Nuclear DNA Helicase II Unwinds both DNA and RNA†

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ABSTRACT: Nuclear DNA helicase II (NDH II) has been purified to near-homogeneity by exploiting its high affinity to poly[(rI)·(rC)]–agarose. The purified enzyme was obtained as two catalytically active forms of 130- and 100-kDa molecular mass, respectively. After treatment with cyanogen bromide, the separated polypeptides displayed very similar digestion patterns. Thus, the 100-kDa form most likely is a proteolytic product of the 130-kDa polypeptide. For DNA unwinding, NDH II could use any of the four rNTPs or dNTPs with K_m values between 20 and 100 μM. DNA unwinding was stimulated up to 20-fold by substrates that contained single-stranded 3′-tails. NDH II-catalyzed DNA unwinding was strongly inhibited by RNA, but was little affected by DNA. The strongest RNA inhibitor, poly[(rI)·(rC)], was also the strongest effector of the NTPase activity of NDH II. The binding constant for poly[(rI)·(rC)] binding was about 2×10^7 M^{-1}; the minimal binding site size was determined as 16 nucleotides. In agreement with its high affinity to RNA, NDH II unwound double-stranded RNA. RNA unwinding required the presence of a nucleoside triphosphate and a divalent cation (Mg^{2+}). Thus, like the prototypic replicative helicase large T antigen of simian virus 40, NDH II may function in both DNA and RNA unwinding.

The opening of double-stranded DNA (dsDNA)[1] is crucial for the processes of DNA replication, DNA repair, DNA recombination, and possibly DNA transcription. A class of enzymes, the DNA helicases, catalyzes DNA unwinding at the expense of chemical energy. So far a variety of DNA helicases have been isolated and characterized from organisms as divergent as bacteria, bacteriophages, viruses, and eukaryotic cells. A common feature of all these enzymes is their DNA-dependent nucleoside-5′-triphosphatase (NTPase) activity which is postulated to drive both the translocation of the enzyme along the template strand and the concomitant unwinding of duplex structures [for recent reviews, see Kornberg and Baker (1991), Lohman (1993), Matson (1991), Matson and Kaiser-Rogers (1990), and Thömmes and Hübscher (1990a)].

In mammalian tissues, as many as nine different DNA helicases have been identified [see, e.g., Thömmes *et al.* (1992) and references cited therein]. However, physiological functions for these enzymes in the various processes of DNA metabolism have not yet been assigned. In our search for cellular DNA helicases that may be involved in DNA replication, we have isolated and partially characterized two copurifying enzymes from calf thymus nuclei (Zhang & Grosse, 1991) and a third one from bovine cytosolic extracts (Zhang & Grosse, 1992). One of these, nuclear DNA helicase II (NDH II), seems exceptional since it shares some biochemical properties with the SV40 large T antigen. Like the viral helicase, NDH II catalyzes DNA unwinding with a 3′ to 5′ polarity and displays a low selectivity for the use of the four rNTPs or dNTPs. Here we extend that analogy by showing that DNA unwinding is stimulated by 3′- and 5′-tailed duplex substrates that resemble replication forks. Furthermore, we show that NDH II binds tightly to both single-stranded and double-stranded RNA. In agreement with its RNA binding properties, NDH II promotes NTP-dependent RNA unwinding. Taken together, these results suggest that NDH II may have multifunctional roles in various processes of cellular DNA and RNA metabolism.

MATERIALS AND METHODS

Materials. Most of the materials used have been described elsewhere (Zhang & Grosse, 1991, 1992). The untailed 22-mer oligodeoxyribonucleotide 5′-ACTCTAGAGGATCCCCGGGTAC-3′, the 45-mer oligodeoxyribonucleotide with a 5′ single-stranded region (underlined) 5′-GTTATTGCATGAAAGCCCGGCTGACTCTAGAGGATCCCCGGGTAC-3′, and the 45-mer with 3′ tail (underlined) 5′-ACTCTAGAGGATCCCCGGGTACGTTATTGCATGAAAGCCCGGCTG-3′ were synthesized on a 380 B DNA synthesizer from Applied Biosystems by using the phosphoroamidite method. Plasmids pGEM-C4 and pGEM-MO1 (Scheffner *et al.*, 1989) were kind gifts of Dr. Hans Stahl (University Konstanz, Germany). MS-2 phage RNA was from Boehringer-Mannheim. Other polyribo- and polydeoxyribonucleotides were obtained from Pharmacia.

Affinity Chromatography and NDH II on Poly[(rI)·(rC)]–Agarose. Nuclear DNA helicase II was purified from nuclear extracts of calf thymus up to the chromatography step on heparin–Sepharose (without the ATP–agarose step) exactly as described before (Zhang & Grosse, 1991). NDH II-containing fractions from heparin–Sepharose were collected and dialyzed against buffer A (20 mM potassium phosphate, pH 7.8, 10 mM $Na_2S_2O_5$, 7 mM 2-mercaptoethanol, 1 mM PMSF, 10% (v/v) glycerol, and 50 mM NaCl]. After dialysis, the enzyme solution (containing about 2 mg of protein) was loaded onto a 0.79 $cm^2 \times 3.2$ cm column of poly[(rI)·(rC)]–agarose (Pharmacia) that had been equilibrated with 25 mL of buffer A. The column was washed with 7.5 mL of buffer A and developed with a 25-mL gradient from 50 to 600 mM

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[1] Abbreviations: ds, double-stranded; NTPase, nucleoside-5′-triphosphatase; ss, single-stranded; NDH II, nuclear DNA helicase II; SV40, simian virus 40; T-ag, large tumor antigen; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; v/v, volume by volume; w/v, weight by volume; NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; NMP, nucleoside monophosphate; UV, ultraviolet; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid.

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NaCl in buffer A. Thereafter, the column was washed with 7.5 mL of buffer A containing 2 M NaCl. Fractions of 1 mL were collected and assayed for poly[(rI)·(rC)]-dependent ATPase activity. Active fractions were combined and dialyzed against buffer B [30 mM potassium phosphate, pH 7.8, 10 mM $Na_2S_2O_5$, 7 mM 2-mercaptoethanol, and 50% (v/v) glycerol]. The enzyme was stored at −20 °C.

NTPase Assays. The NTPase reaction mixture (10 μL) contained 20 mM Tris-HCl, pH 7.5, 10% glycerol, 3.5 mM $MgCl_2$, 100 μg/mL bovine albumin, 5 mM dithiothreitol, 0.25–2.5 mM [^{3}H]NTP (100–200 cpm/pmol), 50 μg/mL nucleic acid (if not stated otherwise), and 2 μL of the enzyme fraction (10–100 ng of NDH II). Following incubation at 37 °C for 30 min, 2 μL of stop solution was added to give a final concentration of 50 mM EDTA, 10 mM NTP, 10 mM NDP, and 10 mM NMP. One microliter of this mixture was spotted onto a poly(ethylenimine)–cellulose thin-layer plate (CEL 300 PEL; Macherey-Nagel, Düren, Germany), and ascending chromatography was performed in a solution of 0.5 M LiCl/1 M formic acid. The radioactive material in the positions of NTP, NDP, and NMP, as identified under UV light, was excised and quantified by liquid scintillation counting. One unit of ATPase activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 nmol of ATP into ADP in 60 min at 37 °C.

Preparation of DNA Helicase Substrates. Twenty picomoles of DNA primer was 5′-labeled by incubation with 60 μCi of [γ-^{32}P]ATP and 20 units of T4 polynucleotide kinase (Sambrook *et al.*, 1989). The labeled oligonucleotide was mixed with M13mp18 ssDNA (20 pmol) in 80 μL of annealing buffer (6 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, 50 mM NaCl, and 1 mM dithiothreitol). The annealing mixture was heated for 5 min to 100 °C, then incubated for 30 min at 60 °C and for another 30 min at 37 °C, and then cooled down to room temperature. Free oligonucleotide was removed by gel filtration over a 2-mL Biogel A-5m (Bio-Rad, Richmond, CA) column.

Preparation of the RNA Helicase Substrate. The substrate used in the RNA unwinding assays was prepared according to Scheffner *et al.* (1989). Plasmid pGEM-C4 (0.5 μg) was linearized by digestion with *Pvu*II, and the incubated with SP6 RNA polymerase in the presence 50 μCi of [α-^{32}P]GTP (3000 Ci/mmol) to produce a labeled 194-nucleotide-long RNA transcript. An unlabeled transcript, 130 nucleotides in length, was synthesized by T7 RNA polymerase on the *Hin*dIII-linearized plasmid pGEM-M01 (1.0 μg). The RNA transcription reactions were performed according to the conditions described by the supplier of the kit (Boehringer-Mannheim). The 2 RNA transcripts, which share a complementary region of 17 nucleotides, were hybridized in a buffer containing 80% formamide, 0.4 M NaCl, 1 mM EDTA, and 40 mM PIPES, pH 6.4. Annealing was achieved by heating the RNA mixture to 80 °C for 15 min followed by incubation for 3 h at 50 °C. Nonincorporated nucleotides and the annealing buffer were removed by chromatography over a 1-mL Sephadex G 50 column (Nick-Column, Pharmacia).

DNA Unwinding Assay. The DNA helicase reaction mixture (10 μL) contained 20 mM Tris-HCl, pH 7.5, 10% glycerol, 3.5 mM $MgCl_2$, 100 μg/mL serum albumin, 5 mM dithiothreitol, 45 μM (nucleotide) DNA substrates, 3 mM ATP, and enzyme (1 μL). After 30-min incubation at 37 °C, the reaction mixture was chilled on ice, and 3 μL of stop solution [1% (w/v) SDS, 200 mM EDTA, 50% (v/v) glycerol, 0.1% (w/v) bromophenol blue, and 0.1% (w/v) xylene cyanol FF] was added. The reaction mixture was electrophoresed through a nondenaturing 15% polyacrylamide gel (8 × 10 cm) in TBE buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA). After electrophoresis, the reaction products were visualized by autoradiography.

RNA Unwinding Assay. The RNA unwinding assay was performed as described (Scheffner *et al.*, 1989). The reaction mixture (10 μL) contained 30 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1.5 mM dithiothreitol, 30 μg/mL serum albumin, 0.5 unit/mL RNase inhibitor (Boehringer-Mannheim), 3 mM ATP, 2 ng of radioactively labeled RNA substrate, and 0.5–1 μg of enzyme. Incubations were at 37 °C for 30 min. Reactions were stopped by the addition of 0.1 volume of 3% SDS and 150 mM EDTA. The reaction mixture was electrophoresed through a 10% SDS–polyacrylamide gel (Laemmli, 1970); thereafter, the reaction products were visualized by autoradiography.

Other Methods. SDS–polyacrylamide gel electrophoresis was performed according to Laemmli (1970). Tricine–SDS–polyacrylamide gel electrophoresis was carried out according to Schägger and von Jagow (1987). For silver staining, we followed the method of Blum *et al.* (1987). Electrophoresis under native conditions was conducted on a 20% polyacrylamide gel in 5 mM Tris/borate, pH 7.5, 0.5 mM EDTA for 20 h at 4 V/cm at room temperature. The nitrocellulose filter retention assay was done as described elsewhere (Grosse *et al.*, 1986). Peptide mapping by CNBr was performed in the presence of SDS as described by Nikodem and Fresco (1979). Protein concentrations were determined by measuring the absorption at 280 nm and assuming that 1 absorption unit/cm is equivalent to 1 mg/mL protein.

RESULTS

Copurification of DNA Helicase II and a Poly[(rI)·(rC)]-Stimulated ATPase Activity. NDH II was purified until the ATP–agarose purification step, exactly as described earlier (Zhang & Grosse, 1991). From this column, a DNA unwinding activity was eluted at about 250 mM NaCl (Figure 1A). SDS–polyacrylamide gel electrophoresis of the active fractions displayed two predominant protein bands with molecular masses of 130 and 100 kDa (Figure 1B). The eluted fractions were also assayed for ATPase activity in the presence of poly[(rI)·(rC)] and M13 ssDNA. These measurements revealed an elution pattern for the ATPase that was congruent with the DNA unwinding activity. The coincidence of ATPase and DNA unwinding activity was observed for all polynucleotides tested so far, which indicates that ATPase and DNA unwinding activities are localized on the same polypeptide(s) (see below). It seems noteworthy that the ATPase was stimulated stronger by poly[(rI)·(rC)] than by M13 ssDNA (Figure 1A).

Affinity Chromatography of Nuclear DNA Helicase II on Poly[(rI)·(rC)]–Agarose. The strong stimulation of the ATPase activity suggested a strong binding capability of NDH II to poly[(rI)·(rC)]. To demonstrate RNA binding directly, we loaded partially purified NDH II from heparin–Sepharose (see Materials and Methods) onto poly[(rI)·(rC)]–agarose. Salt-gradient elution of this column revealed two distinct RNA-stimulated ATPase peaks. The first peak eluted at 0.2–0.25 M NaCl, while the second one came off at 0.6 M NaCl (Figure 2A). SDS–polyacrylamide gel electrophoresis of the two eluted fractions revealed a separation of the two polypeptides associated with NDH II activity (Figure 2B).

Hydrodynamic Properties of the Separated Polypeptides of Nuclear DNA Helicase II. The separation of the two

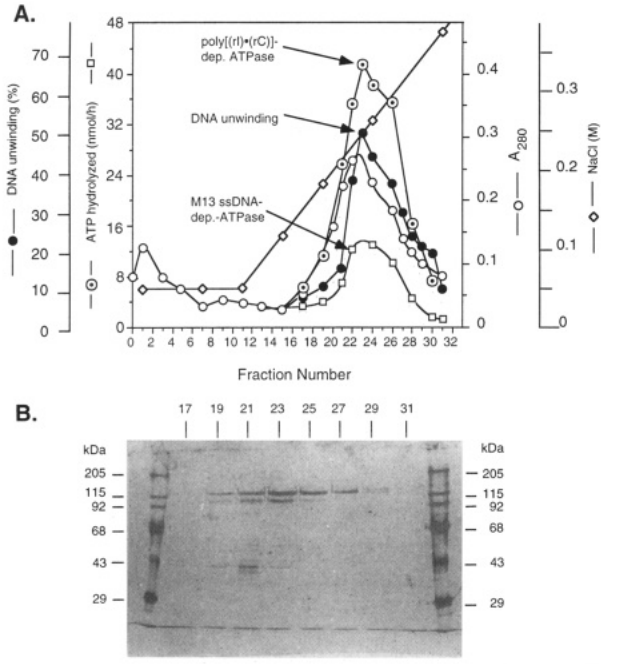

FIGURE 1: Chromatography of nuclear DNA helicase II on ATP–agarose. (A) Nuclear DNA helicase II was chromatographed on a 0.79 cm^2 × 2.5 cm ATP–agarose column as described (Zhang & Grosse, 1991). Fractions of 1 mL were collected, and 2 μL of each fraction was assayed for nucleic acid-dependent ATPase and DNA helicase activities. ATPase activities were measured in the presence of 150 μM M13 ssDNA or poly[(rI)·(rC)]. DNA unwinding was measured with a 45 μM sample of the 3′-tailed substrate shown in Figure 4. The RNA- and DNA-dependent ATPase and DNA helicase activities of NDH II coeluted between 0.2 and 0.3 M NaCl. The symbols used and their meanings are as follows: (◊) concentration of NaCl (M); (○) absorption at 280 nm; (●) DNA unwinding activity; (⊙) poly[(rI)·(rC)]-dependent ATPase activity; (□) M13 ssDNA-dependent ATPase activity. (B) One microliter of each eluted fraction was separated on a 8.5% SDS–polyacrylamide gel; thereafter, the gel was stained with silver.

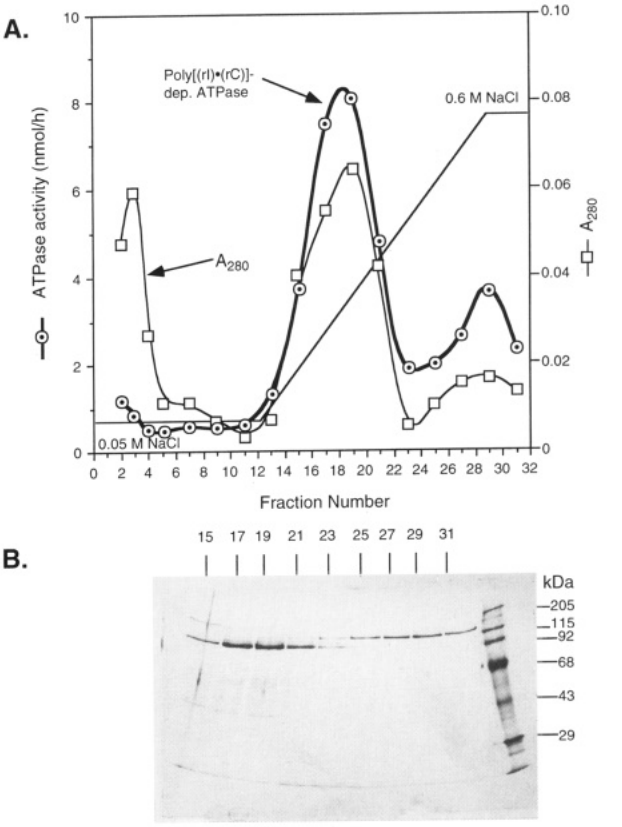

FIGURE 2: Chromatography of nuclear DNA helicase II on poly-[(rI)·(rC)]–agarose. NDH II was purified on a poly[(rI)·(rC)]–agarose column as described under Materials and Methods. (A) Two microliters of each fraction was assayed for ATPase activity in the presence of 150 μM poly[(rI)·(rC)] (⊙). Two ATPase activity peaks were identified, one eluting at 0.2–0.3 M NaCl and the other at >0.6 M NaCl. (B) One microliter of each eluted fraction was separated on a 8.5% SDS–polyacrylamide gel; then the gel was stained with silver.

polypeptides of NDH II allowed an independent analysis of their hydrodynamic properties. On isokinetic sucrose gradients, a sedimentation coefficient of 6.5 S was determined for the 100-kDa polypeptide, while the 130-kDa peptide migrated at 7.0 S. Gel filtration on Ultrogel AcA 44 gave Stokes' radii of 45 Å for the 130-kDa form and 40 Å for the 100-kDa polypeptide (data not shown). By applying the method of Siegel and Monty (1966) and assuming a partial specific volume of 0.725 mL/g, molecular masses of 107 and 130 kDa were obtained for the two forms of the native enzyme. The frictional ratios, f/f_0, were calculated to be 1.28 for the 100-kDa form and 1.34 for the 130-kDa form, which points to largely globular structures for both forms of NDH II. The hydrodynamic data indicate a monomeric structure for NDH II in solution. This does not exclude, however, that dimers or higher order oligomers may be formed when NDH II binds to DNA or RNA. The isoelectric pH for both polypeptides was measured by two-dimensional polyacrylamide gel electrophoresis to be between 6.5 and 7.0 (data not shown), indicating similar charge properties for the two polypeptides.

Peptide Mapping of the 130- and *100-kDa Proteins of Nuclear DNA Helicase II.* Affinity chromatography on poly-[(rI)·(rC)]–agarose provided a simple tool for the separation of both forms of NDH II. This allowed us to determine whether the two polypeptides were related to each other. Therefore, the separated 130-kDa form and the 100-kDa form of NDH II were individually digested by CNBr in the presence of SDS (Nikodem & Fresco, 1979). The resulting digestion fragments displayed similar patterns on Tricine–SDS–polyacrylamide gels (Figure 3). Moreover, digestion experiments with *N*-chlorosuccinimide in the presence of urea (Lischwe & Ochs, 1982) gave very similar fragments for both forms of NDH II (data not shown). Hence, we conclude that the smaller form of NDH II most likely is a proteolytic product of the larger one. The following experiments were performed with the combined fractions 25–27 of the ATP–agarose-purified enzyme as shown in Figure 1 that contained more than 80% of the 130-kDa form of NDH II.

DNA Unwinding Catalyzed by Nuclear DNA Helicase II. Basic enzymological features of NDH II have been described earlier (Zhang & Grosse, 1991). Here we have asked whether the DNA unwinding reaction can be stimulated by replication fork-like structures. Therefore, DNA unwinding was analyzed on substrates that contained a 3′-extension, a 5′-extension, and no extension at all (untailed) (Figure 4). After 10-min incubation at 37 °C, the substrate with the 3′ tail was about 20-fold and the substrate with the 5′ tail was about 3–4-fold more efficiently unwound than the untailed substrate (Figure 4). Thus, substrates with replication fork-like structures were preferentially unwound.

K_m Values for (d)NTPs during DNA Unwinding. DNA unwinding was measured with the 3′-tailed substrate in the presence of different amounts of the four rNTPs and dNTPs. As previously observed (Zhang & Grosse, 1991), DNA unwinding was possible with any of the four (d)NTPs. An evaluation of the unwinding reaction at various (d)NTP concentrations gave apparent K_m values for (d)ATP between 20 and 50 μM, whereas all the other (d)NTPs were used with

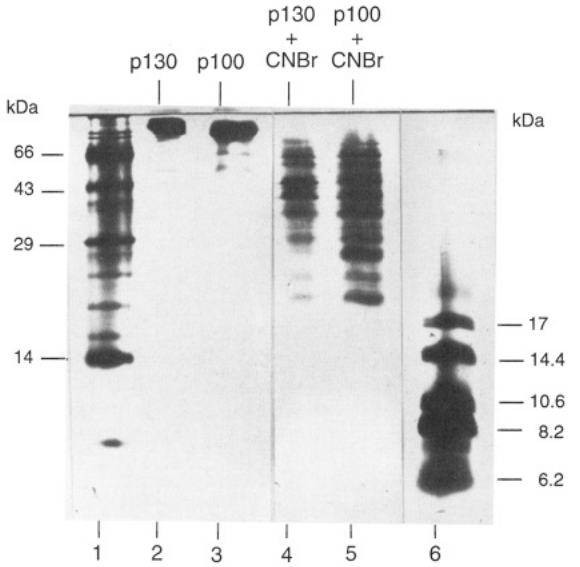

FIGURE 3: Peptide mapping of the two proteins of nuclear DNA helicase II. The 130- and 100-kDa proteins (40 μg of each) of NDH II were digested with CNBr in the presence of SDS. The degraded polypeptides were separated on a 12% Tricine–SDS–polyacrylamide gel and stained with silver. Lane 1, bovine albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and lysozyme (14 kDa) served as molecular mass markers. Lane 2, undigested 130-kDa polypeptide of NDH II. Lane 3, undigested 100-kDa polypeptide. Lane 4, CNBr-digested 130-kDa polypeptide. Lane 5, CNBr-digested 100-kDa polypeptide. Lane 6, myoglobin fragments (Sigma) were used as low molecular mass markers. The corresponding molecular masses are indicated on the right-hand side of the figure.

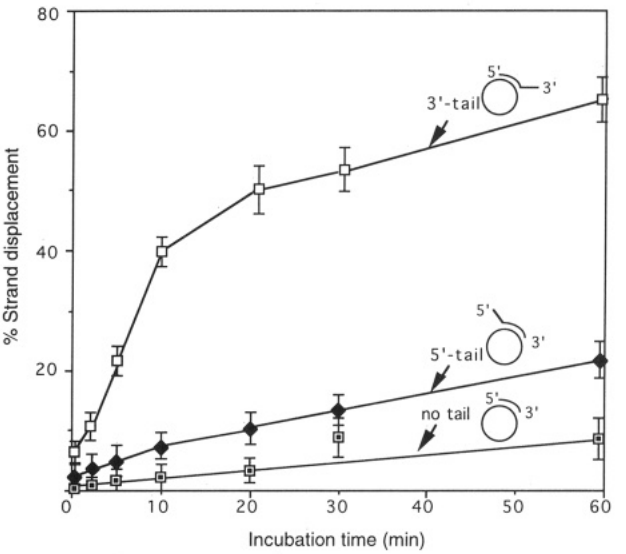

FIGURE 4: Utilization of tailed and untailed substrates for DNA unwinding catalyzed by nuclear DNA helicases II. Three different ^{32}P-labeled substrates containing a primer with complementary 22 nucleotides, a 45-mer primer with a 23-mer single-stranded 3′-tail, and a 45-mer primer with a 23-mer 5′-tail were used to analyze the unwinding activities of NDH II. Enzyme, DNA substrate, and ATP were present at 75 nM, 45 μM, and 3 mM, respectively. After the times indicated, samples were taken and separated on a nondenaturing 15% polyacrylamide gel. The gel was exposed to autoradiography; then the corresponding bands for substrate and product DNA were excised from the gel and counted in scintillation fluid. The error bars indicate the standard deviations from the means of three experiments.

apparent K_m values between 90 and 120 μM (data not shown).

ATPase Activity of Nuclear DNA Helicase II Is More Stimulated by RNAs than by DNAs. In accordance with the DNA unwinding function of NDH II, its NTPase activity was stimulated by M13 ssDNA. The specific activity was measured to be 1700 mol of ATP hydrolyzed per mole of enzyme per hour (units per mole of enzyme), while without DNA a specific activity of only 120 units/mol of enzyme was determined. In general, RNA templates stimulated the ATPase activity of NDH II more efficiently than DNA templates. The strongest effector was poly[(rI)·(rC)], resulting in a specific activity of 4200 units/mol of enzyme (Table 1).

Hydrolysis of Different Nucleotides by the RNA-Stimulated NTPase of Nuclear DNA Helicase II. The hydrolysis of the four dNTPs and rNTPs by nuclear DNA helicase II was studied in the presence of 150 μM poly[(rI)·(rC)]. With this RNA as cofactor, NDH II hydrolyzed dCTP and rCTP with a rate of 5200 units/mol of enzyme. The hydrolysis of ATP and dATP was less efficient, 4200 units/mol of enzyme (see above), for both nucleoside triphosphates; GTP, dGTP, and dTTP were even less efficiently hydrolyzed with rates of 1500, 1600, and 1200 units/mol of enzyme, respectively. It should be pointed out that in the presence of DNA NDH II preferred to hydrolyze ATP (Zhang & Grosse, 1991).

Nucleic Acid Binding Affinity of Nuclear DNA Helicase II. As a first approach to study the binding affinities of NDH II to nucleic acids, we measured the inhibitory effects of various nucleic acids on the DNA unwinding activity catalyzed by this enzyme (Figure 5). In agreement with the data obtained for the stimulation of NTPase activity, DNA unwinding was most strongly inhibited by poly[(rI)·(rC)]; the DNA helicase was also strongly inhibited by MS-2 RNA and poly(rI), while M13 ssDNA and poly(dI) were less efficient competitors. On the other hand, M13 dsDNA, poly(dT), and poly(rU) had no or only little inhibitory effects on NDH II-catalyzed DNA unwinding (Figure 5).

Table 1: Nucleic Acid-Dependent ATPase Activity of NDH II[a]

poly-nucleotide	sp act. [mol of ADP formed (mol of NDH II)$^{-1}$ h^{-1}]	poly-nucleotide	sp act. [mol of ADP formed (mol of NDH II)$^{-1}$ h^{-1}]
without DNA	120 ± 25	poly(dA)	140 ± 20
activated DNA[b]	850 ± 101	MS2 ssRNA	2700 ± 160
M13 ssDNA	1700 ± 141	poly[(rI)·(rC)]	4200 ± 208
M13 dsDNA[c]	150 ± 20	poly(rI)	2050 ± 100
poly[(dI)·(dC)]	550 ± 60	poly(rC)	170 ± 12
poly(dI)	966 ± 120	poly[(rA)·(rU)]	1750 ± 180
poly(dT)	450 ± 35	poly(rA)	500 ± 100
poly(dC)	240 ± 28	poly(rU)	780 ± 150

[a] The rate of ATP hydrolysis was measured as described under Materials and Methods. Nucleic acid was present at 150 μM (nucleotide); NDH II was used at 100 nM. Standard deviations from the means of three independent measurements are given. [b] Activated DNA was prepared to 25% acid solubility as described by Aposhian and Kornberg (1962). [c] M13 dsDNA represented supercoiled M13mp18 DNA (Boehringer-Mannheim).

Filter Binding Assays for Measuring the Binding Constant of NDH II and Poly[(rI)·(rC)]. A direct measure for NDH II binding to poly[(rI)·(rC)] was obtained by using a nitrocellulose filter binding assay. Two micromolar NDH II was sufficient to retain 2 μM (nucleotide) poly[(rI)·(rC)] to more than 90% on the filter surface (Figure 6A). Under otherwise identical conditions, half-maximal retention was obtained at about 100 nM NDH II. The binding stoichiometry was calculated as 15 bases per molecule of NDH II (Figure 6A). Titration experiments with various amounts of NDH II and poly[(rI)·(rC)] gave an affinity binding constant of about 2.2×10^7 M^{-1} (Figure 6B). The presence or absence of 1 mM ATP/Mg^{2+} had no apparent influence on the binding constant (data not shown).

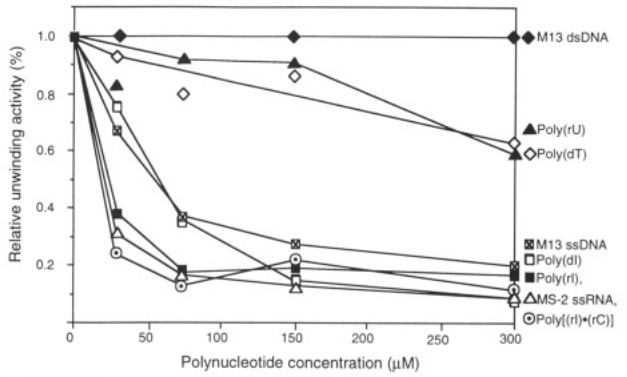

FIGURE 5: DNA unwinding catalyzed by nuclear DNA helicase II in the presence of nucleic acid competitors. 45 μM 3′-tailed substrate was incubated with the indicated amounts of nucleic acid competitors and 3 mM ATP for 5 min at 37 °C. The reaction was started by adding NDH II to a final concentration of 153 nM per assay. After 10-min incubation at 37 °C, the fraction of unwound oligonucleotide was determined by polyacrylamide gel electrophoresis and subsequent counting of the excised bands. Under these conditions, about 70% of the substrate DNA became unwound in the absence of competitor nucleic acids.

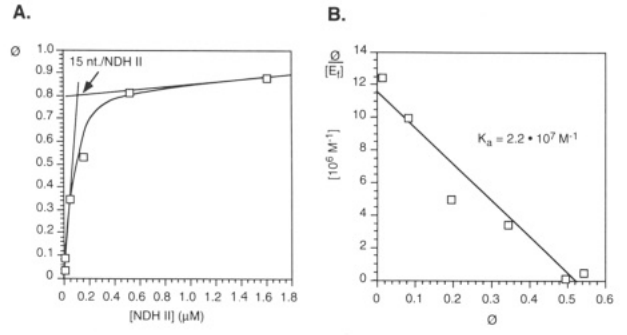

FIGURE 6: Nitrocellulose filter retention assay for NDH II binding to poly[(rI)·(rC)]. (A) Two micromolar (nucleotides) samples of ^{32}P-labeled poly[(rI)·(rC)] were incubated with the indicated concentrations of NDH II in 10 μL of binding buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 50 μg/mL serum albumin for 15 min at room temperature. The mixture was then passed through alkaline-treated nitrocellulose filters. The retained radioactivity was determined by scintillation counting. (B) Scatchard analysis of the binding data of NDH II and poly-[(rI)·(rC)].

Determination of the Minimal Binding Site Size of NDH II by Gel Mobility Shift Experiments. The binding stoichiometry was calculated to be about 15 nucleotides per mole of enzyme (Figure 6A). This value might reflect the minimal occupation site size for NDH II binding to RNA. To determine the minimal binding site size directly, we conducted a gel mobility shift experiment. Poly(rI) was partially degraded by alkaline hydrolysis. The resulting oligo(rI) "ladder" was then incubated with saturating amounts of NDH II. The resulting complexes were separated on a native polyacrylamide gel (Figure 7). NDH II–oligonucleotide complexes were not able to penetrate the polyacrylamide gel, while oligonucleotides that were not efficiently retained by the helicase resulted in a ladder that ended at 16–17 nucleotides (Figure 7, lane C). From the stoichiometry of binding to poly[(rI)·(rC)] (Figure 6A) and the gel retardation shift experiment with oligo(rI) (Figure 7), a minimal binding length of 16–17 nucleotides per molecule of NDH II is concluded.

Identification of an RNA Unwinding Activity of Nuclear DNA Helicase II. The strong binding of NDH II to both ssRNA and dsRNA in conjunction with the strong RNA-dependent ATPase of this enzyme led us to assume that RNA unwinding might be an additional function of NDH II. The

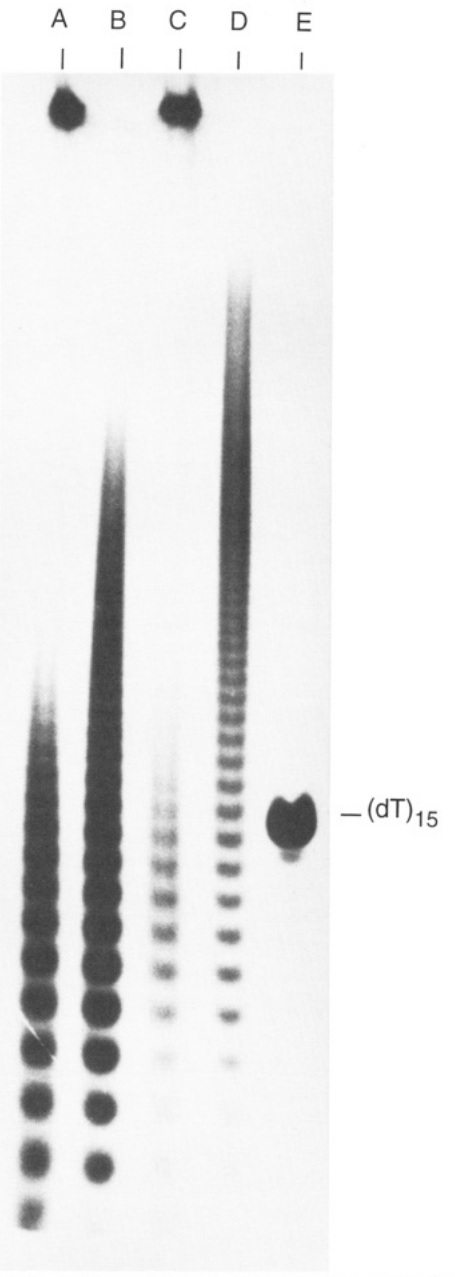

FIGURE 7: Gel shift analysis for determination of the nucleotide occupation size of NDH II. Poly(rI) was degraded by treatment with 0.3 M KOH for 20 s (lane B) and 2 min (lane D) at 56 °C; 0.3 μM (nucleotide) samples of the resulting RNA ladders were phosphorylated with γ-^{32}P and incubated with 3 μM NDH II (lanes A and C) or 50 μg/mL serum albumin (lanes B and D) in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol for 15 min at room temperature. Ten microliters of each reaction mixture was separated on a 20% native polyacrylamide gel. After electrophoresis, the gel was subjected to autoradiography. Lane E indicates the position of the size marker $(dT)_{15}$.

RNA unwinding activity of NDH II was measured directly by using a partial dsRNA substrate as shown in Figure 8A. NDH II unwound RNA if one of the four rNTPs or dNTPs was present (Figure 8B). The unhydrolyzable ATP analogs adenyl-5′-yl imidodiphosphate, App(NH)p, and adenyl-5′-yl β,γ-methylenediphosphate, App(CH_2)p, did not support RNA unwinding (data not shown). Unexpectedly, RNA unwinding was measurable without added divalent cations, but was completely inhibited in presence of 10 mM EDTA (Figure 8C). This inhibition was overcome by the addition of 10 mM $MgCl_2$ (data not shown), which demonstrated that a divalent

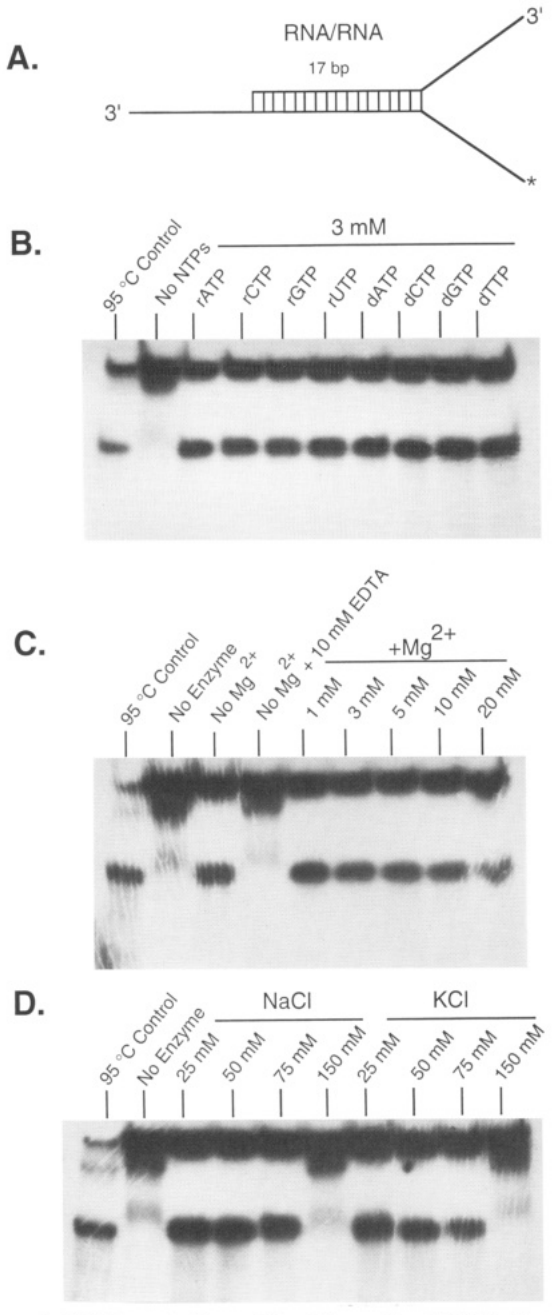

FIGURE 8: RNA unwinding catalyzed by nuclear DNA helicase II. (A) An RNA duplex with a 17-nucleotides-long complementary region was constructed by *in vitro* transcription. The radioactively labeled strand is indicated by an asterisk. (B) RNA unwinding of NDH II was measured in the absence or presence of a 3 mM sample of the four rNTPs or dNTPs, (C) at different concentrations of Mg^{2+}, (D) and at different concentrations of NaCl and KCl. Enzyme was present at 500 nM.

cation was mandatory for RNA unwinding. Interestingly, RNA unwinding was slightly stimulated by the addition of up to 75 mM NaCl (Figure 8D), while at the same concentration DNA unwinding was completely inhibited (Zhang & Grosse, 1991). Thus, RNA unwinding was less salt-sensitive than DNA unwinding. The RNA unwinding reaction required about 10-fold more enzyme than the DNA unwinding reaction. However, the dsRNA substrate was used at a concentration of only 1.87 nM, which is, as inferred from the equilibrium binding constant, much lower than the putative K_m value.

DISCUSSION

Until now nine apparently different DNA helicases have been isolated and characterized from calf thymus glands (Li *et al.*, 1992; Siegel *et al.*, 1992; Thömmes *et al.*, 1992; Thömmes & Hübscher, 1990b; Zhang & Grosse, 1991, 1992). Three enzymes named α-helicase or helicase A (M_r 48 000; Thömmes & Hübscher, 1990b), δ-helicase (M_r 56 000; Li *et al.*, 1992), and ϵ-helicase or helicase E (M_r 100 000; Siegal *et al.*, 1992) have been suggested to be involved in DNA replication because of their copurification with the replicative DNA polymerases α, δ, and ϵ. NDH II is different than these helicases in terms of its molecular mass (130/100 kDa), its low nucleotide selectivity, and its apparent nuclear localization (Zhang & Grosse, 1991). Because of its 3′ to 5′ unwinding direction, NDH II is also distinct from three further DNA helicases (helicases B, C, and D) which unwind DNA with 5′ to 3′ polarity (Thömmes *et al.*, 1992). As shown here, NDH II preferred DNA substrates with replication fork-like termini, suggesting that this enzyme may be involved in DNA replication. By contrast, the 3′ to 5′ DNA helicase E (Siegal *et al.*, 1992) as well as the cytosolic DNA helicase, CDH, were not stimulated by replication fork-like structures (S. Zhang, unpublished observations), which might suggest a role in DNA repair for the latter two enzymes.

NDH II acted as a ssRNA and dsRNA binding protein. This was most clearly shown by using poly[(rI)·(rC)]–agarose as affinity material for the purification of NDH II. While ATP–agarose-purified NDH II consisted of two polypeptides of 130 and 100 kDa, the utilization of RNA–agarose allowed the purification of the individual polypeptides to apparent homogeneity. Subsequent peptide mapping with either CNBr or *N*-chlorosuccinimide revealed that the 100-kDa polypeptide was most likely a degradation product of the 130-kDa protein.

With the proposed role for NDH II in DNA replication, the question arose whether the associated RNA binding and RNA-stimulated NTPase activities were of enzymological importance. Here we have shown that NDH II also unwound RNA. RNA unwinding required ATP/Mg^{2+}, and it was abolished by unhydrolyzable ATP analogs. This strongly suggests that RNA unwinding is an energetically driven process. RNA unwinding required about 10-fold more NDH II than DNA unwinding. However, because of the limited availability of *in vitro* transcribed substrate, RNA unwinding had been conducted at a suboptimal concentration that was at least 25-fold lower than the equilibrium dissociation constant. Furthermore, it is possible that cofactors, such as RNA binding proteins and/or specific RNA sequences, facilitate the RNA unwinding reaction. Thus, RNA unwinding is an enzymatic feature of NDH II that may be important for the living cell.

Recently a novel RNA helicase, designated as RNA helicase A, has been isolated from the nuclei of HeLa cells (Lee & Hurwitz, 1992). This enzyme has many properties in common with the enzyme described here. It has a comparable molecular weight and similar hydrodynamic properties, the same directionality of unwinding, and a preference of RNA over DNA substrates. Furthermore, it hydrolyzes all four dNTPs and rNTPs with low K_m values and with a slight preference of dCTP to the other (d)NTPs. Although RNA helicase A does not unwind duplexes when the 3′ tail is composed of ssDNA, its ATPase is significantly stimulated by poly(dT) and poly(dI). Furthermore, DNA binding of RNA helicase A has been shown by complex formation and affinity chromatography on ssDNA–cellulose (Lee & Hurwitz, 1992). In spite of this, it has been argued that a poor association between RNA helicase A and DNA substrates prevents translocation on DNA and thereby DNA unwinding (Lee & Hurwitz, 1992). However, as we have shown earlier, NDH

II-catalyzed DNA unwinding is salt-sensitive (Zhang & Grosse, 1991). Therefore, it might be possible that a DNA unwinding capability of RNA helicase A has remained undetected because of different assay conditions. Alternatively, RNA helicase A and nuclear DNA helicase II may be highly related enzymes, perhaps isoenzymes, with a specialized function in RNA processing for RNA helicase A, and a function in both RNA and DNA metabolism for NDH II.

The finding of DNA and RNA unwinding catalyzed by one and the same enzyme is not without precedence. The viral replicative DNA helicase, SV40 large T antigen, binds efficiently to RNA (Darlix *et al.*, 1984; Khandjian *et al.*, 1982; Michel & Schwyzer, 1981; Schwyzer *et al.*, 1983) and, moreover, also unwinds RNA (Scheffner *et al.*, 1989, 1991). There are some further biochemical similarities between NDH II and SV40 T-ag such as the same directionality of unwinding (Goetz *et al.*, 1988; Wiekowski *et al.*, 1988; Zhang & Grosse, 1991), the preference of 3′-tailed primer ends (SenGupta & Borowiec, 1992; Wiekowski *et al.*, 1988), and the use of all (d)NTPs as nucleotide cofactors at comparably low K_m values (Scheffner *et al.*, 1991; Weiner *et al.*, 1991). Thus, NDH II exhibits some biochemical features that have been previously documented only for T-ag, with the most distinguished property that it can unwind both DNA and RNA duplexes. From the sum of enzymological similarities, it is certainly not possible to assume that NDH II is a cellular counterpart of the T-ag helicase. Therefore, investigations at a genetic and cell biological level are necessary to gain further insight into the physiological function(s) of NDH II. The easy preparation of large amounts of NDH II, as described here, should facilitate the production of antibodies, which in turn will be helpful for cell biological studies and the cloning of the NDH II-encoding gene. These issues are currently under investigation.

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