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The purified yeast pre-mRNA splicing factor PRP2 is an RNA-dependent NTPase

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Unlike autocatalyzed self-splicing reactions, nuclear pre-mRNA splicing requires *trans*acting macromolecules and ATP. A protein encoded by the *PRP2* gene of *Saccharomyces cerevisiae* is required, in conjunction with ATP, for the first cleavage-ligation reaction of pre-mRNA splicing. In this study, we have purified two forms of the *PRP2* gene product with apparent molecular weights of 100 kDa and 92 kDa, from a yeast strain overproducing the protein. Both proteins were indistinguishable in their ability to complement extracts derived from a heat-sensitive *prp2* mutant. Futhermore, we show that the PRP2 protein is capable of hydrolyzing nucleoside triphosphates in the presence of single-stranded RNAs such as poly(U). However, purified PRP2 by itself did not unwind double-stranded RNA substrates. The fact that an RNA-dependent NTPase activity is intrinsic to PRP2 may account for the ATP requirement in the first catalytic reaction of pre-mRNA splicing.

Key words: ATPases/DEAD-box proteins/pre-mRNA splicing/*Saccharomyces cerevisiae*/splicing factors

Introduction

RNA splicing, the biochemical process leading to the removal of introns from precursor RNAs, is an essential step in the expression of genetic information (Abelson, 1979). Unlike autocatalyzed self-splicing reactions, nuclear pre-mRNA splicing requires numerous *trans*acting factors and ATP (Guthrie, 1991). In mammalian and yeast cells, a large RNA-protein complex, called the spliceosome, forms prior to the actual catalytic reactions. The spliceosome contains the pre-mRNA as well as four small nuclear ribonucleoprotein particles and a number of protein factors (Lührmann, 1988; Steitz *et al.*, 1988; Green, 1991). This complex is the structure in which the two step pre-mRNA splicing reactions take place. In the first step, the 5'-splice site is cleaved with the formation of a branched intron-exon 2 lariat. In the second step the cleavage of the 3'-splice site and ligation of the two exons occur (Maniatis and Reed, 1987; Sharp, 1987). Finally, the ligated exons are released from the spliceosome as mature mRNAs. ATP is required for both spliceosome assembly and cleavage-ligation steps (Guthrie, 1991). However, it is not clear why and how the energy of ATP hydrolysis is utilized in the pre-mRNA splicing process.

Studies of pre-mRNA splicing in the yeast *Saccharomyces cerevisiae* have provided clues to factors that utilize ATP in the splicing process. More than twenty temperature-sensitive mutants defective in pre-mRNA processing (*prp* mutants) have been isolated (Hartwell *et al.*, 1970; Vijayraghavan *et al.*, 1989). At the restrictive temperature, *prp* mutants accumulate pre-mRNAs, introns or splicing intermediates, suggesting that the mutated gene products cannot execute certain step(s) in the pre-mRNA splicing process. Indeed, extracts derived from many *prp* mutants are heat-sensitive for splicing *in vitro*, indicating a direct involvement of the *PRP* gene products in splicing (Lustig *et al.*, 1986; Vijayraghavan and Abelson, 1990; Company *et al.*, 1991). In addition, many *PRP* genes have been cloned by complementation and their DNA sequences have been determined (reviewed in Ruby and Abelson, 1991). Protein sequence analysis reveals that five PRP proteins (PRP2, Chen and Lin, 1990; PRP5, Dalbadie-McFarland and Abelson, 1990; PRP16, Burgess *et al.*, 1990; PRP22, Company *et al.*, 1991; PRP28, Strauss and Guthrie, 1991) contain sequence motifs found in a family of putative RNA-dependent ATPases and RNA helicases, the 'DEAD box' family (Linder *et al.*, 1989; Wassarman and Steitz, 1991). Three proteins in the DEAD-box family [the eukaryotic translation initiation factor (eIF) 4A (Rozen *et al.*, 1990), the human protein p68 (Hirling *et al.*, 1989) and the cylindrical inclusion (CI) protein of plum pox virus (Lain *et al.*, 1990)] have been shown to unwind double-stranded RNAs *in vitro* upon hydrolysis of nucleoside triphosphates (NTPs). Another protein, Srm of *Escherichia coli* (Nishi *et al.*, 1988), hydrolyzes ATP in the presence of RNA but has not been shown to possess RNA unwinding activity. Therefore it was postulated that these putative PRP ATPases may be responsible for the utilization of ATP, possibly to unwind duplex RNA during pre-mRNA splicing (see Guthrie, 1991). Indeed, an RNA-dependent ATPase activity copurified with the PRP16 protein (Schwer and Guthrie, 1991). However, RNA helicase activity has not as yet been observed with any isolated PRP protein.

We are interested in understanding the involvement of ATP in pre-mRNA splicing, especially the first catalytic reaction which requires the action of the *PRP2* gene product (Lin *et al.*, 1987). In addition to the seven conserved motifs found in all DEAD-box proteins, PRP2 shares additional homology with PRP16 and PRP22 (Chen and Lin, 1990). In fact, most amino acid residues are conserved among PRP2, PRP16 and PRP22, except for the N-terminal one-third of the proteins (Company *et al.*, 1991). In this report, we describe the purification and characterization of the PRP2. We found that purified PRP2 is a single-stranded RNA-dependent NTPase but lacks RNA helicase activity with the duplex RNA substrates used.

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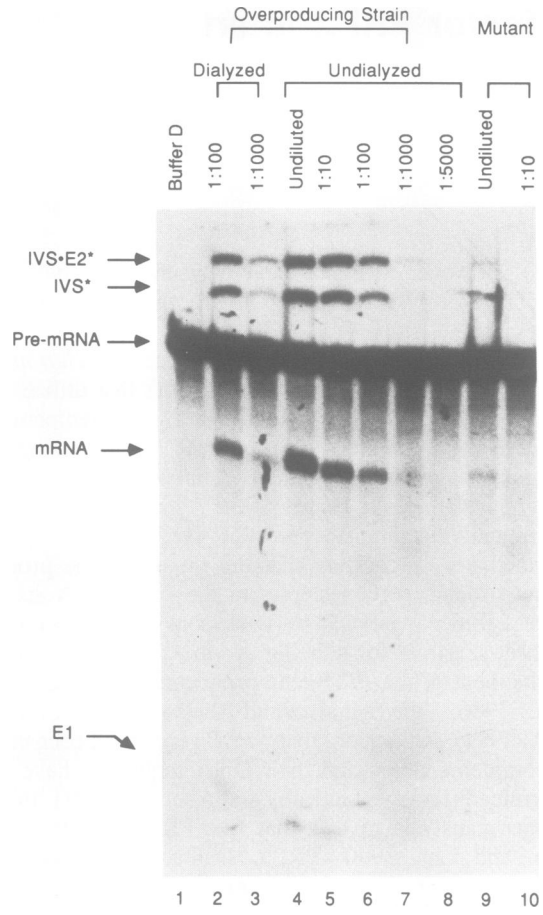

Fig. 1. *In vitro* complementation of heat-inactivated extracts prepared from *prp2* mutant cells. Nucleic acids from complementation reactions were separated on a denaturing polyacrylamide gel and ^{32}P-labeled RNAs were visualized by autoradiography. Individual RNA species marked on the left are the substrate of the reaction (pre-mRNA), two reaction intermediates, lariat intron−exon 2 (IVS-E2*) and exon 1 (E1) and two products, lariat intron (IVS*) and ligated exon 1−exon 2 (mRNA). Inactivated mutant extracts were complemented with buffer D (lane 1), a dialyzed extract (fraction A1) from the strain overproducing PRP2 at dilutions of 1:100 (lane 2) and 1:1000 (lane 3), an undialyzed extract (fraction B1) from the overproducing strain either undiluted (lane 4) or diluted at 1:10 (lane 5), 1:100 (lane 6), 1:1000 (lane 7), 1:5000 (lane 8) and an undialyzed non-heat-treated extract from a *prp2* mutant either undiluted (lane 9) or diluted 1:10 (lane 10). Lane numbers are marked at the bottom of the figure.

Results

Purification of the PRP2 protein

We have previously shown that pre-mRNA splicing carried out with extracts isolated from temperature-sensitive *prp2* mutants can be abolished by heat-treatment; splicing can then be restored by adding back fractions containing the wild-type *PRP2* gene product (Lustig *et al.*, 1986). This sensitive *in vitro* complementation assay allowed us to purify active PRP2 from a yeast strain containing multiple copies of *PRP2* and, as a result, overproducing the protein (Last and Woolford, 1986; Lin *et al.*, 1987). As shown in Figure 1, extracts prepared from the overproducing strain complemented heat-inactivated mutant extracts even at dilutions of 1:1000 (lanes 3 and 7). However, extracts isolated from a mutant strain lacking the wild-type *PRP2* gene supported splicing only when undiluted extracts were used (Figure 1, lane 9). The complementing activity (the PRP2 splicing activity) in extracts prepared from the overproducing strain was estimated to be 50 to 200 times higher than the activity in wild-type yeast extracts (Lin *et al.*, 1987; data not shown). We subsequently purified the PRP2 protein from the overproducing strain using two purification protocols. Both procedures yielded final fractions possessing similar specific activities of PRP2 with similar yield (Table I).

The purity of individual fractions containing PRP2 was analyzed by SDS−PAGE (Figure 2). A protein with an apparent molecular weight of 100 kDa copurified with PRP2 complementing activity using protocol A (Figure 2A, lanes 4−8), while a 92 kDa protein was purified using protocol B (Figure 2B, lanes 3 and 4). Neither the 100 kDa nor the 92 kDa protein was present in fractions derived from the mutant strain (Figure 2B, lanes 7 and 8), indicating that both protein species are related to the *PRP2* gene product. In order to demonstrate that the 100 kDa and the 92 kDa proteins were indeed PRP2, proteins were transferred after SDS−PAGE onto a membrane and probed with anti-PRP2 antibodies. The antibody was affinity purified from anti-sera raised against a synthetic peptide containing the last 13 amino acid residues of PRP2. As shown in Figure 3, both proteins were recognized by the antibody preparation (lanes 1−5), while none was detected in fractions prepared from the mutant (lanes 6−8). From these observations, we conclude that the 100 kDa and 92 kDa proteins in fractions containing PRP2 complementing activity are indeed the gene product

Table I. Purification of the PRP2 Protein

Fraction	Volume (ml)	Protein[a] (mg)	Units/μl[c]	Total activity	Yield (%)	Specific activity	Purification (fold)
Prototol A							
A1. Crude Extract, dialyzed	70	1890	2000	140 000	100	74	1
A2. Phophocellulose	28	7.3	750	21 000	15	2900	39
A3. Ammonium sulfate	0.8	0.28	10 000	8000	5.7	29 000	390
A4. Gel filtration	4.5	0.045[b]	500	2300	1.6	50 000	680
Protocol B							
B1. Crude Extract, undialyzed	80	760	100	80 000	100	105	1
B2. Ammonium sulfate	2.5	32.5	10 000	25 000	31	770	7.3
B3. Gel filtration	21	2.73	750	16 000	20	5800	55
B4. Phosphocellulose	3.5	0.14[b]	750	2600	3.2	19 000	180
B5. Poly(U)−agarose	2	0.03[b]	750	1500	1.9	50 000	480

[a]The amount of protein in a solution was determined by using the Bradford color assay and BSA as standards.
[b]Estimated from the intensity of the protein bands in an SDS−PAGE gel after coomassie blue or silver staining.
[c]One unit is the minimal amount of PRP2 required to complement the splicing defect of heat-inactivated *prp2* mutant extracts under the standard splicing conditions.

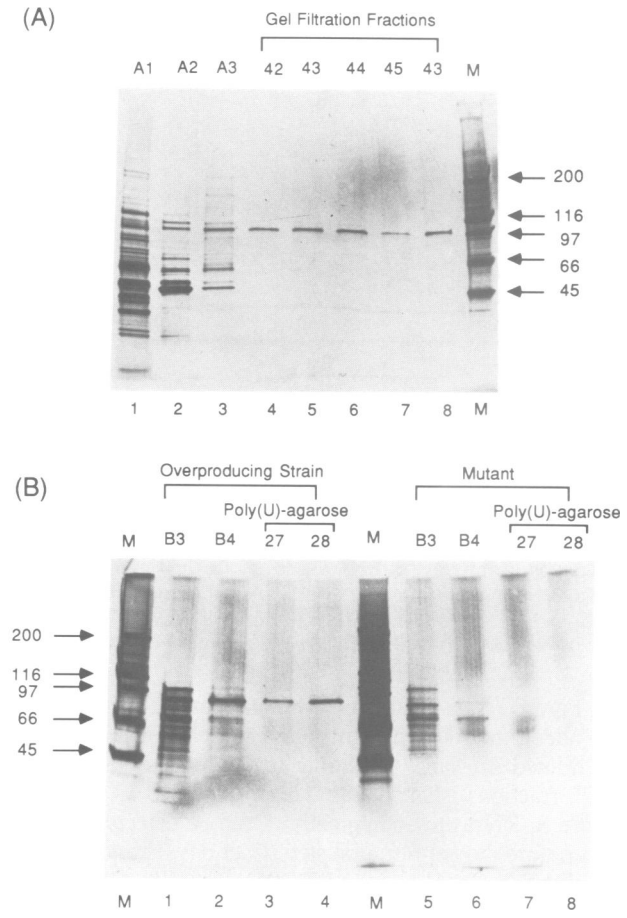

Fig. 2. SDS−PAGE analysis of fractions isolated during the purification of PRP2 by using protocol A (**A**) or B (**B**) (see Table I). Protein samples were analyzed on SDS−PAGE gels and visualized after silver staining. Protein standards were loaded in lanes marked with M and their apparent molecular weight in kDa are shown. (A) 0.1 μl of fraction A1 (lane 1); 2 μl of fraction A2 (lane 2); 1 μl of fraction A3 (lane 3); 10 μl each of gel filtration fractions 42 (lane 4), 43 (lane 5), 44 (lane 6), 45 (lane 7), and 43 (lane 8). (B) Samples (10 μl each) in lanes 1−4 were derived from the overproducing strain while lanes 5−8 were derived from the mutant strain; fraction B3 (lanes 1 and 5); fraction B4 (lanes 2 and 6); poly(U)−agarose fractions 27 (lanes 3 and 7) and 28 (lanes 4 and 8). Lane numbers are marked at the bottom of the figure.

of *PRP2*. The difference in molecular weight of the two forms of the PRP2 protein is not clear. However, both proteins appear to be equally active in supporting splicing in heat-inactivated extracts prepared from *prp2* mutants.

PRP2 is an RNA-dependent nucleoside triphosphatase (NTPase)

The predicted PRP2 protein sequence bears homology to other known RNA-dependent ATPases (Chen and Lin, 1990). To assay for ATPase activity, [α-^{32}P]ATP was incubated with PRP2 fractions in the presence or absence of poly(A) or poly(U). As shown in Figure 4, ATP was hydrolyzed to ADP by fraction B5 [the poly(U)−agarose fraction, see Table I] derived from the overproducing strain only in the presence of RNA (lanes 5 and 6). The RNA-dependent ATPase activity in fraction B5 is due to the presence of PRP2 or a tightly-associated protein, since the activity is absent in the corresponding fraction isolated from the mutant (Figure 4, lanes 8 and 9). To demonstrate further that the RNA-dependent ATPase is an intrinsic activity of

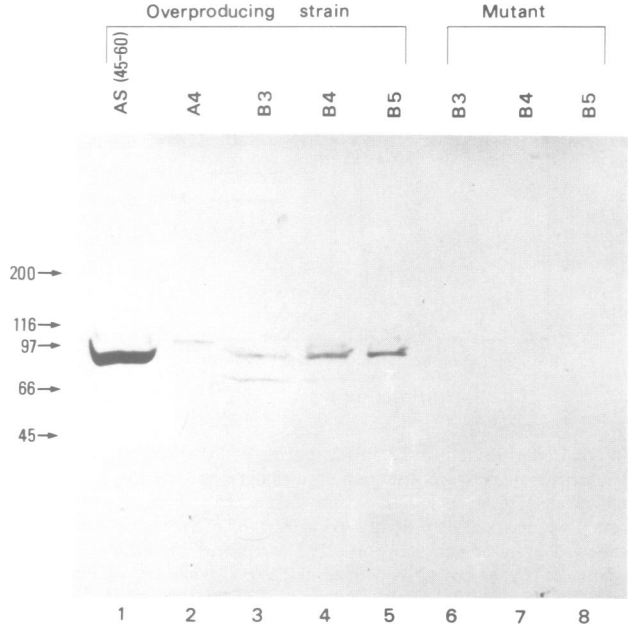

Fig. 3. Immunoblot (Western) analysis of fractions isolated from the strain overproducing PRP2 protein (lanes 1−5) and the mutant strain (lanes 6−8). Protein samples (10 μl of each fraction) were separated by SDS−PAGE, electrotransferred onto a membrane and probed with affinity purified anti-PRP2 antibodies. The ammonium sulfate (45−60) fraction from protocol A (lane 1); fraction A4 (lane 2); fraction B3 (lanes 3 and 6); fraction B4 (lanes 4 and 7); and fraction B5 (lanes 5 and 8). The molecular weight (in kDa) of the protein standards are shown on the left. Lane numbers are marked at the bottom of the figure.

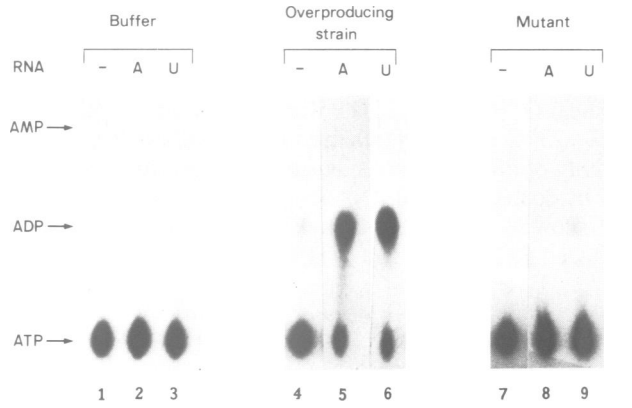

Fig. 4. TLC analysis of ATP hydrolysis catalyzed by PRP2 in the presence or the absence of RNA. The positions of AMP, ADP and ATP on the TLC plates are marked with arrows. The protein samples (1 μl used in 10 μl reaction) used in the assay were: no protein added (lanes 1−3), fraction B5 from the overproducing strain (lanes 4−6) and fraction B5 from the mutant strain (lanes 7−9). RNA used were: no RNA added (marked with −; lanes 1, 4 and 7), 2.5 μg/μl of poly(A) (marked with A; lanes 2, 5 and 8) and 0.5 μg/μl of poly(U) (marked with U; lanes 3, 6 and 9). Lane numbers are marked at the bottom of the figure.

PRP2, we assayed individual fractions derived by gel filtration chromatography of PRP2 fraction A3 (the ammonium sulfate fraction, see Table I). Both PRP2 complementing and RNA-dependent ATPase activities coeluted in fractions 42, 43 and 44 (Figure 5). Furthermore, when analyzed by SDS−PAGE, these fractions contained near homogeneous 100 kDa PRP2 protein (Figure 2A,

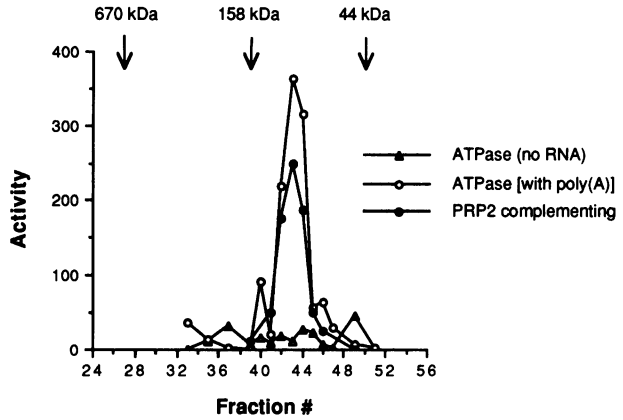

Fig. 5. Copurification of the PRP2 splicing and the RNA-dependent ATPase activities by gel filtration chromatography (fraction A3). Individual fractions were assayed for their activity to complement *prp2* mutant extracts and their ability to catalyze ATP hydrolysis with or without 5 μg/μl of poly(A). The PRP2 complementing activities are expressed in units per μl as defined in Table I, while the ATPase activities are expressed as the fraction of ATP hydrolyzed in the reaction multiplied by 1000. The elution profile of the protein standards are marked with arrows.

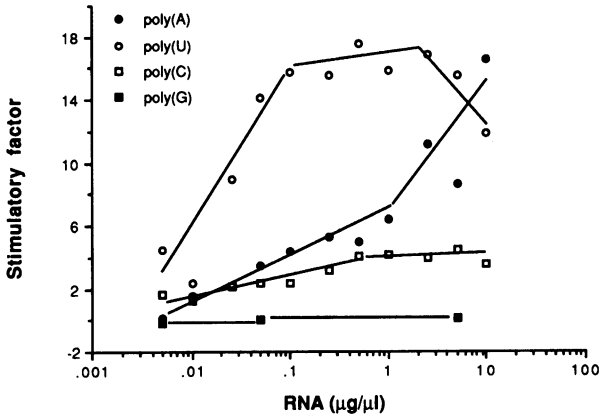

Fig. 6. The effect of our RNA homopolymers on the ATP hydrolysis catalyzed by PRP2 protein. The RNA-dependent ATPase activity of PRP2 in fraction B5 were assayed in the presence of 100 μM ATP and one of the four RNA homopolymers at various concentrations (in μg per μl of reaction). The stimulatory factor was the amount of ADP produced in the presence of RNA divided by the amount of ADP produced (4.2 pmol/μl reaction) when RNA was omitted. In the presence of 0.1–5 μg of poly(U), ~8–12 pmol of ATP were hydrolyzed/s/μg of PRP2 protein at 30°C.

lanes 4–6). Since the predicted molecular weight of PRP2 is 100 kDa (Chen and Lin, 1990), these results indicate that the active form of the PRP2 protein purifies as a monomer. Therefore, we conclude that the RNA-dependent ATPase activity is an intrinsic property of PRP2.

We further investigated the effect of four RNA homopolymers on the ATPase activity of PRP2 (Figure 6). Poly(U) was most effective while poly(A) was effective only at high concentrations. Poly(C) was a weak stimulator of the ATPase activity and poly(G) was inactive at all concentrations tested (Figure 6). In additional experiments, we found that PRP2 hydrolyzed dATP as efficiently as ATP, whereas GTP, CTP and UTP were hydrolyzed by PRP2 with 70% to 30% efficiency (relative to ATP) and that the ATPase activity of PRP2 was not simulated by single-stranded DNA nor by double-stranded RNA such as poly(A)–poly(U) (data not shown). These results indicate that PRP2 is a single-stranded RNA-dependent NTPase.

The purified PRP2 protein does not unwind double-stranded RNAs

Since three proteins with which PRP2 shares sequence homology are ATP-dependent RNA helicases, we decided to analyze PRP2 for RNA unwinding activity using *in vitro* synthesized RNAs. A ^{3}H-labeled RNA strand of 98 nucleotides and a ^{32}P-labeled RNA strand of 38 nucleotides were prepared. The two RNAs were hybridized to form a partially double-stranded RNA with 29 bp flanked by single-stranded regions at the ends (Figure 7A). This double-stranded RNA substrate has been used to detect RNA helicases with either a 5′–3′ or 3′–5′ polarity (Claude *et al.*, 1991). As shown in Figure 7B, the duplex RNA can be unwound to release the ^{32}P-labeled strand by heat (lane 2), by RNA helicases isolated from HeLa extracts (lane 3; Claude *et al.*, 1991), by yeast splicing extracts (lane 4) or by fractions A1 (lane 5), A2 (lane 6) and A3 (lane 8) that contain PRP2 complementing activity. However, no RNA unwinding activity was detected with the most purified fraction of PRP2 (the gel filtration fraction, A4; Figure 7B, lane 10), even though more splicing activity was added than was present in the samples of A2 (lane 6) and diluted A3 (lane 9). Similar results were obtained with PRP2 fractions isolated following protocol B as shown in Figure 8; RNA unwinding activity can be detected in fractions before the gel filtration step (fractions B1 and B2), but not after this step (fractions B3–B5). These results suggest that a factor responsible for the RNA helicase activity, either by itself or in combination with PRP2, was separated from PRP2 during gel filtration chromatography. As yet we have not identified this factor. However, we found that fractions isolated from *prp2* mutant having little PRP2 splicing activity displayed a similar profile of RNA helicase activity (Figure 8, lanes 6–8) as those fractions isolated from the strain overproducing PRP2 protein (lanes 1–3). It appears that the RNA unwinding activity detected in cruder fractions (fractions B1 and B2) either from the overproducing strain or the mutant strain is largely unrelated to PRP2 since fractions lacking PRP2 splicing activity are equally active. The lack of RNA unwinding activity in purified PRP2 fractions is not due to the presence of inhibitors, since RNA helicases isolated from HeLa cells were still active in unwinding duplex RNA in the presence of PRP2 fractions (data not shown).

The simplest interpretation of these results is that PRP2 cannot unwind the duplex RNA examined under the experimental conditions used. However, our results are also consistent with the scenario that PRP2 requires additional factor(s) to unwind duplex RNA. Thus, overproducing the PRP2 protein would not drastically increase the RNA helicase activity in such extracts.

Discussion

We have shown here that the *PRP2* gene product is an RNA-dependent NTPase since the activity that complements heat-inactivated *prp2* mutant extracts and the activity that hydrolyzes ATP in the presence of RNA copurified with the protein recognized by the anti-PRP2 antibodies. Further-

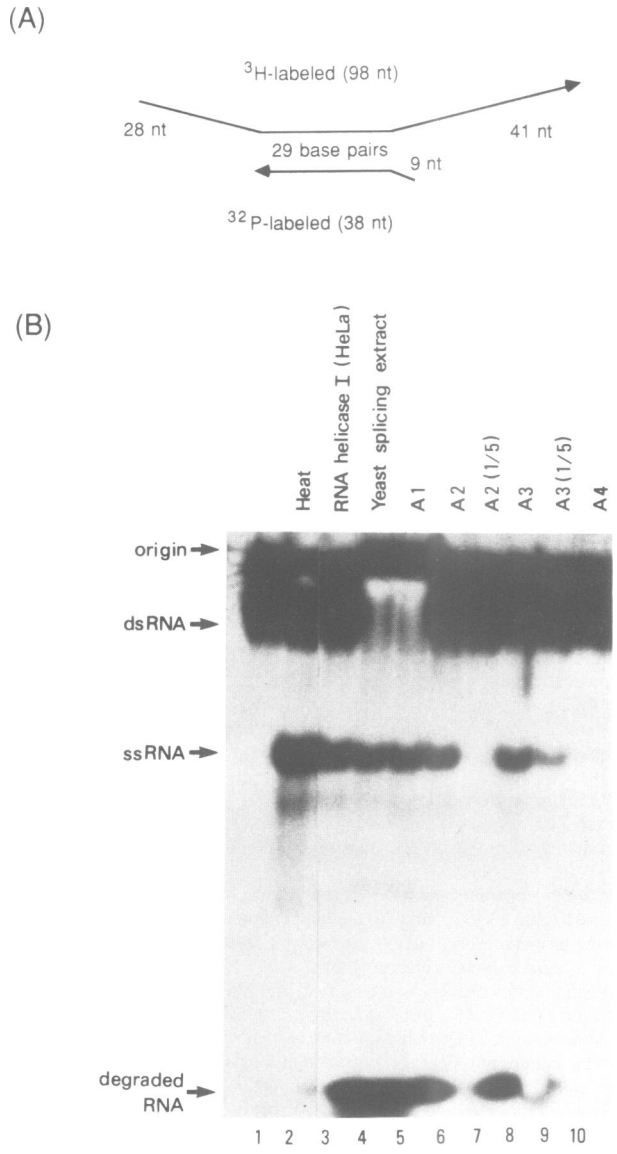

Fig. 7. Analysis of RNA unwinding activity in fractions isolated from the PRP2 overproducing strain using protocol A. (**A**) A diagram depicts the partially double-stranded RNA substrate used in the helicase assay. The polarity of the RNA is indicated by the arrow with the arrow head as the 3′ end. nt, nucleotides. (**B**) The RNA helicase reaction mixture was separated on a nondenaturing gel and ^{32}P-labeled RNAs were visualized by autoradiography. The origin of gel electrophoresis, the double-stranded (ds) RNA substrate, the single-stranded (ss) RNA product, and some degraded RNAs are marked with arrows. Protein samples used in the reaction were: no protein added (lane 1), no protein added and the reaction mixture was heated to 95°C for 1 min before loading (lane 2), 5 units of RNA helicase I [fraction SG2 from HeLa cells (Claude *et al.*, 1991); lane 3], 1 μl of a wild-type yeast splicing extract (lane 4), 1 μl of fraction A1 (2000 units of PRP2; lane 5); 1 μl of fraction A2 (750 unit of PRP2; lane 6), 1 μl of fraction A2 at 1:5 dilution (150 units of PRP2; lane 7), 1 μl of fraction A3 (10000 units of PRP2; lane 8), 1 μl of fraction A3 at 1:5 dilution (2000 units of PRP2; lane 9) and 5 μl of fraction A4 (2500 units of PRP2; lane 10). Lane numbers are marked at the bottom of the figure.

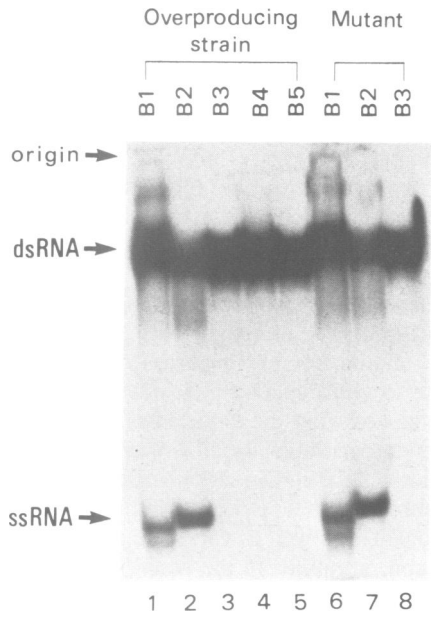

Fig. 8. Analysis of RNA unwinding activity in fractions isolated from the PRP2 overproducing strain (lanes 1–5) and from the mutant strain (lanes 6–8) using protocol B. The assay conditions were identical to that described in Figure 7 except that CTP was used instead of ATP; more RNA unwinding activities in all fractions were observed with CTP. The origin, dsRNA, and ssRNA marked on the left are the same as in Figure 7. Protein samples (1 μl each) used in the reaction were: fraction B1 (lanes 1 and 6), fraction B2 (lanes 2 and 7), fraction B3 (lanes 3 and 8), fraction B4 (lane 4) and fraction B5 (lane 5). Lane numbers are marked at the bottom of the figure.

more, the RNA-dependent ATPase activity was not detected in the corresponding fraction isolated from a *prp2* mutant strain. We have also analyzed PRP2 for RNA unwinding activity using *in vitro* synthesized partially double-stranded RNA substrates. It appears that purified PRP2 could not catalyze the unwinding of double-stranded RNA substrates under the experimental conditions, suggesting that it lacks RNA helicase activity or requires additional factors for this activity.

The molecular weight of PRP2 predicted from sequence data is 99 824 Da (Chen and Lin, 1990). However, PRP2 with apparent molecular weights of 100 kDa and 92 kDa were purified using two different protocols (Table I; Figure 2). We have not yet determined the reasons for this difference. It is likely that the 92 kDa protein is a proteolytic product of the 100 kDa protein. Consistent with this idea, small amounts of a 100 kDa protein and a 70 kDa protein were detected by the anti-PRP2 antibody in fractions containing the 92 kDa protein (Figure 3, lanes 3 and 4). The missing fragments in the 92 kDa and 70 kDa proteins are most likely from the N-terminus since both proteins were recognized by the antibodies raised against the very C-terminal portion of PRP2. A 100 kDa protein and other minor species have also been detected in extracts isolated from PRP2 overproducing yeast strains by anti-PRP2 antibodies (Last and Woolford, 1986; Lee *et al.*, 1986). However, it is also possible that the 100 kDa and the 92 kDa proteins have different modifications which affect their mobilities during SDS–PAGE. In any event, both proteins appear equally active in splicing and ATP hydrolysis.

PRP2 can hydrolyze all four nucleoside triphosphates in the presence of single-stranded RNA. This property is in agreement with the observation that any of the four NTPs can support the PRP2-dependent splicing reaction occurred in a gradient purified prp2Δ spliceosome (our unpublished results). [The prp2Δ spliceosome contains unspliced pre-

mRNA and can be isolated by glycerol gradient sedimentation of a splicing reaction mixture containing heat-inactivated *prp2* mutant extracts (Lin *et al.*, 1987).] In contrast, in unfractionated extracts prepared from wild-type yeast cells, splicing reaction is inefficient when UTP, CTP or GTP (rather than ATP) is used (Lin *et al.*, 1985). It appears that other NTPs can substitute efficiently for ATP in the PRP2-dependent reaction (the first cleavage−ligation) but not in other ATP-dependent steps in splicing such as spliceosome assembly.

We failed to detect RNA unwinding activity with purified PRP2 preparations although PRP2 contains all seven sequence motifs found in three ATP-dependent RNA helicases (see Introduction). However, it is possible that PRP2 cannot unwind the duplex RNA substrate under the experimental conditions used. Therefore, we have systematically changed the conditions of helicase assay. The changes include substituting ATP with CTP, GTP, UTP or one of the four dNTPs, as well as varying magnesium, manganese and salt concentrations in the reaction. We have included single-stranded DNA binding proteins isolated from *Escherichia coli* or HeLa cells to trap any single-stranded RNA product and we have also used two different double-stranded RNA substrates (Claude *et al.*, 1991) in the assay. None of these modifications supported any significant RNA unwinding activity with purified PRP2 preparations (data not shown). Since the duplex RNA substrates and reaction conditions we used are similar to those employed by others to demonstrate RNA helicase activity in eIF4A (Rozen *et al.*, 1990), human p68 (Hirling *et al.*, 1989) or viral CI protein (Lain *et al.*, 1990), these results suggest that if PRP2 is indeed an RNA helicase, it may have stringent substrate requirements or specific reaction conditions.

It is also possible that the lack of RNA unwinding activity in purified PRP2 is due to the separation of a factor during gel filtration chromatography. Interestingly, the RNA helicase activity of the eIF-4A is active only in the presence of another initiation factor eIF-4B (Rozen *et al.*, 1990). In addition to PRP2, a factor termed bn has been shown to be required for splicing in the prp2Δ spliceosome purified from heat-inactivated *prp2* mutant extracts (Lin *et al.*, 1987). However, we cannot detect RNA helicase activity in a mixture combining PRP2 and a partially purified fraction containing bn (data not shown). We are currently carrying out reconstitution experiments to identify the putative factor(s).

PRP2 binds to the spliceosome prior to the first cleavage−ligation reaction (King and Beggs, 1990). We found that this binding reaction was ATP-independent but the presence of ATP is required to carry out the first cleavage−ligation reaction in the spliceosome (Lin *et al.*, 1987; our unpublished results). Similarly, the second cleavage−ligation in the spliceosome requires binding of PRP16 and the presence of ATP (Schwer and Guthrie, 1991). Since both PRP proteins are RNA-dependent ATPases, it is possible that by binding to the spliceosome at specific sites these extrinsic factors hydrolyze ATP upon interacting with an unidentified RNA molecule. The energy derived from ATP hydrolysis may trigger the spliceosome to undergo conformational changes that may not involve unwinding of RNA. Alternatively, energy may be necessary to separate base pairs between certain RNAs in the spliceosome for splicing to proceed. Hence, the RNA helicase activity of PRP2 may only be activated when the protein is bound to the spliceosome. In either hypothesis, PRP2 may function to ensure that splicing occurs only when correct intron sequences or structures are recognized within the spliceosome. A similar proof reading role in splicing has been proposed to PRP16 (Burgess *et al.*, 1990). Studies of extrinsic splicing factors like PRP2 and PRP16 in yeast may eventually lead to a better understanding of alternative or regulated pre-mRNA splicing in higher organisms (Green, 1991; Maniatis, 1991).

Materials and methods

Materials

The following reagents and materials were commerically obtained: zymolyase 100T (Seikagoku Kogyo Co.); Dounce glass homogenizers (Kontes Glass Co.); proteinase K and the four protease inhibitors (PMSF, benzamidine, leupeptine and pepstatin A) are from Boehringer Mannheim; columns for liquid chromatography, protein standards for SDS−PAGE and gel filtration chromatography, 4−15% polyacrylamide gradient SDS−PAGE gels for protein analysis, Bradford protein assay reagents, phosphocellulose (Cellex-P), Affi-Gel 15, goat anti-rabbit (H+L) alkaline phosphatase conjugate and the BCIP/NBT substrate of alkaline phosphatase were all from Bio-Rad; Ultrogel AcA 34 (IBF Biotechniche); heparin (US Biochemicals); PVDF (phenyl methanesulfonyl fluoride) Immobilon-P membrane (Millipore); polyethyleneimine−cellulose TLC (thin layer chromatography) plates (J.T.Baker); radioactive isotopes (NEN, DuPont); pre-stained protein molecular weight markers, complete and incomplete Freund's adjuvants, poly(U)−agarose and RNA homopolymers were from Sigma; single-stranded DNA was prepared from salmon testes DNA (Sigma) according to Schiestl and Gietz (1989).

Yeast growth media

Rich YPD, synthetic minimal SD media and amino acid supplements were prepared according to Sherman *et al.* (1986).

In vitro complementation assay

Yeast strain SS304, carrying the temperature-sensitive *prp2-1* allele, was used to prepare *prp2* mutant extracts for assaying PRP2 splicing activity by *in vitro* complementation (Lustig *et al.*, 1986). The procedure for the preparation of extracts was modified from those of Lin *et al.* (1985) and Lustig *et al.* (1986). Cells were grown to late logarithmic phase at room temperature in YPD medium. Before harvesting the cells, PMSF was added to the culture to a final concentration of 1 mM and the culture was further incubated for 30 min; following centrifugation, the cells were washed with 50 mM Tris−HCl and 0.2 mM EDTA, pH 8.0. Spheroplast formation and KCl extraction were carried out as described by Lin *et al.* (1987). The splicing activity of mutant *prp2* extracts was inactivated by incubation at 37°C for 30 min in the presence of 2 mM $MgCl_2$ and 20 mM potassium phosphate (pH 7.3) (Lin *et al.*, 1987). The ^{32}P-labeled substrate pre-mRNA used in the *in vitro* splicing reaction was prepared from SP6-actin (Lin *et al.*, 1987). The assay of PRP2 splicing activity was carried out in reaction mixture (5 μl) containing 2.5 mM $MgCl_2$, 60 mM potassium phosphate (pH 7.3), 2.4% polyethylene glycol 8000, 2 mM ATP, 5000 Cerenkov c.p.m. of ^{32}P-labeled pre-mRNA molecules, 2 μl of a heat-inactivated SS304 extract and 0.5 μl of complementing fractions (or their dilutions) in buffer D [20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)−KOH (pH 7.9), 0.2 mM EDTA, 0.5 mM DTT (dithiothreitol), 50 mM KCl, 20% (vol/vol) glycerol]. After 30 min at 23°C, reactions were stopped by the addition of 1 μl of a stop mixture (2.5% SDS, 2.5 mg/ml heparin and 2.5 mg/ml proteinase K) followed by incubation at 65°C for 10 min. Gel loading dye (6 μl of 95% formamide, 2 mM EDTA, 0.02% bromophenol blue and 0.02% xylene cyanol) was added and the mixture was loaded onto a denaturing 7.5% polyacrylamide (29:1, acrylamide:bisacrylamide) gel. In some cases RNA was isolated from the splicing reaction by the addition of 200 μl of stop buffer [50 mM sodium acetate (pH 5.3), 1 mM EDTA and 0.1% SDS] followed by phenol−chloroform extraction and ethanol precipitation (Lin *et al.*, 1985).

Purification of the PRP2 protein

The PRP2 protein was purified from a *prp2* mutant strain containing the wild-type *PRP2* gene on a high copy number plasmid (RL92/pJDB207-RN2-1) (Last *et al.*, 1986). Cells were initially grown overnight at 30°C in SD medium containing 20 mg/l uracil. Leucine was omitted from the medium to select cells retaining the plasmid. A 50 ml overnight culture was then used to inoculate 1 l of SD medium containing uracil. After 24 h at 30°C, the entire 1 l culture was used to inoculate 12 l

of YPD media and incubation continued at 30°C until the A_{600} of the culture reached 2–4. Cells were harvested by centrifugation and cell extracts were prepared and stored in aliquots without dialysis at −80°C until needed (Lin *et al.*, 1985). Control extracts were prepared from the strain RL92/pJDB207 using the same conditions except that the culture was grown at room temperature. Before purification, frozen extracts were quickly thawed in a 30°C waterbath with gentle shaking and were immediately chilled on ice. All purification steps were carried out at 4°C following either of the two protocols described below.

Protocol A The crude extract was dialyzed against buffer D and a 70 ml portion (Fraction A1) was loaded onto a 70 ml phosphocellulose column equilibrated with buffer A [20 mM HEPES–KOH, (pH 7.4), 0.5 mM DTT, 10% glycerol, 0.5 mM EDTA, 0.5 mM PMSF, 1 mM benzamidine, 1 μM leupeptine and 1 μM pepstatin A] containing 50 mM KCl. After washing the column with 140 ml of buffer A containing 50 mM KCl, PRP2 was eluted with 900 ml of a linear gradient (50–500 mM) of KCl in buffer A. Fractions containing the majority of PRP2 were pooled (Fraction A2) and subjected to ammonium sulfate precipitation. Solid ammonium sulfate was added to 28 ml of Fraction A2 to 25% saturation. After stirring the mixture for 30 min, it was centrifuged at 17 000 r.p.m. for 30 min in a Sorvall SS34 rotor. The pellet was resuspended in a minimal volume of buffer A containing 50 mM KCl and is referred to as the AS(0–25) fraction. The supernatant was fractionated further by adding ammonium sulfate to 35% saturation. After centrifugation, the pellet was resuspended in buffer A [AS(25–35) fraction]. Fractions AS(35–45) and AS(45–60) were obtained as described; these two fractions contained most of the PRP2. The AS(35–40) fraction (Fraction A3), which had the highest specific activity of PRP2, was further fractionated by gel filtration chromatography. Fraction A3 (0.8 ml) was loaded onto a column (1.6 cm × 100 cm) containing Ultrogel AcA34 (linear fractionation range between 20 kDa and 350 kDa) in buffer AG [20 mM HEPES (pH 7.4), 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 0.5 mM PMSF, 10% glycerol and 300 mM KCl] and 2.25 ml fractions were collected at a flow rate of 4.5 ml/hr. The PRP2 protein behaved as a 100 kDa monomer under these conditions. Two fractions (#43 and #44) containing most of the PRP2 activity were pooled as fraction A4.

Protocol B The undialyzed extract (Fraction B1) was subjected to ammonium sulfate fractionation using a back extraction procedure. Solid ammonium sulfate was added to 80 ml of extract to 60% saturation. After centrifugation, the pellet was washed in buffer BA [20 mM HEPES (pH 7.4), 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.01% NP-40 and 10% glycerol] containing 50 mM KCl and 60% ammonium sulfate and then dissolved in 35 ml of buffer BA containing 50 mM KCl and 45% ammonium sulfate. The insoluble material recovered by centrifugation was re-extracted with 15 ml of the same 45% ammonium sulfate buffer and recentrifuged. Protein in the two supernatants was reprecipitated with ammonium sulfate and dissolved in a minimum volume of buffer BA containing 50 mM KCl to give the AS(60–45) fraction. Similarly, the material insoluble in 45% ammonium sulfate was sequentially back-extracted to obtain fractions AS(45–30), AS(30–20) and AS(20–0). The AS(45–30) fraction (B2) was fractionated further by gel filtration chromatography. A 2.5 ml portion of B2 was loaded on an Ultrogel AcA34 column (2.5 cm × 100 cm) in buffer BG (buffer BA with 5 mM DTT) containing 300 mM KCl and 4.1 ml fractions were collected at a flow rate of 8.2 ml/h. Five fractions containing the majority of the PRP2 activity were pooled and dialyzed against buffer BP (same as buffer BG except the pH was 7.7) containing 50 mM KCl to give fraction B3. An 18 ml portion of B3 was loaded onto a 2 ml phosphocellulose column in buffer BP containing 50 mM KCl. The column was then washed and step eluted by using 5 ml of buffer BP containing KCl (100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 500 mM and 1 M). PRP2 activity eluted at 150–200 mM KCl. Fractions containing PRP2 were pooled (fraction B4), diluted with an equal volume of buffer BP and loaded onto a 1.5 ml poly(U)–agarose column equilibrated in buffer BP plus 50 mM KCl. The column was washed in buffer BP plus 100 mM KCl and bound proteins were step eluted by using 4.5 ml of buffer BP containing KCl (150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM, 500 mM and 1 M). PRP2 activity eluted at 200–250 mM KCl. Two fractions (#27 and #28) containing most of the PRP2 activity were pooled to give fraction B5.

Protein analysis by SDS–PAGE

Protein fractions were analyzed on 4–15% polyacrylamide gradient SDS–PAGE gels following the manufacturer's (Bio-Rad) instruction. After electrophoresis, proteins were visualized with Coomassie blue or silver staining (Wray *et al.*, 1981).

Immunizations

A 14mer peptide bearing the sequence, Y-K-D-L-I-D-D-K-T-N-R-G-R-R, corresponding to the C-terminal end of PRP2, except for the first tyrosine residue (Chen and Lin, 1990) either free or coupled to KLH (Keyhole limpet hemacyanin), was synthesized and kindly provided by Dr Ambrose Jong (USC Medical School). New Zealand white rabbits were given multiple subcutaneous injections using 300 μg of the 14mer–KLH conjugate emulsified in complete Freund's adjuvant. Subsequent injections of 200 μg of the conjugate emulsified with incomplete Freund's adjuvant were done every 4 weeks. Bleeding of the rabbits was performed 1 week after injection. Blood was allowed to clot overnight at room temperature; the mixture was then centrifuged at 3200 r.p.m. for 15 min; sodium azide was added to the supernatant to 0.02% and the serum was stored at −20°C.

Antibody purification

The purification of the antibodies from sera was carried out according to Harlow and Lane (1988). The 14mer peptide (28 mg) was coupled to 67 mg of BSA (bovine serum albumin) in PBS (phosphate buffered saline, pH 7.2) containing 0.1% glutaraldehyde. After incubation for 1 h, the coupling solution was brought to 200 mM glycine in PBS and dialyzed against 50 mM MOPS (4-morpholinepropanesulfonic acid), pH 7.5. The peptide–BSA conjugate was then coupled to 4 ml of Affi-Gel 15 in 100 mM triethylamine (pH 10.5) for 4 h at 4°C with gentle agitation. The gel slurry was packed into a chromatography column and washed successively with 10 mM Tris–HCl (pH 7.5), 100 mM glycine (pH 2.5), 10 mM Tris–HCl (pH 8.8), 100 mM triethylamine (pH 11.5) and finally 10 mM Tris–HCl (pH 7.5). IgG molecules were purified from the serum by adding 2 vol of 60 mM sodium acetate (pH 4.0) and 0.7 ml of caprylic acid per 100 ml of the original serum. After centrifugation, the supernatant containing IgG antibodies was dialyzed against PBS (pH 7.5) and loaded on the peptide–BSA column. The column was washed with 500 mM NaCl, 10 mM Tris–HCl (pH 7.5) and the antibody eluted with 40 ml of 100 mM glycine (pH 2.5) which was collected in 4 ml of 1 M Tris–HCl (pH 8). The column was requilibrated in 10 mM Tris–HCl (pH 8.8) and the antibody which remained bound was eluted with 40 ml of 100 mM triethylamine (pH 11.5) which was collected in 4 ml of 1 M Tris–HCl (pH 8). The two antibody fractions were pooled and precipitated with 50% ammonium sulphate; the pellet was dissolved in PBS (0.05 volumes of the eluate) and stored in 0.02% sodium azide at −20°C.

Immunoblot (Western) analysis

After electrophoresis, proteins were transferred to membranes and probed with antibodies according to Harlow and Lane (1988). The SDS–polyacrylamide gel was equilibrated with two changes of transfer buffer [25 mM Tris–HCl (pH 8.3) and 192 mM glycine] for 15 min each of 4°C. PVDF membrane, filter paper and pads were also soaked in the transfer buffer for 30 min. A gel-PVDF membrane sandwich was formed with the gel facing the cathode side and the membrane facing the anode side. The electro-blot was carried out at 4°C using Bio-Rad mini-electrophoretic transfer cell at 30 V overnight and then at 100 V for 1 h. The PVDF membrane blot was incubated with Blotto (5% wt/vol nonfat dry milk in PBS) for 2 h at room temperature with gentle shaking. The purified antibodies were then added at a dilution of 1:250. After 3 h of further incubation, the blot was washed with PBS and then with TBS (Tris-buffered saline). The blot was incubated with goat anti-rabbit (H+L) alkaline phosphatase conjugate (1:2000 dilution) for 2 h in TBS containing 5% non-fat milk, followed by washing with TBS. The immunoblot was developed with fresh alkaline phosphatase solution [66 μl of NBT (100 mg nitro blue tetrazolium in 2 ml 70% dimethylformamide) and 33 μl of BCIP (100 mg bromochloroindolyl phosphate in 2 ml 100% dimethylformamide) per 10 ml of alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris–HCl (pH 9.5)] for 15 min. Development was stopped by washing with 20 mM EDTA in PBS.

Nucleoside triphosphatase assay

ATPase assays were carried out essentially as described by Shibata *et al.* (1983). Reaction mixtures (10 μl) contained 2 mM DTT, 5 mM $MgCl_2$, 0.5 mM EDTA, 50 mM KCl, 100 μg/ml BSA, 1 μM unlabeled ATP (unless specified), 500 nCi of [α-^{32}P]ATP, 0–10 μg/μl various RNAs as indicated and 1 μl of protein samples. Incubation was at 30°C for 30 min and the reaction was stopped by the addition of 6 μl of a mixture containing 3 mM each of ATP, ADP and AMP, and 25 mM EDTA. Samples (5 μl) were spotted onto a polyethyleneimine–cellulose TLC plate pre-washed with 1 M methanol. The plates were developed with 1 M formic acid and 0.5 M LiCl. After the solvent front had reached the 10 cm mark, the plates were washed in 1 M methanol. The positions of ATP, ADP and AMP were located by short wave UV illumination. Dried TLC plates were exposed to X-ray film and radioactive spots were excised and counted in a Beckman LS5000TD

scintillation counter. All RNAs used were purified from commercial preparations by phenol extraction, ethanol precipitation and then dissolved in water.

RNA helicase assay

RNA unwinding activity in protein samples was analyzed according to Claude *et al.* (1991). Partially double-stranded RNA substrates were prepared by forming heteroduplexes between run-off transcripts derived from pSP65, pGEM1 and pGEM3 (Claude *et al.*, 1991). The unwinding reaction mixture (20 μl) contained 40 mM Tris−HCl (pH 7.4), 1 mM $MgCl_2$, 3 mM ATP, 2 mM DTT, 150 mM NaCl, 50 μg/ml BSA, 2−5 units of RNasin, 50 fmol of RNA substrate and protein samples. The reaction was carried out at 30°C for 20 min, stopped by adding 4 μl of loading buffer and incubated at room temperature for 15 min. One half of each reaction (12 μl) was analyzed by electrophoresis on a 17% polyacrylamide (acrylamide:bis-acrylamide = 29:1). 1 × TBE, non-denaturing gel, followed by autoradiography (Claude *et al.*, 1991).

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