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Single-Stranded DNA-Dependent ATPase from HeLa Cells That Stimulates DNA Polymerase α–Primase Activity: Purification and Characterization of the ATPase†

Jamboor K. Vishwanatha*,‡ and Earl F. Baril§
Department of Biochemistry, University of Nebraska Medical Center, Omaha, Nebraska 68198, and Cell Biology Group, The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545
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ABSTRACT: A single-stranded DNA-dependent ATPase that cofractionates during the early stages of purification of a multiprotein DNA polymerase α complex from HeLa cells has been purified to homogeneity. The ATPase is part of a 16S multienzyme DNA polymerase α complex that is fully active in SV40 DNA replication in vitro. The ATPase hydrolyzes ATP to ADP in a reaction that is completely dependent on the presence of DNA. DNA in single-stranded form is strongly preferred as a cofactor, and polydeoxynucleotides with adenine or thymidine residues are highly effective. Glycerol gradient sedimentation showed that the purified ATPase sedimented at an $s_{20,w}$ of 7 S, and polyacrylamide gel electrophoresis under denaturing conditions reveals two polypeptides with relative molecular weights of 83 000 and 68 000. Both of these polypeptides have purine nucleotide binding sites as revealed by photoaffinity cross-linking experiments. ATP binds to the two subunits more efficiently than GTP, and CTP or UTP does not cross-link with the two polypeptides. DNA synthesis catalyzed by purified HeLa cell DNA polymerase α–primase is stimulated in the presence of ATPase and ATP at an optimum concentration of 2 mM. Analysis of the DNA product by gel electrophoresis indicates that with poly(dT) but not phage M13 DNA as template the ATPase overcomes a lag and decreases the length of nascent DNA chains synthesized by the DNA polymerase α–primase complex.

Many reactions in DNA metabolism such as replication, repair, and recombination are driven by chemical energy derived from the hydrolysis of ATP. Enzymes catalyzing the hydrolysis of ATP in a DNA-dependent reaction have been found in prokaryotes and eukaryotes (Kornberg, 1980; Hachmann & Lezius, 1976; Assairi & Johnston, 1979; Cobianchi et al., 1979; Boxer & Korn, 1980; DeJong et al., 1981; Hyodo & Suzuki, 1981; Thomas & Meyer, 1982; Biamonti et al., 1983; Tawaragi et al., 1984; Hubscher & Stalder, 1985; Hockensmith et al., 1986; Sugino et al., 1986; Tsurimoto & Stillman, 1990). Most of these enzymes are DNA-dependent

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*Author to whom correspondence should be addressed at the Department of Biochemistry, University of Nebraska Medical Center, 600 S. 42nd St., Omaha, NE 68198-4525.
‡University of Nebraska Medical Center.
§The Worcester Foundation for Experimental Biology.

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enzymes and act in conjunction with other proteins in DNA synthesis. Stimulation of DNA polymerase α and β activities mediated by DNA-dependent ATPases has been shown in many cases. DNA replication is a tightly regulated process that involves multiple stages during which many protein–protein and protein–DNA interactions occur (Kornberg, 1980, 1988; Campbell, 1986). The involvement of DNA-dependent ATPase as an accessory protein for DNA polymerase has been shown in bacteriophage T4 DNA replication (Cha & Alberts, 1988). Recently, a protein complex, called RF-C, that is necessary for coordinating leading- and lagging-strand DNA replication during SV40 DNA replication in vitro, was shown to have DNA-dependent ATPase activity (Tsurimoto & Stillman, 1989, 1990).

In our studies on the multiprotein DNA polymerase α complex from HeLa cells (Vishwanatha et al., 1986), we observed a DNA-dependent ATPase activity that cosedimented with the multiprotein DNA polymerase α complex. The DNA-dependent ATPase is part of the 16S multienzyme complex which is capable of T-antigen-dependent replication of SV40 DNA in vitro (Baril et al., 1988). The ATPase has been purified to homogeneity, and we have characterized its physical and enzymic properties and its action on the HeLa cell DNA polymerase α–primase complex.

Materials and Methods

Materials

^{3}H- and ^{32}P-labeled ribo- and deoxyribonucleoside triphosphates were obtained from ICN (Irvine, CA). Unlabeled ribo- and deoxyribonucleotides, oligonucleotides, and calf thymus DNA were obtained from Pharmacia P-L Biochemicals (Milwaukee, WI). Synthetic polynucleotides were obtained from Midland Certified Reagents (Midland, TX). Pancreatic DNase I activated calf thymus DNA was prepared by a published procedure (Baril et al., 1977). (Diethylaminoethyl)cellulose (DEAE-cellulose)[1] (DE-52) was purchased from Whatman. Native and denatured calf thymus DNA–cellulose matrices were prepared according to the procedure of Alberts and Herrick (1971). ATP–agarose, type III, was obtained from Pharmacia. Poly(ethylenimine)–cellulose (PEI–cellulose) thin-layer chromatogram, CEL 300 PEI, was obtained from Brinkmann Instruments. [γ-^{32}P]8-N_3ATP (18.1 Ci/mmol) and [γ-^{32}P]8-N_3GTP (15.7 Ci/mmol) prepared and purified by the procedure of Potter and Haley (1983) and [γ-^{32}P]5-N_3UTP (4.7 Ci/mmol) prepared and purified by the method of Evans and Haley (1987) were kindly provided by Dr. Boyd Haley, University of Kentucky.

Methods

Growth of Cells. HeLa S_3 cells were grown in suspension cultures in Joklik's minimal essential medium supplemented with 3.5% each of calf serum and fetal bovine serum. Cells were harvested at a density of (0.5–1.0) × 10^6 cells/mL, while they were in the log phase of growth. Cells were judged free of mycoplasma contamination by previously published procedures (Chiu & Baril, 1975; Novak & Baril, 1978).

Subcellular Fractionation Procedure. The separation of cell homogenates into the nuclear and postmicrosomal supernatant fractions was according to a published procedure (Vishwanatha et al., 1986). The nuclear extract and the postmicrosomal supernatant fractions were combined and dialyzed for 4 h against 4 L of buffer A [50 mM Tris-HCl,[1] pH 7.5, 1 mM EDTA,[1] 1 mM DTT,[1] and 10% (v/v) glycerol]. This served as the starting material for purification of the ATPase.

ATPase Assays. The DNA-dependent ATPase assay was performed by two procedures. Throughout the enzyme purification, ATPase activity was monitored by the conversion of [γ-^{32}P]ATP to a charcoal-nonadsorbable form. In a reaction volume of 25 μL, the enzyme fraction was incubated with 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM DTT, 1 mM [γ-^{32}P]ATP (2000–3000 cpm/nmol), and 5 μg of heat-inactivated bovine serum albumin with or without 0.5 μg of DNA as a cofactor. After a 30-min incubation at 30 °C, 75 μL of a 10% (v/v) suspension of activated charcoal in 0.25 N HCl, 0.025 M KH_2PO_4, and 0.025 M $Na_4P_2O_7$ was added, and the mixture was allowed to stand on ice for 5 min. After centrifugation at 2000*g* for 5 min, the supernatant was withdrawn and radioactivity determined by liquid scintillation spectrometry. Background radioactivity in the absence of enzyme and at zero time in the reaction were subtracted from experimental results.

The second procedure for ATPase assay utilized PEI–cellulose thin-layer chromatography. The reaction was set up as above except that [2,8-^{3}H]ATP (100–150 cpm/pmol) was substituted for [γ-^{32}P]ATP. Following a 30-min incubation at 30 °C, the tubes were chilled in an ice/H_2O bath, and 2 μL of 0.1 M EDTA, pH 7.0, was added. Five microliters of the reaction product was applied to a PEI–cellulose thin-layer chromatogram and was overlaid with 2 μL of a mixture of 2 mg each of ATP, ADP, and AMP per milliliter. The chromatogram was developed by ascending chromatography with 1 M lithium chloride at room temperature. The areas corresponding to the standards were scraped off and solubilized with 0.5 mL of a PEI–cellulose extraction solution (0.2 M KCl in 0.1 N HCl) for 60 min at 50 °C. The radioactivity in the solubilized material was determined by liquid scintillation spectrometry. When different ribo- or deoxyribonucleotides were substituted for ATP in the assay, the corresponding markers were used in PEI chromatography of the product.

Other Enzyme Assays. DNA polymerase α activities with DNase I activated calf thymus DNA, heat-denatured calf thymus DNA, and poly(dA)$_{1000}$·(dT)$_4$ were performed as previously described (Vishwanatha et al., 1986). Stimulation of polymerase α activity by DNA-dependent ATPase was measured under polymerase α incubation conditions with the addition of 2 mM ATP.

DNA primase and deoxyribonuclease activities were measured as previously described (Vishwanatha et al., 1986). Topoisomerase I was measured according to a published procedure (Ishii et al., 1983) using pBR322 plasmid DNA as substrate. DNA helicase activity was measured according to a published procedure (Jongeneel et al., 1984). Coupled RNA–DNA synthesis reactions were performed as previously described (Vishwanatha & Baril, 1986).

Analysis of nascent RNA–DNA chains synthesized by DNA polymerase α–primase in the presence or absence of DNA-dependent ATPase was performed on polyacrylamide gels (0.25 mm × 33 cm × 42 cm) containing 8% acrylamide [with a 20:1 ratio of acrylamide to *N*,*N*′-methylenebis(acrylamide)], 8 M urea, 100 mM Tris–borate, pH 8.3, and 1 mM EDTA (Sanger & Coulson, 1978). Electrophoresis was carried out at 2000 V for 90 min. Gels were exposed to Kodak

[1] Abbreviations: DEAE-cellulose, (diethylaminoethyl)cellulose; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; KPO_4 buffer, contains equimolar K_2HPO_4 and KH_2PO_4; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; 8-N_3ATP, 8-azidoadenosine 5′-triphosphate; 5-N_3UTP, 5-azidouridine 5′-triphosphate; 8-N_3GTP, 8-azidoguanosine 5′-triphosphate.

Table I: Purification of HeLa Cell DNA-Dependent ATPase[a]

fraction	volume (mL)	total protein (mg)	total act. (units)	sp act. (units/mg)	x-fold purification
(I) native DNA-cellulose	9.8	4.40	23240	5269	1
(II) denatured DNA-cellulose	10.7	1.90	10861	5800	1.1
(III) ATP-agarose	4.8	0.30	4323	12872	2.5
(IV) glycerol gradient	2.3	<0.01	407	354000	67.2

[a] The starting material was 28 g (wet weight) of HeLa cells. Due to the presence of high amounts of DNA-*independent* ATPase activity in earlier fractions of the purification, the native DNA–cellulose fraction is taken as fraction I in this table. One unit of ATPase activity is defined as the hydrolysis of 1 mmol of ATP in 1 h at 30 °C.

X-Omat AR films with Cronex-plus intensifying screens at −70 °C.

Photoaffinity Labeling of ATP Binding Site. Photoaffinity labeling was performed by a published procedure (Potter & Haley, 1983) with modifications. DNA-dependent ATPase (fraction III) was dialyzed extensively against 25 mM Tris-HCl, pH 7.5, 25 mM NaCl, and 5% (v/v) glycerol using a Centricon 30 microconcentrator (Amicon Corp., Danvers, MA) to remove free thiol-reducing agents that interfered with photolabeling of the protein. The dialyzed protein fraction was incubated in a 50-μL reaction consisting of 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 20 μM final concentration of either [γ-^{32}P]8-N_3ATP or [γ-^{32}P]8-N_3GTP, and 40 μM [γ-^{32}P]5-N_3UTP. The reaction was held on ice for 10 s followed by photolysis for 30 s with a UVS-11 mineralight lamp (max output 254 nm) from a distance of 1 cm (lamp output at 1 cm is approximately 5000 μW/cm^2 at 252 nm). After photolysis (or 30-s incubation on ice for the nonphotolysis control), 25 μL of a protein solubilizing mix was added, and the samples were subjected to electrophoresis on a 8% polyacrylamide slab gel under denaturing conditions. After the gel was stained with Coomassie blue to identify the protein bands, the gel was destained, dried, and exposed to Kodak XAR-5 film with a Cronex-plus intensifying screen at −70 °C. In experiments where protection of photoaffinity labeling by different NTPs was studied, the reactions were also performed at various concentrations of the respective NTPs.

Electrophoresis. Polyacrylamide gel electrophoresis under denaturing conditions was performed as previously described (Lamothe et al., 1981) with a 10% separating gel and a 3% stacking gel. Protein bands were detected by the Bio-Rad silver-staining procedure.

Other Methods. Fatty acid free bovine serum albumin was heat-inactivated for 3 h at 55 °C at a concentration of 1 mg/mL. Buffers were prepared at 20 °C as concentrated stock solutions. Ionic strengths of buffers were monitored by an Orion Research conductivity meter (Model 101). The protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard.

Results

Purification Procedure. A DNA-dependent ATPase activity from the combined nuclear extract and postmicrosomal supernatant of HeLa cells was purified to homogeneity by the procedure summarized in Table I.

The combined nuclear extract and postmicrosomal supernatant was chromatographed over a DEAE-cellulose column (35-mL volume) that had been previously equilibrated in buffer A. The column was then washed with 8 column volumes of buffer A followed by 8 column volumes of buffer A containing 0.15 M KCl. The column wash contained about 70% of the loaded protein and the majority of the DNA-*independent* ATPase activity (data not shown). All of the DNA-*dependent* ATPase activity, the majority of DNA polymerase α activity, and 15% of the protein that was applied to the DEAE-cellulose column were eluted by buffer A containing 0.15 M KCl. Since this fraction contained some DNA-independent ATPase activity, it was not used as the starting material for calculating the enzyme purification data (Table I).

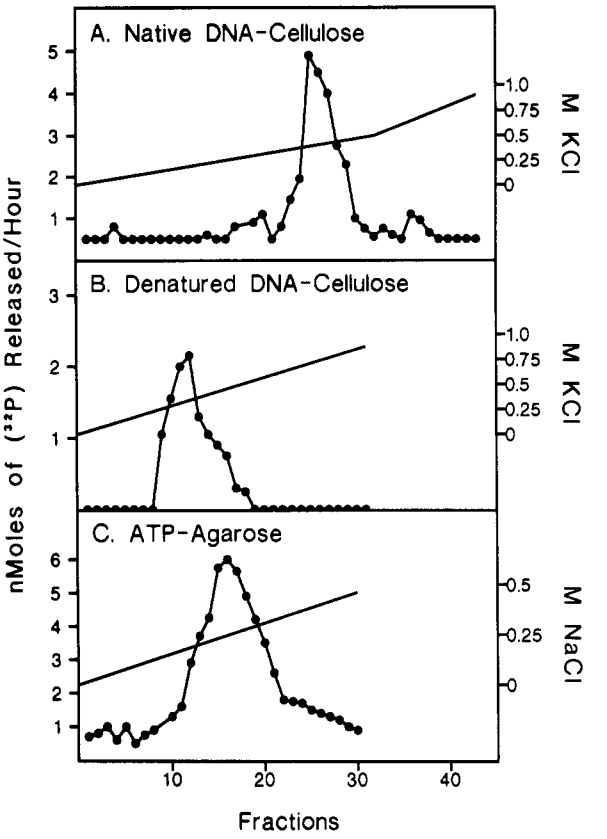

FIGURE 1: Purification of DNA-dependent ATPase. The combined nuclear extract and postmicrosomal supernatant from 28.5 g of HeLa cells (1800 mg of protein) was chromatographed on a 35-mL column of DEAE-cellulose (Whatman DE-52) equilibrated in buffer A. After the column was washed with 8 column volumes of buffer A, DNA-dependent ATPase was eluted with 8 column volumes of buffer A containing 0.15 M KCl. The DEAE-cellulose eluate was loaded onto an 8-mL column of native calf thymus DNA–cellulose (panel A) equilibrated in buffer A containing 0.15 M KCl. After being washed with 8 column volumes of the equilibration buffer, the column was eluted by a linear gradient of 0.15–1.0 M KCl in buffer A (10 column volumes). An aliquot of each fraction (2 mL) was assayed for ATPase activity. The fractions corresponding to the peak of ATPase activity were pooled, dialyzed against buffer A, and applied to a 4-mL column of denatured calf thymus DNA–cellulose (panel B) equilibrated in buffer A. After the column was washed with 8 column volumes of buffer A, the column was eluted by a linear gradient of 0–1 M KCl in buffer A (10 column volumes), and 1.2-mL fractions were collected. Fractions corresponding to the peak of ATPase activity were pooled, dialyzed against buffer A, and applied to a 1.5-mL column of ATP–agarose. The column was developed with a 0–0.5 M NaCl gradient in buffer A, and 0.5-mL fractions were collected. ATPase activity was measured by monitoring the release of ^{32}P from [γ-^{32}P]ATP by the charcoal adsorption assay as described under Materials and Methods.

The ATPase activity eluting from DEAE-cellulose with buffer A containing 0.15 M KCl was loaded onto a native calf thymus DNA–cellulose column (8 mL) that was previously equilibrated in buffer A containing 0.1 M KCl. The column was washed with 8 column volumes of buffer A containing 0.15 M KCl and then eluted by an 80-mL linear gradient of 0.15–1.0 M KCl in buffer A. Fractions of 2.0 mL were collected, and an aliquot of each fraction was assayed for

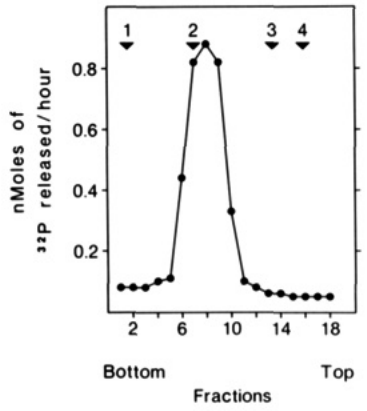

FIGURE 2: Glycerol gradient centrifugation of ATPase. DNA-dependent ATPase (fraction III, Table I) was precipitated with ammonium sulfate. The precipitate was dissolved in a small volume of 0.02 M KPO_4, pH 7.5, and 1 mM DTT and layered on a 10–30% glycerol gradient containing 0.05 M KPO_4, pH 7.5, 200 mM $(NH_4)_2SO_4$, 1 mM DTT, 1 mM EDTA, and 1 mM PMSF and prepared in polyallomer tubes for the Beckman SW41 rotor. Centrifugation was at 35000 rpm for 41 h. Fractions were collected from the bottom of the tubes, and aliquots were assayed for ATPase activity.

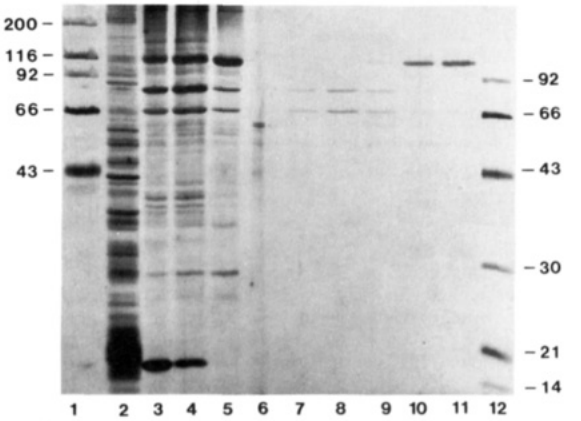

FIGURE 3: Denaturing gel electrophoresis of ATPase. Fractions at various stages of purification of the DNA-dependent ATPase were subjected to electrophoresis on a 10% separating and a 3% stacking polyacrylamide gel containing SDS.[1] Lanes 1 and 12 contain molecular weight markers whose relative molecular weights ($\times 10^{-3}$) are indicated. The other lanes contain 10 μg of protein of combined nuclear extract and postmicrosomal supernatant (lane 2), 10 μg of protein of fraction I (lane 3), 10 μg of protein of fraction II (lane 4), 10 μg of protein of fraction III (lane 5), and 3–5 μg of protein of glycerol gradient fractions 6–11 (lanes 6–11), respectively.

DNA-dependent ATPase activity (Figure 1A). All of the DNA-*independent* ATPase activity appeared in the column wash. DNA-*dependent* ATPase activity eluted as a single peak of activity at around 0.4 M KCl. The fractions containing this activity were pooled and dialyzed against 4 L of buffer A for 12 h (fraction I, Table I). This fraction was used as the starting material in calculating the purification of the DNA-dependent ATPase.

Fraction I was loaded onto a 4-mL column of denatured calf thymus DNA–cellulose equilibrated with buffer A. After the column was washed with 8 column volumes of buffer A, the column was eluted by a 40-mL linear gradient of 0–1 M KCl in buffer A, and fractions of 1.2 mL were collected. The DNA-dependent ATPase activity eluted as a single peak at about 0.3 M KCl. The active fractions were pooled and dialyzed for 12 h against 4 L of buffer A (fraction II, Table I).

Fraction II was chromatographed on a 1.5-mL column of ATP–agarose. Since fraction II was susceptible to nonspecific adsorption that resulted in a considerable loss of activity, the ATP–agarose matrix was first washed with buffer A containing 2 mg/mL heat-inactivated bovine serum albumin followed by buffer A containing 2 M KCl before equilibration with buffer A. After loading of fraction II, the column was washed with 8 column volumes of buffer A and eluted by a 15-mL linear gradient of 0–1 M KCl in buffer A. Fractions of 0.5 mL were collected, and an aliquot from each was assayed for DNA-dependent ATPase activity (Figure 1C). Active fractions from this column were pooled and dialyzed against buffer A for 12 h (fraction III, Table I).

Fraction III was precipitated with 80% saturation of ammonium sulfate; the precipitate was collected by centrifugation and resuspended in 20 mM KPO_4 buffer, pH 7.6, containing 1 mM DTT. The suspended fraction was loaded onto a 10–30% glycerol gradient containing 50 mM KPO_4 buffer,[1] pH 7.6, 200 mM $(NH_4)_2SO_4$, 1 mM DTT, and 1 mM EDTA prepared in polyallomer tubes for the Beckman SW41 rotor. After centrifugation for 41 h at 35000 rpm and 4 °C, fractions (15 drops) were collected by puncturing the bottom of the tube, and an aliquot of each fraction was assayed for DNA-dependent ATPase activity (Figure 2). DNA-dependent ATPase activity sedimented as a single peak with an $s_{20,w}$ value of 7 S. The gradient fractions containing ATPase activity were pooled, dialyzed against buffer A containing 50% glycerol, and stored at −80 °C (fraction IV, Table I). When stored under these conditions, the enzyme remains stable for over 4 months. It is also stable to repeated freeze–thawing.

DNA topoisomerase I activity cofractionates with the DNA-dependent ATPase through the denatured calf thymus DNA–cellulose chromatography (fraction II) stage. However, the topoisomerase is separated from the ATPase on the ATP–agarose column. The purified fraction IV does not contain detectable DNA polymerase α, DNA primase, deoxyribonuclease, phosphodiesterase, or helicase activities.

Molecular Structure of the Enzymes. Analysis of the proteins in various stages of purification by denaturing gel electrophoresis (Figure 3) shows that DNA-dependent ATPase is made up of two subunits of 83000 and 68000 daltons. A 108000-dalton protein copurifies with the DNA-dependent ATPase through several chromatographic steps. The 108000-dalton protein, however, is separated from the DNA-dependent ATPase during glycerol gradient centrifugation and does not appear essential for the catalytic activity of the enzyme. The peak fraction (fraction 8) of ATPase activity from the glycerol gradient exhibits only two bands of 83000 and 68000 daltons during denaturing polyacrylamide gel electrophoresis (Figure 3, lane 8). These two bands comprise greater than 95% of the protein that was loaded in lane 8 of the gel. There is no similarity between this ATPase and the ATPase activity associated with the RF-C complex (Tsurimoto & Stillman, 1990).

Photoaffinity Cross-Linking of ATP and Other Nucleotides to the ATPase. In order to determine which of the two subunits of ATPase has the nucleotide binding site, we performed photoaffinity labeling of fraction III by the procedure described under Materials and Methods, and the results are presented in Figure 4. We used fraction III instead of the purified fraction IV for the photoaffinity labeling experiments because of practical difficulties. Photoaffinity labeling is done in the absence of reducing agents to prevent inhibition of photolabeling by nitrene scavenging (Evans & Haley, 1987). We find considerable loss of fraction IV during various dialysis procedures to remove the reducing agents due to protein adhering to the dialysis membrane. We did not encounter these problems with fraction III. With [γ-^{32}P]8-N_3ATP as the photo-

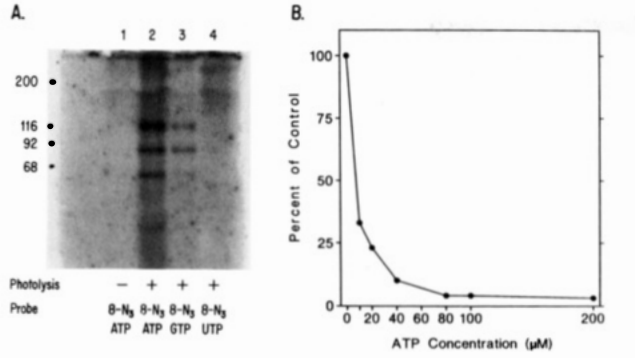

FIGURE 4: Photoaffinity labeling of nucleotide binding sites. (A) DNA-dependent ATPase (fraction III) was incubated with 20 μM [γ-^{32}P]8-N_3ATP (lanes 1 and 2), 20 μM [γ-^{32}P]8-N_3GTP (lane 3), or 20 μM [γ-^{32}P]5-N_3UTP (lane 4) at 2 °C for 10 s followed by photolysis (lanes 2–4) for 30 s or continued incubation on ice (lane 1) as described under Materials and Methods. The samples were electrophoresed on an 8% polyacrylamide gel under denaturing conditions. The gel was dried and exposed to Kodak X-Omat AR film at −70 °C with a Cronex-plus intensifying screen. (B) Protection of the nucleotide binding site by ATP. DNA-dependent ATPase (fraction III) was incubated with 20 μM [γ-^{32}P]8-N_3ATP and the various indicated concentrations of ATP to protect the nucleotide binding site from photolabeling. The experiment was conducted as described above. The autoradiogram was scanned with a laser densitometer, and the intensity of the radiolabeled 83 000- and 68 000-dalton polypeptides was measured. The results are presented as percent of control. The control tube was set up with labeled 8-N_3ATP and without added ATP.

label, three polypeptides are labeled in fraction III (lane 2, Figure 4A), migrating at 108, 83, and 68 kDa on the gel. The 83- and 68-kDa subunits correspond to the DNA-dependent ATPase (Figure 3). No labeling was seen in the absence of photolysis (lane 1, Figure 4A), demonstrating that labeling is not due to phosphorylation of the proteins with [γ-^{32}P]8-N_3ATP. When [γ-^{32}P]8-N_3GTP was used as the photolabel, the same three polypeptides are labeled, but to a lesser extent than with [γ-^{32}P]8-N_3ATP (compare lanes 2 and 3, Figure 4A). The 83 000-dalton polypeptide is labeled more intensely than the other two polypeptides with [γ-^{32}P]8-N_3GTP. No labeling was seen with [γ-^{32}P]5-N_3UTP (lane 4, Figure 4A), indicating that the nucleotide binding by the enzyme is specific for purine bases.

To establish more rigorously the purine binding sites, ATP protection experiments were performed (Figure 4B). The addition of increasing amounts of ATP (0–200 μM) to photolabeling reactions containing 20 μM [γ-^{32}P]8-N_3ATP resulted in a decrease of photolabeling of the 83 000- and 68 000-dalton polypeptides and not the 108 000-dalton polypeptide. Thus, the ATP binding sites on the 83 000- and 68 000-dalton polypeptides are authentic nucleotide binding sites. Addition of 200 μM GTP also results in decreased labeling by [γ-^{32}P]8-N_3ATP. No decrease in photolabeling was observed in the presence of 200 μM CTP or UTP, indicating the specificity of the binding for purine nucleotides. The photolabeling of the 108 000-dalton peptide with [γ-^{32}P]8-N_3 ATP and the lack of protection by ATP indicate that the 108 000-dalton peptide is a DNA binding protein. DNA binding sites seem to attract 8-N_3ATP during photolabeling, but do not protect with ATP (B. E. Haley, personal communication). The 108 000-dalton polypeptide does not contribute to the enzymatic activity at the ATPase.

Requirements for Optimal Activity. The DNA-dependent ATPase has an absolute requirement for a divalent cation for its activity. The most effective in this regard is $MgCl_2$ with an optimum concentration of 2 mM, while $MnCl_2$ and $ZnCl_2$ are only about half as effective.

Table II: Efficiency of Various DNAs as Cofactors of the HeLa DNA-Dependent ATPase[a]

DNA cofactor	% act.	DNA cofactor	% act.
no DNA	10	poly(dA)·poly(dT)	162
calf thymus DNA, single stranded	100	poly(dA-dT)·poly(dA-dT)	148
calf thymus DNA, double stranded	58	$poly(dC)_{1000}$	67
calf thymus DNA, DNase I activated	62	$poly(dG)_{1000}$	10
		poly(dC)·poly(dG)	90
M13 DNA, single stranded	113	poly(U)	25
$poly(dA)_{1000}$	83	dT_3	0
$poly(dT)_{1000}$	168	dT_{10}	0

[a] DNA-dependent ATPase (fraction IV) was incubated with 0.5 μg of the indicated template under the conditions described under Materials and Methods. The product was subjected to thin-layer chromatography on PEI–cellulose plates, and the radioactivity in the ADP spot was counted by liquid scintillation spectrometry. One hundred percent value corresponds to 156 pmol of ADP produced in 30 min at 30 °C.

In the presence of 2 mM $MgCl_2$, the optimum KCl concentration is 150 mM. The ATPase activity is not inhibited by KCl at concentrations up to 300 mM, while 70% inhibition occurs at 450 mM KCl concentration. The optimum pH in Tris-HCl buffers is between 7.0 and 8.5.

The DNA-dependent ATPase activity requires a thiol reducing agent and is completely inhibited in the presence of 10 mM *N*-ethylmaleimide. Inhibitors of other DNA-metabolizing enzyme activities, such as aphidicolin, novobiocin, and nalidixic acid, do not inhibit the DNA-dependent ATPase.

Nucleoside Triphosphate Specificities. The DNA-dependent ATPase catalyzes the hydrolysis of ATP and dATP with nearly equal efficiencies. It is totally inactive with CTP or UTP while the rate of hydrolysis of GTP is about 30% of that of ATP. The products of the hydrolysis of ATP as analyzed by PEI–cellulose thin-layer chromatography are inorganic phosphate and ADP.

DNA Cofactor Requirement. The highly purified DNA-dependent ATPase is dependent on DNA as a cofactor for maximal activity (Table II). Single-stranded linear or circular DNA was the most effective, while duplex or DNase I nicked duplex DNA were only about half as effective. Among the synthetic templates, poly(dT) was the most efficient followed by poly(dA) and poly(dC), while poly(dG) was ineffective. Poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT) were both highly effective as cofactors. Poly(dC)·poly(dG) was more effective than either poly(dC) or poly(dG) alone. Oligonucleotides such as $(dT)_3$ and $(dT)_{10}$ or ribohomopolynucleotides such as poly(U) were ineffective as cofactors.

Hockensmith et al. (1986) have purified a calf thymus DNA-dependent ATPase that prefers a primer-template junction as its effector. We tested DNA template-primers such as poly(dA)·$(dT)_{10}$ at various molar ratios of template to primer as well as a DNA–RNA hybrid such as poly(dT)·$(A)_{10}$. No significant differences were seen in the stimulation of the DNA-dependent ATPase activity with either an RNA or a DNA primer hybridized to DNA at various template:primer molar ratios.

The ability of the purified ATPase to bind to radiolabeled DNA was tested by a nitrocellulose filter binding assay. The DNA-dependent ATPase binds to single-stranded and double-stranded DNA, as well as poly(dA) and poly(dT).

Stimulation of HeLa Cell DNA Polymerase α. In the procedure for purification of a multiprotein form of HeLa cell DNA polymerase α (Vishwanatha et al., 1986), we observed DNA-dependent ATPase activity that cofractionated with the polymerase α complex in early stages of the purification. Therefore, the ability of ATPase to stimulate DNA polymerase α was tested. As shown in Figure 5, DNA-dependent ATPase

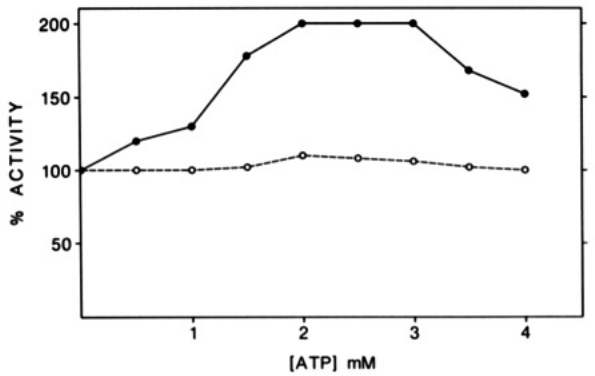

FIGURE 5: Stimulation of DNA polymerase α by DNA-dependent ATPase. HeLa cell DNA polymerase α_2 (Vishwanatha et al., 1986) was incubated with (open circles) or without (closed circles) 1.0 unit of DNA-dependent ATPase (fraction IV) in the presence of denatured calf thymus DNA and the indicated amounts of ATP under the DNA polymerization conditions described under Materials and Methods. Incorporation of [^{3}H]dTMP to DNA was measured by TCA[1] precipitation of labeled DNA. One hundred percent activity represents 22 pmol of [^{3}H]TMP incorporated in 30 min at 35 °C.

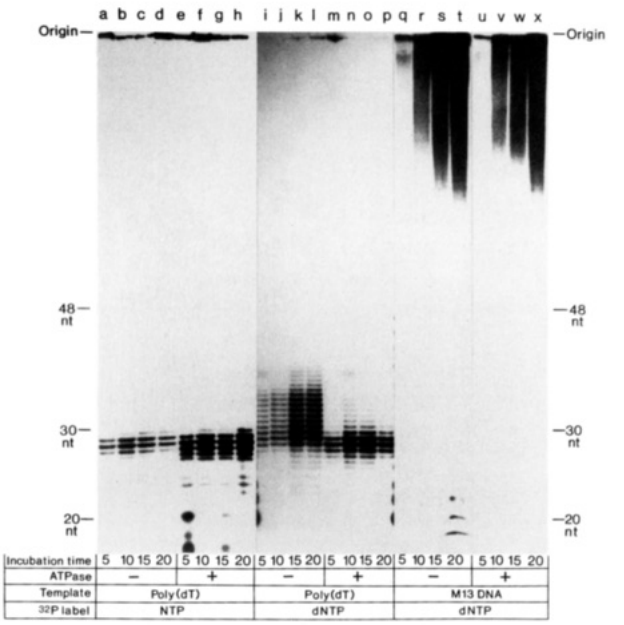

FIGURE 6: Analysis of nascent RNA–DNA chains synthesized by DNA polymerase α–primase. HeLa cell DNA polymerase α–primase was incubated under conditions for synthesis of nascent RNA–DNA chains with poly(dT) (lanes a–p) or M13 DNA (lanes q–x) as templates. Identical reactions with (lanes a–h, m–p, u–x) or without (lanes a–d, i–l, q–t) DNA-dependent ATPase were set up, and aliquots of the reaction were removed at 5-min intervals. The reaction was stopped by the addition of 15 mM EDTA, and DNA was precipitated by adding 0.4 volume of 5 M ammonium acetate and 2 volumes of 2-propanol (Maniatis et al., 1982). The precipitate was suspended in a small volume of 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Electrophoresis and autoradiography were performed as described under Materials and Methods. The mobility of radiolabeled oligonucleotide marker is indicated at the sides of the figure.

stimulated HeLa cell DNA polymerase α activity about 2-fold with denatured DNA as template. ATP was required for this stimulation with an optimum concentration between 1.5 and 2.5 mM. Stimulation of polymerase α activity by the ATPase was also observed with synthetic DNA at low primer to template ratios, such as poly(dA)$_{1000}$·(dT)$_4$ (100:1 ratio) (data not shown). Stimulation of polymerase α was not dependent on the presence of the primer recognition proteins C_1 and C_2 (Vishwanatha et al., 1986).

Nascent RNA–DNA chains synthesized by HeLa DNA polymerase α–primase in the presence or absence of DNA-dependent ATPase were analyzed on 8% polyacrylamide–8 M urea gels, and the results are presented in Figure 6. With poly(dT) as template in the presence of [α-^{32}P]ATP, DNA polymerase α–primase synthesized RNA–DNA chains 20–30 nucleotides long (Figure 6, lanes a–h). However, the lag in nascent DNA synthesis (lane a) is eliminated in the presence of the ATPase (lane e). When [α-^{32}P]dATP was used as the label and poly(dT) as the template (lanes i–p), a reduction in chain length, as well as an absence of the lag, is also seen in addition to elimination of the lag when ATPase is added. However, no differences were observed when M13 DNA was used as template (lanes q–x).

Discussion

In this report, we describe the structure and catalytic properties of the simple species of DNA-dependent ATPase that was purified to homogeneity from HeLa cell extracts. The ATPase is part of a large multienzyme complex, sedimenting at 16 S, that has been shown to be active in replicating SV40 DNA replication in vitro (Baril et al., 1988). The enzyme is composed of two subunits of 83 000 and 68 000 daltons. This is in excellent agreement with the native molecular size of 150 000 daltons ($s_{20,w}$ = 7) estimated from velocity gradient sedimentation. Photoaffinity labeling experiments with azidoadenosine triphosphate showed that both subunits bind ATP equally well. They also bind GTP to some degree although the binding was greater to the 83 000-dalton polypeptide than to the 68 000-dalton polypeptide. Thus, it is not clear at this time if both subunits are essential for the ATPase activity or if the catalytic site resides on one or both of the subunits. A single species of DNA-dependent ATPase from KB cell nuclei was isolated by Boxer and Korn (1980) with a polypeptide of 75 000 daltons whose catalytic properties were similar to those reported here. DeJong et al. (1981), however, demonstrated the presence of at least two distinct DNA-dependent ATPases in human KB cells, one of which (ATPase I) has similar catalytic properties to the DNA-dependent ATPase that we have isolated from HeLa cells. The KB cell ATPase I had a native molecular size of 140 000 daltons and is composed of two subunits of 71 000 and 66 000 daltons. A second DNA-dependent ATPase (ATPase II) isolated by DeJong et al. (1981) was composed of a single polypeptide of 78 000 daltons whose physical properties correspond more to the KB cell DNA-dependent ATPase isolated by Boxer and Korn (1980). The 140 000-dalton (DeJong et al., 1981) and the 75 000-dalton DNA-dependent ATPases from KB cells were unable to displace DNA strands in an assay for helicase activity, but the 75 000-dalton ATPase was reported to stimulate DNA polymerase α and β activities on nicked duplex DNA molecules.

DNA-dependent ATPase associated with helicase activity has been reported for calf thymus (Hubscher & Stalder, 1985) and HeLa (Biamonti et al., 1983) cells. The HeLa cell DNA-dependent ATPase in the early stages of its purification stimulated DNA polymerase α activity on poly[d(A-T)]·poly[d(A-T)] template and showed a duplex DNA unwinding activity with a 3′ to 5′ polarity on the unwound strand. However, the polymerase α stimulatory and DNA unwinding activities were lost upon purification of the enzyme. The purified ATPase was identified as a single polypeptide of 68 000 daltons, and the loss of the associated stimulatory and helicase-like activities was attributed to separation of a 28 000-dalton cofactor. We have been unable to demonstrate ATP-dependent helicase activity with the purified DNA-dependent ATPase from HeLa cells using a variety of substrates

known to function in the assay of prokaryotic helicase. A 108 000-dalton polypeptide cofractionates with the DNA-dependent ATPase but can be separated by glycerol gradient centrifugation. The 108 000-dalton polypeptides bound ATP in the photo-cross-linking experiments. The 108 000-dalton polypeptide is not essential for ATPase activity and for stimulation of DNA polymerase α–primase. We have not investigated the role of this protein in DNA replication any further.

DNA-dependent ATPases have been shown to function in a variety of reactions in DNA replication in prokaryotes other than DNA unwinding, such as formation of "initiation complexes", intrinsic primase activity, etc. (Kornberg, 1981, 1982). The function of DNA-dependent ATPase in eukaryotes is less clear. A DNA-dependent ATPase that prefers primer-template junction as an effector has been isolated from calf thymus (Hockensmith et al., 1986). It was proposed that the ATPase may function in the elongation step of DNA replication by promoting the recognition of primer-template junctions. Tsurimoto and Stillman (1989, 1990) have identified a DNA-dependent ATPase activity associated with RF-C complex that is necessary for SV40 DNA replication in vitro. RF-C complex is thought to be involved in template-primer recognition (Tsurimoto & Stillman, 1990). However, RF-C is composed of multiple subunits, and assignment of a subunit that has ATPase activity has not been made. The DNA-dependent ATPase activity that we have purified from HeLa cells stimulates the DNA polymerase α with single-stranded DNA templates. It copurifies with a multiprotein DNA polymerase α_2 complex that has primer recognition proteins associated with it. The ATPase allows the DNA polymerase α–primase complex to overcome a lag in the initiation of synthesis on single-stranded DNA templates. It is possible that ATPase promotes an interaction of DNA polymerase α–primase with the DNA template-primer in a tertiary complex in an ATP-dependent manner. This could represent one of the stages in the formation of an initiation complex in vivo involving interactions with other proteins in DNA replication. Further studies will be required to elucidate the physiological role of the DNA-dependent ATPase in DNA replication.

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