In this report, I describe the identification of an RNA helicase named RH70. Mass spectrometric analysis reveals that RH70 is DDX17, a DEAD protein with a demonstrated RNA-dependent ATPase activity (16). RH70 functions as an RNA helicase, unwinding duplex RNA in both the 5′–3′ and the 3′–5′ directions. When the duplex region is increased to 29 bp, RNA substrate cannot be unwound by RH70. Interestingly, the presence of a 3-nucleotide mismatch in the middle of a 31-bp duplex leads to an efficient unwinding by RH70. Our observations suggest that RH70 is a bidirectional and catalytic helicase. The possible involvement of RH70 in pre-mRNA splicing is also discussed.

EXPERIMENTAL PROCEDURES

Reagents and Enzymes—All nucleotides were purchased from Amer sham Biosciences. Radioactive nucleotides such as [α-32P]ATP and [α-32P]GTP were obtained from ICN. TLC plates (PEI-cellulose F) used in the ATPase assay were from EM Industry. RNase inhibitor was obtained from Promega. SP6 and T7 polymerases were from Stratagene or New England Biolabs (NEB). All restriction nucleases used to make DNA template for in vitro transcription were purchased from NEB.

Helicase and ATPase Assays—RNA substrates in Figs. 2–4 were essentially the same as described previously (10, 12). The 5′-tailed substrate (Fig. 4A) was prepared from pHGEM3 plasmid, and bulged substrate, shown in Fig. 4B, was prepared using pSP65 cut with XhoI (lower RNA strand) and pGEM3 linearized with PvuII (upper RNA strand) (detailed procedure available upon request). The specific radioactivity of RNA substrate was adjusted to 100,000 cpm/50 fmol of RNA. In vitro reactions for the helicase and ATPase activities were carried out for 30 min at 37 °C following the procedures described previously (10, 12).

Co-purification of U1snRNP and RH70—All purification steps were carried out at 4 °C. Nuclear extracts (2.6 g) were prepared from 60 liters of HeLa suspension culture according to the procedure described by Dignam et al. (17). As summarized in Fig. 1A, nuclear extracts were fractionated through six successive chromatography steps using buffer A (20 mM Hepes-KOH, pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) and an indicated concentration of KCl. At each step, bound proteins were eluted by linearly increasing the concentration of KCl in

The unwinding of nucleic acids (either DNA or RNA) is an essential step in DNA replication, repair, recombination, transcription, RNA processing and transport, and translation (1). Helicase proteins mediate the disruption of hydrogen bonds of duplex DNA, the inter-intramolecular duplex RNA molecules, or the dissociation of proteins from nucleic acid (2). Coupled with their helicase activity, all helicase proteins possess intrinsic NTP hydrolytic (NTPase) activity that can be stimulated by nucleic acids (2). For examples, Rhb, a DEAD-box protein in *Escherichia coli*, is involved directly in mRNA degradation, functioning as an RNA helicase in a multiprotein degradosome complex (3). The eIF4A/eIF4B complex, in conjunction with eIF4F, is believed to melt the 5′–proximal secondary structure of mRNA, generating ssNA regions that facilitate ribosome binding, an important reaction governing the efficiency of RNA translation (4).

Recent studies on their mechanism of action indicate that most helicases, whether monomeric or multimeric, possess two functional domains, one for interaction with single-stranded nucleic acid (ssNA) and the other for interaction with double-stranded nucleic acid (dsNA) (5–8). The spatial and functional arrangements of these NA binding domains determine the directional movement of the helicase relative to the bound ssNA in either the 5′ to 3′ or 3′ to 5′ direction. If multiple ssNA or dsNA binding domains are present in a single protein, or if the helicase functions as an oligomer, bidirectionality can be observed in the unwinding reaction, as seen with p68 (9) and eIF4A complexed with eIF4B (4). Most helicases exhibit a preferential substrate specificity for either DNA or RNA molecules (2). Unlike some DNA helicases that function in a processive manner (13, 14), it is believed that RNA helicases are inherently catalytic, unwinding only short stretches of duplex (15). In addition to directional movement on NA, the substrate specificity and processivity are likely to be dictated by the molecular nature of the NA binding domains of individual helicases.

In this report, I describe the co-purification of a novel 70-kDa RNA helicase (RH70) and U1snRNP through six column steps. Peptide sequence analysis by mass spectrometry and Edman degradation revealed that RH70 is the previously reported DDX17. Biochemical characterization of RH70, obtained by partial separation from U1snRNP, yielded the following results. (a) RH70 mediates the unwinding of duplex RNA but not DNA in an ATP-dependent manner. (b) Both the RNA-dependent ATPase and RNA helicase activities of RH70 are highly specific for ATP, exhibiting an apparent *Km* of 0.5 mM. (c) RH70 catalyzes the unwinding of duplex RNA containing single-stranded regions at either the 5′- or 3′-end. Its association with U1snRNP and ATP specificity suggest a role for RH70 in pre-mRNA splicing, in particular, at the early stages of the splicing reaction involving U1snRNP.
buffer A. Fractions containing U1snRNP, determined by the presence of U1snRNA as well as U1snRNP-specific 70-kDa protein, were pooled. The salt concentration of pooled fractions is described in Fig. 1A. Eluant composition was examined with the indicated amount of pooled fractions by 12% SDS-PAGE (Fig. 1B). S-Sepharose chromatography yielded about 43 mg of protein, which was dialyzed overnight to 0.1 M KCl and loaded onto a single-stranded DNA cellulose column (12 ml). No protein was detected in the flow-through, and bound proteins were eluted in 50 ml of buffer A containing an increasing concentration of KCl (50–500 mM). Of 72 fractions collected, fractions 19–34 were enriched in U1snRNP. They were pooled (8 mg) and dialyzed overnight to 50 mM KCl and 15 mM MgCl₂ in buffer A. Subsequently, they were loaded onto a Q-Sepharose column (2 ml). Bound proteins were eluted using buffer A (30 ml) containing 15 mM MgCl₂ and an increasing concentration of KCl (50–700 mM). Pooled 0.1 M KCl fractions (2.64 mg) contained RH70, whereas U1snRNP was highly enriched in 0.25 M KCl fractions (3.0 mg). An aliquot (100 µg) of pooled Q-Sepharose fractions containing RH70 was loaded on top of a linear glycerol gradient (5 ml, 20–40%) in buffer A containing 0.2 mM KCl and 0.05% Nonidet P-40. After centrifugation at 45,000 rpm for 24 h at 4 °C in a Sorvall AH630 rotor, 30 fractions were collected from the tube bottom. Aliquots (0.5 µl) were used in both RNA helicase and poly(U)-dependent ATPase assays; 16–µl aliquots were analyzed on a 10% SDS-polyacrylamide gel and visualized by Coomassie staining. Protein concentration was determined using Bradford reagent (Bio-Rad).

RESULTS

Co-purification of RH70 and U1snRNP—Several RNA-dependent ATPases and RNA helicases were detected in various column fractions during the purification of U1snRNP, but only one was co-purified with U1snRNP enriched in ssDNA cellulose fractions. Pooled ssDNA fractions contained two distinct proteins of about 70 and 22 kDa in addition to all known components of U1snRNP such as 70 kDa, A, B, C, D, E, F, and G (Fig. 1B, lane 6). Subsequent Q-Sepharose chromatography resulted in partial separation of 70-kDa protein and complete removal of 22-kDa protein from U1snRNP (Fig. 1B, lanes 6–8). For the purpose of their identification, 70- and 22-kDa proteins were subjected to tryptic digestion followed by Edman degradation and mass spectrometry. These studies revealed that the 22-kDa protein is cyclophilin B (CyB), whereas the 70 kDa protein, named RH70, is DDX17, which exhibits extensive sequence similarity to p68, a prototypic DEAD helicase (data not shown) (16).

Because U4/6 snRNP extracts interact with cyclophilins (18,19), I explored the possibility that co-purification of CyB and U1snRNP might be due to their physical interaction in a Mg²⁺-sensitive manner. However, immunoprecipitation of HeLa nuclear extract with IgGs specific for either U1 70 kDa or CyB did not yield any evidence indicating an interaction between CyB and U1snRNP (data not shown).

Association of RH70 with U1snRNP—As U1snRNP is purified from heparin-Sepharose through ssDNA cellulose, RH70 seems also to be enriched relative to other proteins (Fig. 1B, lanes 4–6). In contrast to CyB, which is absent from the final U1snRNP preparation, the continued co-purification of RH70 with U1snRNP may suggest their stable interaction (lane 8). To test this possibility, RH70 and U1snRNP were analyzed by immunoprecipitation using Y12 monoclonal antibodies (mAb) specific for the B/B’ and D polypeptides of U1snRNP. As shown in Fig. 1C, neither RH70 nor CyB reacted with Y12 mAb (lanes 7–9). In contrast, RH70 present in U1snRNP was immunoprecipitated by Y12 mAb (lanes 4–6). There was no noticeable influence of MgCl₂ or ATP on the reactivity of RH70 and U1snRNP with Y12 mAb. Thus, it is very likely that a certain fraction of RH70 forms a stable complex with U1snRNP. It is important to note that p68 RNA helicase, which is highly homologous to RH70, also interacts with U1snRNP-5′-splice site complex during pre-mRNA maturation (20) (see “Discussion”).

RH70 Possesses RNA-dependent ATPase and RNA Helicase Activities—Pooled RH70 fractions (Fig. 1B, lane 7) were subjected to sedimentation analysis as described under “Experimental Procedures.” The helicase activity on the glycerol gradient migrated faster than cytochrome C (CYC) but slower than bovine serum albumin (BSA, 68 kDa, 4.3 S) (Fig. 2). The calculated sedimentation coefficient of RH70 was 4.0 S (Fig. 2C), and the protein co-migrating with the helicase activity was 70 kDa in size (Fig. 2A), suggesting that RH70 might exist as a monomer in solution. In addition to the helicase activity, RNA-dependent ATPase activity also co-sedimented with 70-kDa protein fractions eluted at 0.25 M KCl. For a better resolution of RH70- and U1-specific 70-kDa protein (70K), parts of the stained gel (squares) are magnified 2-fold. Protein subunits of U1snRNP, including 70K, A, B, B’, C, D, E, F, and G, are indicated. C, aliquots of pooled Q-Sepharose fractions enriched in U1snRNP and RH70 were immunoprecipitated with Y12 mAbs as described under “Experimental Procedures.” Aliquots (50%) of the IP pellets were analyzed on 12% SDS-polyacrylamide gel and visualized by Coomassie staining. Lanes: 1, U1snRNP (8 µg); 2, RH70 (1.2 µg); 3–6, IP pellets obtained with U1snRNP; 7–10, IP pellets obtained with RH70; 3 and 7, with 15 mM MgCl₂ and ATP; 4 and 8, with 15 mM MgCl₂; 5 and 9, with 3 mM MgCl₂ and 1 mM ATP; 6 and 10, IP pellets obtained without Y12 mAb; 11, IP pellet obtained with neither U1snRNP nor RH70.

U1snRNP with Y12 mAb. Thus, it is very likely that a certain fraction of RH70 forms a stable complex with U1snRNP. It is important to note that p68 RNA helicase, which is highly homologous to RH70, also interacts with U1snRNP-5′-splice site complex during pre-mRNA maturation (20) (see “Discussion”).
phosphate (P_i) in the presence of RNA but 2 pmol of P_i in the absence of RNA after a 30 min-incubation at 37 °C.

Requirements for the Helicase Activity of RH70—Helicase reactions were carried out using 40 ng of RH70 (pooled glycerol gradient fractions) under the standard condition described under “Experimental Procedures.” Helicase activity was absolutely dependent on MgCl_2 and ATP. The addition of either EDTA (5 mM) or nonhydrolyzable ATP analogs such as App(CH_2)p and App(NH)p instead of ATP resulted in more than 90% inhibition of the maximal activity (data not shown). To determine their $K_m$, varying concentrations (0, 0.2, and 1 mM) of each nucleotide was added to helicase reactions. As shown in Fig. 3, the helicase activity was stimulated by higher ATP concentration (lanes 4-5) but was not supported by other nucleotides (lanes 6-11). No helicase activity of RH70 was observed with dsDNA substrate (data not shown). These results indicate that RH70 functions as an RNA helicase, preferentially utilizing ATP.

RH70 Catalyzes the Unwinding of dsRNA Bidirectionally—The directionality of helicase activity is defined by the strand on which an enzyme binds and translocates. To determine the directionality of RH70, two different dsRNA substrates containing single-stranded regions exclusively at the 5'-or 3'-end were compared in the helicase reaction containing 40 ng of RH70 (Fig. 3). RH70 efficiently unwound both 5'- and 3'-tailed dsRNA (compare left and right panels in Fig. 3). In Fig. 2, although helicase activity was measured with 5'-tailed dsRNA substrate, essentially the same result was obtained with 3'-tailed dsRNA substrate. These results indicate that RH70 catalyzes the unwinding of dsRNA bidirectionally.

The processivity of helicase reflects the probability that the helicase will perform the next unwinding step with a unique step size (15). Thus, the processivity of RH70 was determined using a new 5'-tailed dsRNA containing a duplex region comparable with that of the 3'-tailed dsRNA substrate. As shown in Fig. 4A, with an increase in duplex length from 15 to 26 bp, the efficiency of the unwinding reaction drastically decreased. These results suggest that the unwinding of 5'-tailed dsRNA by RH70 is highly catalytic. It is also possible that the length of ssRNA regions, which is much longer in 3'-tailed dsRNA (78 and 82 nucleotides) than in 5'-tailed dsRNA (26 and 20 nucleotides), might also influence the unwinding reaction catalyzed by RH70.

The directionality of RH70 was further explored using two
additional dsRNAs, including one containing shorter ssRNA regions but a 3'-nucleotide mismatch in the middle of a duplex region (Fig. 4B). RNA helicase A (RHA) efficiently catalyzed the unwinding of both dsRNAs (compare lanes 4–5 and 12–13). Unlike RH70, RHA did not unwind 5’-tailed substrate at all (compare lanes 20–21 and 23–24). These results indicate that the presence of a mismatch does not significantly influence RHA and that the availability of ssNA regions at the 3'-end is important for RHA to be effective as a helicase. Despite the presence of comparable ssNA regions at both 3'- and 5'-ends, the unwinding by RH70 was insignificant with dsRNA containing a longer duplex (29 bp) (lanes 7–8). Interestingly, the presence of a mismatch in the duplex region allowed effective unwinding of an RNA substrate that was otherwise poorly utilized by RH70 (compare lanes 7–8 and 15–16). These results indicate that, unlike RHA, RH70 unwinds dsRNA in a highly catalytic and bidirectional mode.

**DISCUSSION**

Here I report that RH70 co-purified with U1snRNP is DDX17, a DEAD family member (16). Employing sedimentation analysis, I also demonstrate that RH70 possesses both RNA-dependent ATPase and RNA helicase activities. As reported previously for its ATPase activity, ATP is the only nucleotide that supports the helicase activity of RH70. Our study also shows that RH70 catalyzes the unwinding of dsRNA in both the 5’ to 3’ and 3’ to 5’ directions, consistent with its sequence identity (about 70%) to p68, which mediates the unwinding of dsRNA in a bidirectional manner (9).

This study establishes that the presence of a mismatch in a duplex region can be important for an efficient unwinding of dsNAs by catalytic helicases. It can be predicted that if the helicase dissociates after partial disruption of a duplex region, no ssNA product will be observed because of the re-annealing of partially unwound duplex region. This so-called “abortive unwinding” is depicted in Fig. 5A. The prevention of partially melted regions from re-annealing would be important for the productive generation of ssNA regions. As seen in the unwinding of the SV40 viral replication origin, mediated by T antigen with the assistance of single-stranded DNA-binding protein (SSB) (21, 22), the above can be achieved by a particular ssNA-binding factor. Even in the absence of ssNA-binding factor, however, the use of a mismatch can turn abortive unwinding of dsNA to productive unwinding, as shown in Fig. 5B.
Helicase protein translocates along dsDNA with a defined unwinding step size. A recent study demonstrated that a DExH (Asp-Glu-x-His) protein, NPH-II, unwinds dsDNA with a step size of roughly 6 bp (15), which is similar to that of UvrD DNA helicase, 4–5 bp (23). If a certain helicase dissociates from dsDNA after a single round of translocation with a step size of about 4–6 bp, a productive unwinding reaction would hardly be detected in vitro. To date, not all DEAD/DEAH family members have been shown to possess helicase activity in vitro (2, 24), perhaps because of the requirement for a specific substrate or assisting factors. My observation suggests an additional possibility; helicases, previously reported to be nonfunctional, may be highly catalytic in vitro in such a way that partially unwound duplex regions are re-annealed before the second engagement of the helicase. As demonstrated for RH70 in this study, the use of dsDNA, consisting of a series of mismatches between multiple duplex regions of varying base pairs, will enable us to test the above possibility and also experimentally determine the maximum step size of catalytic helicases.

Pre-mRNA splicing involves five snRNPs and numerous non-snRNP proteins (25, 26). A number of conformational changes occur during pre-mRNA splicing, including the formation and disruption of RNA base pairs and protein-RNA and protein-protein interactions. At least eight splicing factors belonging to the DEAH ATPase/helicase family have been identified in yeast (26). The ATP requirement for pre-mRNA splicing can be attributed in part to energy-consuming steps mediated by these DEAH proteins. For example, two DEAH proteins, PRP22 and PRP43, mediate the dissociation of the spliceosome complex releasing mRNA as well as splicing factors, which is essential for the recycling of splicing factors for new rounds of pre-mRNA splicing (27–29). It is intriguing to note that PRP2 and PRP16, the two relatively well characterized DEAH proteins, show a broad range of nucleotide specificity (30, 31), whereas pre-mRNA splicing is strictly dependent on ATP (25). In addition to various processes mediated by known DEAH proteins, yet unknown energy-requiring reactions may take place in the earlier stages of pre-mRNA splicing, mediated by proteins highly specific for ATP. In fact, a protein kinase activity was observed with immunoaffinity-purified U1snRNP (32), and a similar kinase activity (SRPK1) was purified from HeLa cells (33). However, SRPK1 seems to function as a negative regulator of pre-mRNA splicing (34). My observation makes it plausible to consider the following scenario as an alternative explanation for the ATP specificity of pre-mRNA splicing. As soon as pre-mRNAs are synthesized by RNA polymerase II, RH70 may assist U1snRNP to recognize and interact with the 5′-splice site through its helicase activity functioning in the 5′ to 3′ direction. In this regard, it is informative to note that p68 RNA helicase, which is highly homologous to RH70, interacts with the U1.5′-splice site duplex (20). A detailed functional study on RH70 and p68 in the context of pre-mRNA splicing could elucidate the nucleotide specificity of pre-mRNA splicing and perhaps clarify the molecular basis of U1snRNP interaction with the 5′-splice site.

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