabilized by 3 min. Densitometric analysis of the smear of products identified the peak at approximately 1.6 kb with half-maximal intensity on either side of the peak at approximately 0.75 and 3.0 kb (not shown). Hence, the processivity of pol II in the presence of  $\beta$ ,  $\gamma$  complex and SSB is approximately 1.6 kb. A 3-kb product synthesized over 2 to 3 min indicates an intrinsic velocity for pol II between 20 and 30 nucleotides per s. This agrees with the velocity determined in Fig. 3. A control analysis using the pol III  $\alpha\epsilon$  complex, which is known to be processive over the length of this template, is shown in the three rapid time points on the right side of the gel.

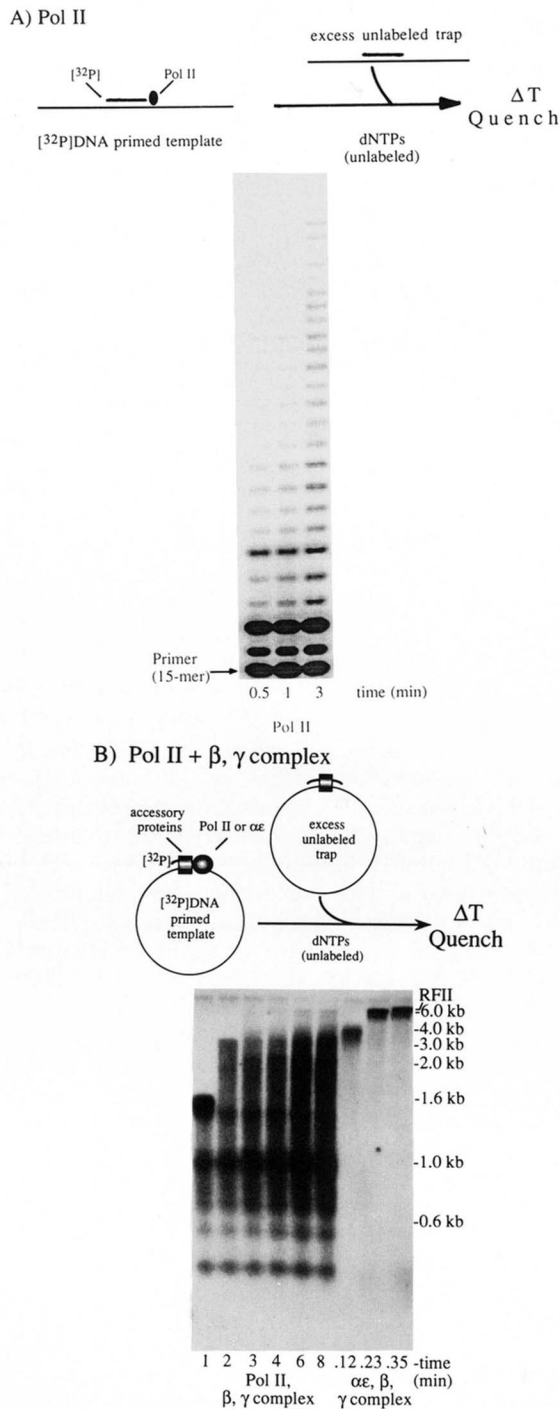
An experiment identical to Fig. 4 was performed in which SSB was present but the accessory proteins were omitted. No products greater than 200 nucleotides were observed in the alkaline gel even after 22 min of replication (data not shown). Therefore, under these conditions, pol II, in the absence of accessory proteins, gives no significant signal in the agarose gel. Analysis in a denaturing polyacrylamide gel of the pol II extension products in the absence of accessory proteins showed the products increased in size steadily throughout the time course and reached a length distribution of 50–180 nucleotides after 22 min of replication (data not shown). All the templates had been extended, consistent with the distributive action of pol II in the absence of accessory proteins. This analysis of pol II processivity on primed M13 mp18 was not extended to the case of  $\beta$ ,  $\gamma$  complex in the absence of SSB since the accessory proteins do not significantly stimulate pol II in the absence of SSB (see Fig. 2).

**Effect of Accessory Proteins on Nucleotide Incorporation and Bypass of Abasic Sites by pol I, II, and III**—pol III and pol II have been implicated in SOS-induced error prone DNA repair synthesis. Since the accessory proteins enhance processivity of both polymerases, it was important to determine whether those proteins could affect the ability of the polymerases to insert nucleotides opposite a well-defined abasic (apurinic/aprimidinic) template lesion and/or stimulate extension be-

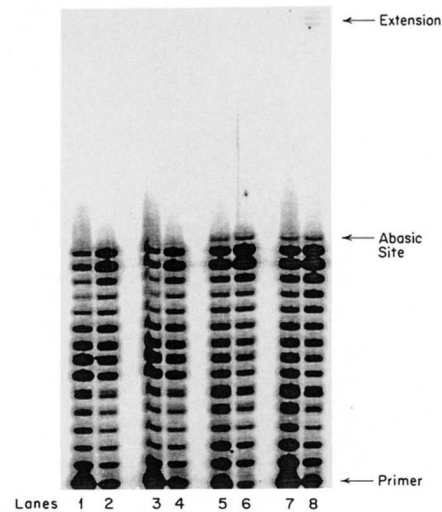
yond the site of the lesion. A synthetic 60-mer oligonucleotide containing a single reduced abasic site 30 nucleotides from the 5' end was extended by terminal deoxynucleotidyltransferase and annealed to a  $^{32}$ P end-labeled primer 15 nucleotides upstream of the lesion. Stoichiometric levels of both pol II and  $\alpha\epsilon$  were assayed with and without the addition of  $\beta$ ,  $\gamma$  complex for insertion and bypass at the abasic site. The results are shown in Fig. 5. pol II exhibited slight misincorporation of a nucleotide opposite the abasic site with little extension beyond the abasic site (Fig. 5, lanes 5 and 6). Addition of accessory proteins had little effect on misinsertion; however, bypass of the abasic site increased from barely detectable to about 2% bypass (Fig. 5, lane 8). No  $\beta$ ,  $\gamma$  complex effect was seen in the absence of SSB (data not shown). In contrast, pol III exhibited no detectable misincorporation or bypass with or without the accessory proteins (Fig. 5, lanes 1–4).

The synthetic oligonucleotide template is not optimal for analyzing the role of processivity subunits because the replication blocking lesion is so close the primer (15 nucleotides). To avoid this difficulty, the 60-mer oligonucleotide containing a single abasic site was ligated into linear  $\phi$ X174 DNA, allowing us to investigate translesion replication when the blocking lesion is more distant from the primer terminus. The assay used to measure polymerase incorporation and bypass of abasic sites with the longer template is shown in Fig. 6A. A single abasic site was introduced at a unique location. The  $\phi$ X174 DNA was primed 86 nucleotides upstream from the abasic lesion with a  $^{32}$ P end-labeled 20-mer oligonucleotide; replication by the various polymerases and accessory proteins was initiated as described under "Experimental Procedures," and the products were resolved on denaturing gels. The size of the replication products indicates whether synthesis is completely blocked at the abasic site (85 nucleotide product), if a nucleotide can be incorporated opposite the abasic site (86 nucleotide product), or if the lesion can be bypassed (116 nucleotide product). However, if the misincorporation opposite the abasic site is very limited, the resolution of this band from the intense band before the lesion is very difficult.

The results of the various polymerase reactions are shown in Fig. 6B. Bypass of the abasic lesion was very inefficient for



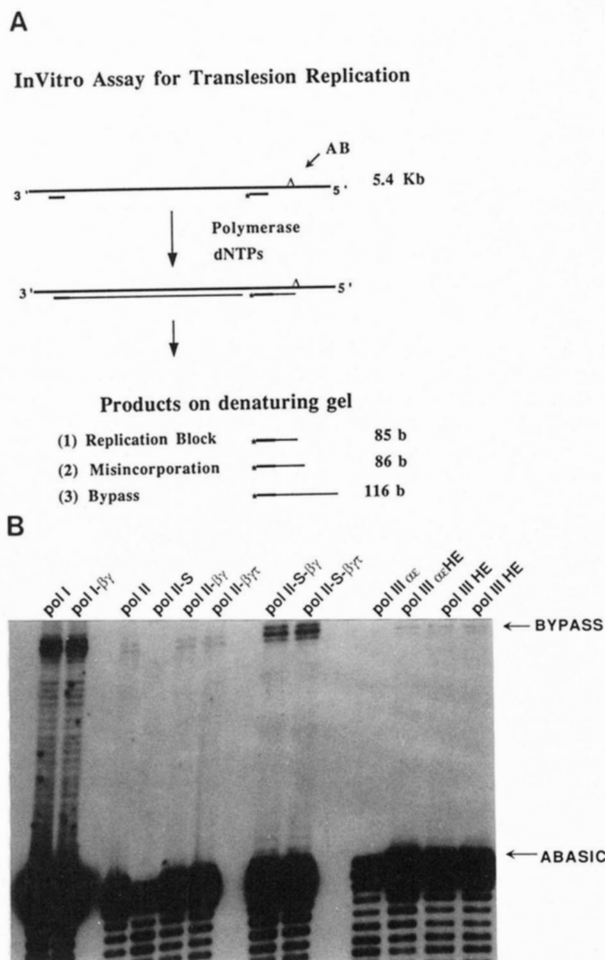
**FIG. 4. Analysis of the processivity of pol II in the presence and absence of  $\beta$ ,  $\gamma$  complex.** A, processivity of pol II in the absence of accessory proteins was determined by an excess DNA challenge assay (see scheme at top). pol II (10 nM) was preincubated for 5 min at 37 °C with TdT extended synthetic oligonucleotide (10 nM) primed with  $^{32}$ P end-labeled DNA 15-mer in 10  $\mu$ l of buffer A containing 0.5 mM ATP and 120  $\mu$ M each dGTP and dATP. Replication was initiated upon addition of challenge DNA (100 nM) in 10  $\mu$ l of buffer A containing 120  $\mu$ M each dCTP and dTTP. At the times indicated, 5- $\mu$ l aliquots were removed and quenched with 10  $\mu$ l of EDTA/formamide. Reactions were analyzed by electrophoresis at 2000 V for 2 h in a 10% polyacrylamide gel containing 8 M urea. The gel was dried, exposed to a phosphorimager cassette overnight, and scanned on a Molecular Dynamics phosphorimager. B, the processivity of pol II in the presence of accessory proteins was determined by a DNA excess challenge assay using M13 mp18 (see scheme at the top). pol II (1.5 nM),  $\beta$  (11 nM as dimer), and  $\gamma$  complex (0.9 nM) were preincubated for 6 min at 37 °C with M13 mp18 ssDNA (1.7 nM as circles) primed with a  $^{32}$ P end-labeled DNA 30-mer and "coated" with SSB (0.6  $\mu$ M



**FIG. 5. Effect of  $\beta$  and  $\gamma$  complex stimulation of pol II and  $\alpha\epsilon$  on insertion and bypass of abasic sites on synthetic oligonucleotides.** Phosphorimager tracing of a 10% polyacrylamide gel showing primer extension by pol II and  $\alpha\epsilon$  on a template containing an abasic site. Reconstitution reactions were performed as described in Fig. 1 using 50 nM  $\alpha\epsilon$  (lanes 1–4) or pol II (lanes 5–8), and 50 nM SSB-coated template, except that the template contains a single reduced abasic site 15 nucleotides from the end of the primer terminus.  $\beta$  (50 nM) and  $\gamma$  complex (10 nM) were added in lanes 3, 4, 7, and 8. Lanes 1 and 2,  $\alpha\epsilon$ , 30-s reaction (lane 1) and 1-min reaction (lane 2); lanes 3 and 4,  $\alpha\epsilon\beta\gamma$  complex, 30 s (lane 3) and 1 min (lane 4); lanes 5 and 6, pol II, 30 s (lane 5) and 1 min (lane 6); pol II/ $\beta\gamma$  complex, 30 s (lane 7) and 1 min (lane 8).

all three DNA polymerases, and so the gel is overexposed to show the differences. Because of the extremely limited misincorporation, we were unable to make a reliable comparison of this aspect of the system among the polymerases. The extent of bypass was estimated from a densitometer tracing of this exposure (for bypass) compared to a lower exposure in which the relative amount of blocked replication product could be measured. Significant lesion bypass (approximately 3%) was observed for pol I (Fig. 6B, lane 1). There was no effect of  $\beta$ ,  $\gamma$  complex on pol I-dependent bypass at the abasic site (lane 2). A barely detectable bypass of the abasic lesion was observed for pol II alone (Fig. 6B, lane 3). Addition of SSB protein inhibited both replication and bypass by pol II (Fig. 6B, lane 4), an effect also seen on synthetic oligonucleotides (data not shown). The accessory proteins alone had little effect on bypass of the abasic site catalyzed by pol II (Fig. 6B, lanes 5 and 6). In contrast, addition of SSB,  $\beta$ ,  $\gamma$  complex increased replication up to and exceeding the abasic lesion (approximately 2%) (Fig. 6B, lanes 7 and 8). A third pol III accessory protein,  $\tau$ , which increases the processivity

as tetramer) in 141  $\mu$ l of buffer A containing 0.5 mM ATP and 60  $\mu$ M each dCTP and dGTP. Replication was initiated upon addition of an excess DNA challenge mixture consisting of 5.9 nM M13 mp18 ssDNA primed with the same DNA 30-mer (but unlabeled) which also contained a preinitiation complex upon a 6-min incubation at 37 °C with SSB (1.2  $\mu$ M as tetramer),  $\beta$  (11 nM as dimer), and  $\gamma$  complex (2 nM) in 159  $\mu$ l of buffer A containing 60  $\mu$ M each dCTP and dGTP, and 120  $\mu$ M each dATP and dTTP. At the times indicated, 42- $\mu$ l aliquots were removed and quenched with 40  $\mu$ l of 1% sodium dodecyl sulfate, 40 mM EDTA. Reactions were analyzed by electrophoresis at 40 V for 20 h in a 1.2% alkaline agarose gel. The gel was neutralized, dried, and exposed to x-ray film. The three lanes on the right side of the gel are time points from a control reaction which was performed in a similar manner except pol III  $\alpha\epsilon$  complex replaced pol II. The positions of the size standards visualized by ethidium bromide staining induced fluorescence after neutralization of the alkaline gel are marked on the right of the gel.



**FIG. 6. Bypass at a single abasic lesion in  $\phi$ X174 DNA by *E. coli* DNA polymerases I, II, and III.** *A*, *in vitro* assay for translesion replication using a 5.4-kb linear ssDNA template with an abasic site at a defined location. The doubly primed substrate is replicated by the polymerases indicated. Only the products of replication from the end-labeled primer can be visualized on the autoradiograms of denaturing acrylamide gels. Replication blocked at a base before the abasic site gives a product of 85 bases, misincorporation opposite the abasic site gives a product of 86 bases, and the complete bypass product is 116 bases. *B*, translesion replication reactions using the three *E. coli* DNA polymerases were performed as described under "Experimental Procedures." Lanes from left to right: pol I (Klenow fragment); pol I (Klenow) +  $\beta$  +  $\gamma$  complex; pol II; pol II + Ssb; pol II +  $\beta$  +  $\gamma$  complex; pol II +  $\beta$  +  $\gamma$  complex +  $\tau$ ; pol II + Ssb +  $\beta$  +  $\gamma$  complex; pol II + Ssb +  $\beta$  +  $\gamma$  complex +  $\tau$ ;  $\alpha$  subassembly of pol III;  $\alpha\epsilon$  complex + Ssb +  $\beta$  +  $\gamma$  complex; pol III holoenzyme (naturally purified).

of pol III core (28), was assayed with pol II using the abasic site template (Fig. 6B, lanes 6 and 8). Addition of  $\tau$  had no effect on bypass of the abasic site. In addition, three different assemblies of pol III were assayed on the abasic lesion-containing template: the complete 10-subunit holoenzyme (HE, lanes 11 and 12), the two subunit  $\alpha\epsilon$  (lane 9), and the eight-subunit  $\alpha\epsilon\beta\gamma$  complex (lane 10). Of the enzymes tested, the three forms of pol III showed the most restricted bypass at the abasic lesion (less than 0.5%). The absence of significant bypass by the pol III assemblies depended on the presence of the exonuclease ( $\epsilon$ ) subunit; readily detectable, although limited, bypass was observed for the  $\alpha$  subunit alone or for the  $\alpha\beta\gamma$  complex form of the enzyme.<sup>3</sup> The results of Fig. 6 are consistent with our earlier demonstration that bypass of abasic lesions occurs to some extent for pol I and pol II with very little bypass by pol III (36).

## DISCUSSION

**pol III Accessory Proteins and Processivity of pol II**—The recent demonstrations that DNA polymerase II of *E. coli* is part of the SOS regulon (13, 22, 23) and is capable of bypassing abasic sites in template DNA molecules (22) has renewed interest in the function and biochemical properties of this enzyme. Early studies suggested that DNA synthesis by pol II could be stimulated by addition of pol III accessory proteins (25); more recently, Hughes *et al.* (26) showed that pol II activity was markedly enhanced by the presence of  $\beta\gamma$  complex.

With highly purified DNA polymerase II, we have investigated the functional interaction of pol II with the sliding clamp formed by the  $\beta\gamma$  complex accessory proteins of the pol III holoenzyme, using as templates synthetic oligonucleotides and long single-stranded viral DNA with and without unique abasic lesions. We have concluded that the primary effect of  $\beta\gamma$  complex is to increase pol II processivity between 150- and 600-fold. Similarities in the effects of  $\beta\gamma$  complex on pol II and pol III include: requirement for SSB protein; a minimum template-primer length requirement for assembly of the active complex; optimized stimulation in activity at approximately stoichiometric levels of  $\beta\gamma$  complex with each polymerase. However, there is also an important difference in that the accessory proteins enhance pol III processivity to a far greater extent than pol II. The pol III complex is able to catalyze complete replication of M13 mp18 ssDNA circles within about 20 s, whereas the pol II- $\beta\gamma$  complex cannot complete a full round of M13 mp18 synthesis without dissociation and even at saturation requires between 2 and 4 min to complete the circular template (Fig. 3). Under assay conditions in which pol II is approximately stoichiometric with primer, pol II alone incorporates nucleotides at a rate of approximately 1 per s. This rate is increased 20-fold in the presence of a stoichiometric amount of  $\beta\gamma$  complex and SSB. This correlates well with the observed processivity for the pol II/ $\beta\gamma$  complex (Fig. 4). At 20 nucleotides per s, a residence time of 2–3 min predicts a product of 2.4–3.6 kb, well within the 0.75–3-kb processivity measured in Fig. 4.

The faster optimized rate of synthesis for pol III compared to pol II may reflect differences in the biological functions of the two enzymes; pol III for rapid chromosomal replication, and pol II perhaps for specialized "limited extent" synthesis accompanying repair. The stoichiometry required to achieve optimal stimulation of pol II by  $\beta\gamma$  complex suggests that the accessory protein-pol II interactions are specific, and therefore, likely to be biologically important. For comparison, we find that pol I and bacteriophage T4 polymerase also exhibit more rapid DNA synthesis in the presence of  $\beta\gamma$  complex, but the degree of stimulation is small and occurs only at extreme substoichiometric levels of the polymerase (~50 mol of complex per mol of pol I or T4 polymerase).<sup>2</sup> There are no obvious conserved amino acid sequences or domains that might imply a structural relationship between pol II and the  $\alpha$  subunit of pol III. Thus, interactions of pol II and pol III  $\alpha$  subunit with  $\beta\gamma$  complex are likely to involve external non-catalytic regions containing common tertiary structural elements.

**Activity of DNA Polymerases at a Replication-blocking Lesion**—Although pol II has been shown to incorporate nucleotides opposite abasic sites *in vitro*, it extends these inefficiently (22). Since it has been suggested that factors which increase polymerase processivity may play a role in translesion replication (37–40), we investigated the effect of pol III accessory proteins on bypass by pol I, pol II, and pol III of one particular lesion, an abasic site. Addition of accessory

proteins to *E. coli* polymerases I and III had little or no effect on lesion bypass, whereas addition to pol II resulted in increased lesion bypass. Misincorporation and bypass were markedly less for pol III than for pol II (Figs. 5 and 6). It is important to note that abasic site bypass is extremely inefficient for all polymerases. Since genetic data implicate pol III in lesion bypass and pol III holoenzyme has extraordinary processivity, factors other than (or in addition to) increased processivity are likely to be required for translesion replication by pol III.

Although recent observations suggest a specialized role for pol II in one or more DNA repair pathways requiring SOS induction, pathways requiring intervention of pol II remain to be elucidated. We have recently constructed a new insertion mutation in the pol II gene which removes both transcription and translation start signals and more than 50% of the 5' portion of the gene.<sup>2</sup> In preliminary experiments, we find that this mutant appears to have a normal growth rate. Thus, it now appears virtually certain that pol II is not required for replication of *E. coli*; however, the observation that the activity of pol II is stimulated significantly by stoichiometric levels of the pol III accessory proteins suggests that pol II might utilize these interactions *in vivo*. The availability of mutants should allow investigations into the possible role of pol II in DNA repair synthesis, either cooperatively with or as a backup to pol I and III.

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