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Xenopus laevis Ovarian DNA Helicase I: A 3′ to 5′ Helicase That Unwinds Short Duplexes[†]

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ABSTRACT: A novel DNA helicase isolated from *Xenopus laevis* ovaries [Poll, E. H. A., & Benbow, R. M. (1988) *Biochemistry 27*, 8701–8706] was characterized biochemically. The directionality of DNA unwinding was determined to be 3′ to 5′. A short 3′ ssDNA tail adjacent to duplex DNA was required for DNA unwinding; the minimum length of this tail was between four and nine bases. Only short duplex DNA regions were unwound: duplex DNA of 16 base pairs was readily unwound, whereas a 26 base pair duplex was not. Longer duplex regions were unwound in the presence of *Escherichia coli* single-strand DNA binding protein if, in addition, the duplex region was flanked by an unpaired 3′ or 5′ tail and the substrate resembled a branched replicative intermediate. *X. laevis* DNA helicase I exhibited high affinity for ssDNA, moderate affinity for dsDNA, and no affinity for RNA. DNA unwinding activity was stimulated by monovalent cations, with an optimal concentration of 150 mM for NaCl or KCl or 125 mM for Na_xPO_4 or K_xPO_4. The ATP analog ATPγS inhibited the DNA unwinding and copurifying DNA-dependent ATPase activity, whereas AMPPCP and AMPPNP moderately inhibited DNA unwinding activity and had little effect on the copurifying DNA-dependent ATPase activity. CTP was a relatively strong inhibitor of DNA unwinding activity, but GTP, UTP, dCTP, dGTP, or TTP showed moderate or no inhibition. The copurifying DNA-dependent ATPase activity was not inhibited by CTP, GTP, UTP, dCTP, dGTP, or TTP.

DNA helicases catalyze separation of the strands of duplex DNA with concomitant hydrolysis of nucleoside 5′-triphosphates (Geider & Hoffman-Berling, 1981; Lohman, 1993). All DNA helicases discovered to date utilize DNA-dependent NTPase activity to supply energy for unidirectional translocation of the enzyme along one strand of duplex DNA. DNA helicases translocate in either a 3′ to 5′ or a 5′ to 3′ direction with respect to the DNA strand to which the enzyme is bound. Some DNA helicases preferentially unwind if a forklike structure is adjacent to the duplex DNA (Li *et al.*, 1992; Lohman 1993), whereas others are inhibited by such structures (Turchi *et al.*, 1992).

Eukaryotic DNA helicases have been identified in *Lilium* (Hotta & Stern, 1978), yeast (Sugino *et al.*, 1986; Sung *et al.*, 1987), calf thymus (Hübscher & Stalder, 1985; Thömmes & Hübscher, 1990; Downey *et al.*, 1990; Zhang & Grosse, 1991; Bambara & Jesse, 1991; Siegal *et al.*, 1992; Turchi *et al.*, 1992; Li *et al.*, 1992), mouse cells (Seki *et al.*, 1987), human cells (Tuteja *et al.*, 1990, 1991, 1992, 1993; Seo *et al.*, 1991), and *Xenopus laevis* ovaries (Poll & Benbow, 1988). The biological roles of most eukaryotic DNA helicases have not been established, although substrate requirements have been determined by assaying for strand displacement from structures such as gapped or nicked duplex DNA, or from structures resembling replication forks. DNA helicase E from calf thymus, for example, is a DNA helicase with specificity for gapped and nicked substrates: this suggests that it may play a role in excision repair (Turchi *et al.*, 1992).

In this study, we characterized the substrate requirements and biochemical properties of *X. laevis* ovarian DNA helicase I. We examined the length of fragment displaced and the directionality of DNA unwinding, and determined the minimum size of the 3′ single-stranded DNA (ssDNA)[1] tail adjacent to duplex DNA required for DNA unwinding. *Escherichia coli* ssb was shown to stimulate displacement of longer duplex fragments from branched substrates. *X. laevis* DNA helicase I was active at forklike structures and unwound duplex DNA with an absolute requirement for hydrolysis of either ATP or dATP (Poll & Benbow, 1988). The *X. laevis* DNA helicase I unwinding activity was tightly associated with a DNA-dependent ATPase and dATPase activity. The effects of ATPγS, AMPPCP, and AMPPNP as well as CTP and NaCl on DNA unwinding activity were compared with the effects of these compounds on the copurifying DNA-dependent ATPase activity.

EXPERIMENTAL PROCEDURES

Materials

Enzymes. X. laevis ovarian DNA helicase I was purified as described by Poll and Benbow (1988). The experiments

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[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); AMPPNP, adenylyl 5′-β,γ-imidodiphosphate); AMPPCP, adenylyl 5′-(β,γ-methylenediphosphonate); *E. coli* ssb, *Escherichia coli* single-strand DNA binding protein; HSSB, human single-strand binding protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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in this study were carried out with fraction VII enzyme. *X. laevis* DNA polymerases α_1 and α_2 were purified as described by Kaiserman and Benbow (1987). Klenow fragment of *E. coli* DNA polymerase I and *Eco*RI restriction enzyme were from New England Biolabs, Inc. T4 DNA polynucleotide kinase was from Amersham.

Chemicals and Column Resins. Heparin grade 1 and novobiocin were purchased from Sigma. Electrophoresis reagents and Bio-gel A-5m were from BioRad. *E. coli* ssb was from USB. Nuclease-free bovine serum albumin, MS2 RNA, aphidicolin, and the ATP analogues ATPγS, AMP-PNP, and AMPPCP were from Boehringer Mannheim Biochemicals. Nonradioactive nucleotides and Sephadex G-25 were from Pharmacia/LKB Inc. Nucleotides were from NEN-Dupont or ICN. Camptothecin and VM26 were obtained as a gift from Dr. Van Narayanan of the National Cancer Institute.

Oligonucleotides and DNA. Oligonucleotides were synthesized at the Iowa State University Nucleic Acid Research Facility. The oligonucleotide 5′-TCCCAGTCACGACG(T)$_{26}$ was used for preparation of the standard DNA helicase substrate. The first 16 bases of this oligonucleotide are complementary to bases 6325–6309 of M13mp18 DNA. Native calf thymus DNA (type I, Sigma) was extracted with phenol before use. Denatured calf thymus DNA was prepared by boiling native calf thymus DNA (1 mg/mL) for 10 min followed by rapid cooling in an ice bath. *ϕX174* ssDNA was purchased from Bethesda Research Laboratories. M13mp18 ssDNA and M13mp18 RF I DNA were purified according to standard procedures (Bayne & Dumas, 1978).

Methods

DNA Unwinding Substrates. Thirty picomoles of each oligonucleotide was end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (Sambrook *et al.*, 1989), purified on a NENSORB (Dupont) column, and lyophilized. Each oligonucleotide was then mixed with 10 μg of M13mp18 ssDNA in 25 μL of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 200 mM NaCl, boiled for 5 min, and slowly cooled to room temperature. The molar ratio of oligonucleotide to M13 DNA was 7:1. Nonhybridized oligonucleotides were removed by chromatography on a 2-mL Bio-gel A-5m column. Substrates were concentrated with a Centricon 30 concentrator (Amicon) according to the manufacturer, followed by chromatography on a second 2-mL Bio-gel A-5m column. The 26-mer and 50-mer duplex substrates were purified with two successive Chroma Spin-1000 columns (Clontech). Substrates produced by both methods were used directly in DNA unwinding assays.

Directionality Substrates. A 430 pmol sample of the oligonucleotide 5′-CTCGAATTCGTAATCA was hybridized to 100 μg of M13mp18 ssDNA (molar ratio 10:1), generating a cleavable *Eco*RI restriction site (Figure 1). Hybrids were purified and concentrated as above. Forty-eight micrograms of M13 hybrid DNA was cleaved with 2400 units of *Eco*RI for 3 h at 37 °C. Digestion was monitored by gel electrophoresis. Ten micrograms of cleaved M13 DNA was boiled and hybridized to 50 pmol of ^{32}P-end-labeled oligonucleotide 5′-GTACCGAGCTCGAATT (as above), resulting in a substrate with a long 3′ ssDNA tail; another 10 μg was hybridized to 50 pmol of ^{32}P-end-labeled oligonucleotide 5′-CGTAATCATGGTCATA, resulting in a substrate with a long 5′ ssDNA tail (Figure 1). The molar ratio of oligonucleotide to M13 DNA was 12:1. Unhybridized oligonucleotides were removed as described above. Trace amounts of circular M13 DNA may be present in the linear substrates due either to incomplete hybridization of the initial oligonucleotide or to incomplete cleavage of the M13-oligonucleotide hybrid by *Eco*RI.

Substrates with 3′ Tails of Different Lengths. These substrates were prepared essentially as described (Wiekowski *et al.*, 1988). Thirteen picomoles of the 12-mer 5′-TCACGACGTTGT was hybridized to the indicated oligonucleotide (see Figure 3) in 10 μL of 10 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, and 200 mM NaCl, as described above (molar ratio 1:2.5). The 12-mer primer was elongated in the presence of [α-^{32}P]dATP and 5 units of Klenow fragment of *E. coli* DNA polymerase I as described (Stahl *et al.*, 1986) to label the substrates. Unincorporated deoxynucleotides were removed by Sephadex G-25 chromatography on a 2-mL column.

DNA Unwinding Assays. The standard assay (15 μL) contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM dithiothreitol, 2 mM $MgCl_2$, 50 μg/mL bovine serum albumin, 2 mM ATP, and approximately 10 ng of substrate unless otherwise indicated. The reaction was terminated after 30 min at 37 °C by addition of 2 μL of 10% sodium dodecyl sulfate (SDS) and 4 μL of a 20% Ficoll/0.01% bromophenol blue solution. Samples were loaded on 7% (M13 substrates) or 15% (oligonucleotide substrates) polyacrylamide gels, subjected to electrophoresis in 89 mM Tris–borate, 2 mM EDTA, and 0.1% SDS for 2 h at 125 mA, dried, and analyzed. DNA unwinding was quantitated by excising the bands and determining radioactivity by liquid scintillation counting in Liquifluor (NEN) or by scanning the gels and quantitating on a PhosphorImager (Molecular Dynamics). Percentage DNA unwinding was determined as $(X-B)/(100-B)$, where X is the percentage of unwound oligonucleotide and B is the percentage of nonhybridized oligonucleotide present in the original substrate. One unit of DNA helicase activity is defined as the amount of enzyme resulting in 40% unwinding of 10 ng (4 fmol) of the substrate 5′-TCCCAGTCACGACG(T)$_{26}$-(M13mp18) in 30 min at 37 °C (Poll & Benbow, 1988).

ATPase Assays. Assays (10 μL) contained 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 50 μg/mL bovine serum albumin, 2 mM [α-^{32}P]-ATP [$(1–6) \times 10^6$ cpm per reaction], and 20 μg/mL M13mp18 ssDNA. Reactions were terminated after 60 min at 37 °C by the addition of 2 μL of stop solution (50 mM EDTA, 10 mM ATP, 10 mM ADP, and 10 mM AMP). Two milliliters of the reaction mixture was spotted on a poly(ethylenimine) (PEI) plate (Brinkmann). Analysis was performed as described by Hübscher and Stalder (1985). Eight units of DNA helicase unwinding activity hydrolyzed approximately 1400 pmol of ATP/h.

Heat Inactivation. Sixty units of *X. laevis* DNA helicase I was incubated at 50 °C for the indicated time; DNA unwinding and DNA-dependent ATPase activities were immediately determined in duplicate for each time point.

RESULTS

Directionality of DNA Unwinding. Substrates to determine the directionality of DNA unwinding were prepared as illustrated in Figure 1. Using these substrates, *X. laevis* DNA helicase I efficiently displaced a 16-mer oligonucleotide from the 3′ to 5′ substrate with the same kinetics as from circular M13 DNA (Figure 2A,B). *X. laevis* DNA helicase I was not able to efficiently displace a 16-mer oligonucleotide from the 5′ to 3′ substrate (Figure 2C,D), whereas the same oligonucleotide hybridized to circular M13 DNA was completely displaced. The 3′ to 5′ directionality of DNA unwinding

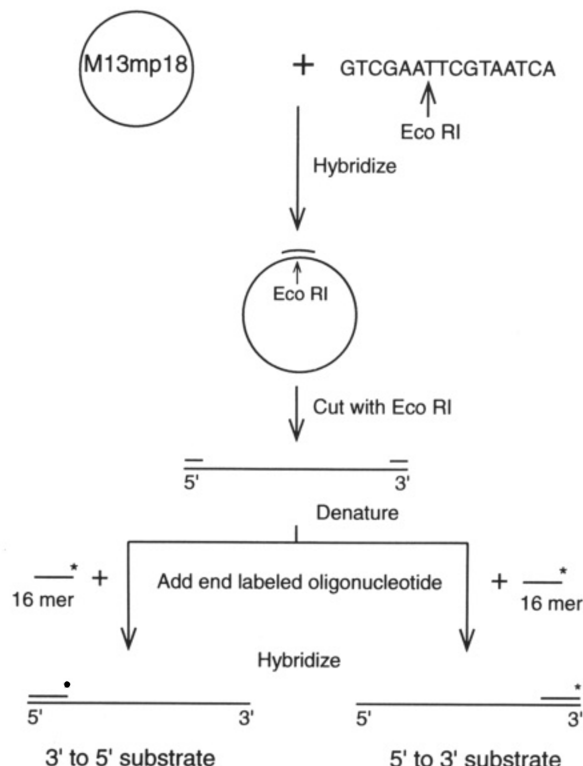

FIGURE 1: Preparation of substrates to determine the directionality of unwinding. A 16-mer oligonucleotide was hybridized to M13mp18 ssDNA, generating an *Eco*RI restriction enzyme cleavage site. (The M13mp18 DNA is not represented on the same scale as the oligonucleotides.) The resulting duplex region was cleaved with *Eco*RI. The linearized M13 ssDNA was denatured and hybridized at a molar ratio of 1:12 with ^{32}P-labeled oligonucleotides complementary with either the 3′ end or the 5′ end of the molecule. For the 3′ to 5′ directionality substrate, the sequence for the complementary strand was 5′-GTACCGAGCTCGAATT-3′. The sequence for the 5′ to 3′ directionality substrate was 5′-CGTAATCATGGTCATA-3′.

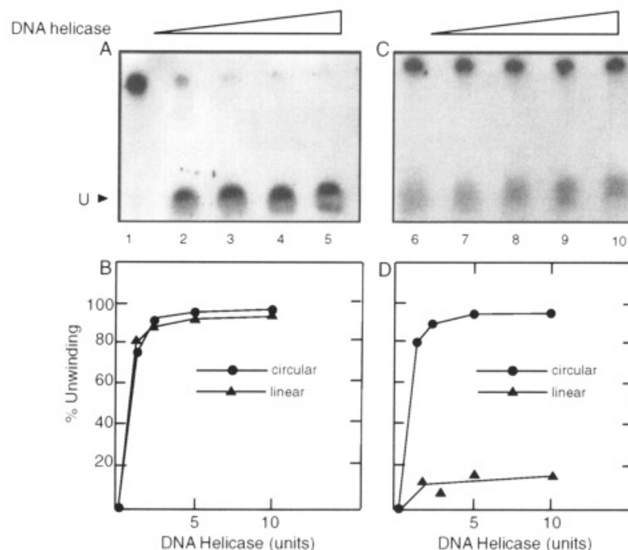

FIGURE 2: Determination of the directionality of unwinding by *X. laevis* DNA helicase I. Substrates were prepared as described in Figure 1 and under Experimental Procedures. Approximately 5 ng of substrate was used in each reaction. (A and C) Representative gels showing unwinding of 3′ to 5′ and 5′ to 3′ linearized substrate, respectively, at progressively higher concentrations of *X. laevis* DNA helicase I. U indicates unwould fragments. (B and D) Quantitation of unwinding. (B) (●) M13 circular ssDNA hybridized to 5′-GTACCGAGCTCGAATT; (▲) 3′ to 5′ directionality substrate: M13 linearized ssDNA hybridized to 5′-GTACCGAGCTCGAATT. (D) (●) M13 circular ssDNA hybridized to 5′-CGTAATCATGGTCATA; (▲) 5′ to 3′ directionality substrate: linearized M13 ssDNA hybridized to 5′-CGTAATCATGGTCATA.

DNA Helicase (units)	A (9; 5′ 3′)	B (4; 5′ 3′)	C (5′ 3′)
1	57 %	0 %	0 %
2	69 %	0 %	0 %
4	86 %	1 %	7 %
7	89 %	6 %	6 %

FIGURE 3: Size requirement for the 3′ ssDNA region adjacent to duplex DNA. The 12-mer 5′-TCACGACGTTGT was annealed to 5′-TTTTACAACGTCGTGACTCTCTCTC (A), 5′-TTTTACAACGTCGTGACTCT (B), and 5′-TTTTACAACGTCGTGA (C). The partial hybrids were 5′-end-labeled and purified as described under Experimental Procedures. The unhybridized 3′ ssDNA tails were nine (A), four (B), and zero (C) bases long. The percentage unwinding of each substrate with increasing amounts of enzyme is shown.

established by the data in Figure 2 was confirmed using small oligonucleotide substrates (Figure 3, and unpublished observations).

Size Requirement for the 3′ ssDNA Tail Adjacent to Duplex DNA. In order to determine the minimum length of ssDNA extending 3′ beyond the duplex region required for DNA unwinding, substrates with different length 3′ ssDNA tails were prepared and assayed for DNA unwinding as described under Experimental Procedures. A 3′ ssDNA tail of nine bases was sufficient to support 89% unwinding of duplex DNA (Figure 3, substrate A), whereas a substrate with a 3′ tail of four bases (Figure 3, substrate B) or a blunt-ended duplex substrate (Figure 3, substrate C) was able to support only about 6% unwinding.

Inhibition of DNA Unwinding Activity by Nucleic Acids. Increasing concentrations of ssDNA, dsDNA, and RNA were added to the standard DNA unwinding reaction in order to determine the relative affinity of *X. laevis* DNA helicase I for various nucleic acids. As shown in Figure 4, M13 ssDNA and calf thymus ssDNA both efficiently competed with the DNA helicase substrate (60% inhibition at 2.5 ng/μL nucleic acid). Calf thymus dsDNA and double-stranded M13 DNA also competed, though less efficiently (30% inhibition at 12.5 ng/μL). The greater inhibition by calf thymus dsDNA relative to M13 RFI DNA at higher concentrations probably was the result of ssDNA regions in native calf thymus DNA (Seki *et al.*, 1986). As shown in Figure 4, comparable amounts of MS2 RNA did not compete with the DNA helicase substrate.

Inhibitors of DNA Unwinding and DNA-Dependent ATPase Activity. The effects of potential inhibitors of DNA unwinding and ATPase activity on *X. laevis* DNA helicase I are shown in Table 1. DNA unwinding was not inhibited by VM26 or novobiocin, compounds which inhibit DNA topoisomerase II (Wang & Liu, 1990; Ikeda, 1990), or by camptothecin, which inhibits topoisomerase I (Drlica & France, 1980). Aphidicolin, an inhibitor of DNA polymerases α, δ, and ϵ, also did not inhibit DNA unwinding. Likewise, heparin had no effect on DNA unwinding and DNA-dependent ATPase activity (Table 1). The ATP analogs ATPγS, AMPPCP, and AMPPNP did not support DNA unwinding (<1% unwinding) when substituted for ATP in the DNA unwinding reaction. ATPγS was a strong inhibitor of DNA unwinding activity (88% at 50 μM) and of DNA-dependent ATPase activity (58% at 50 μM) (Table 1). In contrast, the ATP analogues AMPPNP and AMPPCP showed only a moderate inhibition of DNA unwinding (40% and 18%, respectively, at 5 mM). AMPPNP

Table 1: Effects of Potential Inhibitors on DNA Unwinding and ATPase Activities of *X. laevis* DNA Helicase I[a]

inhibitor	max unwinding (%)	residual ATPase act. (%)	inhibitor	max unwinding (%)	residual ATPase act. (%)
VM26 (10 mM)	101	98	ADP (5 mM)	27	ND[b]
novobiocin (100 μM)	100	94	AMP (5 mM)	80	ND
camptothecin (10 mM)	103	100	GTP (2 mM)	98	101
aphidicolin (20 μg/mL)	100	97	GTP (9 mM)	51	99
heparin (50 μg/mL)	99	104	TTP (2 mM)	87	103
ATPγS (50 μM)	12	42	CTP (2 mM)	21	109
AMPPNP (2 mM)	95	100	UTP (2 mM)	97	100
AMPPNP (5 mM)	60	96	UTP (9 mM)	74	100
AMPPCP (2 mM)	98	120	dGTP (2 mM)	63	101
AMPPCP (5 mM)	82	123	dCTP (2 mM)	85	97

[a] Unwinding and DNA-dependent ATPase values are relative to 100% for the standard substrate using standard unwinding conditions. [b] ND, not determined.

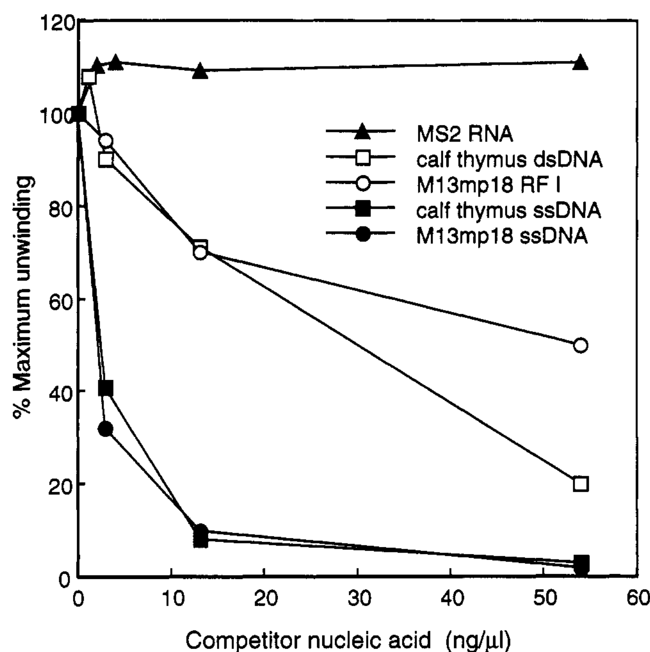

FIGURE 4: Inhibition of DNA unwinding by competitor nucleic acids. Assays were carried out as described under Experimental Procedures with competitor nucleic acids added to the indicated final concentrations. Each assay was performed with 0.5 unit of *X. laevis* DNA helicase I and contained 2 ng of substrate. Enzyme activity in the absence of added competitor nucleic acids was arbitrarily designated as 100%.

did not significantly inhibit DNA-dependent ATPase activity. Surprisingly, 5 mM AMPPCP showed 20% stimulation of DNA-dependent ATPase activity (Table 1). Addition of ADP inhibited DNA unwinding by 73% at 5 mM, and AMP caused 20% inhibition at 5 mM. ATP and dATP were the only common nucleoside triphosphates that supported DNA unwinding (Poll & Benbow, 1988). Addition of other nucleoside triphosphates to the standard DNA unwinding assay, however, did result in partial inhibition of DNA unwinding at high concentrations (Table 1 and Figure 5A).

Effect of Monovalent Cations on DNA Unwinding and DNA-Dependent ATPase Activity. In contrast to most other eukaryotic DNA helicases which were either inhibited or not affected by monovalent cations, DNA unwinding activity of the *X. laevis* DNA helicase I was markedly stimulated by monovalent cations (Poll & Benbow, 1988). The optimal salt concentrations for DNA unwinding activity were 150 mM NaCl, 150 mM KCl, 125 mM Na_xPO_4, or 125 mM K_xPO_4. In contrast to DNA unwinding activity, however, the associated DNA-dependent ATPase activity was relatively constant throughout a range of salt concentrations up to 150 mM.

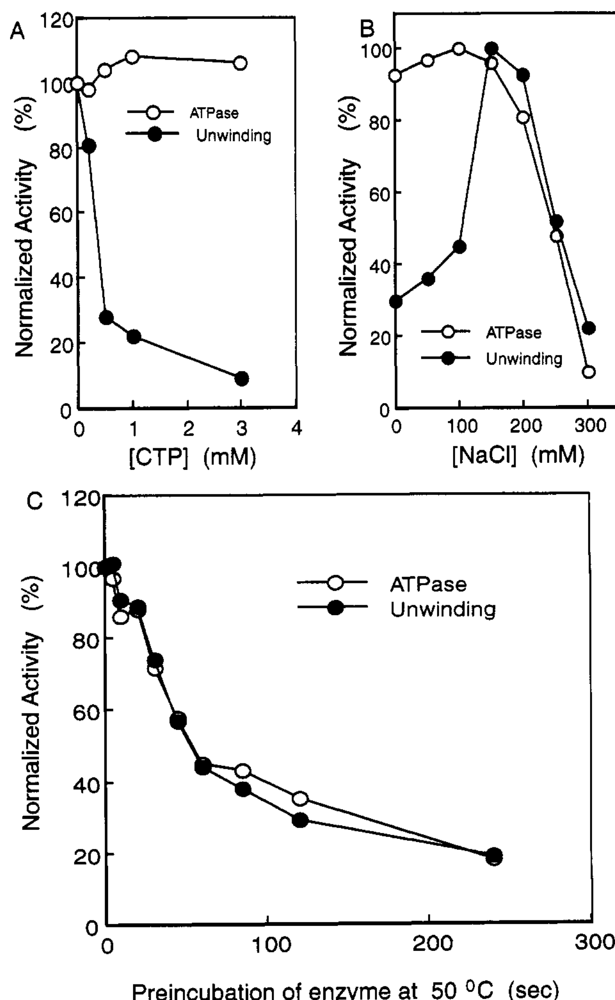

FIGURE 5: Comparison of DNA unwinding versus DNA-dependent ATPase activities. (A) Effect of [CTP]. (B) Effect of [NaCl]. (C) Heat inactivation. *X. laevis* DNA helicase I was incubated for the indicated times at 50 °C. After incubation, the enzyme was immediately assayed in duplicate for DNA unwinding activity (1.5 units) and DNA-dependent ATPase (8 units) activity (0.5 h at 37 °C). Data shown are the average of two experiments performed independently as described under Experimental Procedures. Activity without preincubation was 100%.

Activity then decreased at higher salt concentrations in parallel with DNA unwinding activity (Figure 5B).

Relationship between DNA Unwinding and DNA-Dependent ATPase Activities. As shown in Figure 5A, although CTP was a relatively strong inhibitor of DNA unwinding, it slightly stimulated ATPase activity. Similarly, the DNA unwinding activity, but not the associated DNA-dependent

Table 2: Unwinding of Different Length Oligonucleotides Hybridized to M13 ssDNA by *X. laevis* DNA Helicase

length	sequence	Hel I	ssb	% unwinding[a]
16-mer	5′-TCCCAATTCTGCGAAC	+	no	85
	5′-GTACCGAGCTCGAATT	+	no	81
	5′-GATCCCCGGGTACCGA	+	no	90
	5′-GGATCCCCGGGTACCG	+	no	79
26-mer	5′-TCCCAATTCTGCGAACGAGTAGATTT	+	yes	9
	5′-TCCCAATTCTGCGAACGAGTAGATTT	+	no	4
+5′ tail	5′-$(A)_{50}$TCCCAATTCTGCGAACGAGTAGATTT	+	yes	60
	5′-$(A)_{50}$TCCCAATTCTGCGAACGAGTAGATTT	+	no	<3
+3′ tail	5′-TCCCAATTCTGCGAACGAGTAGATTT-$(A)_{50}$	+	yes	44
	5′-TCCCAATTCTGCGAACGAGTAGATTT-$(A)_{50}$	+	no	8
50-mer	5′-TCCCAATTCTGCGAACGAGTAGATTTAGTTTGACCATTAGATACATTTCG-3′	+	yes	<2
+5′ tail	5′-$(N)_{25}$TCCCAATTCTGCGAACGAGTAGATTTAGTTTGACCATTAGATACATTTCG-3′	+	yes	<2
+3′ tail	5′-TCCCAATTCTGCGAACGAGTAGATTTAGTTTGACCATTAGATACATTTCG$(N)_{25}$-3′	+	yes	<2

[a] Based on the absolute unwinding of each substrate as defined under Experimental Procedures and as shown in Figure 2.

ATPase activity, was affected by salt concentrations up to 150 mM (Figure 5B). The differential sensitivities of DNA unwinding and DNA-dependent ATPase activities to various compounds (Table 2 and Figure 5A,B) raised the possibility that these activities might copurify rather than correspond to intrinsic activities of *X. laevis* DNA helicase I. This possibility was further investigated by measuring thermal inactivation of the activities (Figure 5C). Incubation at 50 °C inactivated both activities in parallel, consistent with the hypothesis that both the DNA unwinding and DNA-dependent ATPase activities are intrinsic to the native enzyme. Moreover, these activities were very heat-stable in comparison to *X. laevis* DNA polymerase α_1 and α_2 activities, which were each completely inactivated after 1 min at 40 °C.

Stimulation of Strand Displacement by E. coli ssb. *X. laevis* DNA helicase I was previously shown to unwind an oligonucleotide of 16 bases hybridized to M13 ssDNA (Poll & Benbow, 1988). The unwinding of longer duplex regions was investigated to determine the maximal length of fragment that was displaced. The oligonucleotides shown in Table 2 were hybridized to M13mp18 and used to assay for DNA unwinding by *X. laevis* DNA helicase I. A duplex DNA of 26 bp was not unwound by *X. laevis* DNA helicase I, even when an excess of enzyme (10 units) was used. Addition of an ATP-regenerating system to the DNA unwinding assay did not increase processivity. However, inclusion of *E. coli* ssb in the reaction (25 μg/mL) stimulated unwinding of the 26-mer duplexes tested. Duplexes flanked by a 5′ or 3′ tail showed a higher percentage unwinding. Figure 6 shows stimulation of *X. laevis* DNA helicase I by *E. coli* ssb. Other accessory proteins such as *X. laevis* DNA polymerase primase α_1 or *X. laevis* DNA polymerase α_2 did not stimulate unwinding of the 26 base pair duplex region by the *X. laevis* DNA helicase (less than 1% unwinding with 10 units DNA helicase). In contrast to unwinding of 26-mer duplexes, unwinding of 50-mer duplexes was not stimulated by ssb, even when a 3′ or 5′ flanking region was present.

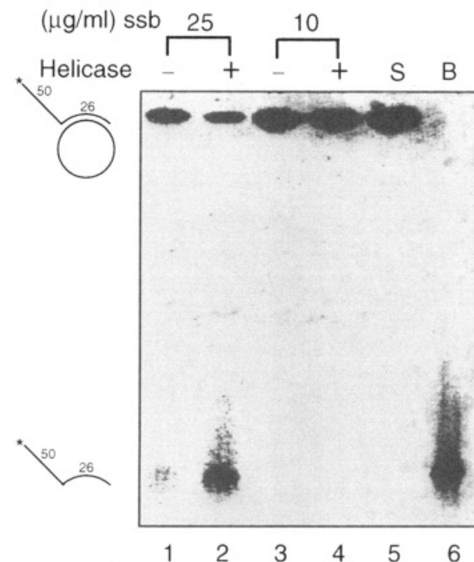

FIGURE 6: Effect of *E. coli* ssb on unwinding. Approximately 5 ng of ^{32}P-labeled M13mp18 76-mer substrate (a 26-mer duplex with a 50-base 5′ tail) was used in the reaction. The indicated concentrations of ssb were preincubated with the substrate before addition of *X. laevis* DNA helicase I. The standard assay was used except that 25 mM NaCl was substituted for 150 mM because ssb preferentially stimulated unwinding at the lower salt concentrations. Lane 1, no helicase, 25 μg/mL ssb; lane 2, 5 units of helicase, 25 μg/mL ssb; lane 3, no helicase, 10 μg/mL ssb; lane 4, 5 units of helicase, 10 μg/mL ssb; lane 5 is a no enzyme control; lane 6 is a heat-denatured control.

DISCUSSION

In this study, we have shown that the directionality of DNA unwinding by *X. laevis* DNA helicase I is 3′ to 5′. This directionality is shared by human DNA helicases I, III, and V (Tuteja *et al.*, 1993) and by calf thymus DNA helicases A (Thömmes & Hübscher, 1990) and E (Turchi *et al.*, 1992). The minimum required length of the 3′ ssDNA tail adjacent to the duplex DNA region is between five and nine bases *in vitro* for efficient unwinding. SV40 T antigen DNA helicase requires less than five bases for nonspecific DNA unwinding, and has no requirement for a 3′ ssDNA tail when the duplex DNA contains an SV40 DNA replication origin (Wiekowski *et al.*, 1988; Goetz *et al.*, 1988). In contrast, *E. coli* DNA helicase I requires a single-stranded region of approximately 200 nucleotides of ssDNA adjacent to duplex DNA (Kuhn *et al.*, 1979). Human DNA helicases II and IV require a single-stranded region of over 84 bases adjacent to duplex DNA to support unwinding (Tuteja *et al.*, 1993).

DNA unwinding by *X. laevis* DNA helicase I is supported by hydrolysis of ATP or dATP, and is not supported by ATP analogs or other nucleoside triphosphates (Poll & Benbow, 1988). Addition of ATP analogs or nucleoside triphosphates to the DNA unwinding and DNA-dependent ATPase assays gives rise to complex effects (Table 1). ATPγS inhibits both DNA unwinding and DNA-dependent ATPase activities. At high concentrations of ATPγS, however, the residual ATPase activity is 4 times higher than the residual DNA unwinding activity. ATPγS was previously reported to inhibit DNA unwinding by SV40 T antigen (Goetz *et al.*, 1988), and to inhibit the DNA-dependent ATPase activity of yeast RAD III protein (Sung *et al.*, 1987a). CTP significantly inhibits

the DNA unwinding reaction, but slightly stimulated the ATPase reaction. Yeast RAD III protein DNA-dependent ATPase activity is inhibited by CTP but stimulated by dCTP (Sung *et al.*, 1987). CTP does not inhibit DNA unwinding by SV40 T antigen (Goetz *et al.*, 1988), although T antigen exhibits an affinity for CTP (Scheffner *et al.*, 1989).

It should be noted that the DNA unwinding activity of *X. laevis* DNA helicase I is far more sensitive to inhibitors than the DNA-dependent ATPase activity. Similar data have been obtained for the SV40 T antigen DNA helicase (Stahl *et al.*, 1986) and HSSB-dependent DNA helicase from HeLa cells (Seo *et al.*, 1991). For example, T antigen specific antibodies that block the ATPase function block the DNA unwinding activity, but not necessarily vice versa. DNA unwinding requires hydrolysis of ATP, but ATP hydrolysis does not necessarily require DNA unwinding. A possible explanation for the differential effects of inhibitors on the *X. laevis* DNA helicase I DNA-dependent ATPase and DNA unwinding activities might be the presence of multiple ATPase activities in the most purified fraction. This is not likely, however, for the following reasons. The enzyme was extensively purified: during the last chromatographic step, the DNA-dependent ATPase activity copurifies with the DNA unwinding activity [Figure 1 of Poll and Benbow (1988)] and elutes at a high salt concentration from ssDNA–cellulose. The DNA unwinding and DNA-dependent ATPase activities cosediment in low- and high-salt glycerol gradients and coelute on Sephacryl S-300 (Poll & Benbow, 1988; unpublished observations). In addition, the DNA-dependent ATPase and DNA unwinding activities share many properties (Table 1), and have similar affinities for ssDNA, dsDNA, and RNA. Finally, incubation of *X. laevis* DNA helicase I at 50 °C results in parallel inactivation of the DNA unwinding and DNA-dependent ATPase activities, consistent with the hypothesis that both activities are intrinsic to the enzyme.

Most differential effects of inhibitors on DNA unwinding and DNA-dependent ATPase activities are probably due to conformational alterations. The ATP analogs and CTP might change the binding of DNA helicase to its substrate, resulting in differential effects on DNA unwinding and DNA-dependent ATPase activities. Strong association of enzyme with its substrate might induce an increase in ATPase activity (since there is no need for additional binding events) and a decrease in DNA unwinding activity.

It had been previously shown that *E. coli* DNA primase, in addition to *E. coli* ssb, was necessary to stimulate unwinding of long stretches of duplex DNA by the *E. coli dnaB* protein DNA helicase (Lebowitz & McMacken, 1986). More recently, DNA unwinding by a DNA helicase from HeLa cells was shown to be dependent on stimulation by HSSB or *E. coli* ssb at high enzyme concentrations (Seo *et al.*, 1991). The *X. laevis* DNA helicase unwinds only short stretches of duplex DNA *in vitro* (Table 2). It can unwind a 16-mer annealed to M13mp18 ssDNA, regardless of whether mismatched tails are present at the 5′ end, 3′ end, or neither (Poll & Benbow, 1988). With the addition of *E. coli* ssb, *X. laevis* DNA helicase I is able to unwind a 26-mer, with more efficient unwinding occurring when a 5′ or 3′ mismatched tail is present (Table 2). These data are consistent with a slight stimulation by eukaryotic RP-A (single-strand DNA binding protein) of the displacement of larger fragments by DNA helicase E from calf thymus (Turchi *et al.*, 1992). We cannot rule out the possibility that *X. laevis* DNA helicase I unwinds even longer duplex regions *in vivo*. Although the length of fragment unwound by *X. laevis* DNA helicase I is only slightly increased by *E. coli* ssb, it is not unreasonable that more specific accessory proteins in *X. laevis* embryos could further increase its processivity.

Table 3: Properties of *X. laevis* DNA Helicase I

size (kDa)	62 and 75, or 107[a]
directionality	3′ to 5′
blunt-end duplex unwinding	no
preference for forklike structure	yes
max length unwound (bp)	50 > 26[b]
length of free ssDNA on substrate (bases)	9 > 4
cation requirement	$Mg^{2+} > Mn^{2+}$
preferred (d)NTP	ATP, dATP
affinity	ssDNA > dsDNA ≫ RNA
molecules of ATP hydrolyzed (molecule of enzyme)$^{-1}$ s^{-1}	1.4×10^{-3}

[a] The molecular mass of 107 kDa is based on the molecular mass of the most abundant polypeptide in fraction VII on SDS–PAGE gels; 62 and 75 kDa are based on coeluting peptides summing to the native molecular mass of 144 kDa calculated from the *s* value and Stokes radius (Poll & Benbow, 1988). [b] With *E. coli* ssb.

We do not know the biological role of *X. laevis* ovarian DNA helicase I. A physiological role in DNA replication might seem unlikely since *X. laevis* DNA helicase I unwinds only short duplexes *in vitro*. Eukaryotic DNA helicases implicated in replication often unwind duplexes hundreds of base pairs in length: HSSB-dependent human DNA helicase was found to unwind over 300 base pairs (Seo *et al.*, 1991). Some prokaryotic DNA helicases unwind duplexes thousands of base pairs long. It is not known why eukaryotic DNA helicases exhibit lower processivities, but this may reflect a fundamental difference in replication strategies. Strand separation in eukaryotes may be uncoupled from strand synthesis (Benbow *et al.*, 1985, 1986, 1992), whereas in prokaryotes strand separation is tightly coupled to strand synthesis at replication forks.

The properties of *X. laevis* DNA helicase I (Table 3) are similar in some respects to those of human DNA helicase V and HSSB-dependent DNA helicase from HeLa cells (Tuteja *et al.*, 1993; Seo *et al.*, 1991). The HeLa cell HSSB-dependent DNA helicase (Seo *et al.*, 1991) shares a number of characteristics in common with *X. laevis* DNA helicase I, but also differs in several significant ways. Both enzymes translocate in the 3′ to 5′ direction. Both enzymes do not displace relatively short oligonucleotides of length 30 (HSSB-dependent DNA helicase) or 26 (*X. laevis* DNA helicase I) nucleotides in the absence of ssb, although processivity was only slightly increased (to 26 nucleotides) for the *X. laevis* enzyme even in the presence of ssb. Furthermore, both enzymes show inhibition by ATPγS and AMPPNP, and both elute from ssDNA–cellulose at 0.6 M NaCl. However, whereas CTP has an inhibitory effect on *X. laevis* DNA unwinding activity (79% at 2 mM), CTP has a stimulatory effect (in the absence of ATP) on HSSB-dependent DNA unwinding activity at concentrations higher than 1 mM. In addition CTP, UTP, dATP, and dCTP support unwinding by HSSB-dependent DNA helicase, but not by *X. laevis* DNA helicase I. Mn^{2+} cannot substitute for Mg^{2+} as cofactor for the HSSB-dependent DNA helicase, but can for *X. laevis* DNA helicase I. HSSB-dependent DNA helicase can unwind DNA fragments of 55 nucleotides and longer in the presence of HSSB, whereas *X. laevis* DNA helicase I does not unwind a 50-mer, even in the presence of ssb. Finally, HSSB-dependent DNA helicase unwinds blunt-end duplexes, wheres *X. laevis* DNA helicase does not.

Human DNA helicase V (Tuteja *et al.*, 1993) and *X. laevis* DNA helicase I also share numerous characteristics. Both human DNA helicase V and *X. laevis* DNA helicase I catalyze

strand displacement in the 3′ to 5′ direction. Second, they both exhibit low processivity: human DNA helicase V can unwind a 17-mer annealed to M13 ssDNA, regardless of the presence or absence of mismatched tails at either the 5′ or the 3′ end. When the duplex region is increased to 25 base pairs, it is not unwound. In comparison, *X. laevis* DNA helicase I unwinds a 16-mer with or without mismatched tails and is unable to unwind a 26-mer with or without mismatched tails in the absence of *E. coli* ssb. Furthermore, both enzymes have the same cation requirements, both can use Mn^{2+} in place of Mg^{2+}, and both use only ATP or dATP hydrolysis to support DNA unwinding. Finally, both enzymes are inhibited at 200 mM NaCl, although *X. laevis* DNA helicase I is stimulated between 100 and 150 mM NaCl, a characteristic of human DNA helicase I (Tuteja *et al.*, 1990). Both enzymes have a high affinity for ssDNA: human DNA helicase V elutes from ssDNA–Sepharose at approximately 0.85 M KCl, whereas *X. laevis* DNA helicase I elutes at 0.65 M KCl from ssDNA–cellulose. These similarities suggest a possible correspondence between the respective enzymes.

However, there are also some significant differences in the properties reported for human DNA helicase V and *X. laevis* DNA helicase I. Most notably, the rate at which ATP is utilized by human DNA helicase V (14 molecules of ATP hydrolyzed per molecule of enzyme per second) is substantially higher than the rate it is utilized by *X. laevis* DNA helicase I (1.4×10^{-3} molecules of ATP hydrolyzed per molecule of enzyme per second) (Table 3). The latter rate of hydrolysis is closer to the value described for human DNA helicase III, and most other eukaryotic DNA helicases. The molecular mass of human DNA helicase V is 92 kDa, which differs somewhat from those of the possible subunits of *X. laevis* DNA helicase I (Table 3). In spite of these differences, we believe *X. laevis* ovarian DNA helicase I most closely resembles human DNA helicase V of all eukaryotic DNA helicases described to date.

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