PRP28, a 'DEAD-box' protein, is required for the first step of mRNA splicing *in vitro*

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ABSTRACT

We previously reported the isolation of *PRP28*, a gene in *Saccharomyces cerevisiae* whose activity is required for the first step of nuclear mRNA splicing *in vivo* (1). Sequence analysis revealed that *PRP28* is included in the 'DEAD-box' gene family, members of which are thought to function as ATP-dependent RNA helicases (2). Genetic interactions led us to suggest that *PRP28* is functionally associated with the U4/U5/U6 snRNP. We have now purified the PRP28 protein from *S. cerevisiae* and demonstrated that it is required for the first step of splicing *in vitro*. Interestingly, PRP28 is not a stably associated snRNP protein. Strand displacement assays indicate that PRP28 does not exhibit RNA helicase activity, suggesting that an additional factor or factors may be required for its activation.

INTRODUCTION

Pre-messenger RNA (pre-mRNA) splicing requires many factors, including five small nuclear ribonucleoproteins (snRNPs), each composed of a specific complement of proteins and a particular snRNA (3, 4), and numerous non-snRNP proteins (5). Over 30 pre-mRNA processing (*PRP*) genes essential for splicing have been identified genetically in *S. cerevisiae*. The factors associate with the substrate pre-mRNA and with one another in a highly ordered fashion to facilitate the removal of an intron in the large complex known as the spliceosome (6, 7, 8, 9, 10, 11). In contrast, autocatalytic group II RNAs, which splice by the same chemical mechanism, are able to fold via extensive structure that is encoded in the substrate RNA, without the aid of accessory factors *in vitro* (12, 13). Therefore, it has been suggested that the machinery required for nuclear pre-mRNA splicing supplies information for folding the unstructured RNA into a splicing-competent configuration (14, 15, 16). Elucidating the details of the interactions between the *trans*-acting factors and the pre-mRNA is therefore crucial to understanding the mechanism of nuclear pre-mRNA splicing.

Both nuclear pre-mRNA and group II introns are removed in two successive steps which covalently modify the substrate RNA

(16, 17, 18, 19). The first step yields free 5' exon and the intron-3' exon in branched form (lariat intermediate). The second step yields mature mRNA and liberates the free intron, still in lariat form. The chemical reaction itself does not require energy input since ATP is dispensable for splicing of group II introns *in vitro* (12, 13). In contrast, ATP is required for pre-mRNA splicing at many steps during spliceosome assembly (6, 9, 20, 21, 22, 23, 24). A number of splicing proteins have been shown to contain putative ATP-binding domains (1, 25, 26, 27, 28, 29). These proteins fall into two related classes. The DEAD-box proteins contain all of the signature motifs of the founding member (30) eukaryotic translation initiation factor (eIF4A) (1, 27). The DEAH-box proteins contain motifs which are related to but distinct from the DEAD-box motifs (e.g. DEAH vs. DEAD, GRAGR vs. HRIGR) (25, 26, 28); in addition, they share a unique 3' domain not found in DEAD-box proteins. The putative ATP-binding domain in both DEAH and DEAD proteins contains the motif G/AX₄GKT; this sequence is known to bind the β/γ phosphate of the NTP molecule in crystal structures of several ATP- and GTP-binding proteins (31, 32, 33). To understand the role of ATP in spliceosome assembly, it will be essential to determine whether the DEAD/H proteins use ATP to unwind secondary structure (like eIF4A) or perhaps promote different kinds of conformational changes.

Examination of spliceosome assembly *in vitro* has demonstrated that early steps include association of U1 with the pre-mRNA and formation of a commitment complex, followed by addition of U2 (34, 35, 36). Next, U4/U6 (a single particle) and U5 join the spliceosome, apparently together (7, 9). Following the association of all five snRNPs, U4 is destabilized from the spliceosome (7, 9, 10, 37, 38). Since U4 and U6 are normally held together by 24 intermolecular base pairs (39, 40, 41, 42) this step is likely to require the action of an energy-dependent RNA helicase. Finally, an ATPase (PRP2) associates with the spliceosome and facilitates the first covalent modification of the pre-mRNA (23, 43, 44).

We previously reported the isolation of *PRP28*, a gene whose activity is required for the first step of splicing *in vivo* (1). Sequence analysis revealed that PRP28 is a member of the DEAD family of proteins. Genetic interactions suggesting that PRP28

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is closely associated with the U4/U5/U6 snRNP led us to propose a model in which PRP28 unwinds the U4/U6 helix in the context of the U4/U5/U6 snRNP in the spliceosome. We have now identified the PRP28 protein, purified it from *S. cerevisiae*, and demonstrated that it is required for the first step of splicing *in vitro*. PRP28 is not a stably associated snRNP protein. As with other members of the DEAD-box gene family, highly purified preparations of PRP28 display RNA-dependent ATPase activity. Interestingly, PRP28 does not appear to exhibit RNA helicase activity, suggesting that an additional factor or factors are required for its activation.

MATERIALS AND METHODS

Plasmids and strains

pHD20. 2.4 kb *EcoRI* fragment containing *PRP28* cloned into *EcoRI* site of pSE 360 (*PRP28*, *URA3*, *CEN*) (1).

pHD25. 2.4 kb *EcoRI* fragment (as in pHD20) containing *PRP28* cloned into *EcoRI* site of pSE 362 (*PRP28*, *HIS3*, *CEN*).

pHD30. 2.3 kb fragment containing *PRP28* cloned into *BamHI* site of pGAC24 (69) (*PRP28*, *LEU2*, 2 μ). This fragment was obtained by using *ExoII* to generate a deletion of 105 nucleotides at the 5' end of the 2.4 kb fragment containing *PRP28* (above). This was carried out on pBJ3-1, a plasmid identical to pBJ3-2 (1) except that the orientation of *PRP28* is reversed. Following generation of the 5' noncoding-deleted plasmid (pBJ17), the 2.3 kb *BamHI* fragment was cloned into pGAC24.

Production of antibodies

Antibodies were prepared using a fusion protein containing residues 6–240 of the PRP28 protein fused to *E. coli* TrpE. This was constructed by cloning the 700 nte. *ClaI*–*BstEII* fragment of pHD20, filled in with Klenow (USB), into the filled-in *BamHI* restriction site of pATH3 (70), which expresses TrpE. The hybrid protein was induced with indoleacrylic acid, purified as previously described (70), and used to immunize rabbits (BabCO, Inc., Berkeley, Ca.). The first immunization was with 500 μ g of protein and three subsequent immunizations were with 250 μ g each. Protein concentrations were determined by comparing different amounts of sample with known amounts of BSA (SIGMA) on a Coomassie-stained protein gel. Polyclonal antiserum was purified using Protein A Sepharose (Pharmacia).

Western blot analysis

Proteins from splicing extracts were separated by SDS-PAGE and transferred to nitrocellulose. The filter was incubated with α -PRP28 (1:500) or pre-immune serum (1:500), followed by goat α -rabbit secondary antibodies conjugated to alkaline phosphatase (1:3000) (BIORAD). The blot was developed in 100 mM Tris–Cl, pH 9.5, 100 mM NaCl, 5 mM Mg Cl₂, 3 mg/ml NBT, and 3 mg/ml BCIP (GIBCO BRL). Yeast extracts were prepared from yEJS103 (*MATa1*, *his3*, *ura3-52*, *leu2*, *lys2*, *trp1*, *prp28::TRP1*) transformed with either a plasmid bearing a single copy of *PRP28* (pHD25) or an overexpression 2 μ plasmid in which *PRP28* is under the control of the GPD promoter (pHD30). 250 μ g of protein was loaded in each lane. Protein concentrations were determined according to Bradford (71), using BSA (SIGMA) to determine a standard curve.

Immunodepletion, *in vitro* complementation, and determination of specific activities

To immunodeplete extracts, 70 μ l of wild type yeast splicing extract was incubated with 15 μ l of Protein A-purified α -PRP28 (17 mg/ml) on ice for one hour. 190 μ l of Protein A Sepharose (Pharmacia) (0.1g/ml) was washed with PBS and briefly centrifuged. The extract/antibody mix was then added to the beads and incubated for one hour at 4°C on a nutator. The beads were removed by centrifugation and 4.4 μ l of the supernatant assayed for activity in a standard splicing reaction at 25°C (10 μ l) (72). For complementation, one μ l of water was replaced with one μ l of the fraction to be assayed.

To quantitate specific activities, we first determined when the levels of splicing products were increasing linearly. We then chose a point (15 minutes) during this period and carried out the complementation assay for this length of time. Fractions were serially diluted and assayed for activity. We used the C303/305 precursor so the products of step one would not proceed through step two, and we could thus quantitate the amount of lariat intermediate produced. This was done with a phosphorimager, and normalized to the amount of precursor in each lane. One unit of activity was defined as the amount of lariat intermediate produced by a 1:80 dilution of the 40% ammonium sulfate fraction. The activities of the other fractions were determined by the dilution required to yield this same amount of activity.

Purification of PRP28

Sixteen liters of yEJS103 containing pHD30 were grown in YEPD to an optical density of 3.0 and 65 mls splicing extract was prepared according by the standard method except that lyticase was used instead of zymolyase. The 40% ammonium sulfate fraction was prepared as described (73), by back-extracting the 70% ammonium sulfate precipitate. The 40% pellet was dissolved in 3 mls of buffer D (20 mM Hepes, pH 7.6, 0.5 mM DTT, 0.2 mM EDTA, 20% glycerol)/50 mM KCl and dialyzed against buffer D/50 mM KCl for 4 hours. This material was loaded onto a 11 ml DEAE Sephadex column (Pharmacia) and washed with 23 mls buffer D/50 mM KCl. The flowthrough/wash was collected and loaded onto a 4 ml Affigel Blue (Cibacron Blue) (Sigma) column and washed with 12 mls of buffer D/250 mM KCl. The fraction enriched for PRP28 was eluted with 12 mls buffer D/500 mM KCl and dialyzed against buffer D/50 mM KCl. This was then loaded onto a 1.2 ml Carboxymethyl Sepharose column (Pharmacia) and washed with 3.6 mls buffer D/100 mM KCl. Purified PRP28 was then eluted with 3.6 mls buffer D/250 mM and dialyzed against buffer D/50 mM KCl.

Fractions were separated by 10% SDS-PAGE and visualized by silver staining (74).

Hydroxyapatite chromatography (IBF biotechnics) was carried out on a 500 μ l column. 500 μ l of the CM250 fraction was loaded and washed with 1 ml buffer D/75 mM KCl, and then with 1.5 ml buffer D/350 mM KCl. This step was employed to purify away an RNase which was complicating interpretation of results from the helicase assay. The column was then equilibrated with 2.5 mls buffer D/75 mM KCl. Fractions from three 1 ml phosphate step washes (buffer D/75 mM KCl plus 50, 100, and 150 mM Na₃PO₄, were collected).

A 2 ml, 5–20% sucrose gradient (in buffer D/no glycerol) was loaded with 50 μ l of protein (0.110 mg/ml) which had been diluted with 150 μ l buffer D/no glycerol. This protein was prepared by a similar procedure to that outlined above; the affigel

blue and carboxymethyl sepharose fractions collected were both 100–500 mM KCl. The most highly purified material looked the same as that shown in figure 3 on a silver stained gel. Gradients were spun in a Beckman TL100 ultracentrifuge using a TL55 rotor for 12 hours at 55000 rpm and 170 μ l fractions were collected from the top. Molecular weight standards were run in parallel on an identical gradient, TCA precipitated, separated on a 10% SDS-PAGE and visualized by Coomassie Blue stain.

Helicase assays

Partial double stranded (ds) RNA substrates were prepared by run-off transcription of pSP65 (Promega) (cut with XbaI) in the presence of rNTPs with α -³²P-GTP and transcription of the pGEM1 plasmid (Promega) (cut with PvuII) in the presence rNTPs and cold GTP. The transcription reactions (400 μ l) contained 500 μ M each of ATP, CTP, UTP, 50 μ M GTP (250 μ M for cold), 100 μ M 7MeGpppG, 20 μ g template, 20 mM DTT, 350 units RNAGuard (Pharmacia), 10 μ l SP6 polymerase (Pharmacia), 1 mCi α -GTP (or 250 mM cold GTP, see above), 80 μ l 5X transcription buffer (Pharmacia). They were incubated at 38°C for one hour. An additional 5 μ l SP6 polymerase was added and the reactions were incubated for another hour. Partially double stranded substrate was prepared as in Lee and Hurwitz (49).

The helicase assay was carried out in reaction mixtures (20 μ l) containing 20 mM Hepes-KOH, pH 7.6, 2 mM DTT, 50 mM KCl, 1 mM MgCl₂, 0.1 mg/ml BSA, 1 μ l (35 units) RNAGuard, 1.25 mM ATP, 50 fmol of ds RNA substrate, and 4 μ l of the column or 5 μ l of the sucrose gradient fractions. Mixtures were incubated for 30 minutes at 30°C, then stopped by the addition of 5 μ l of a mixture containing 0.1 M Tris-HCl, pH 7.4, 20 mM EDTA, 0.5% SDS, 0.1% NP40, 0.1% bromphenol blue, 0.1% xylene cyanol, and 50% glycerol. The reactions were loaded on a 1.5 mm thick, 13.5 cm long 17% polyacrylamide

(30:1) gel and electrophoresed at 10 mA for 3 hours. Gels were then autoradiographed.

RESULTS

Polyclonal antibodies recognize PRP28

We raised polyclonal antibodies to a TrpE-PRP28 fusion protein made in *E.coli*. This antigen was constructed by ligating 700 nucleotides from the 5' end of the coding region of PRP28 to TrpE (see materials and methods). The resultant fusion protein should contain 228 amino acids of PRP28. This region lies mostly outside of the highly conserved region ('DEAD-box' and related motifs) which is shared by other splicing factors (eg.: PRP5); thus, antibodies against this portion of the protein were predicted to be specific for PRP28. A western blot of splicing extract probed with immune serum reveals a protein of the predicted molecular weight for PRP28 (67 kD), which is not detected with pre-immune serum (figure 1, lanes 1 and 3). To confirm that this band represents PRP28, we examined an equivalent amount of protein from the same strain, harboring a plasmid which constitutively overexpresses PRP28 (lanes 2 and 4). As shown in lane 2, the 67 kD band (and breakdown products) is intensified in extract from the strain overexpressing PRP28. We estimate this to be approximately 100-fold overexpression based on a comparison of ammonium sulfate fractions enriched for PRP28 in which there is an easily visible band even with the non-overexpressed strain (data not shown). Strains bearing the overexpression plasmid display no growth defect, which indicates that 100-fold overexpression of PRP28 is not detrimental to the cell.

PRP28 is required for step 1 of splicing *in vitro*

We have previously shown that a conditional (cold-sensitive) allele of PRP28 causes accumulation of pre-mRNA under nonpermissive conditions *in vivo*. To determine whether this phenotype reflects a direct role in splicing, we examined the effects of α -PRP28 antibodies on an *in vitro* splicing reaction at 25°C. As shown in figure 2, immunodepletion of a splicing extract with α -PRP28 antibodies (lanes 4 and 10) but not pre-immune serum (lanes 3 and 9) blocks the first step of splicing *in vitro*. The lefthand side of the panel shows splicing with wild type actin pre-mRNA as the substrate. The righthand side of the panel shows splicing with substrate which can not proceed through the second step of the reaction because of a mutation at its 3' splice site (C303/305) (45); pre-mRNA as well as lariat intermediate and 5' exon (products of step one) are indicated. We used this substrate in all subsequent experiments since it simplifies analysis by blocking the reaction immediately after the step at which PRP28 acts. This enables quantitation of lariat intermediate without having to take into account its turn-over into mRNA and excised intron (as in a wild type intron). These experiments demonstrate that the first covalent modification of the pre-mRNA is inhibited in extracts that have been depleted of PRP28.

Purification of PRP28 from *S.cerevisiae*

In order to determine whether the effect of immunodepletion (above) is due solely to loss of PRP28, we sought to restore splicing activity with purified PRP28. It is possible to rescue *in vitro* splicing in PRP28-immunodepleted extract by supplementing the reaction with fractions from a yeast splicing extract which

Figure 1. α -PRP28 serum recognizes a protein of the predicted molecular weight for PRP28. Western blot of splicing extract prepared from a strain containing a single copy plasmid bearing PRP28 (lanes 1 and 3) and a strain containing a high copy plasmid overexpressing PRP28 (lanes 2 and 4). Lanes 1 and 2 are probed with α -PRP28 serum and lanes 3 and 4 are probed with pre-immune serum. Positions of molecular weight markers are shown on the right.

are incompetent for splicing on their own. (Compare lanes 4, 5 and 10, 11 in figure 2). Using this complementation assay for function and standard purification techniques, we have obtained highly purified PRP28 from *S. cerevisiae* overproducing PRP28 (complementation in lanes 5 and 11 is with the most highly purified fraction shown in fig. 3, below). Since the most highly purified fraction can complement immunodepleted extract, we conclude that PRP28 is directly involved in splicing. The purification scheme and table are shown in figure 3 and table 1. Splicing extract was precipitated with 40% ammonium sulfate, and the pellet fraction (after resuspension and dialysis) was chromatographed on a DEAE Sephadex column. PRP28-enriched material appeared in the flowthrough, and was subsequently subjected to chromatography on an Affigel Blue (Cibacron Blue) column. This material, which displays affinity for proteins that bind to nucleotides (46) retains PRP28, which elutes between 250 and 500 mM KCl. After dialysis, this eluate was applied to a Carboxymethyl Sepharose column and eluted between 100 and 250 mM KCl. As can be seen in the last lane of figure 3, PRP28 has been purified to apparent homogeneity based on a standard silver staining assay. However, by using a more sensitive staining procedure (Coomassie staining, followed by silver staining) it is possible to see small amounts of contaminating proteins (not

shown). Because of the behavior of PRP28 on both DEAE and carboxymethyl sepharose resins, we conclude that it is basic (all of these procedures were carried out at pH7), as predicted by the sequence analysis program, DNA Strider (predicted pI of 10).

Specific activities were obtained as described in materials and methods. We estimate that we have obtained at least a 57-fold purification of the ammonium sulfate fraction. The splicing assay is very difficult to quantitate reliably. Table 1 therefore represents a very conservative estimate of the degree of purification ($57\times$ increase in specific activity). Factoring in the enrichment in the ammonium sulfate fraction compared to the ammonium sulfate fraction from a non-overproducing strain ($100\times$), this corresponds to a 5700-fold purification from wild type extract.

PRP28 does not copurify with RNA helicase activity

PRP28 has been predicted to exhibit ATP-dependent RNA helicase activity because it contains a highly conserved region of approximately 300 amino acids (DEAD-box and related motifs). This is based on the biochemical activities of eIF4A, the prototype of this class of proteins, which requires eIF4B for its activity (30), and p68, a second member of this family, which can unwind duplex RNA on its own (47). It was therefore of interest to determine whether PRP28 can unwind duplex RNA in an ATP-dependent fashion.

We employed an assay that has been used to detect unwinding activity by SV40 T antigen and RNA helicase I and RNA helicase A, two Hela helicases (48, 49). The substrate for this assay is

Figure 2. Immunodepletion with α -PRP28 serum inhibits the first step of splicing *in vitro* and this defect can be rescued by purified PRP28. Products of splicing reactions carried out with wild type precursor (lanes 1–6) and C303/305 (lanes 7–12) are shown on the left; the positions of precursor, lariat intermediate, free lariat and exon 1 are diagrammed (mRNA is obscured by degradation products). Exon 1 from wild type precursor is shorter than that from C303/305 and is not shown. Splicing assays were carried out in the presence of precursor alone (lanes 1 and 7), extract (lanes 2 and 8), pre-immune-depleted extract (lanes 3 and 9), α -PRP28-depleted extract (lanes 4 and 10), α -PRP28-depleted extract plus purified PRP28 (lanes 5 and 11), and purified PRP28 (lanes 6 and 12).

Figure 3. Purification of PRP28. Silver stained gel of 20 μ g splicing extract (lane 1), 20 μ g 40% ammonium sulfate pellet (fraction I) (lane 2), 2 μ g DEAE Sephadex eluate (lane 3), 2 μ g Affigel Blue eluate (lane 4), 2 μ g Carboxymethyl Sepharose eluate (lane 5). Positions of molecular weight markers are shown on the left.

a partially duplexed RNA (28 base pairs) in which only the bottom strand is labeled (diagrammed in figure 4A). Helicases often require a single stranded region of nucleic acid for binding (50), and most translocate either in a 5' to 3' or 3' to 5' direction (defined by the strand on which the helicase is bound) (50). The substrate in our assay contains both free 5' and 3' ends, and should therefore be useable by both types of helicases. Unwinding is indicated by increased mobility of the labelled RNA on a native gel. The most highly purified fraction of PRP28 shown in figure 3 (lane 5, carboxymethyl sepharose fraction) contains RNA-unwinding activity. In order to determine whether PRP28 is responsible for this activity, we subjected this fraction to further chromatographic procedures. Figure 4A shows three assays of fractions from a hydroxyapatite column: a silver-stained protein gel (top), complementation of *in vitro* splicing (band representing lariat intermediate is shown in the middle panel), and RNA helicase activity (band representing unwound RNA is shown in the bottom panel). The protein and complementation activity peak in the 100 mM phosphate fraction (lanes 2), while the unwinding activity peaks in the 150 mM phosphate fraction (lanes 3).

A similar result is shown in figure 4B, following sucrose gradient fractionation of a highly purified PRP28 fraction (purified almost identically to material in figure 3, lane 5; see materials and methods). Again, the three panels show protein, *in vitro* complementation, and RNA unwinding activity profiles (top of the gradient is fraction 1, bottom of the gradient is fraction 12). Here PRP28 protein and complementation activity peak in fraction 6, while RNA unwinding activity is offset to the right, peaking in fractions 7 and 8.

These results show that PRP28 does not possess RNA helicase activity under these assay conditions. It is also formally possible that an inhibitory factor is present in the protein preparation. We can not rule out the possibility that the RNA unwinding activity seen is dependent on PRP28 since, as yet, we have been unable to obtain fractions that contain unwinding activity but are completely free of PRP28. Attempts to achieve such samples by immunodepleting purified protein preparations were unsuccessful.

Highly purified fractions of PRP28 contain RNA-dependent ATPase activity

A second activity that is exhibited by ATP-dependent RNA helicases is RNA-dependent ATPase activity. This has been difficult to assay reliably in the most purified samples of PRP28 because quantitation is difficult due to low activity. However, we have seen reproducible RNA-dependent ATPase activity with the most highly purified (hydroxyapatite chromatography and sucrose gradient) fractions containing PRP28 (data not shown). Unfortunately, we have been unable to obtain convincing results

Table 1. Protein purification.

Fraction	Protein (mg)	Activity yield (%)	Specific activity (units/mg)
Extract	1104		ND
Amm. Sulfate	36	100*	83
DEAE Seph.	6.2	140	676
Affigel Blue	2.2	230	3125
CM Seph.	0.819	130	4760

*Since the activity could not be assayed in the crude extract (ND), this value was set arbitrarily at 100%.

Figure 4. PRP28 does not cofractionate with RNA helicase activity. (A) Fractions from hydroxyapatite chromatography on a silver stained protein gel (top), activity in splicing complementation assay (middle), and activity in strand displacement assay (bottom). Lanes 1: 50 mM Na₃PO₄, lanes 2: 100 mM Na₃PO₄, lanes 3: 150 mM Na₃PO₄. (B) Fractions from sucrose gradient sedimentation on a silver stained protein gel (top), activity in splicing complementation assay (middle), and activity in strand displacement assay (bottom). The gradient is shown from top to bottom (left to right). Native Cytochrome C (MW=12.4 kD, 2.5S) sedimented in fraction three, BSA (MW=66 kD, 4.5S) sedimented in fraction 6, Aldolase (161 kD, 8.5S) sedimented in fraction 9, and Catalase (247 kD, 11.5S) sedimented at the bottom of the gradient (fraction 12).

showing that there is only one RNA-dependent ATPase in these fractions. The ATPase activity rises with the appearance of PRP28 in both the hydroxyapatite and sucrose gradient steps, but does not fall off as expected. We know, however, that this activity is not due solely to the RNA unwindase (figure 4) for a number of reasons. Slightly more RNA-dependent ATPase activity elutes in the 100 mm hydroxyapatite fraction than in the 150 mm fraction; in contrast, the 150 mm fraction shows the peak in unwinding activity. Furthermore, the ATPase activity is inhibited to different extents by different ATP analogs (including AMP-PNP and AMP-PCP, which are nonhydrolyzable), while unwinding activity is supported equally well by these analogs (data not shown). This indicates in addition that the RNA unwindase present in our prep would not necessarily be expected to exhibit ATPase activity since it does not need to hydrolyze ATP in order to unwind RNA duplexes. (Note that since helicase activity is defined as RNA unwinding activity that requires hydrolysis of an NTP, this unwinding activity is novel.) At present we cannot distinguish between several plausible explanations for the ATPase activity profiles from hydroxyapatite chromatography and sucrose gradient fractions; possibilities include the presence of inhibitors/activators or more than one RNA-dependent ATPase. Attempts to show PRP28-specific ATPase activity by immunoprecipitation have been unsuccessful. In summary, our results are consistent with PRP28 possessing RNA-dependent ATPase activity.

DISCUSSION

PRP28 is a non-snRNP protein required for the first step of splicing *in vitro*

Using polyclonal antibodies we have identified the protein encoded by PRP28, developed an *in vitro* assay for its activity, purified it from yeast, and shown that it is required for the first step of splicing *in vitro*, thus demonstrating direct involvement in this process. Splicing activity in extracts immunodepleted for PRP28 can be rescued by highly purified protein. Therefore, PRP28 is not a tightly bound, integral snRNP protein. That is, if PRP28 was stably bound to an snRNA, immunodepletion would coprecipitate this snRNA (and associated proteins); in this case, addition of purified PRP28 would not restore splicing activity because the other components of the snRNP would still be missing. The conclusion that PRP28 is not a snRNP protein is also consistent with the observation that immunoprecipitation of PRP28 does not detectably coprecipitate any of the five snRNAs known to be involved in pre-mRNA splicing (data not shown). It will be of interest to determine the precise step in splicing at which PRP28 acts.

PRP28 does not display ATP-dependent RNA helicase activity

PRP28 has been proposed to be an ATP-dependent RNA helicase, based on its inclusion in the DEAD-box gene family. These genes share an extensive region of homology over a span of about 300 amino acids, including the hallmark Asp-Glu-Ala-Asp (DEAD) motif for which the family is named (51). They have been found in organisms from *E. coli* to mammals and are involved in a large number of diverse cellular processes (52, 53, 54, 55, 56, 57, 58, 59, 60, 61). The founding member, eIF4A, is thought to use its RNA unwinding capability to unravel secondary structure in mRNA during translation initiation (30, 62, 63). It requires another initiation factor, eIF4B, for its activity in many assays: 1) RNA binding (64), 2) RNA cross-linking (62, 65), 3) RNA-

dependent hydrolysis of ATP (64, 66), 4) ATP-dependent unwinding of artificial substrates (30), and 5) deprotection of mRNA to nuclease (62). The other member of this gene family which has been tested for helicase activity (p68) appears to be enzymatically active on its own in an *in vitro* unwinding assay (47); its biological function is unknown. We have found that PRP28 does not display RNA helicase activity under the assay conditions tested. It is inviting to speculate that, like eIF4A, PRP28 requires one or more additional factor(s) to activate its helicase activity. For example, PRP28 may require some protein associated with U4, U5, U6, or the spliceosome itself (in some particular conformation) to trigger helicase activity. PRP24, a U6 protein (68), is an attractive candidate for such an activator. Previously we identified a synthetic lethal interaction between *prp28-1* (a cold-sensitive allele) and *prp24-1* (a temperature-sensitive allele); that is, at a temperature permissive for *S. cerevisiae* strains containing each individual allele (25°C), the double mutant combination was inviable. One explanation for the synthetic lethality conferred by the *prp28-1*, *prp24-1* combination is that the wild type proteins function synergistically. If, for example, these proteins are in fact a helicase and a helicase activator, reduction of either activity alone would be expected to interfere with unwinding, thereby slowing cell growth; however, reduction of both activities simultaneously might completely abolish unwinding and cause lethality. Alternately, it is possible that PRP28 does not function as an RNA helicase or that we have not found the appropriate assay conditions.

Another activity exhibited by ATP-dependent RNA helicases is hydrolysis of ATP in an RNA-dependent fashion (RNA-dependent ATPase). We find this activity in our most pure PRP28 fractions. However, we have been unable to determine whether there is only a single RNA-dependent ATPase in these fractions (see results). While our results are consistent with the possibility that PRP28 possesses RNA-dependent ATPase activity, we have not demonstrated this definitively. This issue may be resolved with the identification of a factor that activates helicase activity; such a factor would likely stimulate ATPase activity as well.

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