Purification of a Calf Thymus DNA-Dependent Adenosinetriphosphatase That Prefers a Primer-Template Junction Effector†

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ABSTRACT: A purification procedure has been developed that resolves four chromatographically distinct DNA-dependent ATPase activities from calf thymus tissue. One of these activities has been purified to a nearly homogeneous protein, as judged by polyacrylamide gel electrophoresis. This protein has a specific activity of 18 μmol of ATP hydrolyzed per minute per milligram of protein and is active only in the presence of a DNA effector. The DNA-dependent ATPase activity is greatest in the presence of DNA containing a 3′-hydroxyl primer-template junction with a segment of adjacent single strand, i.e., a DNA polymerase substrate. Primer-template effectors that have had the 3′-hydroxyl group eliminated by the addition of a dideoxyribonucleotide are less active as cofactors for ATP hydrolysis than effectors which retain the 3′-hydroxyl group. Other DNAs can serve as cofactors, but with a reduced rate of ATP hydrolysis. DNA cofactors which are single stranded are much more effective at promoting ATPase activity than completely double-stranded cofactors, although the effectiveness of single-stranded DNA decreases as the length of the oligonucleotide decreases. An RNA/DNA hybrid does not promote ATPase activity. These data suggest that ATPase A may be involved in the recognition of primer-template junctions and the elongation phase of DNA synthesis.

The role of DNA-dependent ATPases in DNA replication and recombination has been established in procaryotes by a number of genetic and enzymatic approaches (Kornberg, 1980, 1982). DNA-dependent ATPases have been identified in several mammalian systems. A partial list includes DNA-dependent ATPase from (a) mouse (Hachmann & Lezius, 1976; Hyodo & Suzuki, 1981; Tawaragi et al., 1984), (b) rat (Yaginuma & Koike, 1981; Thomas & Meyer, 1982), (c) cow (Otto, 1977; Assairi & Johnston, 1979; Hubscher & Stalder, 1985), and (d) human (Cobianchi et al., 1979; Boxer & Korn, 1980; De Jong et al., 1981) cells. Only two of these enzymes have been purified to near the homogeneity (Boxer & Korn, 1980; Thomas & Meyer, 1982).

The absence of defined genetics in these mammalian systems has hindered progress in determining the role of these DNA-dependent ATPases. Nevertheless, the available data suggest that the DNA-dependent ATPase from human KB cells plays a role in DNA replication by stimulating synthesis by DNA polymerases α and β (Boxer & Korn, 1980). The human EUE cell enzyme appears to have an involvement in DNA replication on the basis of its ability to unwind double-stranded DNA and stimulate α-DNA polymerase on a poly(dA-dT) template (Cobianchi et al., 1979). Novikoff hepatoma ATPase III stimulates the homologous DNA polymerase β, thereby suggesting a role for this ATPase in DNA repair (Thomas & Meyer, 1982).

Studies in procaryotes have demonstrated a number of different DNA-dependent ATPases present in a single organism (Kornberg, 1980, 1982). On the basis of this observation, eucaryotes might be expected to have more than one such activity. Thomas and Meyer (1982) have identified five chromatographically distinct DNA-dependent ATPase activities from Novikoff hepatoma tissue. Multiple species of DNA-dependent ATPase activities have also been identified in mouse (Tawaragi et al., 1984) and calf thymus (Assairi & Johnston, 1979) tissues, but no evidence regarding a role in DNA metabolism has been established.

We have expanded our studies of the calf thymus DNA replication system (Hockensmith & Bambara, 1981; Wierowski et al., 1983; Wahl et al., 1984) by identifying four chromatographically distinct DNA-dependent ATPase activities from this system. We have purified one enzyme to near homogeneity and have demonstrated the ability of the preparation to recognize primer-template junctions, a structure essential for DNA replication.

Materials and Methods

Materials. All chemicals were of reagent grade. Low molecular weight standards for sodium dodecyl sulfate (SDS)[1]-polyacrylamide gels were purchased from Bio-Rad. Blue dextran was purchased from Pharmacia. BSA and trimonocyclohexylammonium phosphoenolpyruvate were purchased from Sigma Chemical Co.

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[1] Abbreviations: PEG, poly(ethylene glycol); DEAE, diethylaminoethyl; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; SDS, sodium dodecyl sulfate; UP1, calf thymus unwinding protein 1; AMPPNP, 5′-adenylyl imidodiphosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Enzymes. *Escherichia coli* DNA polymerase I was purchased from New England Biolabs. Pancreatic deoxyribonuclease I was purchased from Worthington Biochemicals. Micrococcal nuclease was purchased from Boehringer Mannheim Biochemicals. S1 nuclease and *E. coli* RNA polymerase were purchased from Miles Laboratories. The restriction enzymes *Sau*3A, *Hae*II, and *Hae*III (and their respective reaction buffers) were purchased from International Biotechnologies, Inc. Pyruvate kinase, trypsinogen, and pancreatic ribonuclease A were purchased from Sigma Chemical Co. *E. coli* exonuclease III was a gift of Dr. L. Loeb (University of Washington). *E. coli* DNA ligase was a gift of Dr. I. R. Lehman (Stanford University).

Calf thymus DNA polymerase α was isolated essentially as described by Holmes et al. (1974, 1975). Calf thymus DNA polymerase β was isolated essentially as described by Chang (1974). Calf thymus unwinding protein (UP1) was purified according to Herrick and Alberts (1976).

Nucleotides and Polynucleotides. Deoxynucleoside triphosphates and monophosphates were purchased from Sigma Chemical Co. [^{3}H]dTTP (40–60 Ci/mmol), [^{3}H]dGTP (10 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), and [α-^{32}P]dATP (880 Ci/mmol) were purchased from New England Nuclear Corp. [^{3}H]ATP (21 Ci/mmol) was purchased from Amersham Corp. Native, ColE1 DNA and native, pBR322 DNA were isolated according to Blair et al. (1972). Native, single-stranded DNA from bacteriophages fd and M13mp9 was prepared by the method of Sadowski and Hurwitz (1969a,b). Preparation of replicative form I fd (RFI) DNA was done by the method of Model and Zinder (1974) with the following alterations: cell lysis was carried out by the method of Reuben et al. (1974), and preparation of the DNA-containing supernatant solution was carried out by the method of Blair et al. (1972). Calf thymus DNA and herring sperm DNA were purchased from Sigma Chemical Co. *E. coli* tRNA was purchased from Boehringer Mannheim Biochemicals. Polynucleotides, oligonucleotides, and dideoxyribonucleotides were purchased from Pharmacia. The oligo(dT)-tailed poly(dA) was a gift of Dr. James J. Crute (Stanford University) and was prepared by tailing $(dA)_{4000}$ at the 3′-hydroxyl end with oligo(dT) by the method of Kato et al. (1967).

ColE1 DNA was labeled according to the procedure of Sakakibara and Tomizawa (1974) and isolated according to Blair et al. (1972). Native, single-stranded fd DNA was labeled and isolated by the method of Yamamoto and Alberts (1970).

Nick translation of fd RFI DNA with [^{32}P]dATP (880 Ci/mmol) was done by the method of Davis et al. (1980). Reactions were terminated by incubation at 70 °C for 10 min. Unreacted [^{32}P]dATP was removed by passing the 2.0-mL reaction volume through a column (1.5 × 47 cm) of Sephadex G-100 equilibrated in 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 1 mM EDTA. ^{32}P-Labeled, primer DNA fragments were prepared by digesting the nick-translated, fd RF DNA (200 μM) with pancreatic DNase I (500 ng/mL) for 15 min at 37 °C. This method yields fragments approximately 100 nucleotides long after boiling to separate DNA strands, as judged by thin-layer chromatography on poly(ethylenimine)–cellulose plates (Brinkmann Co.) developed in 1.2 M LiCl and 7 M urea. Primed fd DNA was prepared by annealing these ^{32}P-labeled fragments to unlabeled fd phage DNA and purifying the product, employing the method of Sherman and Gefter (1976).

Heat denaturation of DNA was accomplished by boiling native DNA at 1 mg/mL for 10 min followed by rapid cooling in an ice bath. Nicked, calf thymus DNA was prepared as described by Uyemura and Lehman (1976). Gapped, calf thymus DNA was prepared according to Hockensmith and Bambara (1981). Micrococcal nuclease treated, calf thymus DNA was prepared by the method of Bambara et al. (1978).

Polynucleotide concentrations are expressed as molarity of nucleotides.

Chromatography Resins. Native DNA–cellulose and denatured DNA–cellulose were prepared by using herring sperm DNA and calf thymus DNA, respectively, and Whatman CF-11 cellulose was prepare according to the methods of Alberts and Herrick (1971). ATP–agarose type 4 was purchased from P-L Biochemicals (ATP coupled through the ribose ring to a six-carbon chain that is coupled to the agarose). Phosphocellulose (P-11) and DEAE-cellulose (DE-52) were purchased from Whatman. Sephacryl S-200 and Sephadex G-100 were purchased from Pharmacia.

Polyacrylamide Gel Electrophoresis. Gel electrophoresis in the presence of SDS was performed essentially as described by Dreyfuss et al. (1984) using a 4% stacking gel and a 12.5% separating gel. Gels were stained with silver according to the method of Morrissey (1981).

Nondenaturing, pH 8.8, gel electrophoresis was performed by using the system of Laemmli (1970) but in the absence of sodium dodecyl sulfate. Gels were stained with Coomassie blue. To recover activity, gels were prerun overnight, loaded and run at 4 °C, and immediately sliced after electrophoresis. Slices were extracted in the standard assay mixture (described below) at 37 °C, and free phosphate was determined after 4 h.

Gel electrophoresis was also performed in a pH 5.5 system. Slab gels containing 7.5% polyacrylamide, 0.05% *N,N′*-methylenebis(acrylamide), 3.1 μL/mL TEMED, and 0.31 mg/mL ammonium persulfate were prepared in 60 mM potassium hydroxide and 62.5 mM acetic acid (pH 5.5). A stacking gel of 2.5% polyacrylamide, 0.625% *N,N′*-methylenebis(acrylamide), 3.1 μL/mL TEMED, and 0.31 mg/mL ammonium persulfate in 60 mM potassium hydroxide and 62.5 mM acetic acid (pH 5.5) was used. Running buffer was 0.31% β-alanine and 14 mM acetic acid. Protein samples were prepared in running buffer, and electrophoresis was toward the cathode. The tracking dye was 0.05% methyl green. Staining and assaying for activity are described above.

Standard ATPase Assay. The colorimetric assay measures the release of inorganic phosphate from ATP and is a modification of the method of King (1932). In addition, the reaction contains a modification of the ATP-regenerating system described by Schuster et al. (1975). Reactions (100 μL) contained 50 mM Tris–H_2SO_4, pH 7.5, 1 mM $MgSO_4$, 5 mM β-mercaptoethanol, 500 μg/mL BSA, 2 mM trimonocyclohexylammonium phosphoenolpyruvate, 30 μg/mL pyruvate kinase, 2 mM ATP, 200 μM denatured, calf thymus DNA, and ATPase A as indicated in the figure legends. Incubations were at 37 °C. The reaction was terminated by the addition of 350 μL of water and 225 μL of 10% SDS. Phosphomolybdic acid is formed by the addition of 180 μL of 2.5% ammonium molybdate in 35% perchloric acid. The phosphomolybdic acid is then reduced by the addition of 45 μL of a reagent containing 0.2%, 1-amino-2-naphthol-4-sulfonic acid, 12% sodium metabisulfate, and 2.4% sodium sulfite. The development of color is time dependent, and assays were always read at 720 nm at exactly 10 min. One unit of ATPase activity is defined as the amount of enzyme that can hydrolyze 1 μmol of ATP/min at 37 °C.

Table I: Purification of Calf Thymus Gland DNA-Dependent ATPase A

	fraction	protein (mg)	act. (units)	sp act. (units/mg)	yield (%)	purification (x-fold)
(I)	crude extract	209000[a]	(81.2)[b]	(0.0004)	(100)	(1)
(II)	PEG_{8000} pptn	7460	(81.2)	(0.011)	(100)	(27.5)
(III)	native DNA–cellulose	258	81.2	0.31	100	775
(IV)	denatured DNA–cellulose	15.2	11.7	0.77	14	1930
(V)	ATP–agarose	6.30	10.0	1.59	12[c]	3980
(VI)	phosphocellulose	1.61	4.02	2.50	5.0	6250
(VII)	Sephacryl-S-200	0.059	1.07	18.00	1.2	45000

[a] The purification shown was begun with 3675 g of calf thymus glands. [b] Fraction III is the first fraction with which it is possible to identify DNA-dependent ATPase activity. The values in parentheses have been estimated by assuming 100% recovery of the activity during the initial steps of this purification. [c] Substitution of polypropylene tubes for glass tubes is critical for maintaining good yields after fraction IV.

A second ATPase assay was used to determine the products resulting from the ATP hydrolysis. The enzyme fraction was incubated with [^{3}H]ATP, and the resulting products were spotted on a poly(ethylenimine)–cellulose plate. After the plate was developed, spots corresponding to AMP, ADP, and ATP were removed and counted. Details of this assay were described previously (Hockensmith & Bambara, 1981).

Polymerization Assays. Assays to document the absence of polymerase activities were performed with 0.027 unit of DNA-dependent ATPase A. The measurement of DNA polymerase α synthetic activity was performed as described previously (Hockensmith & Bambara, 1981). The assay of polymerase β synthetic activity was performed by the method of Stalker et al. (1976). Terminal deoxynucleotidyl transferase activity was assayed according to Bollum et al. (1974) using $(dA)_{40-60}$ and [^{3}H]dGTP. All reactions were assayed for acid-insoluble radioactivity as described earlier (Wu et al., 1974).

The RNA polymerizing reaction (45 μL) contained 60 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 5 mM β-mercaptoethanol, 500 μg/mL BSA, 200 μM denatured, calf thymus DNA, and UTP, GTP, CTP, and [^{3}H]ATP (1.0 Ci/mmol) each at 200 μM. After incubation for 30 min at 37 °C, the acid-insoluble radioactivity was determined as described by Wu et al. (1974) with the following exception: Dissolution of precipitates was accomplished by using 0.5 mL of 1 M Tris-HCl, pH 8.5, to minimize hydrolysis of the RNA.

Nuclease Assays. DNA exonuclease and double-stranded DNA endonuclease assays (with the without ATP) were performed as described by Hockensmith and Bambara (1981) using 0.027 unit of DNA-dependent ATPase A. Single-stranded endonuclease assays were performed under the same conditions as double-stranded endonuclease assays with two exceptions: ^{3}H-labeled, native, single-stranded, fd DNA was used, and centrifugation was for 6.5 h.

Topoisomerase Assay. Reactions (90 μL) were performed by using 0.027 unit of DNA-dependent ATPase A under standard ATPase conditions, but using 100 μM supercoiled ColE1 DNA. Incubation was at 37 °C with 25-μL aliquots removed at 10, 20, and 40 min. The DNA aliquot was brought to 1% SDS, 5% sucrose, and 50 μg/mL bromophenol blue. The sample was loaded onto a 1% agarose gel in the Tris–acetate buffer of Davis et al. (1980). Electrophoresis and staining were performed according to Davis et al. (1980).

DNA Ligase Assay. The substrate for ligation, [^{3}H]d(A-T)$_n$, and the ligation reaction were prepared essentially as described by Modrich and Lehman (1970). When ATP was substituted for nicotinamide adenine dinucleotide, the ATP concentration was 1 mM. Reactions were performed with 0.027 unit of DNA-dependent ATPase A and incubated at 37 °C for 30 min.

Duplex DNA Unwinding Assay. The assay for unwinding of duplex DNA measured the generation of acid-soluble nucleotides by digestion of unwound (single-stranded) DNA by S1 nuclease. The unwinding reaction (90 μL) contained 60 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 5 mM β-mercaptoethanol, 500 μg/mL BSA, 1 mM ATP, 2.5 μg of UP1, 8 mM K_2SO_4, 1% (w/v) glycerol, 35 μM ^{32}P-primed fd DNA, and 0.027 unit of ATPase A. Incubation was for 30 min at 37 °C. The incubation was continued for 5 min following the addition of an equal volume of 4 M NaCl and addition of 20 μg of denatured calf thymus DNA. Digestion by S1 nuclease (500 units) was performed as described by Yarranton and Gefter (1979). Acid-insoluble radioactivity was determined as described by Wu et al. (1974).

The method of Matson et al. (1983) was also employed for measurement of helicase activity.

Renaturation Assay. The DNA renaturation assay measured the increase in resistance to S1 nuclease as duplex DNA is formed. The substrate for renaturation was boiled, nick-translated, fd RF DNA. The reaction conditions and S1 nuclease digestion were identical with those for the unwinding assay, with two exceptions: the DNA concentration was 12 μM, and UP1 was absent.

Autophosphorylation and Polynucleotide Kinase Assay. Reactions (10 μL) were performed with 0.001 unit of DNA-dependent ATPase A. The reactions contained 25 mM Tris–acetate, pH 7.5, 60 mM potassium acetate, 6 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10 pmol of [γ-^{32}P]ATP (3000 Ci/mmol), and either 200 μM $(dT)_{16}$ or 200 μM $(dT)_{16}$·poly(dA) (1:5) or no DNA. Incubations were at 37 °C for 1 h. Samples were then prepared and separated by electrophoresis on SDS–polyacrylamide gels as described above or were denatured in formamide, and electrophoresis was conducted through a 20% polyacrylamide gel with a 30:1 ratio of acrylamide to *N,N'*-methylenebis(acrylamide) in 50 mM Tris–borate, pH 8.3, 8 M urea, and 1 mM EDTA. Following electrophoresis, the polyacrylamide gels were covered with plastic wrap and overlaid with Kodak X-Omat AR film and a Dupont Cronex Lightening Plus intensifying screen.

Strand Exchange Assay. Assays were performed according to West et al. (1981, 1982).

Other Methods. Buffers were routinely prepared as 1 M stock solutions and pH adjustments made at 20 °C using a Beckman Expandomatic SS-2 pH meter. Ionic strengths of buffers were determined by using a Model 31 conductivity bridge from Yellow Springs Instrument Co. Protein was determined by the method of Bradford (1976) using BSA as the standard.

Purification of the DNA-Dependent ATPase. We have resolved four chromatographically distinct species of DNA-dependent ATPase activity and purified the major activity to near homogeneity by the procedure summarized in Table I. All steps were performed at 0–4 °C, and all buffers contained 5 mM β-mercaptoethanol.

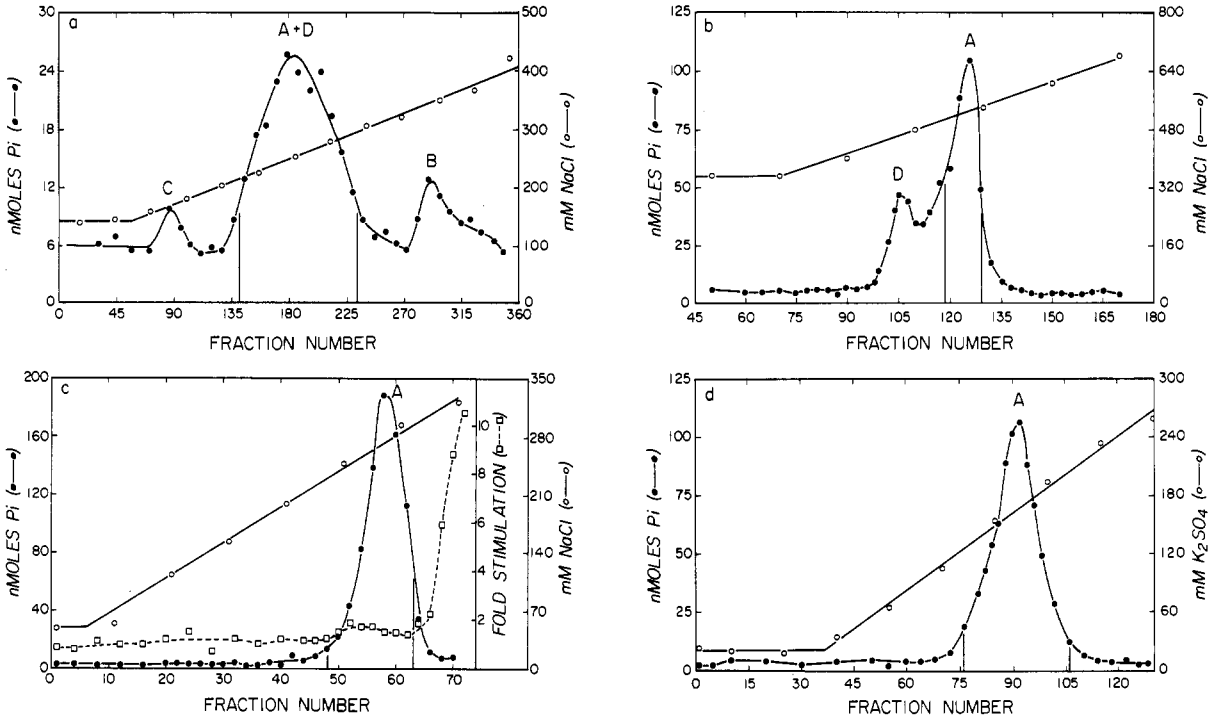

FIGURE 1: Fractionation of calf thymus DNA-dependent ATPases by column chromatography. All column fractions were assayed by incubating 10 μL of the fraction in the standard assay mixture described under Materials and Methods for 1 h at 37 °C. Vertical lines denote combined fractions. (a) Fraction II of the calf thymus ATPase preparation was fractionated over native, herring sperm DNA–cellulose. The ATPase activity eluting from this column is very dilute, and, as a result, the background level of P_i appears high. The base line shown is background and should not be interpreted as "trailing" of the ATPase activity peaks. (b) Fraction III ATPase fractionated on denatured, calf thymus DNA–cellulose. (c) Fraction IV ATPase fractionated on ATP–agarose. The reaction for measuring stimulation of DNA polymerase α (45 μL) contained 60 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 5 mM β-mercaptoethanol, 500 μg/mL BSA, 50 μM poly(dA-dT), 1 mM ATP, 40 μM dATP and dTTP (4.0 Ci/mmol), 0.45 unit of α-C-DNA polymerase, and 5 μL of the separation fraction. Acid-insoluble radioactivity (Wu et al., 1974) was determined after incubation at 37 °C for 30 min. (d) Fraction V ATPase fractionated on phosphocellulose.

(*A*) *Preparation of Thymus Gland Extract.* Thymus glands were obtained from calves within 15 min of slaughter, held on wet ice (typical glands weighed approximately 200 g each), and used for an enzyme preparation within 2 h. Glands were trimmed free of connective tissue and fat and cut into 20–40-g pieces. The pieces were blended in 175-g portions with 225 mL of frozen buffer A [20 mM Tris-HCl, pH 8.8, 5% (w/v) glycerol, 1 mM EDTA, and 50 mM NaCl] and 300 mL of buffer A (4 °C) in a Waring blender [run at half-speed with five bursts (10 s) at full speed] for a total of 2 min.[2] The homogenate was sedimented for 30 min at 9000 rpm (13500*g*) in a Sorvall GS-3 rotor. The resulting supernatant solution was filtered through cheesecloth (fraction I). A poly(ethylene glycol) precipitation was performed on fraction I as described earlier (Herrick & Alberts, 1976) to remove nucleic acids from the extract. The resulting supernatant solution from a typical preparation (3000–3500 g of tissue) was dialyzed for 16 h against three changes of 33 L of buffer A without glycerol and sedimented for 30 min at 9000 rpm (13500*g*) in a Sorvall GS-3 rotor (fraction II).

(*B*) *Native DNA–Cellulose Chromatography.* Fraction II was loaded at 900 mL/h onto a native, herring sperm DNA–cellulose column (6.4 × 32 cm) previously equilibrated with buffer A. The column was then washed with 2 L of buffer A containing 150 mM NaCl followed by a 6-L linear gradient of 150–500 mM NaCl in buffer A (18 mL per fraction). This procedure yields three peaks of DNA-dependent ATPase activity: (A + D), B, and C (Figure 1a). The majority of activity, (A + D), elutes in a symmetrical peak centered around 255 mM NaCl. The fractions with this activity were combined as denoted by the vertical lines in Figure 1a (fraction III). All DNA-independent ATPase activity flows through this column. There is no detectable DNA-independent ATPase activity from any subsequent column.

(*C*) *Denatured DNA–Cellulose Chromatography.* Fraction III was loaded directly onto a buffer A equilibrated, denatured, calf thymus DNA–cellulose column (2.5 × 20 cm) at 100 mL/h. After adsorption of the protein, the column was washed with 400 mM NaCl in buffer A, and the DNA-dependent ATPase activity was subsequently eluted with a 1-L linear gradient of 400–800 mM NaCl in buffer A (4.5 mL per fraction). Two peaks of enzymatic activity were evident, A and D (Figure 1b), eluting at 530 and 465 mM NaCl, respectively. Preliminary work with these two peaks of activity suggests that the enzymatic activity of the A peak is more stable than the D peak activity. Further purification of the D peak by ATP–agarose and phosphocellulose chromatographies (followed by concentration) resulted in a fraction that lost all enzymatic activity after 24 h at 4 °C. In addition, the A peak represents the great majority of the DNA-dependent ATPase activity. Consequently, the A peak fractions were combined and dialyzed for 10 h against two 3-L changes of AGB buffer [20 mM Tris-HCl, pH 7.5, 20% (w/v) glycerol, 1 mM EDTA, and 50 mM NaCl] (fraction IV).

(*D*) *ATP–Agarose Chromatography.* Fraction IV was loaded at 15 mL/h onto a column of DEAE-cellulose (1.6 × 5 cm) coupled to a column of ATP–agarose (1.9 × 5.3 cm), each equilibrated in AGB buffer. The DEAE-cellulose removes any residual DNA fragments from the enzyme fraction but does not bind the enzyme, thereby allowing the enzyme to flow onto the ATP–agarose and bind. The DEAE-cellulose column was washed with 15 mL of AGB buffer, uncoupled

[2] Unpublished observations from our studies of calf thymus DNA polymerase α indicated that this procedure results in less proteolysis than methods that involve freezing and thawing of the tissue.

from the ATP–agarose column, and discarded. The ATP–agarose column was further washed with 15 mL of AGB buffer and then eluted with a 150-mL linear gradient of 50–400 mM NaCl in AGB buffer (2.8 mL per fraction). A single symmetrical peak of DNA-dependent ATPase activity elutes at 270 mM NaCl (Figure 1c). Rapid losses (5–10% per hour) of enzymatic activity occur at this and subsequent stages of purification when glass or polystyrene tubes are used for collecting fractions. These losses can be minimized by using polypropylene tubes for collecting fractions and by using the rapid colorimetric ATPase assay described under Materials and Methods. The enzymatic activity is stable in dialysis tubing (95–100% recovery after 12 h), and consequently, all fractions were combined and dialyzed as quickly as possible. Fractions had to be combined carefully to exclude a factor that stimulates DNA polymerase α and begins to elute from this column at 300 mM NaCl[3] (Figure 1c). The factor had to be removed quantitatively because it cannot be resolved from the DNA-dependent ATPase on the next columns. The combined fractions were dialyzed for 10 h against two (1 L) changes of S buffer (20 mM Tris-HCl, pH 7.5, 20% glycerol, 1 mM EDTA, and 20 mM K_2SO_4) (fraction V).

(*E*) *Phosphocellulose Chromatography*. Fraction V was loaded at 20 mL/h onto a column of phosphocellulose (1.9 × 5.3 cm) equilibrated in S buffer. Following a wash with S buffer, the DNA-dependent ATPase was eluted with a 200-mL linear gradient of 20–300 mM K_2SO_4 in S buffer (1.8 mL per fraction) (Figure 1d). The fractions with enzymatic activity, which routinely eluted in a symmetrical peak at 165 mM K_2SO_4, were combined as rapidly as possible and concentrated by using an Amicon type PM-10 membrane in a stirred ultrafiltration cell (fraction VI).

(*F*) *Sephacryl-S-200 Chromatography*. Fraction VI was loaded at 6 mL/h onto a column of Sephacryl-S-200 (1 × 114 cm) equilibrated in AGB buffer plus 200 mM NaCl, and 0.7-mL fractions were collected. The column was calibrated with blue dextran for the void volume and BSA (M_r 67 000), trypsinogen (M_r 24 000), and RNase A (M_r 14 300) as markers. The DNA-dependent ATPase activity elutes as a symmetrical peak, and its position of elution yields a Stokes radius consistent with a molecular weight of 68 000 for a symmetrical protein. The fractions containing enzymatic activity were combined as rapidly as possible and concentrated by using an Amicon type PM-10 membrane in a stirred ultrafiltration cell (fraction VII).

Results

Enzyme Stability. The DNA-dependent ATPase activity of fractions VI and VII is remarkably stable when stored in polypropylene tubes at concentrations greater than 30 μg/mL. When held at 37 °C, the enzyme (37 μg/mL in S buffer containing 165 mM K_2SO_4) retains greater than 80% of its original activity after 11 days and greater than 30% after 21 days. At 20 °C, the enzyme retains greater than 80% of its original activity after 11 days and greater than 55% after 21 days. After storage at 4 °C for 3 months or at −80 °C for 3 years, no activity loss was observed. The enzyme is stable to freeze–thawing and is routinely stored at −80 °C.

Evidence for Purification to Near Homogeneity. Electrophoresis of fraction VII DNA-dependent ATPase A in SDS–polyacrylamide gels reveals a single major protein species when stained with silver nitrate (Figure 2). The protein has an estimated molecular weight of 69 000 from the electrophoretic analysis, a number in good agreement with the estimate from the Sephacryl-S-200 gel filtration. Attempts to renature DNA-dependent ATPase A from SDS–polyacrylamide gels using the method of Rosenthal and Lacks (1977) were unsuccessful.

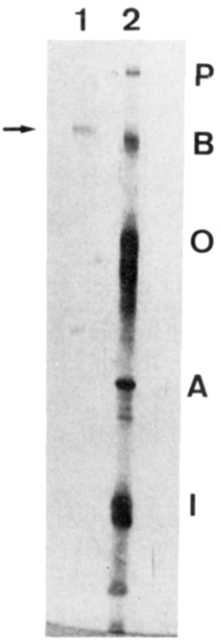

FIGURE 2: SDS–polyacrylamide gel. (Lane 1) DNA-dependent ATPase A (0.7 μg) was denatured and subjected to electrophoresis in a 12.5% polyacrylamide gel as described under Materials and Methods. (Lane 2) Standard proteins are indicated as follows: phosphorylase B (P), BSA (B), ovalbumin (O), carbonic anhydrase (A), and soybean trypsin inhibitor (I).

ATPase A fails to enter native, 2.5% polyacrylamide gels, pH 8.8, when loaded to run either toward the anode or toward the cathode. The ATPase will enter native, 7.5% polyacrylamide gels, pH 5.5, when loaded to run toward the cathode and migrates as a single major species. Analyses of unstained sister tracks from the gel at pH 5.5 have failed to demonstrate recovery of ATPase activity.

Absence of Contaminating Enzymatic Activities. Fractions VI and VII DNA-dependent ATPase A were found to be free of any other enzymatic activities. The results of our studies show that the following activities can be excluded to levels of 10^{-6}–10^{-8} of the ATPase activity: DNA polymerase, terminal deoxynucleotidyl transferase, endonuclease, exonuclease, and RNA polymerizing activities. In addition, no DNA ligase or DNA topoisomerase activities were detectable.

Properties of the Enzyme. The properties of fractions VI and VII DNA-dependent ATPase A are identical except for the appearance of a 30 000-dalton contaminating polypeptide on polyacrylamide gels of fraction VI.

(*A*) *Molecular Weight*. As noted above, the molecular weight of the DNA-dependent ATPase A appears to be between 68 000 and 69 000.

(*B*) *Reaction Requirements*. DNA-dependent ATPase A requires a divalent cation and a polydeoxyribonucleotide for hydrolysis of ATP or dATP. ATPase A hydrolyzes ATP or dATP to the corresponding ribonucleoside diphosphate and inorganic phosphate, and there is no detectable generation of ribonucleoside monophosphate. There also is no detectable hydrolysis of GTP, CTP, UTP, dGTP, dCTP, or dTTP.

The DNA-dependent ATPase has a broad pH optimum of 7.0–8.5. The buffer routinely used to assay for ATPase activity is Tris–H_2SO_4, pH 7.5. A reaction system buffered at this

[3] Characterization of proteins that stimulate DNA polymerase activity will be considered in a later paper.

Table II: Polynucleotide Effector Specificity of ATPase A

nucleic acid[a]	nmol of P_i formed[b] (% of control)
none	<1.0
DNAs	
denatured calf thymus DNA	(100)
phage fd DNA	79
phage M13mp9 DNA	70
ColE1 plasmid DNA	12
native calf thymus DNA	67
nicked calf thymus DNA	87[c]
gapped calf thymus DNA	107[d]
micrococcal nuclease treated calf thymus DNA	40
synthetic polydeoxyribonucleotides	
poly(dA)	5.2
poly(dT)	11
poly(dA)–oligo(dT) tailed	38
poly(dA)·poly(dT)	73
poly(dA-dT)·poly(dA-dT)	150
poly(dG-dC)·poly(dG-dC)	110[e]
poly(dI-dC)·poly(dI-dC)	150[e]
$p(dT)_{16}$·poly(dA), 1:5	66
oligodeoxyribonucleotides	
$p(dA)_{40-60}$	9.6
$p(dA)_{19-24}$	7.2
$p(dA)_{12-18}$	4.2
$p(dA)_3$	5.1
$p(dT)_{12-18}$	5.0
$p(dT)_{10}$	3.5
$p(dG)_{12-18}$	2.8
deoxyribonucleotides	
dAMP	2.7
dAMP, dCMP, dGMP, dTTP	1.5
ribonucleotides (oligomers, polymers)	
$(rU)_{10}$	<1
poly(rA)	2.4
poly(rU)	2.5
$(rU)_{10}$·poly(dA), 1:5	2.4
$(rU)_{17}$·poly(dA), 1:5	<1
E. coli tRNA	3.2

[a] Each nucleic acid was present at a concentration of 200 μM (nucleotide) in the standard ATPase reaction mixture with 0.005 unit of fraction VI or VII ATPase A. [b] ATPase A hydrolyzed 131 nmol of ATP per hour using denatured, calf thymus DNA as the effector. Values less than 3% are considered to be indistinguishable from background (see legend to Figure 1). [c] Nicked calf thymus DNA had an average of 1 3′-hydroxyl terminus every 1350 nucleotides. [d] Gapped calf thymus DNA had an average of 1 3′-hydroxyl terminus every 674 nucleotides with an average gap of 90 nucleotides. [e] Effector concentration was 25 μM.

pH was chosen because our previous analyses of calf DNA polymerase α were performed at this pH (Hockensmith & Bambara, 1981). Furthermore, pH 7.5 allows the effective use of an ATP-regenerating system consisting of pyruvate kinase and phosphoenolpyruvate. The ATP-regenerating system facilitates initial reaction velocity measurements of ATP hydrolysis.

The ATPase requires a divalent cation for any detectable ATP hydrolysis activity. In the presence of a constant concentration of 2 mM ATP, magnesium ion is the preferred cation, at an optimum level of 1 mM. Manganese ion at 1 mM can substitute for magnesium ion, giving 86% of the activity observed with magnesium. Substitution of 1 mM Co^{2+} or calcium for magnesium results in 66% and 17% of the maximum activity observed with magnesium, respectively. The requirement of a divalent cation for ATP hydrolysis suggests that the substrate is not ATP but is the metal–ATP complex. The metal–ATP complex concentration is affected by the metal concentration, ATP concentration, and pH of the reaction but can be readily calculated by using eq 1 from Ahlers et al. (1975). Double reciprocal plots of $1/V$ vs. $1/[MgATP]$ gave a straight line from which a K_m value of 0.79 mM for MgATP was calculated. The rate of hydrolysis of MgdATP is 85% of that for MgATP (both at 0.83 mM) and proceeds with a similar K_m (0.63 mM).

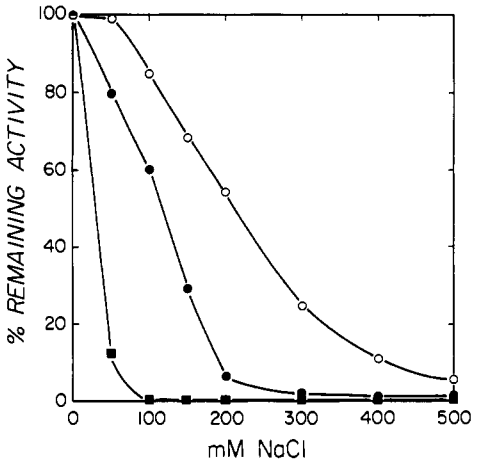

FIGURE 3: Salt inhibition of DNA-dependent ATPase A activity. Standard ATPase reactions (100 μL) were performed with increasing amounts of NaCl added. Each reaction contained 0.005 unit of fraction VI or VII ATPase A and was incubated for 1 h. DNA cofactors were poly(dA-dT) (●), denatured calf thymus DNA (○), and ColE1 plasmid DNA (■).

(C) Polynucleotide Cofactor Requirement. The presence of a polynucleotide is essential for ATP hydrolysis by ATPase A (Table II). Different polynucleotides satisfy this requirement to varying degrees, with their relative efficiencies indicated in Table II. Single-stranded, natural DNAs (such as heat-denatured, calf thymus DNA or phage DNA) are preferred over double-stranded DNAs as cofactors for ATP hydrolysis. Natural DNAs with partial single-stranded character (gapped calf thymus DNA) also allow high levels of ATP hydrolysis. The ATPase activity seen with the nicked, calf thymus DNA could be accounted for by the presence of a small population of single-stranded regions (presumably generated during isolation of native, calf thymus DNA). At the high DNA concentrations used, these single-stranded regions could serve as an adequate substrate for the enzyme.

Table II shows that single-stranded homopolymer DNAs are less efficient than natural DNAs as cofactors for ATP hydrolysis. Nevertheless, hydrolysis is detectable with poly(dA) or poly(dT). ATP hydrolysis decreases as shorter length polymers are used as cofactors. There was no distinct size requirement cutoff below which no hydrolysis of ATP occurs, but hydrolysis approached zero when cofactors smaller than trinucleotides were tested. No polyribonucleotide tested permitted significant ATP hydrolysis nor did the addition of RNA primers [oligo(rU)] to DNA templates [poly(dA)].

The data of Table II also show that homopolymers such as poly(dA)·poly(dT), poly(dA-dT), poly(dG-dC), or poly(dI-dC) are very good cofactors for ATPase activity. They are, in fact, much more effective than poly(dA) or poly(dT). This result suggests that, while single-stranded DNA may be preferred as a cofactor over completely double-stranded DNA, DNA cofactors with both single- and double-stranded regions may be the most effective cofactors.

Additional suggestive evidence for the importance of having both single- and double-stranded regions in the DNA effector comes from salt titrations of ATPase activity using various DNA effectors. Increasing salt concentration has different effects on ATP hydrolysis depending on the polynucleotide used as an effector for the hydrolysis reaction. Three DNA effectors were examined: ColE1 DNA, poly(dA-dT), and denatured, calf thymus DNA (Figure 3). The reaction with

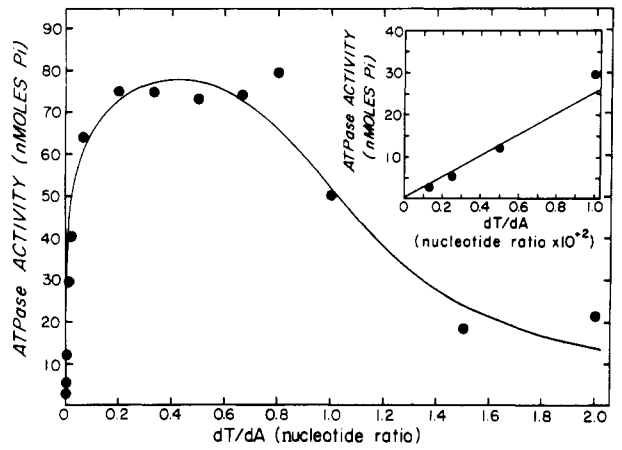

FIGURE 4: $(dT)_{16}$ titration of poly(dA). Standard ATPase reactions with an additional 100 mM NaCl were performed by holding the total DNA concentration constant at 200 μM but varying the ratio of thymidine to adenosine. Each reaction contained 0.003 unit of ATPase A and was incubated for 1 h.

ColE1 DNA is the most sensitive to the added salt. ATPase activity in this reaction is undetectable when 100 mM NaCl is added. The reaction containing poly(dA-dT) is less sensitive to added salt and retains 6%, of the ATPase activity seen under standard salt conditions after the addition of 200 mM NaCl. The ATPase activity with denatured, calf thymus DNA is the least sensitive to salt, decreasing to 54% of the activity of the standard reaction when 200 mM NaCl is added. We presume that the salt is causing structural changes, such as alterations in the availability of single-stranded regions, in the polynucleotide effectors, thereby decreasing their effectiveness as cofactors. Possible reasons for the differential salt sensitivity of these polynucleotide cofactors are discussed later.

The importance of having both single- and double-stranded character in the polynucleotide effector was tested by using two different substrates. The first substrate was a $p(dT)_{16}$-primed poly(dA) template, a model primer-template junction. The data of Table II show that a 1:5 molar ratio of thymidine residues to adenosine residues (T:A) yields ATPase activity that is more than 12-fold greater than the activity of the ATPase when either oligo(dT) or poly(dA) is used alone. At this T:A ratio, the DNA effector is expected to have considerable single-stranded structure. We varied the T:A ratio, increasing the number of primer-template structures as the amount of single-stranded structure diminishes (Figure 4). As Figure 4 shows, the ATP hydrolysis of ATPase A is greatly enhanced as the T:A ratio grows from 1:800 through 1:5. However, as the T:A ratio grows beyond 1:1, the ATPase activity drops dramatically, corresponding to the decrease in the amount of the single-stranded component of the primer-template. The addition of $(dC)_{10}$ to a $(dT)_{16}$·poly(dA) mix at a T:A ratio of 1:1.5 did not alter the ATPase activity (Table III). The titration shown in Figure 4 is sensitive to the salt concentration of the solution (Table III).

The second substrate used to examine the importance of having both single- and double-stranded DNA in the effector molecule used for ATP hydrolysis was oligo(dT)-tailed poly(dA) (Table II). This molecule is a snapback molecule that generates a duplex region with a 3′-hydroxyl group adjacent to a single-stranded region of template DNA. The tailed template is a much better effector (>5-fold) for ATP hydrolysis than the parent poly(dA) molecule.

Elimination of the 3′-hydroxyl terminus of this effector by the addition of a dideoxyribonucleotide reduced the ATP hydrolysis by ATPase A by more than 60% (Table III). The importance of a 3′-hydroxyl group was further demonstrated by the addition of dideoxyribonucleotides to a poly(dA-dT) effector molecule. Again, the activity of the ATPase was reduced in the absence of a 3′-hydroxyl group.

Table III: Structural Components of ATPase A Polynucleotide Effectors

nucleic acid	nmol of P_i formed[a] (% of control)	nmol of P_i formed, 100 mM added NaCl (% of control)
none	<1.0	<1.0
denatured calf thymus DNA	100	81
poly(dA)	5.2	4.4
$p(dT)_{16}$		1.5
$p(dT)_{16}$·poly(dA), 1:100[b]	42	18
$p(dT)_{16}$·poly(dA), 1:20	47	
$p(dT)_{16}$·poly(dA), 1:10	53	
$p(dT)_{16}$·poly(dA), 1:5	66	48
$p(dT)_{16}$·poly(dA), 1:1.5		39
$p(dT)_{16}$·poly(dA), 1:1	68	37
$p(dT)_{16}$·poly(dA), 1.5:1		9.6
$p(dT)_{16}$·poly(dA), 5:1	56	5.7
$p(dT)_{16}$·poly(dA) + $d(C)_{10}$, 1:1.5:1.25[c]		38
poly(dA)–oligo(dT) tailed		26[d]
poly(dA)–oligo(dT) tailed, 3′-terminated with a dideoxynucleotide		10[d]
poly(dA-dT)·poly(dA-dT)	151	76
poly(dA-dT)·poly(dA-dT), 3′-terminated with a dideoxynucleotide		37
ColE1 plasmid DNA	12	<1.0
*Sau*3A-digested pBR322 DNA		6.3[e]
*Hae*II-digested pBR322 DNA		4.2[e]
*Hae*III-digested pBR322 DNA		<1.0[e]

[a] Footnotes *a* and *b* from Table II are applicable. [b] Molar ratios of thymidine residues to adenosine residues are given. [c] Molar ratios of thymidine to adenosine to cytidine residues are given. [d] The added salt was at 25 mM rather than 100 mM. [e] The number of 3′-hydroxyl ends was held constant at 0.25 μM, and consequently, the nucleotide concentration was 25 μM except for the *Hae*II-digested pBR322 DNA, which was 50 μM in nucleotide.

The structure of the DNA in the region adjacent to the 3′-hydroxyl terminus was examined by cutting pBR322 DNA with three different restriction enzymes. These enzymes were *Sau*3A (leaving a recessed 3′ end), *Hae*II (leaving a recessed 5′ end), and *Hae*III (leaving a blunt end). The data in Table III show that the blunt-ended DNA is no better as an effector of ATP hydrolysis than a closed-circular DNA molecule, while the *Sau*3A-restricted DNA is a much better effector.

(D) Effects of Inhibitors on ATPase Activity. ATPase A is very sensitive to *N*-ethylmaleimide, retaining 5% of its original activity in the standard assay with a denatured, calf thymus DNA effector after a 10-min incubation with 10 mM *N*-ethylmaleimide (in a reaction containing 5 mM β-mercaptoethanol). Novobiocin (up to 200 μg/mL), which inhibits *E. coli* DNA gyrase (Gellert et al., 1976), had no detectable effect on ATPase A. Aphidicolin (up to 200 μg/mL), which inhibits all forms of DNA polymerase α (Fry, 1983), had no effect on ATPase A. AMPPNP is a nonhydrolyzable analogue of ATP and a potent inhibitor of mitochondrial ATPases (Schuster et al., 1975). DNA-dependent ATPase A cannot hydrolyze AMPPNP, but the AMPPNP is a moderately effective inhibitor, producing 50% inhibition when the concentration of AMPPNP equals that of the ATP.

Tests for Catalytic Activities of ATPase A. ATPase A did not stimulate calf thymus α-C-DNA polymerase or β-DNA polymerase in assays using nicked, gapped, or denatured, calf thymus DNA or poly(dA-dT).

Tests for DNA unwinding, polynucleotide kinase, renaturation, strand exchange, and autophosphorylation activities yielded negative results.

Discussion

A purification procedure that resolves four chromatographically distinct DNA-dependent ATPase activities from calf thymus has been devised. When denatured, calf thymus DNA is used as the effector for the ATPase reaction, one of these activities is significantly greater than any of the others. We have purified the protein responsible for this activity to near homogeneity, as evidenced by its appearance as a single band on SDS–polyacrylamide gel electrophoresis. The protein is a 69 000 molecular weight monomer with a specific activity of 18 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$. This specific activity is the highest reported activity of any mammalian DNA-dependent ATPase.

The enzyme does not appear to have deoxyribonuclease, DNA or RNA polymerizing, topoisomerase, DNA helicase, DNA ligase, DNA unwinding, terminal deoxynucleotidyl transferase, polynucleotide kinase, autophosphorylating, DNA renaturing, or strand exchange activities. Furthermore, it does not appear to directly stimulate the activities of calf α- or β-polymerases.

An unusual characteristic of calf ATPase A is its stability. This enzyme can retain ATPase activity for days at 37 °C in the absence of ATP or DNA. The stability, size, and template preference of ATPase A make it unlikely that the majority of previously described eucaryotic DNA-dependent ATPases are identical with or analogous to ATPase A.

There has been partial purification of a DNA-dependent ATPase by Yamada and co-workers (Watanabe et al., 1981; Enomoto et al., 1984; Tawaragi et al., 1984) that is similar to the DNA-dependent ATPase A reported in this paper. The earliest report details a partial purification of three chromatographically distinct DNA-dependent ATPases from calf thymus tissue. One of these, the A2 enzyme, elutes from DNA–cellulose similarly to our ATPase A and has a similar preference for poly(dA-dT) as an effector. A more extensive analysis of multiple ATPases was reported later using mouse cells as the source of enzyme (Tawaragi et al., 1984). Four DNA-dependent ATPases were separated by column chromatography. Of the four, only the C_3 ATPase shows substrate specificity similar to our ATPase A. ATPase C_3 appears as two bands on an SDS–polyacrylamide gel, and its specific activity is approximately 30-fold lower than our ATPase A. ATPase C_3 may certainly be the same enzyme as our ATPase A, although less pure. The ability of both mouse ATPase C_3 and calf thymus A to utilize poly(dA-dT) as the best effector for DNA-dependent ATP hydrolysis is certainly curious. However, we have extended our studies to show that the effector structure for ATPase activity is a primer-template junction.

Our results show that DNAs containing both double- and single-stranded regions are the best substrates for ATPase activity. Two excellent substrates in the standard assay are denatured, calf thymus DNA and poly(dA-dT) (or any DNA alternating copolymer). Both, presumably, have double- and single-stranded regions. Poly(dA-dT) loses its ability to act as a cofactor for ATP hydrolysis when 200 mM NaCl is added to the standard assay mix, while the denatured, calf thymus DNA retains its ability to act as a cofactor at much higher salt concentrations. This difference is almost certainly caused by the stabilization of hairpin structures in the templates. All regions of poly(dA-dT) are complementary to all other regions. The calf thymus DNA has varying levels of complementarity, ranging from easily formed hairpins to sequences present very infrequently in the DNA. When the NaCl supplement reaches 200 mM, the poly(dA-dT) must have attained almost completely double-stranded character, and therefore become a poor substrate for ATPase activity. At very high NaCl additions (300–500 mM), the loss of ATP hydrolysis, using the calf DNA as the effector, is most likely related to the decreased binding of the protein to the DNA template and not to significant changes in the structure of the DNA.

Evidence for the loss of ATPase activity because of salt stabilization of double-stranded structures was also provided by examination of the cofactor activity of ColE1 plasmid DNA. ColE1 DNA is negatively supercoiled in its native state, and the mechanical strain energy stored in each molecule tends to unwind local regions of the DNA double helix (Fisher, 1981). An increase in ionic strength should decrease the frequency of local unwinding. Addition of only 100 mM NaCl presumably makes the template totally double stranded and no longer a substrate for ATP hydrolysis by ATPase A.

Both nicked, calf thymus DNA and gapped, calf thymus DNA were much better effectors of ATPase activity than the native calf thymus DNA, indicating the importance of termini in the DNA effectors. However, the gapped DNA was a better effector than the nicked DNA, suggesting that a single-stranded region adjacent to the free DNA end is important for optimal effector usage by the ATPase A.

We continued our inquiries into the nature of the DNA effector by using homopolymeric DNAs as model effectors. DNA-dependent ATPase A does not utilize either poly(dA) or $(dT)_{16}$ as good effectors. However, $(dT)_{16}$-primed poly(dA) is a good effector. Since the melting temperature of this effector is close to the temperature of our standard assay (Cassani & Bollum, 1969), we increased the NaCl concentration by an additional 100 mM, thereby increasing the melting temperature of the nucleic acid complex. If poly(dA) is titrated with $(dT)_{16}$, two observations can readily be made: (1) Initially the rate of ATP hydrolysis increases as the amount of primer-template complex increases, indicating the importance of the double strandedness. (2) As the titration approaches and passes a 1:1 molar ratio of thymidine to adenosine, the rate of ATP hydrolysis decreases, thereby demonstrating the importance of having a single-stranded region of template adjacent to the duplex region of the DNA. The addition of $(dC)_{10}$ to the $(dT)_{16}$·poly(dA) reaction demonstrates that the decrease in ATP hydrolysis at T:A ratios greater than 1:1 is not the result of free DNA ends competing for binding of the enzyme. The importance of the ionic strength is shown by decreasing the salt concentration at greater than 1:1 ratios of thymidine to adenosine. At the lower salt concentrations, the $(dT)_{16}$ melts off the poly(dA), or alternatively, the protein may be allowed to disrupt the hydrogen bonding of some of the $(dT)_{16}$ molecules to the poly(dA), thereby increasing the apparent efficiency of the DNA effector.

The effectiveness of the DNA effector can be diminished by blocking available 3′-hydroxyl termini by the addition of a dideoxyribonucleotide. The need for 3′-hydroxyl termini was also suggested by the lowered efficiency of calf thymus DNA as an effector of ATPase activity after nicking with micrococcal nuclease, which leaves a 3′-phosphate rather than a 3′-hydroxyl.

We believe that calf thymus ATPase A, with its particular size and apparent ability to recognize primer-template junctions, represents a unique class of eucaryotic DNA-dependent ATPases. This class may be analogous to the gene 44/62 and 45 products of the bacteriophage T4 DNA replication system.

These gene products form a DNA-dependent ATPase that specifically recognizes primer-template junctions as effectors for ATPase activity (von Hippel et al., 1983) and are essential for normal T4 DNA replication (Morris et al., 1979; Nossal & Peterlin, 1979). The specificity of ATPase A suggests that it may be involved in the elongation phase of DNA synthesis, much like the gene 44/62 and 45 products in bacteriophage T4. Considering that one or more of these calf thymus DNA-dependent ATPases are likely to be important components of the DNA replication or recombination machinery in this organism, we are continuing to expand our efforts to purify and characterize these enzymes.

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Registry No. ATPase, 9000-83-3; Mg-ATP, 1476-84-2; Mg-dATP, 74386-14-4.

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Properties of Two Forms of DNA Polymerase δ from Calf Thymus†

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ABSTRACT: Purified calf thymus DNA polymerases δ I and II each have an associated 3′ to 5′ exonuclease but otherwise resemble DNA polymerase α in size, biochemical kinetic parameters, and the presence of DNA primase [Crute, J. J., Wahl, A. F., & Bambara, R. A. (1986) *Biochemistry 25*, 26–36]. Here we demonstrate a functional association of polymerase and exonuclease with each δ form. Furthermore, we show that the exonuclease can be dissociated from DNA polymerase δ I but does not appear to be removable from DNA polymerase δ II. Polymerases δ I, δ II, and α are equally sensitive to the inhibitor aphidicolin, suggesting a similarity in active site structure. In comparison with DNA polymerase α and δ II, DNA polymerase δ I has intermediate sensitivity to 2-(*p*-*n*-butylanilino)-2′-deoxyadenosine 5′-triphosphate (BuAdATP) or N^2-(*p*-*n*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate (BuPdGTP). The activity of the DNA primase of the δ II enzyme is insensitive to BuAdATP whereas 1.0 μM of this inhibitor will decrease the activity of the DNA primase of the α and δ I enzymes approximately 50%. Two monoclonal antibodies that potently inhibit DNA polymerase α are only slightly inhibitory to DNA polymerase δ I and are ineffective at inhibiting DNA polymerase δ II. DNA polymerase δ II had been previously found to be nearly inactive on nuclease-treated calf thymus DNA, relative to its activity on homopolymeric DNA. We find that addition of purified calf histone proteins or spermidine can greatly enhance synthesis by this enzyme on activated calf DNA.

We have recently purified two forms of DNA polymerase δ (I and II) from calf thymus using ion-exchange and substrate affinity chromatography (Crute et al., 1986a). They are polymerases that contain a 3′ to 5′ exonuclease activity and differ from each other by chromatographic behavior and template preference. They have been designated δ-class polymerases on the basis of their similarities of the δ polymerase described earlier (Byrnes et al., 1976; Lee et al., 1984; Byrnes, 1984). Examination of the δ polymerases has revealed that they share many properties with calf DNA polymerase α, which we have purified using an immunoaffinity method (Wahl et al., 1984). The three enzymes, DNA polymerases α, δ I, and δ II, have similar molecular weight and axial ratio, have an associated DNA primase activity, display similar extents of processive synthesis, and are stimulated by ATP apparently through the same mechanism (Wahl et al., 1984; Crute et al., 1986a). Crute et al. (1986b), however, demonstrated that these enzymes differ in their photosensitization to the drug hematoporphyrin derivative. Also, the compounds BuPdGTP[1] and BuAdATP have been used to differentially inhibit DNA polymerase α and δ forms (Lee et al., 1985; Crute et al., 1986a).

The increase in DNA polymerase α activity in replicating tissue and its sensitivity to the DNA replication inhibitor aphidicolin argue for its major role in DNA replication (Pedrali-Noy & Spadari, 1979). Sensitivity of DNA polymerase δ to aphidicolin (Crute et al., 1986a; Lee et al., 1984; Byrnes, 1984) is comparable to that of DNA polymerase α. However, the relative activity of DNA polymerase δ in proliferating vs. nonproliferating cells has not yet been determined. Moreover, the 3′ to 5′ exonuclease activity could be necessary for the maintenance of high-fidelity DNA replication. Therefore, DNA polymerase δ could also have a central role in DNA replication.

In general, prokaryotic DNA polymerases have an associated 3′ to 5′ exonuclease activity, which functions to remove misincorporated nucleotides during DNA replication. This

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; BuPdGTP, N^2-(*p*-*n*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate; BuAdATP, 2-(*p*-*n*-butylanilino)-2′-deoxyadenosine 5′-triphosphate; DEAE, diethylaminoethyl; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IgG_1, immunoglobulin G_1; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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