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DNA-Dependent Adenosinetriphosphatase B from Mouse FM3A Cells Has DNA Helicase Activity†

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Received October 7, 1986; Revised Manuscript Received January 7, 1987

ABSTRACT: We have detected at least four forms of DNA-dependent ATPase in mouse FM3A cell extracts [Tawaragi, Y., Enomoto, T., Watanabe, Y., Hanaoka, F., & Yamada, M. (1984) *Biochemistry 23*, 529-533]. The purified fraction of one of the four forms, ATPase B, has been shown to have DNA helicase activity by using a DNA substrate which permits the detection of limited unwinding of the helix. The DNA substrate consists of single-stranded circular *fd* DNA and the hexadecamer complementary to the *fd* DNA, which bears an oligo(dT) tail at the 3′ terminus. The helicase activity and DNA-dependent ATPase activity cosedimented at 5.5 S on glycerol gradient centrifugation. The helicase required a divalent cation for activity ($Mg^{2+} \simeq Mn^{2+} > Ca^{2+}$). The optimal concentrations of these divalent cations were 5 mM. The requirement of divalent cations of the DNA helicase activity was very similar to that for the DNA-dependent ATPase activity of ATPase B. The helicase activity was absolutely dependent on the presence of a nucleoside triphosphate. ATP was the most effective cofactor among the ribo- and deoxyribonucleoside triphosphates tested, and considerable levels of helicase activity were observed with other ribo- and deoxyribonucleoside triphosphates. The efficiency of a nucleoside triphosphate to serve as cofactor for the helicase activity correlated with the capacity of the nucleotide to serve as substrate for the DNA-dependent ATPase activity. The nonhydrolyzable ATP analogues such as adenosine 5′-*O*-(3-thiotriphosphate) were not effective for the helicase activity. The helicase displaced the hexadecamer with no tail as well as the hexadecamer bearing the 3′ or 5′ tail. The efficiency of displacement was almost the same among the three substrates.

On the replication of duplex DNA, DNA helicase action is required for unwinding the double strand in advance of a replication fork. It has been indicated that the bacteriophage T7 gene *4* protein, the T4 gene *41* protein, and the *Escherichia coli rep* protein are required for the replication of the chromosome of coliphages T7, T4, and *φX174* RF, respectively (Kornberg, 1980). The *E. coli dnaB* protein is also essential for the replication of oriC plasmid containing the replication origin of *E. coli* chromosome (Ogawa et al., 1985). Biochemical analysis of these proteins in vitro has indicated that they have DNA helicase activity and this activity is coupled with hydrolysis of nucleoside 5′-triphosphate in a DNA-dependent manner (Kornberg, 1980; Yarranton & Gefter, 1979; Venkatesan et al., 1982; Matson et al., 1983; LeBowitz & McMacken, 1986).

To find analogous proteins participating in eukaryotic DNA replication, eukaryotic DNA-dependent ATPases have been isolated from various sources (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; Hyodo & Suzuki, 1981; Yaginuma & Koike, 1981; Thomas & Meyer, 1982; Brewer et al.,

† This work was supported in part by Grants-in-Aid for Scientific Research and for Cancer Research from the Ministry of Education, Science, and Culture of Japan and by the Naito Foundation.

0006-2960/87/0426-2924$01.50/0

1983). The lily enzyme (Hotta & Stern, 1978) is the only one that exhibits helicase activity assayed by the method using nuclease S1 developed by Abdel-Monem and Hoffmann-Berling (1976).

In recent years, we have isolated four forms of DNA-dependent ATPase from mouse FM3A cells and characterized in detail one of the four forms, ATPase B (Watanabe et al., 1982; Tawaragi et al., 1984; Enomoto et al., 1984; Seki et al., 1986). In the previous study (Seki et al., 1986), we could not detect helicase activity by the S1 method with the purified DNA-dependent ATPase B. However, the assay system seems to be too insensitive to detect a limited unwinding of the duplex. More recently, Hübscher and Stalder (1985) have reported the detection of DNA helicase activity with a calf thymus DNA-dependent ATPase by the method using a helicase substrate which permits the detection of limited unwinding of the helix. In this study, we have constructed similar helicase substrates and presented evidence by using these substrates that the DNA-dependent ATPase B has DNA helicase activity.

Materials and Methods

Materials. [γ-^{32}P]ATP was prepared with Promega Biotec's γ prep synthesis system from ADP and ^{32}P-labeled inorganic phosphate. [^{32}P]P_i and [^{3}H]dTTP were purchased from Amersham Corp. Nucleoside triphosphates, ADP, and AMP were obtained from Yamasa Biochemicals. 5′-Adenylyl methylenediphosphate (AMP-PCP),[1] AMP-PNP, and ATP-γ-S were purchased from Boehringer Mannheim. Soluene-350 was obtained from Packard Instrument Co. Inc. T4 polynucleotide kinase was purchased from Takara Biochemicals. Terminal deoxynucleotidyltransferase was obtained from Pharmacia P-L Biochemicals. Single-stranded circular *fd* DNA was prepared according to the method of Herrmann et al. (1980). The 16-base-long oligonucleotide 5′-*ACCAGAAGGAGCGGAA*-3′ complementary to *fd* DNA and the 33-base-long oligonucleotide 5′-ACGCCTTGTTTCTTACC*ACCAGAAGGAGCGGAA*-3′, bearing a 17-base-long noncomplementary sequence at the 5′ side, were generously supplied by Drs. H. Gushima and M. Takayama (Yamanouchi Pharmaceutical Co., LTD). The 5′ termini of two oligonucleotides are OH.

Purification of DNA-Dependent ATPase B. DNA-dependent ATPase B was purified from mouse FM3A cells by sequential column chromatography on DEAE-cellulose (Brown), DE-52 (Whatman), phosphocellulose, hydroxylapatite, single-stranded DNA–cellulose, and phosphocellulose to a specific activity of 51 000 units/mg of protein as described previously (Seki et al., 1986). One unit of activity is defined as the amount of enzyme that hydrolyzes 1 nmol of ATP/h at 37 °C. The purified fraction contained no detectable DNA polymerase β, DNA polymerase γ, endonuclease, and DNA topoisomerase type I and type II activities (Seki et al., 1986).

DNA-Dependent ATPase Assay. The assay for DNA-dependent ATPase activity was performed as described previously (Watanabe et al., 1981).

Preparation of Helicase Substrates. DNA concentrations are expressed in molarity of molecules. Eighty picomoles of the hexadecamer or the 33-mer was incubated with 10 units of T4 polynucleotide kinase at 37 °C for 60 min in the presence of 20 pmol of [γ-^{32}P]ATP (3600 Ci/mmol) in order to label the 5′ end of the oligonucleotides. A portion of the ^{32}P-labeled hexadecamer was oligo(dT)-tailed with terminal deoxynucleotidyltransferase. The reaction was carried out in the reaction mixture containing 200 mM potassium cacodylate, pH 7.2, 40 mM KCl, 1 mM $CoCl_2$, 0.5 mg/mL BSA, 0.5 mM dithiothreitol, 300 pmol of dTTP, 20 pmol of the ^{32}P-labeled hexadecamer, and 0.6 unit of terminal deoxynucleotidyltransferase. The mixture (20 μL) was incubated at 37 °C for 120 min, and the reaction was stopped by chilling the reaction mixture and adding 1 μL of 0.2 M Na_3EDTA. The size of the products was determined by electrophoresis in a 20% polyacrylamide–7 M urea gel. The average size of the products was 23 bases long. To form the short duplex, 20 pmol of the hexadecamer, the oligo(dT)-tailed hexadecamer, or the 33-mer was annealed to 25 pmol of single-stranded circular *fd* DNA. The DNAs were mixed, and ammonium acetate was added at a final concentration of 2 M. The DNAs were precipitated with ethanol and dissolved in 180 μL of the annealing buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 M NaCl, and 1 mM dithiothreitol. The annealing mixture was heated at 100 °C for 4 min to denature the DNA and then incubated at 67 °C for 60 min followed by 37 °C for 30 min. The mixture was cooled to room temperature, and EDTA was added to the mixture at a final concentration of 25 mM. The mixture was loaded onto 4.8 mL of a 5–20% sucrose gradient containing 50 mM Tris-HCl, pH 7.5, 5 mM Na_3EDTA, and 0.1 M NaCl. Centrifugation was performed at 37600*g* for 12 h at 4 °C to remove the oligonucleotides that did not anneal to the *fd* DNA. The sucrose gradient purified DNAs were used directly for DNA helicase assay. ^{3}H-Labeled oligo(dT)-tailed hexadecamer was prepared essentially according to the method reported by Hübscher and Stalder (1985). The average size of the products was about 30 bases long.

Helicase Assay. The standard reaction mixture (20 μL) contained 50 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 5 mM ATP, 0.5 mg/mL BSA, 0.017 pmol of the ^{32}P-labeled helicase substrate or 0.034 pmol of the ^{3}H-labeled helicase substrate (4000 cpm), and the purified DNA-dependent ATPase B fraction. Incubation was carried out at 37 °C. The reaction was terminated by chilling the reaction mixture to 0 °C and adding 5 μL of the solution containing 75 mM Na_3EDTA, 5% Sarkosyl (w/v), 0.1% bromophenol blue, and 30% (v/v) glycerol. The sample was allowed to stand at 0 °C for 20 min, and 10 μL of the sample was loaded onto a 6% polyacrylamide gel in TBE buffer (89 mM Tris–borate, pH 8.2, and 2 mM EDTA) and electrophoresed. Autoradiography was performed at −80 °C using Kodak X-Omat-AR film. When the ^{3}H-labeled substrate was used for helicase assay, the portion of the acrylamide containing displaced oligonucleotides and that containing nondisplaced helicase substrates were cut out, and the radioactivity in the gel was extracted with 1 mL of Soluene-350 at 55 °C for 12 h and counted in toluene-based scintillation fluid. The helicase activity was calculated by the following formula: $X = P/(P + S)$. P is the cpm of the displaced oligonucleotides; S is the cpm of nondisplaced substrates. The helicase activity was normalized with the positive and negative controls by the following formula: DNA helicase activity (%) = $100[(X_{sample} - X_n)/(X_p - X_n)]$. X_n is the negative control assayed at 37 °C with no enzyme; X_p is the positive control when the reaction mixture containing no enzyme was heated in boiling water for 1 min.

Results

Detection of DNA Helicase Activity in Purified DNA-Dependent ATPase B Fraction. In order to detect a limited unwinding, we have constructed a helicase substrate consisting

[1] Abbreviations: AMP-PCP, 5′-adenylyl methylenediphosphate; AMP-PNP, 5′-adenylyl imidodiphosphate; ATP-γ-S, adenosine 5′-*O*-(3-thiotriphosphate); BSA, bovine serum albumin; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride.

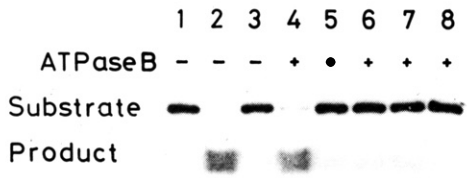

FIGURE 1: Detection of DNA helicase activity. The DNA helicase substrate (0.017 pmol of *fd* DNA) containing ^{32}P-labeled hexadecamer bearing the 3′-dT tail was incubated in the standard reaction mixture with or without 9 units of DNA-dependent ATPase B for 20 min. Electrophoresis and autoradiography were performed as described under Materials and Methods. Lane 1, incubated at 0 °C without the enzyme; lane 2, incubated at 100 °C for 1 min without the enzyme; lane 3, incubated at 37 °C without the enzyme; lane 4, incubated at 37 °C with the enzyme; lane 5, incubated at 37 °C with heat-inactivated enzyme; lane 6, incubated at 0 °C with the enzyme; lane 7, incubated at 37 °C with the enzyme without ATP; lane 8, same as lane 4 except that the ATP was replaced by ATP-γ-S.

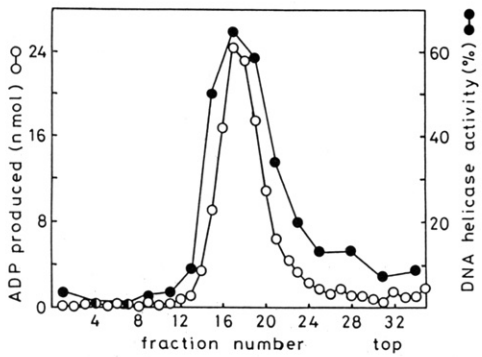

FIGURE 2: Cosedimentation of DNA-dependent ATPase activity and DNA helicase activity. Two hundred microliters of the purified DNA-dependent ATPase fraction (3600 units, 51 000 units/mg of protein) was dialyzed against 40 mM KCl in buffer 2 (20 mM potassium phosphate, pH 7.5, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 0.25 mM PMSF, 1% ethanol, and 20% ethylene glycol). The dialysate was layered onto a 4.8-mL linear gradient of glycerol from 15% to 35% (v/v) in buffer 2 containing 40 mM KCl and 0.5 mg/mL BSA. Centrifugation was performed at 258000*g* for 20 h at 4 °C. An aliquot of each fraction (15 μL) was incubated at 37 °C for 90 min for the DNA-dependent ATPase assay (○), and 6 μL of each fraction was incubated at 37 °C for 30 min with the ^{3}H-labeled helicase substrate (0.034 pmol) for the helicase assay (●) as described under Materials and Methods.

of single-stranded circular *fd* DNA and hexadecamer complementary to the *fd* DNA, which is labeled with ^{32}P at the 5′ end and bears an oligo(dT) tail at the 3′ terminus. When the helicase substrate was incubated at 37 °C with the purified DNA-dependent ATPase B fraction in the presence of ATP, the ^{32}P-labeled oligonucleotide was displaced from the *fd* DNA (Figure 1, lane 4). The displacement activity was destroyed when the ATPase was inactivated by heating in boiling water for 2 min (lane 5). No detectable displacement activity was observed when the reaction was carried out at 0 °C (lane 6). The displacement of the fragment was dependent on the presence of ATP (lane 7), and replacement of ATP by the nonhydrolyzable ATP analogue adenosine 5′-*O*-(3-thiotriphosphate) prevented the helicase activity (lane 8).

DNA-Dependent ATPase B Is a Helicase. The purified DNA-dependent ATPase B was subjected to glycerol density gradient centrifugation. The ATPase sedimented at 5.5 S in the gradient containing 40 mM KCl as described previously (Watanabe et al., 1982). As shown in Figure 2, DNA helicase activity cosedimented with the DNA-dependent ATPase activity, suggesting that the DNA helicase activity is an intrinsic property of the DNA-dependent ATPase.

To confirm further that the DNA-dependent ATPase has DNA helicase activity, the requirement of nucleoside triphosphate for the helicase activity was examined. As shown in Table I, the efficiency of a nucleoside triphosphate to serve as cofactor for the helicase activity correlated with the capacity of the nucleotide to serve as substrate for the DNA-dependent ATPase activity.

Table I: Nucleotide Specificity for DNA-Dependent ATPase Activity and DNA Helicase Activity[a]

DNA-dependent ATPase activity		DNA helicase activity			
nucleotide	%	nucleotide	%	nucleotide	%
ATP	100[b]	ATP	100[b]	dTTP	32
CTP	35	CTP	45	ADP	3
GTP	38	GTP	48	AMP	0
UTP	36	UTP	45	AMP-PCP	2
dATP	74	dATP	60	AMP-PNP	4
		dCTP	20	ATP-γ-S	2
		dGTP	54	none	2

[a] DNA-dependent ATPase activity was assayed with 26 units of DNA-dependent ATPase B in the presence of 0.26 pmol of single-stranded circular *fd* DNA and a 5 mM sample of the indicated nucleoside triphosphate as described under Materials and Methods. Incubations were carried out at 37 °C for 90 min. DNA helicase reactions were carried out at 37 °C for 10 min with 5 units of DNA-dependent ATPase B in the presence of 0.034 pmol of the ^{3}H-labeled helicase substrate and a 5 mM sample of the indicated nucleotide. [b] The values indicate the percent of the activity in the presence of ATP as 100%. The 100% values correspond to 39 nmol/90 min and 52% for DNA-dependent ATPase activity and for DNA helicase activity, respectively.

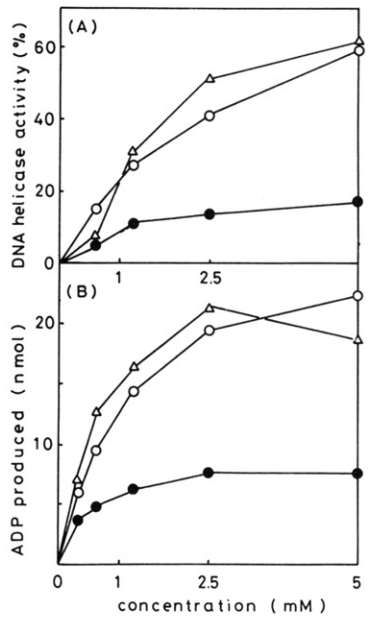

FIGURE 3: Effect of divalent cations on the DNA helicase activity and DNA-dependent ATPase activity of ATPase B. (A) The effect of divalent cations on the DNA helicase activity was examined with 5 units of ATPase B in the presence of 5 mM ATP. Helicase reactions were carried out for 10 min at 37 °C with 0.034 pmol of the ^{3}H-labeled helicase substrate. The activity is normalized as described under Materials and Methods. (B) The effect of divalent cations on the DNA-dependent ATPase activity was examined with 15 units of ATPase B in the presence of 5 mM ATP. Reactions were performed for 90 min at 37 °C as described under Materials and Methods. $MgCl_2$ (○), $MnCl_2$ (△), $CaCl_2$ (●).

Characterization of DNA Helicase Activity. The helicase required a divalent cation for activity. In the presence of 5 mM ATP, the optimal concentrations of Mg^{2+}, Mn^{2+}, and Ca^{2+} were 5 mM. The maximum activity obtained with Mn^{2+} was almost the same as that with Mg^{2+}. The efficiency of Ca^{2+} was lower than that of Mg^{2+}, and Ca^{2+}, at an optimal level, supported 30% of the maximal activity obtained with Mg^{2+}

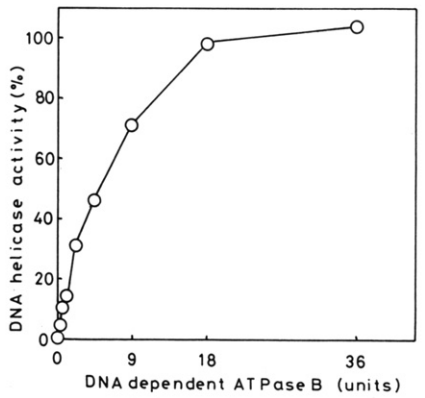

FIGURE 4: DNA helicase activity as a function of the amount of ATPase B. Increasing amounts of DNA-dependent ATPase B were incubated at 37 °C for 30 min in the reaction mixture containing 0.034 pmol of the ^{3}H-labeled helicase substrate. The activity is normalized as described under Materials and Methods.

(Figure 3A). The requirement of divalent cations for the helicase activity was very similar to that for the DNA-dependent ATPase activity of ATPase B (Figure 3B).

ATP was the most effective cofactor among ribo- and deoxyribonucleoside triphosphates tested (Table I). ADP and AMP were not effective for the DNA helicase activity. The nonhydrolyzable ATP analogues AMP-PCP, AMP-PNP, and ATP-γ-S were also not capable of serving as cofactor for the helicase activity, suggesting that hydrolysis of nucleoside triphosphate is essential for the enzyme to exhibit DNA helicase activity.

The optimal concentration of NaCl was 75 mM, and the inhibition of enzyme activity was observed at concentrations higher than 100 mM.

Under optimal conditions, the displacement reaction continued for up to 15 min and reached a plateau by further incubation. DNA helicase activity increased with the amount of DNA-dependent ATPase activity up to 18 units (Figure 4).

Existence of Single-Stranded DNA on Both Strands at the Border of the Duplex Region Is Not Essential for the Helicase Activity. It is known that the helicase activities of the *E. coli dna B* protein (LeBowitz & McMacken, 1986) and bacteriophage T7 gene *4* protein (Matson et al., 1983) require single-stranded DNA on both strands at the border of the duplex region. To examine whether the helicase activity of DNA-dependent ATPase B requires single-stranded DNA on both strands at the border of the duplex region, three kinds of DNA substrates were used to assay the helicase activity. The helicase displaced the hexadecamer with no tail as well as the hexadecamer bearing the 3′ or 5′ tail. Figure 5 shows the time courses of the displacement of the three substrates. It is clear from the result that the efficiency of displacement is almost the same among the three substrates.

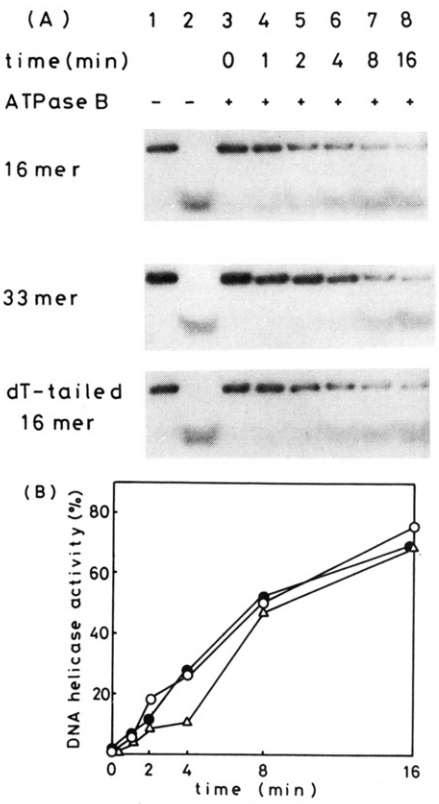

FIGURE 5: Substrate structure required for the helicase activity. (A) Autoradiograms of electrophoresis gels showing the displacement helicase activity. Helicase reactions were carried out at 37 °C for the indicated periods with 5 units of ATPase B in the presence of 0.017 pmol of helicase substrates. The helicase substrates used are ^{32}P-labeled hexadecamer annealed to *fd* DNA, ^{32}P-labeled 33-mer containing a 17-base-long noncomplementary sequence at the 5′ side annealed to *fd* DNA, and ^{32}P-labeled hexadecamer bearing an oligo(dT) tail at the 3′ end annealed to *fd* DNA. Lane 1, incubated at 0 °C without the enzyme; lane 2, heated at 100 °C without the enzyme; lane 3, incubated for 0 min at 37 °C with the enzyme; lane 4, 1 min; lane 5, 2 min; lane 6, 4 min; lane 7, 8 min; lane 8, 16 min. (B) Time courses of the helicase activity with various substrates. The values were determined by the densitometric tracings of the autoradiograms and normalized as described under Materials and Methods. 16-mer (○); 33-mer (△); dT-tailed 16-mer (●).

Discussion

The purified DNA-dependent ATPase B fraction used here was devoid of contaminating endodeoxyribonuclease, DNA polymerase β and γ, or topoisomerase type I and type II activities. In addition, although a very low level of DNA polymerase α activity was detected in the purified fraction, the remaining polymerase activity was separated from the ATPase by glycerol gradient centrifugation under low-salt conditions as described previously (Watanabe et al., 1982; Seki et al., 1986). The property of DNA-dependent ATPase B to hydrolyze the four ribonucleoside triphosphates and dATP (Table I) distinguishes the enzyme from the other three DNA-dependent ATPases from FM3A cells, which hydrolyze only ATP, or ATP and dATP (Tawaragi et al., 1984). Cosedimentation of DNA helicase activity with DNA-dependent ATPase activity (Figure 2) suggests that ATPase B has helicase activity. The observation that the efficiency of a nucleoside triphosphate to serve as cofactor for the helicase activity correlates with the capacity of the nucleotide to serve as substrate for the DNA-dependent ATPase activity further supports the above possibility.

It is reported that the helicase activities of the *E. coli dna B* protein (LeBowitz & McMacken, 1986) and bacteriophage T7 gene *4* protein (Matson et al., 1983) require single-stranded DNA on both strands at the border of the duplex region, that is, a replication fork like structure. However, the results shown in Figure 5 indicated that the helicase reported here did not require a replication fork like structure. The observation is not surprising because many proteins showing DNA helicase activity such as *E. coli* helicases, I, II, and III, *rep* protein, T4 *dda* protein, and T4 gene *41* protein do not require single-stranded DNA on both strands at the border of the duplex region (Yarranton & Gefter, 1979; Geider & Hoffmann-Berling, 1981; Venkatesan et al., 1982).

The in vivo functions of ATPase B are presently unknown. Stahl et al. (1986) have shown that SV40 large T antigen,

which is known to possess ATPase activity and to be essential for SV40 DNA replication, has DNA helicase activity. Recently, two DNA-dependent ATPases have been purified from calf thymus (Hübscher & Stalder, 1985) and yeast cells (Sugino et al. 1986). DNA-dependent ATPase III from yeast, which has been shown to have DNA helicase activity, stimulates DNA polymerase I in vitro. The calf thymus DNA-dependent ATPase associated with one form of DNA polymerase α holoenzyme (Ottiger & Hübscher, 1984) has been also shown to have DNA helicase activity (Hübscher & Stalder, 1985). From the observation of the association of the helicase with a DNA polymerase α holoenzyme, it has been speculated that the helicase might act in concert with DNA polymerase α at the leading strand, possibly pushing the replication fork ahead of the polymerase. In a previous study, we found that DNA-dependent ATPase B was associated with DNA polymerase α during the early step of the purification and that the ATPase could be separated from DNA polymerase α by glycerol gradient centrifugation under low ionic strength conditions (Watanabe et al., 1982). The calf thymus helicase can also be separated from DNA polymerase α by velocity sedimentation under conditions similar to those reported by us. In addition, the DNA-dependent ATPase from FM3A cells and calf thymus have similar s values (5.5–6.5 S). These observations seem to suggest that the mouse and calf thymus ATPases are similar enzymes.

ACKNOWLEDGMENTS

We thank Dr. Y. Ishimi for helpful discussions. We also thank H. Ohno for her help in preparing the manuscript.

Registry No. ATP, 56-65-5; CTP, 65-47-4; GTP, 86-01-1; UTP, 63-39-8; dATP, 1927-31-7; dGTP, 2564-35-4; ATPase, 9000-83-3.

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