

Purification and Properties of Wild-type and Exonuclease-deficient DNA Polymerase II from *Escherichia coli**

(Received for publication, February 21, 1995, and in revised form, April 19, 1995)

Hong Cai[‡], Hong Yu[‡], Kevin McEntee[§], Thomas A. Kunkel[¶], and Myron F. Goodman[‡]

From the [‡]Department of Biological Science, Hedco Molecular Biology Laboratories, University of Southern California, Los Angeles, California 90089-1340, the [§]Department of Biological Chemistry and the Molecular Biology Institute, UCLA School of Medicine, Los Angeles, California 90024, and the [¶]Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

Wild-type DNA polymerase II (pol II) and an exonuclease-deficient pol II mutant (D155A/E157A) have been overexpressed and purified in high yield from *Escherichia coli*. Wild-type pol II exhibits a high proofreading 3'-exonuclease to polymerase ratio, similar in magnitude to that observed for bacteriophage T4 DNA polymerase. While copying a 250-nucleotide region of the *lacZα* gene, the fidelity of wild-type pol II is high, with error rates for single-base substitution and frameshift errors being $\leq 10^{-6}$. In contrast, the pol II exonuclease-deficient mutant generated a variety of base substitution and single base frameshift errors, as well as deletions between both perfect and imperfect directly repeated sequences separated by a few to hundreds of nucleotides. Error rates for the pol II exonuclease-deficient mutant were from ≥ 13 - to ≥ 240 -fold higher than for wild-type pol II, depending on the type of error considered. These data suggest that from 90 to >99% of base substitutions, frameshifts, and large deletions are efficiently proofread by the enzyme. The results of these experiments together with recent *in vivo* studies suggest an important role for pol II in the fidelity of DNA synthesis in cells.

Three DNA polymerases have been identified in *Escherichia coli* (1). Pol I¹ is involved in repair of damaged DNA and processing RNA-primed Okazaki fragments (1), while pol III is required for replication of the bacterial genome (2). Although pol II was discovered more than 24 years ago (3), its role in either replication or repair has been difficult to define. It has been shown that pol II expression is regulated by the *lexA* repressor, as part of the SOS regulon, where the locus of the structural gene for pol II is coincident with the DNA damage-inducible *dinA* gene (4, 5). *In vitro* studies have established that DNA pol II activity (6, 7) and processivity (8) are strongly stimulated by DNA pol III accessory proteins suggesting that the two polymerases might share overlapping roles in the cell. An *E. coli* strain containing a deletion in the pol II structural gene exhibited an increase in adaptive mutation rate (9), suggesting a role for pol II in localized DNA synthesis or repair in nondividing cells (10). Recently, we have shown that replacement of wild-type *polB* with an exonuclease-deficient *polB* al-

lele increases the rate adaptive mutation in *E. coli*.²

As a consequence of these newly-established pol II phenotypes (10, 12),² biochemical investigations of pol II (7, 8, 13, 14) are especially timely and important to pursue. Pol II is classified as a type II, human α -like polymerase (4) having considerable sequence similarity to bacteriophage T4 pol. The enzyme contains both polymerase and 3'-exonuclease activities within a single 89.9-kDa polypeptide chain (4, 15, 16). In this paper, we describe the purification and biochemical properties of wild-type pol II and an exonuclease-deficient pol II derivative (pol II ex1).

EXPERIMENTAL PROCEDURES

Chemicals—All nonradioactive dNTP substrates were purchased from Pharmacia LKB Biotechnology Inc. Radioactive [γ -³²P]ATP (4000 Ci/mmol), [α -³²P]dNTP (4000 Ci/mmol), and [methyl-³H]TTP (20 Ci/mmol) were purchased from ICN Radiochemicals, Inc. (Irvine, CA). Diethylaminoethyl-cellulose (DE52) and phosphocellulose (P-11) resins were obtained from Whatman BioSystems Inc.

Enzymes—Phage T4 DNA polymerase (3.5×10^6 units/mg (17), 1 unit is that amount of enzyme required to incorporate 1 pmol of labeled dTMP into nucleic acid product in 1 min at 37 °C) was kindly provided by Dr. L. Reha-Krantz (University of Alberta, Canada). A measurement of T4 pol specific activity was also carried out at 30 °C for direct comparison with published data; the value obtained, 2×10^6 units/mg is consistent with the data in Ref. (17). Klenow fragment (7000 units/mg) was purchased from Pharmacia LKB Biotechnology Inc. Pol II wild-type and a 3'-exonuclease-deficient pol II derivative were purified as described below.

Primer/Template—Single-stranded M13 DNA template was prepared as described (18). Primers were synthesized using an Applied Biosciences DNA synthesizer by Lynn Williams (Kenneth Norris Jr. Comprehensive Cancer Center, University of Southern California, Los Angeles) and used after purification by polyacrylamide gel electrophoresis. Primer sequence p5, 5'-ATTAATCCTTTGCCCG-3', is complementary to the numbered positions on wild-type M13 strand: 4640–4657. Primer p15C (5'-ATGATTAAGACTCCTTA-3') can be annealed to template TGG-1(3'-TACTAATTCTGAGGAATGGTGTGATCATACAATC-GT-5'). The sequence of primer Prsp2 is 5'-GATCAGTCCTGTACGG-TACTGACTGACC-3'.

Bacteria Strain and Plasmid—*E. coli* JM109 (*recA1 Δ(lac-proAB⁺) F'(traD36 proAB⁺ lacI^q lacZΔM15)*) was kindly provided by Dr. Miriam Susskind, University of Southern California. Plasmid pPROK-1 was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA).

Construction of Pol II Overproducing Plasmid (pHY400) and a Pol II 3'-Exonuclease Mutant (D155A/E157A) Overproducing Plasmid (pHC700)—A 2.4-kb DNA fragment containing the *dinA/polB*³ open reading frame was obtained from plasmid pSH100 by polymerase chain reaction amplification of the *polB* coding region (4). The original "inefficient" GTG translation initiation codon was changed to ATG using a polymerase chain reaction primer containing the appropriate base

* This research was supported by National Institutes of Health Grants GM21422, GM42554, and GM29558. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ The abbreviations used are: pol I, DNA polymerase I from *E. coli*; pol II, *E. coli* DNA polymerase II; pol III, *E. coli* DNA polymerase III; KF, Klenow fragment of pol I; pol II ex1, an exonuclease-deficient pol II mutant (D155A/E157A); T4 pol, bacteriophage T4 DNA polymerase; dNTP, deoxyribonucleoside triphosphate; kb, kilobase pair.

² Foster, P. L., Gudmundsson, G., Trimarchi, J. M., Cai, H., and Goodman, M. F. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, in press.

³ The *polB* gene originally described in Refs. 42 and 43 corresponds to the DNA polymerase II structural gene (Z.-H. Qiu, K. McEntee, and M. F. Goodman, manuscript in preparation).

change. This 2.4-kb polymerase chain reaction fragment was subcloned into the pPROK-1 vector (a 4.6-kb plasmid vector containing a P_{lac} promoter) to give a 7.0-kb plasmid construct, pHY400. The sequence of *polB* was confirmed by DNA sequence analysis. The expression of *polB* is under the control of the P_{lac} promoter that is regulated by Lac I^a. The *polB* gene containing substitutions D155A/E157A was engineered using standard oligonucleotide-directed mutagenesis procedures (19). Mutations in the plasmid were screened initially by restriction endonuclease mapping (the mutant oligonucleotide encoding the alanine substitution introduced a new restriction site for *Afl*III endonuclease) and later by DNA sequencing of the *polB* gene. A 2.4-kb fragment corresponding to the *polB* open reading frame containing the desired mutations was inserted into the pPROK-1 vector resulting in a 7.0-kb plasmid, pHC700.

Cell Growth and Cell Lysis.—*E. coli* JM109 cells carrying the *polB* gene on the overproducing plasmid pHC700 (wild-type pol II) or pHC700 (3'-exonuclease-deficient pol II, *i.e.* pol II ex1) were grown in LB with 50 µg/ml ampicillin in a 180-liter fermenter at 37 °C. The overproduction of pol II protein was induced by adding isopropyl β-D-thiogalactoside to the cells at midlog phase (*A*₅₉₅ about 0.8) to a final concentration of 0.4 mM. Two hours after isopropyl β-D-thiogalactoside induction, cells were harvested and resuspended in a volume of storage buffer (sterile 50 mM Tris-HCl, pH 7.5, 10% sucrose) equal to the wet weight of the cells in grams. Cells were rapidly frozen by resuspension in liquid nitrogen and were stored at -70 °C. Lysis buffer (50 mM Tris-HCl, pH 7.5, 10% sucrose, 0.1 M NaCl, 15 mM spermidine) was added to frozen cells to achieve a final concentration of 0.2 g of cells/ml. Cells were thawed at 4 °C and the pH was adjusted to 7.7 with 2 M Tris base. Lysozyme was added to a final concentration of 0.2 mg/ml. The cell slurry was incubated for 1 h at 4 °C, 4 min at 37 °C, and centrifuged at 11,800 rpm for 1 h in a Sorvall GSA rotor. The supernatant (Fraction I) was decanted and kept on ice.

Purification of Pol II.—Ammonium sulfate was added slowly to Fraction I, to a final concentration of 30% (w/v), and the suspension was kept at 4 °C overnight, without stirring. The suspension was centrifuged in a Sorvall GSA rotor (11,800 rpm, 40 min) and the supernatant was discarded. A volume of PC/25 buffer (50 mM Tris-HCl, pH 7.5, 20% glycerol, 1 mM EDTA, 5 mM dithiothreitol, 25 mM NaCl) equal to one-fifth to one-tenth of the original volume of Fraction I was added to the ammonium sulfate pellet and the protein was gently resuspended (Fraction II). Fraction II was dialyzed against PC/25 buffer until the conductivity reached a value equivalent to that of 40 mM NaCl. After dialysis, Fraction II was diluted with PC/25 buffer to a protein concentration of approximately 10 mg/ml and then loaded onto a P-11 phosphocellulose column, 5 × 70 cm (inner diameter × length). The column was washed with 1 column volume of PC/25 buffer and an additional column volume of PC/200 buffer (the same components as buffer PC/25 except that the NaCl concentration was 200 mM) to elute DNA polymerase III. Pol II protein was eluted with a gradient of 200–500 mM NaCl in buffer PC (8 column volumes). The pol II fractions (eluting between 225 and 250 mM NaCl) were pooled to give Fraction III. Fraction III was dialyzed against PK20 buffer (20 mM potassium phosphate, pH 6.8, 15% glycerol, 1 mM EDTA, 5 mM dithiothreitol) until the conductivity reached that of PK30 buffer (30 mM potassium phosphate, pH 6.8) and loaded on a DEAE column, 5 × 70 cm (inner diameter × length). The DEAE column was washed with 2-column volumes of PK20 followed by elution with an 8-column volume gradient of 20–350 mM potassium phosphate (PK20 to PK350). The pol II fractions (typically eluting at 100–140 mM potassium phosphate) were pooled as Fraction IV and stored at -70 °C. The procedure used to purify the exonuclease deficient pol II mutant was the same used for wild-type pol II. The specific activity and recovery at each step of purification for wild-type pol II is contained in Table I.

Deoxyribonucleotide Incorporation Assay.—The deoxynucleotide incorporation activity of pol II was measured using [*methyl*-³H]TTP and activated salmon sperm DNA as described (20, 21). Reactions contained 2.5 mM dithiothreitol, 20 mM Tris-HCl, pH 7.5, 7.3 mM MgCl₂, 6 mM spermidine/HCl, 1 mg/ml bovine serum albumin, 1.1 mM activated DNA, 60 µM dATP, dCTP, dTTP, dGTP, and [³H]dTTP. One unit of enzyme catalyzed the incorporation of 1 pmol of [³H]TTP into acid insoluble material in 1 min at 37 °C. T4 pol deoxynucleotide incorporation activity was measured by the same method except the reaction buffer contained 67 mM Tris-HCl, pH 8.8, 16.7 mM (NH₄)₂SO₄, 0.5 mM dithiothreitol, 6.7 mM MgCl₂, and 167 µg/ml bovine serum albumin.

Protein Assay.—Protein concentration was measured by the Bradford assay using materials and protocol supplied by Bio-Rad. Silver staining of protein gels were carried out using protocols and materials supplied in the ICN Rapid Ag-Staining Kit.

TABLE I
Purification of *E. coli* DNA polymerase II from *E. coli* JM109
Cells were induced with isopropyl-1-thio-β-D-galactopyranoside to overproduce pol II.

| Fraction | Volume | Protein concentration | Specific activity | Recovery |
|-----------------------|--------|-----------------------|---|----------|
| | ml | mg/ml | 10 ³ units/ mg ^a | |
| I. Crude lysate | 1000 | 12 | 0.50 | 1.0 |
| II. Ammonium sulfate | 250 | 14 | 1.5 | 0.85 |
| III. Phosphocellulose | 340 | 1.0 | 14 | 0.78 |
| IV. DEAE | 1000 | 0.3 | 18 | 0.78 |

^a One unit of enzyme catalyzes the incorporation of 1 pmol of [³H]dTTP into acid-insoluble material in 1 min at 37 °C.

Active Site Titration of Purified Pol II Protein Using DNA-Heparin Trap.—5' End-labeling of primer p15C was carried out in a labeling reaction (20 µl) containing 50 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, and 0.5 mM dithiothreitol, 3.0 µM primer, 0.3 µM [^γ-³²P]ATP, and 10 units of T4 polynucleotide kinase. The labeling reaction was performed at 37 °C for 1 h and terminated by heating it to 90 °C for 5 min. 2 µl of 100 mM EDTA (dissolved in 5 mM Tris-HCl, pH 8.0) was added to the labeling reaction to chelate Mg²⁺ ions. Template TGG-1 was added to the above solution and conditions were adjusted to attain 17 mM Tris-HCl, pH 7.5, 0.7 mM MgCl₂, 0.17 mM dithiothreitol, 1.0 µM primer, 1.25 µM template TGG-1, and 3 mM EDTA. Annealing was carried out by heating the annealing reaction mixture to 90 °C for 3 min and then cooling to room temperature over a 1-h time period. Under these conditions, more than 96% of annealed primer could be utilized by polymerase as judged by the primer extension experiment described below (data not shown). A combination of 0.1 mg/ml heparin sulfate and 0.66 mg/ml calf thymus DNA, prepared as described (22) was completely effective in trapping free (*i.e.* dissociated) pol II. For a typical reaction (20 µl), 4 µl of enzyme was preincubated with 8 µl of primer/template/dNTP mixture for 1 min at 37 °C to form enzyme-primer-template complexes, and primer extension was initiated with 8 µl of MgCl₂/trap mixture. The final concentrations were 3–50 nM polymerase, 50 mM Tris-HCl, pH 7.5, 50 nM primer/template DNA, 50 µg/ml bovine serum albumin, 0.5 mM dithiothreitol, 60 µM dNTPs, 7.3 mM MgCl₂, 0.1 mg/ml heparin sulfate, and 0.66 mg/ml calf thymus DNA. Following incubation, the reaction was quenched by addition of 20 mM EDTA (40 µl) in 95% formamide. The efficiency of the trap was determined by preincubating pol II with the trapping mixture before addition of labeled primer/template. Separation of ³²P-labeled primers of different lengths was carried out by gel electrophoresis, and the fraction of primer molecules extended by addition of 1 or more nucleotides was determined using a PhosphorImager (Molecular Dynamics). The amount of primer extended represents the minimum fraction of active enzyme-primer-template complex and therefore determines a lower limit for the fraction of active pol II (see "Results"). The minimum fraction of active pol II is given by the slope of the curve obtained by plotting the amount of primer extended (nm) versus the amount of enzyme (nm) in the reaction.

Measurement of Exonuclease Activity by Primer Degradation.—5' End-labeling and gel electrophoresis were carried out as described (23). To measure 3'-exonuclease activity, 20 µl total reaction volume, 50 nM primer p5 (as single-stranded DNA substrate) was incubated with pol II, T4 pol, or KF. The concentrations of the three enzymes were adjusted to an equivalent of 0.04 pol II units. Incubations were carried out for different times at 37 °C and reactions were terminated as described above. Reaction buffers for pol II and KF contained 50 mM Tris-HCl, pH 7.5, 50 µg/ml bovine serum albumin, 0.5 mM dithiothreitol, and 7.3 mM MgCl₂. The reaction buffer for T4 pol was given above. The rate of primer degradation was determined from the slope of the linear region of a plot of percent primer degraded versus time. A comparison of the exonuclease activities of wild-type pol II and pol II ex1 (D155A/E157A) was carried out by the same method, except that primer Prsp2 was used as the substrate (28-mer, at a concentration of 180 nM) and 0.4 units of wild-type pol II (or 4 units of pol II ex1) was present in the reaction mixture (40 µl total volume).

Turnover Assay.—Primer/template p5-M13 was used for measuring dNTP → dNMP turnover during DNA synthesis. The reaction conditions were the same as those used to measure 3'-exonuclease activity by primer degradation except the primer p5 was unlabeled and [^α-³²P]dCTP or [^α-³²P]dTTP (4000 Ci/mmol, 3.3 µM) and 95 µM dATP were used to allow primers to be extended by addition of 3 nucleotides.

TABLE II

Fidelity of wild-type and exonuclease-deficient forms of *E. coli* DNA polymerase II

Reactions (25 μ l) contained 20 mM Tris-HCl buffer, pH 7.5, 2 mM dithiothreitol, 10 mM MgCl₂, dATP, dTTP, dGTP, and dCTP as indicated, 30 fmol of (150 ng) gapped M13mp2 DNA and 5–14 units of DNA polymerase II. Reactions were incubated for 60 min at 37 °C and terminated by adding EDTA to 15 mM. Analysis of 15 μ l of the reaction by agarose gel electrophoresis showed that all reactions filled the gap to the extent that the DNA migrated coincident with the fully double-stranded RFII standard (e.g. see Ref. 24). The mutant frequency of uncopied DNA ranges from 5.1 to 7.0 $\times 10^{-4}$.

| Presence of Exo | Plaques scored | | Mutant frequency ($\times 10^{-4}$) |
|------------------|----------------|--------|--|
| | Total | Mutant | |
| 50 μ M dNTPs | | | |
| + | 35,344 | 54 | 15 |
| – | 7,201 | 149 | 210 |
| 1 mM dNTPs | | | |
| + | 3,836 | 30 | 78 |
| – | 2,669 | 108 | 400 |

0.4 pol II units were used to measure turnover of the correctly inserted nucleotide (dCTP), and 1.2 pol II units were used to measure turnover of the incorrectly inserted nucleotide (dTTP). The concentrations of T4 pol and KF were adjusted to have the same number of polymerase units that was used for pol II, i.e. the nucleotide insertion rates were adjusted to be the same for all three enzymes. Product dNMP was separated from unreacted dNTP and contaminating dNDP by electrophoresis on an 8% polyacrylamide gel for 1 h at 2000 V. The rate of turnover was determined from the slope of the linear region of a plot of the amount of dNMP formed versus time. Contaminating levels of radioactive dNDP in dNTP substrates were typically about 0.5–1% and were easily resolved from the product dNMP (see, e.g. Fig. 4); levels of contaminating dNMP in freshly prepared substrates were negligible compared to the amount of product formed during the reaction.

Forward Mutagenesis Assay—The assay, described in detail in Ref. 24, measures errors in the wild-type *lacZa* gene of M13 mp2. Correct polymerization during synthesis to fill a 390-base single-stranded gap produces DNA that yields dark blue M13 plaques upon transfection of an appropriate *E. coli* host strain. A wide variety of errors at many different sites are scored as lighter blue or colorless plaques. After sequence analysis of a collection of independent mutants, errors rates per detectable nucleotide incorporated were calculated as described (24). The DNA polymerase reaction conditions and description of product analysis are in the legend to Table II. *E. coli* strains, bacteriophage M13 mp2, and the sources of materials used for the M13 mp2 fidelity assay have been described (24).

RESULTS

E. coli JM109 cells carrying the *polB* gene under Ptac control on a high copy number plasmid were used to overproduce wild-type pol II and the 3'-exonuclease-deficient pol II derivative (pol II ex1). A typical preparative scale purification starting with 300 g of dry cells yielded about 300 mg of purified pol II (Table I). Approximately 75–80% of the enzyme activity was recovered with a corresponding 35–40-fold purification (Table I). The degree of purification and recovery of polymerase activity for the exonuclease-deficient enzyme was similar to wild-type pol II (data not shown). Fractions from each step of the purification were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by silver staining (Fig. 1). Each enzyme preparation appeared to be at least 95% chromatographically pure. Crystals suitable for high resolution x-ray diffraction have been obtained from the fraction IV material for the wild-type protein (25). The specific activity of wild-type pol II was routinely 3-fold higher than for the pol II ex1 enzyme suggesting that the Asp¹⁵⁵ to Ala and Glu¹⁵⁷ to Ala mutations in the exonuclease domain, that reduce levels of exonuclease to 1/1000th the level of wild-type, appeared to cause a small reduction in the polymerization rate of the enzyme.

A titration using increasing levels of pol II at a constant primer/template DNA concentration was carried out to deter-

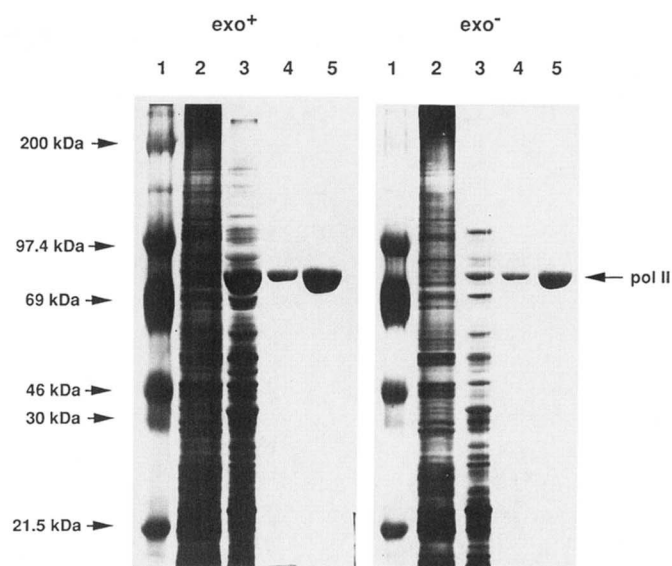


FIG. 1. Silver-stained polyacrylamide gel showing protein bands during purification of *E. coli* wild-type and exonuclease-deficient (exo) pol II. Lane 1, prestained molecular weight markers; lane 2, F1 crude lysate (17 μ g total protein); lane 3, F2 ammonium sulfate fraction (20 μ g total protein); lane 4, phosphocellulose column fraction (5 μ g total protein); lane 5, DEAE-cellulose column fraction (5 μ g total protein). The purification procedure is described under "Experimental Procedures." The specific activity and recovery at each stage of purification for the wild-type pol II is given in Table I.

mine the fraction of pol II (fraction IV) that was active (Fig. 2A). Pol II was allowed to bind to primer/template DNA and extend a ³²P-labeled primer molecule, at most once, by carrying out the reaction in the presence of excess unlabeled DNA and heparin to trap free polymerase molecules ("Experimental Procedures"). The primer extension reaction in the absence of trap is shown in lane 1, where pol II was able to engage and elongate primer/template DNA multiple times. The effectiveness of the trap is seen in lane 8, where the addition of trap prior to initiation of the polymerization reaction, by addition of Mg²⁺, was successful in eliminating detectable elongation of ³²P-labeled primer strands. Increased levels of pol II resulted in increased primer utilization (lanes 3–7), and the fraction of primer/template DNA extended is plotted as a function of pol II concentration in Fig. 2B. From the slope of the line we conclude that at least 50% of the pol II molecules were active. The spots corresponding to unelongated primers (P) are overexposed (Fig. 2A), but we have verified by integrating all of the bands on the gel that the disappearance of intensity in the P band is converted quantitatively into primer extension bands. Since not all polymerase binding events are productive, i.e. a bound pol II-DNA complex might dissociate prior to elongation followed by trapping of the dissociated enzyme, the observed molar ratio of extended DNA/pol II (~0.5) represents a lower limit for the fraction of active pol II (Fig. 2B). The same criteria was used to verify that at least half of the purified exonuclease-deficient pol II ex1 (D155A/E157A) molecules were active (data not shown).

A Comparison of Exonuclease Activities of Pol II, T4 Pol, and Pol I(KF)—It was of interest to compare the polymerase and 3'-exonuclease proofreading activities of pol II and bacteriophage T4 pol because both enzymes are class B polymerases (human α -like polymerases) sharing five highly conserved sequence motifs (4). Wild-type T4 pol has an exceptionally high 3'-exonuclease activity and is able to excise 10–20% of correctly paired nucleotides (26–28), depending on the identity of the base pair and sequence context (29). Equal polymerase units of pol II, T4 pol, and KF were incubated in the presence of a 5'-³²P-labeled single-stranded 17-mer without dNTPs (Fig. 3).

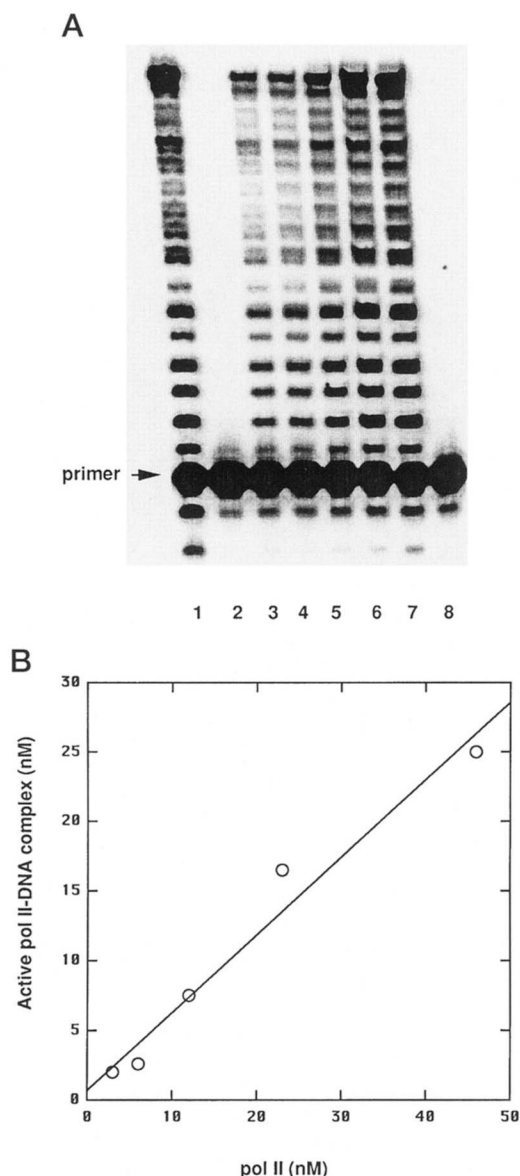


FIG. 2. **Active site titration of pol II.** *A*, lane 1, primer extension was carried out using 2.5 nM pol II in the absence of DNA-heparin trap (see "Experimental Procedures"); lane 2, 46 nM pol II incubated with labeled primer/template DNA and all four dNTP substrates; lane 3, primer extension was carried out using 3 nM pol II in the presence of a DNA-heparin trap; lanes 4–7, same as lane 3 using pol II at 6, 12, 24, 46 nM, respectively; lane 8, 46 nM pol II was preincubated with DNA-heparin trap and all four dNTP substrates and the reaction was then initiated by addition of Mg^{2+} . The primer/template DNA was present at 50 nM. Lane 2 serves as a control showing that trace amounts of Mg^{2+} present in the primer/template DNA solution are insufficient to cause measurable primer elongation. Lane 8 is a control demonstrating the effectiveness of the DNA-heparin trap; preincubation with trap eliminates elongation of 5'- ^{32}P -labeled primer molecules. *B*, plot showing the amount of active pol II-DNA complex (given by the amount of primer extended) versus the amount of pol II present in the reaction. The minimum fraction of active purified pol II, given by the slope of the line, is equal to 50%.

Degradation of the 17-mer occurred more rapidly for pol II than T4 pol, with full-length input primer disappearing within 1 min of incubation at 37 °C. In the case of KF, the primer remained essentially intact for incubations of up to 10 min. Degradation rates were determined to be 6.9×10^{-13} mol/min, 4.7×10^{-13} mol/min, and 1.5×10^{-15} mol/min for pol II, T4 pol, and KF, respectively.

A measurement of 3'-exonuclease activity under synthesiz-

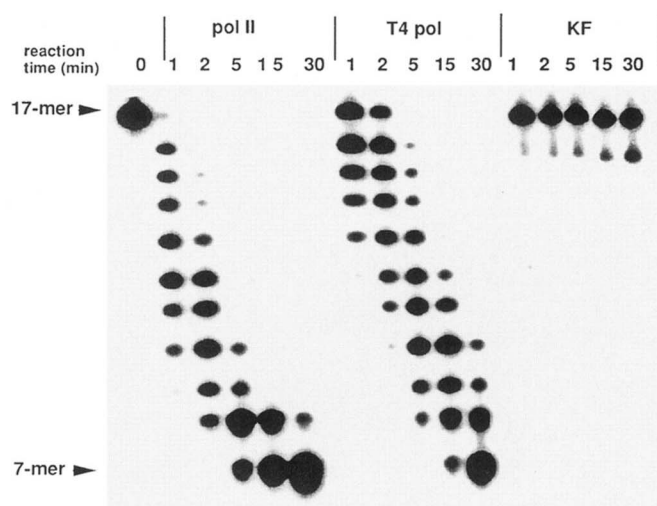


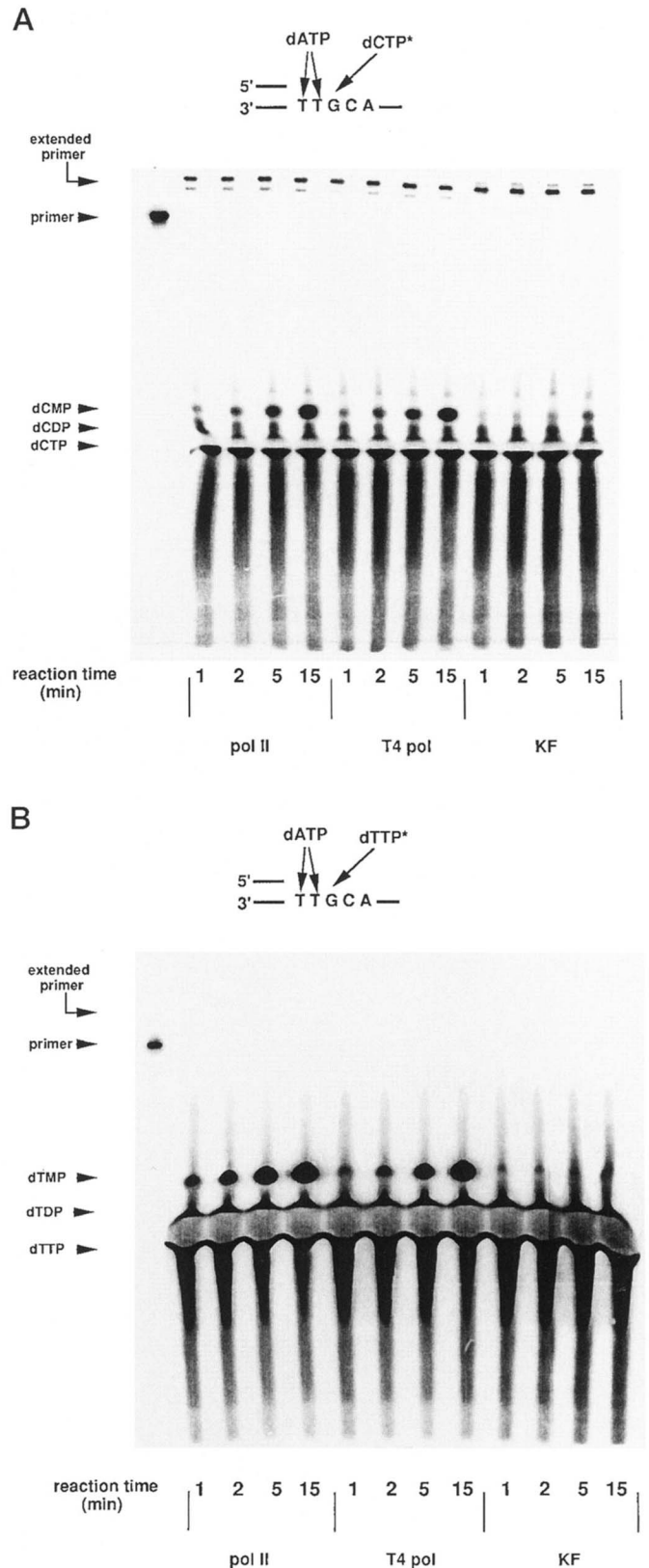
FIG. 3. **Comparison of pol II, T4 pol, and KF 3'-exonuclease activities on single-stranded DNA.** A 5'- ^{32}P -labeled single-stranded DNA oligonucleotide (17-mer) was incubated with pol II, T4 pol, and KF for the reaction times shown. The concentrations of the three enzymes were adjusted to have the same number of polymerase units, equivalent to 0.04 pol II units. The first lane on the left-hand side indicates the position of undegraded 5'- ^{32}P -labeled single-stranded 17-mer.

ing conditions (dNTP \rightarrow dNMP turnover), was carried out for insertion of a correct (dCTP, Fig. 4A) and an incorrect (dTTP, Fig. 4B) deoxynucleotide opposite G by pol II, T4 pol, and KF. An increase in dNTP \rightarrow dNMP turnover as a function of incubation time was observed for each enzyme. Using equal polymerase units, turnover of both correct and incorrect inserted dNMP's were slightly larger for pol II than T4 pol, with both polymerases exhibiting far greater proofreading activity than KF. The radioactive spot migrating in between the dense spot of unreacted α - ^{32}P -labeled dNTP substrate and product dNMP is dNDP, which likely resulted from the spontaneous hydrolysis of dNTP. The level of dNDP is about 0.5% of the input dNTP and remained constant during the course of the reaction. A small amount of stable correct incorporation of dCMP was observed as an extended primer band (Fig. 4A), but there was no detectable band corresponding to the stable misincorporation of dTMP (Fig. 4B). Turnover rates of the correct nucleotide (dCTP) were about 6.7×10^{-13} mol/min, 5.6×10^{-13} mol/min, and 1.1×10^{-14} mol/min for pol II, T4 pol, and KF, respectively (Fig. 4A). For the incorrect nucleotide (dTTP), turnover rates were 5.6×10^{-13} mol/min, 2.3×10^{-13} , and 1.5×10^{-14} for pol II, T4 pol, and KF, respectively (Fig. 4B). The rates of turnover of incorrect nucleotide per polymerase unit were less than correct nucleotide because dNTP \rightarrow dNMP turnover measures insertion followed by excision, and insertion of incorrect nucleotides occur much less frequently than correct nucleotides. For these experiments, a 3-fold higher concentration of each of the polymerases was used for measurements of incorrect compared to correct turnover.

Exonucleolytic Degradation of Single-stranded DNA—We compared the ability of wild-type pol II and the pol II ex1 mutant (D155A/E157A) to degrade single-stranded DNA (Fig. 5). During incubations with equivalent polymerase units, degradation of a 28-mer was detected within a 5-s incubation with the wild-type enzyme, but was just barely detectable after 80 s with the exonuclease-deficient mutant (Fig. 5). The residual level of 3'-exonuclease activity in the mutant enzyme was calculated to be approximately 0.1% of the wild-type activity, based on the fraction of 28-mer remaining intact.

Fidelity of Wild-type and Exonuclease-deficient Forms of Pol II—The fidelity of both pol II forms was determined during

FIG. 4. Comparison of pol II, T4 pol, and KF dNTP \rightarrow dNMP turnover activities. A, incorporation and excision of a correct deoxynucleotide [α - 32 P]dCTP opposite template G on the unlabeled primer/template DNA shown at the top of the figure. Incorporation of labeled dCTP is indicated by the location of the extended primer band corresponding, primarily, to the addition of three nucleotides. Turnover of the dCTP substrate is indicated by the dCMP radioactive spot. The heavy radioactive spot migrating at the bottom of the gel corresponds to unreacted dCTP, and the spot migrating between the unreacted dCTP and dCMP product is dCDP contaminant present in the dCTP substrate. B, excision of an incorrectly inserted deoxynucleotide [α - 32 P]dTTP opposite template G on the unlabeled primer/template shown at the top of the figure. No stable incorporation of dTTP is observed as shown by the absence of a detectable radioactive band at the location denoted by "extended primer." Turnover of the dTTP substrate is indicated by the dTMP radioactive spot. The radioactive spot migrating at the bottom of the gel is unreacted dTTP, and the spot located between dTTP and dTMP product is dTDP contaminant present in dTTP. Reactions using pol II, T4 pol, and KF were carried out for the indicated times. The position of the unextended primer (5'- 32 P-labeled 17-mer) is shown in the left-hand lane of each gel. The concentrations of the three enzymes used for turnover of the correct nucleotide (dCMP) were adjusted to have the same number of polymerase units, equivalent to 0.4 pol II units. Three-fold higher concentrations of each polymerase (1.2 pol II units) were used to measure turnover of the incorrect nucleotide (dTTP).



synthesis to fill a 390-nucleotide single-stranded gap containing the *lacZ α* complementation reporter gene sequence. Gap-filling synthesis by the wild-type polymerase in a reaction containing 50 μ M dNTPs generated products that had a *lacZ* mutant frequency that was elevated about 2-fold compared to an uncopied control DNA substrate (Table II). When a wild-type pol II reaction was performed using 1 mM dNTPs, the

mutant frequency increased to a value more than 10-fold higher than the uncopied control value. This increase is consistent with stimulation of polymerization at the expense of exonucleolytic proofreading (28, 30). The 2-fold higher mutant frequency observed at 1 mM dNTPs is consistent with a dNTP-dependent increase in extension of mispaired and misaligned intermediates (31). In comparison, pol II ex1 generated sub-

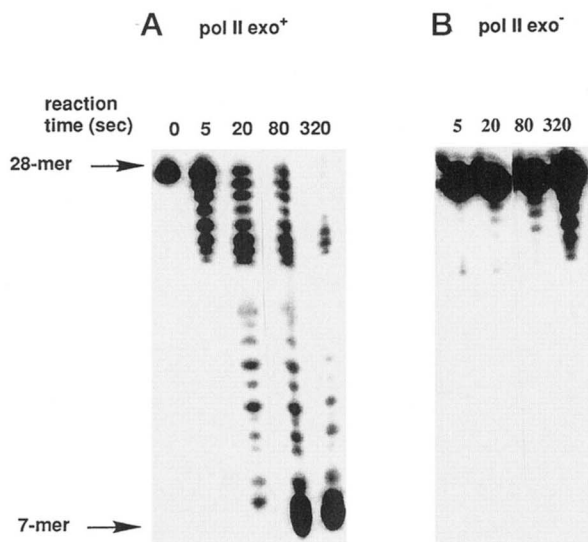


FIG. 5. Comparison of 3'-exonuclease activities of wild-type pol II and an exonuclease-deficient (*exo*⁻) pol II mutant (D155A/E157A). A, wild-type pol II (0.4 polymerase units) and B, pol II *exo*⁻ mutant (4 polymerase units) were used to hydrolyze a single-stranded 5'-³²P-labeled single-stranded DNA oligomer as described under "Experimental Procedures." The DNA oligomer (28-mer) was present at a concentration of 180 nM.

stantially higher *lacZ* mutant frequencies than did the wild-type enzyme at both dNTP concentrations (Table II).

In order to determine the nature of the errors generated by pol II *exl* and to determine quantitative error rates and the contribution of the exonuclease to fidelity, collections of independent *lacZ* mutants from reactions containing 50 μ M dNTPs were analyzed by DNA sequence analysis. The data are summarized in Table III, and the distribution of mutants containing single base substitutions and frameshifts are shown in Fig. 6. Of 54 mutants from reactions catalyzed by wild-type pol II, 33 contained a single C→T transition mutation. These mutants, which account for most of the increase in mutant frequency above the control value (Table II) are likely the result of cytosine deamination, with wild-type pol II "correctly" inserting dAMP opposite template U residues. Most of the other classes of mutants from the wild-type pol II reaction occurred at background frequencies, suggesting that they were not the result of synthesis errors *in vitro*. The frequency data were used to calculate (see legend to Table III) "less than or equal to" error rates per detectable nucleotide polymerized, for the single base errors and the two nucleotide deletions.

The only mutants observed in the wild-type pol II collection having a frequency above that of the uncopied control values were the eight deletions of more than two nucleotides (Fig. 7, top). None of these mutants has been observed previously in sequence analysis of over 200 mutants from uncopied controls, suggesting that they are errors generated by wild-type pol II. These mutants had lost between 49 and 268 nucleotides and in each instance the deletion end points were flanked by short repeated sequences. One particular mutation, a 182-nucleotide deletion between a perfect 7-base pair direct repeat, was recovered four times in this small collection. Only one of the eight mutants was flanked by perfect repeat shorter than 5 base pairs, and even here the repeat homology is higher if formation of G-T pairs in the repeat is permitted (see legend to Fig. 7 and "Discussion").

The collection of mutants from the pol II *exl* reaction was much more diverse, containing a variety of single base substitutions and frameshifts and deletions of two or more nucleotides (Table III, Figs. 6 and 7). Rates calculated for the single-

base substitution and frameshift errors and two-base deletions are higher by values ranging from ≥ 13 -fold to ≥ 240 -fold for *exo*-pol II than for wild-type pol II (Table III), consistent with a role for the exonuclease in proofreading each of these classes of errors. Among deletions involving the loss of more than two bases, 10 had lost more than 20 nucleotides, and each of these had substantial but often imperfect homology at the deletion end points (Fig. 7, bottom). The others (not shown) had lost fewer than 10 nucleotides and most had limited homology at the end points.

DISCUSSION

Although DNA polymerase II was discovered in 1970 (3) its precise role in DNA replication or repair has been difficult to establish. Pol II contains both 5'-polymerase and 3'-exonuclease activities (1) on a single 89.9-kDa polypeptide chain (4, 5, 32). It has been shown that pol II can bypass abasic lesions *in vitro* (20) and is regulated by the LexA repressor (4, 5, 20) as part of the SOS regulon of *E. coli* (33). Recent experiments by Tessman and Kennedy (12) suggest that pol II may be required for bypassing abasic lesions *in vivo* under conditions where heat shock proteins are not induced. We have presented evidence for the involvement of pol II in the repair of or tolerance to oxidative damage and in influencing the rate of adaptive mutation (10).² The purification and properties of wild-type pol II and an exonuclease-deficient pol II mutant (pol II *exl*) provide additional insights into the role of this polymerase in cells.

A purification scheme has been presented, which makes use of an overexpression system for wild-type pol II and exonuclease-deficient enzyme, that can be carried out rapidly, yielding large quantities of highly purified polymerase that has been used to prepare single crystals suitable for x-ray diffraction analysis (25). Based on an active site titration of our preparations (Fig. 2), we have determined that at least 50% of the purified wild-type and exonuclease-deficient pol II molecules were active in extending primer/template DNA in the presence of a DNA-heparin trap. This 50% value is a minimum estimate of the fraction of active enzymes molecules since the assay does not detect those enzymes that dissociated from the primer/template DNA before incorporating a deoxynucleotide.

The specific activity of our most highly purified pol II fraction (Fraction IV) was calculated to be 1.8×10^4 units/mg. Recently, another laboratory has reported a specific activity for purified DNA polymerase II which is nearly 11,000 times larger (13). This reported value is undoubtedly in error since their steady state rate exceeds the fastest measured presteady state synthesis rate by 4–5-fold (34). Our specific activity measurements are consistent with values for other DNA polymerases. As we have previously reported, DNA polymerase II can be made more processive by the addition of DNA polymerase III accessory factors which increase the specific activity of pol II by preventing dissociation from the DNA template (8).

Bacteriophage T4 polymerase contains a highly active 3'-exonuclease proofreading activity (26). Pol II and T4 pol have been classified as group B " α -like" polymerases because of their similarity in sequence to five conserved regions of eucaryotic pol α (4, 35). It is of considerable interest to compare the relative levels of nuclease/polymerase activities for pol II and T4 pol, not only because of their sequence similarities, but also because of their effects on mutagenesis. Mutations in T4 pol that decreased nuclease-to-polymerase ratios *in vitro* were shown to confer mutator phenotypes for base substitution mutations *in vivo* and conversely mutants that increased nuclease/polymerase ratios conferred antimutator phenotypes (17, 26, 36, 37).

Pol II appeared to degrade single-stranded DNA about 1.5-fold more rapidly than T4 pol and 400–500-fold more rapidly

TABLE III
Average error rates by class

| Type of mutation | Wild-type | | | Exonuclease-deficient | | | Exo ⁻ Exo ⁺ |
|--------------------------|-----------|------------------------------|------------------------------------|-----------------------|------------------------------|------------------------------------|--------------------------------------|
| | Mutants | M.F. ($\times 10^{-4}$) | Error rate ($\times 10^{-6}$) | Mutants | M.F. ($\times 10^{-4}$) | Error rate ($\times 10^{-6}$) | |
| Base substitutions | | | | | | | |
| C \rightarrow T | 33 | 9.2 | $\leq 61^a$ | 5 | 11 | 73 | 1 |
| Others | 5 | 1.4 | $\leq 2.0^a$ | 16 | 34 | 48 | ≥ 24 |
| One-base addition | 0 | ≤ 0.3 | $\leq 0.3^a$ | 2 | 4 | 4 | ≥ 13 |
| Deletions | | | | | | | |
| One-base in runs | 1 | 0.3 | $\leq 1.1^a$ | 7 | 15 | 57 | ≥ 50 |
| One-base, non-reiterated | 1 | 0.3 | $\leq 0.3^a$ | 33 | 71 | 76 | ≥ 240 |
| Two-base | 0 | ≤ 0.3 | $\leq 0.3^a$ | 10 | 21 | 18 | ≥ 70 |
| Two-base | 8 | 2.2 | | 18 | 39 | | |
| Other ^b | 0 | ≤ 0.3 | | 6 | 13 | | |
| Spontaneous ^c | 6 | 1.7 | | 1 | 2 | | |

^a Error rates per detectable nucleotide incorporated were calculated as described (Ref. 24). There are 25, 118, 44, 155, 199, and 199 detectable sites (in the order listed), respectively, for each error for which a rate is calculated. Since the mutant frequencies with wild-type pol II are not greater than those obtained with uncopied DNA (e.g., see Ref. 44), the error rates are given as " \leq " values.

^b Includes mutants having a variety of sequence changes.

^c These are interpreted to be of spontaneous origin, as their frequency is similar to that of uncopied DNA and the mutants contain either deletions known to result from recombination (11), deletions whose end points are outside the gap being filled, or mutants for which no change was found in the lacZ α mutational target within the single-stranded gap.

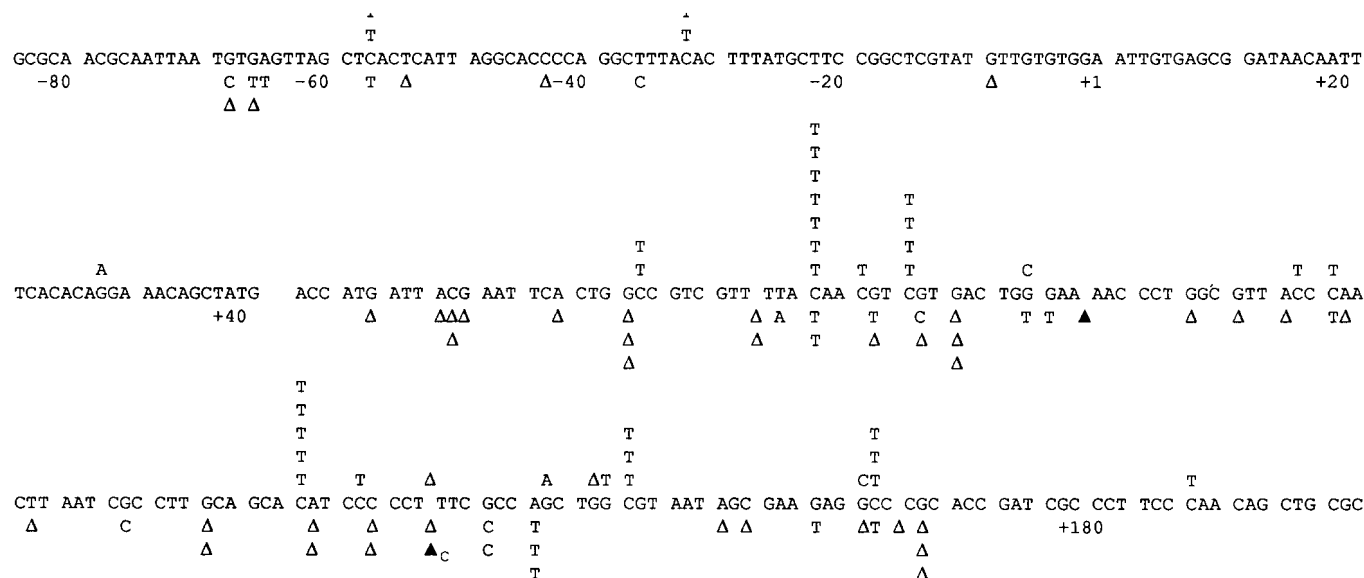


FIG. 6. Spectra of single-base errors by *E. coli* DNA polymerase II. Three lines of primary DNA sequence for the lacZ α -complementation gene in M13 mp2 are shown. The sequence is that of the viral (+) template strand. Position +1 is the first transcribed base. The spectrum above the lines of sequence is of mutants generated by wild-type enzyme, while that below the lines of sequence is of mutants generated by the exonuclease-deficient polymerase (pol II ex1). In both cases the reactions contained 50 μ M of each dNTP. Substitutions are indicated by a single letter and are the new base found in the viral DNA. For frameshifts, the loss of a base is indicated by a Δ , while the addition of a base is indicated by an \blacktriangle . When frameshifts occur at iterated nucleotides, it is not possible to distinguish which base was lost or added.

than KF, when equivalent polymerase units were used for each enzyme (Fig. 3). The relative 3'-exonuclease activities for the three polymerases were also determined under DNA synthesizing conditions by measuring the turnover of [α - 32 P]dNTP to [α - 32 P]dNMP (Fig. 4). The rates of turnover of correctly inserted dCMP opposite G were roughly similar for pol II and T4 pol, and about 60-fold greater than observed for KF (Fig. 4A). Turnover rates of misinserted dTMP opposite G were similar for pol II and T4 pol and approximately 20-fold greater than for KF (Fig. 4B). Thus pol II and T4 pol have essentially the same ratio of nuclease/polymerase activities even though the specific activity of T4 pol is approximately 100-fold greater than pol II, which undoubtedly reflects its role as the replicative polymerase.

A pol II exonuclease-deficient (pol II ex1) derivative was constructed by introducing two amino acid changes (D155A/E157A) analogous to those made for a proofreading-deficient mutant of bacteriophage T4 (D112A/E114A). The exonuclease-

deficient mutant pol II exhibited about a 1000-fold reduction in exonuclease activity compared to wild-type pol II (Fig. 5), and a 3–4-fold reduction in polymerase activity was also observed. The residual exonuclease activity that represents 0.1% of the normal level may be due to contaminant wild-type DNA polymerase II encoded by the chromosome.

Wild-type pol II is highly accurate, having base substitution and frameshift error rates of $\leq 10^{-6}$ (Table III). In contrast, the exonuclease-deficient polymerase generated a variety of different errors during DNA synthesis *in vitro*. Assuming that the two amino acid substitutions in the conserved exonuclease motif that are responsible for diminished exonuclease activity do not affect the selectivity of the polymerase, the differences in error rates between the two polymerases under identical reaction conditions represent the contribution of proofreading to fidelity for each type of error. The data suggest that single base substitutions, single base additions, and a variety of deletion errors are proofread by the highly active intrinsic 3'-exonucle-

FIG. 7. Deletion mutations recovered from reactions with wild-type and exonuclease-deficient pol II.

Shown are lacZ mutants recovered from pol II reactions wherein 22 or more nucleotides have been deleted. Nucleotide +1 is the first transcribed nucleotide of the gene. The deletion end points are indicated by slashes, with the number of nucleotides deleted as listed and the homology at the deletion end points indicated in the last column. For example, the first deletion listed for wild-type pol II had lost 268 nucleotides, from nucleotide -126 through nucleotide 142, and a perfect 8-base pair direct repeat sequence was present at the deletion end points. Complementarity at the end points is indicated by capital letters, while non-complementary nucleotides are indicated by lower case letters. Underlined lower case letters indicate positions where a G-T or T-G mispair would be located in a putative misaligned intermediate formed during synthesis proceeding from right to left using the template strand shown. Numbers in parentheses under "Homology" indicate homologies obtained by including the involvement of these two types of mispairs. Numbers under "Bases Lost" indicate the number of times the particular mutation was recovered.

| Wild-Type Pol II | | Bases Lost | Homology |
|----------------------|-------------------|------------|------------|
| -126 | 142 | 268 | |
| taatg/CAGCTGGCac | ttcgc/CAGCTGGCgt | | 8/8 |
| 97 | 145 | 49 | |
| aaacc/CTGGCGTtAc | gccag/CTGGCGTaAt | | 7/7 |
| -123 | 59 | 182 (x4) | |
| tgcag/CTGGCacgac | attca/CTGGCcgctcg | | 5/5 |
| -103 | 58 | 161 | |
| tcccg/ACTGGaaagC | aattc/ACTGGccgtC | | 5/5 |
| -111 | 101 | 212 | |
| gacag/GTTtCCCgAC | ctggc/GTTaCCCaAC | | 6/7 (9/10) |
| Exo-Deficient Pol II | | | |
| -180 | 145 | 325 | |
| ccacc/CTGGCGccca | gccag/CTGGCGtaat | | 6/6 |
| -152 | 165 | 317 (x2) | |
| ctctc/CCCGCgCgtt | agagg/CCCGCaCcga | | 5/5 (7/7) |
| -115 | 25 | 140 | |
| gcacg/ACAGGtttCc | ttcac/ACAGGaaaCa | | 5/5 |
| 58 | 84 | 27 | |
| aattc/ACTGGccgtC | tcgtg/ACTGGgaaaa | | 5/5 |
| -174 | 183 | 357 | |
| tggcg/CCCAAtAcgc | ccctt/CCCAAGct | | 6/7 (7/7) |
| 61 | 82 | 22 | |
| ttcac/TGgCcGtctg | cgctg/TGaCtGggaa | | 4/6 (5/6) |
| -182 | 166 | 348 | |
| aacca/CCcCtggcgc | gaggc/CCgCaccgat | | 3/4 |
| 56 | 102 | 47 | |
| acgaa/TTcaCtggCc | tggcg/TTacCaaCt | | 3/5 (7/9) |
| -14 | 33 | 47 | |
| cggct/CgtaTgTtgt | ggaaa/CagcTaTgac | | 3/7 (5/7) |

ase activity of pol II. The substitutions likely result from direct misinsertion followed by mispair extension by the polymerase, while models involving misaligned template/primers initiated by strand slippage (38) or base misinsertion (39) have been presented that can account for the one- and two-base frameshift errors observed at repetitive and non-reiterated sequences (Table III).

An unexpected and interesting result from the present study was the frequent generation of errors involving loss of a large number of nucleotides flanked by directly repeated sequences (Fig. 7). The simplest model to account for these involves pol II synthesis through the first repeat, disruption of repetitive terminal base pairs, formation of base pairs involving the newly-made DNA and the downstream direct repeat, and continued synthesis. Deletions consistent with this model have been found in previous studies of the fidelity of several different exonuclease-deficient DNA polymerases (reviewed in Refs. 40 and 41). However, here they are even observed above background mutant frequencies in the lacZ mutant collection from the exonuclease-proficient pol II reaction (Fig. 7, top). In 7 of 8 such mutants, perfect direct repeat homology involving 5, 7, or 8 base pairs was present at the deletion end points, with the eighth example being homologous for 9 of 10 base pairs (if one G-T pair is allowed). This suggests that, if the degree of homology is sufficient, deletions between direct repeats can be gen-

erated by wild-type pol II despite the presence of a highly active exonuclease that successfully proofreads point mutations.

The frequency of deletions between direct repeats is substantially higher in the exonuclease-deficient pol II mutant collection (Table III) and, among those observed, several may involve imperfect direct repeats (Fig. 7, bottom). Intermediates involving imperfect repeats would contain mismatches in the double-stranded template/primer region that could slow polymerization and lead to proofreading by the wild-type enzyme. The frequency and specificity data thus suggest that some direct repeat-dependent polymerase errors are in fact proofread. It is even possible that the absence of exonuclease activity may promote such deletion errors, by a mechanism wherein unedited nucleotide misinsertion generates a terminus that frays and realigns with a downstream sequence that is more homologous by virtue of the original misinsertion. This idea is supported by numerous observations suggesting that one-base deletions are initiated by misinsertion followed by realignment with a downstream sequence (for review, see Ref. 40).

Our results demonstrate that the 3'-proofreading exonuclease of DNA polymerase II plays an important role in rectifying replication errors that can lead to frameshifts and deletions *in vitro*. Recently we have obtained evidence that strains deficient in the exonuclease function of pol II show a mutator phenotype in nondividing cells.² These biochemical and genetic results

suggest a fundamental role for DNA polymerase II in maintaining the sequence integrity of DNA.

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