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DNA helicase from mammalian mitochondria

(DNA replication/DNA unwinding/Rep helicase)

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ABSTRACT **In spite of the fact that a DNA helicase is clearly required for the predominately leading-strand synthesis occurring during mammalian mtDNA replication, no such activity has heretofore been identified. We report the characterization of a mammalian mitochondrial DNA helicase isolated from bovine brain tissue. The sucrose gradient-purified mitochondria in which the activity was detected had less than 1 part in 2500 nuclear contamination according to Western blot analysis using nuclear- and mitochondrial-specific probes. Mitochondrial protein fractionation by DEAE-Sephacel chromatography yielded a DNA helicase activity dependent upon hydrolysis of ATP or dATP but not other NTPs or dNTPs. The mitochondrial helicase unwound 15- and 20-base oligonucleotides but was unable to unwind 32-base or longer oligonucleotides, and the polarity of the unwinding is 3′-to-5′ with respect to the single-stranded portion of the partial duplex DNA substrate. This direction of unwinding would place the bovine mitochondrial helicase on the template strand ahead of DNA polymerase γ during mtDNA replication, a situation analogous to that of the Rep helicase of *Escherichia coli* during leading-strand DNA synthesis of certain bacteriophages.**

Mammalian mitochondria contain multiple copies of a 16.5-kilobase (kb) double-stranded DNA genome (mtDNA) (reviewed in refs. 1 and 2). Recent observations suggest that mtDNA copy number may be primarily responsible for regulation of mitochondrial gene expression in mammalian tissue (3, 4). The proteins used for mitochondrial DNA replication, clearly important in controlling mtDNA copy number, are encoded by the nucleus and imported into the organelle. To date, much of the biochemistry of mtDNA metabolism has not been determined. Therefore, to understand that process, we are assembling and investigating components of the bovine mtDNA replication system.

Utilization of duplex DNA for DNA replication requires its unwinding to provide a single-stranded template for DNA polymerase (reviewed in refs. 5 and 6). Enzymes that catalyze this reaction, DNA helicases, act in an NTP/dNTP-dependent manner to separate the two strands of a DNA helix. DNA helicases have been isolated from a number of organisms, including *Escherichia coli*, its bacteriophages, eukaryotic viruses, yeast, lily, *Xenopus*, mouse, cow, and human (5–11). The only presently identified DNA helicase found in mitochondria is involved in DNA repair and recombination in yeast (9, 12). To date no mitochondrial DNA helicase from mammals or any higher eukaryote has been identified.

In this paper, we identify and partially purify a mitochondrial DNA helicase activity from purified bovine mitochondria. This activity unwinds partially duplex DNA in an ATP- or dATP-dependent manner and shows a 3′-to-5′ polarity of movement. Based on these properties, its potential role in the unidirectional synthesis of mtDNA is addressed.

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MATERIALS AND METHODS

Isolation of Bovine Brain Mitochondria and Nuclei. All procedures were carried out at 4°C or on ice. Mitochondria were isolated from bovine brain tissue as described by Hauswirth *et al.* (13) with the modifications described. Approximately 900 g of freshly harvested bovine brain tissue was liquefied in 2.4 liters of MSB (50 mM Tris·HCl, pH 7.5/210 mM mannitol/70 mM sucrose) containing 3 mM $CaCl_2$ in a Waring blender and homogenized in a Potter–Elvehjem tissue grinder. Homogenate was subjected to centrifugation at 1600 × *g* for 10 min; the supernatant was collected for centrifugation as above. Supernatant from the second spin was brought to a final concentration of 20 mM EDTA and centrifuged at 17,700 × *g* for 45 min. The pellet was washed in MSB/10 mM EDTA and resuspended in 120 ml of sucrose buffer (50 mM Tris·HCl, pH 7.5/584 mM sucrose/10 mM EDTA). Aliquots (10 ml) were layered over 1 M/1.5 M sucrose step gradients containing 10 mM Tris·HCl, pH 7.5/5 mM EDTA and were then subjected to centrifugation in a Beckman SW27 rotor at 22,000 rpm for 20 min. Mitochondria present at the 1 M/1.5 M sucrose interface were collected, diluted with an equal volume of MSB/10 mM EDTA, and pelleted at 23,300 × *g* for 15 min. Purified mitochondrial pellets were frozen in a dry ice/ethanol bath and stored at −70°C.

Nuclei were isolated from 70 g of bovine brain tissue according to Gorski *et al.* (14) except that after resuspension in the nuclear lysis buffer, the nuclei were directly stored in aliquots at −70°C.

Western Blot Analysis. Protein samples were electrophoresed on SDS/12.5% polyacrylamide gels and immunoblotted as described by Choi and Dreyfuss (15). The 1D8 antibody was generously provided by M. S. Swanson (University of Florida), the anti-Scl70 antibody and the HMS antibody were purchased from Immunovision (Springdale, AR), and secondary antibodies were from Amersham. Specific proteins were detected by using an enhanced chemiluminescence Western blotting detection system (Amersham). Low molecular weight protein standards were from Bio-Rad.

Fractionation of Mitochondrial Proteins. All procedures were carried out at 4°C or on ice. Purified mitochondria, ≈20 ml, were resuspended in 40 ml of buffer A [25 mM Tris·HCl, pH 8.0/5 mM $MgCl_2$/1 mM EDTA/10% (vol/vol) glycerol/0.1 mM benzamidine/10 mM sodium bisulfite/0.5 mM phenylmethanesulfonyl fluoride/2 mM 2-mercaptoethanol], brought to a final concentration of 0.5 M KCl and 1.5% Triton X-100, and homogenized by 20 strokes in a glass Dounce homogenizer, and the suspension was cleared by centrifugation in a Beckman SW28 rotor at 28,000 rpm for 60 min. The supernatant was collected, diluted by addition of buffer A to a final volume of 500 ml, and loaded onto a 400-ml DEAE-Sephacel column (Pharmacia LKB) (2.6 × 75 cm) previously equilibrated with buffer A + 50 mM KCl. The column was washed with 3 column volumes of equilibration buffer and

Abbreviation: hnRNP, heterogeneous nuclear ribonucleoproteins.
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proteins were eluted with 1 column volume of buffer A + 0.6 M KCl. Ten-milliliter fractions were collected and stored at −70°C.

Preparation of DNA Helicase Substrates. The plasmid pBSMCB-2 contains the mitochondrial sequences 16201–14291 (16) inserted into the pBSM13+ (Stratagene) *Bam*HI site. Single-stranded pBSMCB-2 was obtained by using VCSM13 helper phage (Stratagene) by the protocol supplied. Synthetic oligonucleotides were supplied by the DNA Synthesis Core of the Interdisciplinary Center for Biotechnology Research (University of Florida). The 20-base oligonucleotide (TTAAGCTCGTGATCTAATGG) is complementary to the bovine mitochondrial light strand sequence 16144–16163 (16). The 31-base oligonucleotide (TCGAGCTCGGTACCCGGGGATCCCTGCCTAG) is complementary to sequences spanning the *Sma* I site of the single-stranded plasmid pBSMCB-2.

Three substrates were prepared. *Substrate 1.* Twenty nanograms of the 20-base oligonucleotide, labeled at its 5′ terminus by using T4 polynucleotide kinase (United States Biochemical) and [γ-^{32}P]ATP (ICN), and 1 μg of single-stranded pBSMCB-2 were incubated at 70°C for 3 min in 10 mM Tris·HCl, pH 7.5/5 mM NaCl (20-μl reaction volume) and slowly cooled (45 min) to room temperature. The reaction mixture was diluted to 2 ml with TE buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA), concentrated in a Centricon 100 (Amicon), and washed twice with 2 ml of TE buffer. *Substrate 2.* Eighty nanograms of the 31-base oligonucleotide, labeled at its 5′ end as described above, and 4 μg of single-stranded pBSMCB-2 were annealed as described above (see Fig. 5*A*). The annealed DNA was digested overnight with *Sma* I (United States Biochemical) and then extracted successively with phenol/chloroform (1:1, vol/vol), chloroform, and 1-butanol and placed under reduced pressure for 10 min. Free label and unannealed oligonucleotide were removed as described above. *Substrate 3.* Eighty nanograms of the 31-base oligonucleotide and 4 μg of single-stranded pBSMCB-2 were annealed as described above, 3′ end labeled by incubation with 100 μCi of [α-^{32}P]dCTP (3000 Ci/mmol; Amersham; 1 Ci = 37 GBq) and 4 units of Klenow fragment of DNA polymerase I (United States Biochemical) for 15 min at room temperature and purified as described above.

Helicase Assay. DNA helicase activity assays were based on those of Venkatesan *et al.* (17) and LeBowitz and McMacken (18). Unless otherwise indicated, reaction mixtures (15 μl) contained 40 mM Tris·HCl at pH 7.5, 2 mM $MgCl_2$, bovine serum albumin at 50 μg/ml, 50 mM potassium glutamate, 20 mM Na_2HPO_4, 5 mM dithiothreitol, 4 mM ATP, 2.5 ng of ^{32}P-labeled DNA substrate, and 3 μl of column fraction. Mixtures were incubated at 37°C for 30 min; reactions were stopped by addition of 4 μl of 75 mM EDTA/0.15% bromphenol blue/0.15% xylene cyanol/60% (vol/vol) glycerol; and products were analyzed by electrophoresis on nondenaturing 8% polyacrylamide gels (or nondenaturing 12% gels for directionality experiments). Helicase activity was quantitated by using a Microtek Scanmaker model MRS 600ZS and IMAGE quantification software from National Institutes of Health Research Services. One unit of helicase activity is defined as 50% unwinding of 2.5 ng of DNA helicase substrate in 30 min.

RESULTS

Purified Bovine Mitochondria Contain No Detectable Nuclear Contamination. Because nuclei contain active DNA helicases, it was important to determine whether our mitochondrial preparations were essentially free of nuclear protein. Mitochondria and nuclei were isolated from bovine brain tissue and analyzed by Western blotting using antibodies specific for nuclear or mitochondrial antigens. The 1D8 antibody is a mouse ascites monoclonal antibody to the 66- to 68-kDa, human, nuclear-specific, M class of heterogeneous nuclear ribonucleoproteins (hnRNP) (M. S. Swanson, personal communication; ref. 19). As shown in Fig. 1*A*, species of the expected size were present with 5 μg of whole cell homogenate protein (lane 1) and with 5 μg and 0.25 μg of nuclear extract protein (lanes 2 and 3). In contrast, M-hnRNP were not detected in lanes with 5 μg or 25 μg of purified mitochondrial extract protein (lanes 6 and 7). Upon longer exposure of the Western blot (Fig. 1*B*), the M-hnRNP band was clearly visible with 0.01 μg of nuclear extract (lane 5) but was still not detected in either mitochondrial lane. Similar results were obtained with a human polyclonal antibody to topoisomerase 1 (anti-Scl70), a nuclear-specific 100-kDa protein (20) (Fig. 1*C*; compare lanes 2 and 3). Presence of enriched mitochondrial protein in the mitochondrial lanes was verified by using a human polyclonal antibody (HMS antibody) specific for several mitochondrial proteins: the dihydrolipoamide acetyltransferase component (74 kDa) of the pyruvate dehydrogenase complex, the dihydrolipoamide acetyltransferase component (52 kDa) of the α-keto acid dehydrogenase complex, and a third polypeptide (39 kDa), believed to be a breakdown product of the 74-kDa protein (21). Bands corresponding to the expected sizes were seen in the mitochondrial lane (Fig. 1*D*, lane 3), to a lesser extent in the cell homogenate lane (lane 1), but not in the nuclear lane (lane 2). In this analysis the intensity of a band corresponding to a specific protein indicates the relative concentration of that protein in the solution. Since M-hnRNP were detected in a 1:500 dilution of nuclear extract but not with a 2500-fold

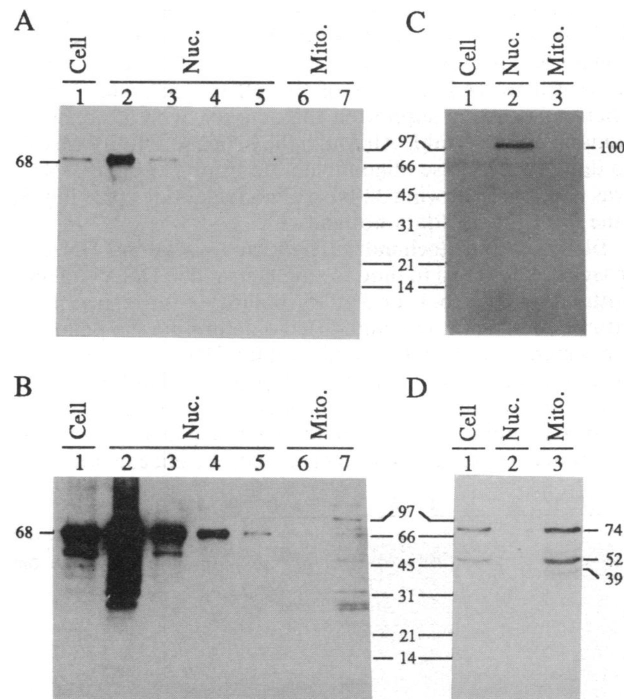

FIG. 1. Western blot analysis to determine the purity of sucrose gradient-purified mitochondria from nuclear contamination. (*A*) Cell homogenate (lane 1, 5 μg of protein), nuclear extract (lanes 2–5, 5 μg, 250 ng, 50 ng, and 10 ng of protein, respectively), and mitochondrial extract (lanes 6 and 7, 5 μg and 25 μg of protein, respectively) probed with 1D8 (M-hnRNP) antibody. (*B*) Longer exposure of *A*. At this exposure cross-reaction of antibody with proteins other than those that migrate as M-hnRNP is detected (see lane 7). (*C*) Cell homogenate (lane 1, 5 μg of protein), nuclear extract (lane 2, 5 μg of protein), and mitochondrial extract (lane 3, 5 μg of protein) probed with anti-Scl70 (topoisomerase I) antibody. (*D*) As in *C*, but probed with anti-HMS (mitochondrial) antibody. The molecular masses (kDa) of protein standards are indicated in the center of the figure, and the expected marker proteins are indicated on the sides.

greater amount of mitochondrial protein, the isolated mitochondria have less than 1 part in 2500 nuclear contamination. This result strongly suggests that any helicase activity detected from this mitochondrial preparation will be of mitochondrial, not nuclear, origin.

Identification of DNA Helicase Activity from Sucrose Gradient-Purified Mitochondria. Total mitochondrial protein (360 mg) was fractionated by chromatography on a DEAE-Sephacel column. Individual fractions collected during the column elution were tested for DNA helicase activity (Fig. 2). A helicase activity was detected in fractions 2–9 (eluted at 0.15–0.40 M KCl) as indicated by an increase in the signal intensity of the displaced 20-base oligonucleotide in the presence of ATP (dissociation of the 20-mer from the single-stranded circular plasmid) along with a decrease in the signal intensity of the 5.1-kb circular DNA. Approximately 34,000 units of activity and 95.1 mg of protein were contained in the active fractions, yielding a specific activity of 360 units/mg of protein.

Characterization of the Mitochondrial DNA Helicase Activity. Helicase activity was titratable (Fig. 3, lanes 3–8), sensitive to heating (lane 19), and dependent on hydrolysis of ATP, since activity was not supported by the ATP analog adenosine 5′-*O*-(3-thiotriphosphate) (Pharmacia LKB), even at concentrations 4-fold greater than that of the optimal ATP concentration (lanes 9–13). Fig. 3 also demonstrates a requirement for divalent cation, in this case Mg^{2+}, since addition of EDTA at 2 mM or greater inhibited the activity and at 1 mM partially inhibited the activity (lanes 14–18). All reaction mixtures contained 2 mM $MgCl_2$. As shown in Fig. 4, both ATP and dATP were able to support activity at concentrations as low as 1 mM, with ATP only slightly preferred to dATP. None of the other nucleoside triphosphates, however, supported the activity even at concentrations as high as 8 mM. Although the helicase activity was able to unwind a 20-base oligonucleotide from a circular DNA, it was not able to unwind 32-base oligonucleotides (see Fig. 5*B*, lane 3) or longer (data not shown).

Direction of Mitochondrial Helicase Unwinding. DNA helicases are believed to bind to single-stranded DNA and move with either a 5′-to-3′ or 3′-to-5′ polarity with respect to the strand on which it is bound (5). To determine the polarity of movement, two linear partially duplex DNA substrates (substrates 2 and 3) were constructed as outlined in Fig. 5*A* such that they contained a large single-stranded DNA region (approximately 5.1 kb) for helicase binding and a short region of double-stranded DNA on each end. A helicase translocating in a 3′-to-5′ direction would unwind the oligonucleotide labeled at its 5′ end; one translocating in a 5′-to-3′ direction would unwind the oligonucleotide labeled at its 3′ end. The results in Fig. 5*B* indicate the mitochondrial helicase has a 3′-to-5′ polarity, since it unwound the 15-base oligonucleotide labeled at its 5′ end (lanes 7 and 15) but not the 17-base oligonucleotide labeled at its 3′ end (lanes 11 and 15). These results also demonstrate that the helicase cannot act at a blunt-ended DNA duplex, since the 17-base oligonucleotide labeled at its 3′ end was not unwound.

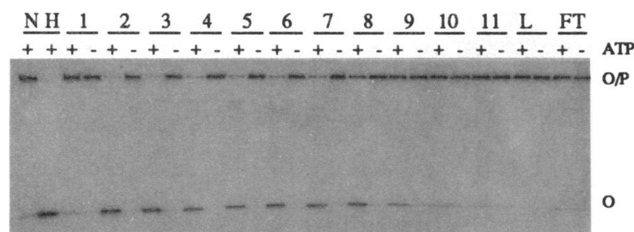

FIG. 2. Mitochondrial DNA helicase activity is present in fractions eluted from a DEAE-Sephacel column. Aliquots of individual fractions (1–11), mitochondrial lysate (L), and column flow-through (FT) were assayed for activity in the presence (+) or absence (−) of ATP, using DNA substrate 1. N refers to substrate incubated with column buffer only, and H refers to substrate heat denatured just prior to loading. The positions of oligonucleotide/single-stranded plasmid complex (O/P) and free oligonucleotide (O) are indicated at the side. The bands between O/P and O in fractions 4–6 + are believed to be due to oligonucleotides unwound from the plasmid by helicase but with their gel migration altered by proteins that have bound to them as in a band-shift assay. For quantitation they are included with O.

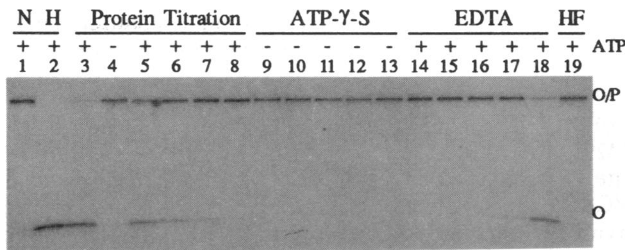

FIG. 3. Mitochondrial DNA helicase activity was sensitive to dilution, adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S), EDTA, and heating in the DNA helicase assay using substrate 1. Lanes 1 and 2 are as in Fig. 2. Undiluted pooled DEAE-Sephacel column fractions (lanes 3 and 4) were 2-fold serially diluted in column buffer to 0.50, 0.25, 0.12, and 0.06 times initial concentration (lanes 5–8, respectively) and assayed. Helicase was assayed in the presence of ATP-γ-S at concentration of 16, 8, 4, 2, or 1 mM (lanes 9–13, respectively). Helicase was assayed in the presence of EDTA at a concentration of 16, 8, 4, 2, or 1 mM (lanes 14–18, respectively). Pooled fractions were heated in boiling water for 5 min prior to addition to reaction mixture (lane 19, HF). Assays were performed in the presence (+) or absence (−) of ATP. The positions of oligonucleotide/single-stranded plasmid complex (O/P) and free oligonucleotide (O) are indicated at the side.

DISCUSSION

The Identified Helicase Is an Intrinsic Mitochondrial Activity. From bovine tissue, specific DNA helicases have been identified that copurify with the nuclear DNA polymerases α, δ, and ε (6, 22) and they could, along with other as-yet-unidentified nuclear helicases, contaminate the purified mitochondrial preparations. Since it is not yet possible to distinguish biochemically between a nuclear and a mitochondrial helicase, it was first necessary to demonstrate the purity of the isolated mitochondria. Western blot analysis detected nuclear antigens in as little as 10 ng of bovine brain nuclear protein, but nuclear antigens were not detected in as much as 25 μg of total mitochondrial protein. Similar results were obtained with an antibody to a second nuclear protein, topoisomerase I. The results indicate that the isolated mitochondria have less than 1 part in 2500 nuclear contamination

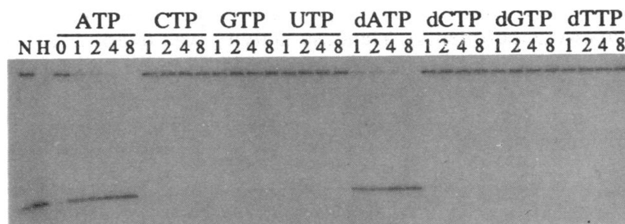

FIG. 4. Mitochondrial DNA helicase activity was supported by ATP or dATP but not by other nucleoside triphosphates. Various concentrations of each NTP and dNTP were tested for their ability to support DNA helicase activity in reaction mixtures containing pooled DEAE-Sephacel column fractions and DNA substrate 1. Numbers refer to the concentration (mM) used in the reaction. N and H are as in Fig. 2. The positions of oligonucleotide/single-stranded plasmid complex and free oligonucleotide are as described above.

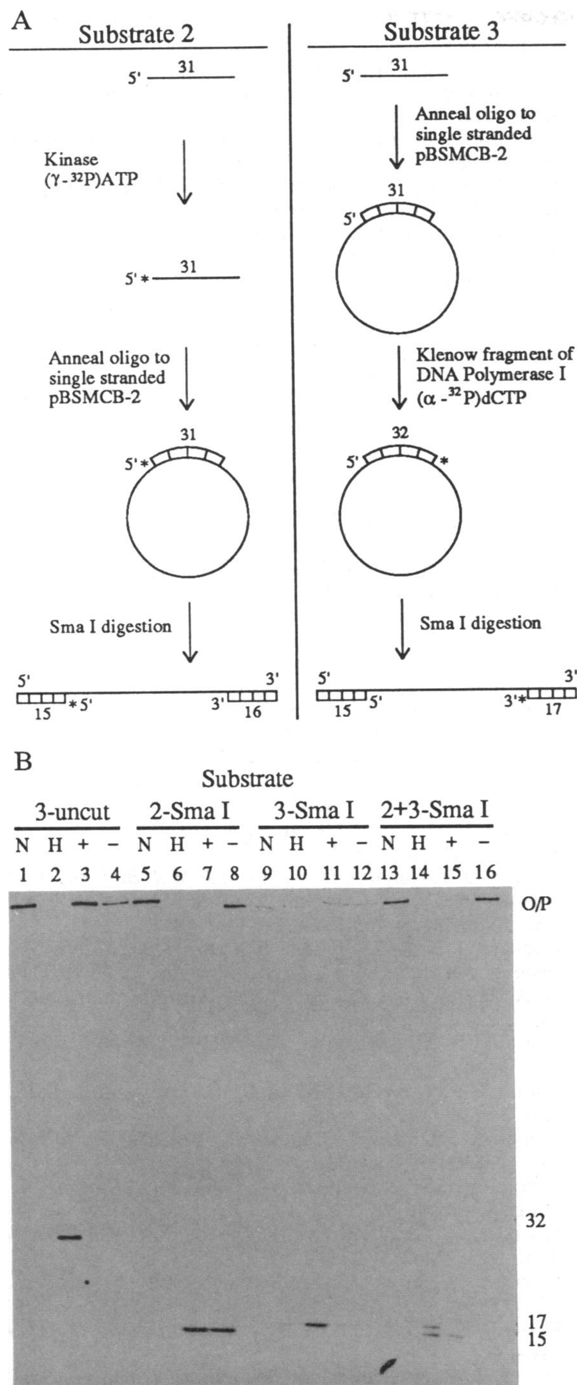

FIG. 5. Mitochondrial DNA helicase moves with a 3′-to-5′ polarity. (*A*) Schematic diagram of the assay DNA substrates used as described in *Materials and Methods*. (*B*) Helicase assays were performed with undigested substrate 3 (lanes 1–4), *Sma* I-digested substrate 2 (lanes 5–8), *Sma* I-digested substrate 3 (lanes 9–12), and an equal mixture of *Sma* I-digested substrates 2 and 3 (lanes 13–16). Templates were incubated with buffer only (N, lanes 1, 5, 9, and 13), heat denatured (H, lanes 2, 6, 10, and 14), or incubated with pooled DEAE-Sephacel fractions in the presence (+, lanes 3, 7, 11, and 15) or absence (−, lanes 4, 8, 12, and 16) of ATP. Reaction products were electrophoresed in a nondenaturing 12% polyacrylamide gel. The positions of oligonucleotide/single-stranded plasmid complex (O/P) and free oligonucleotide lengths are indicated at the side.

and strongly suggest that any helicase activity subsequently detected is due to an authentic mitochondrial activity.

Comparison with Other DNA Helicases. The NTP/dNTP requirements for DNA helicase activities are known to vary from helicase to helicase. For instance, mouse helicase can utilize any of the NTPs or dNTPs but prefers ATP (23), and the calf thymus helicase that copurifies with DNA polymerase α prefers ATP but can also utilize dATP, CTP, and dCTP (22). As for the calf thymus helicase that copurifies with DNA polymerase ε (6) and the *E. coli* Rep helicase (24), the bovine mitochondrial helicase activity is supported only by ATP or dATP, with the mitochondrial activity slightly preferring ATP over dATP. The mitochondrial helicase activity was able to unwind a 20-base oligonucleotide from a circular DNA but not a 32-base oligonucleotide. Other DNA helicases also possess limited capabilities in unwinding fully complementary oligonucleotides; *E. coli* DnaB (25) and the HeLa cell helicase IV (10) are limited to 33- and 25-base oligonucleotides, respectively. It has been suggested (24, 26) that this may be due to the fact that the natural templates for these helicases are likely to be replication forks—that is, duplex DNAs already partially unwound at one end.

Recently, the yeast *PIF1* gene product has been shown to be a DNA helicase used in repair and recombination of the mtDNA (9, 12). It is unlikely we have isolated its bovine mitochondrial counterpart, since the PIF1 helicase translocates in a 5′-to-3′ direction (9), opposite to the bovine mitochondrial helicase. Furthermore, since in mammalian mitochondria no evidence for removal of pyrimidine dimers or recombinational DNA repair has been found (27–30), these mechanisms may be absent from mammalian mitochondria. Nucleotide excision enzymes have been identified in mammalian mitochondria, but these enzymes may actually function in degradation and elimination of defective mtDNA (31).

Potential Role of Mammalian Mitochondrial Helicase in mtDNA Replication. Only indirect evidence suggests the characteristics required for a mitochondrial DNA helicase involved in replication. However, several features of the mammalian mtDNA replication strategy are similar to those of the circular single-stranded DNA phages G4, ϕX174, and Ff (a group of phages that includes M13, fd, and f1; reviewed in ref. 32). Specifically, the D-loop mechanism, proposed as the mode of mammalian mtDNA replication (33, 34) and receiving substantial subsequent experimental support (refs. 35–37; reviewed in ref. 2), bears similarity to the bacteriophage rolling circle mechanism of DNA replication during stages II and III of viral replication. Both replication intermediates contain extensive, displaced, single-stranded DNAs originating from a unidirectional origin with initially only one DNA strand utilized as a template for DNA synthesis (2, 32, 38). The displaced strand is not used as a template until parental DNA unwinding has proceeded a significant way around the genome: in mammalian mitochondria, ⅔ of the genome (34, 39, 40); in phage G4, ½ of the genome (41); in Ff phages, completely around the genome (42). Thus, in both mitochondria and specific stages of viral strand synthesis of single-stranded phage, replication appears to occur primarily by continuous leading-strand DNA synthesis and therefore may require functionally similar DNA helicases.

The *E. coli rep* gene product, a helicase with 3′-to-5′ polarity of movement (43), has been shown to be essential for stage-II (42, 44, 45) and stage-III (46–48) viral strand replication but not for stage-I phage replication (32) or for replication of the bacterial chromosome (49). The similarities of replicative intermediates suggest that the mitochondrial DNA helicase used in replication is likely to have properties similar to the *E. coli* Rep helicase. If this is the case, it would be expected that the mitochondrial helicase would have a 3′-to-5′ polarity of movement which, indeed, is the polarity of the mitochondrial helicase activity we have identified. Furthermore, as indicated above, both Rep helicase and the mitochondrial activity utilize ATP and dATP but not the

other NTPs or dNTPs, suggesting additional biochemical similarity.

Other DNA helicases essential for replication with 3′-to-5′ polarity are the eukaryotic viral helicases, simian virus 40 large tumor antigen (50–52), and polyoma virus large tumor antigen (53). Other eukaryotic helicases that may be involved in DNA synthesis and translocate in the 3′-to-5′ direction have been isolated from the calf thymus (6, 22). Conversely, there are three known prokaryotic helicases essential for replication that translocate in the 5′-to-3′ direction, *E. coli* DnaB (18), T7 gp4 (26), and T4 gp41 (17).

The reported bovine DNA helicase activity is, to our knowledge, the first documented mammalian mitochondrial DNA helicase. A comparison of its characteristics with those of the *E. coli* Rep protein suggests its possible role in mtDNA replication. Although we have no evidence for additional mitochondrial helicases with 5′-to-3′ polarity or a second helicase with 3′-to-5′ polarity, we cannot exclude their existence. However, their absence is consistent with current views of mammalian mtDNA replication (2), which would require only a single 3′-to-5′ helicase for the strictly leading-strand synthesis involved. Further purification and characterization of the activity we have detected may resolve these questions.

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