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Biochemistry

The subunits of activator 1 (replication factor C) carry out multiple functions essential for proliferating-cell nuclear antigen-dependent DNA synthesis

(DNA polymerases δ and ε)

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ABSTRACT **p37 and p40 are two cloned gene products of the five-subunit human cellular DNA replication factor activator 1 (A1) protein complex (also called replication factor C). Here, we describe the solubilization, purification, and characterization of these two proteins that were overproduced in *Escherichia coli*. Using a nitrocellulose filter binding assay, we demonstrated that the purified A1 p37 protein associated with DNA preferentially at the primer terminus, a property resembling that of the A1 complex. We also show that in the presence of relatively high levels of salt, the recombinant p37 protein alone activated DNA polymerase ε but not polymerase δ in catalyzing the elongation of DNA chains. The p40 protein specifically associated with cellular p37 and proliferating-cell nuclear antigen (PCNA) present in HeLa cell cytosolic extract. The addition of purified p40 protein abolished the *in vitro* polymerase δ-catalyzed DNA elongation reaction dependent on both PCNA and A1. However, this inhibition was reversed by excess polymerase δ, suggesting a specific interaction between the polymerase and the p40 protein. Thus, while p37 binds DNA at the primer end and has a specific affinity for pol ε, p40, which binds ATP, interacts with PCNA and pol δ. These activities are essential for the DNA elongation reactions that lead to the synthesis of leading-strand DNA and the maturation of Okazaki fragments.**

Activator 1 [A1, also known as replication factor C (RF-C)] is a multisubunit protein complex from human cells that is required for the proliferating-cell nuclear antigen (PCNA)-dependent DNA polymerase (pol) δ-catalyzed synthesis of simian virus 40 DNA *in vitro* (refs. 1 and 2; for reviews, see refs. 3 and 4). Purified A1 contains five polypeptides of 145, 40, 38, 37, and 36.5 kDa; binds to DNA at the primer terminus; and forms a stable complex with PCNA in the presence of ATP or adenosine 5′-[γ-thio]triphosphate (5, 6). Under conditions that lead to A1-catalyzed ATP hydrolysis, the A1/PCNA/DNA complex associates with pol δ, which then efficiently elongates DNA chains (6). More recently, A1, together with PCNA and human single-stranded DNA-binding protein (HSSB), was shown to activate the pol ε-catalyzed elongation of DNA chains from primed templates at physiological salt concentrations (7, 8).

We have reported the cDNA cloning and expression of p40 and p37, two subunits of the A1 complex (9, 10). Their cDNA sequences revealed that both subunits contain a putative ATP-binding site and share significant homology with their functional counterparts, the T4 phage gene product 44 and the γ and τ proteins encoded by the *Escherichia coli dnaZX* gene. The bacterially expressed A1 p40 was shown to bind ATP after ultraviolet crosslinking (9), a result consistent with that of Tsurimoto and Stillman (11). The addition of polyclonal antibodies against p40 alone, or polyclonal antibodies against p37 together with low levels of anti-p40, blocked the *in vitro* pol δ-catalyzed DNA elongation reaction dependent on both PCNA and A1. This suggests that these two subunits may both be indispensable for the function of the A1 complex. In this report, we describe the further biochemical characterization of A1 p37 and p40.

MATERIALS AND METHODS

Enzymes and Antibody Reagent. Enzymes were purified as described (2, 12–14), as were polyclonal antibodies against p37 and p40 (9, 10).

Solubilization and Purification of A1 p37 and p40. The procedures for the overproduction of A1 p37 and p40 were described previously (9, 10). After isopropyl β-D-thiogalactopyranoside induction, a 1-liter culture of *E. coli* was harvested and lysed by addition of 20 ml of 0.05 M Tris·HCl, pH 8.0/10% sucrose/0.1% lysozyme/0.6% Brij 58/10 mM dithiothreitol (DTT)/10 mM EDTA and incubation for 45 min at 0°C. After sonication and centrifugation at 17,000 × *g* for 10 min, the pellets were resuspended in 6 M urea/20 mM DTT and incubated for 30 min at 37°C (p40 extract, 30 mg of protein; p37 extract, 135 mg of protein). The urea-containing extracts were diluted 3-fold in buffer A [0.05 M Tris·HCl, pH 7.8/0.6 mM phenylmethylsulfonyl fluoride/5 mM EDTA/10% (vol/vol) glycerol/0.1% Triton X-100] and chromatographed on DEAE-Sepharose [column dimensions: for p40, 1.5 × 3.2 cm (5.7 ml); for p37, 1.5 × 7.5 cm (13 ml)] preequilibrated with buffer A/2 M urea/5 mM DTT (buffer A*). After washing with 2 column volumes of buffer A*, protein was eluted with either a 60-ml linear gradient of 0–0.4 M NaCl in buffer A*, for the preparation of p40, or 3 column volumes of buffer A* plus 0.05 M and subsequently 0.4 M NaCl, for p37. Most of the p40 and p37 was recovered in the flowthrough (p40, 9.3 mg of protein; p37, 70 mg) as determined by SDS/PAGE. Both the p40 and p37 flowthrough fractions were precipitated with solid ammonium sulfate to 50% saturation (29.1 g/100 ml). After centrifugation the pellets were resuspended in buffer B (40 mM potassium phosphate, pH 7.0/5 mM DTT/10% glycerol/0.05% Brij 58). The yield in this step was 3.6 mg of protein for p40 (3 ml) and 40 mg for p37 (20 ml). The semisoluble suspensions were loaded directly onto hydroxylapatite [column dimensions: for p40, 1.0 × 2.1 cm (1.6 ml); for p37, 1.5 × 2.9 cm (5.1 ml)] preequilibrated with 10 column volumes of buffer B. The hydroxylapatite-bound p40 or p37 was eluted with buffer B/6 M urea. The yield was 1.3 mg of p40 and 23 mg of p37. After dialysis against 1 liter of buffer D (0.025 M Tris·HCl, pH 7.5/1

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Abbreviations: pol, DNA polymerase; PCNA, proliferating-cell nuclear antigen; A1, activator 1; RF-C, replication factor C; HSSB, human single-stranded DNA-binding protein; BSA, bovine serum albumin; DTT, dithiothreitol.

mM EDTA/0.01% Nonidet P-40/10% glycerol/0.5 M NaCl/5 mM DTT/0.2 mM phenylmethylsulfonyl fluoride) for 14–16 hr at 4°C, both proteins were soluble and the fractions were further concentrated by using Centriflo-CF25 membrane cones (Amicon).

DNA-Binding Assay. Nitrocellulose filter DNA-binding assays were performed as described (2). For this purpose, 20 fmol of 5′-^{32}P-labeled DNA (2–4 × 10^3 cpm/fmol) was incubated with A1 p37, as indicated, in 0.1 ml of binding buffer [0.025 M Hepes·NaOH, pH 7.5/5 mM $MgCl_2$/1 mM DTT/0.01% bovine serum albumin (BSA)/0.15 M NaCl] for 10 min at 37°C. The reaction mixture was then filtered through alkaline-treated filters, which were washed with three 1-ml aliquots of the binding buffer lacking BSA. Radioactivity of the dried filters was measured by liquid scintillation counting.

Assay of pol ε. pol ε (0.1 unit) was incubated with A1 p37 in reaction mixtures (30 μl) containing 40 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM DTT, 0.01% BSA, 5% glycerol, 0.1 μg of $(dA)_{4000}$·$(dT)_{12-18}$ (20:1), and 33 μM [^{3}H]dTTP (200–300 cpm/pmol) in the absence or presence of 0.175 M potassium glutamate for 30 min at 37°C. The acid-insoluble material formed was measured by liquid scintillation counting. For antibody neutralization experiments, p37 (1 μg) was first incubated with either preimmune or immune serum for 30 min at 0°C in reaction mixtures containing the same components as described above but lacking DNA and [^{3}H]dTTP. Then pol ε (0.1 unit) was added with 0.1 μg of DNA, 33 μM [^{3}H]dTTP, and 0.175 M potassium glutamate, and the mixture was further incubated for 30 min at 37°C. In experiments testing reversal of inhibition, purified p37 (as indicated) was added with pol ε (0.1 unit), DNA (0.1 μg), [^{3}H]dTTP (33 μM), and potassium glutamate (0.175 M), following the initial incubation of p37 (1 μg) with anti-p37 serum (2 μl). The mixture was further incubated for 30 min at 37°C.

Preparation of p40- or p37-Linked Affi-Gel. Purified p40 (23 mg, 9 mg/ml) or p37 (32 mg, 4.3 mg/ml) was dialyzed for 20 hr against 750 ml of coupling buffer (50 mM Hepes·NaOH, pH 7.5/5 mM DTT/0.1 M NaCl) and mixed with 2 ml of Affi-Gel 15, or Affi-Gel 10 (Bio-Rad) prewashed with the same coupling buffer. The mixture was incubated with rocking for 24 hr at 4°C, followed by treatment with ethanolamine at pH 8.0 (0.1 mmol per ml of gel) for another 20 hr at 4°C. The beads were washed with 50 mM Hepes·NaOH, pH 7.5/2 M NaCl and stored in phosphate-buffered saline at 4°C. Approximately 11.4 mg of p40 or 8 mg of p37 was crosslinked to 2 ml of the gel. A mock Affi-Gel 10 column was prepared using the same procedure without p40 or p37.

Incubation of p40- or p37-Linked Affi-Gel with HeLa Cytosolic Extract. The p40- or p37-linked Affi-Gel or the mock-treated Affi-Gel (1 ml in each case), equilibrated with buffer E [25 mM Tris·HCl, pH 7.5/1 mM EDTA/0.01% Nonidet P-40/10% glycerol/1 mM DTT/0.2 mM phenylmethylsulfonyl fluoride containing antipain (0.4 μg/ml) and leupeptin (0.2 μg/ml)] plus 0.025 M NaCl, was mixed with HeLa cytosolic extract (150 mg of protein) (12). The mixture, after incubation with rocking for 15 hr at 4°C, was packed into a column (0.7 × 0.5 cm) and then washed with 40 ml of buffer E/0.025 M NaCl. The bound proteins were eluted in sequential steps (5 column volumes per step) of 0.5 M NaCl and then 5 M LiCl, both in buffer E. Similar amounts of protein (0.2 mg) were eluted in each step (0.5 M NaCl or 5 M LiCl) from either the mock or the p37-linked Affi-Gel column. However, 6 and 0.56 mg of protein were eluted with 0.5 M NaCl and 5 M LiCl, respectively, from the p40-linked Affi-Gel column. To reduce the nonspecific protein present in the fractions, the eluted protein from each salt step was rebound to 1 ml of the p40-Affi-Gel column in buffer E/0.025 M NaCl for 4 hr at 4°C. In the case of the 0.5 M NaCl fraction (6 mg of protein reloaded), 1.1 mg of protein did not bind to the second column. After the column was washed with buffer E/0.025 M NaCl (10 column volumes) until no further protein was detected in the eluate, elution successively with buffer E plus 0.1 M (30 ml), 0.25 M (15 ml), and 0.5 M (5 ml) NaCl yielded 3, 0.044, and 0.05 mg of protein, respectively. In the case of the 5 M LiCl fraction, where 0.56 mg of protein was reloaded, 0.2 mg of protein passed through the column. After the column was washed with 10 ml of buffer E/0.025 M NaCl, elution successively with buffer E containing 0.5 M NaCl (30 ml), 2 M NaCl (10 ml), and 5 M LiCl (10 ml), yielded 150, 2, and 4 μg of protein, respectively. The p40-Affi-Gel-eluted fractions used for the immunoblot assays described in Figs. 4 and 5*A* were derived from the second binding treatment. Specifically, fractions designated as F.T., 0.1 M, 0.25 M, and 0.5 M were from the second binding step using the 0.5 M NaCl eluate obtained from the initial binding treatment. The fraction designated 2 M was from the second binding step using the 5 M LiCl eluate obtained from the initial binding procedure.

Immunoblot Assay. After SDS/10% PAGE, proteins were transferred to nitrocellulose membrane paper. After blocking with 5% BSA (type V) and 1% gelatin for 14–16 hr, the membrane was incubated for 1 hr with the primary antibody, at the concentrations indicated, in phosphate-buffered saline plus 0.05% Tween-20 and 1% gelatin. Antibody–antigen complexes were detected with a biotin/avidin-based, alkaline phosphatase-linked immunodetection system (Vector Laboratories).

Binding of PCNA to p40-Affi-Gel. Approximately 0.62 mg of HeLa PCNA in buffer E/0.025 M NaCl, purified as described (12), was mixed with 0.5 ml of the p40-Affi-Gel beads. The mixture was incubated with rocking for 4 hr at 4°C. The beads were packed into a column (0.7 × 1.4 cm) and washed with 20 ml of buffer E/0.025 M NaCl, and bound proteins were eluted with 0.1 M NaCl in buffer E; 0.5-ml fractions were collected with the latter buffer.

Assay of pol δ. The assay of pol δ was carried out (2) in reaction mixtures (30 μl) containing 40 mM Tris·HCl (pH 7.8), 7 mM $MgCl_2$, 0.5 mM DTT/0.0167% BSA, 50 ng of $(dA)_{4000}$·$(dT)_{12-18}$ (20:1), 2 mM ATP, 33 μM [^{3}H]dTTP (300 cpm/pmol), 0.5 μg of HSSB, 0.1 μg of PCNA, 30 ng of A1, and 0.2 unit of pol δ. After 60 min at 37°C, acid-insoluble material was measured by liquid scintillation counting. To examine the effect of A1 p37 or p40, various amounts of the purified bacterial protein were incubated in the above reaction. For the reversal experiment, various amounts of pol δ, or A1, or PCNA were preincubated with 0.68 μg of p40 in reaction mixtures containing the same components as described above but lacking dTTP, PCNA, A1, and pol δ, all of which were added after a period of 30 min on ice. Mixtures were then incubated for 60 min at 37°C.

RESULTS

Solubilization and Purification of A1 p37 and p40. We previously reported the overproduction of both A1 p37 and p40 in *E. coli* using the pET expression system (9, 10). However, neither of the expressed proteins was soluble unless extracted with solutions containing 6 M urea. Removal of urea by dialysis resulted in precipitation of both proteins. We succeeded in solubilizing both proteins by using a modified chromatographic procedure developed for the isolation of the DnaJ protein of *E. coli* (15), as described in *Materials and Methods*. This procedure yielded nearly homogeneous preparations of p37 and p40 (Fig. 1). The 36- and 25-kDa polypeptide bands in the preparation of A1 p40 were probably proteolyzed or prematurely terminated products of p40, since they reacted with antibodies specific for p40 on immunoblotting (data not shown).

A1 p37 Binds DNA Preferentially at Primer Ends. We examined the p37 and p40 for their ability to bind DNA. For

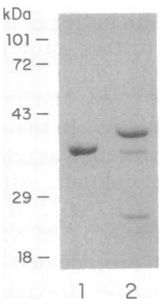

FIG. 1. SDS/PAGE analysis of purified A1 p37 and p40. Six micrograms of purified p37 (lane 1) or p40 (lane 2) was subjected to SDS/10% PAGE and then stained with 0.25% Coomassie brilliant blue.

this purpose, the purified recombinant protein was incubated with 5′-^{32}P-labeled $(dT)_{12-18}$ or $(dA)_{4000}$ alone, or with the two annealed. The influence of increasing amounts of p37 on DNA binding is shown in Fig. 2. While no binding of $(dT)_{12-18}$ was observed, binding of $(dA)_{4000}$ was detected. However, incubation of p37 (0.5 μg) with $(dA)_{4000}$ annealed to $(dT)_{12-18}$ at nucleotide ratios of 20:1 and 4:1 resulted in 3- and 6-fold more DNA binding than with $(dA)_{4000}$ alone. This indicated that A1 p37 binds DNA preferentially at the primer terminus.

The binding of p37 to single-stranded DNA was confirmed by three additional observations. First, p37 was quantitatively adsorbed to single-stranded DNA-cellulose at low salt concentrations (0.025 M NaCl) and eluted at higher salt concentrations (0.15–0.2 M NaCl). Second, renaturation of p37 isolated from an excised SDS/polyacrylamide gel slice yielded ≈30% of the DNA-binding activity (10 fmol bound per μg of p37) of the untreated p37 (30 fmol bound per μg of p37) (data not shown). Third, binding of p37 to a heat-denatured 400-nucleotide DNA fragment (196 fmol of DNA per μg of p37) was 15-fold higher than binding to the untreated double-stranded DNA (13 fmol/μg) (data not shown).

There are multiple factors that may account for the low DNA-binding efficiency observed with the cloned p37 protein (0.1%; p37 calculated as a monomer on a molar basis). For example, the other subunits (p145, p40, p38, and p36.5) of A1 may be required for more efficient binding. In addition, it is possible that only a portion of the p37 protein was renatured into molecules active in DNA binding during its solubilization and purification. Glycerol gradient fractions active in DNA binding sedimented faster than the p37 monomer (data not shown). This suggested that multimers, rather than the monomer (present as the major form of p37), bind DNA. SDS/PAGE and Western blotting analyses showed that the multimeric structures consisted of p37.

No DNA binding activity was detected with purified A1 p40 under the assay conditions used for p37.

A1 p37 Stimulates pol ε Activity in the Presence of Salt. The A1 complex and PCNA function as accessory elongation factors for both pol δ and, in the presence of 0.17 M potassium glutamate, pol ε. These polymerases, which alone exhibit only a weak DNA binding activity, associated with primer ends complexed with A1 and PCNA in the presence of ATP. We examined the effect of purified A1 p37 on pol ε with multiprimed $(dA)_{4000}$·$(dT)_{12-18}$ (20:1) in the absence or presence of 0.17 M potassium glutamate. As shown in Fig. 3*A*, p37 had no significant stimulatory effect on pol ε activity in the absence of salt, though the rate of elongation was reduced 20–30% at the highest concentration of p37 added. However, in the presence of salt, which completely inhibited pol ε activity, p37 markedly activated pol ε-catalyzed dTMP incorporation to 30–40% of the activity observed without salt. Under these conditions, p37 alone exhibited no polymerase activity. The stimulatory effect of p37 on pol ε activity in the presence of salt was confirmed by antibody neutralization experiments (Fig. 3 *B* and *C*). The addition of anti-p37, but not preimmune serum, abolished the p37-dependent pol ε activity (Fig. 3*B*). This effect, however, was reversed by the addition of purified p37 protein (Fig. 3*C*). The p37-dependent pol ε activity observed in the presence of salt was unaffected

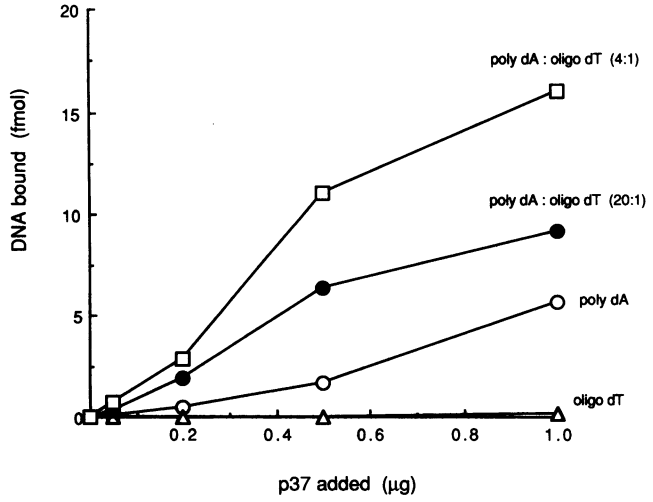

FIG. 2. Interaction of A1 p37 with DNA. The nitrocellulose filter DNA-binding assay was described in *Materials and Methods*. Various amounts of purified p37 were incubated with 20 fmol of 5′-^{32}P-labeled $(dA)_{4000}$·$(dT)_{12-18}$ (4:1, □; 20:1, ●), $(dA)_{4000}$ (○), or $(dT)_{12-18}$ (△).

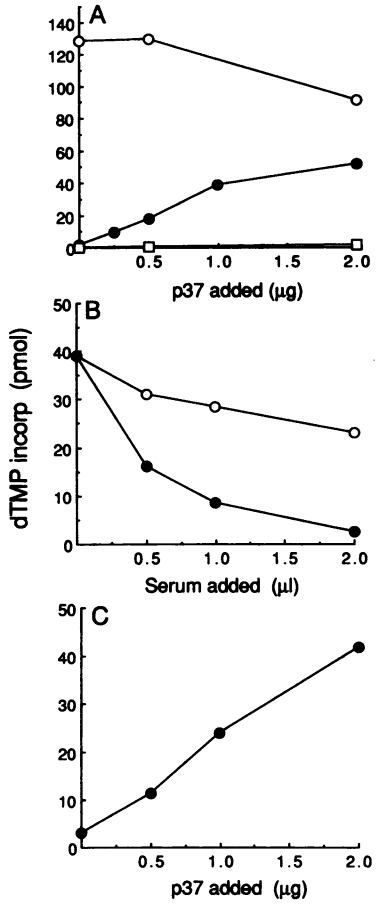

FIG. 3. Influence of A1 p37 on pol ε activity with $(dA)_{4000}$·$(dT)_{12-18}$. (*A*) Purified p37, in amounts as indicated, was incubated with (○, ●) or without (□) pol ε, in the absence (○) or presence (●, □) of 0.175 M potassium glutamate. (*B*) Various amounts of preimmune (○) or immune (●) serum raised against p37 were incubated with 1 μg of p37 for 30 min prior to the addition of pol ε. (*C*) Following the initial incubation of anti-p37 serum (2 μl) with p37 (1 μg), various amounts of p37 were added along with pol ε.

by the addition of ATP, PCNA, or HSSB, alone or in combination (data not shown).

A1 p40 Interacts with Cellular A1 p37 and PCNA. To examine the interaction of the A1 p40 or p37 subunit with other proteins, Affi-Gel columns linked with p40 or p37 were prepared and examined for their ability to bind proteins present in HeLa cytosolic extract. SDS/PAGE analyses indicated that the cellular proteins eluted from the p37-linked column were similar to those from mock-treated Affi-Gel columns (data not shown). However, chromatography of crude cell extract on p40-linked Affi-Gel resulted in the elution of a unique array of proteins compared with those eluted from mock-treated Affi-Gel columns. Fig. 4 shows an immunoblot of these protein fractions probed with anti-p37 antibodies. Significant amounts of cellular p37 were detected in material that was eluted between 0.25 and 0.5 M NaCl (lane 6). Much lower levels of p37 were detected in the flow-through, 0.025–0.1 M, and 0.1–0.25 M NaCl fractions (lanes 3–5), in amounts comparable to that observed from a mock-treated column (lane 8). p37 was present in the 0.5–2 M NaCl eluate (lane 7); only one-third as much total protein was loaded in lane 7 compared with the other lanes. These data suggest that the recombinant p40 protein interacted with the cellular p37.

The proteins eluted from the p40 column were also analyzed for the presence of PCNA (Fig. 5*A*). PCNA was detected in the 0.025–0.1 M fraction (lane 4) in amounts significantly higher than that present in the flowthrough (lane 3). Low levels of PCNA were detected in a fraction obtained from the p37-linked column (lane 6), but no PCNA was observed with eluates from the mock-treated column (lane 5).

To further establish the direct interaction of A1 p40 with PCNA, purified PCNA was passed through the p40-Affi-Gel column. Fig. 5*B* shows a SDS/PAGE analysis of such an experiment. Only very low levels of PCNA were detected in the flowthrough (lane 2) in the presence of 0.025 M NaCl, indicating that PCNA was almost quantitatively adsorbed. After extensive washing with buffer containing 0.025 M NaCl, the bound PCNA was efficiently eluted (70% recovery) with 0.1 M NaCl (lanes 3–12). Thus, p40 directly interacts with PCNA.

A1 p40 Interacts with pol δ. We examined the effect of either p37 or p40 on pol δ-catalyzed DNA synthesis using a multiprimed $(dA)_{4000}$·$(dT)_{12-18}$ template, a reaction requiring both PCNA and A1 (2). pol δ activity was nearly abolished by the addition of p40, but not by p37 (Fig. 6*A*). However, this inhibition was markedly reversed by increasing amounts of pol δ (Fig. 6*B*). When subsaturating levels of pol δ were used (<0.2 unit), p40 (0.23 μg) inhibited dTMP incorporation

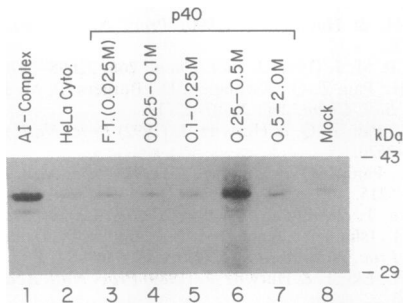

FIG. 4. Immunoblot assay with anti-p37 polyclonal antibodies. Purified A1 (75 ng, lane 1), HeLa cytosolic extract (0.5 μg, lane 2), various fractions [F.T. (flowthrough, 0.025 M NaCl) and 0.025–0.1 M, 0.1–0.25 M, 0.25–0.5 M, 0.5–2.0 M NaCl] derived from the p40-Affi-Gel column (lanes 3–6, 0.5 μg of protein; lane 7, 0.15 μg of protein), and 0.5 μg of protein from the mock-treated column (lane 8) were subjected to immunoblot assay with anti-p37 polyclonal antibodies (1:1000 dilution).

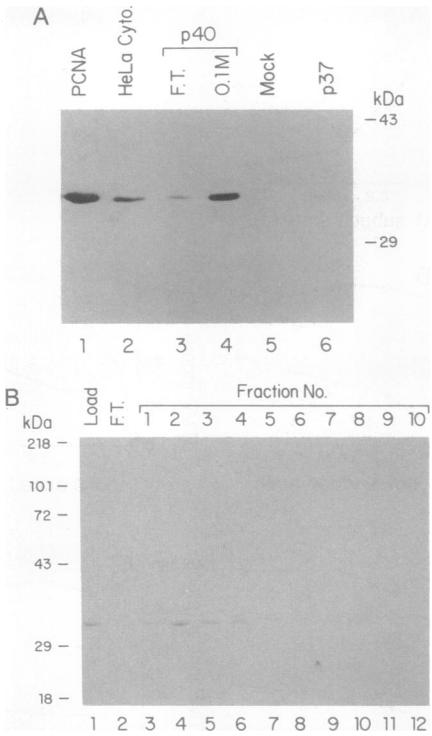

FIG. 5. Interaction of p40 with PCNA. (*A*) Immunoblot assay with PCNA antibodies. Purified PCNA (0.1 μg, lane 1), HeLa cytosolic extract (3 μg, lane 2), the flowthrough (F.T.) obtained after incubation of the HeLa extract with the p40-linked Affi-Gel beads (3 μg, lane 3), the fraction eluted with 0.1 M NaCl (3 μg, lane 4) from the p40-linked Affi-Gel column, and protein eluted with 0.5 M NaCl from either the mock-treated column (3 μg, lane 5) or the p37-linked Affi-Gel column (3 μg, lane 6) were subjected to immunoblot assay with PCNA antibodies (Boehringer Mannheim, 10 μg/ml). (*B*) SDS/PAGE analysis of PCNA fractions after binding to the p40-Affi-Gel column. Protein fractions (2 μl) containing purified PCNA, prior to (lane 1) or after (lane 2) binding to the p40-Affi-Gel column, along with the 0.1 M NaCl eluate (lanes 3–12) of the beads, were subjected to SDS/10% PAGE followed by 0.25% Coomassie brilliant blue staining.

>60% (Fig. 6*C*). However, when saturating amounts of pol δ (>0.2 unit) were used, the same amount of p40 protein inhibited DNA synthesis only 30%. These data indicate that pol δ is the limiting factor in the p40-mediated inhibition and reversal, suggesting that there is an interaction between the polymerase and p40. Neither A1 or PCNA efficiently restored pol δ activity (Fig. 6 *D* and *E*).

DISCUSSION

The human A1 (RF-C) is a multimeric protein complex containing five different subunits—p145, p40, p38, p37, and p36.5. The cDNA sequences of p40 (9), p37 (10), and p38 (M.C. and J.H., unpublished observations) have been determined, as well as a number of peptide sequences derived from p145 and p36.5. These results indicate that these five subunits can be distinguished from one another by sequence and are unique polypeptides. Some similarity in subunit composition between human A1 and its counterpart in *Saccharomyces cerevisiae* has been recently shown (16, 17).

The A1 complex contains multiple enzymatic activities. (*i*) It functions as a primer-recognition factor that, when complexed to DNA, forms a stable complex with PCNA in the presence of ATP (6). (*ii*) Its intrinsic DNA-dependent ATPase activity is required for the association of pol δ and pol ε (in the presence of salt) with the primer/template/A1/PCNA complex, resulting in DNA chain elongation (6). (*iii*) The combined action of A1 and PCNA blocks the further elongation of DNA

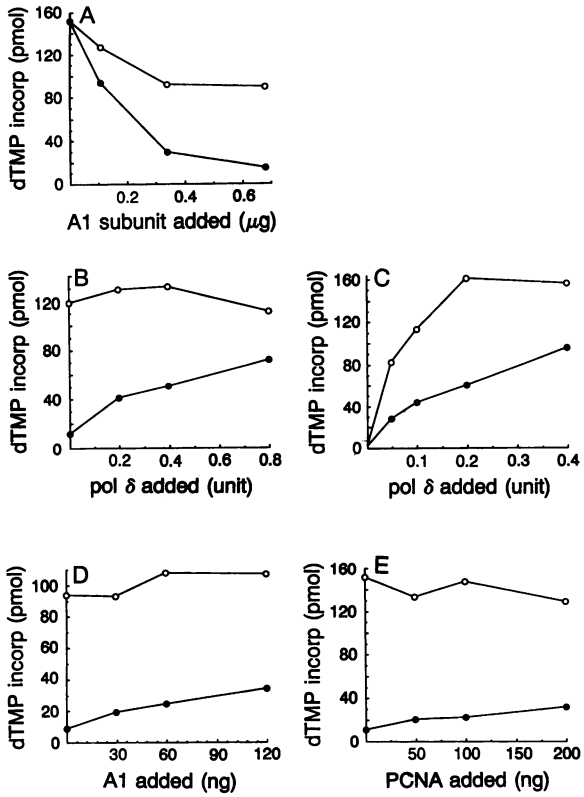

FIG. 6. Interaction of p40 with pol δ. (*A*) Effect of p37 (○) or p40 (●) on pol δ activity is shown. (*B, D,* and *E*) Various amounts of pol δ (*B*), A1 (*D*), or PCNA (*E*) were added to the complete reaction mixture in the absence (○) or presence (●) of p40 (0.68 μg). (*C*) Various amounts of pol δ were incubated with other reaction components in the absence (○) or presence (●) of p40 (0.23 μg).

chains by pol α in the purified *in vitro* simian virus 40 DNA replication system, resulting in the formation of short Okazaki fragments, 35–50 nucleotides in length (18). The binding of A1 and PCNA at the primer terminus recruits pol δ and possibly pol ε as well, leading to a more processive synthesis of the leading-strand DNA, and to the maturation of Okazaki fragments to chains 150–250 nucleotides in length.

Here, we have shown that the A1 p37 subunit resembles A1 in binding to DNA preferentially at the primer terminus. However, A1 binds to $(dA)_{4000}\cdot(dT)_{12-18}$ nearly 100-fold more efficiently than p37. Possible reasons for this discrepancy have been discussed in the text. Tsurimoto and Stillman (11) have reported that the p140 subunit of RF-C can be UV-crosslinked to a DNA fragment containing a primer. It is presently unclear whether p37 and p145 are functionally redundant in binding DNA, or whether they interact with each other, possibly along with other subunits, to achieve a more efficient and specific binding to the primer terminus.

We have shown that the recombinant p37 can partially substitute for the combined action of A1, PCNA, HSSB, and ATP in restoring pol ε activity in the presence of salt. This was unexpected, since the A1 complex alone did not activate pol ε under such conditions. We propose that p37, complexed with DNA at a primer junction as a result of its intrinsic primer binding activity, recruits pol ε in the presence of salt, which otherwise does not bind to DNA. Such "recruiting" activity may be masked when p37 is present in the complex with the other four subunits. It is conceivable that the interaction of A1 with PCNA, followed by ATP hydrolysis, results in conformational changes in A1 that unmask the p37 protein, which then interacts with pol ε. Significant amounts of p37 (>10 pmol) are required to activate pol ε. Thus, it is possible that large amounts of A1, at the same molar level used with p37 (not practical at present), could obviate the requirements for PCNA, HSSB, and ATP. The activation of pol ε by p37 is surprising, since the A1- and PCNA-dependent pol δ activity with the same template was unaffected by this recombinant protein. It was difficult to examine the effects of p37 on pol α activity using a multiprimed DNA template, since pol α catalyzes DNA chain elongation poorly, although we observed a small stimulation of pol α activity by p37.

It was previously reported that the p40 subunit, either in the RF-C (A1) complex (11) or as the purified recombinant protein (9), was capable of binding ATP (shown by UV-crosslinking experiments). This binding activity, however, was inhibited by the addition of purified PCNA, suggesting that PCNA interacts with p40 and thus prevents it from being bound to ATP. In this paper, we have presented direct evidence for an interaction between PCNA and p40. Purified PCNA, as well as PCNA present in the crude HeLa cell cytosolic extract, was efficiently and specifically bound to p40-linked Affi-Gel beads. The bound PCNA was eluted with 0.1 M NaCl, in either case, suggesting a weak interaction. It is not known whether other subunits of A1 strengthen this interaction, or whether a weak interaction in this case may be advantageous for disassembly and the recycling of the A1/PCNA complex at the primer terminus.

The observations reported here suggest that p37 and p40 play different roles in the pol δ- and pol ε-catalyzed DNA elongation reactions. We propose that the p37 subunit, and possibly the p145 subunit, is required for the binding of A1 to the primer terminus on both leading and lagging strands. The p40 subunit is required for the binding of PCNA at the primer junction, which blocks the action of pol α. The p37 protein specifically recruits pol ε and facilitates further elongation of Okazaki fragments to complete lagging-strand DNA synthesis. The p40 protein is required for the binding of pol δ, which requires ATP hydrolysis catalyzed by other subunits(s) of A1 that remain to be identified, on the leading strand to initiate a processive elongation reaction.

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