BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **219,** 134–139 (1996)
ARTICLE NO. 0194

DNA-Helicase Activity from Sea Urchin Mitochondria

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Received December 15, 1995

As a step toward the characterization of the main components of mitochondrial DNA replication apparatus in sea urchin, we report the identification of a DNA-helicase activity in *Paracentrotus lividus* mitochondria. The activity was detected in a protein fraction obtained by fractionating on DEAE-Sephacel a lysate of gradient purified mitochondria from *Paracentrotus lividus* eggs. The mitochondrial helicase unwound, in the presence of ATP and Mg^{++}, a 39-base oligonucleotide annealed to single-stranded M13mp18 (+) DNA. Its direction of movement is 3′ to 5′ with respect to the single stranded portion of the partial duplex DNA substrate. This polarity is similar to that exhibited by the *Escherichia coli rep* helicase and by the helicase from bovine brain mitochondria. These features suggest that the sea urchin mitochondrial helicase could function in enabling the polymerization of the H-strand during mitochondrial DNA replication.

Animal mitochondrial (mt) DNA is a multicopy, closed-circular double-stranded molecule of 15–17 kbp (for review see refs. 1, 2). Although early studies from Clayton's group allowed to elucidate the mechanism of mammalian mt DNA replication (for review see refs. 3, 4), the knowledge of the biochemical steps involved in mt DNA metabolism is still lacking. Sea urchin is a system in which several protein factors involved in the mt DNA replication have been already characterized. They include three sequence specific double-stranded DNA-binding proteins (5–8) and one single-stranded DNA-binding protein (Roberti et al. in preparation). The first of these factors was found in *Paracentrotus lividus:* it is a 40 kDa polypeptide that binds specifically two homologous sequences on mt DNA (5, 6). The two binding sites are located at the end of the non-coding region, where the H-strand replication origin was mapped (9), and at the ND5/ND6 gene boundary, respectively. The other two proteins (25 kDa and 18 kDa) were found in *Strongylocentrotus purpuratus;* they interact with two distinct sequences, located at the boundary of ATPase 6 and Co III genes and containing the major pause site of mt DNA replication (7, 8). Recently we identified and purified from *P. lividus* egg mitochondria (Roberti et al. in preparation) a 15 kDa polypeptide, having an estimated native molecular mass of 60 kDa, which binds selectively to single-stranded DNA. This protein probably plays a role in maintaining the displaced strand in a single-stranded configuration, during mt DNA replication.

In order to shed light on the mt DNA replication mechanism in sea urchin, we have undertaken the identification and characterization of other components of the mt DNA replication apparatus. The unwinding of duplex DNA is a prerequisite to provide the DNA polymerase with the single-stranded DNA template for DNA replication and repair. A class of enzymes, DNA-helicases, uses the hydrolysis energy of ATP to disrupt the hydrogen bonds that hold the two strands of duplex DNA together, thus causing DNA unwinding (10). Enzymes with helicase activity have been described in many systems, including *E. coli* and its bacteriophages, eukaryotic viruses, and nuclei of yeast, lily, *Xenopus,* mouse and human (10–16). Only two mitochondrial DNA-helicase activities have been identified to date. The first, described in yeast (17), is involved in mt DNA

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Abbreviations: mt, mitochondrial; bp, base pairs; SDS, sodium dodecyl sulfate; nt, nucleotides.

0006-291X/96 $18.00

recombination and repair; the second, found in bovine mitochondria (18), probably plays a role in mt DNA replication.

Here we report the identification and partial purification of a DNA helicase activity from *P. lividus* mitochondria. This activity unwinds the DNA duplex in an ATP-dependent manner and has a 3′ to 5′ polarity of movement.

MATERIALS AND METHODS

Isolation and Fractionation of Sea Urchin Mitochondria

All procedures were carried out at 4C or on ice. Mitochondria were isolated from 50 g of sea urchin eggs by differential centrifugation and then purified by sucrose gradient sedimentation as previously described (19). Mitochondrial pellets (about 100 mg of proteins) were resuspended in Buffer A [25 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 20% (v/v) glycerol and 0.1 μg/ml of each of the following protease inhibitors: antipain, chymostatin, elastinal, leupeptin and pepstatin (ACELP)], to a final protein concentration of 10 mg/ml. Mitochondrial lysis was carried out by adding 1% Nonidet P-40 in the presence of 500 mM KCl. The lysate was centrifuged at 44,000 rpm for 2 h at 4°C in a Beckman 70.1 Ti rotor. The supernatant was diluted with buffer A to 50 mM KCl and loaded onto a 20 ml DEAE-Sephacel column (Pharmacia, Inc.), previously equilibrated with buffer B (10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 7.5% (v/v) glycerol, 1 mM dithiothreitol, 0.2 mM PMSF, 0.1 μg/ml ACELP) containing 50 mM KCl. The column was washed with 3 volumes of buffer B and proteins were eluted with buffer B containing 0.6 M KCl. Fractions of 1 ml were collected and used for the DNA-helicase activity assay. Protein concentration was measured by using the Biorad assay.

Preparation of DNA Helicase Substrates

Single stranded M13mp18 (+) DNA was purchased from Pharmacia. Synthetic oligonucleotides were obtained from Pharmacia and MedProbe. Three substrates were prepared. *Substrate 1* was obtained by annealing a 36-mer, complementary to the polylinker (5′ CAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTC 3′, position 6236–6271), with M13mp18 (+) DNA. For its preparation 0.66 pmol of M13mp18 (+) DNA were combined with 1 pmol of the oligonucleotide in 10 μl of buffer C (50 mM NaCl, 6.6 mM $MgCl_2$, 1 mM dithiothreitol, 6.6 mM Tris-HCl pH 7.6) and incubated for 1 hr at 37°C. DNA labelling was performed in 20 μl of buffer C in the presence of 500 μM dGTP, 30 μCi of α [^{32}P] dATP (3000 Ci/mmol) and 3 units of Klenow DNA-polymerase (Boehringer) for 15 min at 25°C. Chase followed, by incubating for 5 min at 25°C in the presence of 500 μM dATP and finally the reaction was stopped by adding 50 mM EDTA. Then the reaction volume was brought to 100 μl with buffer C and free label was removed by spin-column chromatography on Sephadex G-50 (Pharmacia). At the end of the reaction the length of the oligonucleotide was 39 nt. *Substrate 2* was made by annealing, as described above, 1 pmol of 17-mer M13 universal sequencing primer (5′ GTAAAACGACGGCCAGT 3′) with 0.66 pmol of M13mp18 (+) DNA. The DNA was labelled in the presence of 3 units of Klenow DNA polymerase, 500 μM dCTP, 500 μM dATP and 30 μCi of α [^{32}P] dGTP. Chase with 500 μM dGTP and Sephadex G-50 purification were performed as above. At the end of the reaction the length of the oligonucleotide was 24 nt. *Substrate 3* was used for the directionality assay. It was obtained by annealing 1 pmol of the 36-mer, used for substrate 1 construction, with 0.66 pmol of M13mp18 (+) DNA. The DNA was then digested overnight at 37°C, in 20 μl, with 20 units of BamHI. Labelling was performed in 30 μl in the presence of 3 units of Klenow DNA-polymerase, 500 μM dATP and 40 μCi of α [^{32}P] dGTP for 15 min at 25°C. Chase with 500 μM dGTP and Sephadex G-50 purification were performed as above. At the end of the reaction two oligonucleotides of 18 nt and 23 nt, annealed to M13mp18 (+) DNA, were obtained (see fig. 3A).

Helicase Assay

Helicase activity was tested by adding, in a final volume of 20 μl, 4 μl of 5 × helicase buffer (200 mM Tris-HCl pH 7.5, 250 μg/ml bovine serum albumin, 250 mM potassium glutamate, 25 mM dithiothreitol, 20 mM ATP), 4 μl of protein fraction and 5–10 ng of [^{32}P] labelled substrate. ATP was omitted in control samples. The mixture was incubated for 20 min at 20°C and the reaction was stopped with one volume of 80 mM EDTA, 1% (w/v) SDS, 20% (v/v) glycerol, 0.15% (w/v) bromophenol blue and xylene cyanol. Reaction products were analyzed by electrophoresis on 10% non-denaturing polyacrylamide gel. In some cases (see figure legends) the 5 × helicase buffer contained also 12.5 mM $MgCl_2$ and/or 100 mM Na_2HPO_4.

RESULTS

Mitochondria from *P. lividus* eggs were purified on sucrose gradient, lysed with Nonidet P-40 and centrifuged at 130,000 × *g*. The supernatant, made 50 mM in KCl, was applied to a DEAE-Sephacel column eluted with 0.6 M KCl buffer. Fig. 1 shows the result of a DNA-helicase assay

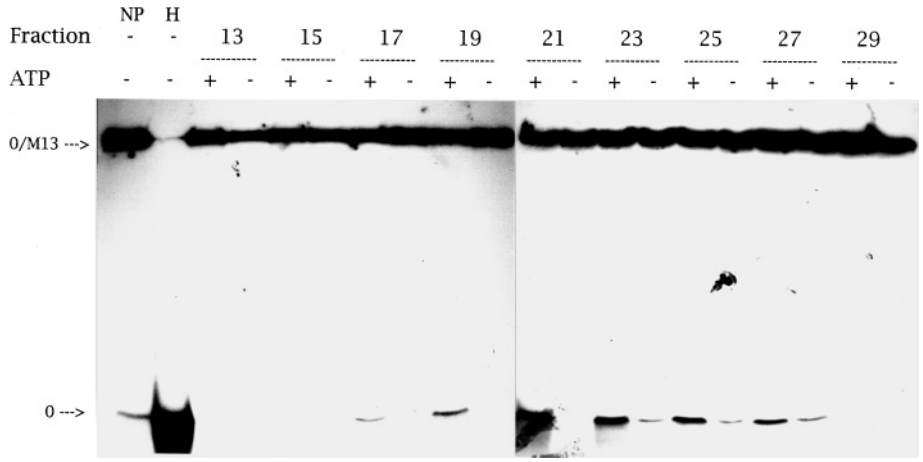

FIG. 1. Assay of *P. lividus* mt DNA-helicase activity in fractions eluted from a DEAE-Sephacel column. Individual fractions (4 μl) were tested for helicase activity using as substrate a 3′ end-labeled 39-mer (substrate 1) annealed to single-stranded M13mp18 (+) DNA. The activity was tested in the presence (+) or absence (−) of 4 mM ATP. NP refers to substrate incubated with elution buffer only and H refers to substrate denatured 5 min at 100°C before loading. The positions of unwound oligonucleotide (O) and of the oligonucleotide–DNA complex (O/M13) are indicated at the side of the figure.

performed using 4 μl of the fractions collected from the DEAE column. The assay was performed at a $MgCl_2$ concentration of 2 mM, due to the 5-fold dilution of the 4 μl protein aliquot which contained 10 mM $MgCl_2$. Fractions 21–27, eluting at 0.3–0.4 M KCl, contained a helicase activity, as indicated by the ability to partially unwind, in the presence of 4 mM ATP, a 39-base oligonucleotide annealed to single-stranded M13mp18 (+) DNA (substrate 1). The active fractions were pooled and tested in different conditions, using as substrate a 24-base oligonucleotide annealed to circular single-stranded DNA (substrate 2). The experiment, reported in fig. 2, shows that the activity, detected in the presence of 4 mM ATP and 2 mM $MgCl_2$ (lanes 4 and 8), was abolished by the addition of 5 mM EDTA (lane 9). The increase of magnesium concentration up to 4.5 mM (lanes 3 and 7) seemed to reduce the intensity of the displaced band; this was probably due to the activation of Mg-dependent nucleases present in the protein fraction containing the helicase activity. Finally we found that the activity of the enzyme was slightly increased by the presence of 20 mM Na_2HPO_4 in the reaction mixture (lane 7) and that ATP was not replaceable by other nucleoside triphosphate (experiments not shown).

DNA helicases are believed to bind to single stranded DNA and move either with a 5′ to 3′ or with a 3′ to 5′ polarity, with respect to the strand on which they are bound (10). To determine the polarity of the movement a partially duplex linear DNA substrate (substrate 3, shown in fig. 3A) was used. It consisted of a large single-stranded DNA molecule having a short duplex region of 18 nt and 23 nt on either end. If the helicase polarity had been 3′ to 5′, the displaced oligonucleotide would have been the 18-mer; in the opposite case the the displaced oligonucleotide would have been the 23-mer. Fig. 3B shows that the unwound oligonucleotide has a size of 18 nt indicating that the *P. lividus* mt helicase moves in the 3′ to 5′ direction.

DISCUSSION

In this paper we report the identification and characterization of a DNA helicase activity from *P. lividus* egg mitochondria; this is the first identified mt helicase from invertebrates, to date. The activity requires ATP and Mg^{++} and does not seem to depend on the size of the annealed oligonucleotide, at least up to a length of 39-base.

The helicase could be a component of mt DNA replication apparatus in sea urchins. Mt DNA

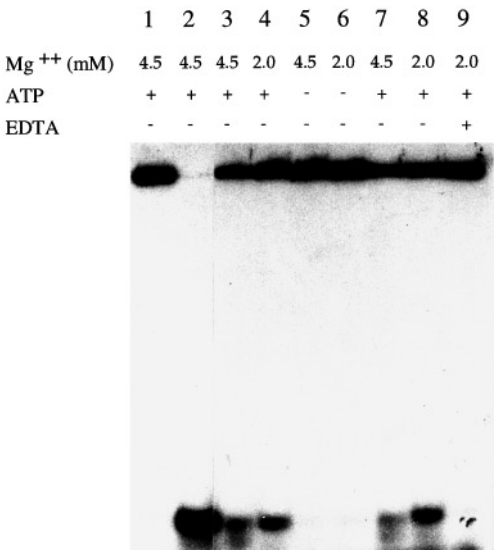

FIG. 2. Effect of EDTA and Mg^{++} on the *P. lividus* mt DNA-helicase activity. DNA-helicase activity was determined on a 4-μl aliquot (about 4 μg of proteins) of pooled fractions, using as substrate a 3′ end-labeled 24-mer annealed to M13mp18 (+) DNA (substrate 2). Lane 1, substrate incubated with elution buffer only; lane 2, substrate denatured 5 min at 100°C before loading; lanes 3–9, substrate incubated with proteins. In lanes 7 and 8 the incubation buffer also contained 20 mM Na_2HPO_4

replication has been investigated prevalently in mammals (for review see refs. 3, 4). In this system, as well as in circular single-stranded DNA phages, DNA replication takes place by formation of replication intermediates containing a displaced DNA helix originating from the unidirectional replication origin. This strand is not used as template until parental DNA unwinding has proceeded to a significant extent around the genome, that is ⅔ in mammalian mtDNA (3), ½ in the phage G4 DNA (20) and completely in Ff phages (21). Therefore in these systems the continuous synthesis of the leading strand should require DNA helicases with similar properties. This expectation was confirmed by the finding that the bovine mt helicase and the *E. coli rep* gene product, a helicase essential for stages II and III of viral strand replication, both move in 3′ to 5′ direction (18, 21–25). Early studies of Matsumoto et al. (26) suggested that also in sea urchins the mt DNA synthesis could take place via the expansion of molecules with a small displaced loop (D-loop). The finding of a sea urchin mt helicase with a 3′ to 5′ polarity suggests that the *P. lividus* enzyme is similar to the bovine one and that both might be involved in the synthesis of the mt DNA leading strand during replication.

The sea urchin mt helicase might be involved also in the regulation of the mt DNA replication during embryonic development. In sea urchins mt DNA synthesis takes place during oogenesis, stops in mature oocytes, eggs or early embryos and resumes when the feeding larval (pluteus) stage is reached (27). The replication block might be due to *trans*-acting factors, whose binding to the mt DNA replication origin could make it unavailable to mt DNA replication enzymes. A similar situation was described in *E. coli* where the binding of the DNA termination protein TBP blocks replication, preventing the action of *dna B* helicase (28). In animal mitochondria two proteins that might have a role in the regulation of mtDNA copy number have been described. The first, found in cow by Hauswirth et al. (29), binds selectively a conserved sequence element placed at the end of the D-loop. This element is thought to be associated with the termination of most of the H-strand initiation events that take place at the H-strand replication origin (30). The second protein is the 40 kDa DNA-binding protein that we have purified from *P. lividus* eggs (5, 6). One of its binding sites comprises the end of the small triplex region which in sea urchin (9) is composed of an RNA primer

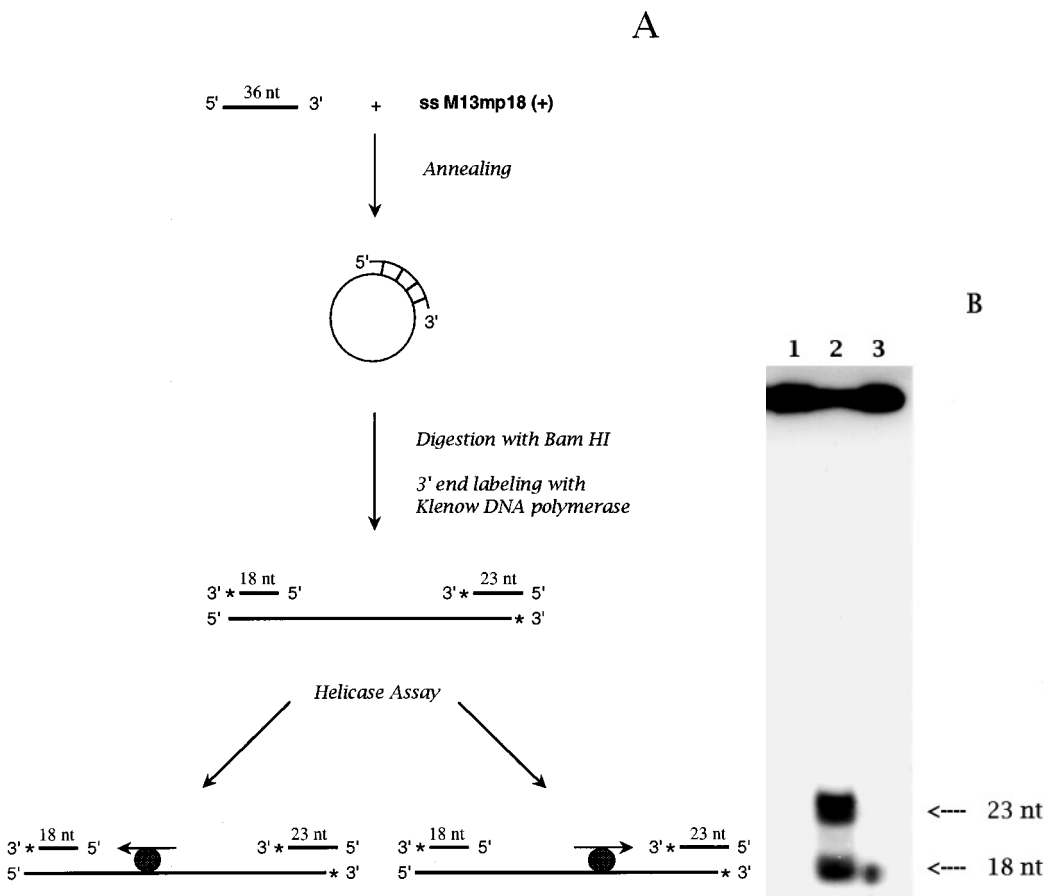

FIG. 3. Direction of movement of *P. lividus* mt DNA-helicase. A. Schematic diagram illustrating the assay and the substrate used (substrate 3). B. Helicase assay. 1, substrate incubated with elution buffer; 2, substrate denatured 5 min at 100°C before loading; 3, substrate incubated with 4 μl of pooled protein fractions.

of about 80 nt and of a small tract of nascent DNA (15–25 nt). The 40 kDa DNA-binding protein could regulate the mt DNA replication in sea urchin by interfering with the action of the mt DNA helicase. The further purification and characterization of the DNA helicase activity here reported and the availability of *P. lividus* 40 kDa DNA-binding protein will allow us to set up experiments addressed to directly test this hypothesis.

ACKNOWLEDGMENTS

This work was supported by grants from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (40% and 60%), Progetto Finalizzato ''Ingegneria Genetica'' CNR and HCM Programme of European Community. The technical assistance of Mr. F. Milella and D. Munno is gratefully acknowledged.

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