Kinetic Analysis of Nucleotide Incorporation by Mammalian DNA Polymerase δ*

Heidi J. Einolf‡ and F. Peter Guengerich§

From the Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

Received for publication, February 15, 2000
Published, JBC Papers in Press, March 16, 2000, DOI 10.1074/jbc.M001291200

The kinetics of nucleotide incorporation into 24/36-mer primer/template DNA by purified fetal calf thymus DNA polymerase (pol) δ was examined using steady-state and pre-steady-state kinetics. The role of the pol δ accessory protein, proliferating cell nuclear antigen (PCNA), on DNA replication by pol δ was also examined by kinetic analysis. The steady-state parameter $k_{\text{cat}}$ was similar for pol δ in the presence and absence of PCNA (0.36 and 0.30 min$^{-1}$, respectively); however, the $K_m$ for dNTP was 20-fold higher in the absence of PCNA (0.067 versus 1.2 μM), decreasing the efficiency of nucleotide insertion. Pre-steady-state bursts of nucleotide incorporation were observed for pol δ in the presence and absence of PCNA (rates of polymerization ($k_{\text{pol}}$) of 1260 and 400 min$^{-1}$, respectively). The reduction in polymerization rate in the absence of PCNA was also accompanied by a 2-fold decrease in burst amplitude. The steady-state exonuclease rate of pol δ was 0.56 min$^{-1}$ (no burst, 10$^{-3}$-fold lower than the rate of polymerization). The small phosphorothioate effect of 2 for correct nucleotide incorporation into DNA by pol δ-PCNA indicated that the rate-limiting step in the polymerization cycle occurs prior to phosphodiester bond formation. A $K_{D\text{DTP}}$ value of 0.93 μM for polδ-dNTP binding was determined by pre-steady-state kinetics. A 5-fold increase in $K_{D\text{NA}}$ for the pol δ-DNA complex was measured in the absence of PCNA. We conclude that the major replicative mammalian polymerase, pol δ, exhibits kinetic behavior generally similar to that observed for several prokaryotic model polymerases, particularly a rate-limiting step following product formation in the steady state (dissociation of oligonucleotides) and a rate-limiting step (probably conformational change) preceding phosphodiester bond formation. PCNA appears to affect pol δ replication in this model mainly by decreasing the dissociation of the polymerase from the DNA.

—

Polimerase (pol) 1 δ is the major polymerase involved in chromosomal replication in eukaryotic cells (1, 2). pol δ is essential for leading and lagging strand DNA synthesis and has also been shown to be involved in DNA repair processes such as long patch base excision repair, nucleotide excision repair, and mismatch repair (3–6). The other eukaryotic polymerases involved in eukaryotic genomic DNA replication are pol α and pol ε (1). pol α is a moderately processive polymerase that is involved in synthesis of RNA-DNA and DNA-DNA primers on the leading and lagging strand (7). pol ε is required for DNA replication in vivo; however, it is not required for in vitro SV40 DNA replication, supporting the other proposed roles of pol ε in lagging strand synthesis, DNA repair, and cell cycle check point control (6, 8, 9). pol δ, therefore, has been identified as the enzyme involved in processive replication of the majority of the eukaryotic genome. Understanding the basic replication reaction by mammalian pol δ should aid in the elucidation of the mechanisms underlying the effects of other proteins or DNA structure on genomic DNA replication and repair.

Eukaryotic pol δ is a heterodimer consisting of 125- and 50-kDa protein components (10). The larger subunit contains the catalytic domain as well as the 3’ → 5’ exonuclease site (11, 12). The smaller 50-kDa subunit is essential for the binding to and stimulation of pol δ by its accessory protein, PCNA (13). In the absence of PCNA, DNA pol δ is a relatively non-processive polymerase. The activity and processivity of pol δ is enhanced by PCNA up to 100-fold, and this characteristic has been used to distinguish pol δ from other mammalian DNA polymerases (14, 15). Mammalian PCNA is a trimeric protein that encircles the DNA and acts as a sliding clamp for pol δ (16). With chromosomal or circular DNA, PCNA requires a clamp loading protein, RF-C, and hydrolysis of ATP to encircle the DNA, after which pol δ can associate with PCNA and DNA (6). RF-C dissociates from the pol δ-PCNA complex after PCNA encircles the DNA and is not thought to influence DNA synthesis by pol δ (8). RF-C is not required for PCNA loading onto shorter primer/templates.

The role of PCNA in pol δ replication is likely to be stabilization of the pol δ-DNA interaction to maintain the processivity of the polymerase during genomic replication. PCNA has been reported to stabilize the pol δ-template-primer binary complex under static conditions by 3 orders of magnitude (17). PCNA also has been reported to affect the steady-state kinetics of nucleotide incorporation into poly(dA)·oligo(dT) primer/templates by pol δ by decreasing the $K_m$ (6-fold) and by increasing the $k_{\text{cat}}$ for dTTP incorporation by 2-fold (7). Steady-state re-
actions involving DNA polymerases and oligonucleotides are rather uninformative because $k_{cat}$ is dominated by the rate of enzyme release from DNA, the slowest step in the polymerization reaction (Fig. 1, step 7). Although the meaning of the steady-state $K_m$ (for dNTP) is unclear, the ratio $k_{cat}/K_m$ can be used to evaluate the efficiency of nucleotide incorporation. Many DNA polymerases complete a single turnover reaction within 100 ms; therefore, pre-steady-state kinetic approaches are required to examine individual rate constants within the reaction cycle (Fig. 1). Pre-steady-state kinetics can provide a quantitative measure of the actual rate of polymerization phosphodiester bond formation and preceding steps (Fig. 1, steps 2–4) as well as dNTP and DNA binding constants. Such studies can be used to directly determine the kinetics of nucleotide insertion and effects of PCNA on replication by pol δ.

In this study we successfully isolated and purified pol δ from fetal calf thymus in sufficient quantities for a detailed examination of steady-state and pre-steady-state kinetics of nucleotide incorporation into primer/template DNA. The use of pre-steady-state rapid quench kinetic analysis enabled examination of the role of PCNA on the kinetics of nucleotide incorporation by pol δ.

**EXPERIMENTAL PROCEDURES**

**Materials**—Primer and template oligonucleotides, poly(dA), and oligo(dT)$_{12-15}$ were purchased from Midland Certified Reagent Co. (Midland, TX). The primer and template oligonucleotides were purified as described previously (19). First trimester fetal calf thymus was obtained from Pel-Freez (Rogers, AR), and unlabeled Ultra Pure Grade dNTPs were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). The rho-substituted dNTPs, ([γ-32P]dATP and [3H]dUTP) were purchased from United States Biochemical Corp., and [γ-32P]dATP and [3H]dUTP were obtained from NEN Life Science Products. The bacterial expression vector pT7/PCNA (expressing human PCNA) was a generous gift of Dr. B. Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) obtained from Prof. E. Fanning (Vanderbilt University, Nashville, TN).

**Preparation of Hydroxypatinate**—Hydroxypatinate was synthesized by dropwise addition of 0.5 M CaCl$_2$ (1.5 liters) into 0.5 M sodium phosphate, pH 6.6 (1.75 liters) with stirring. The mixture was stirred for an additional 1 h after all the reactants were combined, the precipitate was allowed to settle, and the liquid was decanted. H$_2$O (5 liters, containing 20 μM phenolphthalein) was added to the precipitant, and NH$_4$OH was added to keep the mixture basic. The hydroxypatinate suspension was boiled for 30 min and the liquid was decanted. The hydroxypatinate was washed six times with 3 liters of 5 mM sodium phosphate (pH 6.6), washed once with 3 liters of H$_2$O, and resuspended in 0.2 M potassium phosphate (pH 7.0) with 0.05% NaN$_3$ for storage at 4 °C.

**Expression and Purification of Human PCNA**—Human PCNA was expressed and purified as described (20) with modifications. *Escherichia coli* BL21(DE3)pLysS cells were transformed with pT7/PCNA and grown in LB medium containing 50 μg of ampicillin ml$^{-1}$ and 20 μg of chloramphenicol ml$^{-1}$ to $A_{600} = 0.5$. A 1-ml culture of the bacterial cells was induced with 0.4 μM isopropyl-β-D-thiogalactoside for 3 h and harvested by centrifugation (3000 × g, 15 min). The cells were resuspended in Buffer A containing 25 mM NaCl (Buffer A: 25 mM Tris-HCl, pH 7.5, containing 1.0 mM DTT, 1.0 mM EDTA, 10 mM NaH$_2$PO$_4$, 1.0 mM PMSF, 1.0 μg of aprotinin ml$^{-1}$, and 1.0 μg of leupeptin ml$^{-1}$) and then added to 0.10 M NaCl in Buffer B (Buffer B: 25 mM Tris-HCl, pH 7.5, containing 1.0 mM DTT, 1.0 mM EDTA, and 10 mM NaH$_2$PO$_4$). The presence of PCNA in the fractions was determined by 15% SDS-polyacrylamide gel electrophoresis (21, 22) and silver staining (23). The PCNA-containing fractions were pooled, adjusted to 1.5 M NaCl, and loaded onto a phenyl-Sepharose column (1.5 × 11 cm) equilibrated with Buffer A plus 0.10 M NaCl. The column was washed with the equilibration buffer and then eluted at 0.4 ml min$^{-1}$ with a 200-ml linear gradient of 0.10–0.50 M NaCl in Buffer B (Buffer B: 25 mM Tris-HCl, pH 7.5, containing 1.0 mM DTT, 1.0 mM EDTA, and 10 mM NaH$_2$PO$_4$). The presence of PCNA in the fractions was determined by 15% SDS-polyacrylamide gel electrophoresis (21, 22) and silver staining (23). The PCNA-containing fractions were pooled, adjusted to 1.5 M NaCl, and loaded onto a phenyl-Sepharose column (1.5 × 11 cm) equilibrated with Buffer B containing 1.5 M NaCl. The column was washed with the equilibration buffer and then eluted at 0.5 ml min$^{-1}$ with a 200-ml linear gradient decreasing from 1.5 M to 0.10 M NaCl in Buffer B. Fractions containing electrophoretically homogeneous PCNA were pooled and concentrated using a Centriloc concentrator (Amicon, Beverly, MA). The concentration of PCNA was estimated by the calculated ε$_{280}$ value of 17.7 m M$^{-1}$ cm$^{-1}$.

**pol δ Activity Assay**—The activity of pol δ was monitored during its purification as described previously (24) with minor modifications. Aliquots (3 μl) of the fractions collected from the pol δ purification steps were added to 60 μl of a mixture containing poly(dA)oligod(T)$_{12-15}$ (base ratio of 10:1, final A$_{260}$, 0.023), 44 mM bis-Tris-HCl (pH 6.5), 44 mM dTPP (1 μCi of [3H]TTP), 6.6 mM Mg$_2$Cl$_2$, 2.5% glycerol (v/v), 2.6 m M of BSA and 57 m M PCNA. The reaction mixture was incubated at 37 °C for 15 min and quenched with 2 ml of cold 5% Cl$_2$CO$_2$H (w/v) containing 20 mM sodium pyrophosphate. The precipitated DNA was collected by centrifugation and resuspended in 5% C$_2$H$_5$OH and then with C$_2$H$_6$OH. The filters were dried, and the radioactivity was determined by scintillation counting.

**Purification of pol δ**—pol δ was purified as referenced by Podust et al. (25) with modifications. Due to the relatively high reaction volumes of the rapid quench instrument used to measure pre-steady-state kinetics of nucleotide incorporation by pol δ (>2-fold higher than steady-state reactions), the purification procedure for pol δ was optimized for purity and high yield. pol δ was purified to homogeneity from fetal calf thymus by the entire procedure was done within 5 days each time in order to minimize deterioration.

**First trimester fetal calf thymus** (750 g) was lysed in a Waring blender with 1.5 liters of Buffer C containing 50 mM potassium phosphate, pH 7.4 (Buffer C; 10% glycerol (v/v), 2 mM EDTA, 10 mM NaH$_2$PO$_4$, 10 mM benzamidine HCl, 0.5 mM PMSF, 1.0 μg of leupeptin ml$^{-1}$, 10 μg of aprotinin ml$^{-1}$, 4 μg of antipain ml$^{-1}$, 2 μg of pepstatin A ml$^{-1}$, 10 μg of soybean trypsin inhibitor ml$^{-1}$, and 1.0 μg of trans-epoxyuccinyl-1-leucylamido(4-guanidino)butane (E64) ml$^{-1}$).

The lysate was centrifuged at 9000 × g for 30 min, and the supernatant was filtered through 4 layers of cheesecloth. The filtrate was batch-absorbed for 0.5–1 h to phosphocellulose gel (800 ml) that had been pre-cycled and equilibrated in 50 mM potassium phosphate buffer (pH 7.4) containing 10% glycerol (v/v), 2 mM EDTA, and 10 mM NaH$_2$PO$_4$. The phosphocellulose was pelleted by centrifugation (1500 × g, 10 min) and resuspended in Buffer C containing 50 mM potassium phosphate (pH 7.4) and poured into a 5-cm diameter column. The column was washed with Buffer C containing 100 mM potassium phosphate (pH 7.4) at a flow rate of 1.25 ml min$^{-1}$, and the polymerase was eluted by isocratic elution with Buffer C containing 350 mM potassium phosphate

*Relatively large amounts of pol δ were required for these pre-steady-state kinetic experiments. Methods for expression of either p125 (26) or a hybrid of p125 and p55 (7, 27) have been described. An advantage of these heterologous expression systems is the absence of the inactive 116-kDa degradation product seen in thymus preparations (28). However, the expression levels in the baculovirus system are low and the purification scheme is nearly as complex as with the thymus preparation, so we elected to use fetal calf thymus as the source of our pol δ.*
Kinetics of pol δ Nucleotide Incorporation

(pH 7.4). Active fractions were combined and dialyzed against 10 liters of a buffer containing 10% glycerol (v/v), 10 mM NaH₂SO₄, 1.0 mM EDTA, 0.5 mM PMSF, and 1.0 mM DTT (pH 7.0) for 1 h and then dialyzed against a fresh 10 liters of the same buffer for an additional 1 h. The dialyze was loaded onto a heparin-Sepharose column (1.5 × 17 cm) equilibrated in Buffer D (Buffer D: 20 mM potassium phosphate (pH 7.5), 10% glycerol (v/v), 1.0 mM EDTA, 10 mM NaH₂SO₄, 0.5 mM PMSF, 1.0 mM DTT, 10 µg of leupeptin ml⁻¹, and 10 µg of aprotinin ml⁻¹) at a flow rate of 1.25 ml min⁻¹. The column was washed with Buffer D containing 0.10 M NaCl and eluted at a flow rate of 0.3 ml min⁻¹ with a 300-ml linear gradient of 0.10 M NaCl to 0.70 M NaCl in Buffer D. The polymerase activity in the fractions was determined in the absence and presence of PCNA. The fractions that showed the most stimulation by PCNA were combined and dialyzed against 10 liters of 20% glycerol (v/v), 0.5 mM PMSF, and 1.0 mM DTT (pH 7.0) for 1 h. The protein was then dialyzed against a fresh 10 liters of 20% glycerol (v/v), 0.5 mM PMSF, and 1.0 mM DTT (pH 7.0) for an additional 1 h. The dialyze was loaded (1 ml min⁻¹) onto a hydroxylapatite column (1 × 19 cm) equilibrated with Buffer E containing 20 mM KCl (Buffer E: 20 mM potassium phosphate (pH 7.0), 20% glycerol (v/v), 0.5 mM PMSF, and 1.0 mM DTT). The column was washed with Buffer E containing 20 mM KCl and eluted (isocratically) with Buffer E containing 600 mM KCl. Activity of pol δ was assayed in a volume twice the reaction volume to dilute the KCl in the reaction (high concentrations of KC1 are known to interfere with pol δ activity).³ The fractions containing the greatest PCNA-stimulated activity were combined and dialyzed against 10 liters of 10 mM Tris-HCl buffer (pH 8.0) containing 10% glycerol (v/v), 0.5 mM PMSF, 1.0 mM EDTA, and 1.0 mM DTT for 1 h. The protein was then dialyzed for an additional 1 h with a fresh 10 liters of dialysis buffer. The protein was loaded onto a DEAE-Sepacel column (1 × 13 cm) equilibrated in Buffer F (50 mM Tris-HCl (pH 8.0) containing 1.0 mM EDTA, 10% glycerol (v/v), 0.5 mM PMSF, and 1.0 mM DTT) at a flow rate of 0.5 ml min⁻¹. The column was washed with Buffer F, and the polymerase was eluted with a 120-ml linear gradient of 0–500 mM NaCl in Buffer F. The fractions containing the greatest PCNA-stimulated activity were combined and dialyzed against 10 liters of Buffer G containing 15 mM potassium phosphate, pH 7.0 (Buffer G: 10% glycerol (v/v), 10 mM NaH₂SO₄, 0.5 mM PMSF, and 1.0 mM DTT). The protein was then dialyzed for another 1 h with a fresh 10 liters of the dialysis buffer. The polymerase was loaded onto a hydroxylapatite column (1 × 6 cm) equilibrated in Buffer G containing 15 mM potassium phosphate, pH 7.0. The column was washed with the equilibration buffer, and pol δ was eluted with a 50-ml linear gradient of 15–200 mM potassium phosphate (pH 7.0) in Buffer G. Fractions containing purified pol δ were combined and concentrated with a Centriplus concentrator and stored in small aliquots at −70 °C. pol δ was purified from contaminating polymerases and other proteins to yield the p125 and p50 subunits and to a 116-kDa degradation product of p125 (28) (Fig. 2). This degradation product has been seen for other preparations of pol δ from tissue (7, 29, 30). The amount of pol δ was determined by quantitative amino acid analysis of the individual polypeptides of pol δ in the Vanderbilt facility. The individual subunits of pol δ were separated by SDS-polyacrylamide gel electrophoresis (Fig. 2), transferred onto a polyvinylidene fluoride membrane, and stained with Coomassie Brilliant Blue 250 (30); the bands corresponding to each subunit were excised and submitted for quantitative amino acid analysis, with calculations based on amino acid composition. The amount of loss of protein during the gel and transfer was determined by quantitative amino acid analysis. Typical rate enhancement of final pol δ preparations by PCNA was 30–70-fold in the standard assay with poly(dA·oligo(dT)) (see above).

³ C.-K. Tan, personal communication.

End Labeling of Primer and Primer/Template Annealing—Oligonucleotide primers were 5′-end-labeled with [γ-³²P]ATP (3000 Ci mmol⁻¹) and T4 polynucleotide kinase and annealed to the template in a ratio of 1:1.5 (primer:template) (19). The oligonucleotides used in these studies are shown in Table I.

Steady-state Reactions—pol δ containing 0.13 pmol of the limiting 125-kDa subunit of pol δ was added to a mixture (5-µl volume) containing annealed 5′-32P-labeled 24/36G-mer primer/template (200 nM), 0.8 µM PCNA, 0.4 mg of BSA ml⁻¹, 50 mM bis-Tris-HCl (pH 6.5), and 2 mM DTT. The reactions were initiated with the addition of an equal volume of Buffer H (50 mM bis-Tris-HCl (pH 6.5) and 12.5 mM MgCl₂) and dNTP (2× final concentration) at varying concentrations. Final concentrations were 0.2 mM bis-Tris-HCl (pH 6.5), 0.5 mM dATP, 1.0 mM DTT, 100 mM 24/36G-mer, 13 mM pol δ, 0.4 µM PCNA, 6 mM MgCl₂, and 1.0 mM DTT in 10 µl. The reactions were run in triplicate at 37 °C and quenched with 20 µl of 20 mM EDTA (pH 7.4) after 10 min. The products were separated by denaturing polyacrylamide gel electrophoresis (16% acrylamide (w/v), 1.5% bisacrylamide (w/v), 8.0 × urea), and the amount of primer extended was quantitated utilizing a model 400E PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and Image software version 3.3. The kₚᵣ and Kᵥ values were estimated by non-linear regression using a lcgut computer program (Biometics, Princeton, NJ).

Determination of pol δ Exonuclease Rate—The exonuclease rate of pol δ was determined in steady-state kinetic experiments. pol δ (26 nM), 200 nM 24/36G-mer, and 0.8 µM PCNA were incubated in a solution containing 50 mM bis-Tris-HCl (pH 6.5), 0.4 mg of BSA ml⁻¹, and 2 mM DTT in 5 µl. An equal volume of a solution containing 12 mM MgCl₂ and 50 mM bis-Tris-HCl (pH 6.5) was added to initiate the reaction. Final concentrations were 50 mM bis-Tris-HCl (pH 6.5), 0.2 mg of BSA ml⁻¹, 100 nM primer/template, 13 mM pol δ, 0.4 µM PCNA, 6 mM MgCl₂, and 1.0 mM DTT. Reactions were run in triplicate at 37 °C and quenched with 20 µl of 20 mM EDTA (pH 7.4) after 2–300 s. Exonuclease products (23/36G-mer and 22/36G-mer) were quantitated as described above, and the exonuclease rate was determined by linear regression.

Pre-steady-state Kinetics—Pre-steady-state rapid-quetch experiments were performed using a KinTek Quench Flow Apparatus (model RQF-3, KinTek Corp., Austin, TX). Reactions were initiated by rapid mixing of dNTP (in Buffer H) with a primer/template/pol δ solution at 37 °C. The final concentrations of the reactants were 9.2–69 nM pol δ, 50 mM bis-Tris-HCl (pH 6.5), 0.4 mg of BSA ml⁻¹, and 2 mM MgCl₂. The values for the burst amplitude were measured to determine Kᵥ and kₚᵣ by fit to a hyperbola (31). The kinetic Kᵥ and kₚᵣ values for dCTP binding to pol δPCNA DNA—The kinetic Kᵥ and kₚᵣ values for dCTP binding to the pol δPCNA-24/36G-mer complex was estimated by pre-steady-state rapid-quetch analysis. The dNTP concentration dependence of the pre-steady-state burst rate was examined by varying the concentration of dCTP and measuring the rate of dCTP incorporation by pol δ into 24/36G-mer duplex DNA. The values of kₚᵣ were determined by single-exponential analysis as described above. The pre-steady-state rates were plotted against [dNTP], and the data were fit to the hyperbola kₚᵣ = kₚᵣ(DNTP)/(DNTP + Kᵣ) to determine Kᵣ (31).

Determination of Kᵥ and Kᵥ for dNTP—The Kᵥ values for pol δ binding to DNA or PCNA/DNA complexes were determined by pre-steady-state rapid quench analysis. DNA concentration was varied, and the changes in the burst amplitude were measured to determine Kᵥ for

Fig. 2. SDS-polyacrylamide gel electrophoresis of pol δ. DNA polymerase δ was purified as described under "Experimental Procedures." The subunits (numbers on the left) are shown as 125 and 50 kDa with a 116-kDa degradation product of the 125-kDa subunit (28). The numbers on the right (kDa) are from Mr standards.
binding DNA or DNA/PCNA substrate. The $K_d$ was determined by fitting the data to the quadratic equation $[E+D] = 0.5(E + E + D)^2 - [0.25K_d + E + D]^2 = E, D]^{1/2}$ where $E =$ total enzyme, $D =$ total DNA, and $K_d =$ dissociation constant for the reaction $E + D = E-D$.

### RESULTS

**Steady-state Kinetics of dCTP Incorporation Opposite G by pol δ**—The steady-state kinetics of elongation of the 5'[^22]P-labeled 24/36G-mer duplex DNA (Table I) to a 25/36G-mer product were measured by examining dCTP concentration dependence on the steady-state rate of incorporation using a large excess of 24/36G-mer (100 nM) relative to pol δ (13 nM) in the reactions. The concentration of pol δ used in the reactions was calculated on the basis of the amount of the limiting 125-kDa subunit of pol δ determined by quantitative amino acid analysis. The steady-state rate constant $k_{cat}$ was 0.36 min$^{-1}$, which is an upper limit for the slowest step of single nucleotide incorporation in multiple turnover reactions. The steady-state $K_m^{dCTP}$ was determined to be 0.067 μM.

PCNA is known to increase the processivity of pol δ replication (14, 15). When PCNA was eliminated from the reactions, the steady-state rate constant remained similar to that of reactions containing PCNA, $k_{cat}$ 0.30 min$^{-1}$, and $K_m$ increased 20-fold to a value of 1.2 μM.

**3' → 5' Exonuclease Activity of pol δ**—The rate of exonuclease product formation was examined by incubating the 24/36G-mer with pol δ and PCNA and then initiating the reactions with MgCl$_2$ for various times (20–300 s) in the absence of dNTPs. The rate of formation of exonuclease products (23/36G-mer and 22/36G-mer) was determined to be 0.56 min$^{-1}$ (Fig. 3). This rate of exonuclease activity is 10$^9$-fold lower than the rate of polymerization (see below) and therefore did not interfere with the pre-steady-state single turnover kinetics of pol δ. No burst of activity was observed, and the first reaction cycle required ~100 s.

**Pre-steady-state Kinetics of dCTP Incorporation Opposite G by pol δ**—The rate of polymerization ($k_{pol}$) can be measured by rapid quench kinetics and includes rates of dNTP binding, any conformational change, and phosphodiester bond formation (Fig. 1). The incorporation of dCTP into 24/36G-mer by pol δ in the presence of PCNA shows biphase kinetics consisting of an initial burst followed by a linear phase (Fig. 4). The burst phase represents the rate of single nucleotide incorporation in the first turnover of the enzyme, and the rate of polymerization ($k_{pol}$) was determined to be 1260 min$^{-1}$ by single exponential analysis. The much higher rate of the pre-steady-state burst phase compared with the steady-state rate constant indicates that the rate-limiting step in multiple turnover reactions follows phosphodiester bond formation. The amplitude of the burst corresponds to the concentration of active enzyme present in the reactions, i.e. ~65% of the pol δ is active in these reactions.

In the absence of PCNA, the rate of polymerization decreased to 400 min$^{-1}$, ~3-fold slower than reactions containing PCNA. The amplitude of the burst phase was also decreased in the absence of PCNA by ~2-fold, suggesting that PCNA is involved in formation of an active pol δ complex capable of nucleotide incorporation.

**Determination of Elemental Effect**—The replacement of the α-phosphate group of a nucleotide triphosphate with a phosphorothioate can reduce the rate of single nucleotide incorpor-

### Table I

| Primer/template sequence |
|--------------------------|
| 24/36G-mer              |
| 5'-GCC TCG AGC CAG CCG CAG CAG  |
| 3'-GGG AGC TCG GTC GCC GTC GTC GCT GCT GCG GCT |

---

**Fig. 3. Exonuclease rate of pol δ, pol δ (13 nM) was incubated with PCNA (0.4 μM) and 100 nM 24/36G-mer, and the reactions were initiated with 6 mM MgCl$_2$ and quenched with EDTA at various times. The reactions were run in triplicate, and the rate of exonuclease activity was determined by linear regression from a plot of the time-dependent formation of 23/36G-mer and 22/36G-mer exonuclease products.**

**Fig. 4. Pre-steady-state rapid quench kinetics of nucleotide insertion by pol δ, pol δ (9.2 nM) was incubated with 100 nM 24/36G-mer in the absence (■) or presence of PCNA (180 nM) (●) and then mixed with 200 μM dCTP solution in the rapid quench instrument. The reactions were quenched with EDTA at various times and the rate of polymerization ($k_{pol}$) was determined by single exponential analysis. The solid line is a fit to the burst equation described under “Experimen
tal Procedures.” For analysis of a phosphorothioate elemental effect, pol δ (9.2 nM) was incubated with the 24/36G-mer (100 nM) and PCNA (180 nM) and then mixed with a 200 μM αdSdCTP solution (○).**

**Fig. 5.** Pre-steady-state rapid quench kinetics of nucleotide insertion by pol δ, pol δ (9.2 nM) was incubated with 100 nM 24/36G-mer in the absence (■) or presence of PCNA (180 nM) (●) and then mixed with 200 μM dCTP solution in the rapid quench instrument. The reactions were quenched with EDTA at various times and the rate of polymerization ($k_{pol}$) was determined by single exponential analysis. The solid line is a fit to the burst equation described under “Experimen
tal Procedures.” For analysis of a phosphorothioate elemental effect, pol δ (9.2 nM) was incubated with the 24/36G-mer (100 nM) and PCNA (180 nM) and then mixed with a 200 μM αdSdCTP solution (○).
role of PCNA on DNA replication by polymerases, the first (Table II). In steady-state reactions of dNTP incorporation into 24/36-mer DNA primer/templates was examined the exonuclease rate, because the exonuclease activity rate cannot possible that the incorporation rate should be corrected by addition of A

quench kinetics. The burst rates from greater than the apparent k:

were plotted against [dCTP], was determined by examination of the DNA concentration dependence of the burst amplitude for incorporation of dCTP by pol δ into 24/36G-mer or PCNA-24/36G-mer complexes. A fixed amount of pol δ was incubated with increasing concentrations of 24/36G-mer or 24/36G-mer-PCNA, and the reactions were initiated with the addition of saturating dCTP for 0.1 s; the reaction time allowed for maximal burst amplitudes with negligible effect from multiple turnovers of the enzyme. The Kd, DNA, determined by plotting the burst amplitudes against the concentration of DNA and then fitting the data to the quadratic equation (Fig. 6), was determined to be 64 nM in the presence of PCNA and 300 nM in the absence of PCNA, indicating that PCNA increases the affinity of the pol δ-DNA interaction ~5-fold.

DISCUSSION

The goal of this work was to analyze elementary steps in the pol δ reaction leading to correct nucleotide incorporation. The role of PCNA on DNA replication by pol δ was also examined by steady-state and transient pre-steady-state kinetics.

The steady-state kinetics of pol δ-catalyzed nucleotide incorporation into 24/36-mer DNA primer/templates was examined first (Table II). In steady-state reactions of dNTP incorporation by polymerases, the kcat is dominated by the slow dissociation rate of the DNA from the enzyme rather than the actual polymerization rate. The slow kcat for pol δ (0.36 min−1)4 is similar to other replicative DNA polymerases such as HIV-1 RT (kcat = 1.8 min−1) and T7 (kcat = 4.4 min−1) (37) and is consistent with the importance of having a slow dissociation from DNA for highly processive replicative DNA polymerases. In the absence of PCNA, the kcat of nucleotide incorporation by pol δ was unaffected (0.36 versus 0.30 min−1)4 but the Km increased 20-fold (0.067 versus 1.2 μM), similar to a previous report of a greater effect of PCNA on Km in pol δ that includes values for nucleotide incorporation by pol δ into poly(dA)-oligo(dT) primer/templates (7). However, PCNA was also reported to produce a 2.5-fold increase in kcat for pol δ-directed incorporation of a single dNTP into a 21/30-mer substrate (7). The effect of PCNA on the ratio of kcat/Km indicated a lower incorporation efficiency for dCTP nucleotide incorporation into the 24/36G-mer than for dTTP insertion into poly(dA)-oligo(dT) primer/templates found in previous studies (7). This difference may reflect the importance of PCNA in reactions where more than a single nucleotide is inserted into DNA, e.g. dTTP incorporation into poly(dA)-oligo(dT). The pol δ insertion efficiency of 5.4 μM−1 min−1 is similar to other replicative polymerases such as HIV-1 RT and pol T7 (6 and 2.3 μM−1 min−1, respectively) (37). The insertion efficiency decrease of 20-fold in the absence of PCNA is dominated by the increase in Km, but the physical interpretation of Km for a polymerase reaction is unclear (38).

4 The estimated steady-state of exonuclease activity (0.56 min−1) is greater than the apparent kcat for dNTP incorporation (Table II). It is possible that the incorporation rate should be corrected by addition of the exonuclease rate, because the exonuclease activity rate cannot exceed the incorporation rate. We have not made such a correction (i.e. 0.36 + 0.56 = 0.92 min−1) in presence of PCNA; Table II because we do not know if exonuclease activity is the same in the presence of dNTP. The comparisons point out the usefulness of using pre-steady-state kinetics.

5 The meaning of the polymerase steady-state Km value is not known (18). It is defined as the concentration of dNTP that produces a rate
The polymerization rate, \( k_{pol} \), corresponds to the first rapid incorporation of a single nucleotide (burst phase) preceding multiple turnovers of the enzyme (Fig. 4). The rate of incorporation with a polymerase at high dNTP concentration is governed by the rate of conformational change after dNTP binding or the rate of phosphodiestere bond formation (Fig. 1, steps 3 and 4). The intrinsic exonuclease activity of pol \( \delta \) was slow (0.56 min\(^{-1}\)), and the exonuclease products formed in the time scales of the pre-steady-state kinetic analysis were negligible due to the rates of polymerization being 10\(^3\)-fold higher. A burst of nucleotide incorporation into the 24/36G-mer was found for pol \( \delta \) (Fig. 4) in the presence or absence of PCNA. The polymerization rate \( (k_{pol}) \) measured for pol \( \delta \) in the presence of PCNA was 1260 min\(^{-1}\), and this rate was attributed to the rate of the conformational change following dNTP binding rather than a rate-limiting chemistry step, because of the observed phosphorothioate elemental effect of 2. Rate-limiting conformational change steps have also been proposed for correct nucleotide incorporation by pol T7, pol \( \Gamma \), HIV RT, and bacteriophage T4 DNA polymerase on the basis of phosphorothioate elemental effects of <4 (31, 33, 34, 40) (but not pol \( \Pi \) (Ref. 39)), suggesting a common polymerization mechanism for many DNA polymerases, including mammalian replicative polymerases.

PCNA affected the single turnover kinetics of pol \( \delta \) by increasing the rate of polymerization by 3-fold and by increasing the amount of active pol \( \delta \)-DNA complex (as measured by the amplitude of the burst) by 2-fold (Figs. 4 and 6). The effect of PCNA on the transient state kinetics indicates that PCNA can affect the active conformation of pol \( \delta \) to some extent; however, PCNA may be more important for multiple turnovers of the enzyme (processivity), due to the greater effect of PCNA on the steady-state kinetics of nucleotide incorporation. To examine whether the primer/templates chosen were optimal for nucleotide incorporation by pol \( \delta \) and PCNA, longer primer templates were used (36/48G-mer and 36/60G-mer) and the pre-steady-state kinetic analysis of dCTP incorporation was examined for pol \( \delta \) in the presence of PCNA (results not shown). The longer primer templates decreased both the rate and burst amplitude of nucleotide incorporation by \( \delta \)-PCNA, as seen previously for bacteriophage T4 polymerase (associated with an increase in nonspecific binding of the polymerase and the DNA) (41). pol \( \delta \) and PCNA are predicted to occupy approximately 20 base pairs on duplex DNA, suggesting that the 24/36-mer used in these studies is sufficient for the accommodation of these proteins (42).

The difference in the dissociation constants (\( K_{d}^{DNA} \)) of pol \( \delta \) and DNA with and without PCNA was 5-fold (Fig. 6). The role of PCNA in a single nucleotide incorporation event by pol \( \delta \) mainly involves the affinity of the interaction of the polymerase and the DNA, as predicted. The fact that these effects of PCNA are modest (at the most 5-fold) indicate that PCNA may be most important for the processivity of pol \( \delta \) measured in reactions with multiple turnovers of the enzyme.

Under static conditions, the pol \( \delta \)-PCNA-DNA complex has been reported to have a long half-life (\( t_{1/2} = 2.65 \) h) and the absence of PCNA greatly reduced the apparent \( t_{1/2} \) (2000-fold) (17). The \( K_{d}^{DNA} \) determined in the present study indicated only a 5-fold difference in the binding constant of the pol \( \delta \)-DNA complex in the presence and absence of PCNA. The difference in the results can be explained in part by the difference in the experimental procedure. Our \( K_{d}^{DNA} \) was not determined under static conditions and reflects a true dissociation constant under conditions of active polymerization (presence of dNTP). Significant destabilization of the complex has been reported to occur in the presence of dNTP, as determined qualitatively (43). However, the loss of affinity is not explained by nucleotide incorporation (43) but by the interaction of pol \( \delta \) with dNTP. The presence of dNTP in this study may have greatly reduced the stability of the pol \( \delta \)-DNA interaction compared with studies examining this interaction in the absence of dNTP. A \( t_{1/2} \) of 2.65 h (in the presence of PCNA) corresponds to a maximum steady-state \( k_{cat} \) of 0.03 h\(^{-1}\) (for incorporation into the 21/30-mer) (43), 3 orders of magnitude too slow (Table II).

A minimal mechanism of correct nucleotide incorporation by pol \( \delta \)-PCNA is shown in Fig. 7. We conclude that the catalytic mechanism of pol \( \delta \) is similar to that of other polymerases such as HIV-1 RT, pol T7, and human pol \( \gamma \), the only other processive mammalian polymerase that has been studied using pre-steady-state kinetic approaches (44). Kinetic parameters for correct nucleotide incorporation by several DNA polymerases including E. coli pol I and II, HIV-1 RT, pol T7, human pol \( \gamma \), and fetal calf thymus pol \( \delta \) (37, 39, 44) are compiled in Table III. The processivity estimated for pol \( \delta \) is 7-fold higher than any of the other polymerases examined. In comparison with the other mammalian replicative polymerase that has been examined, mitochondrial pol \( \gamma \), pol \( \delta \) was found to have a 30-fold higher processivity factor and considerably greater affinity for dNTP. The differences may be due to pol \( \gamma \) lacking another factor(s) necessary for processive replication or to inherent differences

### Table II

| Condition | \( k_{cat} \) | \( K_m \) | \( k_{pol} \) | \( K_d^{NTT} \) | \( k_{pol} / K_d^{NTT} \) | \( K_d^{DNA} \) |
|-----------|---------------|----------|-------------|--------------|-----------------|-------------|
| + PCNA    | 0.36 ± 0.01   | 0.067 ± 0.011 | 1280 | 0.93 ± 0.32 | 22 | 64 ± 10 |
| − PCNA    | 0.30 ± 0.02   | 1.2 ± 0.2 | 400 | —           | — | 300 ± 31 |

*Not determined.*
in the cellular roles of these polymerases. The length and volume of DNA to be replicated is much greater for DNA pol δ than for mitochondrial pol γ; thus, the variation of the polymerase processivities may reflect the differences in their cellular demands. We emphasize that the steady-state $k_{\text{cat}}$ determined with oligonucleotides in single nucleotide incorporation experiments is dominated by the DNA $k_{\text{cat}}$ rate (44), and a higher $k_{\text{cat}}$ is misleading in that it is actually associated with lower processivity and probably less efficient replication on long strands of DNA.

In summary, the mechanism of DNA polymerization by pol δ, the major leading strand mammalian polymerase, appears to be very similar to other prokaryotic DNA polymerases studied previously (with the exception of the unusual thio effects on pol II” (Ref. 39)). The values of $K_{\text{OFF}}$, $K_{\text{ON}}$, and $k_{\text{pol}}$ and rate-limiting steps in the reaction mechanism are comparable, as judged by the usual mechanistic criteria. Model replicative prokaryotic DNA polymerases are therefore considered good models for studies of eukaryotic DNA replication, although we recognize that they may show differential effects among each other and with mammalian polymerases in terms of their responses to particular lesions and mispairs (19).

Acknowledgments—We acknowledge the contributions of K. M. Downey, A. G. So, and M. Carastro (University of Miami School of Medicine, Miami, FL) and V. N. Podust, E. Fanning, J. B. Wheeler, and A. N. Mican (Vanderbilt University, Nashville, TN) for their assistance in the purification of pol T7. We also acknowledge E. Howard (Protein Chemistry Core Facility, Vanderbilt University, Nashville, TN) for the bacterial expression vector for human PCNA.

REFERENCES

1. Kornberg, A., and Baker, T. A. (1992) DNA Replication, W. H. Freeman, New York
2. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) DNA Repair and Mutagenesis, American Society for Microbiology, Washington, D. C.
3. Stucki, M., Pascucci, B., Parlanti, E., Fortini, P., Wilson, S. H., Hübscher, U., and Dogliotti, E. (1996) Oncogene 17, 835–843
4. Shrivv, M. K. K., Podust, V. N., Hübscher, U., and Wood, R. D. (1995) Biochemistry 34, 5011–5017
5. Longley, M. J., Pierce, A. J., and Modrich, P. (1997) J. Biol. Chem. 272, 12310–12316
6. Podust, V. N., Georgaki, A., Strack, B., and Hübscher, U. (1992) Nucleic Acids Res. 20, 4159–4165
7. Zhou, J. Q., Tan, C. K., Downey, K. M., and Fisher, P. A. (1996) Biochemistry 35, 8268–8274
8. Johnson, K. A. (1993) Annu. Rev. Biochem. 62, 685–713
9. Einolf, H. J., Schents-Boutaud, N., and Guengerich, F. P. (1998) Biochemistry 37, 13000–13312
10. Fien, K. and Stillman, B. (1992) Mol. Cell. Biol. 12, 155–163
11. Mcconnell, M., Miller, H., Mozzerini, D. J., Qumina, A., Tan, C. K., Downey, K. M., and Fisher, P. A. (1996) Biochemistry 35, 8268–8274
12. Johnson, K. A. (1993) Anal. Biochem. 118, 197–203
13. Downey, K. M., and So, A. G. (1995) Methods Enzymol. 262, 84–92
14. Podust, V. N., Georgaki, A., Strack, B., and Hübscher, U. (1992) Nucleic Acids Res. 20, 4159–4165
15. Zhou, J. Q., Tan, C. K., So, A. G., and Downey, K. M. (1996) J. Biol. Chem. 271, 29740–29745
16. Wu, S. M., Zhang, P., Zeng, X. R., Zhang, S. J., Mo, J. Li, B. Q., and Lee, M. Y. (1998) J. Biol. Chem. 273, 9561–9569
17. Zhang, J., Chung, D. W., Tan, C. K., Downey, K. M., Dave, E. W., and So, A. G. (1991) Biochemistry 30, 11742–11750
18. Jiang, Y., Zhang, S.-J., Wu, S.-M., and Lee, M. Y. W. T. (1995) Arch. Biochem. Biophys. 320, 297–304
19. LeGendre, N., Manfield, M., Weiss, A., and Matsudaira, P. (1993) in A Practical Guide to Protein and Peptide Purification for Microsequencing (Matsudaira, P., ed) pp. 71–101, Academic Press, San Diego
20. Kati, W. M., Johnson, K. A., Jerva, L. F., and Anderson, K. S. (1992) J. Biol. Chem. 267, 25888–25897
21. Benkovci, S. J., and Shroyer, K. J. (1973) in The Enzymes (Boyer, D. P., ed) Vol. 8, pp. 201–236, Academic Press, New York
22. Patel, S. S., Wong, I., and Johnson, K. A. (1991) Biochemistry 30, 511–525
23. Mirzali, V., Henrie, R. N., Marlier, J. F., Johnson, K. A., and Benkovci, S. J. (1995) Biochemistry 24, 4100–4108
24. Herschlag, D., Picirilli, J. A., and Cech, T. R. (1991) Biochemistry 30, 4844–4854
25. Tan, H. B., Swann, P. F., and Chance, E. M. (1994) Biochemistry 33, 5335–5346
26. Purge, L. L., and Guengerich, F. P. (1997) Biochemistry 36, 6475–6487
27. Johnson, K. A. (1995) Methods Enzymol. 249, 38–61
28. Lowe, L. G., and Guengerich, F. P. (1996) Biochemistry 36, 9840–9849
29. Freny, M. W., Sowers, L. C., Miller, D. P., and Benkovci, S. J. (1995) Biochemistry 34, 9185–9192
30. Carpen, T. L., Peliska, J. A., Kaboord, B. F., Frey, M. W., Lively, C., Dahlberg, M., and Benkovci, S. J. (1992) Biochemistry 31, 10984–10994
31. Mozzerini, D. J., Tan, C. K., Downey, K. M., and Fisher, P. A. (1999) J. Biol. Chem. 274, 19862–19867
32. Ng, L., Mcconnell, M., Tan, C. K., Downey, K. M., and Fisher, P. A. (1993) J. Biol. Chem. 268, 13571–13576
33. Graves, F. W., Johnson, A. A., and Johnson, K. A. (1998) Biochemistry 37, 6050–6058