DNA Polymerase ϵ Interacts with Proliferating Cell Nuclear Antigen in Primer Recognition and Elongation†

Giovanni Maga and Ulrich Hübscher*

Department of Veterinary Biochemistry, University of Zürich-Irchel, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

Received September 12, 1994; Revised Manuscript Received October 31, 1994⊗

ABSTRACT: Kinetic analysis of DNA polymerase ϵ in its interaction with the homopolymeric template-primer poly(dA)/oligo(dT) and a singly-primed synthetic oligonucleotide of defined sequence indicated that primer utilization is inhibited by single-stranded DNA. Long single-stranded DNA regions appear to sequester DNA polymerase ϵ via nonproductive binding, thus reducing its catalytic efficiency. Proliferating cell nuclear antigen can reduce this nonproductive effect by increasing the rate of primer binding by DNA polymerase ϵ. Once the complex between DNA polymerase ϵ and the primer is formed, proliferating cell nuclear antigen can increase the rate of nucleotide incorporation. The results suggested a dual role of proliferating cell nuclear antigen in stimulating the activity of DNA polymerase ϵ, namely, first to facilitate primer binding and second to stimulate the synthetic activity itself. A model for the interaction between these two proteins in DNA synthesis is discussed.

In eukaryotes, five different DNA polymerases (pol's)[1] have been identified and termed respectively α, β, γ, δ, and ϵ (Burgers et al., 1990). Three of them, namely, pol's α, δ, and ϵ, have been shown by genetic and biochemical studies to participate in nuclear DNA replication [reviewed in Hübscher and Spadari (1994)]. The identification and characterization of cellular proteins required for SV40 DNA replication *in vitro* indicated that additional factors such as PCNA, RF-C, and RP-A are required for efficient DNA synthesis [reviewed in Wang (1991)]. Initiation of DNA replication involves the synthesis of a primer by the primase activity associated with pol α. Then RF-C, PCNA, and ATP can form a primer recognition complex at the 3′-OH primer on the leading strand. This step results in the displacement of pol α and the binding of pol δ at the leading strand. Pol α then moves to the lagging strand to synthesize the first Okazaki fragment. Pol α elongates the primer only for 30−50 nucleotides (Hurwitz et al., 1990); then probably PCNA and RF-C form a primer recognition complex also on the lagging strand. This results in a switching between pol α and another pol which completes the synthesis of the Okazaki fragment (Tsurimoto et al., 1990; Tsurimoto & Stillman, 1991). The identity of this pol is still uncertain, since both pol δ and pol ϵ could be involved in lagging strand synthesis.

Pol ϵ was initially discovered in the yeast *Saccharomyces cerevisiae* and called pol II (Wintersberger & Wintersberger, 1970). Later it was discovered in higher eukaryotic cells (Crute et al., 1986; Focher et al., 1988, 1989; Syvaoja & Linn, 1989; Morrison et al., 1990; Somashekarappa & Karumathil, 1993) and named pol ϵ in 1990 (Burgers et al., 1990). Like pol δ, it contains in the same polypeptide both DNA polymerase and 3′→5′ exonucleolytic activities (Kesti & Syväoja, 1991). Most compelling among the enzymologic differences between pol ϵ and δ is their response to the auxiliary factor PCNA. In the presence of PCNA, pol δ is highly processive; in its absence, it functions poorly and incorporates only few nucleotides per binding event (Tan et al., 1986; Weiser et al., 1991). In contrast, pol ϵ is processive by itself, and it is apparently unresponsive to the addition of PCNA (Focher et al., 1988, 1989; Morrison et al., 1990; Syvaoja & Linn, 1989; Syvaoja et al., 1990; Bambara & Jessee, 1991; Weiser et al., 1991). In addition, pol ϵ, like pol δ, can form in the presence of RF-C and ATP a stable complex with PCNA at the 3′-OH end of a primer, and this complex is called pol δ or ϵ holoenzyme (Burgers, 1991; Lee et al., 1991; Podust et al., 1992). PCNA itself does not have an intrinsic affinity for DNA, but is able to interact with pol δ on linear DNA in the absence of other auxiliary factors. It depends, however, absolutely on RF-C for loading of PCNA on circular DNA (Burgers & Yoder, 1993). Recently it has been suggested that RF-C is required only for efficient loading of PCNA on the DNA, without participating further in catalysis (Podust et al., 1994), suggesting that the functional properties of pol δ and ϵ holoenzymes are mostly due to their interaction with PCNA.

PCNA has been found in all eukaryotic tissues tested. They include protozoa (Olins et al., 1989), the yeast *S. cerevisiae* (Burgers, 1991) and *S. pombe* (Waseem et al., 1992), *Xenopus* (Leibovici et al., 1990), Drosophila (Yamaguchi et al., 1990), mammalian (Matsumoto et al., 1987), and higher plants (Suzuka et al., 1989). The amino acid sequence of PCNA is highly conserved, indicating an essential and similar role of this protein. Furthermore, yeast PCNA can stimulate mammalian pol δ, and mammalian PCNA can stimulate yeast pol δ, suggesting an exstensive conservation of the protein−protein interaction (Bauer & Burgers, 1988). In its active form, PCNA forms a trimer, which is postulated

† This work was supported by the EMDO Stiftung, Zürich (grant-in-aid to G.M.), the Kanton of Zürich, and the "Bundesamt für Bildung und Wissenschaft", who sponsors the Swiss part of a EU "Human Capital and Mobility Program".

* Correspondence should be addressed to this author. Telephone: 411 257 54 72. FAX: 411 362 05 01.

⊗ Abstract published in *Advance ACS Abstracts,* January 1, 1995.

[1] Abbreviations: pol, DNA polymerase; SV40, simian virus 40; PCNA, proliferating cell nuclear antigen; RF-C, replication factor C; RP-A, replication protein A; ss, single stranded; ds, double stranded; sp, singly primed; BSA, bovine serum albumin; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

0006-2960/95/0434-891$09.00/0

to bind topologically to the DNA in analogy to the β subunit of *Escherichia coli* pol III holoenzyme (Kong et al., 1992). Yeast PCNA and the β subunit have been crystallized, and they have been shown to be structurally superimposable, despite less than 5% primary amino acid sequence conservation [reviewed in Campbell (1993)]. In addition, PCNA is involved also in nucleotide excision repair (Shivji et al., 1992). Since pol ϵ has also been found to be involved in the latter DNA transaction (Nishida et al., 1988), it is likely that a functional interaction between pol ϵ and PCNA might occur. The apparent unresponsiveness of pol ϵ to PCNA on linear DNA, in the absence of RF-C and RP-A, suggested a different mode of interaction of pol δ and ϵ with PCNA. In this paper, we investigated the mechanism of pol ϵ interaction with linear substrates and show that PCNA increases the primer binding as well as the nucleotide incorporation catalyzed by pol ϵ, suggesting a possible role of PCNA as a modulatory factor for pol ϵ in various DNA transactions.

MATERIALS AND METHODS

Chemicals. [^{3}H]dTTP (40 Ci/mmol), [^{3}H]dCTP (40 Ci/mmol), [^{3}H]dATP (40 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were from Amersham, and unlabeled dNTP's were from Boehringer. Whatman was the supplier of the GF/C and DE-81 filters. All other reagents were of analytical grade and purchased from Merck and Fluka.

Nucleic Acid Substrates. The homopolymer poly(dA)$_{400}$ (Pharmacia) was mixed at weight ratios in nucleotides of 5:1, 10:1, 15:1, or 30:1 to the oligomer oligo(dT)$_{12-18}$ (Pharmacia) in 20 mM Tris-HCl (pH 8.0), containing 20 mM KCl and 1 mM EDTA, heated at 65 °C for 5 min, and then slowly cooled at room temperature. d15-mer and d61-mer synthetic oligodeoxyribonucleotides were synthesized at the Institute of Zoology of the University of Zürich and purified to homogeneity on a 20% polyacrylamide gel. The sp d61:d15 was prepared by mixing d15-mer oligonucleotide and d61-mer oligonucleotide in a 1:1 molar ratio in 20 mM Tris-HCl (pH 8.0), containing 20 mM KCl and 1 mM EDTA. The mixture was heated at 90 °C for 3 min, then incubated at 65 °C for 2 h, and cooled slowly at room temperature. 5′-Labeled d15-mer or d61-mer was prepared by enzymatic phosphorylation with T_4 polynucleotide kinase (Boehringer) according to the manufacturer's protocol. Phosphorylated d15-mer oligonucleotide was then annealed to d61-mer as described above, without any isotopic dilution in order to prepare 5′-labeled sp d61:d15 oligonucleotide.

Enzymes and Proteins. DNA polymerase ϵ was purified from fetal calf thymus as described (Weiser et al., 1991) and was composed of two subunits of 145 and 45 kDa, respectively. In our preparation, 1 pmol of pol ϵ corresponded to 2 units of enzyme. One unit of enzyme activity corresponds to the incorporation of 1 nmol of total dTMP into acid-precipitable material in 60 min at 37 °C in a standard assay containing 0.5 μg (nucleotides) of poly(dA)/oligo(dT)$_{10:1}$ and 20 μM dTTP. Human PCNA was overexpressed in *E. coli* strain BL21(DE3) harboring the expression plasmid pT7/PCNA and purified as described by Fien and Stillman (1992).

Steady-State Kinetic Measurements. A reaction mixture contained the following in a final volume of 25 μL: 50 mM Bis-Tris (pH 6.6), 6 mM $MgCl_2$, 0.25 mg/mL BSA, and 1 mM DTT. Pol ϵ, PCNA, template-primer, and nucleotides were added as indicated in the figure legends. All reactions were incubated at 37 °C for 5 min and precipitated with trichloroacetic acid, and the insoluble radioactive material was determined as described (Hübscher & Kornberg, 1979). For the determination of steady-state kinetic parameters for single-nucleotide incorporation into d61:d15-mer, the final volume of the reaction mixtures was 50 μL. Reaction were incubated for 15 min at 37 °C and then stopped with 5 μL of 0.5 M EDTA, pH 8.0; 40 μL samples were taken and spotted onto DE-81 cellulose filters. Filters were immediately immersed in 0.3 M ammonium formate, washed twice in the same buffer, rinsed once in distilled water and once in ethanol, and then dried. Bound radioactivity was determined as described (Focher et al., 1989).

Blanks consisting of 40 μL samples of the reaction mixture incubated without the enzyme were counted together with the samples in a scintillation counter (Beckman LCS 6000) in the automatic background subtracting mode.

Gel-Retardation Assays. Pol ϵ, PCNA, 5′-labeled sp d61:d15 oligonucleotide, or ss 61-mer oligonucleotide were added as described in the figure legend to the standard reaction mixture described above and incubated at 22 °C. After the indicated time, samples were incubated 2 min at 22 °C in the presence of 5% glutaraldehyde, mixed with sample loading buffer (10% w/v sucrose, 0.2% w/v bromophenol blue, and 0.2% w/v xylene cyanol), and subjected to nondenaturing gel electrophoresis at 4 °C in a 8% polyacrylamide gel for 4 h at 250 V in 90 mM Tris−borate buffer/10 mM EDTA, pH 8.0. The gel was fixed with 12% (v/v) methanol and 10% (v/v) acetic acid, washed with distilled water, dried at 80 °C, and exposed to X-ray film (Fuji RX). Radioactive bands corresponding to the bound and free oligonucleotide were excised from the gel, mixed with scintillation cocktail (Emulsifier safe, Packard), and counted. Under our condition, no detectable smear of the bands was observed. Background was determined by counting an excised portion of the gel without radioactivity.

Inhibition of Pol ε Activity on Poly(dA)/Oligo(dT)$_{5:1}$ by Different Competitor DNA Molecules. Reactions were carried out in the standard reaction mixture in the presence of 1 nM pol ϵ and 5 μM [^{3}H]dTTP (8 Ci/mmol) by varing the substrate in the presence of different concentrations of the competitor DNA. Substrate concentrations used in all the experiments were 17, 34, 68, and 170 nM (in terms of 3′-OH ends). Inhibitor concentrations were varied from $0.25K_i$ to $4K_i$.

Short-Time Incorporation Kinetics. Pol ϵ, PCNA, poly(dA)/oligo(dT)$_{10:1}$, and labeled dTTP were added as indicated in the figure legends to the standard reaction mixture described above in a final volume of 50 μL. Reactions were incubated at 37 °C (unless otherwise stated) and stopped at the time intervals indicated in the figure legends with 5 μL of 0.5 M EDTA, pH 8; 40 μL samples were taken and spotted onto DE-81 cellulose filters. Filters were immediately immersed in 0.3 M ammonium formate, washed twice in the same buffer, rinsed once in distilled water and once in ethanol, and then dried. Bound radioactivity was determined as described (Focher et al., 1989).

Steady-State Kinetic Data Analysis. K_m, K_i, K_D, and V_{max} values were calculated according to the Michaelis−Menten equation. Fitting of the experimental data to the equation

Table 1: Sequences of the Synthetic Oligodeoxyribonucleotides Used in This Study

oligodeoxyribonucleotide	sequence
d61-mer template	3′-TCA TGC AGT AGA GCC TGG ACG ACG TAG TGG TAG TGG TAG TGA TCT TAA GCA GCT GGA GCT C-5′
d15-mer primer	5′-AGT ACG TCA TCT CGG-3′

was performed with the computer program "Enzyme Kinetics" (Dog Star Software, Bloomington, IN).

Protein and Nucleic Acid Determination. PCNA concentration was determined according to Bradford (1976). Poly(dA)$_{400}$ and oligo(dT)$_{12-18}$ concentrations were determined spectrophotometrically according to the manufacturer's protocol.

RESULTS

Active Site Titration of Pol ϵ. According to the molecular weight of pol ϵ, we estimated an enzyme concentration (in terms of proteins) of our preparation of 0.25 μM. In order to precisely measure which was the actual active fraction of our enzyme, we performed an active site titration with the sp d61:d15 oligonucleotide (Table 1). The incorporation of a single dAMP residue into the d61:d15-mer catalyzed by pol ϵ in the absence of other dNTPs was biphasic: a burst of product formation was observed followed by a slow steady-state rate. The rapid initial phase of the biphasic time course for single-nucleotide incorporation by pol ϵ was observable at 4 °C (Figure 1a). The amplitude of the burst for single dAMP incorporation increased linearly with increasing concentrations of pol ϵ (Figure 1b). The burst of product formation was not dependent upon preincubation of the d61:d15-mer with the enzyme, suggesting that template-primer binding to the enzyme was not the rate-limiting step. As discussed below, under the conditions used the nonspecific binding to the ss template by pol ϵ was negligible. Since the amount of product formed in the burst phase is generated by a single turnover of the enzyme, its concentration gives an estimate of the pol ϵ active sites concentration in the reaction. From the slope of a replot of the amplitude of the burst *versus* the amount of pol ϵ added (expressed as protein concentration), an active site concentration of 0.4 pmol (±0.08)/pmol of pol ϵ was obtained (Figure 1c), indicating that 40% of our enzyme preparation was active. This value was used in all the subsequent experiments for the calculation of the enzyme concentration and of the k_{cat} values.

Affinity of Pol ϵ for the 3′-OH End Is Reduced by Increasing the Molar Ratio of ss DNA to the Primer. First, pol ϵ was titrated in the presence of 100 nM (3′-OH ends) of poly(dA)$_{400}$/oligo(dT)$_{12-18}$ at different template:primer ratios ranging from 5:1 to 30:1 (in nucleotides). As shown in Figure 2, the incorporation was linear during the time of incubation, and the measured velocities at each time point corresponded to the utilization of less than 10% of the available substrate, thus representing the actual initial rates for nucleotide incorporation. Next the K_m value of pol ϵ for the primer under these conditions was determined, with poly(dA)/oligo(dT) at different template:primer ratios. The K_m values calculated from double-reciprocal plots of the initial velocities measured are listed in Table 2. The K_m values for the primer increased as the template:primer ratio increased, ranging from a value of 28 nM for poly(dA)/oligo(dT) at 5:1 to a value of 100 nM for poly(dA)/oligo(dT) at 30:1. In order to uncover the contribution given by ss DNA to the primer binding reaction of pol ϵ, different concentrations of poly(dA)/oligo(dT)$_{5:1}$ were titrated in the absence or in the presence of free ss poly(dA)$_{400}$ molecules at final ssDNA:primer ratios of, respectively, 10:1, 15:1, and 30:1. Again, the affinity of pol ϵ for the primer decreased as the molar excess of ss DNA over the primer increased. The K_m values listed in Table 2 were comparable with those calculated in the previous experiment, ranging from 30 nM for a ssDNA:primer ratio of 5:1 to 110 nM for a ratio of 30:1.

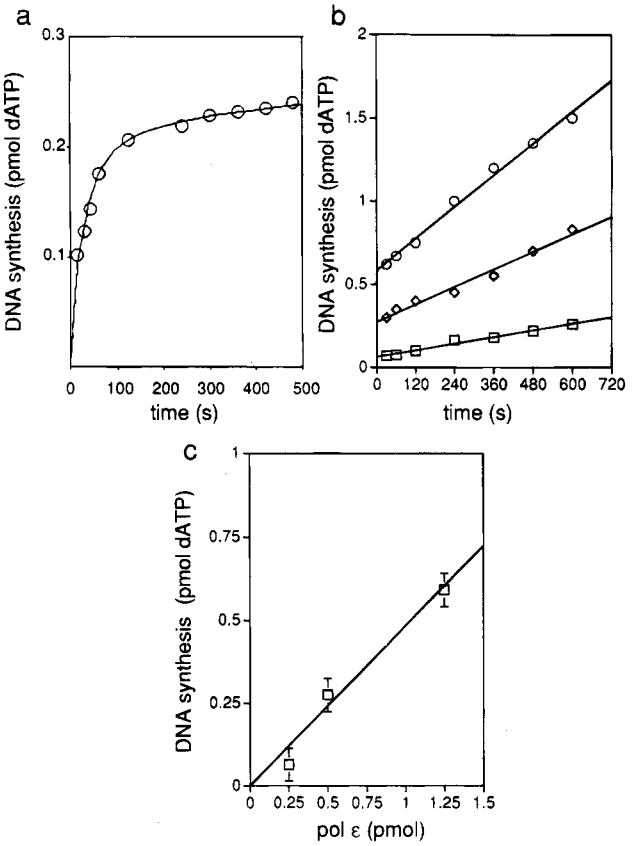

FIGURE 1: Active site titration of pol ϵ. (a) Biphasic time course of single-nucleotide incorporation into d61:d15-mer catalyzed by pol ϵ at reduced temperature. Reactions were carried out at 4 °C as described under Materials and Methods in the presence of 20 nM pol ϵ and 100 nM d61:d15-mer (3-OH ends). Reactions were started by the addition of 2 μM [^{3}H]dATP (40 Ci/mmol). (b) Kinetics of single-nucleotide incorporation into d61:d15-mer catalyzed by pol ϵ. Reactions were carried out as described under Materials and Methods in the presence of 100 nM d61:d15-mer (3′-OH ends), 2 μM dATP, and 0.25 pmol (squares), 0.5 pmol (rhombics), or 1.25 pmol (circles) of pol ϵ (in terms of proteins). (c) Replot of the intercepts extrapolated from the experiments shown in panel b versus pol ϵ concentration.

The Primer Utilization Catalyzed by Pol ϵ Is Inhibited by ss DNA. In order to analyze the inhibition of ss DNA on the primer utilization by pol ϵ, the enzyme was competed with different ss DNA molecules of increasing length in single-strandness. In all cases tested, the inhibition was competitive with the 3′-OH primer (data not shown). As shown in Table 2, the calculated K_i values of the competitor

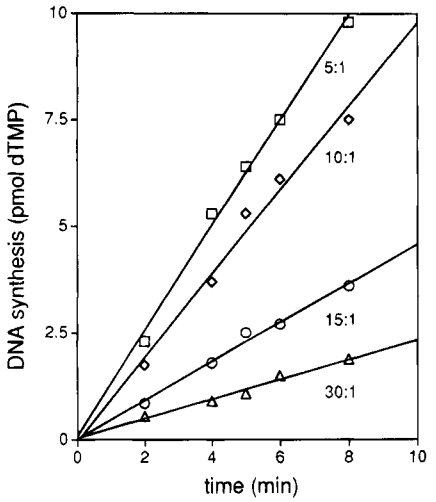

FIGURE 2: Kinetics of dTMP incorporation catalyzed by pol ϵ on poly(dA)/oligo(dT) at different template:primer ratios. Reactions were carried out as described under Materials and Methods in the presence of 1 nM pol ϵ, 100 nM substrate (3′-OH ends), and 5 μM [^{3}H]dTTP (8 Ci/mmol). Poly(dA)/oligo(dT) was tested at the following template:primer ratios (in nucleotides): squares, 5:1; rhombics, 10:1; circles, 15:1; triangles, 30:1.

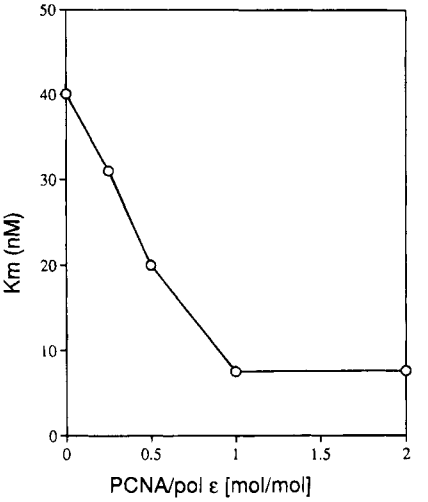

FIGURE 3: PCNA decreases the K_m value of pol ϵ for the primer if poly (dA)/oligo(dT)$_{10:1}$ was used as template. Reactions were carried out as described under Materials and Methods in the presence of 7, 17, 34, 68, and 170 nM template (in terms of 3′-OH ends), 1 nM pol ϵ, and 5 μM [^{3}H]dTTP (8 Ci/mmol).

ss DNA indicated that the affinity of pol ϵ for the ss DNA augmented as the single-strandness length increased. The K_i value for the ss poly(dA)$_{400}$ was found to be 15 μM (in nucleotides). By comparison, a ss 15-mer oligonucleotide showed a K_i value of approximately 1 mM (in nucleotides) and circular ss M13 DNA (7229 nucleotides length) one of 0.2 μM (in nucleotides). In sum, these results supported the hypothesis that the reduction of the affinity of pol ϵ for the primer in the presence of increasing template:primer ratios is due to the competitive inhibition of pol ϵ by the ss DNA.

PCNA Increases the Affinity of Pol ϵ for the Primer. Next the K_m value of pol ϵ for the primer with poly(dA)/oligo(dT) at different template:primer ratios was measured in the presence of human PCNA. The K_m values listed in Table 2 indicated that PCNA increased the affinity of pol ϵ for the primer. The K_m value for the primer with poly(dA)/oligo(dT) at a molar ratio 5:1 was reduced from 28 to 6.5 nM (3′-OH ends), whereas at a molar ratio of 30:1 the K_m value was reduced from 100 to 13 nM. Thus, it appears that, at high molar excess of ss DNA over primer, PCNA stimulated the affinity of pol ϵ for the primer 4−8-fold. In the presence of PCNA, the difference between the K_m values at, respectively, 5:1 and 30:1 template:primer ratios was only 2-fold, whereas in its absence it was nearly 4-fold (Table 2). As shown in Figure 3, the effect of PCNA was dependent upon the concentration of both PCNA and pol ϵ, with a maximal reduction of the K_m value at a 1:1 molar ratio, suggesting a direct protein−protein interaction (see below and also Figure 6).

The Effect of PCNA Is Specific for Primed DNA. Pol ϵ was competed with ss or sp DNA molecules in the absence or in the presence of PCNA. As shown in Table 2, PCNA did not change the K_i values for the inhibition of pol ϵ by ss DNA molecules of different lengths. However, when pol ϵ was competed with different concentrations of a sp d61:d15 oligonucleotide, in the presence of 5:1 poly(dA)/oligo(dT) as the substrate, the resulting inhibition was competitive with the 3′-OH primer and the calculated K_i value was 6 nM (3′-OH ends). If given as nucleotide concentration, this value was 0.45 μM. In comparison, the K_i value for the ss d61-mer (Table 2) was 200 μM (in nucleotides). Thus, the lower K_i value of pol ϵ for the sp d61:d15, with respect to the ss

Table 2: Effect of ss DNA and PCNA on the Affinity of Pol ϵ for the Primer Terminus

			K_m(3′-OH) (nM)		K_i (μM)	
template	base ratio[a]	competitor	−PCNA	+PCNA	−PCNA	+PCNA
poly(dA)/oligo(dT)						
	5:1		28	6.5		
	10:1		40	7.5		
	15:1		75	9.2		
	30:1		100	13		
		ss poly(dA)$_{400}$[b]				
	5:1	none	30	nd[c]		
	5:1	5	45	nd		
	5:1	10	77	nd		
	5:1	25	110	nd		
poly(dA)/oligo(dT)						
	5:1	ss 15-mer			>1000	>1000
	5:1	ss 61-mer			200	200
	5:1	ss poly(dA)$_{400}$			15	17
	5:1	ss M13 mp9			0.2	0.2
	5:1	sp d61:d15			0.45	0.15

[a] Picomoles of nucleotides. [b] Addition of ss poly(dA) expressed as molar excess in molecules of the primers. [c] nd, not determined.

d61-mer, likely reflects the higher affinity of the enzyme for the primer. The same experiment was repeated in the presence of PCNA and pol ϵ in equimolar amounts, and, as shown in Table 2, PCNA reduced both the K_m value of pol ϵ for the substrate poly(dA)/oligo(dT)$_{5:1}$ and the K_i value for the sp d61:d15 oligonucleotide. These results suggested that PCNA specifically increased the affinity of pol ϵ for primed but not for unprimed ss DNA.

Study of the Interaction between Pol ϵ and a Synthetic Singly-Primed Oligonucleotide by a Gel-Retardation Assay. PCNA reduced the apparent equilibrium dissociation constant (K_i) for the primer binding reaction of pol ϵ, but this fact did not permit discrimination of the effect of PCNA on the rate constants for primer binding (k_{on}) and primer dissociation (k_{off}). In fact, a given K_i as the ratio k_{off}/k_{on}, the observed apparent increase in the affinity of pol ϵ for the 3′-OH primer in the presence of PCNA, could be explained either as an increase in the k_{on} value (higher rate of primer binding) or as a decrease in the k_{off} value (increased residence of pol ϵ at the primer end). In order to address this question, we developed a gel-retardation assay which allowed us to monitor directly the formation of the complex between pol ϵ and the primer in the absence of DNA synthesis. As substrate, the sp d61:d15 oligonucleotide was used, since it represented a defined primer-template carrying only one primer for each template molecule. Moreover, we have already shown that the interaction between this substrate and pol ϵ at the concentrations used in our experiments is only due to the binding of the enzyme to the primer (Table 2; compare the K_i values of ss 61-mer and sp d61:d15). As a first experiment, the equilibrium dissociation constant for the primer binding reaction, K_D,[2] was measured. Preformed complexes between pol ϵ and different concentrations of the 5′-end labeled template in the absence or in the presence of PCNA were fixed with glutaraldehyde and separated from the free oligonucleotide by nondenaturing PAGE. The retarded bands as well as the unbound oligonucleotide bands were identified by autoradiography and excised, and the radioactivity was determined by counting. The formation of the complex between pol ϵ and the sp oligonucleotide showed a hyperbolic dependence on the concentration of the primer (Figure 4b). In the absence of PCNA, the K_D value of pol ϵ for the template was 6 nM, whereas in the presence of PCNA this value decreased to 2.2 nM. These values were in good agreement with those previously calculated from the inhibition experiments (Table 2). No supershifts of the retarded bands were detected in the presence of PCNA. This fact was interpreted as an indication that under these conditions (i.e., in the absence of DNA synthesis), PCNA does not form a stable complex with pol ϵ at the 3′-OH end of the primer. We cannot exclude, however, the possibility that glutaraldehyde could not efficiently cross-link PCNA to the pol ϵ−primer complex.

PCNA Does Not Influence the Rate of Dissociation (k_{off}) of Pol ϵ from the Primer End. Next the rate of dissociation of pol ϵ from the primer was measured in the absence and in the presence of PCNA. Pol ϵ and the 5′-end labeled d61:d15 oligonucleotide were incubated in the presence or in the

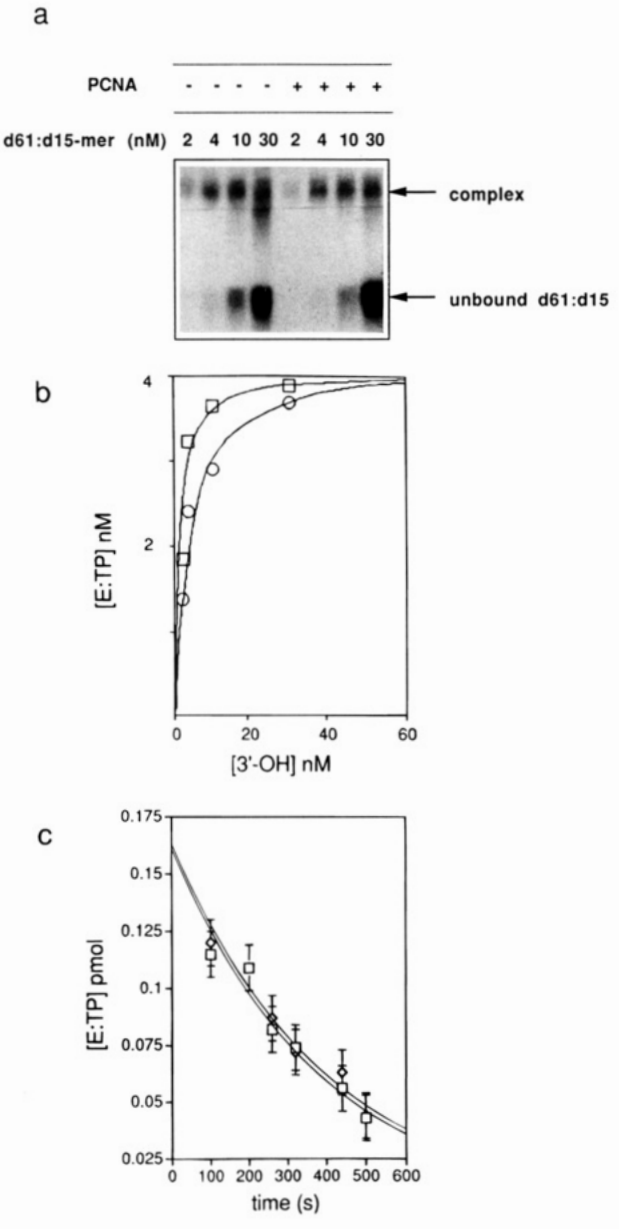

FIGURE 4: PCNA lowers the K_D value of pol ϵ for sp d61:d15 oligonucleotide but does not influence the rate of dissociation (k_{off}) of pol ϵ from the 3′-OH primer. (a) Determination of the K_D value of pol ϵ for sp d61:d15 oligonucleotide by a gel-retardation assay. 4 nM pol ϵ was incubated 5 min at 22 °C with different concentrations of 5′-γ-^{32}P-labeled sp d61:d15 oligonucleotide in the absence or in the presence of 4 nM PCNA under the assay conditions described under Materials and Methods. Gel electrophoresis was performed as described under Materials and Methods. Substrate concentrations tested were 2, 4, 10, and 30 nM (3′-OH ends). (b) Determination of the radioactivity in the area of the bandshift (indicated with arrow as complex) of panel a. Quantitation of the radioactivity was performed as described under Materials and Methods. Data were fitted to the hyperbolic equation: $[E:TP] = [E]_0/(1 + K_D/[TP])$. Circles, in the absence of PCNA; squares, in the presence of PCNA. (c) Determination of the k_{off} value for the complex between pol ϵ and the sp d61:d15 oligonucleotide. 15 nM pol ϵ and 30 nM 5′-γ-^{32}P-labeled sp d61:d15 oligonucleotide were incubated 5 min at 22 °C in the absence (squares) or in the presence (rhombics) of 15 nM PCNA under the assay conditions described under Materials and Methods. 1 μM (in nucleotides) of ss M13 competitor DNA was then added and the incubation continued for 8 min. Samples taken at different time points were fixed and subjected to electrophoresis as described under Materials and Methods. Quantification of the radioactivity corresponding to the complex and to the unbound oligonucleotide was performed as described under Materials and Methods.

[2] We indicated the equilibrium dissociation constant for the primer binding reaction of pol ϵ as K_i when derived from inhibition experiments or as K_D when derived by direct binding studies. In both cases, it represents the same quantity k_{off}/k_{on}.

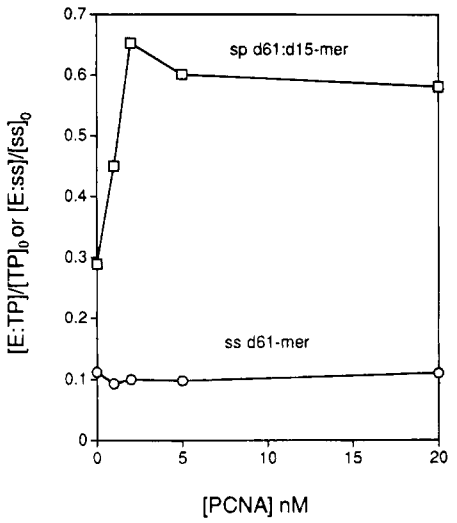

FIGURE 5: PCNA increases the rate of association (k_{on}) of pol ϵ with the primer. 2 nM pol ϵ and 5 nM 5′-γ-^{32}P-labeled sp d61:d15 oligonucleotide (squares) or 100 nM 5′-γ-^{32}P-labeled ss 61-mer oligonucleotide (circles) were incubated 5 min at 22 °C in the absence or in the presence of different amounts of PCNA under the assay conditions described under Materials and Methods. Quantitation of the radioactivity corresponding to the complex and to the unbound oligonucleotide was performed as described under Materials and Methods. PCNA concentrations were 0, 1, 2, 5, and 20 nM.

absence of PCNA to form complexes. The reaction was then chased with ss M13 DNA and incubation continued. Samples were taken at different time points and subjected to electrophoresis. Pol ϵ molecules which dissociated from the primer after chasing were thus sequestered by the competitor DNA. In this way, the amount of radioactivity in the retarded bands at each time point represented the remaining complex. The time course for dissociation of pol ϵ from the primer-template fitted a simple-exponential curve (Figure 4c). From this experiment, a value of 0.0012 s^{-1} was derived for the first-order dissociation constant k_{off}, both in the absence and in the presence of PCNA, estimated in a half-life for the pol ϵ−template complex of 7 min. In order to confirm these results, similar reactions with poly(dA)/oligo(dT)$_{5:1}$ were performed, and at different time points after chasing, the remaining complex was quantitated by a 30 s pulse with labeled dTTP and subsequent acid-precipitation, but without prior fixing of the complexes and electrophoretic separation. The resulting k_{off} value was 0.0015 s^{-1} and in good agreement with the value obtained from the gel-retardation assay (data not shown). PCNA did not change this value, regardless whether added before chasing or together with the competitor ss DNA, suggesting that PCNA did not influence the rate of dissociation of the pol ϵ−template complexes.

PCNA Specifically Increases the Association of Pol ϵ with the sp d61:d15 Oligonucleotide. In order to understand whether the observed decrease of the K_D value for primer binding of pol ϵ in the presence of PCNA reflected a true increase in association between the enzyme and the primer, pol ϵ was preincubated with a 5′-labeled spd61:d15 oligonucleotide or with a 5′-labeled ss d61-mer in the absence or in the presence of different amounts of PCNA. Complexes were then fixed with glutaraldehyde and subjected to nondenaturing PAGE. These results are quantitated in Figure 5. In the presence of PCNA, more than a 2-fold increase in the amount of the complex formed between pol ϵ and the sp d61:d15 oligonucleotide was observed, whereas no effect of

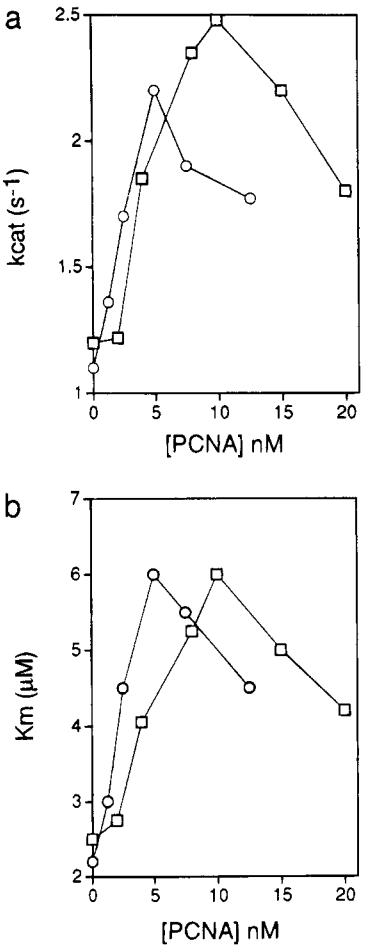

FIGURE 6: PCNA increases the k_{cat} and K_m values for the processive nucleotide incorporation reaction catalyzed by pol ϵ. K_m and V_{max} values were determined by incubating 5 nM (circles) or 10 nM (squares) pol ϵ in the presence of 100 nM poly(dA)/oligo(dT)$_{10:1}$ under the assay conditions described under Materials and Methods with the following different concentrations of [^{3}H]dTTP (8 Ci/mmol): 1, 2, 4, 10, and 15 μM in the absence or in the presence of increasing amounts of PCNA. (a) Effect of different concentrations of PCNA on the k_{cat} value for dTTP incorporation catalyzed by pol ϵ. k_{cat} values were derived by dividing the V_{max} (pmol s^{-1} units) of the reaction by the enzyme concentration. (b) Effect of increasing concentrations of PCNA on the K_m value of pol ϵ for dTTP.

PCNA could be detected on the complex formation between pol ϵ and the ss d61-mer, confirming that PCNA increased the specific association of pol ϵ with a primed DNA but not with an unprimed ss DNA.

PCNA Influences the Nucleotide Incorporation Catalyzed by Pol ϵ on Poly(dA)/Oligo(dT). The effect of PCNA on the K_m value of pol ϵ for the nucleotide substrate was apparently opposite to the one observed in the case of the primer binding reaction. In fact, PCNA increased the K_m value for dTTP with poly(dA)/oligo(dT)$_{10:1}$ as the template. In addition, a proportional increase in the V_{max} of the reaction was evident. This fact suggested that PCNA increased the rate of processive nucleotide incorporation (thereby increasing the k_{cat} value of the reaction) rather than decreased the affinity of pol ϵ for the nucleotide substrate. As shown in Figure 6, both the k_{cat} and the K_m values for dTTP incorporation increased in a PCNA-dependent manner. The relationship to the PCNA concentration was not linear, showing a bell-shaped curve. Increasing the amount of pol ϵ 2-fold did not change the shape of the curve, but resulted

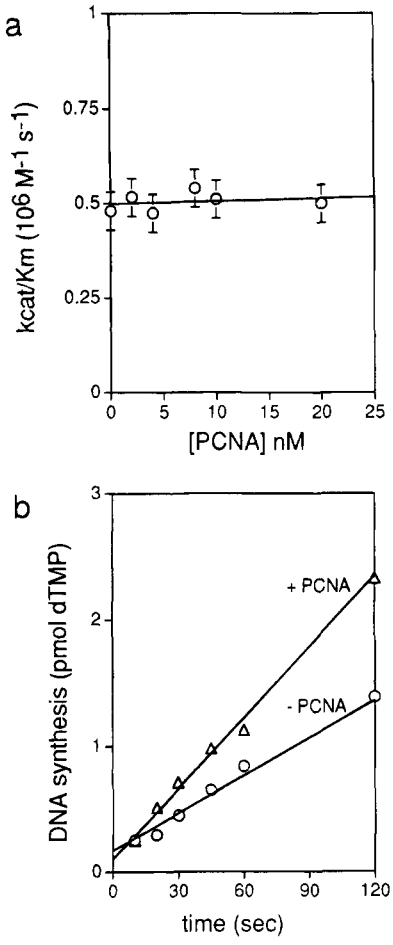

FIGURE 7: PCNA increases the rate of processive nucleotide incorporation catalyzed by pol ϵ: (a) Effect of increasing concentrations of PCNA on the k_{cat}/K_m values for dTTP incorporation catalyzed by pol ϵ. k_{cat} and K_m values were derived by the experiments shown in Figure 6. (b) Single-turnover kinetics for dTMP incorporation catalyzed by pol ϵ with poly(dA)/oligo-$(dT)_{10:1}$. 1 nM pol ϵ and 100 nM poly(dA)/oligo$(dT)_{10:1}$ were preincubated for 2 min at 37 °C under the assay conditions described under Materials and Methods in the absence (circles) or in the presence (triangles) of 1 nM PCNA. Reactions were started by the addition of 10 μM ss M13 competitor DNA and 5 μM [^{3}H]-dTTP (8 Ci/mmol), and the incubation was continued for 2 min. Samples were taken at different time points and quenched with ice-cold 100 mM EDTA. Time points were 10, 15, 30, 45, 60, and 120 s. Errors in determination of the time points due to handling were estimated to be ±2 s.

in a proportional shifting of the maximum to a 2-fold higher PCNA concentration. Maximal increase of the k_{cat} and the K_m values was observed in both cases at a 1:1 molar ratio between pol ϵ and PCNA.

PCNA Increases the Rate of Primer Elongation by Pol ϵ. Figure 7a shows that the apparent second-order constant for binding of dTTP by pol ϵ, k_{cat}/K_m, was not changed by PCNA, suggesting that the binding of PCNA to pol ϵ did not influence the recognition and binding of the nucleotide substrate. A possibility could be that the nucleotide incorporation step in the reaction pathway was affected. In order to test this, the rate of DNA synthesis of preformed pol ϵ−primer complexes during a single primer turnover, in the absence and in the presence of PCNA, was measured. This was possible by adding an excess of ss M13 competitor DNA during the elongation step. Thus, each pol ϵ molecule which dissociated from one primer could not rebind to a new one and initiate a new round of DNA synthesis. As shown in Figure 7b, PCNA increased the rate of processive nucleotide incorporation of pol ϵ during a single turnover of the enzyme.

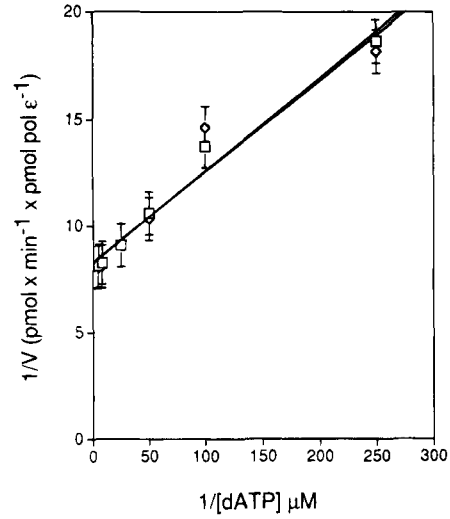

FIGURE 8: PCNA does not influence the K_m and k_{cat} values for single-nucleotide incorporation into d61:d15-mer catalyzed by pol ϵ. Reactions were carried out as described under Materials and Methods in the presence of 20 nM pol ϵ, 100 nM d61:d15-mer (3-OH ends), and increasing concentrations of [^{3}H]dATP (40 Ci/mmol) in the absence (squares) or in the presence (rhombics) of 20 nM PCNA.

PCNA Does Not Affect the K_m and k_{cat} Values for Single-Nucleotide Incorporation into d61:d15-mer Catalyzed by Pol ϵ. The K_m value for single dAMP addition catalyzed by pol ϵ with d61:d15-mer as the template-primer was 5 nM, and the steady-state k_{cat} value was 0.002 s^{-1}. Addition of PCNA together with dATP did not change these values significantly (Figure 8). The difference between the K_m and k_{cat} values, respectively, for processive and single-nucleotide incorporation, can be explained if the rate-limiting step of the single-nucleotide incorporation (k_{cat}) is the dissociation of the enzyme from the primer-template. As shown in Figure 1a, the single-nucleotide incorporation reaction catalyzed by pol ϵ is biphasic: a fast incorporation is followed by a slow rate-determining step. This step is likely to be the dissociation of the enzyme from the template, since the observed k_{cat} value is close to the k_{off} value of 0.0012 s^{-1} (see also Discussion and Scheme 1).

DISCUSSION

The observation that pol ϵ is able, as pol δ, to form a holoenzyme complex with RF-C and PCNA made the difference between these two enzymes subtile (Burgers, 1991; Lee et al., 1991; Podust et al., 1992). Results obtained recently in our laboratory suggested that RF-C is required for efficient loading of PCNA on the DNA without participating further in catalysis (Podust et al., 1994). We have therefore looked for conditions which permitted examination of the effect of the interaction of pol ϵ and PCNA in the *absence* of other auxiliary proteins. Enzymological studies performed in order to elucidate the mechanism of interaction between PCNA and pol δ showed that PCNA increases the low intrinsic processivity of pol δ by stabilizing the enzyme−primer complex, thus preventing spontaneous dissociation from the template (Ng et al., 1991, 1993). This analysis, however, was facilitated by the fact that pol δ *absolutely* requires PCNA for DNA synthesis. On the other hand, pol

ϵ has a high affinity for the template and is very efficient in both primer binding and extension, and these properties were not apparently changed by addition of PCNA under standard assay conditions. To detect subtle changes in the catalytic properties of pol ϵ in response to PCNA, we used in the present studies lower amounts of enzyme than in previous studies (Focher et al., 1988; Syvaoja & Linn, 1989; Weiser et al., 1991; Somashekarappa & Karumathil, 1993), and we searched for stimulation by PCNA in the presence of low concentrations of 3′-OH primer ends. Since pol ϵ has been implicated not only in DNA replication (Morrison et al., 1990) but also in DNA repair (Nishida et al., 1988) and DNA recombination (Jessberger et al., 1993), reactions that *in vivo* require only limited amounts of enzyme and DNA molecules, we argue that the presented approach could reflect a close to physiological situation. Previous attempts to study the functional interaction of PCNA with pol ϵ were complicated by the fact that such an interaction could only be detected in the presence of RF-C, RP-A, and ATP. In order to avoid this problem, poly(dA)$_{400}$/oligo(dT)$_{12-18}$ and sp synthetic oligonucleotides were chosen as the templates because (i) RF-C is not required in order to load PCNA since the templates are linear (Burgers & Yoder, 1993) and (ii) on these templates pol ϵ does not require RP-A for efficient synthesis. Thus, in this system, the effect of PCNA on pol ϵ could be studied in the presence of the DNA template and nucleotides, exclusively.

Primer Binding by Pol ϵ Is Influenced by ss DNA. In order to elucidate the mechanism of primer recognition and binding of pol ϵ, two observations were crucial: (i) that the kinetics of primer utilization by pol ϵ are influenced by the extent of the ss regions of the template; and (ii) that ss DNA molecules inhibited pol ϵ in dependence of their lengths (as reflected by the dependence of their K_i values on the single-strandness length). On the basis of these observations, we propose the model depicted in Figure 9a for the interaction of pol ϵ with the DNA template. The increase in the K_m values of pol ϵ for the primer, observed when the concentration of ss poly(dA) over the 3′-OH ends was raised (Table 2), can be explained by the competitive inhibition of the primer binding reaction by the ss DNA. The fraction of the pol ϵ molecules which is involved in nonproductive binding to ss poly(dA) should then dissociate in order to bind a primer and to start DNA synthesis. Thus, addition of ss DNA to the reaction engaged the enzyme in multiple rounds of association and dissociation, resulting in a decrease of the apparent affinity for the primer.

PCNA Interacts with Pol ϵ both in Primer Binding and in Primer Elongation. Studies of the effect of PCNA on the primer binding and the primer elongation step catalyzed by pol ϵ indicated the following: (i) PCNA increased the affinity of the enzyme for the primer in the presence of increasing molar ratios of ss poly(dA) over the primer (Table 2). (ii) An equimolar amount of PCNA to pol ϵ was required to give maximum stimulation (Figure 3), suggesting a direct protein−protein interaction. (iii) PCNA had no effect on the affinity of pol ϵ for ss DNA (Table 2). (iv) PCNA specifically reduced the equilibrium dissociation constant (K_i) of pol ϵ for primed DNA (Table 2). On the basis of the model shown in Figure 9b, we propose that the effect of PCNA on the primer utilization reaction is to enhance the binding of pol ϵ to the primer, thus decreasing the K_m value. This was confirmed by direct analysis of the kinetics of formation of the complex between pol ϵ and a sp oligonucleotide (Figures 4 and 5). (v) PCNA increased the association of pol ϵ with a sp oligonucleotide without affecting the rate of dissociation of the complex once formed. Again, no effect was found with a ss oligonucleotide. Finally, (vi) the effect of PCNA on the processive primer elongation reaction catalyzed by pol ϵ was a proportional increase in both the K_m value for dTTP incorporation on poly(dA)/oligo(dT) and the V_{max} of the reaction. The observation that the increase of both the k_{cat} and K_m values was dependent upon the concentration of both pol ϵ and PCNA, with the best stimulation at 1:1 molar ratio (Figure 6a,b), again supports the hypothesis of a direct protein−protein interaction. According to the simplified kinetic model in Scheme 1, under processive polymerization conditions, dissociation of the template-primer is not on the reaction pathway, $k_{cat} = k_5$, and the K_m for dNTPs is given by

Scheme 1: Simplified Kinetic Pathway of the Nucleotide Incorporation Reaction of DNA Polymerase ϵ[a]

$$E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{:}TP_n \underset{k_4}{\overset{k_3[dNTP]}{\rightleftharpoons}} E{:}TP_n{:}dNTP \xrightarrow{k_5} E{:}TP_{n+1} \xrightarrow{k_6} E + TP_{n+1}$$

[a] Abbreviations: E, pol ϵ; TP_n, template-primer; dNTP, deoxynucleoside triphosphate.

$$K_m = (k_4 + k_5)/k_3 \quad (1)$$

Under conditions of forced temination of processive polymerization (i.e., single nucleotide addition), the dissociation of the template-primer is rate-limiting, $k_{cat} = k_6$, and K_m is given by

$$K_m = k_6(k_4 + k_5)/k_3k_5 \quad (2)$$

From eq 1 and 2, it follows that in both cases k_{cat}/K_m is given by

$$k_{cat}/K_m = k_3k_5/(k_4 + k_5) \quad (3)$$

Thus, the observed effect of PCNA under processive polymerization conditions could be due to an increase in the k_{cat} value of the reaction, rather than to a decreased affinity for the substrate. In agreement with this interpretation, increasing concentrations of PCNA did not change the apparent second-order rate constant for nucleotide binding, k_{cat}/K_m (Figure 7a). Analysis of dTTP incorporation under conditions which prevented multiple primer elongation by the same pol ϵ molecule showed that PCNA increases the rate of processive polymerization catalyzed by pol ϵ (Figure 7b). The biphasic time course of single-nucleotide incorporation catalyzed by pol ϵ into d61:d15-mer (Figure 1a) suggests that first dNTP binds and is readily incorporated, followed by the rate-determining dissociation of the enzyme from the template-primer. This results in a burst of product formation. Since the amount of product formed in the burst phase is generated by a single turnover of the enzyme, its concentration gives an estimate of the pol ϵ active site concentration in the reaction. The low K_m and k_{cat} values observed for single-nucleotide incorporation into d61:d15-mer (Figure 8) result from the slow steady-state rate k_6 (compare eqs 1 and 2). Its calculated value of 0.002 s^{-1} is in good agreement with the calculated k_{off} value of 0.0012 s^{-1} for dissociation of pol ϵ from the template, supporting

Table 3: Kinetic Parameters for Primer Recognition and Nucleotide Incorporation Catalyzed by Pol ϵ

	−PCNA	+PCNA
E + ssDNA ⇄ E:ssDNA	$K_i = 15\ \mu M$[a]	$K_i = 15\ \mu M$[a]
E + TP[b] ⇄ E:TP	$K_D = 6$ nM[c]	$K_D = 2$ nM[c]
	$k_{off} = 0.0012\ s^{-1}$	$k_{off} = 0.0012\ s^{-1}$
	$k_{on} = 0.2 \times 10^6\ M^{-1}\ s^{-1}$ [d]	$k_{on} = 0.6 \times 10^6\ M^{-1}\ s^{-1}$ [d]
E:TP + dNTP → E:TP$_{+1}$	$k_{cat} = 1.3\ s^{-1}$ [e]	$k_{cat} = 2.5\ s^{-1}$ [e]

[a] For ss poly(dA)$_{400}$. [b] TP, template-primer. [c] For sp d61:d15 oligonucleotide. [d] Calculated from K_D and k_{off}. [e] For dTTP incorporation on poly(dA)/oligo(dT)$_{10:1}$.

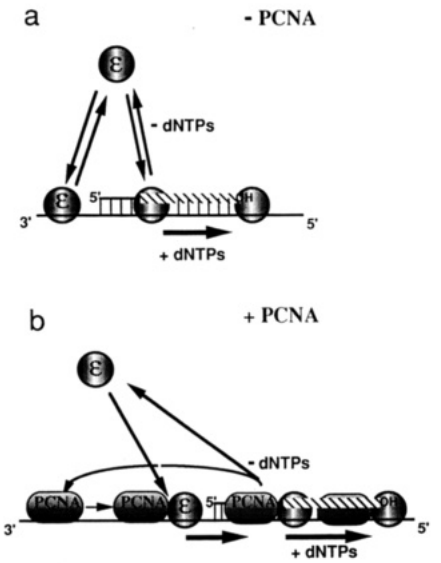

FIGURE 9: Model for the interaction of pol ϵ with the DNA template and primer. (a) In the absence of PCNA; (b) in the presence of PCNA. For details, see text.

the model in Scheme 1 in which under these conditions the rate-determining step is k_{off}. If $k_5 \gg k_4$ (high commitment to catalysis), then from eqs 2 and 3 it is seen that in the steady state $k_m = k_6/k_3$ and $k_{cat}/K_m = k_3$. PCNA did not change the K_m and k_{cat} values for single-nucleotide incorporation, according to the idea that it increases specifically k_5, leaving unaffected k_3 and k_6.

From the k_{cat}/K_m ratio, a value for k_3 of $0.5 \times 10^6\ M^{-1}\ s^{-1}$ is obtained for processive polymerization and of $0.4 \times 10^6\ M^{-1}\ s^{-1}$ for single-nucleotide incorporation. Compared with T_4 pol (Capson et al., 1992), this is a quite slow on-rate constant for dNTP binding to the enzyme−primer complex. It is possible that, as in the case of T_4 pol, $k_4 \gg k_5$. In such a case, however, a variation of k_5 should be reflected by the K_m and k_{cat}/K_m values for single-nucleotide incorporation, which is not the case of pol ϵ. It should be mentioned that k_{cat}/K_m in most cases is only an apparent second-order rate constant, which most likely gives the lower limit for the true on-rate constant for substrate binding. Thus, estimation of k_3 from steady-state parameters could result in an underestimation of the true value. A more complete pre-steady-state kinetic analysis is required to assign precise values to all the rate constants in the kinetic pathway.

A Model for the Interaction between Pol ϵ and PCNA. As reported in this paper, complex formation between PCNA and pol ϵ can facilitate primer binding by the enzyme. A possible explanation for this fact is outlined in Figure 9 and Table 3. We propose that PCNA increases the transfer rate of pol ϵ from nonspecific ss DNA unproductive sites to primer-end productive sites. Since PCNA has no effect on the equilibrium dissociation constant (K_i) of pol ϵ from ss DNA, a function of PCNA could be to "drive" pol ϵ to the primer, thus preventing a time-consuming dissociation of the enzyme from the template. It is conceivable that PCNA, once loaded on the DNA, could act as a "dynamic clamp" for pol ϵ bound to ss DNA, pushing it along the template to meet a 3′-OH end. Once the complex between pol ϵ and the primer is formed, PCNA could either remain associated with pol ϵ or dissociate from it. This decision could be made depending on the presence of dNTPs, and, if present, pol ϵ can immediately start DNA synthesis without pausing at the primer. PCNA would remain associated with pol ϵ, and this specific interaction increases the rate of nucleotide incorporation (Table 3). The observed k_{cat} value for processive nucleotide incorporation catalyzed by pol ϵ in the presence of PCNA ($2.5\ s^{-1}$) is still far from the observed *in vivo* rate of eukaryotic replicative fork movement (50 nt s^{-1}). Albeit less "artificial" than in previous studies, our *in vitro* conditions are still different from the true *in vivo* situation since (i) we used synthetic DNA templates and (ii) we have chosen a simple system in which other cofactors like RF-C and RP-A are missing. It has been shown (Burgers, 1991) that the polymerization rates of both pol δ and pol ϵ approximate to 50 nt s^{-1} when measured on a natural template and in the presence of RF-C and RP-A. Our findings show that PCNA alone is sufficient to increase the rate of processive polymerization of pol ϵ "core" enzyme, but also suggest that other components such as RF-C and RP-A contribute to reach the rate measured for the pol ϵ holoenzyme, according to the idea that the intrinsic properties of DNA polymerases are modulated by their auxiliary factors.

If dNTPs are missing, pol ϵ pauses for a long time at the 3′-OH end before dissociating (the half-life of the complex is approximately 7 min at 22 °C). This value likely reflects the intrinsic high processivity of pol ϵ. Given the processivity as the average number of nucleotides incorporated per binding event, it can be calculated from the ratio of the rate of polymerization to the rate of dissociation of the enzyme−primer complex. In the case of pol ϵ, we obtained a value of 890 in the absence of PCNA, which is consistent with the one estimated, although only approximately, in a previous study (Syvaoja & Linn, 1989). This value was increased up to 2000 in the presence of PCNA. It has been recently shown that the highly processive DNA polymerase holoenzyme of the bacteriophage T_4 has a dissociation rate from the primer-template of $0.003\ s^{-1}$, in the absence of dNTPs (Hacker & Alberts, 1994). This value is close to the one measured in the present study for pol ϵ. Thus, it seems that a slow dissociation rate for the enzyme−primer complex is a common feature for highly processive enzymes.

We propose that, in the absence of dNTPs, PCNA leaves the enzyme−primer complex quickly. Two lines of evidence suggest that PCNA does not form a stable complex with pol

ϵ and the primer in the absence of DNA synthesis: (i) PCNA does not alter the electrophoretic mobility of pol ϵ−primer complexes; and (ii) PCNA does not alter the dissociation rate of pol ϵ from the primer.

The molecular basis of the interaction between pol ϵ and PCNA is still unclear; anyway, it was recently suggested [reviewed in Johnson (1993)] that the rate-limiting step of processive polymerization is a conformational change in the pol−DNA−dNTP complex prior to the chemical step. According to this model, nucleotide binding is a two-step process. In the first step, dNTPs bind quickly to the pol in an "open" conformation. In the second step, the pol conformation changes to a "closed" state which leads to rapid incorporation of the dNTPs. In view of the proposed role of PCNA in the primer elongation reaction catalyzed by pol ϵ, it is tempting to speculate that the protein−protein interaction between pol ϵ and PCNA could facilitate the transition from the "open" to the "closed" state, thus increasing the rate of nucleotide incorporation. The suggestion that PCNA could associate stably only with the pol ϵ−DNA−dNTP complex fits into this model.

It has also been shown recently that the replication-competent multisubunit complex called DNA polymerase holoenzyme of eukaryotic cells has striking functional similarities with the corresponding replicative holoenzyme of the *E. coli* bacteriophage T_4. In particular, it has been proposed that PCNA is the analogue of gp45 of the T_4 system. Protein gp45 has been shown to increase the rate of primer association and elongation by the T_4 DNA polymerase, even in the absence of other accessory proteins [reviewed in Young et al. (1992)]. In this paper, we have shown that PCNA displays similar effects on pol ϵ. The so-called "clocking" model for the T_4 holenzyme system proposes that translocation of the holoenzyme during DNA synthesis is required to maintain its integrity [reviewed in Young et al. (1992)]. Pausing of the polymerase at the 3′-OH end would start a sort of "decay clock" leading to the dissociation of the "sliding clamp" gp45 from the pol. The observation that PCNA and pol ϵ need to be engaged in DNA synthesis in order to form a stable complex suggests that a similar mechanism could function also in eukaryotes and suggests a high level of functional conservation between prokaryotes and eukaryotes.

In conclusion, we propose that PCNA could be responsible for the "fine tuning" of the catalytic activity of pol ϵ at the DNA replication fork as well as in DNA repair and recombination. The fact that our data were obtained with calf thymus pol ϵ and human PCNA argues for the fact that this interaction is general and highly conserved.

ACKNOWLEDGMENT

We thank Silvio Spadari and Vladimir Podust for their critical reading of the manuscript, Birgit Sturzenegger and Röbi Hindges for purifiying human PCNA, and Bruce Stillman for the strain BL21(DE3) harboring the human PCNA expression vector pT7/PCNA.

REFERENCES

Bambara, R. A., & Jessee, C. B. (1991) *Biochim. Biophys. Acta 1088*, 11−24.

Bauer, G. A., & Burgers, P. M. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7506−7510.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Burgers, P. M. J. (1991) *J. Biol. Chem. 266*, 22698−22706.

Burgers, P. M. J., & Yoder, B. L. (1993) *J. Biol. Chem. 268*, 19923−19926.

Burgers, P. M. J., Bambara, R. A., Campbell, J. L., Chang, L. M. S., Downey, K. M., Hübscher, U., Lee, M. Y. W. T., Linn, S. M., So, A. G., & Spadari, S. (1990) *Eur. J. Biochem. 191*, 617−618.

Campbell, J. L. (1993) *J. Biol. Chem. 268*, 25261−25264.

Capson, T. L., Peliska, J. A., Fenn-Kaboord, B., West-Frey, M., Lively, C., Dahlberg, M., & Benkovic, S. J. (1992) *Biochemistry 31*, 10984−10994.

Crute, J. J., Wahl, A. F., & Bambara, R. A. (1986) *Biochemistry 25*, 26−36.

Fien, K., & Stillman, B. (1992) *Mol. Cell. Biol. 12*, 155−163.

Focher, F., Spadari, S., Ginelli, B., Hottiger, M., Gassmann, M., & Hübscher, U. (1988) *Nucleic Acids Res. 16*, 6279−6295.

Focher, F., Gassmann, M., Hafkemeyer, P., Ferrari, E., Spadari, S., & Hübscher, U. (1989) *Nucleic Acids Res. 17*, 1850−1861.

Hacker, K., & Alberts, B. (1994) *J. Biol. Chem. 269*, 24209−24220.

Hübscher, U., & Kornberg, A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6284−6288.

Hübscher, U., & Spadari, S. (1994) *Physiol. Rev. 76*, 259−304.

Hurwitz, J., Dean, F. B., Kwong, A. D., & Lee, S.-K. (1990) *J. Biol. Chem. 265*, 18043−18046.

Jessberger, R., Podust, V. N., Hübscher, U., & Berg, P. (1993) *J. Biol. Chem. 268*, 15070−15079.

Johnson, K. A. (1993) *Annu. Rev. Biochem. 62*, 685−713.

Kesti, T., & Syväoja, J. E. (1991) *J. Biol. Chem. 266*, 6336−6341.

Kong, X. P., Onrust, R., O, D. M., & Kuriyan, J. (1992) *Cell 69*, 425−437.

Lee, S. H., Pan, Z. Q., Kwong, A. D., Burgers, P. M., & Hurwitz, J. (1991) *J. Biol. Chem. 266*, 22707−22717.

Leibovici, M., Gusse, M., Bravo, R., & Méchali, M. (1990) *Dev. Biol. 141*, 183−192.

Matsumoto, K., Moriuchi, T., Koji, T., & Nakane, P. K. (1987) *EMBO J. 6*, 637−642.

Morrison, A., Araki, H., Clark, A. B., Hamatake, R. K., & Sugino, A. (1990) *Cell 62*, 1143−1151.

Ng, L., Tan, C.-K., Downey, K., & Fisher, P. A. (1991) *J. Biol. Chem. 266*, 11699−11704.

Ng, L., McConnell, M., Tan, C.-K., Downey, K. M., & Fisher, P. A. (1993) *J. Biol. Chem. 268*, 13571−13576.

Nishida, C., Reinhard, P., & Linn, S. (1988) *J. Biol. Chem. 263*, 501−510.

Olins, D. E., Olins, A. L., Cacheiro, L. H., & Tan, E. M. (1989) *J. Cell Biol. 109*, 1399−1410.

Podust, V. N., Georgaki, A., Strack, B., & Hübscher, U. (1992) *Nucleic Acids Res. 20*, 4159−4165.

Podust, L. M., Podust, V. N., Floth, C., & Hübscher, U. (1994) *Nucleic Acids Res. 22*, 2970−2975.

Prelich, G., Kotsura, M., Marshak, D. R., Mathews, M. B., & Stillman, B. (1987) *Nature 326*, 471−475.

Shivji, K. K., Kenny, M. K., & Wood, R. D. (1992) *Cell 69*, 367−374.

Somashekarappa, N., & Karumathil, P. (1993) *J. Biol Chem. 268*, 15557−15564.

Suzuka, I., Daidoji, H., Matsuoka, M., Kadowaki, K.-I., Takasaki, Y., Nakane, P. K., & Moriuchi, T. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3189−3193.

Syvaoja, J., & Linn, S. (1989) *J. Biol. Chem. 264*, 2489−2497.

Syvaoja, J., Suomensaari, S., Nishida, C., Goldsmith, J. S., Chui, G. S., Jain, S., & Linn, S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6664−6668.
Tan, C.-K., Castillo, C., So, A. G., & Downey, K. L. (1986) *J. Biol. Chem. 261*, 12310−12316.
Tsurimoto, T., & Stillman, B. (1991) *J. Biol. Chem. 266*, 1950−1960.
Tsurimoto, T., Melendy, T., & Stillman, B. (1990) *Nature 346*, 534−539.
Wang, T.-S. F. (1991) *Annu. Rev. Biochem. 60*, 513−552.
Waseem, N. H., Labib, K., Nurse, P., & Lane, D. P. (1992) *EMBO J. 11*, 5111−5120.
Weiser, T., Gassmann, M., Thömmes, P., Ferrari, E., Hafkemayer, P., & Hübscher, U. (1991) *J. Biol. Chem. 266*, 10420−10428.
Wintersberger, U., & Wintersberger, E. (1970) *Eur. J. Biochem. 13*, 11−14.
Yamaguchi, M., Yasuyoshi, N., Moriuchi, T., Hirose, F., Hui, C.-C., Suzuky, Y., & Matsukage, A. (1990) *Mol. Cell. Biol. 10*, 872−879.
Young, M. C., Reddy, M. K., & von Hippel, P. H. (1992) *Biochemistry 31*, 8675−8690.

BI942160R