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Human DNA Polymerase ϵ: Enzymologic Mechanism and Gap-Filling Synthesis[†]

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ABSTRACT: DNA polymerase ϵ (pol ϵ) was purified to apparent homogeneity from human placentas. The purified enzyme contains a single polypeptide of approximately 170 kDa (apparent mass) and has both DNA polymerase and 3′−5′-exonuclease activities. Competitive inhibition studies indicate that like DNA polymerases α and δ (pol α and pol δ, respectively), free pol ϵ binds single-stranded but not double-stranded DNA. This conclusion was confirmed by sedimentation binding analysis. Also like pol α and pol δ, pol ϵ exhibits induced dNTP inhibition in the presence of template annealed to complementary primer containing a 2′,3′-H (dideoxy)-terminus. Together, these data suggest that pol ϵ follows an ordered sequential ter-reactant mechanism of substrate recognition and binding; it binds template first followed by annealed primer and then template-specified dNTP. Enzymologic studies suggest that in contrast to both pol α and pol δ, pol ϵ functions more efficiently as gap size decreases. This observation is consistent with a specific role for pol ϵ in gap-filling *in vivo*. Gap-filling is essential for both replication and repair.

In the yeast *Saccharomyces cerevisiae*, three different DNA polymerases were shown to be essential for cell proliferation. These are DNA polymerases α, δ, and ϵ (pol α, pol δ and pol ϵ, respectively). Human homologs of yeast DNA polymerases including pol ϵ have been purified, cloned, and sequenced [Chung et al., 1991; for a review of earlier literature, see Wang (1991); for pol ϵ, see Lee and Toomey (1987), Syväoja et al. (1990), Lee et al. (1991), Mozzherin et al. (1992), and Kesti et al. (1993)].

The biochemical properties of the purified DNA polymerases are consistent with their presumed biological roles. For example, purified pol α is only moderately processive and is physically associated with DNA primase (Fisher et al., 1979; Hu et al., 1984). Based on dissection of cell-free viral systems (Waga & Stillman, 1994), it is apparent that *in vivo*, pol α along with DNA primase is required for initiation of DNA synthesis on both leading and lagging strands. In addition, it is required for the multiple rounds of RNA primer generation (i.e., reinitiation) necessary for discontinuous lagging strand replication. Extended processive synthesis would be counterproductive in these contexts; primase activity is essential.

In contrast, purified pol δ along with its auxiliary factor, proliferating cell nuclear antigen (PCNA), is highly processive, incorporating hundreds of nucleotides per binding event (Tan et al., 1986). *In vivo*, it is thought to be responsible for the bulk of continuous leading strand synthesis (Tan et al., 1986; Prelich et al., 1987a,b; Bravo et al., 1987; Prelich & Stillman, 1988); processive synthesis would be highly advantageous in this context. In a cell-free viral system, pol δ was recently demonstrated to be required for lagging strand synthesis as well (Waga & Stillman, 1994). The need for processive synthesis in this context is less clear.

Despite the fact that pol ϵ is essential *in vivo*, at least in yeast, its precise biochemical role remains elusive. This is largely due to the fact that unlike cell proliferation, cell-free replication of viral DNA can apparently occur in the complete absence of pol ϵ [see, e.g., Waga and Stillman (1994)]. As an alternative to dissection of crude cell-free systems, we have pursued in-depth biochemical characterization of near-homogeneous pol ϵ. Like both pol α (Fisher & Korn, 1981; Wong et al., 1986; Ng et al., 1989) and pol δ [Ng et al., 1991; for a review, see Fisher (1994)], pol ϵ follows an ordered sequential ter-reactant mechanism of substrate recognition and binding; template is bound first followed by template-complementary primer and template-specified dNTP. However, unlike these other DNA polymerases, pol ϵ works more efficiently as gap size decreases. This, as has been proposed previously (Podust & Hübscher, 1993), suggests that pol ϵ may play a gap-filling role *in vivo*. Although the requirement for gap-filling during replication would pertain to both leading and lagging strands, discontinuous synthesis on the lagging strand would necessitate far more gap-filling in that context. Gap-filling is also necessary for DNA repair.

EXPERIMENTAL PROCEDURES

Most of the procedures were described previously (Ng et al., 1989, 1991, 1993).

Materials. Unlabeled deoxyribonucleoside and dideoxyribonucleoside triphosphates (dNTPs and ddNTPs, respectively) were from Pharmacia LKB Biotechnology. [α-^{32}P]-dTTP, [α-^{32}P]dATP, and [γ-^{32}P]ATP were from Amersham. N^2-(*p*-*n*-Butylphenyl)-2′-deoxyguanosine 5′-triphosphate (BuPhdGTP) was the generous gift of G. Wright (University of Massachusetts School of Medicine, Worcester, MA). 2′-Deoxythymidine 5′-α-methylenephosphonyl-β,γ-diphosphate (dT[CH$_2$]PP$_2$) was the generous gift of M. Jasko (Engelhardt Institute of Molecular Biology, Moscow, Russia). *Hae*III restriction endonuclease was from New England BioLabs. Terminal deoxyribonucleotidyl transferase was from United States Biochemical. Pol α (calf thymus) was from Molecular

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Biology Resources Inc.; pol δ and PCNA (both from calf thymus) were purified according to published protocols (Lee et al., 1984; Tan et al., 1986; Ng et al., 1991) and were the generous gifts of C.-K. Tan and K. Downey (University of Miami School of Medicine, Miami, FL).

DNA Molecules of Defined Structure. Synthetic oligonucleotide templates and primers of defined sequence were prepared using conventional phosphoramidite chemistry (Takeshita et al., 1987) and purified by high-performance liquid chromatography. Terminal deoxyribonucleotidyl transferase was used to add single ddNMP residues to the 3′-OH terminus of primers as previously described (Fisher & Korn, 1981; Ng et al., 1989), to prepare ^{32}P-labeled $(dT)_{40}$, and also to synthesize the "hook" homopolymer template-primer, $(dC)_{100}-(dG)_{25}$, in which the $(dG)_{25}$ primer is covalently linked to the $(dC)_{100}$ template (Fisher et al., 1981). A mixture of supercoiled and relaxed forms of bacterial phagemid pBS (molar ratio of about 2:1, respectively) was purified on a QIAGEN Mini (100) column (QIAGEN Inc.) according to the manufacturer's instructions. Digestion of pBS to completion with *Hae*III was according to the vendor's instructions and as previously described (Fisher & Korn, 1979). DNase I "activated" calf thymus DNA was prepared as described previously (Fisher et al., 1979) as was activated poly(dAT). M13mp10 single-stranded circular DNA was the generous gift of L. Victorova (Engelhardt Institute of Molecular Biology, Moscow, Russia).

DNA Polymerase Assays. One unit of pol ϵ is defined as the amount of enzyme which catalyzes the incorporation of 1 nmol of dNTP/h at 37 °C with activated poly(dAT) (Lee & Toomey, 1987). Standard reaction mixtures for pol α and pol ϵ contained 40 mM potassium phosphate, pH 7.0, 8 mM $MgCl_2$, 1 mM DTT, and 20 μg/mL bovine serum albumin. Unless otherwise indicated, reactions also contained template-primers as specified and 60 μM of each dNTP. The standard reaction mixture for pol δ contained 40 mM Bis-Tris, pH 6.7, 6 mM $MgCl_2$, 2 mM DTT, 10% glycerol, and 40 μg/mL of bovine serum albumin. Unless otherwise indicated, reactions also contained template-primers as specified and 100 μM of each dNTP. Additional details are provided in the figure legends. Standard assays were for 30 min at 37 °C in a final volume of 10 μL. The DNA product synthesized was determined by spotting reaction mixtures onto Whatman DE-81 filters and washing with a 5% (w/v) solution of $Na_2HPO_4 \cdot 12H_2O$. Kinetic constants (K_m and V_{max}) were determined according to Duggleby (1981) using the Hyper.exe computer program of J. S. Easterby (Liverpool, England) available through shareware on the Internet.

Exonuclease Assays. 3′-5′-Exonuclease was assayed as described (Lee et al., 1984) with ^{32}P-labeled $(dT)_{40}$ (1000 cpm/pmol of nucleotide) as substrate. The final reaction volume was 25 μL, and incubation was for 30 min at 37 °C. ^{32}P-Labeled $(dT)_{40}$ remaining was quantified by spotting 20 μL aliquots of reaction mixtures on Whatman DE-81 filters and washing with a 5% (w/v) solution of $Na_2HPO_4 \cdot 12H_2O$.

RESULTS

Purification of DNA Polymerase ϵ from Human Placentas. Human pol ϵ, purified as described (Mozzherin et al., 1992), has a single 170 kDa subunit. This procedure was simplified. Approximately 30 000 units of partially purified enzyme [step 3 according to Mozzherin et al. (1992)] was applied to a Mono-Q FPLC HR5/5 column in 40 mM potassium phosphate, pH 7.0, with 1 mM DTT and 10 mM sodium bisulfite. The column was eluted by a 40-mL linear gradient of KCl (0.1−0.35 M) in the same buffer. It is possible to distinguish pol α from other DNA polymerases in that pol α is particularly sensitive to inhibition by BuPhdGTP (also see below). Active fractions (2.5 mL each) that were not inhibited by BuPhdGTP were pooled and dialyzed against 40 mM potassium phosphate, pH 7.0, 10 mM sodium bisulfite, 0.5 mM EDTA, 1 mM DTT, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 20% glycerol. The dialyzed pool was applied to a heparin−agarose column (0.5 × 0.6 cm) equilibrated with the same buffer. After the column was loaded, it was washed with 0.2 M KCl in the same buffer, and pol ϵ was eluted with a 1-mL linear gradient of KCl (0.2−0.35 M) followed by 1 mL of 0.35 M KCl. As expected, the final chromatogram demonstrated that pol ϵ contains both DNA polymerase activity and 3′-5′ exonuclease activity (Figure 1).

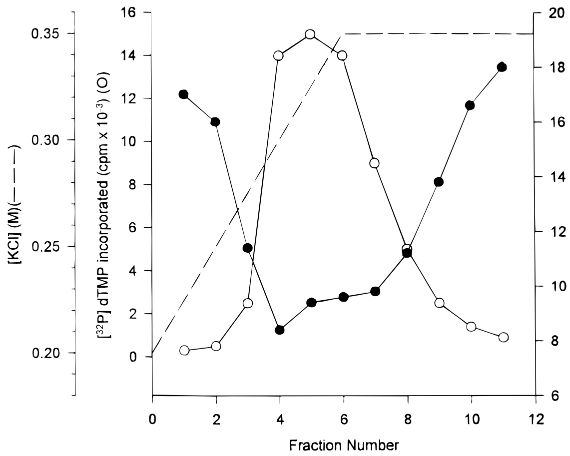

FIGURE 1: Heparin−agarose chromatography of human pol ϵ. The heparin−agarose column was eluted with a 1.0 mL total volume linear gradient of 200−350 mM KCl as indicated (- - -) at a flow rate of 2 mL/h. Fractions of 150 μL each were collected. Additional details are given in the text. Reaction mixtures were 25 μL each. Aliquots (5 μL) of the indicated column fractions were assayed for DNA polymerase activity (○) and 3′-5′-exonuclease activity (●) as described under Experimental Procedures.

Purified pol ϵ was distinguished both biochemically and immunochemically from pol α as well as pol δ (data not shown). Thus, it was not affected by 3 μM BuPhdGTP; purified pol α was inhibited by more than 95% when incubated identically. Similarly, the purified enzyme neither incorporated nor was affected by $dT(CH_2)PP_2$, a potent inhibitor of pol α (Jasko et al., 1995). Finally, purified pol ϵ was not inhibited by SJK 132−20, a neutralizing monoclonal anti-pol α antibody. Purified pol ϵ was not affected by 7 μg/mL calf thymus proliferating cell nuclear antigen (PCNA); under identical conditions of substrate and PCNA, pol δ was stimulated 14-fold. Immunoblot analysis with anti-human PCNA antibodies demonstrated that purified pol ϵ lacked detectable levels of contaminating PCNA. Also in contrast with pol δ, purified pol ϵ was alone able to synthesize DNA efficiently using singly-primed circular M13 as a substrate.

Enzymologic Analysis of the Interaction of Pol ϵ with Nonsubstrate DNA Molecules of Defined Structure. We examined the ability of various nonsubstrate DNA molecules

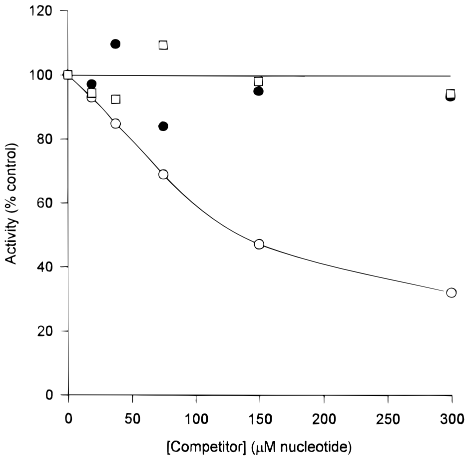

FIGURE 2: Inhibition of human placenta pol ϵ by nonsubstrate DNA molecules of defined structure. Human placenta pol ϵ was incubated in a standard reaction mixture (see Experimental Procedures) with activated DNA at a final concentration of about 0.05 mM nucleotide. $MgCl_2$ was at a final concentration of 8 mM, dTTP was at 10 μM, and each of the other three dNTPs was at 60 μM. [α-^{32}P]dTTP was at 4000 cpm/pmol. Increasing amounts, as indicated on the abscissa, of various nonsubstrate DNA molecules were added as competitors: a mixture of supercoiled and relaxed (about 2:1, respectively) circular duplex pBS (●); blunt-ended linear double-stranded fragments of pBS (□); and single-stranded circular M13mp10 (○). The amount of dTMP incorporated in each reaction is expressed as a percentage of the amount incorporated in the absence of competitor DNA.

of defined structure to inhibit the activity of purified pol ϵ on an activated DNA substrate. Like both pol α (Fisher & Korn, 1979; Wong et al., 1986; Ng et al., 1989) and pol δ (Ng et al., 1991), human placental pol ϵ was unaffected by several types of double-stranded DNA but was strongly inhibited by single-stranded circular DNA (Figure 2). This inhibition was competitive with a DNA substrate (not shown). These results suggest that the active site of free pol ϵ can bind single-stranded but not double-stranded DNA.

Direct Analysis of Binding of Pol ϵ to DNA. To confirm results of inhibition studies presented in Figure 2, direct sedimentation binding experiments were performed. Pol ϵ was incubated with various nonsubstrate DNA molecules of defined structure and then subjected to analysis by ultracentrifugation through linear glycerol gradients, followed by standard DNA polymerase assay of gradient fractions to determine the migration position of the enzyme. Results, shown in Figure 3, were consistent with results of enzyme activity inhibition studies (Figure 2) and indicated that pol ϵ was unable to bind double-stranded DNA, either supercoiled or relaxed (Figure 3A), but was able to bind single-stranded circular DNA (Figure 3B).

Induced Substrate (dNTP) Inhibition and the Ordered Sequential Ter-Reactant Mechanism of Substrate Recognition and Binding by Human Placenta Pol ϵ. We showed for both pol α and pol δ that the presence of a base-paired 2′,3′-dideoxy-terminated primer promotes inhibition by the dNTP complementary to the immediately adjacent (next) template nucleotide (Fisher & Korn, 1981; Wong et al., 1986; Ng et al., 1989, 1991). This phenomenon, known as induced substrate inhibition, is diagnostic for the ordered sequential ter-reactant mechanism of substrate recognition and binding by the polymerase (Cleland, 1979; Fisher & Korn, 1981). To investigate the behavior of pol ϵ, a synthetic hooked oligonucleotide inhibitor was prepared as shown (Figure 4A).

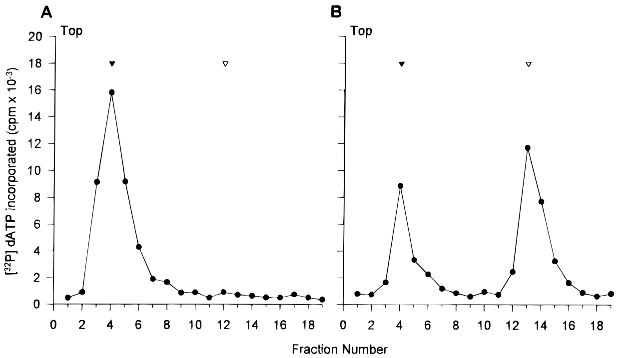

FIGURE 3: Sedimentation binding analysis of the interaction between human placenta pol ϵ and DNA molecules of defined structure. Pol ϵ from human placenta (5 units) was incubated for 3 min at 37 °C in a final volume of 100 μL containing 40 mM potassium phosphate, pH 7.0, 8 mM $MgCl_2$, 20 μg/mL bovine serum albumin, 1 mM DTT, 10% glycerol, and either 0.42 mM (nucleotide) supercoiled double-stranded pBS DNA plus 0.21 mM (nucleotide) relaxed circular double-stranded DNA (A) or 0.42 mM (nucleotide) single-stranded M13mp10 DNA (B). After incubation, samples were layered onto linear 20−40% glycerol gradients containing 40 mM potassium phosphate, pH 7.0, 8 mM $MgCl_2$, 1 mM DTT, and 20 μg/mL bovine serum albumin. Sedimentation was at 4 °C in the SW60 rotor for 7 h at 45 000 rpm. Parallel sedimentation experiments were performed with pol ϵ alone (peak migration position designated by ▼) and DNA alone (peak migration position designated by ▽). After centrifugation, fractions of all gradients (200 μL each) were collected from the top of centrifuge tubes. To 20 μL of each fraction was added 5 μL of 5× concentrated incubation buffer such that the final mixture was 0.05 mM (nucleotide) activated DNA, 1 μM dATP, 80 μM of each of the other three dNTPs, and [α-^{32}P]dATP at 30 000 cpm/pmol. Samples were then incubated for 1 h at 37 °C.

To this synthetic 72-mer was added a single ddCMP residue at the 3′ terminus by terminal deoxyribonucleotidyl transferase. This molecule was designated as 72-mer-ddCMP. The activity of pol ϵ was monitored using the hook homopolymer $(dC)_{100}-(dG)_{25}$ (Figure 4A) as substrate. We used [α-^{32}P]dGTP to monitor incorporation on the $(dC)_{100}-(dG)_{25}$ template-primer, and the effect of each of the other three dNTPs on incorporation with and without 72-mer-ddCMP was measured. Results of these analyses, shown in Figure 4B−D, demonstrate that only dTTP, complementary to the next template position (dAMP) on the 72-mer-ddCMP template-primer, was able to promote increased inhibition by the 72-mer-ddCMP (Figure 4B); template-noncomplementary nucleotides, dCTP and dATP (Figure 4C and Figure 4D, respectively) had no such effect. In the absence of 72-mer-ddCMP, none of these dNTPs including dTTP had any effect on the incorporation by pol ϵ of [^{32}P]dGMP on the hook homopolymer substrate.

Based on results of steady-state kinetics and direct sedimentation binding experiments (Figures 2−4), a model for the mechanism of substrate recognition and binding by pol ϵ from human placenta was formulated. This mechanism, depicted in Figure 5 [drawn according to Cleland (1970)], is identical with that originally proposed after extensive study for human pol α (Fisher & Korn, 1981; Wong et al., 1986), *Drosophila* pol α (Ng et al., 1989), and calf thymus pol δ (Ng et al., 1991). According to this scheme, pol ϵ binds template first, followed subsequently by primer and, ultimately, by template-directed dNTP. Separate binding of template and primer is required to explain competitive inhibition of incorporation on DNA by single-stranded DNA. In the presence of a correctly base-paired

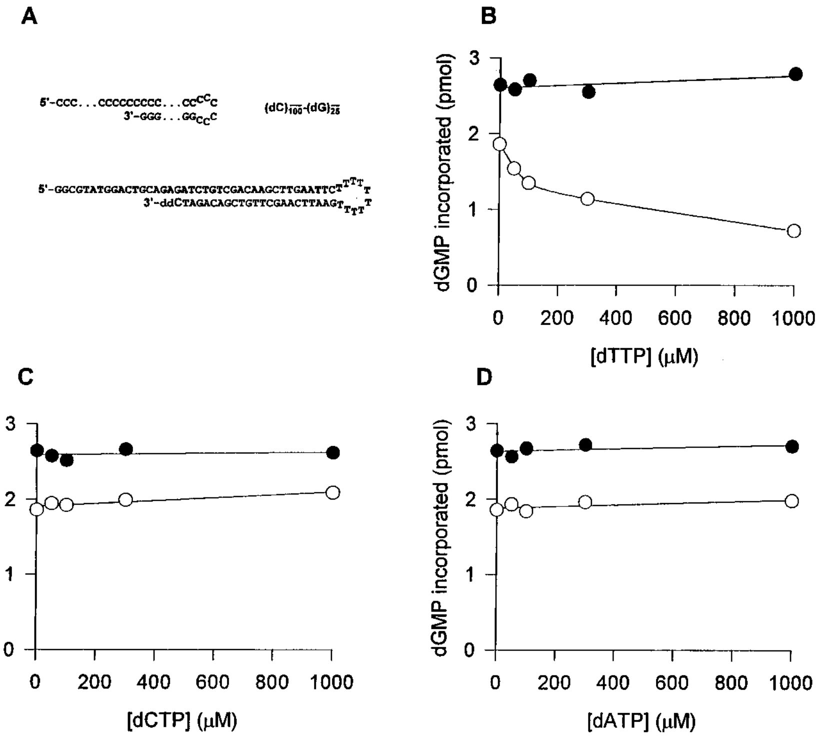

FIGURE 4: Substrate (dNTP) inhibition of pol ϵ from human placenta induced by a dideoxynucleotide-terminated primer annealed to a complementary template. Pol ϵ from human placenta was incubated in a mixture containing 80 mM potassium phosphate, pH 7.0, 5 mM $MgCl_2$, 20 μg/mL bovine serum albumin, 1 mM DTT, 6 μM [α-^{32}P]dGTP (5000 cpm/pmol), and 15 μM (nucleotide) hook homopolymer $(dC)_{100}-(dG)_{25}$ substrate with or without 2 μM (nucleotide) 72-mer-ddCMP competitor. (A) Structure of the hook homopolymer substrate $(dC)_{100}-(dG)_{25}$ and the competitor 72-mer-ddCMP. (B−D) Effect of adding dNTPs, as indicated on the abscissa in each panel, on the incorporation of dGMP on $(dC)_{100}-(dG)_{25}$ in the presence (○) or absence (●) of nucleic acid competitor 72-mer-ddCMP.

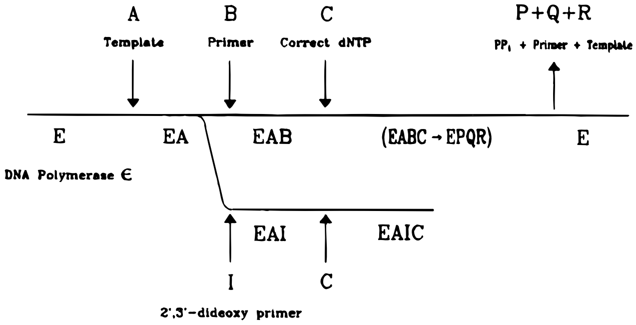

FIGURE 5: Partial steady-state kinetic model of pol ϵ from human placenta. Diagram was constructed according to Cleland (1970). No specific information is available on the order of product release; hence, all products are shown as being released in a single step.

2′,3′-dideoxy-primer, "normal" primer binding occurs, thus allowing subsequent template-directed dNTP recognition. However, catalysis is not possible since the 2′,3′-dideoxy-primer lacks the 3′-OH group necessary for nucleophilic attack on the incoming dNTP. By increasing the concentration of the template-specified dNTP, pol ϵ can be trapped in a dead-end template•dideoxy-primer•dNTP complex.

Enzymologic Comparison of Gap-Filling by Pol α, Pol δ, and Pol ϵ. In vivo, gap-filling is necessary during replication to complete lagging-strand Okazaki fragments before ligation as well as to terminate leading-strand replication. It is also required for DNA repair. To compare the relative abilities of pol α, pol δ, pol δ plus PCNA and pol ϵ to fill gaps, the hooked template and four primers shown (Figure 6) were synthesized. By annealing each of these primers separately to the complementary hooked template (Figure 6), it was possible to generate 4 template-primer molecules with gaps of 10, 7, 4, and 1 nucleotide(s), respectively. For all four, template base composition dictated dATP as the next dNTP for incorporation.

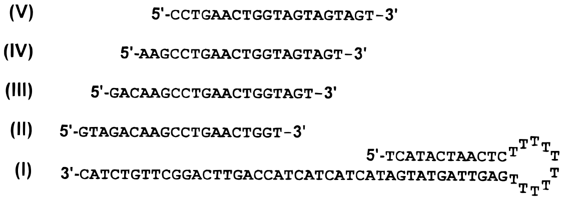

FIGURE 6: Substrates prepared for gap-filling experiments. Hooked 64-mer (I) was synthesized as a common template for all experiments. Four different 20-mer primers (II−V) were prepared, complementary to different regions of the 64-mer template. When annealed to 64-mer, various 20-mers generated gaps of 10 (II), 7 (III), 4 (IV), and 1 (V) nucleotide(s).

For each enzyme, standard enzymologic analysis of V versus [dATP] was performed; V_{max} and K_m (Table 1) were determined from nonlinear regression analysis of the primary data (Duggleby, 1981). Lineweaver−Burk double-reciprocal plots are shown (Figure 7). K_m is a complex term reflecting several rate constants. Hence, changes in K_m, particularly for a multisubstrate enzyme, are difficult to interpret. In contrast, when only a single dNTP is provided, V_{max} reflects only a single rate constant, k_{cat}. As the gap size decreased, the V_{max} of pol α dropped precipitously (decreased more than 6-fold) (Figure 8A; see also Figure 7A). With identical substrates, the V_{max} of pol ϵ increased more than 2.5-fold (Figure 8B; see also Figure 7B). Similar analyses of pol δ and pol δ plus PCNA revealed relatively little effect of gap size on the V_{max} of dAMP incorporation (less than a 1.4-fold change in each case) (Figure 8C,D; see also Figure 7C,D). For all four (pol α, pol ϵ, pol δ, and pol δ plus PCNA), the product of dAMP incorporation on the substrate

Table 1[a]

	pol α		pol ϵ		pol δ		pol δ + PCNA	
gap size	V_{max}[b]	K_m[c]	V_{max}[b]	K_m[c]	V_{max}[b]	K_m[c]	V_{max}[b]	K_m[c]
10	0.96 ± 0.031	2.13 ± 0.189	0.19 ± 0.016	1.20 ± 0.346	0.53 ± 0.119	11.1 ± 3.64	0.76 ± 0.050	7.12 ± 1.037
7	0.70 ± 0.028	1.80 ± 0.207	0.23 ± 0.031	1.48 ± 0.629	0.93 ± 0.377	29.2 ± 15.09	1.18 ± 0.107	9.06 ± 1.541
4	0.71 ± 0.050	3.39 ± 0.503	0.38 ± 0.033	2.28 ± 0.493	0.62 ± 0.104	18.8 ± 4.09	1.04 ± 0.072	6.80 ± 1.019
1	0.15 ± 0.009	1.50 ± 0.472	0.49 ± 0.037	2.50 ± 0.496	0.44 ± 0.107	18.3 ± 5.79	1.04 ± 0.021	1.73 ± 0.154

[a] Values for V_{max} and K_m, plus and minus one standard error, were calculated by nonlinear regression of the primary data (Duggleby, 1981). [b] Values for V_{max} are in pmol/h. [c] Values for K_m are in μM.

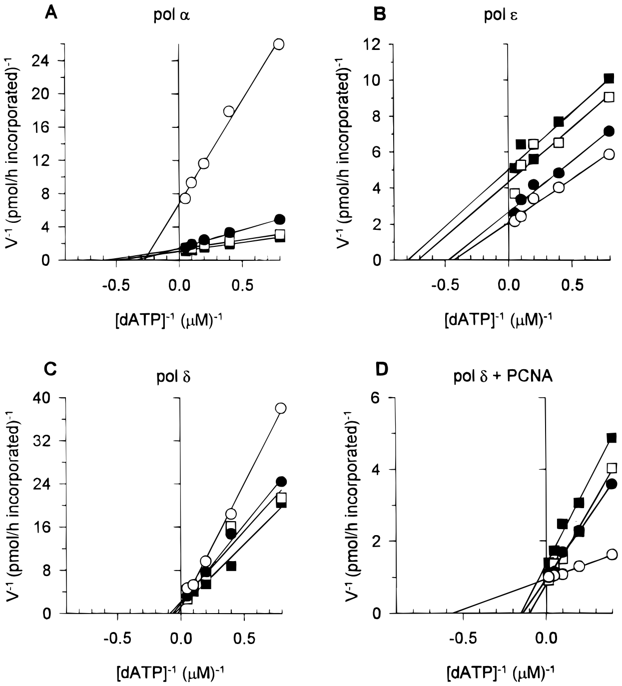

FIGURE 7: Lineweaver−Burk analysis of velocity versus dATP concentration on template-primers with varying gap size. Standard reaction mixtures (Experimental Procedures) were formulated and incubated with enzyme for 30 min at 37 °C. Template-primers with gaps of 10 (■), 7 (□), 4 (●), or 1 nucleotide(s) (○) were used. The concentration of dATP was varied as indicated. (A) Each incubation contained 0.1 unit of pol α. (B) Each incubation contained 0.05 unit of pol ϵ. (C) Each incubation contained 0.02 unit of pol δ. (D) Each incubation contained 0.02 unit of pol δ plus 70 ng of PCNA.

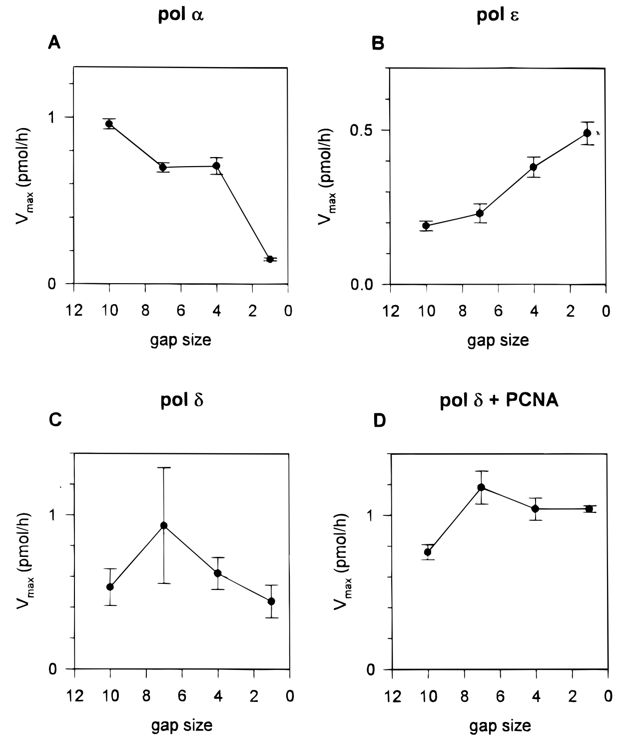

FIGURE 8: Effect of gap size on V_{max} for dATP. V_{max} data from Table 1 are plotted versus template-primer gap size. (A) Pol α. (B) Pol ϵ. (C) Pol δ alone. (D) Pol δ plus PCNA.

with a single-nucleotide gap could be effectively joined (ligated) to the hook template by incubation after polymerase reaction, with T4 DNA ligase (not shown).

DISCUSSION

We showed that human pol ϵ, purified from placenta, follows an ordered sequential ter-reactant mechanism of substrate recognition and binding; template is bound first followed by template-complementary primer and subsequently by template-specified dNTP. While this result was perhaps expected based on comparison with both pol α (Fisher & Korn, 1981; Wong et al., 1986; Ng et al., 1989) and pol δ (Ng et al., 1991), these studies are nevertheless an essential prerequisite to further enzymologic investigation. In addition, it is tempting to speculate that the ordered sequential ter-reactant mechanism is general, at least for replicative DNA polymerases in eukaryotes. Mechanisms elucidated for two putative repair enzymes, *E. coli* DNA polymerase I (McClure & Jovin, 1975) and mouse DNA polymerase β (Tanabe et al., 1979), are also consistent; moreover, reports of dead-end complex formation between enzyme and chemically modified primers suggest that a similar mechanism pertains to mammalian DNA polymerase β (Pelletier et al., 1994), herpes virus DNA polymerase (Reardon & Spector, 1989), and HIV reverse transcriptase (Tong, 1995).

Two experimental results were crucial in establishing this mechanism for human pol ϵ. The first was the demonstration that of nonsubstrate DNA molecules, only single-stranded and not double-stranded DNA competed with DNA for pol ϵ activity. This, in conjunction with results of direct sedimentation binding to single-stranded DNA, demonstrates that free pol ϵ recognizes only template (single-stranded DNA) as its first substrate. The second was induced dNTP inhibition in the presence of a template-annealed 2′,3′-dideoxy-terminated primer. In this context, induced substrate inhibition by the template-specified dNTP is diagnostic for the ordered ter-reactant mechanism (Cleland, 1979; Fisher & Korn, 1981).

The observation that pol ϵ binds to a 2′,3′-dideoxy-terminated primer in a way which permits binding of the next template-specified dNTP also has practical implications. Because the complex between pol ϵ and a dideoxy-terminated primer-template can be stabilized by the template-specified dNTP, dideoxy-terminated primer-templates may provide specific affinity ligands for rapid and gentle enzyme purification. Presumably, pol α and pol δ would copurify with pol ϵ in such a scheme.

What is the biological role of pol ϵ? According to current models of eukaryotic replication (Waga & Stillman, 1994), initiation occurs when pol α-associated DNA primase synthesizes a short RNA primer which is then extended for a short distance by the DNA polymerase activity of pol α. Further growth of the nascent strand is then catalyzed by pol δ in association with auxiliary factors, PCNA and replication factor C. On the leading strand, a single priming event by pol α is apparently sufficient; processive synthesis by pol δ-holoenzyme can account for complete or nearly-complete leading strand replication. Because of the antiparallel nature of DNA strands, lagging strand replication must be discontinuous with multiple pol α-dependent reinitiation events as the fork progresses. After initiation, pol δ-dependent lagging strand replication ensues.

Although pol δ and even pol α can both fill a gap to ligatable completion, at least *in vitro*, we showed that only pol ϵ incorporates deoxyribonucleotide with increased efficiency as gap size is decreased. Hence, pol ϵ is ideally suited to a gap-filling role *in vivo*. Necessarily, this would be much more apparent on the lagging strand where many more gaps occur. Indeed, the reported interaction between pol ϵ and PCNA (Lee et al., 1991; Maga & Hübscher, 1995), though controversial (Chui & Linn, 1995), provides an obvious hypothetical mechanism whereby pol ϵ may become involved in DNA synthesis *in vivo*. To act as a cofactor for both pol δ and pol ϵ, we assume that PCNA has specific binding sites for both enzymes. We also assume that PCNA can only bind one polymerase at a time. If pol δ dissociates from PCNA as gaps near completion, pol ϵ could replace it to complete gap-filling efficiently and thereby free pol δ for extended processive synthesis elsewhere.

To this point, discussion of the biological role of pol ϵ has focused on DNA replication. However, there is a growing body of evidence that PCNA and proteins with which it interacts are also involved in DNA repair (Nichols & Sancar, 1992; Shivji et al., 1992; Stivala et al., 1993; Kvam & Stokke, 1994; Matsumoto et al., 1994). Moreover, factors such as p21 which bind PCNA appear to inhibit replication specifically (Waga et al., 1994). A hypothetical mechanism by which this could occur would be if p21 blocked the pol δ-binding site on PCNA but not the pol ϵ-binding site. We would further speculate that pol ϵ but not pol δ is required for DNA repair. Indeed, gap-filling would be an essential part of any repair mechanism currently thought to operate [see, e.g., Sancar, (1994) and references cited therein].

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