Activator 1 (A1) is a multiprotein complex which is essential for proliferating cell nuclear antigen (PCNA)-dependent DNA polymerase δ (pol δ) activity and efficient in vitro DNA synthesis in the SV40 dipolymerase replication system. In this report, we describe the isolation of A1 from HeLa cytosolic extracts. A1 stimulated pol δ activity in singly primed eX174 DNA or (dA)₄₀₀·oligo(dT)₁₂₋₁₈ in reactions containing PCNA, single-stranded DNA binding protein (SSB), and ATP. Using this assay, A1 has been extensively purified. Purified preparations contained five discrete subunits of 145, 40, 38, 37, and 36.5 kDa. ATP hydrolysis to ADP and Pi is essential for A1-dependent pol δ activity, and we have shown that A1 contains an intrinsic ATPase which is stimulated by DNA. The DNA-dependent hydrolysis of ATP can be stimulated by PCNA and further activated by PCNA plus the human single-stranded DNA binding protein. These stimulatory effects were observed with (dA)₄₀₀·oligo(dT)₁₂₋₁₈, but were not detected with each polydeoxynucleotide alone. Furthermore, A1 formed a complex with (dA)₄₀₀·oligo(dT)₁₂₋₁₈ which could be measured by nitrocellulose binding. No complex with (dA)₄₀₀ or oligo(dT)₁₂₋₁₈ alone was detected by this procedure. Data are also presented which indicate that A1, in conjunction with PCNA, functions as a primer-recognition factor for pol δ, increasing its ability to utilize low levels of primer ends, but it does not increase the size of the DNA products. A1 also markedly reduced the amount of PCNA required for pol δ activity on a multiply primed DNA suggesting that PCNA interacts with A1 at the primer end. These multiple effects of A1 closely resemble the properties of the multisubunit protein RF-C described by Tsurimoto and Stillman (Tsurimoto, T., and Stillman, B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1023–1027).

The SV40 DNA replication system is an excellent model for examining eukaryotic DNA replication in vitro because, with the exception of the SV40 large tumor antigen (T ag), it uses the host machinery for its DNA replication. Cytosolic extracts from monkey or human cells, supplemented with T ag support replication of DNA containing the SV40 DNA origin sequence (Li and Kelly, 1984; Stillman and Gluzman, 1985; Wobbe et al., 1985). Initiation of SV40 DNA replication commences with the ATP-dependent binding of T ag within the origin region; in the presence of ATP, a single-stranded DNA binding protein (SSB) and a topoisomerase I (topo I) capable of relieving positive superhelicity, the intrinsic DNA helicase of T ag catalyzes the bidirectional unwinding of DNA (Dean et al., 1987a, 1987b; Dodson et al., 1987; Wold et al., 1987; Stahl et al., 1986). This pre-DNA synthesis stage is then followed by the initiation of DNA synthesis. The mechanism by which the replication machinery gains entrance to the origin region is presently unclear. However, this system has proved useful for the identification of host proteins that participate in the initiation and elongation of DNA. Recent reviews summarizing the replication system have appeared (Challberg and Kelly, 1988; Stillman, 1988; Borowiec et al., 1990).

We have described an in vitro replication system which supports the T ag-dependent replication of DNA containing the SV40 origin, the SV40 monomonomerase system reconstituted with purified human SSB (HSBB), DNA pol α-primase, and topo I proteins (Wobbe et al., 1987; Ishimi et al., 1988). Due to the lack of well defined mutants which are conditionally blocked in DNA replication, host proteins involved in SV40 replication have been identified through the fractionation of crude cytosolic extracts. With this approach, attempts have been made to reconstitute and reproduce the replication reaction observed with crude extracts since the monomonomerase system is less efficient than the crude extracts in supporting DNA replication. The discovery that PCNA, a putative cell cycle-regulated protein, stimulated replication (Prelitch et al., 1987a, 1987b; Prelitch and Stillman, 1988), suggested that additional host proteins present in crude extracts played a role in SV40 replication. The monomonomerase

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1 The abbreviations used are: T ag, SV40-encoded large tumor antigen; SV40 ori' DNA, DNA containing the SV40 origin of replication; topo, topoisomerase; SSB, single-stranded DNA binding protein; PCNA, proliferating cell nuclear antigen; pol α and pol δ, DNA polymerases α and δ, respectively; E1, elongation inhibitor protein; A1, activator I protein; monomonomerase system; reaction mixtures which include SV40 ori' DNA, T ag, human (H) SSB, topo I, pol α-primase complex, dipolymerase system, reaction mixtures containing the monomonomerase system plus A1 (or E1-A1), pol δ, and PCNA; SS, single-stranded; SSC, single-stranded circular; BSA, bovine serum albumin; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; ATP·S, adenosine 5'-O-(thio)triphosphate; AMPPNP, adenosine 5'-O-(β,γ-imino)triphosphate; AMPCCP, adenosine 5'-O-(α,β-methylene)triphosphate.

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* This work was supported by National Institutes of Health Grant GM 34559. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a postdoctoral fellowship from the Leukemia Society of America.
system was unaffected by PCNA and neutralizing antibodies against this protein. In contrast, SV40 replication catalyzed by crude extracts was blocked by anti-PCNA antibodies and the addition of purified PCNA specifically reversed this effect (Lee et al., 1988). These findings led to the discovery of other proteins, including the PCNA-dependent DNA polymerase δ (called activator 2), A1, and an elongation inhibitor (E1) (Lee et al., 1989a, 1989b). The elongation inhibitor, which has been identified as poly(ADP-ribose) polymerase, preferentially binds at the ends of DNA during chain elongation. This results in the accumulation of small Okazaki-size DNA products which can be elongated by pol δ, but not by pol α, in the presence of A1 and PCNA (Lee et al., 1989a, 1989b; Deen et al., 1990). The combination of PCNA, pol δ, A1 (or A1-E1 complex), and the monoplymerase system, which constitutes the SV40 dipolymerase replication system, is more efficient than the monoplymerase system in chain elongation. However, it is not clear how these two different DNA polymerases actually work at the replication fork. A protein fraction, called RF-C, previously described by Tsurimoto and Stillman (1989b), strongly resembles A1 in its structure and many of its functions.

In this paper, we describe the biochemical characterization of A1 and show that, like RF-C, it binds to primer ends and functions as a primer-recognition factor for pol δ which increases the utilization of low levels of primer ends by PCNA-dependent pol δ. This protein markedly lowers the amount of PCNA essential for pol δ activity. We also demonstrate that A1 contains DNA-dependent ATPase activity which may explain the requirement for ATP hydrolysis for pol δ activity on primed DNA templates.

**Materials and Methods**

Preparation of Proteins and DNAs—The following reagents were obtained commercially: oligo(dT)12-18, oligo(U)18, bacteriophage T4 gene 32 (Pharmacia LKB Biotechnology Inc.), (dA)4Sw.12 oligo(dT)18, and αX174 viral DNA (New England Biolabs). For the preparation of poly(dA) annealed to oligo(dT), 300 μM (as nucleotides) (dA)4Sw. and various concentrations of oligo(dT)12-18 were incubated in 10 mM Tris-HCl, pH 9.0, 5 mM EDTA, and 0.3 M NaCl at 50°C for 60 min prior to use. A 50-μm, centrifugal-purified supernatant from cell cultures (5127-5156 of αX174 viral DNA, was used to prepare single primed αX174 DNA as previously described (Lee et al., 1989a).

Cytosolic extracts of HeLa cells, SV40 ori+ DNA (pSV01AP), human single-stranded DNA binding protein (HSSB; 600 units/mg of protein), pol α-primer-complex (4.0 × 10^5 and 3.5 × 10^5 units/mg of protein, respectively), and top 1 (8.0 × 10^7 units/mg of protein) were all isolated from extracts of HeLa cells as previously described (Wobble et al., 1985; Ishimi et al., 1988; Lee et al., 1989a). TAg was isolated from a Baculovirus expression vector (Mastrangelo et al., 1989), and PCNA (2,000 units/mg of protein) was prepared as previously described (Lee et al., 1989) with the following modifications. The PCNA, obtained after hydroxylapatite chromatography, was further purified by linear NaCl gradient elution (50-400 mM) from a Q-Sepharose column. PCNA was measured by its ability to stimulate pol δ activity in the presence of A1, T4 gene 32, and ATP (see A1 assay below for details). One unit of PCNA supported the incorporation of 1 nmol of dTTP in 60 min at 37°C. The PCNA-dependent pol δ (1,500 units/mg of protein) was purified from HeLa cells using a modified procedure of Lee et al. (Lee et al., 1988). A linear gradient of NaCl (50-500 mM) was used in the single-stranded DNA-cellulose step, and the hydroxylapatite fraction was further purified by heparin-Sepharose column chromatography with a linear gradient of KCl (50-600 mM). One unit of pol δ activity supported the incorporation of 1 nmol of dTTP in the presence of PCNA, SSB, A1, and ATP (dA)4Sw. oligo(dT)12-18 after 60 min of incubation at 37°C. Enzyme Assays—ATPase activity was measured in reaction mixtures (20 μl) containing 25 mM Tris-HCl (pH 7.5), 1 mM DTT, 10 mM MgCl2, 50 μg/ml bovine serum albumin (BSA), 50 μM [γ-32P]ATP (1.5 × 10^6 cpm/mmol), 12.5 μM DNA (as nucleotides and as indicated), and enzyme as indicated. After 30 min at 37°C, aliquots (1-2 μl) were spotted on PEI-cellulose thin layer plates which were developed in 1.0 M formic acid, 0.5 M LiCl for 45 to 60 min. ATP and Pi, located by autoradiography (20-min exposure), were excised and counted. (ADP-ribose) polymerase was measured in reactions (30 μl) containing 25 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 1 mM DTT, 0.15 mM [3H]NAD (12 cpm/pmol), 10% glycerol, 5 μg of histone protein (Sigma, Catalogue No. 5505; 0.5 mg/ml), and 0.25 μg of activated DNA (Sigma, Catalogue No. 4525; 0.025 mg/ml). After 15 min at 30°C in 1.0 ml-insoluble material was determined by filtration through glass fiber filters (GF/C).

DNA Binding Assay—The nitrocellulose filter binding assay was carried out as described (Nagata et al., 1983). The standard assay (0.1 ml) contained binding buffer (25 mM Hapes-NaOH (pH 7.5), 5 mM MgCl2, 1 mM DTT, 100 μg/ml BSA, and 0.1% NaN3) or the following buffer (5.25 mM(dA)4Sw.(dA)5.25 oligo(dT)18) (550 to 700 cpm/ pmol), or ([5-32P])dA4Sw. hybridized to varying amounts of unlabeled oligo(dT)18, and A1. After 10 min at 37°C, the mixture was passed through a nitrocellulose filter (Millipore Corp., HA 0.45 μm) which was then washed three times with 1.5 ml of binding buffer. After drying, the label adsorbed to the filters was measured by liquid scintillation counting.

Assay and Isolation of Al—A1 was assayed by its ability to stimulate pol δ activity in the presence of SSB, PCNA, and ATP (Lee et al., 1989b). One unit of A1 supported the incorporation of 1 nmol of dTTP in the following reactions: following buffer containing 40 mM Tris-HCl (pH 7.8), 7.5 mM MgCl2, 1 mM DTT, 150 μg/ml BSA, 0.1 μg of (dA)4Sw. oligo(dT)18, and 0.5 M KCl, containing a total of 201, containing 1.2 pmol of primer ends, 33.3 μM [3H]dTTP (300 cpm/pmol), 2 mM ATP, 0.2 Unit of pol δ, 1.8 μg of T4 gene 32 (or HSSB), and 0.1 μg of PCNA were incubated at 37°C for 2 h. The amount of acid-insoluble dTTP was determined. When an artificially primed αX174 DNA (240 fmol of DNA molecules) was used as the template, 100 μM concentrations of the other three dNTPs were also added.

A1 was isolated from a 0.7 M phosphocellulose fraction prepared from cytosolic extracts of HeLa cells (30 liters), as described previously (Lee et al., 1989a), and a summary of the purification is shown in procedure 1 of Table I. The 0.7 M phosphocellulose fraction (3.8 mg of protein/ml, 70 ml) was chromatographed on a single-stranded DNA-cellulose column (2.5 × 7.0 cm, 35 ml) equilibrated with buffer A (25 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM DTT, 1.0 mM EDTA, 0.01% (v/v) Nonidet P-40, 0.1 mM PMSF, 200 μg/ml anti-pain, 100 μg/ml leupeptin containing 0.1 M NaCl. The column was successively eluted with 180 ml of buffer A containing 0.1 M, 0.4 M, and 0.8 M NaCl. The 0.8 M NaCl fraction (0.34 mg of protein/ml, 40 ml) was dialyzed against 2 liters of buffer B (0.29 M potassium phosphate (pH 7.0, 1.0 mM DTT, 0.01% Nonidet P-40, 0.1 mg/ml leupeptin, 0.1 mg/ml phenylmethylsulfonyl fluoride) and loaded onto a hydroxylapatite column (1.0 × 4.5 cm) equilibrated with the same buffer. After washing the column with buffer B, A1 was eluted with a gradient of 100 ml of 0.56 to 0.65 M potassium phosphate (pH 7.0) in buffer B. For larger preparations of A1, the procedure shown in procedure 2. A1 was isolated from 50 ml of buffer A containing 0.05% (v/v) in buffer A plus 0.15 M NaCl were centrifuged at 45,000 g for 24 h at 4°C. The pooled glycerol gradient fraction of A1 was aliquoted and stored at -80°C and used in all experiments described except for the ATPase and gel filtration analyses. No decrease in activity of this fraction was detected over a period of at least 2 months.

For larger preparations of A1, the procedure shown in procedure 1, Table I, was modified as shown in procedure 2. A 0.7 M phosphocellulose fraction (1.25 mg of protein/ml, 220 ml) prepared from 90 liters of HeLa cytosol was chromatographed on a hydroxylapatite column (2.5 × 11.5 cm, 58 ml) equilibrated with 450 ml of buffer C (buffer B containing 800 mM NaCl) and eluted with 200 ml of 0.5 M potassium phosphate (pH 7.0), in buffer C, followed by a 0.05-0.6 M potassium phosphate linear gradient (500 ml) in buffer C. Two peaks of A1 activity were reproducibly eluted from the hydroxylapatite column at approximately 0.05 M and 0.09 M potassium phosphate. These two fractions containing 18.7% and 16.8% of the activator 1 activity recovered, were separately purified through the glycerol gradient sedimentation step and subsequently found to be identical with
respect to A1 activity and subunit structure as assayed by SDS-PAGE. The results presented in Table 1, procedure 2, represent the activity found only with the 0.09 M potassium phosphate A1 peak. After dialysis against 4 liters of buffer D (buffer A containing 0.02 M NaCl and 20% sucrose) for 12 h, the 0.09 M potassium phosphate hydroxylapatite fraction (0.97 mg of protein/ml, 40 ml) was chromatographed on a single-stranded (SS) DNA-cellulose column (1.5 x 5 cm, 5.5 ml) equilibrated with 500 ml of buffer A containing 0.025 M NaCl. After washing the column with 5 ml of buffer A containing 0.025 M NaCl, A1 was eluted with a linear gradient (400 ml) from 0.1 M to 1.0 M NaCl in buffer A. In this step, A1 eluted at approximately 0.5 M NaCl and was separated from most of the poly(ADP-ribose) polymerase which eluted at 0.65 M NaCl. After dialysis against 2 liters of buffer D for 5 h, the SS DNA-cellulose fraction was chromatographed on a 1 ml FPLC Mono Q HR5/5 column (Pharmacia LKB Biotechnology Inc.) which was equilibrated with 30 ml of buffer A containing 50 mM NaCl. For larger scale preparations starting with preparations of A1 were free of contaminating ATPase and poly(ADP-ribose) polymerase activities. The Mono Q and Q-Sepharose fractions of A1 were stored at -80 °C and were stable for 6 months. The glycerol gradient sedimentation step was carried out with an aliquot of the fraction from the previous purification step. However, the activity and yield reported were calculated based on the use of the entire fraction.

RESULTS

Purification and Characterization of A1—A1 was first identified as a protein fraction which in conjunction with activator 2 (later identified as PCNA-dependent pol δ) and PCNA, rapidly elongated short DNA chains which accumulated in the SV40 monomonomerase system due to the effects of poly(ADP-ribose) polymerase (elongation inhibitor (El)). It was shown that poly(ADP-ribose) polymerase binds to the ends of DNA chains and prevented their elongation by pol α, resulting in the accumulation of short DNA chains. A1 is most likely identical with the protein fraction RF-C previously described by Stillman and co-workers (1989). RF-C was reported to be essential for coordinated synthesis of both leading and lagging DNA strands in the in vitro SV40 replication system and stimulated both pol α and calf thymus pol δ (Tsurimoto and Stillman, 1989a, 1989b). We have shown that A1 (poly(ADP-ribose) polymerase-A1 complex) preferentially stimulated pol δ (isolated from HeLa cells) but not pol α activity in the presence of PCNA, SSB, and ATP (Lee et al., 1989b). Various SSBs, including those isolated from Escherichia coli, adenovirus, and phage T4 (gene 32) also substituted for human SSB (HSSB) in the stimulation of pol δ (Kenny et al., 1989). However, only HSSB stimulated pol α activity, while the other SSBs were inhibitory with this polymerase. This observation was used to develop a pol δ-specific assay for A1 which has been isolated from cytosolic extracts of HeLa cells as described under “Materials and Methods.” Table I summarizes the purification procedures developed. The low yield of A1 activity obtained in the hydroxylapatite step in procedure 1 was due to its separation into three different peaks which represented a recovery of about 60%. The results are shown for the major A1 peak which was used in subsequent steps.

As shown in Fig. 1, an aliquot of the FPLC-Mono Q A1 fraction described in procedure 2, Table I, was separated on a 15–35% glycerol gradient, and fractions were assayed for A1 activity using the poly(dA)-oligo(dT) assay and the SV40 dipolymerase activity found only with the 0.09 M NaCl. A1 was eluted at approximately 0.5 M NaCl and was separated from most of the poly(ADP-ribose) polymerase which eluted at 0.65 M NaCl. After dialysis against 2 liters of buffer D for 5 h, the SS DNA-cellulose fraction was chromatographed on a 1 ml FPLC Mono Q HR5/5 column (Pharmacia LKB Biotechnology Inc.) which was equilibrated with 30 ml of buffer A containing 50 mM NaCl. For larger scale preparations starting with preparations of A1 were free of contaminating ATPase and poly(ADP-ribose) polymerase activities. The Mono Q and Q-Sepharose fractions of A1 were stored at -80 °C and were stable for 6 months. The glycerol gradient sedimentation step was carried out with an aliquot of the fraction from the previous purification step. However, the activity and yield reported were calculated based on the use of the entire fraction.

### Table I

| Fractionation step | Protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) |
|--------------------|-------------|------------------------|----------------------------|-----------|
| Procedure 1        |             |                        |                            |           |
| HeLa cytosolic extracts | 3.125       | 3,970                  | 15                          | 100       |
| Phosphocellulose    | 266         | 3,970                  | 15                          | 100       |
| SS DNA cellulose    | 13.6        | 4,944                  | 364                         | 124       |
| Hydroxylapatite     | 1.2         | 744                    | 620                         | 18.7      |
| Heparin-Sepharose   | 0.12        | 228                    | 1,900                       | 5.7       |
| Glycolic gradient   | 0.04        | 185                    | 4,625                       | 4.7       |
| Procedure 2        |             |                        |                            |           |
| HeLa cytosolic extracts | 17,438      | 36,300                 | 1,150                       | 100       |
| Phosphocellulose    | 271         | 36,300                 | 1,150                       | 100       |
| Hydroxylapatite     | 16.5        | 6,088                  | 368                         | 16.5      |
| SS DNA cellulose    | 3.6         | 5,600                  | 1,556                       | 15.4      |
| FPLC-Mono Q         | 0.195       | 764                    | 3,920                       | 2.1       |
| Glycolic gradient   | 0.087       | 527                    | 6,045                       | 1.4       |

*Procedure 1 was carried out with HeLa cells isolated from 30 liters of culture; procedure 2 was carried out with material isolated from 90 liters of culture. The glycerol gradient sedimentation step was carried out with an aliquot of the fraction from the previous purification step. However, the activity and yield reported were calculated based on the use of the entire fraction.*

**FIG. 1.** A, co-sedimentation of A1 activity and DNA-dependent ATPase activity upon glycerol gradient centrifugation. An aliquot of the FPLC-Mono Q A1 fraction as shown in procedure 2, Table I, was separated on a 15–35% glycerol gradient as described under “Materials and Methods.” The fractions were collected from the bottom of the gradient. In the SV40 dipolymerase assay (A), each reaction (40 μl) contained 3 μl of each glycerol gradient fraction, 0.87 μg of T ag, 1.1 μg of HSSB, 106 units of topo 1, 0.23 μg of SV40 ori' DNA (pSV01ΔE1P), 0.1 unit of pol δ, 0.1 μg of PCNA, pol α-prime complex (0.02 and 0.04 unit, respectively), and dNTPs, rNTPs, and an ATP-regenerating system as previously described (Lee et al., 1988). Reactions were incubated for 60 min at 57 °C, and acid-insoluble material was determined. A1 activity (O) was assayed in reaction mixtures (30 μl) containing 1 μl of each glycerol gradient fraction, under the conditions described under “Materials and Methods.” The A1 ATPase activity (△) was measured in reaction mixtures (20 μl) containing 2 μl of each glycerol gradient fraction as described under “Materials and Methods.” B, SDS-PAGE analysis of the glycerol gradient fractions. The numbers at the top of the figure indicate the gradient fractions analyzed. The position of the size markers (kDa) are at the left.
ase replication system\(^2\) (Fig. 1). These fractions were also examined for the presence of DNA-dependent ATPase activity. As shown in Fig. 1, these three activities co-sedimented (peaking at fraction 10). The presence of DNA-dependent ATPase activity reflected the essential role played by ATP in the stimulation of pol \(\delta\) (Lee et al., 1989b). The other essential proteins for this effect, PCNA, HSSB, and pol \(\delta\), contained either very low or no detectable ATPase activity (see below).

As shown in Fig. 1B, the peak of A1 activity co-migrated with four polypeptides 145, 40, 38, and 37 kDa (double intensity) in size, consistent with the hypothesis that A1 is a multiprotein complex. Furthermore, the lower, more intensely stained 37-kDa polypeptide could be resolved into a doublet on longer SDS-polyacrylamide gels. Thus, the A1 complex may contain at least five distinct polypeptides of 145, 40, 38, and 37 kDa. Assuming that each subunit is present in equimolar amounts, the complex would be 297 kDa. Tsurimoto and Stillman (1989a) reported the presence of similar protein components in the range of 37–41 and 100–140 kDa in their RF-C fraction.

Gel filtration chromatography was performed to define the native size and structure of the A1 complex. The Q-Sepharose A1 fraction (15 \(\mu\)g) was chromatographed on a Sephacryl S300-SF (Pharmacia) column (0.7 \(\times\) 49 cm, 18.8 ml) at 4 °C in buffer E (buffer A without glycerol) containing 0.25 M NaCl and 0.02% sodium azide. Two peaks of A1 were detected in this procedure. The second A1 peak eluted in a fraction which was approximately one-half the molecular weight of the first peak, consistent with the migration of a monomer and a dimer, respectively, of the A1 complex. Of the total activity recovered, 93% and 41% of the activity was found in the dimer and monomer peaks, respectively. The monomer and dimer peaks eluted in fractions corresponding to a Stokes radius of 69.5 and 92.5 A, respectively. Based on a sedimentation coefficient of 7.5 S, the native molecular mass of the A1 complex in the monomer peak was 287 kDa as determined by the Siegel and Monty equation (Siegel and Monty, 1966).

**ATP and Its Hydrolysis Are Essential for A1-dependent pol \(\delta\) Activity**—Pol \(\delta\) was markedly stimulated by PCNA when a multiply primed DNA such as (dA)\(_{400}\)-oligo(dT)\(_{12-18}\) (nucleotide ratio of 20:1, respectively) was used to measure polymerase activity (Table II). For this effect, A1 (or the pol(ADP-ribose) polymerase-A1 complex) and ATP were also required (Kenny et al., 1989) (Table II). In the absence of an SSBI, omission of either PCNA, ATP, or A1 resulted in the loss of pol \(\delta\) activity. The other rNTPs or dNTPs (with the exception of dTTP) partially substituted for ATP in supporting poly(dT) synthesis; low levels of pol \(\delta\) activity were detected with dATP, CTP, and dGTP. Virtually no activity was observed with ADP, ATP, and ADP plus dNTPs, and other nonhydrolyzable analogues of ATP, suggesting that ATP hydrolysis was essential for pol \(\delta\) activity.

**A1 Is an Accessory Factor for pol \(\delta\) but Not for pol \(\alpha\)**—We have previously shown that the pol(ADP-ribose) polymerase-A1 complex can bind to the ends of DNA chains. In the presence of this complex, primed DNA templates were elongated by pol \(\delta\) but not by pol \(\alpha\) (Lee et al., 1989a, 1989b). Since the glycerol gradient fraction of A1 was free of pol(ADP-ribose) polymerase, we compared the effects of A1, SSBI, PCNA, and ATP on both pol \(\alpha\) and pol \(\delta\) using singly primed \(\phi\)X174 DNA as the primer-template. As shown in Fig. 2, pol \(\alpha\) was stimulated by SSBI about 2-fold, but was unaffected by PCNA, A1, or the combined presence of PCNA and A1. Previous studies with pol(dA)-oligo(dT) showed that

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\(^2\)In the presence of the SV40 origin, T ag, and the other protein components constituting the dipolymerase system (pol \(\alpha\)-primase complex, HSSB, pol \(\delta\), PCNA, and A1), the omission of pol \(\alpha\)-primase results in no detectable DNA synthesis. In the presence of low levels of pol \(\alpha\)-primase, the incorporation of dNMPs can be totally dependent on the addition of the proteins essential for leading strand synthesis, as described in Fig. 1. The products formed with limiting amounts of the pol \(\alpha\)-primase complex are virtually free of Okazaki fragments because only a limited number of pol \(\alpha\) elongated primers are formed. Since pol \(\delta\), in the presence of A1 and PCNA, can utilize low levels of primer ends, the DNA chains formed under these conditions are long (unpublished results).

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**TABLE II**

| Additions | dTMP incorporated in 60 min |
|-----------|-----------------------------|
| Complete  | 123                         |
| -SSBI     | 38.4                        |
| -SSB and A1 | 6.2                      |
| -A1       | 1.9                         |
| -PCNA     | 0.5                         |
| -A1 and PCNA or SSBI and PCNA | <1.0             |
| -ATP and/or add dTTP | <1.0             |
| -ATP + dATP | 38.7                     |
| -ATP + dCTP | 24.9                     |
| -ATP + UTP | 23.4                        |
| -ATP + dGTP | 15.4                     |
| -ATP + CTP | 11.3                        |
| -ATP + GTP | 8.2                         |
| -ATP + ATP\(_\gamma\)S | 6.1                      |
| -ATP + AMPPNP | 4.5                     |
| -ATP + AMPCCP | 2.4                     |
| -ATP + ADP | 2.0                         |

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**FIG. 2.** Effect of A1, PCNA, and SSBI on pol \(\alpha\) and pol \(\delta\) activities with \(\phi\)X174-primed template DNA. Reaction mixtures (30 \(\mu\)l) contained 180 fmol (molecules) of singly primed \(\phi\)X174 DNA, 40 mM Tris-HCl (pH 7.8), 200 \(\mu\)g/ml BSA, 1 mM DTT, 7 mM MgCl\(_2\), 3.3 mM ATP, 100 \(\mu\)M concentration each of dGTP, dTTP, dATP, and 20 \(\mu\)M \(\alpha\)-\(^32\)P)dCTP (20,000 cpm/pmol). Where indicated, 1.0 \(\mu\)g of HSSB, 0.1 \(\mu\)g of PCNA, 60 ng of A1, immunopurified pol \(\alpha\)-primase complex (0.1 unit each), and 0.1 unit of pol \(\delta\) were added. Lanes 1 through 8 contained pol \(\alpha\)-primase, and lanes 9 through 16 contained pol \(\delta\). Reactions were incubated for 30 min at 37 °C after which DNA was isolated and analyzed on a 1.0% alkaline agarose gel (60 V, 14 h).
Pol 6 was strongly stimulated (27-fold) by PCNA, which was particularly evident when the pol α-primase complex/pol DNA template.

Poly(ADP-ribose) polymerase did not stimulate pol δ activity in the presence of A1, SSB, and PCNA with singly primed φX174 DNA or with the multiply primed poly(dA)-oligo(dT). Poly(ADP-ribose) polymerase, however, did increase the rate of chain elongation in the SV40 dipolimerase system. This was particularly evident when the pol α-primase complex/pol DNA template.

Whether poly(ADP-ribose) polymerase plays a direct role in the SV40 dipolimerase system remains to be determined. The apparent ratio of oligo(dT)12-18 was high (data not shown). A more detailed examination of the effects of poly(ADP-ribose) polymerase on T ag-dependent DNA replication will be published elsewhere. Whether poly(ADP-ribose) polymerase plays a direct role in the number of molecules of oligo(dT)12-18 primer ends on the template (Fig. 3A). However, A1 had almost no effect on pol δ activity (plus PCNA) with poly(dA) containing large amounts of primer ends (Fig. 3A). This suggests that A1 facilitates the binding of PCNA-dependent pol δ to primer ends. While A1 lowered the Kₘ of PCNA-dependent pol δ for the number of molecules of oligo(dT)12-18 primer ends on the poly(dA) templates 15-fold (from 31 to 2.4), it did not significantly change the apparent Kₘ of pol α for the number of molecules of primer ends (from 10.5 to 7.3) (Fig. 3, A and B).

With singly primed φX174 DNA, pol δ activity was totally dependent on the presence of A1, and incorporation increased as the level of A1 was elevated (Fig. 4). The size distribution of the products formed was virtually unaffected by increased levels of A1 (Fig. 4). This result suggests that A1 functions to increase the utilization of low levels of primer ends on DNA templates rather than increasing the processivity of pol δ.

A1 Preferentially Binds to Primer Ends—We examined the binding of A1 to primer ends of DNA using a nitrocellulose filter binding assay (Fig. 5). (dA)₄₅₀₀ or oligo(dT)₁₂-₁₈ alone were not adsorbed to the filter in the presence of A1. However, when the poly(dA)-oligo(dT) duplex DNA was used, binding was detected, and increased ratios of primer ((dT)₁₂-₁₈) to template ((dA)₄₅₀₀) resulted in increased binding (Fig. 5A). ATP and ATP-S stimulated the primer binding ability of A1 about 2-3-fold (Fig. 5B). It is interesting to note that the ATnPase activity of A1 was increased by the combination of poly(dA) and oligo(dT), although ATPase activity was stimulated by poly(dA) alone (see below, Table III). The binding of A1 to primer ends was decreased 10-fold when (dA)₄₅₀₀-oligo(U)₁₀-₂₀ was used instead of (dA)₄₅₀₀-oligo(dT)₁₂-₁₈ (Fig. 5C).

A1 Reduces the PCNA Requirement for pol δ-catalyzed Elongation—In the poly(dA)-oligo(dT) system, pol δ was totally inactive in the absence of PCNA. The concentration of PCNA required for pol δ-catalyzed elongation of poly(dA)-oligo(dT), however, was quite substantial (≥0.1 µg) (Fig. 6, A and B), and, as shown in a double reciprocal plot, the apparent Kₘ was 7.85 µg of PCNA/ml and the extrapolated V max was calculated to be 25 pmol of dTMP incorporated. When the effect of PCNA concentration was examined in the presence of A1, there was a marked decrease in the amount of PCNA required for pol δ activity as well as a marked increase in the observed and extrapolated V max. These values were 0.082 µg of PCNA/ml and 67 pmol of dTMP incorporated, respectively. The further addition of HSSB increased the V max to 200 pmol of dTMP incorporated and reduced the apparent Kₘ for PCNA to 0.037 µg/ml.

These results suggest that PCNA can interact with A1.
that the binding of SSB to single strands may reduce the efficiency of action of PCNA. 

...to poly(dA).

The exact role played by SSB in this reaction is unclear, it is likely only in the presence of both A1 and SSB (Fig. 7). While the exact role played by SSB in this reaction is unclear, it is likely that the binding of SSB to single strands may reduce the non-specific binding of A1 and pol δ to free single-stranded regions in the template, reducing the deleterious sequestration of these proteins. In addition, as described below, the direct interaction of HSSB with the A1-PCNA complex on the DNA also may contribute to the marked increase in the efficiency of action of PCNA.

**Activator 1 Contains DNA-dependent Nucleoside Triphosphatase Activity**—As described in Fig. 1, A1 activity co-sedimented with DNA-dependent ATPase activity. The properties of this activity were examined in more detail. In the absence of nucleic acid, all purified preparations of A1 examined catalyzed a low but detectable hydrolysis of ATP to ADP + P. In the presence of DNA and some RNAs, this activity was stimulated (Table III). As shown, φX174 SSC DNA was the best effector of those examined while 6x174 DNA was the best effector of those examined while 6x174 DNA did not increase the A1-catalyzed hydrolysis of ATP. (dA)₄₅₀₀-oligo(dT)₁₂-₁₈ was less effective than φX SS DNA in supporting ATP hydrolysis. The ATPase activity observed with either (dA)₄₅₀₀ or oligo(dT)₁₂-₁₈ alone was lower than the activity observed with the annealed duplex which contained many primer ends. If we assume that the hydrolysis of ATP...
In the presence of DNA marginally stimulated the hydrolysis of dGTP, GTP, and 3'-endo reduced by treatment with pancreatic RNase. In general, all common nucleoside triphosphates were incubated at 37°C. A1-catalyzed hydrolysis of ATP in the absence of DNA and in the presence of poly(U) as an effector was markedly reduced by HSSB and dATP was hydrolyzed more effectively than any other nucleoside triphosphate in the absence of DNA. In the presence of (dA)₄₅₀·oligo(dT)₁₂₋₁₈, dATP and ATP were hydrolyzed most efficiently, followed by dCTP and dGTP; DNA marginally stimulated the hydrolysis of dGTP, GTP, and UTP. As shown in Table II, ATP was the most effective nucleotide that supported poly(dT) synthesis in the elongation reaction, while dATP was only partially effective. Thus, there is a discrepancy between the nucleotides which supported elongation of a primed template in the A1-PCNA-pol δ reaction and the nucleotides cleaved by A1 in the presence of DNA.

Tsurimoto and Stillman (1990) reported that PCNA stimulated the action of RF-C on primed DNA templates. We have observed a similar effect with A1 (Fig. 9A). Under the conditions used, PCNA stimulated the hydrolysis of ATP maximally 3-fold in the presence of (dA)₄₅₀·oligo(dT)₁₂₋₁₈. The stimulation of the ATPase activity of A1 by PCNA was not observed in the absence of added DNA, nor was it detected with poly(dA) or oligo(dT) alone. This suggests that a primed template is essential for PCNA stimulation. However, PCNA also stimulated the ATPase activity of A1 in the presence of φX174 SSC DNA (data not shown). At present, the reasons for this discrepancy are unclear, but it is possible that the SSC DNA contains hairpin structures which may approximate the structure of a primed template.

These observations support the conclusion that there is an interaction of PCNA and A1 on the DNA. We have shown that in the presence of ATP, PCNA can form a stable complex with an A1-primed DNA complex coated with SSB which can be isolated by gel filtration. This complex can bind pol δ which can then catalyze the elongation reaction (Lee and Hurwitz, 1990).

The small but reproducible stimulation of DNA-dependent ATPase activity of A1 by PCNA could be further augmented by HSSB (Fig. 9, B and C). The effect of HSSB was observed only in reactions containing both A1 and PCNA; no stimulation of the ATPase activity was observed in reactions containing only A1 and HSSB (Fig. 9B). No stimulation of ATP hydrolysis by A1 was observed with (dA)₄₅₀·oligo(dT)₁₂₋₁₈ alone in the presence of PCNA and HSSB. This suggests that HSSB may interact with the A1-PCNA complex on the primed DNA template.

**DISCUSSION**

A1, a multiprotein complex which stimulates the in vitro replication of SV40 DNA in the dipolimerase system, was isolated using a pol δ complementation assay. With singly primed φX174 DNA as a template, pol δ activity required PCNA, A1, SSB, and ATP. In contrast, when multiply primed
poly(dT) was used as a template, pol δ activity was readily detected with PCNA alone. However, A1 and ATP were also required for pol δ activity in the presence of SSB. This suggests that PCNA-dependent pol δ can bind to single-stranded regions of DNA which can facilitate its eventual interaction with primer ends only when large numbers of ends are available ((dA)1200-oligo(dT)12-18). In the presence of SSB, the binding of PCNA-dependent pol δ to single-stranded regions is reduced and primer-end binding is governed by the presence of the A1-PCNA complex at primer ends. This complex is most likely specifically recognized by pol δ without the necessary presence of extensive single-stranded regions of DNA.

SSBs from a variety of sources (E. coli, T4 bacteriophage, and adenovirus) could be used to detect pol δ activity; however, these SSBs almost completely inhibited pol α activity and only HSSB stimulated both pol δ and pol α (Kenny et al., 1989). For this reason, the use of T4 gene 32 in the pol δ complementation assay selectively eliminated any incorporation due to pol α. This specific effect has been useful for the isolation of pol δ, PCNA, and A1.

The results presented here support the conclusion that A1 is a primer-recognition factor for PCNA and pol δ for the following reasons: (a) A1 is essential for pol δ activity in the presence of SSB, PCNA, and ATP on a singly or multiply primed DNA; (b) A1 had no effect on pol α activity on primed DNAs or in the SV40 polymerase system; (c) A1 increased the affinity of PCNA-dependent pol δ for primer ends more than 10-fold; (d) A1 strikingly decreased the amount of PCNA required in the pol δ-catalyzed elongation reaction; (e) A1 can directly bind to primer ends; and (f) A1 contains DNA-dependent ATPase activity which is required for pol δ activity. Purified A1 appears to be a multicomponent complex containing five protein bands which migrated as 36.5, 37, 38, 40, and 145 kDa on SDS-polyacrylamide gel electrophoresis and co-sedimented during glycerol gradient centrifugation. Similar observations concerning RF-C were reported by Tsurimoto and Stillman (Tsurimoto and Stillman, 1989a).

The properties of A1 and PCNA resemble the auxiliary factors required for E. coli DNA pol III (¢–¢ complex and dnaN gene product) and T4 DNA pol (products of genes 44/62 and 45) activity. These proteins are essential for the processive action of these DNA polymerases on primed DNA templates, and their action requires ATP hydrolysis. A comparison of their respective ATPase activities indicates that the T (Lee and Walker, 1987; Tsusumi and Kornberg, 1989) and the γ–δ complex, in the presence or absence of the dnaN gene product, each hydrolyzed about 10 ATP molecules/min/molecule of protein (or complex). In contrast, T4 gene 44/62 hydrolyzed approximately 20 molecules of ATP/molecule of complex/min, and, in the presence of a large excess of the T4 gene 45 product, this value increased to 700 molecules of ATP/min/molecule of the 44/62 complex (Jarvis et al., 1989). Assuming that the multicomponent preparations of A1 were pure and contained only active molecules, it hydrolyzed 3.5 molecules of ATP/molecule of A1/min, and this value was increased 3- to 4-fold by PCNA plus HSSB. In this respect, A1 more closely resembles the E. coli factors than the T4 factors.

The observation that the DNA-dependent ATPase activity of A1 was most stimulated by βX174 single-stranded DNA was surprising since A1 binds to primer ends. Recently, it was shown that ¾, the auxiliary factor in the pol III holoenzyme, hydrolyzed ATP in the presence of single-stranded circular DNA much more effectively than with homopolymers or even

3 M. O'Donnell, personal communication.
primed homopolymers (Tsuchihashi and Kornberg, 1989). In this regard, ATPase activity of A1 more closely resembles the E. coli γ-δ and τ auxiliary factors than the T4 factors. The precise DNA structure essential for the A1-dependent hydrolysis of ATP remains to be elucidated.

The effects of PCNA, A1, and SSB on pol δ are unique for each auxiliary factor. On singly primed φX174 DNA, maximal pol δ activity required these three factors, but only PCNA was absolutely essential. Furthermore, the sizes of the products formed were unaffected by SSB or A1. In this respect, the effects of these proteins on pol δ differ from the effects of the auxiliary proteins in the procaryotic systems. The length of products formed with singly primed φX174 DNA by crude pol δ fractions (0.4 M phosphocellulose fraction), however, were much larger7 than the products formed with purified pol δ in the presence of PCNA, A1, and SSB (Fig. 4). This suggests that an additional factor, present in the crude fraction, but missing from the purified components, may contribute to the synthesis of longer DNA chains. Another fundamental difference between the procaryotic replication systems and the SV40 system is that two different DNA polymerases are involved in the latter system while only one polymerase plays a role in replication in the T4 and E. coli systems.

The proposal that pol α is involved in lagging strand synthesis and pol δ is specifically involved in leading strand synthesis is based on several observations. (a) pol α is associated with a DNA primase while pol δ is not (So and Downey, 1988). (b) PCNA and RF-C (A1) have been reported to stimulate only leading strand synthesis (Prelich and Stillman, 1988). (c) pol δ has been shown to utilize RNA primers inefficiently (Lee et al., 1989b). This is consistent with the results shown here, which demonstrate that A1 binds poorly to RNA primers. (d) A1 appears to be specific for pol δ.

Tsukimoto and Stillman (1988a) reported that in reactions containing primed M13 DNA, RF-C stimulated both human pol α and calf thymus pol δ and that calf-thymus pol δ activity was inhibited by E. coli SSB. These reaction mixtures contained RF-C, PCNA, and the 4 dNTPs, but lacked ATP. Under the conditions described here, A1 had no effect on pol α activity, and ATP was essential for pol δ activity. Further studies are needed to clarify these discrepancies between RF-C and A1.

After this work was completed, further studies on RF-C by Tsukimoto and Stillman (1990) appeared. Their results indicated that RF-C, like A1, binds preferentially to a primed DNA template, contains intrinsic DNA-dependent ATPase activity, and is required for PCNA-dependent pol δ elongation of primed DNA templates in the presence of HSSB and ATP. They also indicated that PCNA at high levels stimulated the DNA-dependent ATPase activity of RF-C and that the amino acid sequences of PCNA and T4 gene 45 protein had striking similarities. They concluded that RF-C is analogous to the T4 genes 44-62 protein complex and PCNA is analogous to the T4 gene 45 product. Our results suggest that PCNA directly interacts with A1 and that this interaction markedly reduced the concentration of PCNA essential for pol δ. In fact, the levels of PCNA required in the presence of A1 were lower than that required to stimulate the DNA-dependent ATPase activity of RF-C (Tsukimoto and Stillman, 1990) (Figs. 6 and 7). As we have indicated, A1 and HSSB are not processivity factors since they do not increase the length of DNA chains formed by PCNA-dependent pol δ but increase the number of chains elongated by pol δ. Thus, we believe that there are differences between RF-C and A1 which remain to be clarified. It is evident that more information about the procaryotic and eucaryotic proteins which facilitate elongation of DNA chains will help clarify the basic mechanism of DNA synthesis in all the systems.

Acknowledgments—We thank Nilda Belgado and Barbara Phillips for their assistance.

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