The Isolation and Characterization of an RNA Helicase from Nuclear Extracts of HeLa Cells*

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An RNA helicase, isolated from nuclear extracts of HeLa cells, displaced duplex RNA in the presence of any one of the eight common nucleoside triphosphates. The unwinding reaction was supported most efficiently by ATP and GTP and poorly by dCTP and dTTP. The enzyme activity, purified 300-fold, contained two major protein bands of 80 and 55 kDa when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All fractions that contained RNA helicase activity also possessed single-stranded RNA-dependent nucleoside triphosphatase activity.

Purified RNA helicase fractions displaced a hybrid of U4/U6 RNAs with the same efficiency as it displaced other duplex RNA structures. In contrast, the RNA helicase did not displace duplex RNA/DNA and DNA/DNA structures. Evidence is presented that suggests that this RNA helicase can displace duplex RNA by translocating in both the 3' to 5' and the 5' to 3' directions.

The properties of the RNA helicase described here differ from the deaminase RNA unwinding activity described in Xenopus oocytes (Bass, B. L., and Weintraub, H. (1987) Cell 48, 607–613) and from the p68 HeLa RNA helicase (Hirling, H., Scheffner, M., Restle, T., and Stahl, H. (1989) Nature 339, 562–564).

The alteration of the duplex structure of DNA by DNA helicases has been studied extensively. The unwinding or displacement of double-stranded DNA (dsDNA) by these enzymes results in the production of single-stranded DNA (ssDNA), which can then be used as template for the synthesis, repair, and recombination of DNA (1).

More recently, RNA helicases have been discovered that also produce ssRNA from duplex structures. Examples of such proteins include the mammalian translation initiation factor 4A (eIF-4A), which in the presence of eIF-4B and ATP, unwinds partial dsRNA substrates (2–4). The RNA helicase activity of this protein complex may destabilize double-stranded regions of single-stranded mRNAs to increase their translation efficiency. Other well-characterized RNA helicases include the human p68 protein (5) and the SV40 T-antigen (6). Recently, the cylindrical inclusion (CI) protein encoded by the plum pox virus (PPV), which is a positive ssRNA virus, was shown to contain RNA helicase activity (7–9). It has been suggested that this protein is involved in the RNA replication reaction. All of these enzymes, in addition to displacing dsRNA also contain RNA-dependent nucleoside triphosphatase activity. In addition to eIF-4A, p68, and the CI protein of PPV, a large group of proteins possess strong sequence homology to proteins containing RNA helicase activity. However, it is not known whether they catalyze the displacement of duplex RNA. These proteins include the yeast genes translation initiation factors 1 and 2 and MSS116 (10, 11), the Escherichia coli SrmB protein (which contains RNA-dependent ATPase activity) (12), the Drosophila vas6 gene (13), the mouse PL10 gene (14), and some of the newly discovered yeast prp genes (15, 16), which play important roles in pre-mRNA splicing. Whereas the precise role of a number of these proteins is unknown, their action must be related to RNA metabolism. There is genetic evidence that the yeast mutant MSS116 affects mitochondrial RNA splicing (10).

ATP is required at multiple stages in the assembly of spliceosomes and the splicing of pre-mRNA in vitro in both yeast and mammalian systems (17–20). No other nucleoside triphosphate can replace this requirement (21). The specific reactions that require ATP in the assembly of spliceosomes are presently unknown. However, the role of RNA helicases, which are capable of altering the interactions between dsRNA both intramolecularly and intermolecularly, would be well suited for the sequential steps known to involve interactions between RNAs. An excellent example for an important role of an RNA helicase is in the displacement of the U4/U6 RNAs. There is strong evidence that the U4/U6 small nuclear ribonucleoprotein is a single nucleoprotein particle in which U4 and U6 RNAs are hydrogen-bonded together (22). During the formation of the spliceosome, these RNAs are displaced, and U4 RNA is released from the complex while U6 RNA is retained (18, 23).

Prompted by these considerations, we have initiated the purification and characterization of RNA helicases present in nuclear extracts of HeLa cells. We have detected a variety of different RNA helicases; and in this paper, we describe the purification and the properties of one of these enzymes. This enzyme, in the presence of nucleoside triphosphates, displaced a variety of partial dsRNAs, including the U4/U6 RNAs, and is specific in its requirement for partial dsRNA. RNA/DNA hybrids as well as duplex DNA structures were not displaced.

MATERIALS AND METHODS

Preparation of RNA Helicase Substrates—Partial dsRNA substrates (RNA/RNA) were prepared by run-off transcription of DNA.
Fig. 1. A, standard partial dsRNA substrate used to assay HeLa RNA helicase. The substrate was prepared as described under "Materials and Methods." The sequence of the RNA transcripts is shown. B, RNA substrates used to determine polarity of RNA helicase action. The 3' (upper) and 5' (lower)-tailed substrates were prepared as described under "Materials and Methods." The sequences of the templates with SP6 or T7 RNA polymerase according to the protocols supplied by the enzyme manufacturer. The standard RNA substrate (Fig. 1A) was prepared by SP6 RNA polymerase transcription of the pSP65 plasmid (cut with XhoI) in the presence of rNTPs with [α-32P]GTP and by transcription of the pGem1 plasmid (cut with PstI) in the presence of [32P]GTP. The transcription reactions (0.15-0.4 μl) contained 40 μM Tris HCl (pH 7.9), 6 mM MgCl2, 2 mM spermidine, 10 mM DTT, 500 μM each ATP, CTP, and UTP, 50 μM GTP, 0.1 mCi of [32P]GTP or 1 mCi of [α-32P]GTP, 100 μM T4 polynucleotide kinase (for capping), 100 μg/ml template DNA, 100 units of RNasin and 200 units/ml SP6 RNA polymerase. The mixtures were incubated at 40 °C for 2 h, and the RNA products were extracted twice with phenol and once with chloroform/isoamyl alcohol (24:1), treated with ethanol (2.5 volumes), and then centrifuged for 30 min in an Eppendorf microcentrifuge. The pellets were dried under vacuum for 20 min. The transcripts were resuspended in a solution containing 90% formamide, 10% TBE buffer (Tris, 0.089 M, borate, 0.089 M, EDTA, 0.002 M) 0.92% bromphenol blue, 8 μM urea denaturing gel. Electrophoresis was carried out at 20 mA for 45 min, and the RNA products were located by placing the gel on top of a TLC cellulose plate illuminated with UV light. The regions of the gel containing the RNA transcripts were excised and ground with a glass rod in a 1.5-μl Eppendorf tube containing 0.4 μl of a solution containing 0.5 M ammonium acetate (pH 7.0), 0.1% SDS, and 10 mM EDTA. The mixture was incubated for 2 h at 4 °C with gentle agitation and centrifuged for 2 min, and the supernatant was then precipitated with 2.5 volumes of ethanol. After 1 h at -80 °C, the suspension was centrifuged for 30 min, and the alcohol was removed, and the pellet was dried under vacuum for 2 min. The RNAs were dissolved in 0.1 ml of buffer T (40 mM Hepes (pH 7.0), 0.1 M NaCl, 2 m mM EDTA, 0.092% Nonidet P-40). For the preparation of partial dsRNAs, equimolar amounts (50-150 pmol) of each transcript were mixed together and diluted to 0.2 ml with distilled water (if necessary), and 50 μl of 5 × hybridization buffer (2.5 M NaCl, 0.2 M Hepes (pH 7.0), 5 mM EDTA) was added. The hybridization mixture was incubated at 95 °C for 5 min and immediately switched to a 55 °C water bath and incubated overnight at this temperature. The annealed RNAs were precipitated with ethanol as described before, resuspended in 20 μl of loading buffer (0.025% bromphenol blue, 0.25% xylene cyanol, 0.05% SDS, 1 mM EDTA, and 15% glycerol), and loaded onto an 8% polyacrylamide nondenaturing gel. The RNAs were electrophoresed at 6 mA for 2-3 h, and the duplex RNA band was located by autoradiography. In all experiments, control lanes with nonhybridized transcript were carried out for comparison. The gel slice containing the duplex RNA was eluted as described above. The partial dsRNA was precipitated with ethanol and resuspended in buffer T at a concentration of 50 f mol/μl. Such RNA substrates had a specific activity of 1500-2000 cpm/fmol and could be used for as long as 1 month.

To test the directionality of the RNA helicase, 3'- and 5'-tailed substrates were prepared (Fig. 1B). The 5'-tailed substrate was prepared by transcription of the pSP65 plasmid (cut with XhoI) in the presence of rNTPs with [α-32P]GTP and the pGEM3 plasmid (cut with KpnI) in the presence of rNTPs with [α-32P]GTP. The transcription reactions, purification, and annealing of the transcripts were as described above. The 3'-tailed substrate was prepared by transcription of the pGEM3 plasmid as described by Scheflner et al. (6). The short strand was prepared by T7 RNA polymerase transcription of the pGEM3 plasmid (cut with KpnI) in the presence of rNTPs with [α-32P]GTP. The complementary strand was prepared by transcription with SP6 RNA polymerase of the pGEM3 plasmid (cut with XhoI) in the presence of rNTPs with [α-32P]GTP. The transcription reaction, purification, and annealing of the transcripts were as described above. The specific activity of each substrate was 1000 cpm/fmol.

![Figure 1](image-url)
The yeast clones of U4 and U6 small nuclear RNAs were a generous gift of Drs. P. Fabrizio and J. Abelson (California Institute of Technology) and were originally described by Siliciano et al. (24). The U4/U6 partial dsRNA substrate was prepared in a similar way as described above, except that both the plasmid containing the yeast U4 RNA sequence (cut with Sty1) and the plasmid containing the yeast U6 RNA sequence (cut with SalI) were transcribed with T7 RNA polymerase in the presence of rNTPs with [α-32P]GTP as described above, except for the hybridization, which was carried out at 40 °C. Purification of the RNA transcripts and the duplex RNA was as described above. The specific activity of the U4/U6 partial dsRNA substrate was 1000 cpm/mmol. The proposed structure of the U4/U6 dsRNA (22) is depicted in Fig. 1C.

DNA/DNA and RNA/DNA substrates (Fig. 1D) were prepared by labeling the 5'-end of a synthetic 30-nucleotide deoxyoligonucleotide or oligoribonucleotide of the same sequence with polynucleotide kinase and [γ-32P]ATP. The labeled oligonucleotides were purified by gel electrophoresis and annealed to φX174 circular dsDNA as described above, except that the hybridization was carried out at 45 °C in a volume of 50 μl (overlaid with light mineral oil). The duplex structures were loaded onto 0.5-ml Sepharose 4B columns and eluted with buffer T. The early eluting fractions, containing duplex structures separated from the free oligonucleotide, were pooled and directly used for helicase assays. The specific activity of each substrate was 800 cpm/mmol.

Helicase Assay—Unless otherwise indicated, the assay for RNA helicase was carried out in reaction mixtures (20 μl) containing 40 mm Tris-HCl (pH 7.5), 1 mM MgCl₂, 2 mM DTT, 1 mM poly(A) (as nucleotides), 10 μM [3H]dTTP (400–500 cpm/pmol), and the enzyme fraction. To determine ATPase or GTPase activity, [3H]ATP or [3H]GTP, and the assay conditions were identical. Mixtures were incubated at 30 °C for 60 min after which time 2 μl of each reaction was spotted onto a polyethyleneimine-cellulose plate containing 0.02 μmol each of the nucleoside mono-, di-, and triphosphates of the RNA sequence (cut with Sty1) and the plasmid containing the yeast U4/U6 partial dsRNA substrate was prepared in a similar way as described above. The specific activity of the U4/U6 partial dsRNA substrate was 1000 cpm/mmol. The proposed structure of the U4/U6 dsRNA (22) is depicted in Fig. 1C.

RNA helicase activity was determined by a decrease in the density of 1 pmol of RNA under the above conditions. Background values (ssRNA) represented <1% of the input dsRNA. This value has not been subtracted from the results presented.

dTTPase Assay—The measurement of dTTP cleavage was carried out in reaction mixtures (20 μl) containing 40 mm Tris-HCl (pH 7.5), 1 mM MgCl₂, 2 mM DTT, 1 mM poly(A) (as nucleotides), 10 μM [3H]dTTP (400–500 cpm/pmol), and the enzyme fraction. To determine ATPase or GTPase activity, [3H]ATP or [3H]GTP, and the assay conditions were identical. Mixtures were incubated at 30 °C for 60 min after which time 2 μl of each reaction was spotted onto a polyethyleneimine-cellulose plate containing 0.02 μmol each of the nucleoside mono-, di-, and triphosphates of the labeled nucleotide species as markers. The plates were developed with a solution containing 1 M HCOOH and 0.5 M LiCl and then dried, and the regions corresponding to the mono-, di-, and triphosphate derivatives were located by UV illumination. Excess, and quantitated by scintillation counting. One unit of NTPTase catalyzed the cleavage of 1 pmol of RNA under the above conditions. Background values (ssRNA) represented <1% of the input dsRNA. This value has not been subtracted from the results presented.

Protein Assay—Protein concentration was determined using the Bio-Rad protein assay reagent with BSA as the standard. Protein analysis by SDS-polyacrylamide gel electrophoresis (4% acrylamide stacking gel, 10% acrylamide resolving gel) was carried out as described by Laemmli (25). Protein bands were visualized using the ICN rapid silver stain kit.

RESULTS

Preparation of HeLa RNA Helicase

Ammonium Sulfate Fractionation—Dignam extract was prepared from HeLa cells as previously described (26). The nuclear extract (350 ml, 6 mg/ml protein) obtained from 4.5 × 10⁶ cells was brought to 60% ammonium sulfate saturation by adding solid ammonium sulfate (39 g/100 ml) with continuous stirring at 4 °C for 30 min, and the mixture was then centrifuged at 10,000 rpm for 15 min. The supernatant was removed, and the pellet was washed with a solution containing 60% ammonium sulfate in buffer A (20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.01% Nonidet P-40, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 10% (v/v) glycerol) plus 0.1 M KCl. After centrifugation, the pellet was suspended in a solution (40 ml) containing 40% ammonium sulfate in buffer A plus 0.1 M KCl. After centrifugation, the pellet was re-extracted with the same buffer. The supernatants were pooled and adjusted with solid ammonium sulfate to 60% saturation. After centrifugation, the pellet was saved as the ammonium sulfate 60:40 fraction. The pellet obtained after the extraction with 40% ammonium sulfate was extracted with a solution (40 ml) containing 30% ammonium sulfate in buffer A plus 0.1 M KCl and then centrifuged; the pellet was re-extracted with the same buffer and then centrifuged. The supernatants were pooled and precipitated by addition of solid ammonium sulfate to 40% saturation. After centrifugation, the pellet was saved as the ammonium sulfate 40:60 fraction. The pellet obtained after the extraction with 30% ammonium sulfate was suspended in a solution (40 ml) of 20% ammonium sulfate in buffer A plus 0.1 M KCl and then centrifuged. The pellet obtained was re-extracted with the same buffer. The supernatants obtained after washing with 20% ammonium sulfate were pooled and precipitated by addition of solid ammonium sulfate to 30% saturation. After centrifugation, the pellet was saved as the ammonium sulfate 30:70 fraction. All ammonium sulfate pellets were dissolved in the minimum possible volume of buffer A plus 0.1 M KCl (~20 ml), dialyzed against three changes of buffer A plus 0.1 M NaCl (1 liter each) for a total of 6 h at 4 °C, and then assayed for RNA helicase activity. The ammonium sulfate 30:70 fraction contained the bulk of the enzyme activity and was used for subsequent steps.

ssDNA-Cellulose Chromatography—The ammonium sulfate 30:70 fraction (20 ml) was passed through a ssDNA-cellulose column (21 × 1.7 cm², 35 ml, 5–6 mg of DNA/g of resin) previously equilibrated with buffer A plus 0.1 M NaCl. After washing with 50 ml of the same buffer, the column was successively step-eluted with 50 ml each of buffer A plus 0.25, 0.5, and 1.0 M NaCl. Fractions (5 ml) were collected and assayed for both RNA helicase and dTTPase activities. Although the 0.1, 0.25, and 0.5 M salt steps contained RNA helicase with comparable amounts of RNA-dependent dTTPase activity, the material eluted with 0.5 M NaCl contained RNA helicase with the highest specific activity (Fig. 2). This fraction was used for further purification.

CM-Sepharose Chromatography—The 0.5 M NaCl DNA-cellulose fraction (42 ml) was dialyzed against three changes (500 ml each) of buffer C (20 mM MES (pH 6.0), 0.5 mM EDTA, 0.01% Nonidet P-40, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 10% (v/v) glycerol) plus 0.1 M NaCl. Buffer changes were carried out over 20 ml fractions for 90 min at 4 °C. The dialyzed fraction was loaded onto a CM-Sepharose column (15 × 1.7 cm², 25 ml) previously equilibrated with buffer C plus 0.1 M NaCl. The column was washed with 1 volume of buffer C plus 0.1 M NaCl and then eluted with 160 ml of a linear gradient of buffer C from 0.1 (80 ml) to 1.0 (80 ml) M NaCl, and 3-ml fractions were collected. Helicase activity was recovered in the material that eluted between 0.3 and 0.4 M NaCl. There was poor correspondence between RNA-dependent dTTPase and helicase activities, and multiple peaks of dTTPase and RNA helicase were detected. Fractions 37–41 were pooled and used for the next step.

Sedimentation through Sucrose Gradients—The pooled fractions (18 ml) were placed in a dialysis tube (6–8 kDa
Multiple RNA helicase activities in fractions that were not yield the experiments described here. 

The purification procedure summarized in Table I resulted in a 300-fold enrichment of the RNA helicase activity with a yield of 1%. The relatively low yield reflected the presence of multiple RNA helicase activities in fractions that were not included in the various steps developed. We have found that the multiple peaks of RNA helicase and RNA-dependent dTTPase activities (Fig. 2) represent other distinct RNA helicases, some of which have been characterized, whereas others remain to be further studied (data not shown).

The RNA helicase and RNA-dependent dTTPase activities cosedimented in the second sucrose gradient step (Fig. 3). Gel filtration chromatography and sucrose gradient centrifugation, both in the presence of 1 M NaCl, indicated that the helicase has a Stokes radius of 44 Å and a sedimentation coefficient of -5.4 S. Assuming a partial specific volume of 0.725 cm³/g, a native molecular mass of 100 kDa and a frictional ratio of 1.43 can be calculated (data not shown). The various pools containing RNA helicase activity were subjected to SDS-PAGE analysis (Fig. 4, upper), as were the individual fractions collected from the second sucrose gradient (Fig. 4, lower). As shown in Fig. 3, RNA helicase activity was detected in the second sucrose gradient between fractions 8 and 15, with the peak of activity in fractions 20 and 21. The only protein bands detected that co-migrated with the helicase activity were bands of 80 and 55 kDa, which were also present in the first sucrose gradient fraction (Fig. 4, upper). Whether both protein bands together represent the RNA helicase activity is presently unknown.

The final preparation of RNA helicase (SG2 fraction) was stable for at least 6 months. Preparations stored as small aliquots at -80 °C could be freeze-thawed at least four times with no loss of activity.

**Comments on Purification Procedure and Properties of Helicase Activity**

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1. This study was carried out in buffers containing Nonidet P-40. The influence of this nonionic detergent on the Stokes radius is unknown since we do not know whether the helicase binds Nonidet P-40.
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TABLE I
RNA helicase purification procedure

| Fraction          | Protein | Activity | Specific activity | Recovery | Purification |
|-------------------|---------|----------|-------------------|----------|--------------|
|                    | mg      | units    | units/mg          | %        | -fold        |
| Dignam nuclear extract | 2100    | 6300     | 3                 |          |              |
| Ammonium sulfate 30-20% | 410      | 4000     | 10                | 61       | 3            |
| ssDNA-cellulose   | 42      | 1680     | 40                | 26       | 13           |
| CM-Sepharose      | 2.4     | 225      | 95                | 3.5      | 32           |
| Sucrose gradient I | 0.4     | 129      | 350               | 2.0      | 120          |
| Sucrose gradient II | 0.06    | 60       | 1000              | 1.0      | 330          |

FIG. 4. SDS-PAGE analysis of various RNA helicase fractions. Aliquots of each fraction (0.5 µg of protein), as indicated, were subjected to SDS-10% polyacrylamide gel electrophoresis and visualized by silver staining. The migration of different protein markers (M) is indicated to the left. The markers (in kilodaltons) included: phosphorylase b, 97.4; BSA, 66.2; ovalbumin, 42.7; carbonic anhydrase, 31; and trypsin inhibitor, 21.5. Upper, fractions isolated during purification of RNA helicase; lower, fractions from second sucrose gradient (SG2) subjected to SDS-PAGE analyses. Fractions 19-21 contained the peak of RNA helicase activity. AS, ammonium sulfate.

Requirements for RNA Helicase Activity

The displacement of RNA from the partial dsRNA was completely dependent on ATP and Mg²⁺ (Table II). The RNA helicase activity was heat-labile, sensitive to N-ethylmaleimide treatment, inactivated by proteinase K digestion, and unaffected by micrococcal nuclease treatment. These results indicated that the RNA helicase activity was a protein containing essential SH groups, and RNA (or DNA) did not play a role in the catalytic activity of the helicase. The displacement reaction was slightly stimulated by the addition of ssDNA-binding proteins (SSB) isolated from HeLa cells and from E. coli (Table II).

The RNA helicase activity was supported by all eight common nucleoside triphosphates (Fig. 5). ATP and GTP were the most efficient, whereas dTTP and dCTP were the least effective, and the ATP analogs ATPγS and AMP-PNP did not support dsRNA unwinding. Nucleoside triphosphate concentrations >1 mM were found to be saturating (data not shown). With the purified RNA helicase fraction, the apparent Kₐ for ATP was ~100 µM, whereas the Kₐ for Mg²⁺ was 50 µM. The helicase activity was stimulated 3-fold by NaCl (Table II), and maximal activity was observed at salt concentrations between 75 and 250 mM NaCl. In the presence of 0.3 M NaCl, the reaction was reduced 70%. The optimal pH was broad, ranging between 6.5 and 9.2, with 50% activity remaining at pH 6.0 and 10.0 (data not shown).

Influence of Enzyme Concentration, Time of Incubation, and RNA Substrate on RNA Helicase Activity

With increasing concentration of the helicase (Fig. 6), there was a fairly linear increase in RNA displaced until ~30% of the substrate was utilized (with ~20 ng of protein). Further increase of the enzyme resulted in a gradual increase in RNA displacement.
incubation, 8 fmol of RNA was displaced (Fig. 7, upper). Addition of more RNA substrate (another 50 fmol) at 40 min did not significantly alter the rate of RNA displacement. Addition of more helicase (5 ng) after 40 min of incubation resulted in another burst of activity, indicating that the helicase was either inactivated or sequestered during the reaction. The addition of the E. coli SSB or the multisubunit HSSB had no effect on the RNA displacement reaction described in Fig. 7 (upper).

In the presence of limiting amounts of RNA substrate (5 fmol), 50 ng (500 fmol) of RNA helicase quantitatively unwound the partial dsRNA (Fig. 7, lower). After 15 min of incubation, the addition of more RNA helicase had no further effect, in keeping with the quantitative displacement of the dsRNA. In contrast, the addition of 10 fmol of RNA substrate at 15 min resulted in a rapid burst of helicase activity, which plateaued after quantitative displacement of the duplex RNA. In these reactions, the yield of ssRNA was 70% of the expected value based on the input 32P-labeled RNA substrate. The reasons for this discrepancy are not clear.

**Displacement Polarity of RNA Helicase**

The RNA helicase efficiently displaced both 3' and 5' tailed RNA substrates that lacked any fork structure (Fig. 8). This suggested that the helicase displaced partial dsRNA in both the 5' to 3' and the 3' to 5' directions. The 3' and 5' tailed substrates were displaced more efficiently than the

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**Fig. 5. Helicase unwinding with different nucleoside triphosphates.** RNA helicase assays were carried out as described in the text with 20 ng of the SG2 fraction. The final NTP concentration used in each assay was as follows: lane 1, no NTP; lanes 2, 6, 10, 14, 18, 22, 26, and 30, 5 μM; lanes 3, 7, 11, 15, 19, 23, 27, and 31, 40 μM; lanes 4, 8, 12, 16, 20, 24, 28, and 32, 200 μM; and lanes 5, 9, 13, 17, 21, 25, 29, and 33, 1 mM. The NTP used in each assay is indicated above each lane. Control reactions (−ENZ, no enzyme; Δ, substrate boiled for 10 min) are indicated. Reactions were incubated at 30°C for 20 min, and aliquots were electrophoresed on a 17% polyacrylamide gel. After autoradiography of the dried gel, the regions corresponding to the ds- and ssRNAs were excised and quantitated. The amount of ssRNA formed is listed below each lane.

**Fig. 6. Influence of increasing amounts of RNA helicase on displacement reaction.** Unwinding assays were carried out as described in the text with increasing amounts of the SG2 fraction. Control reactions (−ENZ, no enzyme; Δ, substrate boiled for 10 min) and the amount of the SG2 fraction added are indicated. Reactions were incubated at 30°C for 20 min, and aliquots were loaded onto an 17% acrylamide gel. After electrophoresis and autoradiography, regions of the dried gel corresponding to the ds- and ssRNAs were excised and quantitated.

In the presence of low levels of RNA helicase (5 ng, equivalent to 50 fmol, assuming 100% purity and that all molecules were active), the rate of displacement was linear for ~20 min, after which time the rate decreased. After 60–80 min of
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Fig. 8. Polarity of RNA helicase activity. RNA helicase assays were carried out as described in the text with 50 fmol of the standard RNA substrate or the 3'- or 5'-tailed substrate. The reactions contained 5, 10, or 20 ng of the SG2 fraction and were incubated at 30 °C for 20 min. Control reactions (–ENZ, no enzyme; Δ, boiled substrate; –ATP, reaction with 20 ng of helicase but lacking ATP) for each substrate are also indicated. After electrophoresis and autoradiography, regions of the dried gel corresponding to the ds- and ssRNAs were excised and quantitated. The amount of displaced substrate is indicated for each reaction.

Activity of RNA Helicase with Other Duplex Structures

The purified RNA helicase activity specifically displaced dsRNA under the conditions used (Fig. 9). Using the standard partial dsRNA substrate, helicase fractions displaced ~35% of the substrate in the presence of ATP (Fig. 9, upper). φX174 circular ssDNA, hybridized to either a 30-nucleotide complementary oligodeoxynucleotide (Fig. 9, center) or the identical 30-nucleotide oligoribonucleotide, did not support helicase activity (Fig. 9, lower). The SV40 T-antigen, in the presence of ATP, displaced both the RNA and the DNA hybridized to the φX174 circular ssDNA (Fig. 9, center and lower). Also shown in Fig. 9 (upper), the SV40 T-antigen did not displace the dsRNA in the presence of ATP, but did so in the presence of UTP, in accord with the results of Scheffner et al. (6).

In the generation of the spliceosome complex, it has been reported that the U4/U6 small nuclear ribonucleoprotein coupled to the 55 S complex undergoes a displacement reaction such that only U6 RNA remains associated with the spliceosome (18, 23). To check whether the RNA helicase described here could carry out this displacement reaction, we synthesized the yeast U4 and U6 RNAs both labeled with [α-32P]GTP from plasmid templates provided by Dr. J. Abelson. The proposed structure of the RNA duplex is described in Fig. 1C. When this RNA, which contains two distinct partial duplex regions, was incubated with the RNA helicase, the RNAs were efficiently separated in the presence of a number of different NTPs added (Fig. 10). Similar results were obtained with the SV40 T-antigen in the presence of UTP (data not shown).

RNA Helicase Has No Deaminase Activity

The displacement of dsRNA due to the deamination of adenine residues has been reported by Bass and Weintraub (27). The generation of inosines in the RNA resulted in the decreased stability of the duplex regions. We have shown that the RNA helicase described here does not alter the A residues in the RNA substrates as well as in the displaced strands. For this purpose, the standard duplex RNA was prepared with [α-32P]ATP in place of [α-32P]GTP. We found that the unwinding of the AMP-labeled RNA substrate was dependent on the presence of ATP and Mg²⁺ (in contrast to the Xenopus oocyte displacement system). The unwound RNA and the partial dsRNA were digested with P1 nuclease, and the resulting
mononucleotides were chromatographed on a polyethyleneimine-cellulose thin-layer plate. The dried plates were subjected to autoradiography to determine whether the [32P]AMP residues had been deaminated to [32P]IMP. No detectable [32P] was recovered as IMP, and all of the [32P] present in the digests was recovered as AMP (data not shown).

Characterization of ssRNA-dependent Nucleoside Triphosphatase Activity

As shown in Fig. 3, both RNA helicase activity and the poly(A)-dependent hydrolysis of dTTP cosedimented in sucrose gradients. Other experiments, such as the rate of heat inactivation at 50 °C, insensitivity to micrococcal nuclease, and N-ethylmaleimide sensitivity, also supported the conclusion that these two activities were catalyzed by the same enzyme. We have named this activity described here (data not shown). We have named it RNA helicase I and will refer to it as such below.

Preparations of RNA helicase I contain two major polypeptides of 80 and 55 kDa that cosedimented with helicase activity in sucrose gradients carried out in the presence of high salt (1 M NaCl). Furthermore, these two polypeptides also coeluted with RNA helicase I activity when subjected to gel filtration in the presence of 1 M NaCl (data not shown). Whereas these observations suggest a strong interaction between these two polypeptides, we do not know whether both polypeptides are essential for RNA helicase I activity.

RNA helicase I specifically displaced dsRNA and not double-stranded RNA/DNA or DNA/DNA substrates. RNA helicase I is capable of unwinding substrates with either single-stranded 3' - or 5' -regions, suggesting that the enzyme can move bidirectionally on RNA. There are a number of possible explanations for helicase's bidirectionality. These include the following: (i) Bidirectionality is an intrinsic property of RNA helicase I. (ii) The direction of unwinding is modulated by different forms of the same enzyme. (iii) The possibility exists that more than one RNA helicase is present in the SG2 fraction. (iv) The enzyme can displace fully dsRNA structures and does not require a single-stranded tail for translocation. If explanation iv were the case, it would be difficult to determine the direction of translocation. However, RNA helicase I did not displace dsRNA without single-stranded tails (data not shown); and for this reason, we believe that the helicase does require a ssRNA tail for its activity. Bidirectional unwinding has previously been reported only for eIF-4A, which requires eIF-4B for helicase activity. RNA helicase I differs from eIF-4A plus eIF-4B in that it utilizes a variety of nucleotide activities.

**TABLE IV**

| NDPaneformedfrom: | dTTP | GTP | ATP |
|-------------------|------|-----|-----|
| Poly(A) + Poly(A) | 1.0  | 1.1 | 3.2 |
| Poly(A) + Poly(A) | 1.4  | 2.7 | 12.1|
| Poly(A) + Poly(A) | 3.4  | 5.0 | 21.1|

DISCUSSION

The results presented here describe the isolation of an enzyme activity that unwinds partial dsRNA in the presence of Mg2+ and a nucleoside triphosphate. This RNA helicase activity has been purified at least 300-fold from HeLa nuclear extracts, which may be an underestimation due to the presence of multiple RNA helicase activities in crude fractions. During the purification of this activity, at least six other peaks of RNA helicase activity were detected that differed in their chromatographic properties. Some of these were evident in Fig. 2. We have further characterized only two of these and found that they have distinct properties from the RNA helicase activity described here (data not shown). We have named the activity in the SG2 fraction RNA helicase I, and we will refer to it as such below.

Preparations of RNA helicase I contain two major polypeptides of 80 and 55 kDa that cosedimented with helicase activity in sucrose gradients carried out in the presence of high salt (1 M NaCl). Furthermore, these two polypeptides also coeluted with RNA helicase I activity when subjected to gel filtration in the presence of 1 M NaCl (data not shown). Whereas these observations suggest a strong interaction between these two polypeptides, we do not know whether both polypeptides are essential for RNA helicase I activity.

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**TABLE IV**

| NDP formed from: | dTTP | GTP | ATP |
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| Poly(A) + Poly(A) | 3.4  | 5.0 | 21.1|

The displacement of dsRNA, however, was much lower with dTTP than with ATP or GTP (Fig. 5). This suggests that the requirements for binding RNA and for both binding and breakage of hydrogen bonds differ.
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oxide triphosphates as a source of energy for unwinding, whereas eIF-4A (plus eIF-4B) can utilize only ATP or dATP for unwinding (4). The requirement for NTP also distinguishes RNA helicase I from the Xenopus oocyte deaminase unwinding activity, which does not require any NTP and thus is not a true helicase (27). RNA helicase I also differs from the human p68 RNA helicase since the monoclonal antibody PAb204, which cross-reacts with p68 (5), failed to immunoprecipitate the activity of the SG2 fraction and did not react with this preparation using immunoblotting procedures (data not shown).

As reported for eIF-4A plus eIF-4B (4) and the PPV CI protein (7), the displacement of femtomole levels of dsRNA required the addition of picomole quantities of protein. In contrast, femtomole levels of p68 and RNA helicase I displaced femtomole levels of dsRNA. In the case of RNA helicase I, 50 fmol of enzyme displaced 10 fmol of dsRNA, after which time the reaction came to a halt. It appeared that this effect was due to the loss of RNA helicase activity since the addition of more enzyme rather than more dsRNA resulted in the resumption of ssRNA formation. The requirement for additional trans-acting factor(s) may be additional trans-acting factor(s) may be required for efficient dissociation of the enzyme from the products. In the case of eIF-4A (plus eIF-4B), it has been suggested that the requirement for a large molar excess of the proteins may overcome the dissociation of the eIF-4A and eIF-4B complex, which is the active RNA helicase (4). However, such an explanation is unlikely in the case of the CI protein of PPV since its RNA helicase activity can be carried out with a single polypeptide chain. An alternative explanation is that purified helicases strongly interact with ssRNA and that additional trans-acting factor(s) may be required for efficient dissociation of the enzyme from the products.

To date, RNA helicase and RNA-dependent NTPase activities have been demonstrated for only three other proteins: mammalian translation eIF-4A (plus eIF-4B), which is thought to unwind the secondary structure of mRNA to facilitate ribosome binding (2-4); human nuclear protein p68 (5), which is thought to function in the regulation of cell growth and division (28); and the CI protein, encoded by the human p68 RNA helicase since the monoclonal antibody PAb204, which cross-reacts with p68 (5), failed to immunoprecipitate the activity of the SG2 fraction and did not react with this preparation using immunoblotting procedures (data not shown).

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REFERENCES

1. Matson, S. W., and Kaiser-Rogus, K. A. (1990) Annu. Rev. Biochem. 59, 283-329.
2. Ray, B. K., Lawson, T. G., Kramer, J. C., Cladaras, M. H., Grifo, J. A., Abramson, R. D., Merrick, W. C., and Thach, R. E. (1985) J. Biol. Chem. 260, 7651-7658.
3. Abramson, R. D., Dever, T. E., Lawson, T. G., Ray, B. K. Thach, R. E., and Merrick, W. C. (1987) J. Biol. Chem. 262, 3832-3838.
4. Rozen, F., Edery, I., Meeroevitch, K., Dever, T. E., Merrick, W. C., and Sonnenberg, M. (1990) Mol. Cell. Biol. 10, 1574-1576.
5. Hirling, H., Scheffner, M., Restle, T., and Stahl, H. (1989) Nature 339, 562-564.
6. Scheffner, M., Knippers, R., and Stahl, H. (1989) Cell 57, 955-963.
7. Lain, S., Riechmann, J. L., and Garcia, J. A. (1990) Nucleic Acids Res. 18, 7001-7006.
8. Lain, S., Riechmann, J. L., Garcia, J. A. (1991) J. Virol. 65, 1-6.
9. Lain, S., Riechmann, J. L., Martin, M. T., and Garcia, J. A. (1989) Gene (Amst.) 82, 357-362.
10. Linder, P., and Slonimski, P. P. (1988) Nucleic Acids Res. 16, 10359.
11. Seraphin, B., Simon, M., Boulet, A., and Faye, G. (1989) Nature 337, 81-87.
12. Nishi, K., Morel-Diville, F., Hershey, J. W. B., Leighton, T., and Schneider, J. (1989) Nature 336, 496-498.
13. Lasko, P. F., and Ashburner, M. (1988) Nature 335, 611-617.
14. Leroy, P., Alazi, P., Sasson, D., Wogelmuth, D., and Fellous, M. (1989) Cell 57, 549-559.
15. Dalbadie-McFarland, G., and Abelson, J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4236-4240.
16. Burgess, S., Couto, J. R., and Guthrie, C. (1990) Cell 60, 705-717.
17. Padgett, R. A., Grabowski, P. J., Konarska, M. M., Slizer, S., and Sharp, P. A. (1986) Annu. Rev. Biochem. 55, 1119-1150.
18. Chen, S. C., and Abelson, J. (1987) Genes Dev. 1, 1014-1027.
19. Konarska, M. M., and Sharp, P. A. (1986) Cell 46, 845-855.
20. Pikelney, C. W., Raymond, B. C., and Rosbach, M. (1986) Nature 324, 341-345.
21. Pruzan, R., Fournier, H., Lassota, P., Hong, G. Y., and Hurwitz, J. (1990) J. Biol. Chem. 265, 2804-2813.
22. Brow, D. A., and Guthrie, C. (1988) Nature 334, 213-218.
23. Landon, A. I., Konarska, M. M., Grabowski, P. J., and Sharp, P.
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24. Siliciano, P. G., Brow, D. A., Roibo, H., and Guthrie, C. (1987) Proc. Natl. Acad. Sci. U. S. A. 85, 411-415
25. Laemmli, U. K. (1970) Nature 227, 680-685
26. Dignam, J. D., Lebovitz, R. M., and Roeder, R. D. (1983) Nucleic Acids Res. 11, 1475-1489
27. Bass, B. L., and Weintraub, H. (1988) Cell 55, 1089-1098
28. Iggo, R. D., and Lane, D. P. (1989) EMBO J. 8, 1827-1831
29. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) EMBO J. 1, 945-961
30. Linder, P., Laski, P. F., Ashburner, M., Leroy, P., Nielsen, P. J., Nishi, K., Schnier, J., and Slonimski, P. P. (1989) Nature 337, 121-122
31. Chang, T. H., Arenas, J., and Abelson, J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1571-1575
32. Gorbatenya, A. E., Koonin, E. V., Donchenko, A. P., and Blinov, V. M. (1989) Nucleic Acids Res. 17, 4713-4730
33. Hodgman, T. C. (1988) Nature 333, 22-23
34. Chen, J. H., and Lin, R. J. (1990) Nucleic Acids Res. 18, 6447