DNA Polymerase δ Holoenzyme: Action on Single-Stranded DNA and on Double-Stranded DNA in the Presence of Replicative DNA Helicases†

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ABSTRACT: DNA polymerase δ requires proliferating cell nuclear antigen and replication factor C to form a holoenzyme efficient in DNA synthesis. We have analyzed three different aspects of calf thymus DNA polymerase δ holoenzyme: (i) analysis of pausing during DNA synthesis, (ii) replication of double-stranded DNA in the absence of additional factors, and (iii) replication of double-stranded DNA in the presence of the two known replicative DNA helicases from simian virus 40 and bovine papilloma virus. DNA polymerase δ holoenzyme replicated primed single-stranded DNA at a rate of 100−300 nucleotides/min, partially overcoming multiple pause sites on DNA. While *Escherichia coli* single-strand DNA binding protein helped DNA polymerase δ pass through pause sites, the DNA polymerase δ itself appeared to dissociate from the template in the absence of synthesis or when encountering pause sites. Proliferating cell nuclear antigen likely remained on the template. DNA polymerase δ holoenzyme could perform limited strand displacement synthesis on double-stranded gapped circular DNA, and this reaction was not stimulated either by replication protein A or by *E. coli* single-strand DNA binding protein. DNA polymerase δ holoenzyme could efficiently cooperate with replicative DNA helicases from simian virus 40 (large T antigen) and bovine papilloma virus 1 (protein E1) in replication through double-stranded DNA in a reaction that required replication protein A or *E. coli* single-strand DNA binding protein. Our data are consistent with the role of DNA polymerase δ as the leading strand replicase but also suggest that additional factors [e.g., proteins to pass pause sites and cellular DNA helicase(s)] might be required to achieve replication at a physiological speed.

Studies in many experimental systems over the last 30 years indicated that processes in DNA replication are similar throughout life [e.g., reviewed in Kornberg and Baker (1992) and Hübscher and Spadari (1994)]. At an active DNA replication fork, DNA polymerases (pols)[1] engaged in precise processive and fast action require a set of auxiliary proteins. Such proteins have been identified in *Escherichia coli*, bacteriophage T4, and eukaryotes [reviewed in Hübscher and Spadari (1994)]. In these systems, the high processivity and strong affinity for DNA are not the intrinsic properties of replicative pols themselves, but are provided through interactions with the auxiliary proteins, forming a so-called "sliding clamp" [reviewed in Kuriyan and O'Donnell (1993)].

At least three auxiliary proteins (PCNA, RF-C, and RP-A) are required for DNA synthesis by pol δ, the most likely candidate for the replication of leading strand (Tsurimoto et al., 1990). Although these three proteins are essential for pol δ-catalyzed polymerization, their exact function in this process is not well-defined. We have recently demonstrated that PCNA and RF-C but not RP-A are required for pol δ holoenzyme assembly on a circular DNA substrate lacking abundant single-stranded regions (Podust et al., 1994). Since RP-A appears not to be involved directly in sliding clamp formation, we were interested in understanding in detail the role of RP-A in the pol δ holoenzyme polymerization reaction on ss- and dsDNA templates.

RF-C appears to be a brace protein that enzymatically loads PCNA onto DNA. Biochemical studies might be generalized by the following model of auxiliary protein action (Lee & Hurwitz, 1990; Burgers, 1991; Stillman, 1994). RF-C first binds to DNA in the presence of ATP. Then PCNA binds to the RF-C/DNA complex, forming an unstable intermediate complex. Upon hydrolysis of ATP by the RF-C ATPase, the protein/DNA intermediate undergoes a conformational change resulting in a stable complex called PCNA clamp, which interacts with pol δ and pol ε. Despite the intensive studies on the mechanism of pol δ (ε) holoenzyme formation (Lee & Hurwitz, 1990; Lee et al., 1991; Tsurimoto & Stillman, 1991; Burgers, 1991), the structure and mechanism of action of eukaryotic sliding clamp have yet to be determined.

The functional and structural homologies of auxiliary proteins in *E. coli*, bacteriophage T4, and eukaryotes (O'Donnell et al., 1993; Kuriyan & O'Donnell, 1993;

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[1] Abbreviations: pol, DNA polymerase; PCNA, proliferating cell nuclear antigen; RP-A, replication protein A; RF-C, replication factor C; SSB, single-strand DNA binding protein; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; SV40, simian virus 40; Tag, SV40 large tumor antigen; BPV1, bovine papilloma virus 1; E1, protein E1 of BPV1; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); nt, nucleotide(s).

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Stillman, 1994) suggest that the mechanisms of the clamp formation and assembly of replicative holoenzymes in these three systems might be similar. Recent studies in prokaryotic systems resulted in a model of partial disassembly, when a region of duplex DNA is encountered by the pol holoenzyme. Upon completing an Okazaki fragment, *E. coli* and T4 pols disengage from the clamps, hop off DNA, and reassociate with another clamp at new sites. The original clamps remain on DNA (Stukenberg et al., 1994; Hacker & Alberts, 1994).

In this paper, we have investigated further the role of the auxiliary proteins in the DNA synthesis by pol δ. For that we analyzed (i) the ability of pol δ to overcome pause sites on ssDNA, (ii) the ability of pol δ holoenzyme to replicate into dsDNA, and (iii) the functional interaction of pol δ with the two replicative DNA helicases from SV40 (T antigen) and BPV1 (protein E1) on dsDNA. The data are discussed in comparison with studies on *E. coli* and T4 pol holoenzymes.

MATERIALS AND METHODS

Materials

Enzymes and Proteins. Pols α and δ, PCNA, RF-C, RP-A, and *E. coli* SSB were described in detail in our previous publications (Podust et al., 1992a,b). BPV1 E1 protein was overexpressed in *E. coli* and purified as described (Müller et al., 1994). SV40 Tag was kindly provided by J. Sogo (Zürich). Restriction endonucleases, T4 polynucleotide kinase, and proteinase K were purchased from Boehringer Mannheim.

DNA Substrates. Single-stranded M13(mp11) DNA was prepared according to Sambrook et al. (1989). ssDNA was primed with 40-mer oligonucleotide complementary to nt 7041−7080 of the M13 genome. If indicated, the primer was labeled with polynucleotide kinase and [γ-^{32}P]ATP (3 Ci/μmol). The labeled primer (2-fold molar excess over the template) or unlabeled primer (5-fold excess) was annealed to M13 ssDNA as described (Podust et al., 1992a). A double-stranded gapped DNA substrate was prepared from replicative form I of M13(mp11) DNA (Podust et al., 1994).

Methods

Product Analysis in Alkaline Agarose Gels. Reactions were terminated and treated for 30 min at 37 °C with proteinase K (60 μg/mL) in the presence of 1% (w/v) SDS and 20 mM EDTA (pH 8.0), and the DNA was precipitated with ethanol. Alkaline agarose electrophoresis was performed on 0.8−1.5% agarose gels (as outlined in the figure legends) in a solution containing 30 mM NaOH and 1 mM EDTA. In all experiments, the DNA molecular weight marker VI, base pair range from 154 to 2176 (Boehringer Mannheim), was used.

Replication Assays. All reaction mixtures contained the following standard components: 40 mM Tris-HCl (pH 7.5), 0.2 mg/mL bovine serum albumin, and 1 mM dithiothreitol (buffer TBD). Unless otherwise indicated, incubation of the mixtures was carried out at 37 °C.

Assay To Determine the Half-Life of the PCNA Clamp. Reaction mixtures (15 μL) contained buffer TBD, 10 mM $MgCl_2$, 0.1 mM ATP, 50 ng of primed M13 ssDNA, 20 ng of PCNA, 18 ng of RF-C, and 175 ng of *E. coli* SSB. The samples were mixed on ice and incubated for 5 min at 37 °C, and then 8.5 μL of prewarmed (37 °C) solution containing 6 mM ATPγS, 10 mM $MgCl_2$ in TBD buffer was added. After various times, 1.5 μL of a mixture containing 0.2 unit of pol δ and 420 μM each of dATP, dGTP, dCTP, and 250 μM [^{3}H]dTTP (1600 cpm/pmol) was added to the reaction mixtures and incubation continued for 10 min, and the amount of acid-precipitable radioactivity formed was determined. The data were analyzed assuming first-order kinetics of complex dissociation using the CA-Cricket Graph III program.

DNA Synthesis on Singly-Primed M13 ssDNA under Conditions Preventing PCNA Clamp Reassembly. Reaction mixtures (15 μL) contained buffer TBD, 10 mM $MgCl_2$, 0.1 mM ATP, 50 μM dATP, 50 μM dGTP, 50 ng of primed M13 ssDNA (5′-^{32}P-phosphorylated primer), 20 ng of PCNA, 18 ng of RF-C, 175 ng of *E. coli* SSB, and 0.2 unit of pol δ. The samples were mixed on ice, incubated for 5 min at 37 °C, and then mixed with 15 μL of prewarmed (37 °C) solution containing 4 mM ATPγS, 50 μM dCTP, 50 μM dTTP, and 10 mM $MgCl_2$ in TBD buffer. After incubation for various periods, the reactions were terminated and the products analyzed on an alkaline agarose gel as described above.

DNA Synthesis on Singly-Primed M13 ssDNA under Conditions of SSB Depletion. Reaction mixtures (24 μL) contained buffer TBD, 10 mM $MgCl_2$, 2 mM ATP, 50 μM dATP, 50 μM dGTP, 50 ng of primed M13 ssDNA (5′-^{32}P-phosphorylated primer), 20 ng of PCNA, 18 ng of RF-C, 175 ng of *E. coli* SSB, and 0.2 unit of pol δ. The samples were mixed on ice, incubated for 5 min at 37 °C, and then mixed with 1 μL of prewarmed (37 °C) solution containing 1.5 μg of M13 ssDNA, 50 μM dCTP, and 50 μM dTTP in TBD buffer. After various times, reactions were terminated and analyzed on an alkaline agarose gel as described above.

DNA Synthesis on Singly-Primed M13 ssDNA under Conditions Preventing the Reelongation of the Primers. Two different mixtures (called A and B) were prepared. Mixture A contained in a final volume of 25 μL: TBD buffer, 2 mM ATP, 10 mM $MgCl_2$, 50 μM dATP, 50 μM dGTP, 50 ng of primed M13 ssDNA (5′-^{32}P-phosphorylated primer), 20 ng of PCNA, 18 ng of RF-C, 175 ng of *E. coli* SSB, and 0.1 unit of pol δ (2 milliunits of pol δ per 1 ng of DNA). Mixture B contained in a final volume of 25 μL: TBD buffer, 2 mM ATP, 10 mM $MgCl_2$, 50 μM each of dATP, dGTP, dCTP, and dTTP, 150 ng of primed M13 ssDNA (unlabeled primer 1), 50 ng of PCNA, 27 ng of RF-C, and 525 ng of *E. coli* SSB. Both mixtures were first incubated separately for 5 min, and then 2.5 μL of mixture A containing 2 milliunits of pol δ per 1 ng of DNA was added to 25 μL of mixture B to yield 0.064 milliunits of pol δ per 1 ng of DNA. After various times, the reactions were terminated and analyzed on an alkaline agarose gel as described above.

DNA Synthesis by Pol δ Holoenzyme on a Gapped M13 dsDNA. The assay conditions using gapped dsDNA [for details, see Podust et al. (1994)] were exactly the same as those described for DNA synthesis on primed ssDNA with two modifications: (i) 50 ng of gapped dsDNA was used as the template-primer and (ii) no SSB was added. Reaction mixtures were incubated for various times, and the polymerization reaction was terminated by heating for 10 min at 70 °C. Reaction tubes were cooled to room temperature, NaCl was added to 100 mM, and the DNA was digested with *Eco*RI (2 units) for 40 min at 37 °C and finally treated

with proteinase K (60 μg/mL) in the presence of 1% (w/v) SDS and 20 mM EDTA (pH 8.0). DNA was precipitated with ethanol and analyzed on a 5% PAGE, containing 7 M urea (8 cm × 10 cm × 0.8 mm). Electrophoresis was performed at 20 V/cm until the bromphenol blue dye reached the bottom of the gel. The gel was then fixed in 10% acetic acid containing 12% methanol, washed with distilled water, dried at 80 °C, and exposed to X-ray film.

DNA Synthesis in the Presence of DNA Helicase and Pol δ Holoenzyme on a Gapped M13 dsDNA. Reaction mixtures (25 μL) contained TBD buffer, 2 mM ATP, 7 mM $MgCl_2$, 50 μM each of dATP, dGTP, and dTTP and 15 μM [α-^{32}P]-dCTP (12 000 cpm/pmol) [or 50 μM each of dATP, dGTP, and dCTP and 15 μM [^{3}H]dTTP (1600 cpm/pmol)], 50 ng of gapped M13 dsDNA, 20 ng of PCNA, 18 ng of RF-C, and 0.2 unit of pol δ. If not varied, 200 ng of E1, 500 ng of Tag, 200 ng of *E. coli* SSB, or 300 ng of RP-A was added to the reaction mixtures. After incubation for 1 h, DNA synthesis was analyzed by acid-precipitable material and by alkaline agarose gel electrophoresis. In the latter case, the polymerization reactions were terminated by heating for 10 min at 70 °C. Reaction tubes were cooled to room temperature, [NaCl] was adjusted to 0.1 M, and the DNA was digested with *Eco*RI and *Dpn*I (2 units of each enzyme) for 40 min at 37 °C. DNA was analyzed by alkaline agarose gel electrophoresis as described above.

RESULTS

The Pausing of Pol δ Holoenzyme Is Unaffected When Reloading of PCNA Is Prevented. Analysis of products formed by pol δ holoenzyme on primed ssDNA covered with *E. coli* SSB showed that pol δ pauses at multiple sites during the DNA synthesis. The final length of the products essentially depended on the position of primer on the template. When ssDNA was primed with the oligonucleotide complementary to nt 6255−6284 of M13 genome, products 4000−4500 nt in length were synthesized in 10 min of polymerization reaction (data not shown). If a primer complementary to nt 7041−7080 of the M13 genome was used, three strong DNA synthesis pause sites near the replication start were observed and were called A, B, and C, respectively (Figure 1, lanes 2−7). Pausing was readily seen in the first 1.5−10 min of incubation, which could be overcome, with the exception of site C, after 15 min. Due to the strong pausing, the average rate of DNA synthesis was about 100 nt/min.

Since previous investigation of the loading of RF-C and PCNA onto DNA suggested that RF-C binds initially to the 3′-OH end of the primer, followed by PCNA binding to the RF-C/DNA complex (Lee & Hurwitz, 1990; Tsurimoto & Stillman, 1991; Burgers, 1991), we were first interested in the fate of auxiliary proteins while encountering pause sites by pol δ holoenzyme. As we know from the published data, the labile and reversible tertiary complex PCNA/RF-C/DNA can be formed with ATPγS as well as with ATP (Lee & Hurwitz, 1990; Burgers, 1991). This intermediate complex undergoes further transformation in a reaction requiring ATP hydrolysis. Only the resulting irreversible complex, called the PCNA clamp, was eventually recognized by pol δ in a replication-competent manner. ATPγS, on the other hand, has been shown to be a potent inhibitor of PCNA clamp formation, but did not affect the pol δ holoenzyme action

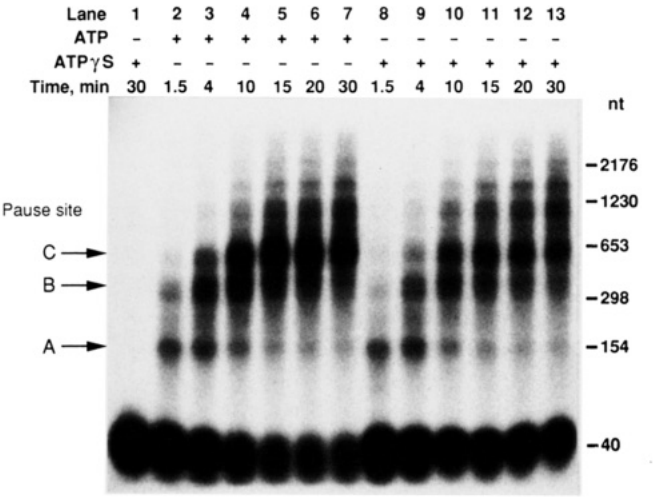

FIGURE 1: Prevention of PCNA reloading does not affect the ability of pol δ holoenzyme to overcome the pause sites. Reaction mixtures (15 μL) containing buffer TBD, 10 mM $MgCl_2$, 0.1 mM ATP, 50 μM dATP, 50 μM dGTP, 50 ng of primed M13 ssDNA (5′-^{32}P-phosphorylated primer), 20 ng of PCNA,18 ng of RF-C, 175 ng of *E. coli* SSB, and 0.2 unit of pol δ were mixed on ice (mixture A). The samples were incubated for 5 min and then mixed with 15 μL of a prewarmed solution containing 4 mM ATP, 50 μM dCTP, 50 μM dTTP, and 10 mM $MgCl_2$ in TBD buffer (mixture B, lanes 2−7) or 4 mM ATPγS, 50 μM dCTP, 50 μM dTTP, and 10 mM $MgCl_2$ in TBD buffer (mixture C, lanes 8−13). Lane 1: mixture A and mixture C were combined on ice before incubation. After various times, the reactions were terminated and DNA products analyzed on a 1.5% alkaline agarose gel.

once the PCNA clamp had been formed with ATP (Yoder & Burgers, 1991; Burgers, 1991). On the basis of these data, we used ATPγS to prevent reassembly of a PCNA clamp after DNA synthesis by pol δ holoenzyme had started. Under conditions used, when ATPγS was added at the beginning of the reaction, it completely inhibited the assembly of pol δ holoenzyme (Figure 1, lane 1). We compared two reactions in which (i) the PCNA clamp could be formed only before DNA synthesis started (Figure 1, lanes 8−13) or (ii) the PCNA clamp could be continuously loaded onto free 3′-OH ends during the polymerization reaction (Figure 1, lanes 2−7). In the latter case, not only the primers initially charged with PCNA clamps but also residual free primers could be elongated, resulting in continuous disappearance of the 40-mer and accumulation of elongated products (Figure 1, lanes 2−7). When the PCNA clamp was assembled in the presence of ATP, the subsequent addition of ATPγS blocked further PCNA loading, but did not significantly affect the length distribution of the polymerization products (Figure 1, compare lanes 2−7 to lanes 8−13). These data suggested that the dissociation of the PCNA clamp does not occur when pol δ traverses the pause site.

PCNA Has a Long Half-Life on Circular DNA. By using the above approach, the half-life of the PCNA clamp loaded on DNA was determined. The reaction mixtures were first preincubated with RF-C and ATP to assemble the PCNA clamp; then a 34-molar excess of ATPγS was added. After incubation for various lengths of time, pol δ was added, and the presence of the PCNA clamp on DNA was monitored by measuring DNA synthesis. When ATPγS was added from the beginning, the amount of acid-insoluble material formed did not exceed the background value (0.2−0.3 pmol). The dependence of DNA synthesis on time followed first-order kinetics with a half-life of the PCNA clamp of 22 min

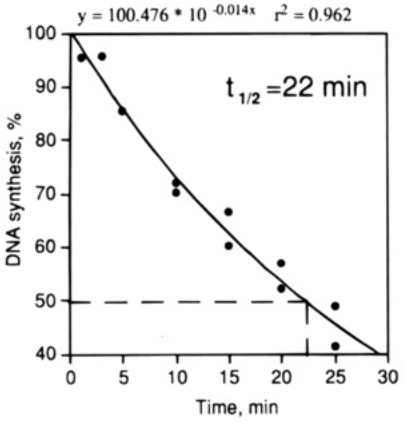

FIGURE 2: PCNA clamp has a long half-life on primed circular ssDNA. The PCNA clamp was assembled on primed circular ssDNA in the presence of RF-C and ATP as outlined under Materials and Methods; then a 34-molar excess of ATPγS over ATP was added to inhibit the RF-C ATPase activity. The mixtures were further incubated at 37 °C for various periods. The activity of the PCNA clamp was measured by its ability to support DNA synthesis by pol δ. An activity of 100% corresponded to 9 pmol of dNMP incorporation. The quantified data were analyzed as a first-order reaction using the CA-Cricket Graph III program.

(Figure 2). These data further indicated that the loaded PCNA clamp remained stably bound on DNA and suggested that no recycling of PCNA is required at the leading strand of the replication fork.

Pausing by Pol δ Holoenzyme Is Enhanced if SSB Is Competed Away from the Template. The studies describing the DNA synthesis by pol δ on singly-primed circular ssDNA demonstrated a simultaneous requirement of RF-C, PCNA, and RP-A (or *E. coli* SSB) (Tsurimoto & Stillman, 1989; Lee et al., 1991; Burgers, 1991; Podust et al., 1992a,b). We have recently demonstrated that the dependence of pol δ holoenzyme assembly on RP-A or *E. coli* SSB was completely eliminated when a circular double-stranded DNA with only a short single-stranded gap was used as substrate (Podust et al., 1994). Thus, it has been assumed that the requirement of RP-A (or *E. coli* SSB) for holoenzyme assembly on ssDNA is due to its ability to eliminate unproductive binding of RF-C to ssDNA. To further dissect the SSB requirement for PCNA clamp assembly from the requirement of SSB for pol δ holoenzyme translocation, the PCNA clamp was first assembled in the presence of RF-C, ATP, and *E. coli* SSB on primed ssDNA under standard conditions. Then a 30-fold molar excess of unprimed ssDNA over primed ssDNA was added. When excess unprimed DNA was added at the beginning of the reaction, it completely inhibited DNA synthesis (Figure 3, lane 2), presumably due to the unspecific binding of RF-C to the ssDNA. However, when ssDNA was added after PCNA clamp assembly occurred, it did not prevent primer elongation *per se* but strongly enhanced stalling of pol δ holoenzyme at the three pause sites A, B, and C (Figure 3, compare lanes 3−8 to lanes 9−14). In control experiments, we (i) preincubated primed DNA with RF-C, PCNA, *E. coli* SSB, and pol δ and then added the excess ssDNA or (ii) preincubated primed ssDNA with RF-C, PCNA, *E. coli* SSB and, separately, unprimed ssDNA with pol δ, and then mixed the samples. Both control experiments showed the same level of DNA synthesis by pol δ when analyzed by scintillation counting of acid-insoluble material and by alkaline agarose gel electrophoresis (data not shown). The latter result suggested that unprimed

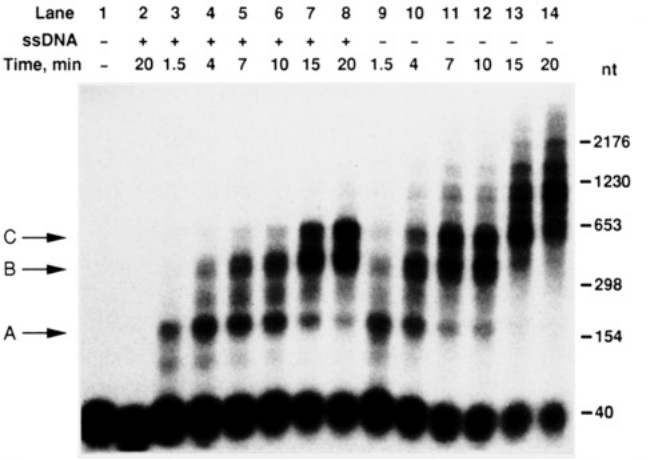

FIGURE 3: Pausing by pol δ holoenzyme is enhanced if *E. coli* SSB is depleted with excess ssDNA. Reaction mixtures (24 μL) contained buffer TBD, 10 mM $MgCl_2$, 2 mM ATP, 50 μM dATP, 50 μM dGTP, 50 ng of primed M13 ssDNA (5′-^{32}P-phosphorylated primer), 20 ng of PCNA, 18 ng of RF-C, 175 ng of *E. coli* SSB, and 0.2 unit of pol δ and were mixed on ice. The samples were incubated 5 min at 37 °C and mixed with 1 μL of prewarmed solution (37 °C) containing 1.5 μg of M13 ssDNA, 50 μM dCTP, and 50 μM dTTP in TBD buffer (lanes 2−8) or 50 μM dCTP, 50 μM dTTP in TBD buffer only (lanes 9−14). Lane 1: the reaction was terminated after preincubation before addition of dCTP and dTTP. Lane 2: ssDNA, dCTP, and dTTP were added to the mixture on ice before incubation. After various times, the reactions were terminated and DNA products analyzed on a 1.5% alkaline agarose gel.

ssDNA has considerably lower affinity for pol δ than ssDNA carrying a PCNA clamp, and, therefore, when present in excess, it does not directly trap pol δ. Whereas sequestration of free RF-C is apparently not essential for pol δ-catalyzed DNA synthesis once PCNA is clamped to DNA (see Figure 1), the most probable target for sequestration by ssDNA is SSB. Indeed, sequestration by excess ssDNA can readily be envisioned to effectively deplete the primed DNA substrate of SSB, and thus, permit restoration of the secondary structure of the template pause sites.

Pausing by Pol δ Holoenzyme Is Reduced When ssDNA Is Heated in the Presence of E. coli SSB. Prior to DNA synthesis, the primed M13 ssDNA was heated for 5 min at 60 °C in the presence of high levels of *E. coli* SSB (DNA: SSB ratio 1:8, w/w). Under these conditions, the 40-mer oligonucleotide primer remained annealed to the ssDNA, as was confirmed by using standard helicase assays (Thömmes et al., 1992) (data not shown). The thermal destabilization in the presence of *E. coli* SSB greatly diminished pausing at sites A and B, but not C (Figure 4, lanes 1−4). Under these conditions, the rate of DNA synthesis by pol δ was about 300 nt/min. The addition of excess ssDNA after preheating of primed DNA in the presence of SSB completely restored the three pause sites for pol δ holoenzyme (compare Figure 3, lanes 3−8, to Figure 4, lanes 5−8).

Pol δ Does Not Form a Stable Holoenzyme and Can Dissociate from the PCNA Clamp at Pause Sites. The stability of pol δ holoenzyme on the whole depends on two factors: the stability of the PCNA clamp itself and the stability of the PCNA clamp/pol δ core linkage. The experiments described above indicated that the PCNA clamp remained stably bound to DNA both in the absence and in the presence of DNA synthesis (Figures 1 and 2). Therefore, the stability of pol δ holoenzyme would reflect the interaction

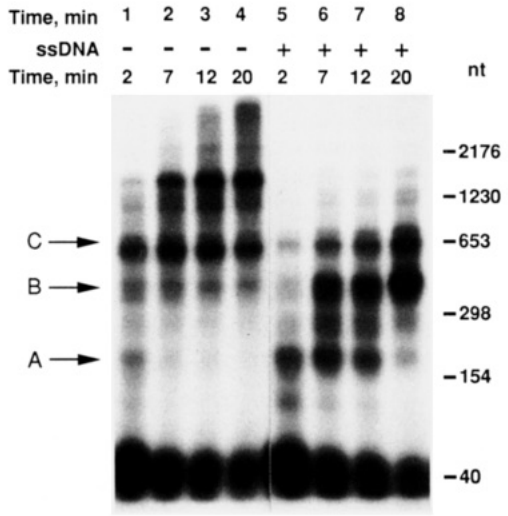

FIGURE 4: Pausing by pol δ holoenzyme is reduced when DNA substrate is preheated in the presence of *E. coli* SSB. The experimental conditions were essentially the same as for Figure 3. Primed M13 ssDNA was incubated for 5 min at 60 °C in the presence of *E. coli* SSB (DNA:SSB ratio 1:8, w/w) and was added to the reaction mixtures after cooling. The samples were incubated 5 min at 37 °C and mixed with 1 μL of prewarmed (37 °C) solution containing 50 μM dCTP and 50 μM dTTP in TBD buffer (lanes 1−4) or 1.5 μg of M13 ssDNA, 50 μM dCTP, and 50 μM dTTP in TBD buffer (lanes 5−8). After various times, the reactions were terminated and DNA products analyzed on a 1.5% alkaline agarose gel.

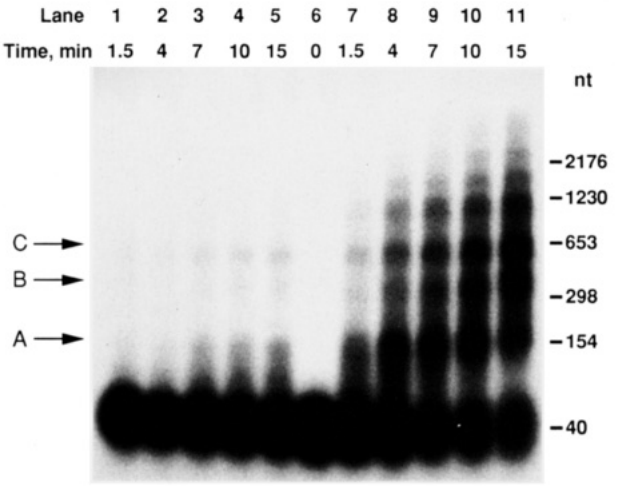

FIGURE 5: Pol δ does not form a stable holoenzyme and can dissociate from the PCNA clamp at pause sites. Pol δ holoenzyme was assembled on a ^{32}P-primed ssDNA substrate (2 milliunits of pol δ per 1 ng of ^{32}P-labeled primed DNA, mixture A), reaction was initiated by addition of missing dNTP's, and 2.5 μL aliquots were taken in various times, terminated, and analyzed on 1.5% alkaline agarose gel (lanes 6−11) as described under Materials and Methods. Separately the PCNA clamp was assembled in the presence of RF-C and ATP on unlabeled primed ssDNA in the presence of all four dNTP's (mixture B). The DNA synthesis was initiated by addition of 2.5 μL of mixture A to 25 μL of mixture B. In this case, the resulting ratio was 64 microunits of pol δ per 1 ng of total primed DNA (lanes 1−5). After various times, the reactions were terminated and DNA products analyzed on a 1.5% alkaline agarose gel.

of pol δ core with the PCNA clamp. In order to study this interaction, pol δ holoenzyme was assembled on a ^{32}P-labeled primed ssDNA substrate (2 milliunits of pol δ per 1 ng of primed DNA) (mixture A), and DNA synthesis was analyzed (Figure 5, lanes 6−11). In a separate experiment, the PCNA clamp was assembled in the presence of ATP and RF-C on unlabeled primed ssDNA (mixture B); 2.5 μL of mixture A and 25 μL of mixture B were combined, resulting (i) in 11-fold dilution of assembled pol δ holoenzyme and (ii) in a ratio of 64 microunits of pol δ per 1 ng of total DNA (Figure 5, lanes 1−5). If the pol δ core forms a long-lived complex with the PCNA clamp, we might expect a similar amount of elongated primer in both cases since the dilution and start of replication occurred after holoenzyme was allowed to form. On the other hand, if free pol δ core molecules dissociated from the ^{32}P-labeled primed DNA, they would be trapped by the 30-fold excess of unlabeled primed DNA charged with a PCNA clamp, resulting in a lower level of labeled products. The dilution of pol δ holoenzyme and the trap of pol δ core together strongly prevented the elongation of labeled primers (Figure 5, lanes 1−5). This indicated that pol δ and the PCNA clamp do not form a long-lived complex; rather, they form a complex which readily dissociates. When preassembled pol δ holoenzyme started the replication in mixture A, most of the products elongated in 1.5 min corresponded to pausing site A (Figure 5, lane 7). Upon longer incubation, pol δ partially overcame this pause site (lanes 8−11). However, the products formed under the pol δ trap conditions corresponded mostly to the first pause site A even after 15 min of incubation (Figure 5, lanes 1−5). Therefore, we conclude that the pause sites do not simply cause the temporary stalling of pol δ holoenzyme, but rather promote the dissociation of pol δ core from the PCNA clamp.

Pol δ Holoenzyme Can Perform Limited Strand Displacement DNA Synthesis on a Circular Gapped dsDNA Template. Pol δ holoenzyme, in contrast to pols α and ε, has been shown to carry out limited strand displacement synthesis on a ssDNA template containing two primers (Podust & Hübscher, 1993). We therefore analyzed this activity of pol δ holoenzyme on a gapped dsDNA substrate (Podust et al., 1994). The increase in product size with time of incubation showed that strand displacement synthesis was slow; no products longer than 190 nt were observed even after 30 min of incubation (Figure 6). Since the assembly of pol δ holoenzyme on this gapped dsDNA substrate did not require RP-A or *E. coli* SSB (Podust et al., 1994), we tested the role of these SSBs in the strand displacement reaction. Neither RP-A nor *E. coli* SSB stimulated the strand displacement reaction catalyzed by pol δ holoenzyme on gapped dsDNA template (Figure 8, lanes 4, 6, 11, and 13).

Pol δ Holoenzyme Can Perform Extensive Synthesis on dsDNA Template in the Presence of Replicative DNA Helicases and RP-A. The inefficient strand displacement reaction catalyzed by pol δ holoenzyme alone (Figure 6) suggested that DNA helicase(s) might be required for replication on double-stranded DNA templates. We therefore tested the two known replicative DNA helicases SV40 Tag and BPV1 E1 for their ability to act cooperatively with pol δ. Tag has been shown to be the only helicase essential for the replication of SV40 DNA *in vitro* (Ishimi et al., 1988). Although E1 has not been extensively characterized, its enzymatic and functional properties are similar to those of Tag (Seo et al., 1993; Yang et al., 1993). As a substrate, a gapped double-stranded circular DNA was used (Podust et al., 1994). Efficient DNA synthesis by pol δ holoenzyme and each of the two DNA helicases was only observed in the presence of a SSB (either RP-A or *E. coli* SSB) (Figures 7 and 8). High levels of RP-A (Figure 7C,F) or *E. coli SSB*

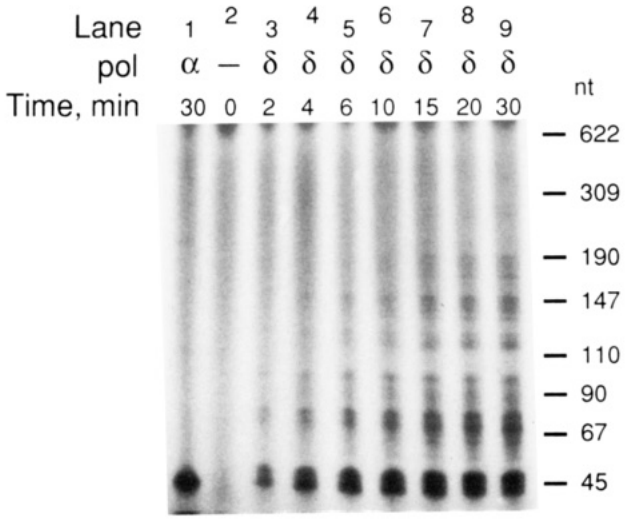

FIGURE 6: Pol δ holoenzyme fills the gap on a circular dsDNA and performs limited strand displacement DNA synthesis. The reaction mixtures contained TBD buffer, ATP, $MgCl_2$, dATP, dGTP, dTTP, and [α-^{32}P]dCTP, gapped dsDNA, PCNA, RF-C, and pol δ in amounts as outlined under Materials and Methods. After various times, the polymerization reaction was terminated by heating for 10 min at 70 °C. DNA was digested with *Eco*RI and finally treated with proteinase K. The samples were analyzed on a 5% PAGE, containing 7 M urea (8 cm × 10 cm × 0.8 mm). Lane1: the DNA synthesis was carried out by pol α (0.3 unit) in the absence of RF-C and PCNA. Lane 2: no enzyme. Lanes 3−9: various reaction times in the presence of pol δ, RF-C, and PCNA. Markers: the DNA fragments resulted from *Hpa*II digestion of pBR322.

(Figure 7B,E) inhibited this reaction. Less E1 (200 ng) than Tag (500 ng) was required for maximal stimulation of DNA synthesis (Figure 7). By analysis of the products on denaturing gels, no difference between RP-A and *E. coli* SSB was detected (Figure 8). The products synthesized in the presence of a SSB and Tag or E1 were up to 7000 nt in length (Figure 8).

DISCUSSION

Analysis of the polymerization products formed by the pol δ holoenzyme revealed multiple pause sites on the ssDNA templates (Figure 1). When the ssDNA was covered with *E. coli* SSB, pol δ holoenzyme could traverse the pause sites after a lag period. When *E. coli* SSB was removed from the DNA after pol δ holoenzyme assembly occurred, pol δ was unable to translocate through the pause sites even after prolongated incubation, obviously due to the restoration of the secondary structure of ssDNA. In agreement with this interpretation, preheating of a primed template in the presence of high levels of *E. coli* SSB destabilized the hairpins and resulted in the partial removal of the pause sites (Figure 4). The incomplete removal of pause sites suggested that some of the pause sites might be caused by primary rather than secondary template structures [see Bedinger et al. (1989) and references cited therein; see Abbotts et al. (1993) and references cited therein]. We conclude that the PCNA clamp, despite its capacity to efficiently load pol δ onto DNA, does not endow the holoenzyme with the capacity to resolve and traverse pause sites.

The DNA replication machinery not only must be assembled; it also must be dismantled when its task is complete. Analysis of the behavior of pol δ holoenzyme at the pause sites can also provide information about the disassembly of the pol δ holoenzyme. The PCNA clamp forms a stable and long-lived complex with DNA with a half-life of 22 min at 37 °C (Figure 2). A comparable half-life was determined for *Saccharomyces cerevisiae* (Burgers, 1991). Pause sites encountered by pol δ do not signal dissociation of the PCNA clamp (Figure 1). On the contrary, our experiments suggested that pol δ itself is easily dissociated from the PCNA clamp in the absence of DNA synthesis. During DNA synthesis, the dissociation of pol δ from the holoenzyme appears to occur preferentially at the pause site (Figure 5). In experiments[2] in which the concentration of pol δ was varied in the presence of fixed concentrations of PCNA, RF-C, and primed ssDNA, we also have observed that pol δ dissociates from the holoenzyme complex at specific, rather than at random, sites. This behavior suggests that pol δ holoenzyme traverses pause sites by a process of distributive "recycling" involving dissociation and reassociation of its various components. Our studies of mammalian replication holoenzyme agree well with a model of partial disassembly of prokaryotic pol holoenzymes (Stukenberg et al., 1994; Hacker & Alberts, 1994). When a region of duplex DNA is encountered by pol holoenzyme, pol core enzyme disengages from the clamp. The clamp itself remains stabily bound to DNA.

A physiologically relevant way to avoid nonspecific pausing and dissociation of the pol δ holoenzyme might be the cooperative action of pol δ with replicative DNA helicases. Two modes of combined actions of pol δ and DNA helicase on dsDNA could be expected: (i) DNA helicase unwinds the DNA, and RP-A binds to the separated strands to prevent reannealing and hairpin (and therefore pausing) formation. Pol δ then elongates the primer along the ssDNA covered with RP-A. (ii) Pol δ follows the helicase "head-to-tail" such that transient single-stranded regions are not formed on the DNA and RP-A is no longer required. These modes of action can be easily distinguished by the requirement for RP-A. As a substrate for combined pol δ /helicase assay, we used dsDNA containing a single defined gap of 45 nt (Podust et al., 1994). This substrate has the following advantages: (i) it allows unidirectional replication started from a defined position; (ii) assembly of pol δ holoenzyme is not dependent on RP-A (or *E. coli* SSB) (Podust et al., 1994); (iii) pol α/primase is not required to start replication; (iv) the substrate contains an entry site for Tag and E1 DNA helicases, since both enzymes unwind in the 3′→5′ direction (Goetz et al., 1988; Wiekowski et al., 1988; Seo et al., 1993; Yang et al., 1993). It has been shown that when Tag starts the unwinding reaction from a 3′-single-stranded extension of a partially double-stranded substrate, it unwinds at least 0.8−1.2 kb duplexes in the absence of SSB (Goetz et al., 1988; Wiekowski et al., 1988). However, our finding clearly indicated that SSB (RP-A and *E. coli* SSB with the same efficiency) was absolutely required for DNA synthesis by pol δ holoenzyme in the presence of replicative DNA helicases (Figures 7 and 8). This suggests that SSB transiently binds the single-stranded regions and thus coordinates the two enzymes, resulting in the synthesis of products up to full length (>7000 nt, Figure 8). The combined action of pol δ holoenzyme and Tag has been demonstrated in the SV40 *in vitro* replication system (Lee

[2] V. N. Podust and U. Hübscher, unpublished data.

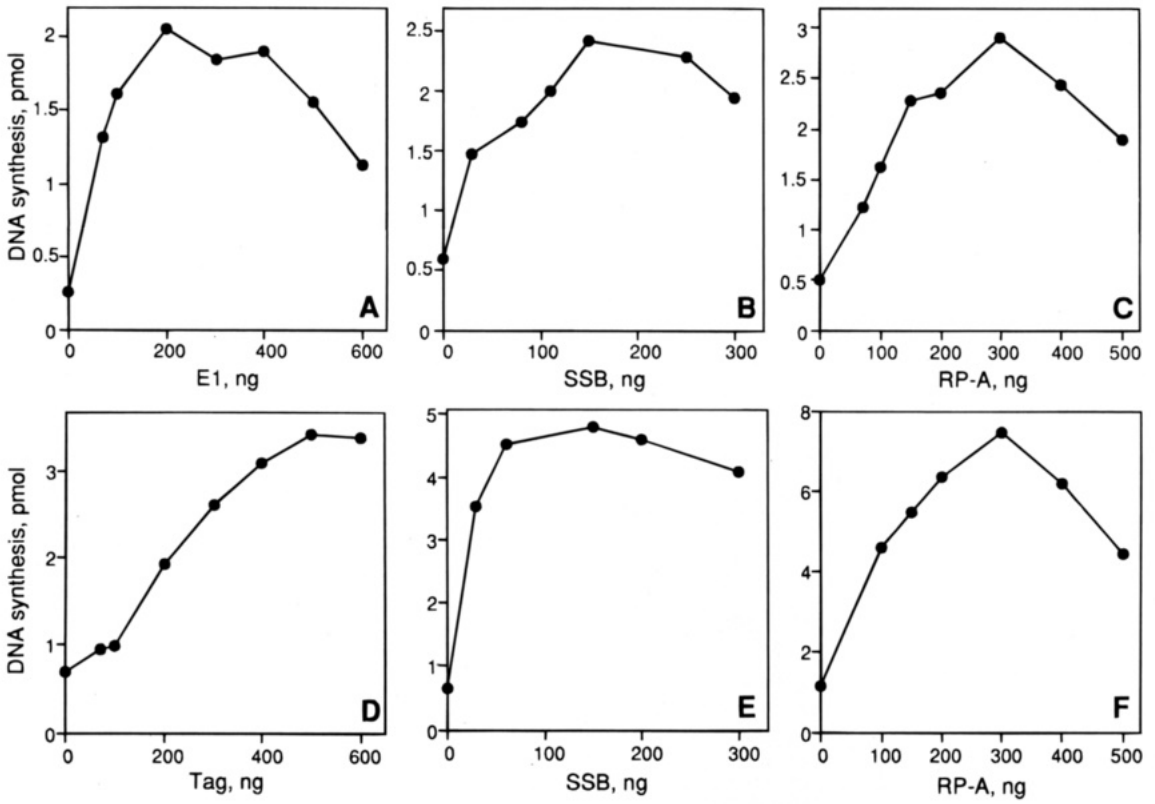

FIGURE 7: Requirements for efficient strand displacement synthesis by pol δ holoenzyme on a gapped dsDNA substrate. The reaction mixtures contained TBD buffer, 2 mM ATP, 7 mM $MgCl_2$, 50 μM each of dATP, dGTP, and dCTP and 15 μM [^{3}H]dTTP (1600 cpm/pmol), 50 ng of gapped M13 dsDNA, 20 ng of PCNA, 18 ng of RF-C, and 0.2 unit of pol δ. If not varied, 200 ng of E1 (panels B, C), 500 ng of Tag (panels E, F), and 200 ng of *E. coli* SSB (panels A, D) were added to the reaction mixture. The reactions were incubated for 1 h and analyzed as acid-precipitable material.

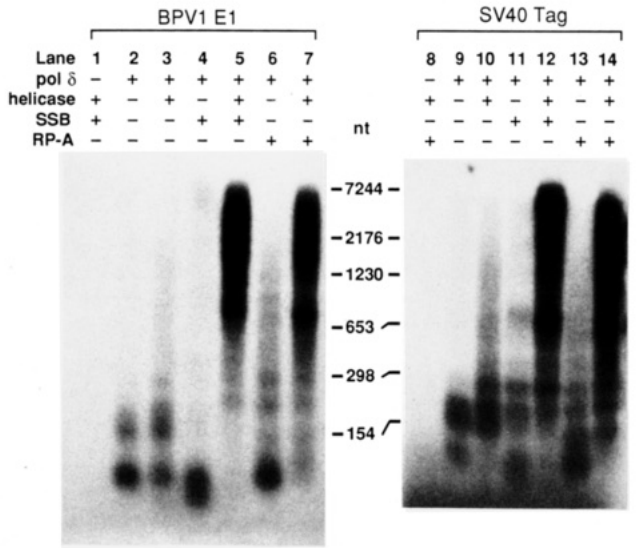

FIGURE 8: Strand displacement DNA synthesis by pol δ holoenzyme on gapped dsDNA is dependent on DNA helicase and a SSB. The reaction mixtures contained TBD buffer, 2 mM ATP, 7 mM $MgCl_2$, 50 μM each of dATP, dGTP, and dTTP and 15 μM [^{32}P]-dCTP (12 000 cpm/pmol), 50 ng of gapped M13 dsDNA, 20 ng of PCNA, 18 ng of RF-C, and, if added, 0.2 unit of pol δ, 200 ng of E1, 500 ng of Tag, 200 ng of *E. coli* SSB, or 300 ng of RP-A. The reactions were incubated for 1 h, DNA was digested with *Eco*RI/*Dpn*I, and the products were analyzed on a 1.5% alkaline agarose gel.

et al., 1989; Tsurimoto et al., 1990). However, the SV40 origin-dependent replication results from several events, namely, (i) primary unwinding of origin, (ii) initiation by pol α/primase, and (iii) elongation by pol δ holoenzyme (Tsurimoto et al., 1990). The absolute requirement of RP-A for the first two events makes the requirement of RP-A for the third step completely indistinguishable. In other origin-independent model systems, previous attempts to show cooperative action of pol δ with cellular helicases were not successful (Seo et al., 1991; Li et al., 1992a,b; Seo & Hurwitz, 1993; Ferrari & Hübscher[3]).

The well-characterized bacteriophage T4 and *E. coli* systems provide another example of pol/helicase cooperation. The functional interaction of T4 pol holoenzyme with the gp41 helicase allowed efficient rolling-circle replication even in the absence of the T4 gp32 SSB, without noticeable pausing and with a rate of DNA synthesis of 250 nt/s (Cha & Alberts, 1989). Similarily, *E. coli* pol III holoenzyme, coupled with DnaB helicase, carried out rolling-circle synthesis in the absence of *E. coli* SSB at a rate of 750 nt/s (Mok & Marians, 1987). The rate of SV40 DNA replication was reported to be 145 nt/min *in vivo* (Tapper et al., 1979) or 200 nt/min *in vitro* (Murakami & Hurwitz, 1993). The two-order of magnitude difference in replication rate between the SV40 and prokaryotic systems might be explained by these different modes of helicase/pol cooperation.

The data described in this paper further our understanding of how pol δ holoenzyme might act in leading-strand DNA synthesis (Tsurimoto et al., 1990). The PCNA clamp loads pol δ onto DNA and makes replication possible under physiological conditions. On the other hand, the possibility remains for pol δ to easily dissociate from the clamp if an obstacle is met (Figure 9). Such obstacles could be physi-

[3] E. Ferrari and U. Hübscher, unpublished data.

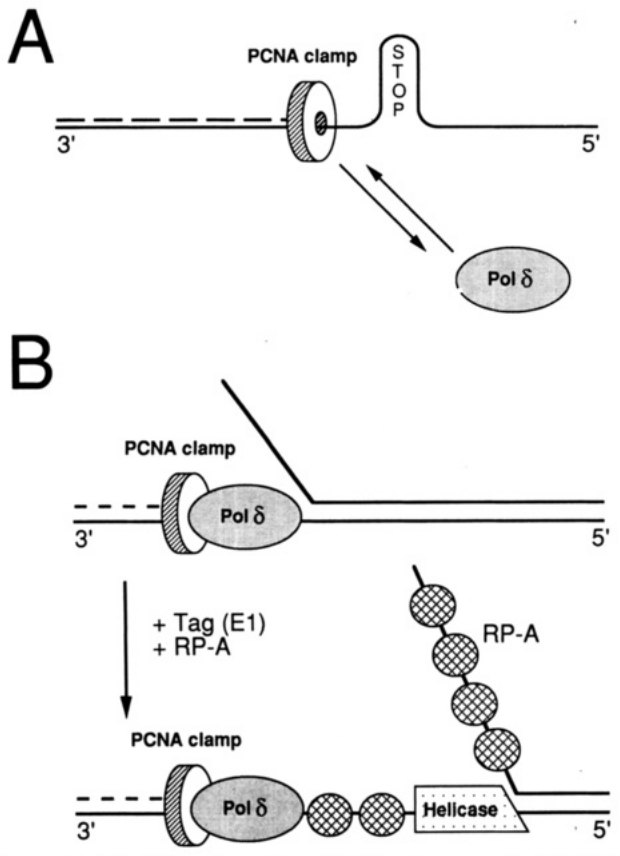

FIGURE 9: Models for the action of DNA polymerase δ. (A) PCNA clamp remains stably bound to DNA both in the absence and during DNA synthesis. Pol δ core dissociates and reassociates with the PCNA clamp at pause sites. (B) Strand displacement DNA synthesis in the presence of a replicative DNA helicase and RP-A. Pol δ holoenzyme (PCNA clamp/pol δ) is able to carry out limited strand displacement DNA synthesis. Efficient strand displacement DNA synthesis can be only observed in the simultaneous presence of DNA helicase and RP-A.

ologically important, for example, the collision of two converging replication forks, meaningful termination signals, or damaged regions on the template. Other cases, such as unspecific pausing or collision with DNA helicase moving at a slower rate than the polymerase, could be regulated by the action of additional factors. One such factor might be RP-A, which is required for the coordinated action of pol δ holoenzyme with Tag or E1 (Figure 9).

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REFERENCES

Abbotts, J., Bebenek, K., Kunkel, T. A., & Wilson, S. H. (1993) *J. Biol. Chem. 268,* 10312−10323.
Bedinger, P., Munn, M., & Alberts, B. M. (1989) *J. Biol. Chem. 264,* 16880−16886.
Burgers, P. M. J. (1991) *J. Biol. Chem. 266,* 22698−22706.
Burgers, P. M. J., & Kornberg, A. (1982) *J. Biol. Chem. 257,* 11468−11473.
Burgers, P. M. J., & Yoder, B. L. (1993) *J. Biol. Chem. 268,* 19923−19926.
Cha, T.-A., & Alberts, B. M. (1989) *J. Biol. Chem. 264,* 12220−12225.
Goetz, G. S., Dean, F., Hurwitz, J., & Matson, S. W. (1988) *J. Biol. Chem. 263,* 383−392.
Hacker, K. J., & Alberts, B. M. (1994) *J. Biol. Chem. 269,* 24221−24228.
Hübscher, U., & Spadari, S. (1994) *Physiol. Rev. 74,* 259−304.
Kornberg, A., & Baker, T. A. (1992) *DNA Replication*, Freeman, New York.
Kuriyan, J., & O'Donnell, M. (1993) *J. Mol. Biol. 234,* 915−925.
Lee, S.-H., & Hurwitz, J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 5672−5676.
Lee, S.-H., Eki, T., & Hurwitz, J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86,* 7361−7365.
Lee, S.-H., Kwong, A. D., Pan, Z.-Q., & Hurwitz, J. (1991) *J. Biol. Chem. 266,* 594−602.
Li, X., Tan, C.-K., So, A. G., & Downey, K. M. (1992a) *Biochemistry 31,* 3507−3513.
Li, X., Yoder, B. L., & Burgers, P. M. J. (1992b) *J. Biol. Chem. 267,* 25321−25327.
Mok, M., & Marians, K. J. (1987) *J. Biol. Chem. 262,* 16644−16654.
Müller, F., Seo, Y.-S., & Hurwitz, J. (1994) *J. Biol. Chem. 269,* 17086−17094.
Murakami, Y., & Hurwitz, J. (1993) *J. Biol. Chem. 268,* 11008−11017.
O'Donnell, M., Onrust, R., Dean, F. B., Chen, M., & Hurwitz, J. (1993) *Nucleic Acids Res. 21,* 1−3.
Podust, V. N., & Hübscher, U. (1993) *Nucleic Acids Res. 21,* 841−846.
Podust, V. N., Georgaki, A., Strack, B., & Hübscher, U. (1992a) *Nucleic Acids Res. 20,* 4159−4165.
Podust, V., Mikhailov, V., Georgaki, A., & Hübscher, U. (1992b) *Chromosoma 102,* S133−S141.
Podust, L. M., Podust, V. N., Floth, C., & Hübscher, U. (1994) *Nucleic Acids Res. 22,* 2970−2975.
Sambrook, J., Fritsch, E. F., & Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Seo, Y.-S., & Hurwitz, J. (1993) *J. Biol. Chem. 268,* 10282−10295.
Seo, Y.-S., Lee, S.-H., & Hurwitz, J. (1991) *J. Biol. Chem. 266,* 13161−13170.
Seo, Y.-S., Müller, F., Lusky, M., Gibbs, E., Kim, H.-Y., Phillips, B., & Hurwitz, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 2865−2869.
Stillman, B. (1994) *Cell 78,* 725−728.
Stukenberg, P. T., Turner, J., & O'Donnell, M. (1994) *Cell 78,* 877−887.
Tapper, D. P., Anderson, S., & DePamphilis, M. L. (1979) *Biochim. Biophys. Acta 565,* 84−97.
Thömmes, P., Ferrari, E., Jessberger, R., & Hübscher, U. (1992) *J. Biol. Chem. 267,* 6063−6073.
Tsurimoto, T., & Stillman, B. (1989) *EMBO J. 8,* 3883−3889.
Tsurimoto, T., & Stillman, B. (1991) *J. Biol. Chem. 266,* 1950−1960.
Tsurimoto, T., Melendy, T., & Stillman, B. (1990) *Nature 346,* 534−539.
Wiekowski, M., Schwarz, M. W., & Stahl, H. (1988) *J. Biol. Chem. 263,* 436−442.
Yang, L., Mohr, I., Fouts, E., Lim, D. A., Nohaile, M., & Botchan, M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 5086−5090.
Yoder, B. L., & Burgers, P. M. J. (1991) *J. Biol. Chem. 266,* 22689−22697.

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