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Mechanism of elongation of primed DNA by DNA polymerase δ, proliferating cell nuclear antigen, and activator 1

(DNA elongation/leading-strand synthesis/simian virus 40 replication/protein–DNA complex formation)

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ABSTRACT **In the presence of a single-stranded-DNA-binding protein (SSB), the elongation of primed DNA templates by DNA polymerase δ (pol δ) is dependent on ATP and two protein factors, activator 1 (A1) and proliferating cell nuclear antigen (PCNA). We have examined the interaction of these proteins with $(dA)_{4500}$·$(dT)_{12-18}$ by measuring their ability to form stable complexes with this DNA. In the presence of ATP, A1, PCNA, and pol δ formed a stable complex with DNA that could be isolated by gel filtration. Incubation of the isolated complex with dTTP resulted in the synthesis of poly(dT). While ATP was required for the formation of this complex, it was not required for the subsequent elongation of DNA. The temporal requirements for complex formation were determined. A1 was found to bind first, followed by the ATP-dependent addition of PCNA to the A1·DNA complex, while pol δ was added last. Each of these complexes could be isolated by gel filtration, indicating that they possessed a high degree of stability. The binding of PCNA to the A1-SSB-coated primed DNA occurred with adenosine 5′-[γ-thio]triphosphate as well as ATP. However, the binding of pol δ to the PCNA·A1·DNA complex was observed only when the latter complex was formed in the presence of ATP. The complete complex was formed after incubation at 37°C for 2 min, whereas no complex was detected after incubation at 0°C. These results indicate that these proteins act in a manner analogous to the accessory proteins that play critical roles in the elongation reaction catalyzed by T4 phage DNA polymerase and *Escherichia coli* DNA polymerase III.**

Even though DNA polymerase δ (pol δ) was identified more than a decade ago in higher eukaryotes (1), its important role in DNA replication has only recently been demonstrated. Proliferating cell nuclear antigen (PCNA), a 37-kDa protein factor which stimulates pol δ activity on multiply primed DNA (2), has been identified as a protein which is required for efficient simian virus 40 (SV40) DNA replication *in vitro* (3, 4). In addition to PCNA, activator 1 protein (A1) is required for pol δ activity in the SV40 replication system as well as in the elongation of primed DNA substrates (5, 6).

The multiprotein complex A1 was originally identified as a factor required for the reversal of the inhibition of SV40 DNA replication by poly(ADP-ribose) polymerase (or elongation inhibitor) (7). Poly(ADP-ribose) polymerase, a 120-kDa protein, preferentially binds at the ends of nascent DNA chains, resulting in the accumulation of small, Okazaki-fragment-sized DNA products. This inhibition can be efficiently reversed by the combined action of A1, PCNA, and pol δ. It was subsequently shown that A1 specifically stimulated pol δ and had little effect on pol α. Replication factor C (RF-C), identified by Tsurimoto and Stillman (8, 9) as a factor required for SV40 DNA replication and pol δ activity on a primed DNA template, is probably identical to A1. RF-C and A1 possess similar complex subunit structures (140, 40, 36, and 34 kDa) and intrinsic DNA-dependent ATPase activity and primer-binding activity (ref. 10; unpublished results). Recent studies indicated that both pol α and pol δ are involved in SV40 DNA replication (6, 8, 11, 12). It has been proposed that pol α is involved in lagging-strand synthesis, while pol δ is involved in leading-strand synthesis (13). This model is based on the findings that pol α is associated with DNA primase, while pol δ lacks such an activity (14), and the pol δ accessory factors, PCNA and A1 or RF-C, affect only leading-strand DNA synthesis (8, 11). A1 was found to be specific for pol δ and had no demonstrable effect on pol α; however, RF-C was reported to stimulate both pol α and pol δ. How these two distinct DNA polymerases act conjointly at the replication fork is presently unclear.

In this study, we investigated the interaction between pol δ, PCNA, A1, and the primed DNA template $(dA)_{4500}$·$(dT)_{12-18}$ coated with single-stranded-DNA-binding protein (SSB) by measuring their ability to form stable protein–DNA complexes that are isolable by gel filtration. Our results indicate that pol δ, PCNA, and A1 form a stable complex with a primed DNA template and its formation requires ATP. The order of addition of these proteins to SSB-coated poly(dA)·oligo(dT) has been determined. A1 binds first, followed by the ATP-dependent addition of PCNA, while the addition of pol δ occurs last. Furthermore, adenosine 5′-[γ-thio]triphosphate (ATP[γS]) substituted for ATP in the binding of PCNA to the A1·DNA complex, but pol δ addition occurred only with the complex formed in the presence of ATP. In the presence of ATP, PCNA interacts with A1 complexed to primed DNA, indicating that PCNA mediates the interaction between A1 and pol δ, presumably at the primer end of the DNA.

MATERIALS AND METHODS

Reagents and Enzymes. $(dA)_{4500}$ and $(dT)_{12-18}$ were from Life Sciences (Saint Petersburg, FL) and Pharmacia–LKB Biotechnology, respectively; ATP and ATP[γS] were from Boehringer Mannheim and Bio-Gel A-5m was from Bio-Rad. Human SSB (650 units/mg of protein) was prepared from HeLa cytosolic extracts as described (15) and pol δ (1.4×10^3 units/mg of protein), PCNA (0.4×10^4 units/mg of protein), and A1 (1.0×10^4 units/mg of protein) were purified from the cytosolic extracts of HeLa cells by procedures to be published elsewhere. Purification was monitored by using the pol δ elongation assay described below. Judging from SDS/polyacrylamide gel electrophoresis followed by silver staining, PCNA, A1, and pol δ were 95%, 80%, and 50% pure, respectively; none of these enzyme preparations contained detectable poly(ADP-ribose) polymerase.

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Abbreviations: SV40, simian virus 40; SSB, single-stranded-DNA-binding protein; PCNA, proliferating cell nuclear antigen; A1, activator 1 protein; RF-C, replication factor C; pol α and pol δ, DNA polymerases α and δ, respectively; ATP[γS], adenosine 5′-[γ-thio]-triphosphate; BSA, bovine serum albumin.

Assays for pol δ, PCNA, and A1. The activities of these proteins were measured in reaction mixtures (20 μl) containing 25 mM Tris·HCl (pH 7.8), 7 mM $MgCl_2$, 2 mM dithiothreitol, bovine serum albumin (BSA) at 50 μg/ml, 2 mM ATP, 33.3 μM [^{3}H]dTTP (300 cpm/pmol), 0.1 μg of $(dA)_{4500}$·$(dT)_{12-18}$ (at a nucleotide ratio of 20:1, respectively), and 1.0 μg of human SSB. For the assay of pol δ activity, reaction mixtures were supplemented with A1 (0.2 unit, 20 ng of protein) and PCNA (0.2 unit, 50 ng of protein); for the assay of A1, reaction mixtures contained PCNA (0.2 unit) and pol δ (0.1 unit, 70 ng of protein); for the assay of PCNA, reaction mixtures contained pol δ (0.1 unit) and A1 (0.2 unit). In all cases, incubation was for 60 min at 37°C followed by the measurement of dTMP incorporation into trichloroacetic acid-insoluble form. One unit of pol δ or PCNA or A1 supports the incorporation of 1 nmol of dTMP under the conditions specified.

RESULTS

The Combination of A1, PCNA, and pol δ Can Form a Stable Complex with Primed DNA. PCNA can stimulate pol δ activity by increasing its processivity on a multiply primed DNA in the absence of ATP. However, in the presence of SSB, pol δ activity required PCNA, A1, and ATP (Table 1). A1 is a multiprotein complex which preferentially binds at the primer-template junction and possesses DNA-dependent ATPase activity (ref. 10 and unpublished data). When multiply primed DNA was used as a template, A1 lowered the amount of PCNA required for pol δ activity about 100-fold (unpublished data), indicating that there is an interaction between A1 and PCNA. These observations prompted us to examine the interaction between PCNA, A1, and pol δ with DNA. For this purpose, pol δ, PCNA, A1 (1.0, 2.0, and 0.6 pmol of each protein, respectively), and ATP were added to a reaction mixture containing SSB-coated multiply primed $(dA)_{4500}$·$(dT)_{12-18}$ (7.2 pmol of primers). After incubation for 2 min at 37°C, the reaction mixture was filtered through a Bio-Gel A-5m column (0.7 × 13.5 cm) at 4°C and the effluent fractions were assayed for pol δ, PCNA, and A1, using the complementation assay for pol δ activity as described in Table 1. Excluded fractions 16 to 20, supplemented with dTTP and ATP, supported poly(dT) synthesis. Approximately 55% of dTMP incorporation expected was observed with these fractions (Fig. 1*A*). The supplementation of these fractions with excess pol δ, PCNA, or A1 increased dTMP incorporation only 30–70%, indicating that pol δ, PCNA, and A1 formed an isolable complex with the DNA.

By using the pol δ complementation assay, proteins that were not in the protein–DNA complex but were in the included fractions could be measured (Fig. 1*B*). As shown, free pol δ and PCNA, peaking in fractions 30 and 32, respectively, were detected; however, no free A1 was observed. Since A1 can bind to a primed DNA template (ref. 10 and unpublished results), these results suggest that all of the added A1 was complexed with DNA. A1 is the critical protein required to form the protein complex with primed DNA, since pol δ and PCNA alone or in combination did not form a stable complex with DNA in the absence of A1 (data not shown).

Table 1. Requirements for pol δ activity with SSB-coated $(dA)_{4500}$·$(dT)_{12-18}$

Reaction mixture	dTMP incorporated in 60 min, pmol
Complete	97.5
Omit PCNA	0.4
Omit A1	3.1
Omit pol δ	0.2
Omit ATP	3.1
Omit ATP, add ATP[γS] (2 mM)	6.1

The complete reaction mixture (20 μl) contained 0.1 μg of $(dA)_{4500}$·$(dT)_{12-18}$ (20:1), human SSB (1.0 μg), A1 (60 ng), pol δ (0.1 unit), PCNA (50 ng), and 2 mM ATP. Other conditions were as described in *Materials and Methods*.

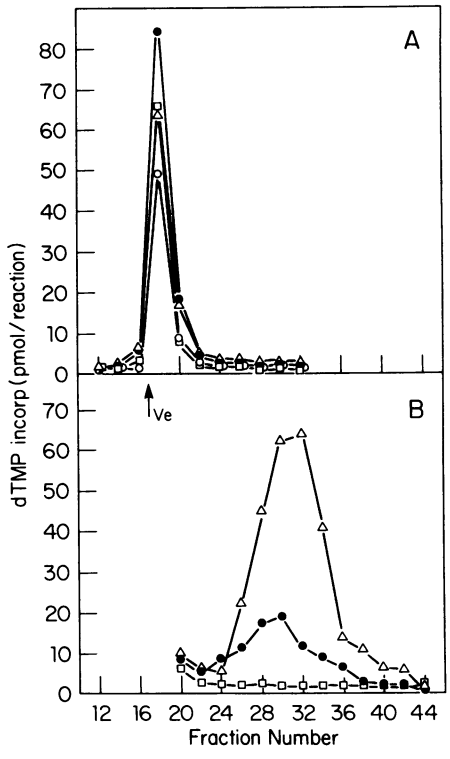

FIG. 1. Isolation of a stable preinitiation complex of pol δ, PCNA, and A1 with SSB-coated DNA by gel filtration. (*A*) A reaction mixture (80 μl) containing 25 mM Tris·HCl (pH 7.5), 2 mM dithiothreitol, 7 mM $MgCl_2$, BSA at 50 μg/ml, 2 mM ATP, 0.6 μg of $(dA)_{4500}$·$(dT)_{12-18}$ (at a nucleotide ratio of 20:1), 6.0 μg of human SSB, 1.2 units of A1, 1.2 units of PCNA, and 0.9 unit of pol δ was assembled on ice. After incubation of the reaction mixture for 2 min at 37°C, the sample was placed on ice and was immediately filtered through a Bio-Gel A-5m column (0.7 × 13.5 cm) at 4°C equilibrated with buffer containing 20 mM Tris·HCl (pH 7.5), 2 mM dithiothreitol, BSA at 100 μg/ml, 7 mM $MgCl_2$, 50 mM KCl, and 5% (vol/vol) glycerol. The column was developed with the same buffer and fractions (140 μl each) were collected at 4°C. The gel filtration step took about 10 min. The arrow indicates the excluded volume (V_e) as measured by blue dextran exclusion (2000 kDa). The fractions were then assayed for their ability to support poly(dT) formation. For this purpose, each eluted fraction (20 μl) was incubated with 33.3 μM [^{3}H]dTTP (300 cpm/pmol) and 2 mM ATP to score for active complex formation (○); each fraction was also supplemented with the nucleotides and 0.1 unit of pol δ (●), 0.2 unit of PCNA (△), or 0.1 unit of A1 (□). After incubation at 37°C for 60 min, dTMP incorporation was measured. (*B*) The included fractions that lacked activity (fractions 20–44), as shown in *A*, were assayed for free PCNA (△), pol δ (●), and A1 (□), using the pol δ complementation assay. In each assay, 20 μl of each column fraction was used.

ATP Is Required for the Formation of a Stable pol δ, PCNA, and A1 Complex with DNA. A1 binds preferentially to a primed DNA in the absence of ATP, although ATP can stimulate the reaction about 2-fold (unpublished data). However, ATP was essential for pol δ activity in the presence of A1, PCNA, and SSB in the $(dA)_{4500}$·$(dT)_{12-18}$ system. To examine the role played by ATP in forming the active complex, reaction mixtures containing these proteins and DNA were incubated in the absence of ATP or in the presence of ATP[γS], a nonhydrolyzable ATP analog, then gel filtered, and the excluded fractions were assayed for activity (Fig. 2). In the absence of ATP, both pol δ and PCNA were not detected in the excluded protein–DNA complex

after gel filtration (Fig. 2A). As expected, A1 was retained in the excluded DNA complex, since these fractions, when supplemented with pol δ, PCNA, and ATP, supported poly(dT) synthesis (Fig. 2B).

Complexes formed in the presence of ATP[γS] had little activity (Fig. 2C). Supplementation of these excluded DNA complex fractions with ATP indicated that PCNA and A1 were present but pol δ was missing completely (Fig. 2D). These results suggest that ATP (and most likely its hydrolysis) is required for pol δ binding to the PCNA·A1·SSB-coated primed DNA. Furthermore, the ATP activation of the complex isolated after incubation with ATP[γS] in the presence of the auxiliary proteins and pol δ (Fig. 2D) suggests that the complex with the nonhydrolyzable analog may be reversible or unstable.

ATP Is Not Required for Subsequent DNA Elongation After Complex Formation. ATP is required to form the complex containing pol δ, PCNA, and SSB-coated DNA (Figs. 1 and 2). Whether ATP plays an additional role after complex formation was examined. For this purpose, the complex of pol δ, PCNA, A1, and SSB-coated DNA was formed in the presence of ATP and then isolated by gel filtration in the absence of ATP. The isolated complex was assayed for its ability to synthesize poly(dT) in the presence or absence of ATP (Fig. 3). The initial rates of DNA synthesis over a 10-min period were identical. In the absence of ATP, the reaction plateaued, while in the presence of ATP, the rate of poly(dT) synthesis was maintained for a longer length of time. This

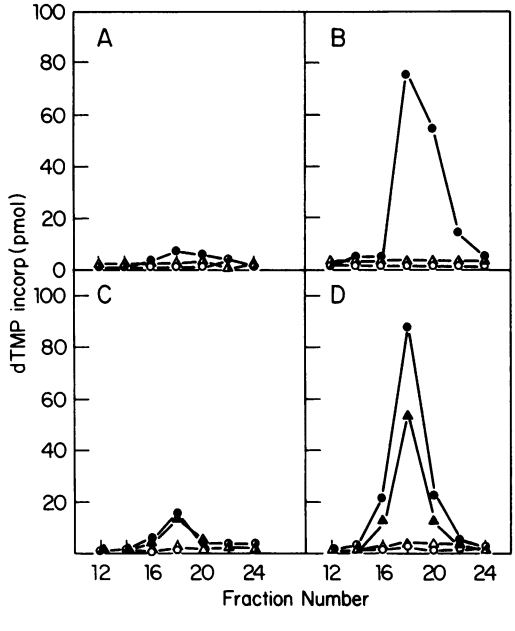

FIG. 2. Effect of ATP on complex formation. In A, a reaction mixture (80 μl), containing the same additions as described for Fig. 1A, with the exception that ATP was omitted, was incubated at 37°C for 2 min and applied to a Bio-Gel A-5m column at 4°C, as described for Fig. 1A. Fractions near the excluded volume (17 μl per assay) were each incubated with 33.3 μM [^{3}H]dTTP (300 cpm/pmol) alone (○) or with [^{3}H]dTTP supplemented with 0.1 unit of pol δ (▲), 0.2 unit of PCNA (△), or 0.1 unit of pol δ plus 0.2 unit of PCNA (●) for 60 min at 37°C in the absence (A) or presence (B) of 2 mM ATP, and then dTMP incorporation was measured. In C and D, reaction mixtures were as described for Fig. 1A, with the exception that ATP[γS] was added in place of ATP. DNA synthesis was measured by incubating eluted fractions (17 μl per assay) with 33.3 μM [^{3}H]dTTP (300 cpm/pmol) (○) or with [^{3}H]dTTP in the presence of 0.1 unit of pol δ (▲), 0.2 unit of PCNA (△), or 0.1 unit of pol δ plus 0.2 unit of PCNA (●) (C). In D, reaction mixtures with the eluted fractions also contained 2 mM ATP, in addition to the proteins described for C. The symbols used in D are the same as those used in C. Incubations were for 60 min at 37°C.

stimulation may reflect the recycling of the proteins, which requires the presence of ATP. Thus, once the complex is formed in the presence of ATP, the elongation reaction catalyzed by the complex does not require ATP.

Stable Protein–DNA Complex Is Formed Rapidly. Solutions containing limiting amounts of pol δ, PCNA, and A1 were mixed with SSB-coated $(dA)_{4500}\cdot(dT)_{12-18}$ in the presence of ATP and incubated at 37°C for 0, 2, and 30 min, followed by the gel filtration at 4°C. As shown in Fig. 4, active complex was formed with the same apparent efficiency after 2 or 30 min of incubation. However, the pol δ–auxiliary protein–DNA complex was not observed in the reaction mixture that was incubated at 0°C.

A1 and PCNA Form a Stable Complex with Primed DNA but A1 and pol δ Do Not. A1 markedly reduced the amount of PCNA required for pol δ activity with $(dA)_{4500}\cdot(dT)_{12-18}$ primer template (unpublished results). This suggested that there is an interaction between PCNA and A1. To further determine the interactions between A1, PCNA, and pol δ with DNA, A1 and PCNA, as well as A1 and pol δ, were incubated separately with SSB-coated $(dA)_{4500}\cdot(dT)_{12-18}$, in the presence or absence of ATP, followed by gel filtration. The proteins present in these protein–DNA complexes were determined by using the pol δ complementation assay (Fig. 5). A1 and PCNA formed an isolable complex with DNA in the presence of ATP (Fig. 5 A and B) as well as in the presence of ATP[γS] (data not shown, but see Fig. 2D). In contrast, when A1 and pol δ were preincubated with DNA, pol δ was not detected in the isolated protein–DNA complex (Fig. 5 C and D). This suggests that PCNA, complexed to the A1 and DNA, is required for pol δ binding. In the absence of A1, neither pol δ nor PCNA alone or in combination formed a stable complex with SSB-coated DNA (data not shown).

DISCUSSION

pol δ carries out the leading-strand DNA synthesis in the replication of SV40 DNA *in vitro*. This activity requires at least two distinct auxiliary protein factors, PCNA and A1, in addition to SSB and ATP. In this paper, we have examined

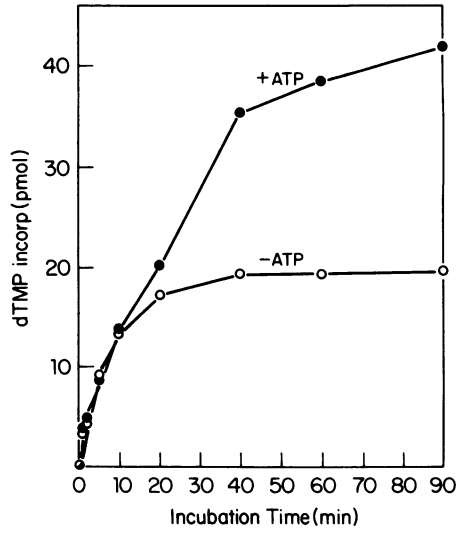

FIG. 3. Effect of ATP on subsequent DNA elongation of isolated complexes. A reaction mixture (80 μl) contained pol δ, A1, PCNA, human SSB, $(dA)_{4500}\cdot(dT)_{12-18}$, and ATP as described for Fig. 1A. The mixture was preincubated for 2 min at 37°C and subjected to gel filtration as described for Fig. 1A. The excluded volume fractions (fractions 16–18) were collected and divided into two tubes (210 μl per tube) and incubated with 33.3 μM [^{3}H]dTTP (350 cpm/pmol) in the presence (●) or absence (○) of 2 mM ATP. At the indicated times, 20-μl aliquots were removed and the amount of acid-insoluble [^{3}H]dTMP was determined.

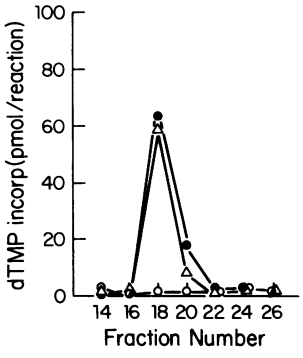

FIG. 4. Effect of incubation on complex formation. Three sets of reaction mixtures (80 μl per reaction) containing the ingredients of Fig. 1A were incubated for 0 (○), 2 (●), or 30 (△) min at 37°C, followed by gel filtration. The reaction mixture and gel filtration procedure were as described for Fig. 1A. The excluded fractions (30 μl per assay) were incubated for 60 min at 37°C in the presence of 33.3 μM [^{3}H]dTTP (300 cpm/pmol) and 2 mM ATP.

how pol δ, PCNA, and A1 interact with each other on a primed template and the role by ATP in the generation of stable protein–DNA complexes which support pol δ activity. The results presented here suggest the following mechanism of DNA elongation of primed DNA template by pol δ (Fig. 6). First, the multiprotein complex, A1, binds to the primer-template junction of human SSB-coated DNA, yielding a stable complex with DNA [A1·DNA complex (I)]. A1 is thought to be a primer-binding protein on the basis of the

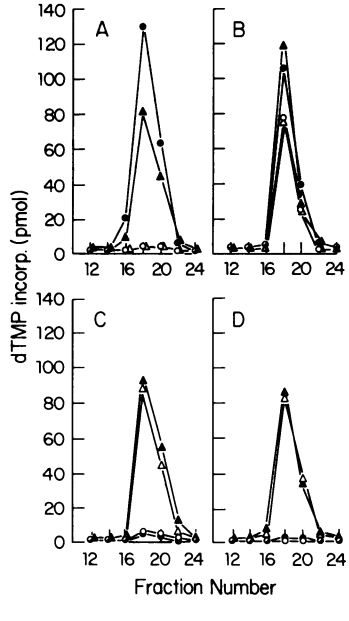

FIG. 5. A1·DNA complex interacts with PCNA but not with pol δ. Reaction mixture (80 μl) contained 25 mM Tris·HCl (pH 7.5), 2 mM dithiothreitol, 7 mM $MgCl_2$, BSA at 50 μg/ml, 0.6 μg of $(dA)_{4500}$·$(dT)_{12-18}$, 6.0 μg of human SSB, 1.2 units of A1, and 1.2 units of PCNA, either in the absence (*A*) or presence (*B*) of 2 mM ATP. Reaction mixtures were incubated for 2 min at 37°C, then filtered as described for Fig. 1A. Void volume fractions (20 μl per assay) were incubated with 33.3 μM [^{3}H]dTTP and 2 mM ATP in the presence of pol δ (○), pol δ + A1 (△), pol δ + PCNA (▲), or pol δ + PCNA + A1 (●) for 60 min at 37°C. In *C* and *D*, reaction mixtures (80 μl) were as described for *A* with the exception that 0.9 unit of pol δ was added and PCNA was omitted. Incubation was carried out in the absence (*C*) or presence (*D*) of 2 mM ATP for 2 min at 37°C. The mixtures were gel filtered and fractions (20 μl per assay) were incubated with 33.3 μM [^{3}H]dTTP and 2 mM ATP in the presence of PCNA (○), PCNA + A1 (●), PCNA + pol δ (△), or PCNA + pol δ + A1 (▲) for 60 min at 37°C.

observation that $(dA)_{4500}$ or $(dT)_{12-18}$ alone does not support the binding of A1, while $(dA)_{4500}$·$(dT)_{12-18}$ does. A1 lowers the affinity of pol δ for primer ends more than 10-fold in the $(dA)_{4500}$·$(dT)_{12-18}$ system (unpublished results). A1 has an intrinsic DNA-dependent ATPase activity and probably forms an ATP-bound A1·DNA complex (II) which, in turn, allows the addition of PCNA to the A1·DNA complex. The binding of PCNA to A1 on the DNA stimulates ATP hydrolysis by A1 (10) and may cause conformational changes in the A1 that position PCNA on the DNA template so it can subsequently interact with pol δ. The nonhydrolyzable ATP analog ATP[γS] supported the formation of the A1·PCNA complex with DNA but did not support the addition of pol δ to the A1·PCNA·DNA complex. After formation of a stable DNA complex of pol δ, PCNA, and A1 in the presence of ATP, the addition of dTTP resulted in DNA synthesis in the absence of additional ATP. Once DNA elongation is completed, the complex of pol δ and its accessory factors can recycle in the presence of ATP. Binding of A1 to primed DNA is clearly the initial step in this pathway, since pol δ or PCNA,

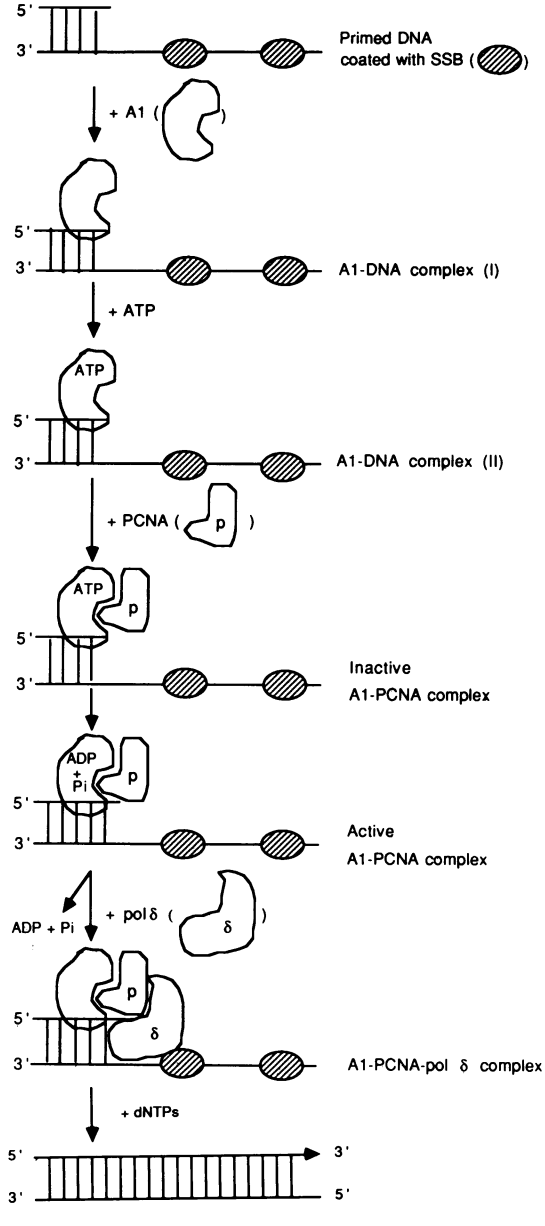

FIG. 6. Proposed model of the interaction of DNA polymerase δ and its accessory factors with a primed DNA template.

alone or in combination, could not complex with DNA in the absence of A1.

The experiments presented here were all carried out with $(dA)_{4500}\cdot(dT)_{12\text{-}18}$. Identical experiments were performed with the singly primed phage ϕX174 single-stranded circular DNA hybridized to a 30-mer complementary to nucleotides 5127–5156. The results obtained with this primed template were basically the same as those obtained with the synthetic polymers.

In the *Escherichia coli* pol III system, the core DNA polymerase III (α and ε), $\gamma\cdot\delta$ complex, and β can form an ATP-dependent complex with primed templates which is capable of processive DNA synthesis in the presence of *E. coli* SSB (16–18). In the T4 phage system, the protein products of genes *45* and *44/62* and T4 DNA polymerase (the gene *43* protein) can form stable complexes with primed DNA in the presence of the T4 gene *32* protein (SSB) and ATP. This complex catalyzes rapid, processive synthesis of DNA (19). Thus, the replicative DNA polymerases in the T4, *E. coli*, and human pol δ systems all form stable complexes with their accessory factors on the DNA in the presence of ATP. It is evident that the action of PCNA and the multisubunit A1 in the pol δ system are similar to the activity of gene *45* and *44/62* products complex in the T4 system and to β and $\gamma\cdot\delta$ complex in the *E. coli* system, respectively. In addition, the phage T4 gene *44/62* product complex contains DNA-dependent ATPase activity which is markedly stimulated by the gene *45* protein (13, 20–23). Similarly, the DNA-dependent ATPase activity of RF-C (A1) was stimulated by PCNA (10). Tsurimoto and Stillman (10) have reported that there is some homology between the amino acid sequence of T4 gene *45* protein and human PCNA. However, neither T4 gene *45* protein nor the β subunit of *E. coli* substituted for PCNA in the activation of pol δ on a primed DNA (data not shown). Moreover, the rate of DNA synthesis in the pol δ system is considerably slower than the rate of DNA synthesis in T4 or *E. coli* systems. At present, it is not clear whether this difference is due to an intrinsic property of the pol δ system or due to the lack of an additional accessory factor(s).

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