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A DNA Helicase from *Xenopus laevis* Ovaries†

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ABSTRACT: A DNA helicase was extensively purified from *Xenopus laevis* ovaries. The most purified fraction was free of DNA topoisomerase, DNA polymerase, and nuclease activities. The enzyme had a Stokes radius of 54 Å and a sedimentation coefficient of 6–7.3 S, from which a native molecular weight of 140000–170000 was calculated. DNA helicase activity required Mg^{2+} or Mn^{2+} and was dependent on hydrolysis of ATP or dATP. Monovalent cations, K^+ and Na^+, stimulated DNA unwinding with an optimum at 130 mM. DNA-dependent ATPase activity copurified with the *X. laevis* DNA helicase. Double-stranded and single-stranded DNA were both cofactors for the ATPase activity, but single-stranded DNA was more efficient. The molecular weight, monovalent cation dependence, cofactor requirements, and elution from single-stranded DNA–cellulose suggest that the *X. laevis* DNA helicase is different from previously described eukaryotic DNA helicases.

Unwinding of duplex DNA is essential for DNA replication, recombination, and repair. The importance of DNA unwinding is highlighted by the identification of seven distinct DNA helicases in *Escherichia coli* (Lahue & Matson, 1988). The only viral protein required for SV40 DNA replication, the large tumor antigen (T-antigen), also is a DNA helicase (Stahl et al., 1986; Dean et al., 1987). DNA helicases contain an intrinsic DNA-dependent ATPase activity that supplies energy for unidirectional translocation along one strand of duplex DNA. This translocation results in DNA unwinding.

The exact physiological role of most DNA helicases is unknown. Some DNA helicases stimulate DNA synthesis by DNA polymerases (Scott & Kornberg, 1978; Sugino et al., 1986). The dnaB protein in *E. coli* has been shown to be the major replicative DNA helicase (Lebowitz & McMacken, 1986). Other *E. coli* DNA helicases have been postulated to function in DNA repair (Matson & George, 1987), DNA recombination (Amundsen et al., 1986), and DNA transfer during bacterial conjugation (Abdel-Monem et al., 1983).

Eukaryotic DNA helicases have been purified from lily, calf thymus, yeast, and mouse (Hotta & Stern, 1978; Hübscher & Stalder, 1985; Sugino et al., 1986; Sung et al., 1987b; Seki et al., 1987, 1988) by first isolating the major DNA-dependent ATPases and then assaying for DNA helicase activity. Most DNA helicases, except the lily enzyme, require single-stranded DNA adjacent to the duplex region to be unwound. Assays using short oligonucleotides hybridized to longer single-stranded DNA were used to identify DNA helicases in calf thymus, yeast, and mouse (Hübscher & Stalder, 1985; Sugino et al., 1986; Seki et al., 1987).

Xenopus laevis ovary has been used for the purification of many DNA replication proteins [for review, see Kaiserman et al. (1989)]. The recently developed cell-free DNA replication systems from *X. laevis* eggs (Lohka & Masui, 1983; Blow & Laskey, 1986; Newport, 1987) combined with the availability of these purified enzymes make *X. laevis* an excellent model system to study eukaryotic DNA replication. Lacking, however, are purified *X. laevis* DNA unwinding proteins.

In this study we describe the detection of a DNA helicase activity from *X. laevis* ovaries in relatively crude enzyme fractions using a partial duplex substrate and the subsequent

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extensive purification of this DNA unwinding activity. The DNA helicase activity copurified with a DNA-dependent ATPase activity. The properties of the *X. laevis* DNA helicase suggest that it is distinct from previously described eukaryotic DNA helicases.

Experimental Procedures

Frogs. Adult *X. laevis* females were obtained from Xenopus I (Ann Arbor, MI).

Chemicals and Column Resins. All reagents were of enzyme grade. Triton X-100, benzamidine, phenylmethanesulfonyl fluoride (PMSF), calf thymus DNA, and the sedimentation standards yeast alcohol dehydrogenase and bovine serum albumin (fraction V) were purchased from Sigma. DEAE-cellulose (DE-52) and cellulose phosphate (P11) were from Whatman Biochemicals. Hydroxylapatite (HA-ultrogel) was from IBF Biotechnics. Single-stranded DNA–cellulose was prepared as described by Alberts and Herrick (1971). The silver stain kit, electrophoresis reagents, and Bio-Gel A-5m were from Bio-Rad. Nuclease-free bovine serum albumin (BSA), MS2 RNA, and ATP analogue adenosine 5′-*O*-(3-thiotriphosphate) were from Boehringer Mannheim. Sephacryl S-300, gel-filtration calibration proteins, and nonradioactive nucleotides were from Pharmacia. Radioactive nucleotides were from NEN–Du Pont.

Oligonucleotides and DNA. M13mp18 DNA was purified according to the procedure of Maniatis et al. (1982). Oligonucleotides were synthesized on a Biosearch 8750 DNA synthesizer and were purified on a Vydac C4 HPLC column (Applied Biosystems Inc. User Bulletin 13). Oligonucleotide 1 (40-mer) is 5′-TCCCAGTCACGACG$(T)_{26}$. The first 16 nucleotides are complementary to bases 6325–6309 of M13mp18 DNA. Oligonucleotide 2 (16-mer), 5′-TCCCAATTCTGCGAAC, is complementary to bases 123–138 of M13mp18 DNA.

Preparation of DNA Helicase Substrate. Oligonucleotide 1 was used for the preparation of helicase substrate except when otherwise stated. A total of 30 pmol of oligonucleotide was end-labeled with T4 polynucleotide kinase (Maniatis et al., 1982), purified on a NENSORB (Du Pont) column, and lyophilized. The oligonucleotide was then mixed with 10 μg of M13mp18 DNA in 32 μL of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.2 M NaCl, and the resultant solution was boiled for 5 min and slowly cooled to room temperature. The molecular ratio of oligonucleotide:M13 DNA was 7:1. Nonhybridized oligonucleotide was removed by chromatography on a 2-mL Bio-Gel A-5m column.

Buffers used during the purification contained 0.1 mM benzamidine, 10 mM sodium bisulfite, 0.5 mM PMSF, and 2 mM 2-mercaptoethanol. Buffer A was 30 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, and 250 mM sucrose. Buffer B was 25 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM EDTA, 5 mM KCl, and 25% (v/v) glycerol. Buffer C was 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM KCl, 20% (v/v) ethylene glycol, and 0.01% (v/v) Triton X-100. Buffer D was 10 mM K_xPO_4 (pH 7.5), 0.1 M KCl, 20% (v/v) ethylene glycol, and 0.01% (v/v) Triton X-100. Buffer E was 20 mM K_xPO_4 (pH 7.5), 0.1 mM EDTA, 0.1 M KCl, 20% (v/v) ethylene glycol, and 0.01% (v/v) Triton X-100. Buffer F was 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.7 M NaCl, 20% (v/v) ethylene glycol, and 0.01% (v/v) Triton X-100.

ATPase assay 1 used during purification was based on a charcoal assay (Abdel-Monem & Hoffmann-Berling, 1976). The assay (100 μL) contained 50 mM Tris-HCl (pH 7.5), 20 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP, 15 μg of BSA, and 20 μg/mL heat-denatured calf thymus DNA. Reactions were for 30 min at 37 °C. Analysis was performed as described by Abdel-Monem et al. (1976).

ATPase assay 2 was used for enzyme characterization. The assay (10 μL) contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM DTT, 2 mM [α-^{32}P]ATP, and 20 μg/mL heat-denatured calf thymus DNA. Reactions were for 30 min at 37 °C and were terminated by addition of 2 μL of a standard solution of 50 mM EDTA, 10 mM ATP, 10 mM ADP, and 10 mM AMP. A total of 2 μL of the reaction mixture was spotted on a poly(ethylenimine) (PEI) plate (Brinkmann). Analysis was performed as described by Hübscher and Stalder (1985).

DNA helicase assay (15 μL) contained 50 mM Tris-HCl (pH 7.5), 130 mM Na_xPO_4 (pH 7.5), 5 mM DTT, 2 mM $MgCl_2$, 50 μg/mL BSA, 2 mM ATP, and 10 ng of substrate (based on A_{260} of M13 DNA). The reaction was terminated after 30 min at 37 °C by addition of 2 μL of 10% sodium dodecyl sulfate (SDS) and 5 μL of 20% Ficoll and 0.01% bromophenol blue solution; the resultant solution was loaded on a 7% polyacrylamide gel (89 mM Tris–borate, 2 mM EDTA, 0.1% SDS), electrophoresed for 2 h at 25 mA, dried, and analyzed by autoradiography. DNA unwinding was quantitated by excising the bands and determining radioactivity by liquid scintillation counting in fluor (Liquifluor, NEN). One unit of DNA helicase activity is defined as the amount of enzyme unwinding 40% of the DNA helicase substrate prepared with oligonucleotide 1 in 30 min at 37 °C.

DNA polymerase assays were performed as described for assay 1 of Kaiserman and Benbow (1987). Incubations were for 30 min at 37 °C.

DNA topoisomerase assays were performed as described by Kaiserman et al. (1988). Reactions were for 30 min at 37 °C.

Purification of DNA Helicase. All manipulations were carried out at 0–4 °C. A total of 527 g of ovaries was washed with ice-cold buffer A and homogenized at a concentration of 1 g wet weight/8 mL of buffer with a Tissumizer from Tekmar (fraction I). The homogenate was centrifuged for 45 min at 4200*g* and the supernatant filtered through eight layers of cheesecloth (fraction II). Fraction II was centrifuged for 90 min at 25000*g* and the supernatant filtered through eight layers of cheesecloth (fraction III). Fraction III was divided in ≈0.9-L portions, each of which was allowed to adsorb overnight to 0.7 L of DEAE-cellulose equilibrated with buffer B. Nonadsorbed proteins were removed by filtration in a funnel fitted with a fritted glass filter; the resin was washed four times with 1 L of buffer B and resuspended in 0.5 L of this buffer. Pooled resins were used to pour a 25 × 10 cm column. The column was eluted with an 11-L gradient of 25 mM–0.4 M Tris-HCl in buffer B, followed by a 5-L wash of 0.4 M Tris-HCl. The flow rate was 0.2 L/h and 110-mL fractions were collected. Fractions were assayed for ATPase and DNA helicase activity; fractions containing DNA helicase activity eluted at 0.1 M Tris-HCl and were pooled (fraction IV). No DNA helicase activity was detected in the flow-through. Fraction IV was dialyzed against buffer C and loaded onto a 30 × 4 cm glass column containing 0.5 L of phosphocellulose equilibrated with buffer C. The column was washed with buffer C and eluted by a 0.9-L gradient of 0.05–1 M KCl in buffer C. The flow rate was 0.1 L/h and 9-mL fractions were collected. DNA-dependent ATPase activity was detected throughout the gradient. ATPase activity in the flow-through was predominantly DNA independent. Fractions containing at least 1 unit of DNA unwinding activity in 0.5 μL were pooled (fraction V). DNA helicase activity eluted

Table I: Purification of a DNA Helicase from *X. laevis* Ovaries[a]

fraction	vol (mL)	total protein[b] (mg)	total units[c]	sp act. (units/mg)
(I) crude extract	5000	132000		
(II) low-speed supernatant	4700	48000		
(III) high-speed supernatant	4500	45000		
(IV) DEAE-cellulose	4200	7980		
(V) phosphocellulose	450	810	900000	1100
(VI) hydroxylapatite	60	300	600000	2000
(VII) ssDNA–cellulose[d]	30	2.2	600000	280000

[a] Based on 527 g of ovarian tissue. [b] Protein in the crude extract was determined by the procedure described by Benbow et al. (1975). [c] DNA unwinding activity could not be quantitated in the DEAE-cellulose pool due to contaminating nuclease activities. [d] The yield from this column also reflects the removal of nuclease activity by this purification step.

at 0.4 M KCl. No DNA helicase activity was detected in the flow-through. Fraction V was dialyzed against buffer D and applied to a 15 × 2 cm column containing 25 mL of HA-ultrogel equilibrated with buffer D. The resin was washed with buffer D and eluted with a 120-mL gradient of 0.01–0.4 M K_xPO_4. The flow rate was 22 mL/h and 1.6-mL fractions were collected. DNA helicase activity eluted at 0.2 M K_xPO_4. The major DNA-dependent ATPase activity coeluted with DNA helicase activity. Fractions containing at least 1 unit of DNA unwinding activity in 0.2 μL were pooled (fraction VI). Fraction VI was dialyzed against buffer E and applied to a 15 × 1.5 cm column containing 12 mL of single-stranded DNA–cellulose. After being loaded, the column was washed with buffer E and eluted with a 60-mL gradient of 0.1–1 M KCl gradient in buffer E containing 1 mM ATP and 1 mM $MgCl_2$. The flow rate was 16 mL/h and 0.8-mL fractions were collected. Fractions containing at least 1.5 unit of DNA unwinding activity in 0.1 μL were pooled (fraction VII). This fraction could be stored at −20 °C for 6 months with a 60% loss of activity.

Determination of Stokes' Radius. A total of 2 mL of fraction VII was applied to a 73 × 2 cm glass column containing Sephacryl S-300 equilibrated with buffer F. The column was calibrated with a Pharmacia gel filtration calibration kit. The flow rate was 6.4 mL/h and 1.9-mL fractions were collected.

Sedimentation Analysis. A total of 200 μL of fraction VII was dialyzed into 5% (v/v) glycerol, 25 mM Tris-HCl (pH 7.5), and 0.5 M or 25 mM NaCl. Then 150 μL was layered on a 5-mL 15%–40% (v/v) glycerol gradient in 25 mM Tris-HCl (pH 7.5), with high or low salt. Centrifugation was in a SW 50.1 rotor at 42000 rpm for 24 h at 4 °C. Sedimentation of catalase (11.3 S) (Pharmacia), yeast alcohol dehydrogenase (7.4 S), and bovine serum albumin (4.9 S) (10 mg/mL) in parallel gradients, monitored by A_{280}, was used to estimate the sedimentation coefficient (Martin & Ames, 1961).

Protein Determination. Protein was measured by using the Bio-Rad protein determination kit with bovine plasma γ-globulin as standard.

RESULTS

Purification of X. laevis DNA Helicase. Preliminary experiments showed that DNA-dependent ATPases were abundant in fractions from various pilot columns. DNA helicase activity, however, could not be detected in fractions from DE-52, phosphocellulose, or HA-ultrogel columns since nuclease activities degraded the helicase substrate and the oligonucleotide. This degradation formed the basis of a very sensitive nuclease assay. Fortuitously, 130 mM Na_xPO_4 was found to inhibit these interfering nucleases (Figure 3A), while also stimulating the DNA helicase activity (see below). DNA helicase was then purified as outlined in Table I. The elution profile of DNA helicase and DNA-dependent ATPase activities from the final single-stranded DNA–cellulose is shown in Figure 1. DNA helicase and a DNA-dependent ATPase activity coeluted at 0.65 M KCl. DNA-dependent ATPase activity was also observed at 0.22 M KCl, but DNA helicase activity was not detected in these fractions. The DNA-dependent ATPase peak eluting at 0.12 M KCl was not found in every purification and did not contain detectable DNA helicase activity. Attempts to further purify the enzyme by chromatography on Affi-Gel Blue (Bio-Rad) or sedimentation in glycerol gradients resulted in rapid loss of enzyme activity. Rechromatography of fraction VII enzyme on phosphocellulose resulted in an enzyme that was extremely sensitive to dilution. Concentration of the DNA helicase by ultrafiltration with an Amicon pressure dialysis cell or by a Centricon microconcentrator (Amicon) also resulted in a low recovery of enzyme activity.

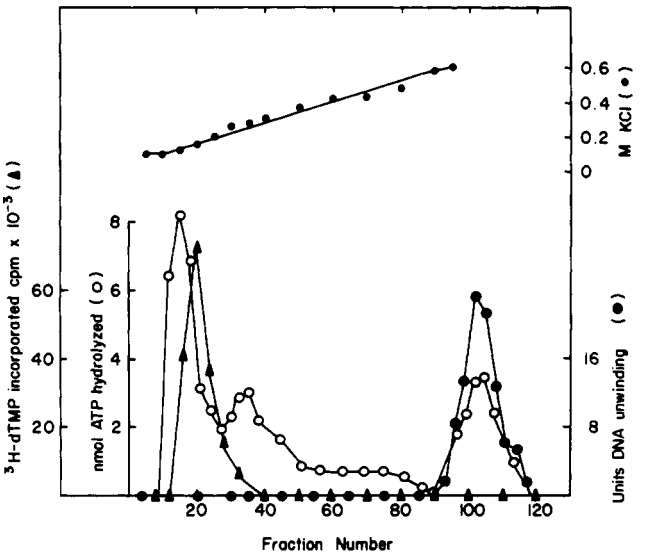

FIGURE 1: Elution profile of DNA helicase, DNA-dependent ATPase, and DNA polymerase activities from single-stranded DNA–cellulose. Fraction VI enzyme was applied to single-stranded DNA–cellulose and eluted as described under Experimental Procedures. DNA unwinding activity (●) was measured in 2-μL aliquots, DNA-dependent ATPase (○) in 20-μL aliquots (assay 1), and DNA polymerase activity (▲) in 10-μL aliquots.

Physical Characterization. DNA helicase activity sedimented both in low and in high salt glycerol gradients at 6–7.3 S. Rapid enzyme activity loss combined with the small linear range of the enzyme (Figure 4) precluded more accurate determinations of the sedimentation value. Earlier fractions of the purification could not be used due to interfering nuclease activity. The Stokes radius of the enzyme was 54 Å (Figure 2A). The native molecular weight calculated according to the procedure of Siegel and Monty (1966), assuming a partial specific volume of 0.74, was 140000–170000. The frictional coefficient was 1.46–1.56. Gel-electrophoretic analysis of Sephacryl column fractions showed eight major protein bands (Figure 2B). The 75- and 62-kDa peptide bands copurified with the DNA helicase activity. It is premature, however, to ascribe the DNA helicase activity to one or both of these polypeptides.

Biochemical Characterization of the DNA Helicase Activity. DNA unwinding activity was inactivated by boiling

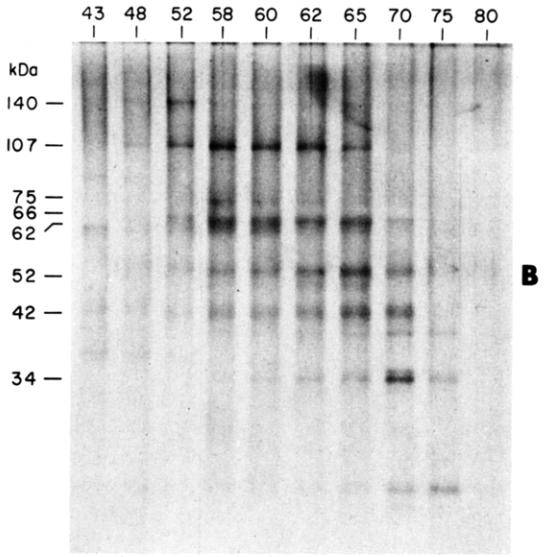

FIGURE 2: (A) Elution profile of DNA helicase activity from Sephacryl S-300. DNA helicase activity was measured in 3-μL aliquots. Arrows indicate elution of the standards: (1) blue dextran, (2) thyroglobulin (85 Å), (3) ferritin (61 Å), (4) catalase (52 Å), (5) aldolase (48 Å), (6) albumin (35.5 Å), (7) ovalbumin (30.5 Å), (8) chymotrypsinogen A (20.9 Å), and (9) ribonuclease (16.4 Å). (B) Gel electrophoretic analysis of proteins. Proteins in 1 mL of the indicated fractions of the Sephacryl S-300 column were precipitated with 10% trichloroacetic acid, analyzed on a 10% Laemmli gel (1970), and visualized by silver staining.

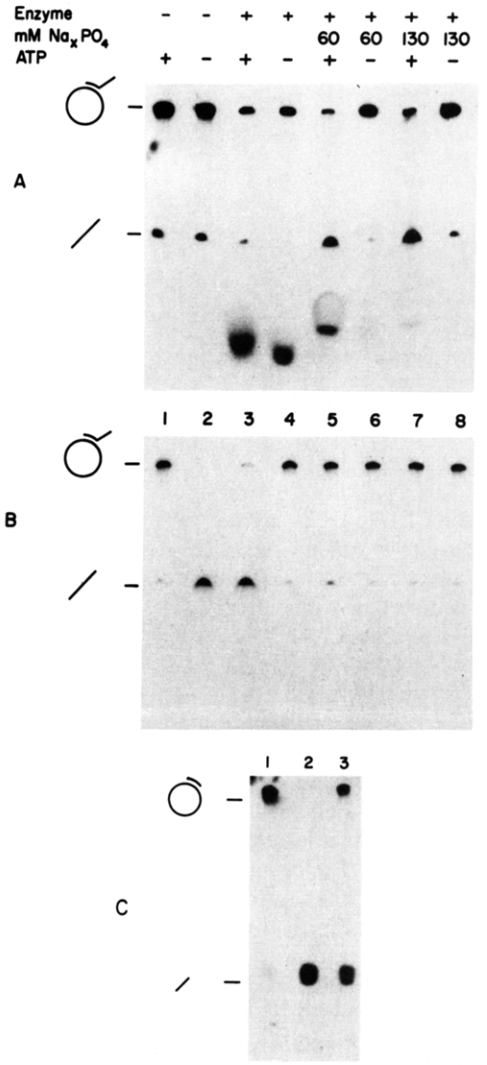

FIGURE 3: (A) Measurement of DNA helicase activity in the presence of nucleases: inhibition of nuclease activity by Na_xPO_4. DNA helicase assays were carried out as described under Experimental Procedures with 4 μL of fraction VI enzyme. The fast migrating band in lanes 3–6 contains degradation products of the substrate caused by nuclease activity. (B) DNA helicase activity: requirements for DNA unwinding. DNA helicase assays were performed with 7 units of fraction VII enzyme: lane 1, no enzyme; lane 2, no enzyme, substrate boiled for 5 min; lane 3, reaction according to that given under Experimental Procedures; lane 4, enzyme boiled for 2 min; lane 5, reaction carried out at 0 °C; lane 6, no $MgCl_2$; lane 7, no ATP; lane 8, 2 mM adenosine 5′-*O*-(3-thiotriphosphate), no ATP. (C) DNA helicase assays with a 16-mer fully hybridized to M13 DNA as substrate: lane 1, no enzyme; lane 2, no enzyme, substrate boiled for 5 min; lane 3, 1 unit of DNA helicase.

the enzyme for 2 min. Incubation for 30 min at 0 °C with 7 units of enzyme showed only 9% DNA unwinding (Figure 3B). DNA helicase activity was dependent on ATP (Figures 3 and 4). dATP could substitute for ATP, in contrast to CTP, GTP, UTP, dCTP, dGTP, and dTTP (<3% DNA unwinding). The ATP analogue adenosine 5′-*O*-(3-thiotriphosphate) did not replace ATP (Figure 3B), suggesting that ATP hydrolysis was necessary for DNA unwinding. A divalent cation was absolutely required (Figure 2B). Mg^{2+} and Mn^{2+}, but not Ca^{2+} or Zn^{2+}, were effective. The optimum concentration for Mn^{2+} was 1 mM and for Mg^{2+} between 2 and 5 mM; DNA unwinding was inhibited slightly at 10 mM $MgCl_2$; Mg^{2+} was more efficient than Mn^{2+} at all concentrations tested (Figure 4C).

DNA helicase activity was stimulated by NaCl, KCl, Na_xPO_4, and K_xPO_4. For all salts tested, the optimal stimulation was at 130 mM, and inhibition was observed at concentrations higher than 200 mM. Under optimal conditions, as described under Experimental Procedures, DNA unwinding was linear for 30 min (Figure 4A) and increased with higher amounts of enzyme (Figure 4B).

Biochemical characterization of the DNA helicase activity was done with a 40-mer (oligonucleotide 1) partially hybridized to M13 DNA as substrate. *X. laevis* DNA helicase, however, also unwound a 16-mer (oligonucleotide 2) fully hybridized to M13 DNA (Figure 3C), suggesting that there is no requirement for single-stranded DNA on both strands of the border of the duplex region.

Biochemical Characterization of DNA-Dependent ATPase Activity. The DNA-dependent ATPase activity copurifying with DNA helicase hydrolyzed both ATP and dATP (Table II). The hydrolysis products were exclusively ADP or dADP, respectively, and orthophosphate. GTP was not hydrolyzed. The K_m for ATP in the ATP hydrolysis reaction was 1 mM. The ATPase activity was dependent on the presence of DNA. Single-stranded DNA and double-stranded DNA were both cofactors for the ATPase activity, but single-stranded DNA

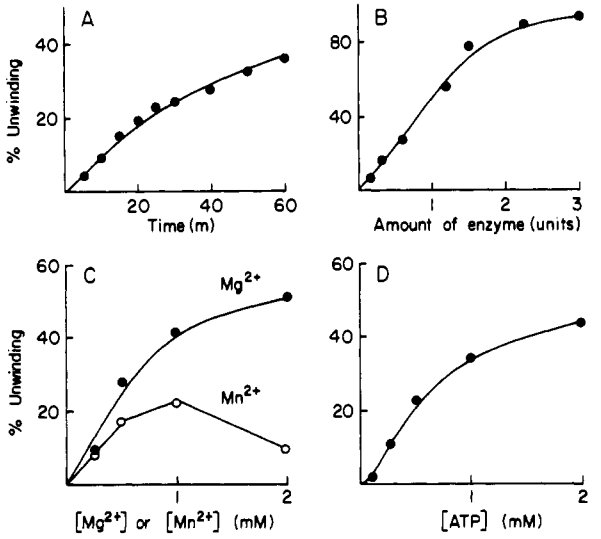

FIGURE 4: DNA helicase activity: effect of reaction parameters on DNA unwinding. Fraction VII enzyme is used. (A) Time dependence of DNA unwinding; 0.6 unit of enzyme. (B) DNA unwinding as function of enzyme concentration. (C) Divalent cation dependence [(●) Mg^{2+}, (○) Mn^{2+}]; 1.3 units of enzyme. (D) ATP dependence; 1 unit of enzyme. dATP (0.5 and 2 mM) supported 25% unwinding.

Table II: Nucleic Acid Cofactor Requirements for the DNA-Dependent ATPase Activity Copurifying with DNA Helicase

cofactor	nmol of ATP hydrolyzed[a]	%[b]	nmol of dATP hydrolyzed[a]
none	0.64	17	0.24
denatured calf thymus DNA	3.74	100	2.09
native calf thymus DNA[c]	3.17	85	nd
single-stranded M13mp18 DNA	4.25	114	nd
M13mp18 form I DNA	1.52	41	nd
MS2 RNA	0.60	16	nd

[a] Assays were performed as described under Experimental Procedures with DNA-dependent ATPase assay 2. The data are given as total reaction products. DNA concentrations were measured by A_{260}. nd = not determined. [b] Hydrolysis of ATP in the presence of denatured calf thymus DNA was arbitrarily set at 100%. [c] Native calf thymus DNA is not completely double stranded.

was more efficient (Table II).

Discussion

In this paper we describe the extensive purification of a DNA helicase from *X. laevis* ovaries. The chromatographic behavior of *X. laevis* DNA helicase and mouse DNA helicase B (Seki et al., 1986, 1987) was similar on DEAE, phosphocellulose, and hyroxylapatite but very different on single-stranded DNA–cellulose. On single-stranded DNA–cellulose the *X. laevis* enzyme eluted at 0.65 M KCl, copurifying with a DNA-dependent ATPase activity, and mouse DNA helicase B eluted at 0.22 M KCl. A *X. laevis* DNA-dependent ATPase activity eluted at 0.22 M KCl, but we were unable to detect DNA helicase activity. However, since previously described eukaryotic DNA helicases are inhibited by 130 mM PO_4^{2-}, necessary in our assays to suppress an interfering nuclease, we cannot conclude that this DNA-dependent ATPase was not a DNA helicase.

The ATPase activity of mouse DNA helicase B and yeast RAD3 protein requires single-stranded DNA as cofactor (Seki et al., 1986; Sung et al., 1987a,b). The DNA helicase activity purified from lily and yeast DNA-dependent ATPase III shows an ATPase activity that can also be supported by double-stranded DNA although only ≈20% as effectively as by single-stranded DNA (Hotta & Stern, 1978; Sugino et al., 1986). The *X. laevis* DNA helicase showed about the same efficiency for double-stranded DNA as the cofactor for the DNA-dependent ATPase activity.

ATP and dATP were the only nucleoside triphosphates that supported DNA unwinding. ATP and dATP were also both hydrolyzed by the DNA-dependent ATPase activity. In contrast, DNA unwinding by mouse DNA helicase B was supported by hydrolysis of all nucleoside triphosphates (Seki et al., 1987). All previously described eukaryotic DNA helicases are inhibited by 130 mM monovalent cations; the *X. laevis* DNA helicase, however, was stimulated severalfold.

The properties of the *X. laevis* DNA helicase, therefore, do not coincide with any of the reported eukaryotic DNA helicases in chromatographic behavior, nucleoside triphosphate requirements, and effect of monovalent cations on the DNA unwinding reaction (Hotta & Stern, 1978; Hübscher & Stalder, 1985; Sugino et al., 1986; Sung et al., 1987b; Seki et al., 1987). *X. laevis* DNA helicase is similar to DNA-dependent ATPase C2 from mouse (Seki et al., 1986) in S value and the use of only ATP and dATP as substrate for the ATPase activity. Seki et al. (1988) have reported as unpublished data that ATPase C2 has DNA helicase activity.

Unwinding of DNA helicase substrate constructed with oligonucleotide 2 shows that the *X. laevis* DNA helicase, like the other eukaryotic DNA helicases, does not require a replication fork like structure for DNA unwinding as was reported for *E. coli* dnaB protein (Lebowitz & McMacken, 1986).

Since there are at least seven different DNA helicases in *E. coli*, multiple DNA helicases in eukaryotes can be expected. It is not known which DNA helicases, if any, are involved in chromosomal DNA replication. Since *X. laevis* ovaries are a rich source of other DNA replication proteins (Laskey et al., 1979; Zierler et al., 1985), it is possible that the DNA helicase reported in this paper plays a role in DNA replication.

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Macrophage Oxidation of L-Arginine to Nitrite and Nitrate: Nitric Oxide Is an Intermediate†

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ABSTRACT: Previous studies have shown that murine macrophages immunostimulated with interferon γ and *Escherichia coli* lipopolysaccharide synthesize NO_2^-, NO_3^-, and citrulline from L-arginine by oxidation of one of the two chemically equivalent guanido nitrogens. The enzymatic activity for this very unusual reaction was found in the 100000*g* supernatant isolated from activated RAW 264.7 cells and was totally absent in unstimulated cells. This activity requires NADPH and L-arginine and is enhanced by Mg^{2+}. When the subcellular fraction containing the enzyme activity was incubated with L-arginine, NADPH, and Mg^{2+}, the formation of nitric oxide was observed. Nitric oxide formation was dependent on the presence of L-arginine and NADPH and was inhibited by the NO_2^-/NO_3^- synthesis inhibitor N^G-monomethyl-L-arginine. Furthermore, when incubated with L-[*guanido*-$^{15}N_2$]arginine, the nitric oxide was ^{15}N-labeled. The results show that nitric oxide is an intermediate in the L-arginine to NO_2^-, NO_3^-, and citrulline pathway. L-Arginine is required for the activation of macrophages to the bactericidal/tumoricidal state and suggests that nitric oxide is serving as an intracellular signal for this activation process in a manner similar to that very recently observed in endothelial cells, where nitric oxide leads to vascular smooth muscle relaxation [Palmer, R. M. J., Ashton, D. S., & Moncada, S. (1988) *Nature (London) 333*, 664-666].

The biosynthesis of NO_2^- and NO_3^- represents an unusual pathway of oxidation in mammals. Initial characterization of this reaction has shown that the pathway is expressed upon immunostimulation of macrophages by the exogenous stimulant *Escherichia coli* lipopolysaccharide (LPS)[1] (Stuehr & Marletta, 1985) and by the endogenous lymphokine IFN-γ (Stuehr & Marletta, 1987a). Murine macrophage cell lines are also capable of carrying out this synthesis after stimulation (Stuehr & Marletta, 1987b). Further characterization with cells in culture revealed that the precursor to NO_2^- and NO_3^- was the amino acid L-arginine (Iyengar et al., 1987; Hibbs et

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[1] Abbreviations: IFN-γ, recombinant murine interferon γ; LPS, *Escherichia coli* lipopolysaccharide; DME, Dulbecco's modified Eagle's media; SMEM, supplemented modified Earle's media; FCS, fetal calf serum; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EDRF, endothelium-derived relaxing factor.

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