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Multiple Deoxyribonucleic Acid Dependent Adenosinetriphosphatases in FM3A Cells. Characterization of an Adenosinetriphosphatase That Prefers Poly[d(A-T)] as Cofactor†

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ABSTRACT: Four chromatographically distinct DNA-dependent ATPases, B, C_1, C_2, and C_3, have been partially purified from mouse FM3A cell extracts. These ATPases are distinguished from each other by their physical and enzymological properties. DNA-dependent ATPases B, C_1, C_2, and C_3 have sedimentation coefficients in 250 mM KCl of 5.5, 5.3, 7.3, and 3.4 S, respectively. ATPases B, C_2, and C_3 hydrolyze dATP as efficiently as ATP, whereas C_1 does not. ATPase B hydrolyzes other ribonucleoside triphosphates with relatively high efficiency as compared to the other three enzymes. ATPase C_3 prefers poly[d(A-T)] to poly(dT) as cofactor, whereas the other three enzymes prefer poly(dT) to poly[d(A-T)]. Among the four ATPases, ATPase C_3 has been highly purified and characterized in detail. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the most purified fraction of ATPase C_3 showed two major bands corresponding to molecular weights of 66 000 and 63 000. The K_m values of the enzyme for ATP and dATP are 0.53 and 0.86 mM, respectively. As cofactor, poly[d(A-T)] is the most effective among the DNAs tested. Heat-denatured DNA and native DNA are also effective but used with less efficiency. Almost no or very little activity has been detected with ribohomopolymers and oligonucleotides. The activity attained with poly(dT) and poly(dA) is 11 and 6% of that with heat-denatured DNA, respectively. When both polymers were added at a molar ratio 1 to 1, very high activity was obtained with these polymers. On the other hand, little activity was observed by the combination of noncomplementary homopolymers such as poly(dT) and poly(dG).

It is well known that ATP is required for DNA replication in various prokaryotic systems. Some of the proteins that have DNA-dependent ATPase activity such as *dnaB* protein, protein *n′*, and *rep* protein have been proved to play important roles in the replication of ϕX174 DNA by a combination of genetic and biochemical approaches (Kornberg, 1980). The requirement of ATP for DNA replication of eukaryotic cells has been also shown with various in vitro systems in permeabilized cells, cell lysate, and isolated nuclei. We have demonstrated an absolute requirement for high levels of ATP for the DNA synthesis in isolated nuclear systems (Tanuma et al., 1980; Nagata et al., 1981), especially for the synthesis of Okazaki fragments (Enomoto et al., 1981, 1983). The molecular basis of the requirement for ATP still remains unclear. To clarify the roles of ATP in the processes of DNA replication, we have paid attention to DNA-dependent ATPases and attempted to purify and characterize them.

In recent years, DNA-dependent ATPases have been isolated from various eukaryotic cells and tissues (Hachmann & Lezius, 1976; Otto, 1977; Hotta & Stern, 1978; Cobianchi et al., 1979; Assairi & Johnston, 1979; Boxer & Korn 1980; Plevani et al., 1980; DeJong et al., 1981; Yaginuma & Koike, 1981; Thomas & Mayer, 1982). We have also isolated three forms of DNA-dependent ATPases from calf thymus (Watanabe et al., 1981). Recently, we have sought analogous enzymes in FM3A cells, which are a good source for the isolation of DNA replication enzymes, because it is possible to obtain a large amount of S phase accumulated cells grown in mice by the method reported by us (Hanaoka et al., 1981). In addition, several mutants related to DNA replication have been isolated from the cells in our laboratory (Nakano et al., 1978; Nishimura et al., 1979; Tsai et al., 1979; Yasuda et al., 1981).

We described here the existence of multiple DNA-dependent ATPases in FM3A cell extracts and the purification and characterization of one form of the ATPases, ATPase C_3, which prefers poly[d(A-T)] as cofactor. Partial purification of another form of the ATPases, ATPase B, has been recently

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published (Watanabe et al., 1982).

Materials and Methods

Chemicals. All chemicals were of reagent grade. Nucleoside triphosphates, AMP-PCP,[1] AMP-PNP, and ATP-γ-S were purchased from Boehringer Mannheim.

Nucleic Acids and Polynucleotides. Calf thymus DNA was obtained from Worthington. Heat-denatured DNA was prepared as follows. The calf thymus DNA was dissolved in a solution containing 10 mM Tris-HCl, pH 7.4, and 1 mM Na_3EDTA at a concentration of 1 mg/mL and denatured by heating for 15 min in boiling water and rapid cooling in ice. Activated DNA was prepared according to the method of Aposhian & Kornberg (1962). Deoxyribohomopolymers, poly[d(A-T)], oligoribo- and oligodeoxyribonucleotides, poly(C), and poly(A) were purchased from P-L Biochemicals. Poly(G) and poly(U) were from Boehringer Mannheim. Yeast tRNA was from Sigma.

Other Materials. DEAE-cellulose was obtained from Brown. Phosphocellulose and carboxymethylcellulose (CM-52) were from Whatman. Hydroxylapatite was from Bio-Rad. DNA–cellulose was prepared with heat-denatured calf thymus DNA according to Alberts & Herrick (1971).

Buffers. Buffer 1 contained 20 mM potassium phosphate buffer, pH 7.5, 0.1 mM Na_3EDTA, 1 mM 2-mercaptoethanol, 0.25 mM phenylmethanesulfonyl fluoride, and 1% (v/v) ethanol. Buffer 2 contained all the components of buffer 1 plus 20% ethylene glycol and 0.01% Triton X-100. Buffer 3 was the same as buffer 2 except the concentration of ethylene glycol was 50% (v/v) instead of 20%. Buffer 4 contained all the components of buffer 1 plus 50% (v/v) glycerol and 0.01% Triton X-100.

Cells. Seven-week-old female C3H/He mice were bred for 5 days after intraperitoneal inoculation with 1×10^6 FM3A cells (clone 28), and then they were injected intraperitoneally with 100 μg of 5-fluoro-2′-deoxyuridine and bred for 16 h in order to enrich S-phase populations (Hanaoka et al., 1981). Cells were harvested from the abdominal cavity and washed twice with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline. The washed cells were suspended in buffer 1 at a concentration of 1×10^8/mL and centrifuged at 2500 rpm for 5 min. The pellet was washed once more with buffer 1 and stored at −80 °C until use.

ATPase Assay. The standard reaction mixture (50 μL) contained 50 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 5 mM ATP, and 25 μg of bovine serum albumin. DNA-dependent and -independent ATPase activities were assayed in the presence or absence of 5 μg of heat-denatured calf thymus DNA. Incubation was carried out for 30 min at 37 °C. The amount of ADP produced was determined by the method of Korn & Yanofsky (1976). One unit of activity is defined as the amount of enzyme to hydrolyze 1 nmol of ATP/h at 37 °C.

Glycerol Density Gradient Centrifugation. Bovine serum albumin was added to the samples at a final concentration of 1 mg/mL in order to stabilize the enzyme, and the samples were dialyzed against buffer 1 containing 5% glycerol and 250 mM KCl. Two hundred microliters of the dialyzate was layered onto a 4.8-mL linear gradient of glycerol from 10 to 30% (v/v) in buffer 1 containing 1 mM dithiothreitol instead of 2-mercaptoethanol, 250 mM KCl, and 0.2 mg/mL bovine serum albumin. Centrifugation was performed for 20 h at 258000g in a Hitachi RPS 65-T rotor at 4 °C. In the parallel run, aldolase (7.6 S), bovine serum albumin (4.4 S), and cytochrome c (1.8 S) were used as markers.

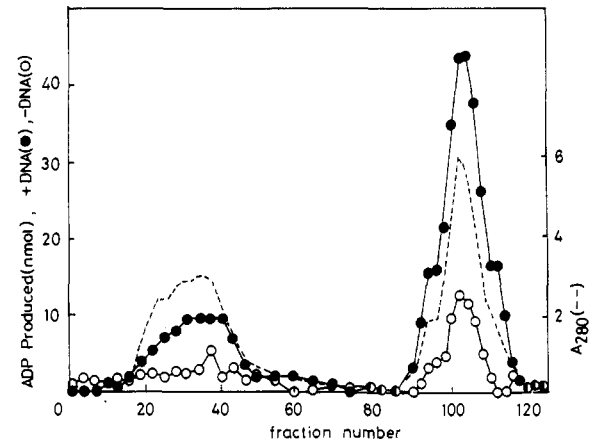

FIGURE 1: Second DEAE-cellulose column chromatography. The first DEAE-cellulose column flow-through fraction (fraction II) was dialyzed and applied onto a second DEAE-cellulose column. Elution was performed as described in the text. An aliquot of each fraction was assayed for ATPase in the presence (●) or absence (○) of heat-denatured calf thymus DNA as described under Materials and Methods. Absorbance at 280 nm (--).

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out essentially according to the procedure of Laemmli (1970). Samples were precipitated as described by Schaffner & Weissmann (1973), dissolved in the sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.001% bromophenol blue), heated at 100 °C for 2 min, and loaded onto a SDS–10% polyacrylamide slab gel. After electrophoresis, the gel was stained with silver as described by Oakley et al. (1980). RNA polymerase B from *Thermus thermophilus* (Seikagaku Kogyo Co.) consisting of β' (M_r 180000), β (M_r 140000), x (M_r 100000), α (M_r 42000), and z (M_r 39000) was used as molecular weight marker.

Quantitation of Protein Amount. Protein concentration was determined by the method of Bradford (1976). Bovine plasma γ-globulin was used as a standard.

Results

Detection and Purification of Multiple DNA-Dependent ATPases. Frozen stock of 5×10^{10} FM3A cells was thawed, suspended in 400 mL of buffer 1, and homogenized by sonication. The sonicate was made 0.3 M in KCl by the addition of $^1/_{10}$th volume of buffer 1 containing 3.3 M KCl. This condition was found to be optimum for the recovery of DNA-dependent ATPases. After being stirred for 30 min at 0 °C, the extract was centifuged at 10000g, and the supernatant was recentrifuged for 1 h at 125000g. The supernatant (fraction I) was loaded on a DEAE-cellulose column (460 mL; 4.5 × 29 cm) equilibrated with 0.3 M KCl, in buffer 1. The flow-through fractions were pooled (fraction II), and Triton X-100 was added to fraction II at a final concentration of 0.01%. Fraction II was dialyzed against buffer 2 containing 50 mM KCl. The dialyzate was loaded on the second DEAE-cellulose column (250 mL; 4.3 × 18.5 cm) equilibrated with 50 mM KCl in buffer 2. The column was washed with 3 bed volumes of the equilibration buffer, and the proteins bound to the column were eluted with 3 bed volumes of 0.4 M KCl in buffer 2. Similar to the case of calf thymus, DNA-dependent ATPase activity was recovered from flow-

[1] Abbreviations: AMP-PCP, 5′-adenylyl methylenediphosphate; AMP-PNP, 5′-adenylyl imidodiphosphate; ATP-γ-S, adenosine 5′-*O*-(3-thiotriphosphate); CM, carboxymethyl; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

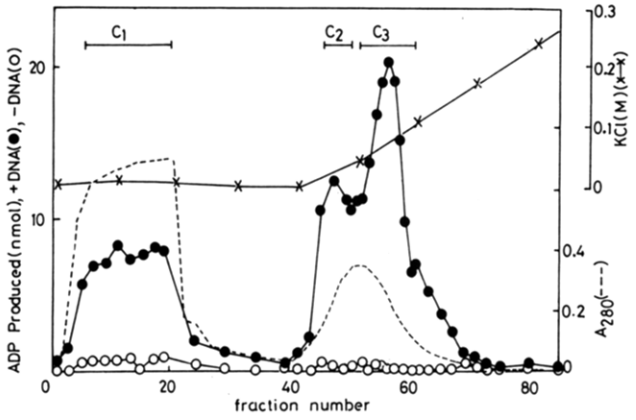

FIGURE 2: CM-cellulose column chromatography. The hydroxylapatite column fraction (fraction V) was dialyzed and applied onto a CM-celulose column. Elution was performed as described in the text. An aliquot of each fraction was assayed for ATPase in the presence (●) or absence (○) of heat-denatured calf thymus DNA as described under Materials and Methods. Absorbance at 280 nm (--). Concentration of KCl (×).

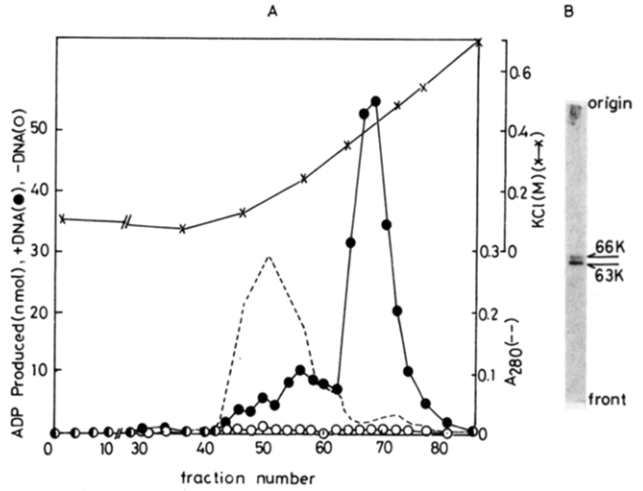

FIGURE 3: Single-stranded DNA–cellulose column chromatography. (A) The CM-cellulose column fraction of DNA-dependent ATPase C_3 (fraction VIII) was applied onto a single-stranded DNA–cellulose column and chromatographed as described in the text. An aliquot of each fraction was assayed for ATPase in the presence (●) or absence (○) of heat-denatured calf thymus DNA. Absorbance at 280 nm (--). Concentration of KCl (×). (B) SDS–polyacrylamide gel electrophoresis. The peak fractions of the DNA–cellulose column (fraction numbers 63–72) were combined (fraction IX), and an aliquot was subjected to SDS–polyacrylamide gel electrophoresis as described under Materials and Methods. Proteins were detected by silver staining.

through fractions (fraction III) and bound fractions (Figure 1). The activity in the bound fractions, which was designated as ATPase B, was further purified by phosphocellulose, hydroxylapatite, native DNA–cellulose, and second phosphocellulose column chromatographies, successively to a specific activity of 28 500 units/mg of protein as described previously (Watanabe et al., 1982). Fraction III was applied onto a phosphocellulose column (75 mL; 2.4 × 16.6 cm) equilibrated with 50 mM KCl in buffer 2. Proteins were eluted from the column with 10 bed volumes of a linear gradient of KCl from 50 mM to 0.8 M in buffer 2. The active fractions eluted at around 0.35 M KCl, were pooled (fraction IV), and applied onto a hydroxylapatite column (20 mL; 2 × 6.8 cm) equilibrated with 0.35 M KCl in buffer 2, and the column was washed with 5 bed volumes of the same buffer. Elution was carried out with 3 bed volumes of buffer 2 containing 0.08 M potassium phosphate buffer, pH 7.5, and 0.35 M KCl. Active fractions eluted from the column were combined (fraction V) and dialyzed against buffer 2. The dialyzate was chromatographed on a CM-cellulose column (5 mL; 1.2 × 4.7 cm) equilibrated with buffer 2. The proteins bound to the column were eluted with 10 bed volumes of a linear gradient of KCl from 0 to 0.3 M in buffer 3. DNA-dependent ATPase activity was divided into flow-through and bound fractions on this column. The bound activity was eluted from the column at KCl concentrations of around 30 and 80 mM. The activities resolved on the CM-cellulose column were designated as C_1 (fraction VI), C_2 (fraction VII), and C_3 (fraction VIII) according to their order of elution from the column (Figure 2). The C_3 activity (fraction VIII) was further purified with a single-stranded DNA–cellulose column (2 mL; 0.9 × 3.2 cm) equilibrated with 80 mM KCl in buffer 3. Elution was carried out with 10 bed volumes of a linear gradient of KCl from 80 mM to 0.8 M in buffer 4. The main peak of ATPase activity was eluted from the column at 0.45 M KCl (fraction IX) (Figure 3). The specific activity of fraction IX was about 40 000 units/mg of protein. The purification of the ATPases is summarized in Table I.

Characterization of DNA-Dependent ATPases of FM3A Cells. From the results shown in the previous section, it is obvious that there exist at least four chromatographically distinct DNA-dependent ATPases in FM3A cell extracts. Table II shows some of physical and enzymological properties of the four enzymes. They were distinguished from each other by their *s* values, their preference for substrates, and their

Table I: Purification of DNA-Dependent ATPases from FM3A Cells[a]

purification step	protein (mg)	total units (×10⁻³)	sp act. (units/mg)	yield (%)
crude extract (I)	4200	1600	390	100
first DEAE-cellulose (II)	3600	2100	570	125
second DEAE-cellulose (III)	1100	550	480	33
phosphocellulose (IV)	260	300	1100	18
hydroxylapatite (V)	45	190	4200	12
CM-cellulose				
C_1 (VI)	32	42	1300	2.6
C_2 (VII)	3.0	22	7300	1.3
C_3 (VIII)	6.1	39	6400	2.4
DNA–cellulose, C_3 (IX)	0.7	27	39000	1.7

[a] ATPase activity was assayed under the standard conditions in the presence or absence of heat-denatured calf thymus DNA. DNA-dependent ATPase activity was determined by subtracting the activity in the absence from that in the presence.

requirement for cofactor DNAs, suggesting that they may be different enzymes.

Characterization of ATPase C_3. The unique preference of ATPase C_3 for poly[d(A-T)] as cofactor distinguishes the enzyme from all of the eukaryotic DNA-dependent ATPases reported previously except DNA-dependent ATPase A_1 and A_2 from calf thymus reported by us (Watanabe et al., 1981). The above fact prompted us to purify highly and to characterize in detail DNA-dependent ATPase C_3. The most purified fraction (fraction IX) sedimented at 3.4 S in a glycerol gradient in the presence of 0.25 M KCl. SDS–polyacrylamide gel electrophoresis of fraction IX showed two major bands corresponding to molecular weights of 66 000 and 63 000 (Figure 3B). The ATPase required a divalent cation for activity. The optimal concentrations of Mg^{2+} and Mn^{2+} were 2.5–5 mM. The efficiency of both ions was similar. The calcium ion was also effective, but the efficiency was lower than those of Mg^{2+} and Mn^{2+}. Monovalent cations, K^+ and Na^+, inhibited the enzyme activity about 30, 60, and 80% at

Table II: Comparison of Properties of DNA-Dependent ATPases from FM3A Cells[a]

property	B	C_1	C_2	C_3
sedimentation coefficient (S)	5.5	5.3	7.3	3.4
K_m for ATP (mM)	0.75	0.69	0.64	0.53
K_m for dATP (mM)	0.56	*b*	1.20	0.86
substrate[c]				
dATP (%)	88	12	73	63
CTP (%)	35	3	9	10
GTP (%)	45	1	9	14
UTP (%)	37	7	7	5
AMP-PCP[d] (%)				0
AMP-PNP[d] (%)				0
ATP-γ-S[d] (%)				9
cofactor[e]				
poly(dT) (%)	111	185	94	11
poly[d(A-T)] (%)	42	33	35	142
none (%)	5	23	4	0

[a] Fraction VI (C_1), fraction VII (C_2), fraction IX (C_3), and highly purified ATPase B (Watanabe et al., 1982) were used for these assays. [b] Not done. [c] Assays were performed in the presence of indicated nucleotides in place of ATP. The values indicate percent of the activity in the presence of ATP as 100%. [d] The concentration of the nucleotide was 1 mM. [e] Assays were performed under the standard conditions except that indicated deoxyribopolymers were added in place of heat-denatured DNA. The values indicate percent of the activity in the presence of heat-denatured DNA as 100%.

Table III: Effect of Various Nucleic Acids on ATPase C_3 Activity[a]

nucleic acid	rel act. (%)	nucleic acid	rel act. (%)
calf thymus heat-denatured DNA	100	poly(A)[b]	5
calf thymus native DNA	65	poly(C)[b]	3
calf thymus activated DNA	54	poly(G)[b]	0
φX174 single-stranded circular DNA	71	poly(U)[b]	5
poly(dA)	6	yeast tRNA[b]	2
poly(dC)	9	$(dA)_{12-18}$	0
poly(dG)	4	$(dT)_{12-18}$	3
poly(dT)	11	$(rA)_{10}$	0
poly[d(A-T)]	142	none	0

[a] The ATPase activity was assayed under the standard conditions for 60 min in the presence of 5 μg of the indicated nucleic acids. The values indicate percent of the activity in the presence of heat-denatured DNA as 100%. [b] 10 μg of the polynucleotide was added.

100, 200, and 360 mM, respectively. The enzyme showed a broad pH optimum from 7.5 to 9.5. Sulfhydryl-protecting reagents such as 20 mM 2-mercaptoethanol and 10 mM dithiothreitol stimulated the enzyme activity about 70 and 110%, respectively.

ATP was the most preferred substrate among the ribonucleoside triphosphates tested. dATP was also efficiently hydrolyzed. Almost no hydrolysis was observed with ATP analogues, AMP-PCP, AMP-PNP, and ATP-γ-S (Table II). The K_m values for ATP and dATP were 0.53 and 0.83 mM, respectively.

Comparison of Various DNAs, RNA, Synthetic Polynucleotides, and Oligonucleotides as Cofactors for ATPase C_3. The presence of DNA was absolutely necessary for the enzyme activity. Heat-denatured DNA was most effective among natural DNAs (Table III). Almost no or very little activity was observed with oligonucleotides, ribo- and deoxyribohomopolymers, and yeast tRNA. The most effective cofactor so far tested was poly[d(A-T)] as described in the previous section. Figure 4 shows ATP hydrolysis as a function of DNA concentration. DNAs used were heat-denatured

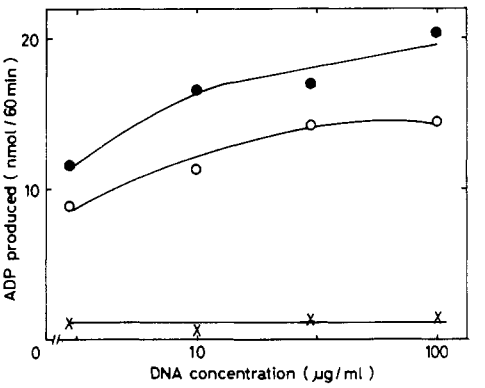

FIGURE 4: DNA-dependent ATPase C_3 activity as a function of DNA concentration. ATPase C_3 (fraction IX, 17 units) was assayed under the standard conditions for 60 min in the presence of various concentrations of heat-denatured DNA (○), poly[d(A-T)] (●), or poly(dT) (×).

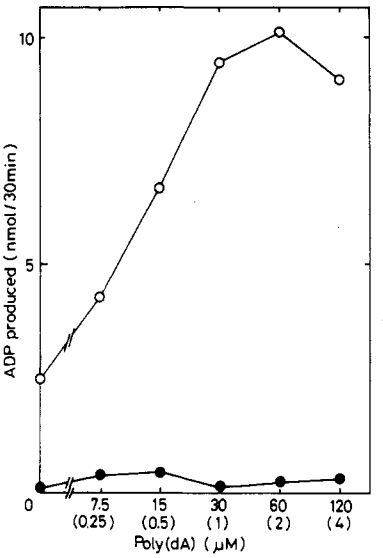

FIGURE 5: DNA-dependent ATPase C_3 activity as a function of poly(dA). ATPase C_3 (fraction IX, 34 units) was assayed under the standard conditions for 30 min in the presence of various concentrations of poly(dA) with (○) or without (●) 30 μM poly(dT). The number in the parentheses indicates the molar ratio of poly(dA) to poly(dT).

DNA, poly[d(A-T)], and poly(dT). The relative efficiency among the DNAs did not change at any concentration tested [poly[d(A-T)] > heat-denatured DNA > poly(dT)]. Time-course experiments revealed that the hydrolysis of ATP was linear with time for up to about 60 min in the presence of these DNAs. Again, the relative activity among these DNAs did not change at any time during the incubation [poly[d(A-T)] > heat-denatured DNA > poly(dT)]. Figure 5 shows the titration curve of poly(dA) in the presence or absence of poly(dT). Poly(dA) was not so effective at any concentration tested. On the other hand, in the presence of poly(dT), a marked increase of the ATPase activity was observed at a molar concentration ratio of poly(dA) to poly(dT) of more than 1. Little activity was attained by the combination of noncomplementary homopolymers such as poly(dT) and poly(dG).

Discussion

It has been revealed from the results reported here that the extracts of FM3A cells contain at least four chromatographically distinct DNA-dependent ATPases. These ATPases differed from each other in their physical and enzymological properties in addition to chromatographic behaviors. It is not

surprising to find multiple DNA-dependent ATPase activities in eukaryotes because multiple species of DNA-dependent ATPase, which are involved in DNA replication, repair, and recombination, have been identified in prokaryotes.

DNA-dependent ATPases have been purified from various eukaryotic cells and tissues. Hachmann & Letius (1976) have isolated a DNA-dependent ATPase from mouse myeloma that has a sedimentation coefficient of 5.5 S. The enzyme preferred single-stranded polydeoxyribonucleotides as cofactor and could hydrolyze both NTPs and dNTPs. These physical and enzymological properties are the same as those of ATPase B reported here (Table II). The identity of the two enzymes is further suggested by their chromatographic behaviors during purification (Watanabe et al., 1982). The DNA-dependent ATPase from KB cell nuclei (Boxer & Korn, 1980) may correspond to ATPase C_1 because of its *s* value of 5.3, preference for cofactor DNAs, substrate specificity, and chromatographic behavior on a DEAE-cellulose column. Partial resemblance between DNA-dependent ATPase III from Novikoff hepatoma (Thomas & Meyer, 1982) and ATPase C_2 has been observed in their physical and enzymological properties, but it is not clear at present whether they are the same enzyme or not.

DNA-dependent ATPase C_3 is unique in its requirement for cofactor DNAs. The most effective cofactor for the enzyme was poly[d(A-T)], and the enzyme showed very low activity with poly(dT) (Table III). None of the DNA-dependent ATPases that have been isolated to date except ATPases A_1 and A_2 from calf thymus (Watanabe et al., 1981) correspond to ATPase C_3 in their requirement for cofactor DNAs.

We have highly purified and characterized in detail ATPase C_3. SDS–polyacrylamide gel electrophoresis of the single-stranded DNA–cellulose fraction showed two major bands corresponding to molecular weights of 66 000 and 63 000. It remains to be clarified whether or not either of the two bands or both possess the enzyme activity.

The physiological function of ATPase C_3 remains unclear. In *Escherichia coli*, a DNA-dependent ATPase that prefers poly[d(A-T)] as cofactor is known as *recBC* enzyme. Unlike *recBC* enzyme, ATPase C_3 has no detectable ATP-dependent endo- or exonuclease activity. Preliminary experiments to investigate the subcellular localization of ATPase C_3 indicated that the enzyme exists in the nucleus. Therefore, it seems likely that the enzyme plays its role in the nucleus. The high K_m value for ATP of ATPase C_3 and the preference of the enzyme for dATP as well as ATP are consistent with our previous observations with nuclear systems that high levels of ATP (millimolar order) are required for the synthesis of Okazaki fragments and the ATP can be substituted with high levels of dATP (Enomoto et al., 1981, 1983). Expecting participation of ATPase C_3 in Okazaki fragment synthesis, we have examined primase and DNA-unwinding activity of the enzyme, but we have not succeeded yet to demonstrate such activities.

Acknowledgments

We thank K. Kawasaki for the collaboration during this study and helpful discussion.

Registry No. ATPase, 9000-83-3; ATP, 56-65-5; dATP, 1927-31-7; poly(dT), 25086-81-1; poly[d(A-T)], 26966-61-0.

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