Further Characterization of DNA Helicase Activity of Mouse DNA-Dependent Adenosinetriphosphatase B (DNA Helicase B)†

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ABSTRACT: The DNA helicase activity of DNA-dependent ATPase B purified from mouse FM3A cells [Seki, M., Enomoto, T., Hanaoka, F., & Yamada, M. (1987) *Biochemistry 26*, 2924–2928] has been further characterized. The helicase activity was assayed with partially duplex DNA substrates in which oligonucleotides to be released by the enzyme were radiolabeled. Oligonucleotides with or without phosphate at the 5′ termini or with a deoxy- or dideoxyribose at the 3′-terminal nucleotides were displaced by this enzyme with essentially the same efficiency and with the same ATP (and dATP) and Mg^{2+} requirements. Thus, there was no strict structure requirement for both ends of duplex regions of substrates to be unwound by the enzyme. Shorter strands were released more readily than longer strands up to the length of 140 bases. The attachment of the enzyme to a single-stranded DNA region was a prerequisite for the neighboring duplex to be unwound; the enzyme-catalyzed unwinding was inhibited competitively by the coaddition of single-stranded DNAs which act as cofactors of the ATPase activity. Their activities as the inhibitor of helicase were well correlated with those as the cofactor of ATPase. The helicase B was found to migrate along single-stranded DNA in the 5′ to 3′ direction by the use of single strands with short duplex regions at both 3′ and 5′ ends as substrate. A possible role of this enzyme in DNA replication in mammalian cells is discussed.

Enzymes having a DNA helicase activity have been shown to be essential for DNA replication in various prokaryotic systems and in the eukaryotic SV40 replication system (Kornberg, 1980; Stahl et al., 1986; Dean et al., 1987). The *Escherichia coli dnaB* protein (LeBowitz & McMacken, 1986), the phage T4 gene 41 protein (Venkatesan et al., 1982), and the T7 gene 4 protein (Matson et al., 1983), all of which exhibit DNA helicase activity, enable primases (the *E. coli dnaG* protein, the T4 gene 61 protein, and the T7 gene 4 protein itself) to synthesize primer RNAs (Kornberg, 1982; Nossal & Alberts, 1983; Richardson, 1983). The helicase–primase combination may be especially effective in DNA synthesis on the lagging strand of duplex DNA.

The *dnaB* protein is also essential for the initiation of the replication of oriC and oriλ plasmids containing the replication origins of *E. coli* and phage λ chromosomes, respectively. In both oriC and oriλ systems, the *dnaB* protein is required for the formation of an origin-specific underwound template, "pre-priming complex" (Baker et al., 1986; Dodson et al., 1986). The *dnaB* protein may function as a helicase during both initiation and elongation phases of replication of the *E. coli* and phage λ chromosomes. The SV40 large tumor antigen (T antigen) has recently been shown to possess a DNA helicase activity (Stahl et al., 1986) and an origin-specific unwinding activity (Dean et al., 1987). The T antigen was also suggested to be involved not only in the initiation but also in the elongation step of SV40 DNA replication (Stahl et al., 1985). These observations suggest that a certain helicase from higher eukaryotic cells, if any, may play a key role in the cellular chromosome replication, which has been the subject of our recent research.

All the prokaryotic helicases so far reported exhibited DNA-dependent ATPase (NTPase) activity. Eukaryotic DNA-dependent ATPases, probably participating in DNA replication, have been isolated from various sources and characterized (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; Watanabe et al., 1981; Thomas & Meyer, 1982; Brewer et al., 1983; Hochensmith et al., 1986). Among them, the lily ATPase (Hotta & Stern, 1978), the calf thymus ATPase (Hübscher & Stalder, 1985), and the yeast ATPase III (Sugino et al., 1986) exhibited DNA helicase activity.

We have recently isolated four forms of DNA-dependent ATPase from mouse FM3A cells, one of which, ATPase B, has been characterized in detail (Watanabe et al., 1982; Tawaragi et al., 1984; Enomoto et al., 1984; Seki et al., 1986). This enzyme will henceforth be referred to as DNA helicase B, since it displayed a DNA helicase activity (Seki et al., 1987). The purpose of the present study is to characterize the helicase activity of this enzyme further with special reference to the size of DNA strands to be displaced and the direction of translocation of the enzyme on the DNA strand.

Materials and Methods

Materials. [γ-^{32}P]ATP was prepared with the Promega Biotec's γ prep synthesis system from ADP and ^{32}P-labeled inorganic phosphate. Phosphorus-32 and [α-^{32}P]dCTP were purchased from NEN and ICN, respectively. Nucleoside triphosphates, ADP, and AMP were obtained from Yamasa Biochemicals; 5′-adenylyl methylenediphosphate (AMP-PCP),[1] AMP-PNP, ATP-γ-S, dideoxyGTP, dideoxyTTP, and *E. coli*

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

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DNA polymerase I Klenow fragment were from Boehringer Mannheim; T4 polynucleotide kinase and *Sma*I endonuclease were from Takara Biochemicals. Single-stranded circular M13mp19 DNA was prepared according to the method of Messing (1983). The 33-base-long oligonucleotides (the 33-mer) with the 5′ terminus of OH, 5′-CGAGCTCGGTACCCGGGGATCCTCTAGAGTCGA-3′ complementary to M13mp19 DNA, were synthesized with a Beckman DNA synthesizer.

Purification of DNA-Dependent ATPase B (DNA Helicase B). DNA helicase B was purified from mouse FM3A cells by sequential chromatographies on DEAE-cellulose (Brown), DE-52 (Whatman), phosphocellulose, hydroxyapatite, single-stranded DNA–cellulose, and phosphocellulose columns 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.

Preparation of 5′-Labeled Helicase Substrate. DNA concentrations are expressed in molarity of molecules. Thirty picomoles of 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 (4000 Ci/mmol) to label the 5′ end of the oligonucleotide. The short duplex was then prepared by annealing 20 pmol of the thus labeled 33-mer to 30 pmol of single-stranded circular M13mp19 DNA in 180 μL of the annealing medium consisting of 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 NaCl, and 1 mM DTT. The annealing mixture was first heated at 100 °C for 4 min to denature the DNAs and then kept at 67 °C for 60 min and allowed to stand at 37 °C for 30 min. These heat treatments were followed by the addition of EDTA and Sarkosyl at final concentrations of 25 mM and 0.25%, respectively. The mixture was incubated at 37 °C for 20 min, chilled in an ice–water bath, and then loaded on 4.8 mL of a 5–20% sucrose gradient containing 50 mM Tris-HCl, pH 7.5, 1 mM Na_3EDTA, and 0.1 M NaCl. After centrifugation at 57000*g* for 14 h at 4 °C, the fraction containing the oligonucleotides annealed to M13mp19 DNA was taken and was used as DNA helicase substrate without further purification.

Preparation of 3′-Labeled Helicase Substrate. Fifteen picomoles of the 33-mer was annealed to 30 pmol of single-stranded circular M13mp19 DNA by heating in 90 μL of the annealing medium as described above. The resulting partial duplex was labeled at the 3′-OH of the 33-mer in the reaction medium (180 μL) consisting of 25 units of DNA polymerase I Klenow fragment, 2.4 μM [α-^{32}P]dCTP (620 Ci/mmol), 1 mM dideoxyTTP, 15 mM Tris-HCl, pH 7.9, 7.5 mM $MgCl_2$, and 1 mM DTT. The mixture was incubated for 20 min as such and then for an additional 15 min with further addition of 50 μM unlabeled dCTP at 25 °C. The procedures adopted for the purification of the 5′-labeled helicase substrate (see above) were applied to purify the M13mp19 DNA annealed to the 36-mer consisting of the original 33-mer and elongated radioactive CCT (dideoxy).

Preparation of Helicase Substrates Containing Duplex Regions of Various Length. Fifteen picomoles of the 33-mer was annealed to 30 pmol of M13mp19 DNA by heating in 90 μL of the medium consisting of 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, and 1 mM DTT as described above. The resulting partial duplex was elongated from the 3′-OH of the 33-mer in the reaction mixture (180 μL) containing 25 units of DNA polymerase I Klenow fragment, 1 μM [α-^{32}P]dCTP (3000 Ci/mmol), 0.5 mM dATP, 0.5 mM dTTP, 0.05 mM dGTP, 0.3 mM dideoxyGTP, 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, and 1 mM DTT. The mixture was incubated at 25 °C for 20 min and then supplemented with 50 μM unlabeled dCTP and 7 units of DNA polymerase I Klenow fragment to be further incubated under the same conditions. The labeled helicase substrates thus prepared were purified as described above.

Preparation of Helicase Substrate Used To Determine the Direction of Translocation. Seven picomoles of the 5′-labeled helicase substrate or the 3′-labeled helicase substrate was dissolved in 50 μL of the *Sma*I digestion buffer containing 10 mM Tris-HCl, pH 8.0, 7 mM $MgCl_2$, 20 mM KCl, 7 mM 2-mercaptoethanol, and 100 μg/mL bovine serum albumin. Sixteen units of *Sma*I endonuclease were added to the mixture. Incubation was performed for 60 min at 30 °C and terminated by the addition of EDTA and NaCl at final concentrations of 8 mM and 0.1 M, respectively. The aliquots of the thus digested DNA were used for DNA helicase assay.

DNA Helicase Assay. The standard reaction mixture (20 μL) consisted of 50 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 5 mM ATP, 0.5 mg/mL bovine serum albumin, and 0.017 pmol (5 μM) of the ^{32}P-labeled helicase substrate. The reaction was started by the addition of the purified DNA-dependent ATPase B fraction and terminated by chilling the reaction mixture to 0 °C followed by the addition of 5 μL of solution containing 75 mM Na_3EDTA, 5% (w/v) Sarkosyl, 0.1% bromophenol blue, and 30% (v/v) glycerol. After being kept at 0 °C for 20 min, a 25-μL aliquot was loaded on a 12% polyacrylamide gel in TBE buffer (89 mM Tris–borate, pH 8.2, and 2 mM EDTA) and subjected to electrophoresis. Autoradiography was performed at −80 °C using Kodak X-Omat AR film. The helicase activity was determined on the basis of densitometric tracings of autoradiograms. The activity was normalized by the following formula: $X = P/(P + S)$, where P (products) is the value for the displaced oligonucleotides and S (substrates) is the value for nondisplaced substrates. The helicase activity is expressed as a percentage of the control value, i.e., $100 \times [(X_{sample} - X_n)/(X_p - X_n)]$, where X_n is the negative control assayed at 37 °C without enzyme and X_p is the positive control where the reaction mixture containing no enzyme was heated in boiling water for 2 min.

RESULTS

DNA Helicase Activities Measured with DNA Substrates Containing Oligonucleotides with Modified 5′ and 3′ Termini. Mouse FM3A DNA-dependent ATPase B was shown to exhibit DNA helicase activity that was measured with a DNA substrate consisting of a single-stranded circular DNA and a complementary oligonucleotide with an oligo(dT) tail at the 3′ terminus (Seki et al., 1987). The complementary oligonucleotide with no tail was also displaced by the helicase. The activity of DNA helicase B was then measured with DNA substrates containing nontailed complementary oligonucleotides with modified 5′ and 3′ termini. The 33-mer (substrate A in Figure 1) was so modified as to have a hydroxy group instead of a phosphate group at the 5′ end and a dideoxynucleotide at the 3′ terminus (36-mer, substrate B in Figure 1). The 33-mer and 36-mer oligonucleotides were annealed to single-stranded circular M13mp19 DNA as shown in Figure 1. DNA helicase B displaced the 33-mer and 36-mer to a similar extent when the reaction mixture was added with ATP (Figure 1, lane 4) or dATP (lane 14) but was ineffective when ATP was omitted (lane 7) or replaced by AMP-PCP, AMP-PNP, ATP-γ-S, ADP, or AMP (lanes 9–13). No helicase activity was observed with any of these two substrates at 0 °C (lane 6), or in the absence of Mg^{2+} (lane 8), or for

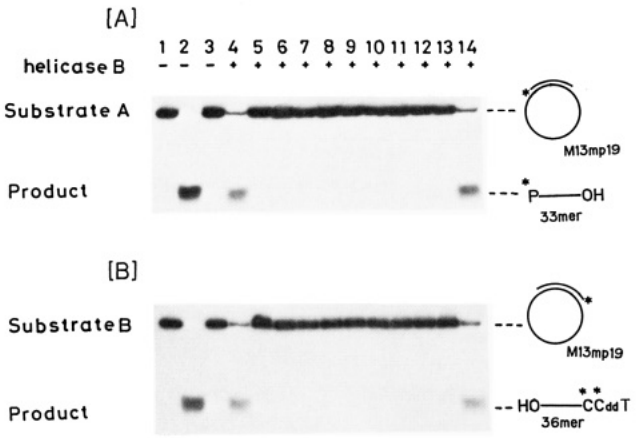

FIGURE 1: DNA helicase activity measured with two substrates possessing different 5′ and 3′ termini of the duplex region. The structures of the substrates and anticipated products are shown on the right-hand side of this figure. The substrates were incubated for 30 min in the standard reaction mixture with or without 57 units of DNA-dependent ATPase B (helicase B). Electrophoresis and autoradiography were performed as described under Materials and Methods. Assay conditions are as follows: lane 1, 0 °C without enzyme; lane 2, 100 °C for 2 min without enzyme; lane 3, 37 °C without enzyme; lane 4, 37 °C with enzyme; lane 5, 37 °C with heat-inactivated enzyme; lane 6, 0 °C with enzyme; lane 7, 37 °C with enzyme in the absence of ATP; lane 8, the same as lane 4 except that 10 mM EDTA was added; lanes 9, 10, 11, 12, 13, and 14, the same as lane 4 except that ATP was replaced by AMP-PCP, AMP-PNP, ATP-γ-S, ADP, AMP, and dATP, respectively.

the enzyme which had been heated in boiling water for 2 min (lane 5). Since no difference was observed in the ability to serve as helicase substrate between the modified (B) and unmodified (A) substrates, it is unlikely that the structure of 5′ and 3′ termini of oligonucleotides in the helicase substrate is strictly required for the helicase activity of DNA helicase B.

Size of Complementary DNA Displaceable by Helicase B from Substrate Duplex. The helicase substrates consisting of M13mp19 DNA and complementary DNA strands of various length terminated by dideoxyGMP were prepared to determine the size of DNA displaceable by the helicase. Dideoxyribonucleotide at the 3′ ends of the DNA strands did not interfere with the susceptibility of these DNA strands to the helicase as shown in Figure 1B. The helicase substrates were incubated for 20 min with increasing amounts of DNA helicase B (Figure 2). Under these conditions, single-stranded DNAs up to about 140 nucleotides in length were released; the longer DNA strands were not released even in the presence of the largest amount (114 units) of the enzyme. It is obvious, in comparison with the positive control (Figure 2, lane 2), that shorter DNA strands were much readily displaced than longer strands. The time course of the reaction was examined with 57 units of the enzyme (Figure 3). No displacement was detected with DNAs of longer than 150 bases even after the longest incubation time of 60 min.

Inhibition of Helicase Activity by the Addition of Single-Stranded DNA. DNA was an essential cofactor for the ATPase activity of the helicase B; single-stranded DNA was preferable to double-stranded DNA in this regard (Enomoto et al., 1984; Seki et al., 1986). It is probable, therefore, that the binding of the helicase to single-stranded regions of substrate DNAs is essential for the enzyme to exhibit helicase activity. The binding may be antagonized by the coaddition of single-stranded DNA which by itself does not serve as a substrate for the helicase. In fact, the helicase activity estimated with a constant amount of substrate was inhibited progressively by the addition of increasing amounts of heat-denatured calf thymus DNA (Figure 4), suggesting that the

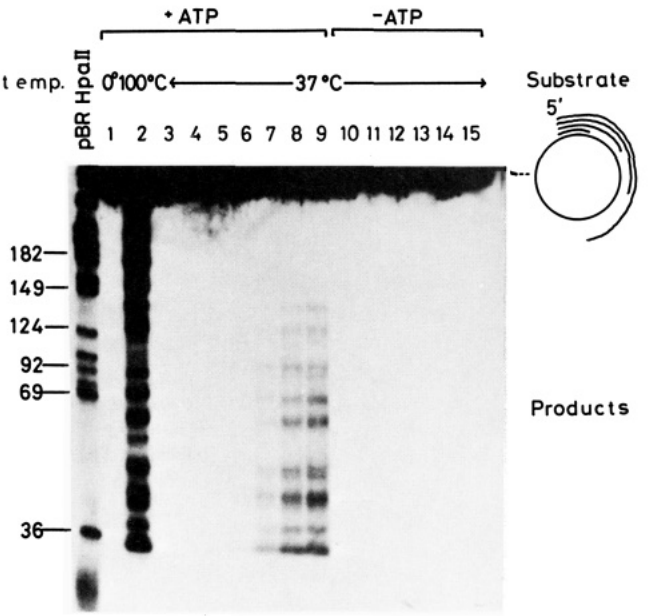

FIGURE 2: DNA helicase B activity with substrates with duplex regions of various length. The DNA substrates (0.017 pmol) having duplex regions of various length were incubated for 20 min with various amounts of DNA helicase B. The left lane shows *Hpa*II-digested ^{32}P-labeled pBR322 markers. Assay conditions are as follows: lane 1, 0 °C without enzyme; lane 2, 100 °C for 2 min without enzyme; lane 3, 37 °C without enzyme; lanes 4, 5, 6, 7, 8, and 9, 37 °C with 3.6, 7.1, 14, 29, 57, and 114 units of DNA-dependent ATPase B, respectively; lanes 10, 11, 12, 13, 14, and 15, 37 °C with 3.6, 7.1, 14, 29, 57, and 114 units of enzyme in the absence of ATP.

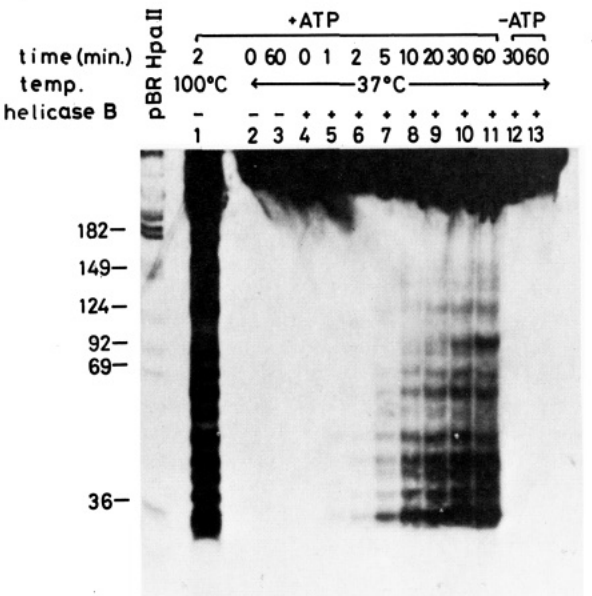

FIGURE 3: Kinetics of unwinding of the DNA substrates having duplex regions of various length by helicase B. The DNA substrates having duplex regions of various length were incubated with 57 units of DNA-dependent ATPase B at 37 °C for various times. The left-side lanes show *Hpa*II-digested ^{32}P-labeled pBR322 DNA markers. Assay conditions are as follows: lane 1, 100 °C for 2 min without enzyme; lanes 2 and 3, 37 °C without enzyme for 0 and 60 min, respectively; lanes 4, 5, 6, 7, 8, 9, 10, and 11, 37 °C with enzyme for 0, 1, 2, 5, 10, 20, 30, and 60 min, respectively; lanes 12 and 13, 37 °C with enzyme in the absence of ATP for 30 and 60 min, respectively.

inhibition is probably due to the competition of the DNA with the substrate. The inhibitory activities of various nucleic acids were compared in Figure 5. Single-stranded DNAs were effective inhibitors, and poly(dT), which is the most effective cofactor for the DNA-dependent ATPase activity (Seki et al., 1986), inhibited the helicase activity most strongly. The order

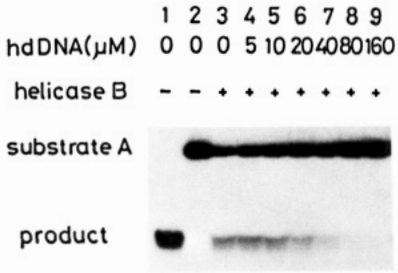

FIGURE 4: Inhibition of helicase activity by the addition of heat-denatured DNA. The 5′-labeled DNA helicase substrate (substrate A, 0.017 pmol = 5 μM) was incubated at 37 °C with 29 units of DNA-dependent ATPase B for 20 min in the presence of various amounts of heat-denatured calf thymus DNA or in its absence. Lane 1, 100 °C for 2 min without enzyme; lane 2, 37 °C without enzyme; lane 3, 37 °C with enzyme; lanes 4, 5, 6, 7, 8, and 9, 37 °C with enzyme in the presence of 5, 10, 20, 40, 80, and 160 μM heat-denatured calf thymus DNA, respectively.

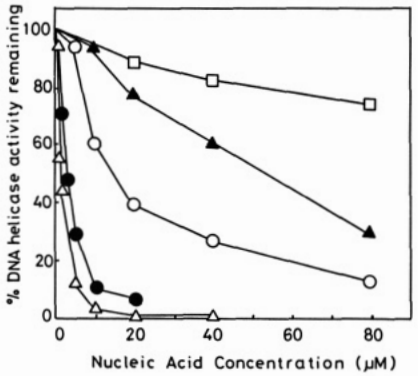

FIGURE 5: Inhibition of helicase activity by the addition of various nucleic acids. The effect of various nucleic acids on the DNA helicase activity with substrate A (see Figure 1) was examined with 29 units of the enzyme. Helicase reactions were carried out for 20 min at 37 °C with 0.017 pmol of (or 5 μM) substrate A. The values were determined by densitometric tracing of the autoradiograms and normalized as described under Materials and Methods. One hundred percent is the value without the addition of nucleic acids: poly[d(A-T)] (□); poly(U) (▲); heat-denatured calf thymus DNA (○); single-stranded circular fd DNA (●); poly(dT) (△).

of potency as inhibitor was poly(dT) > single-stranded circular fd DNA > heat-denatured calf thymus DNA > poly(U). Their concentrations required for 50% inhibition were roughly 1, 2.5, 20, and 50 μM, respectively. Only a slight inhibition was observed with poly[d(A-T)]. Essentially no inhibition was caused by other nucleic acids such as native calf thymus DNA, pBR322 RFI, activated DNA, $(dA)_{12-18}$, $(dT)_{12-18}$, and yeast tRNA (data not shown).

Direction of Translocation of Helicase B. Taking advantage of the fact that the binding of helicase B to single-stranded regions is essential for unwinding of the substrate DNA duplex, the direction of movement of the enzyme on DNA strands was determined by the strategy illustrated in Figure 6. As shown in Figure 6, a pair of linearized helicase substrates (substrates A′ and B′) were prepared by digesting substrates A and B with *Sma*I endonuclease. Since substrates A′ and B′ contain duplex regions at both ends of a long linear molecule, helicase B must bind first to the internal single-stranded regions of these substrates. If the enzyme subsequently moves from 3′ to 5′ along the single-stranded DNA segment, it would displace the radioactive 14-mer from substrate A′. In contrast, the radioactive 22-mer would be librated from substrate B′, if the enzyme migrates in a 5′ to 3′ direction. As shown in Figure 7, helicase B displaced the radioactive 22-mer from substrate B′ (lanes 13 and 16) but did not displace the 14-mer from substrate A′ (lanes 10 and 16). This result strongly suggests

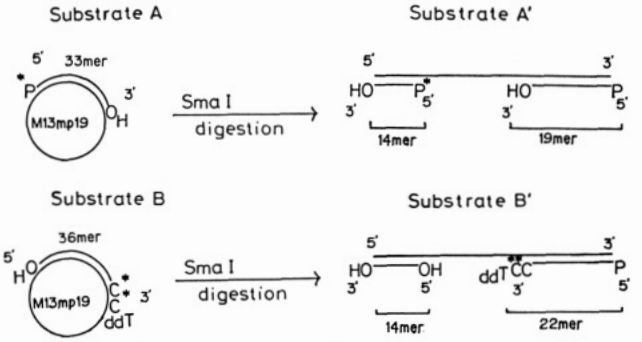

FIGURE 6: DNA helicase substrates designed to determine the direction of translocation of helicase B. The 5′-labeled helicase substrate (substrate A) and the 3′-labeled helicase substrate (substrate B) were digested with *Sma*I endonuclease as described under Materials and Methods to construct linear substrates (A′ and B′). These substrates (substrates A′ and B′) were used in the experiments shown in Figure 7.

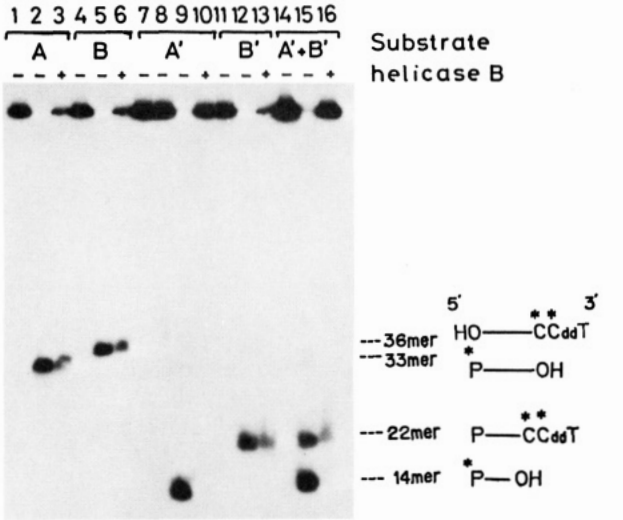

FIGURE 7: Determination of the direction of translocation of helicase B. Substrate A, B, A′, or B′ (0.017 pmol), or the mixture of A′ and B′ (0.017 pmol of each), shown on the top, was incubated with or without 29 units of DNA-dependent ATPase B for 20 min. Lanes 1, 4, 8, 11, and 14, incubated at 37 °C without enzyme; lane 7, incubated at 0 °C without enzyme; lanes 2, 5, 9, 12, and 15, heated at 100 °C for 2 min without enzyme; lanes 3, 6, 10, 13, and 16, incubated at 37 °C with enzyme.

that helicase B moves unidirectionally in a 5′ to 3′ direction along the DNA strand to which it binds.

DISCUSSION

We have shown an earlier paper that the mouse FM3A DNA-dependent ATPase B has a DNA helicase activity (Seki et al., 1987). The helicase was able to displace oligonucleotides with no tail as well as those with an oligo(dT) tail at the 3′ termini. Thus, this helicase does not require single-stranded DNA on both strands at the border of the duplex regions; i.e., the replication fork like structure is not required to perform helix unwinding. In the present study, the helicase activity has been characterized further, and several important properties of this enzyme have been found as follows.

First, oligonucleotides having a dephospho- and dideoxynucleotide at the 5′ and the 3′ terminus, respectively, were as readily displaced as native oligonucleotides from circular M13mp19 DNA (Figure 1). The selective dependence of the enzymic activity on ATP (or dATP) and Mg^{2+} was the same between the native and modified substrates. Thus, there was no strict structure requirement for 5′ and 3′ termini of the oligonucleotides serving as the substrate of this enzyme.

Second, the shorter DNA strands were released more readily than the longer strands from the helicase substrates. The longest DNA displaceable from the duplex DNA by this enzyme was of about 140-base length under the conditions employed in the present study (Figures 2 and 3). The results are compatible with the recent report by Stahl et al. (1986) that the maximal length of DNA displaceable by the SV40 large T antigen was about 150 bases, suggesting that eukaryotic DNA helicases are not capable of unwinding relatively long duplex regions of DNA. We and others (Falaschi et al., 1980; Tawaragi et al., 1984; Seki et al., 1986) failed to detect DNA helicase activity in the DNA-dependent ATPases purified from several eukaryotic cells including calf thymus and mouse FM3A cells. This is probably because DNA strands with longer duplex regions were used as the test substrate when the enzymic activity was studied. In fact, DNA helicase activity was detected in calf thymus DNA-dependent ATPase by using a DNA duplex of a limited length (Hübscher & Stalder, 1985) and in mouse FM3A DNA-dependent ATPases C1 and C2 by using the same DNA duplex shown in Figure 1 (unpublished data) as helicase substrates. In contrast, the lily DNA-dependent ATPase (Hotta & Stern, 1978) and the yeast ATPase (Sugino et al., 1986) were able to unwind DNA duplexes of 500 and 234 base pairs, respectively.

Third, the binding of this helicase to single-stranded DNA regions is a necessary prerequisite for the enzyme to perform helix unwinding. The experimental support for this conclusion was found in Figures 4 and 5 which showed that the helicase activity was inhibited by the addition of single-stranded DNAs but was not affected by double-stranded DNA. The single-stranded DNAs probably competed with the helicase substrate for helicase binding. In fact, poly(dT), which inhibited the helicase activity most strongly among the DNAs tested, was the most effective cofactor for ATPase activity of the enzyme (Enomoto et al., 1984; Seki et al., 1986). Most of the prokaryotic DNA helicases have also been shown to prefer single-stranded DNAs to double-stranded ones as the cofactor for their DNA-dependent ATPase activity and require free single-stranded DNA regions to perform helix unwinding (Yarranton & Gefter, 1979; Kornberg, 1980; Geider & Hoffmann-Berling, 1981; Venkatesan et al., 1982; Matson et al., 1983; LeBowitz & McMacken, 1986; Matson, 1986).

Finally, the present paper is the first, to our knowledge, to show that eukaryotic helicase translocates unidirectionally in a 5′ to 3′ direction along the DNA strand to which the enzyme binds (Figure 7). The direction is the same as that previously observed with prokaryotic helicases such as the T7 gene 4 protein, the T4 gene 41 protein, and the *dnaB* protein (Tabor & Richardson, 1981; Venkatesan et al., 1982; LeBowitz & McMacken, 1986). An additional important property of these prokaryotic helicases was their interaction with primases (or exhibition of primase activity by itself in one case). This property was essential for these enzymes to support the synthesis of the multiple primers needed for production of the lagging strand DNA chains or Okazaki fragments in prokaryotic cells (Kornberg, 1982; Matson et al., 1983; Richardson, 1983; Nossal & Alberts, 1983). Similarly, the eukaryotic FM3A helicase B may also interact with a primase, as strongly suggested by copurification of this enzyme with DNA polymerase α activity (Watanabe et al., 1982), which has been repeatedly shown to be tightly associated with primase activity. Thus, we propose that the DNA-dependent ATPase B processively migrates along the lagging strand template at a replication fork, acting not only as a DNA helicase but also as a coparticipant with primase in the synthesis of Okazaki fragments. This will be the subject of our forthcoming papers.

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Registry No. ATPase, 9000-83-3; 5′-CGAGCTCGGTACCCGGGGATCCTCTAGAGTCGA-3′, 112548-70-6.

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Modes of DNA Cleavage by the *Eco*RV Restriction Endonuclease†

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ABSTRACT: The mechanism of action of the *Eco*RV restriction endonuclease at its single recognition site on the plasmid pAT153 was analyzed by kinetic methods. In reactions at pH 7.5, close to the optimum for this enzyme, both strands of the DNA were cut in a single concerted reaction: DNA cut in only one strand of the duplex was neither liberated from the enzyme during the catalytic turnover nor accumulated as a steady-state intermediate. In contrast, reactions at pH 6.0 involved the sequential cutting of the two strands of the DNA. Under these conditions, DNA cut in a single strand was an obligatory intermediate in the reaction pathway and a fraction of the nicked DNA dissociated from the enzyme during the turnover. The different reaction profiles are shown to be consistent with a single mechanism in which the kinetic activity of each subunit of the dimeric protein is governed by its affinity for Mg^{2+} ions. At pH 7.5, Mg^{2+} is bound to both subunits of the dimer for virtually the complete period of the catalytic turnover, while at pH 6.0 Mg^{2+} is bound transiently to one subunit at a time. The kinetics of the *Eco*RV nuclease were unaffected by DNA supercoiling.

Class II restriction endonucleases recognize specific base sequences in duplex DNA, typically 4–6 bp[1] long, and cleave both strands of the DNA at fixed locations relative to their recognition sites. The only cofactor that they need for phosphodiester hydrolysis is Mg^{2+} ions. These enzymes thus provide test systems for analyzing how a protein can catalyze a reaction at a specific site on DNA. However, different restriction enzymes can display very different reaction mechanisms (Halford et al., 1979; Potter & Eckstein, 1984). At present, at least 615 restriction enzymes have been identified (Kessler & Holtke, 1986) but only 3, *Eco*RI, *Eco*RV, and *Hha*II, have been crystallized to date (Rosenberg et al., 1978; D'Arcy et al., 1985; Chandrasegeran et al., 1986). For the *Eco*RI endonuclease, the structure of its recognition complex with DNA has been elucidated at high resolution (McClarin et al., 1986), and its mechanism of action in solution has been studied extensively (Modrich, 1982; Halford, 1983). However, high-resolution structures of both *Eco*RV and *Hha*II have yet to be reported, though the mechanism of action of *Hha*II has already been analyzed (Kaddurah-Daouk et al., 1985). We describe here studies on the mechanism of action of the *Eco*RV enzyme.

The *Eco*RV restriction endonuclease cleaves DNA specifically at the sequence

5′-G-A-T↓A-T-C-3′
3′-C-T-A↑T-A-G-5′

at the sites marked by arrows (Schildkraut et al., 1984; D'Arcy et al., 1985). Under standard reaction conditions, all other DNA sequences, even those differing by only one bp, are cut at least 10^4 times more slowly (Halford et al., 1986). The amino acid sequence of the *Eco*RV nuclease predicts a polypeptide of M_r 28 600 (Bougueleret et al., 1984). In solution, it exists as a dimer of two such subunits (D'Arcy et al., 1985; Luke et al., 1987). The size and subunit structure of the *Eco*RV enzyme is thus similar to those of *Eco*RI and many other class II restriction enzymes (Modrich, 1982). However, *Eco*RV not only cleaves DNA at a different site from *Eco*RI

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[1] Abbreviations: bp, base pairs; M_r, relative molecular mass; BME, β-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; K_D, equilibrium dissociation constant.

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