The DEAH-box protein PRP22 is an ATPase that mediates ATP-dependent mRNA release from the spliceosome and unwinds RNA duplexes

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Of the proteins required for pre-mRNA splicing, at least four, the DEAH-box proteins, are closely related due to the presence of a central 'RNA helicase-like' region, and extended homology through a large portion of the protein. A major unresolved question is the function of these proteins. Indirect evidence suggests that several of these proteins are catalysts for important structural rearrangements in the spliceosome. However, the mechanism for the proposed alterations is presently unknown. We present evidence that PRP22, a DEAH-box protein required for mRNA release from the spliceosome, unwinds RNA duplexes in a concentration- and ATP-dependent manner. This demonstrates that PRP22 can modify RNA structure directly. We also show that the PRP22-dependent release of mRNA from the spliceosome is an ATP-dependent process and that recombinant PRP22 is an ATPase. Non-hydrolyzable ATP analogs did not substitute for ATP in the RNA-unwinding reaction, suggesting that ATP hydrolysis is required for this reaction. Specific mutation of a putative ATP phosphate-binding motif in the recombinant protein eliminated the ATPase and RNA-unwinding capacity. Significantly, these data suggest that the DEAH-box proteins act directly on RNA substrates within the spliceosome.

Keywords: DEAD/H-box proteins/mRNA splicing/RNA helicase/splicing factors

Introduction

RNA conformation is of elemental importance in RNA-induced catalysis, as well as RNA interactions with other cellular components. Many central components of gene expression and RNA metabolism occur in large ribonucleoprotein complexes, most notably the ribosome and spliceosome. Within these large complexes, alternative conformations of specific RNAs have been demonstrated and the alteration of RNA conformation is believed to play a critical role in enabling, driving and assuring the fidelity of the catalytic reactions these complexes perform (Lodmell and Dahlberg, 1997; Staley and Guthrie, 1998). Therefore, understanding the factors capable of inducing RNA conformational alterations within these complexes

is likely to be central to an overall appreciation of mechanisms by which they execute their intricate and demanding functions.

The spliceosome is a large RNA–protein complex that assembles on pre-mRNA and catalyzes the removal of introns in a two-step reaction involving two successive transesterifications (Moore *et al.*, 1993; Nilsen, 1994). Concurrent with spliceosomal assembly, activation, chemical modification of the pre-mRNA substrate, then mRNA release, there are gross alterations in the constituents and conformations of the spliceosome. An energy source in the form of ATP is required for splicing. However, currently there is no evidence that ATP hydrolysis is required for either of the two chemical steps. Rather, ATP hydrolysis has been shown to occur concomitant with several of the spliceosome structural alterations. Therefore, it is believed that ATP hydrolysis drives conformational rearrangements that enable ordered and accurate identification of the correct splicing signals on the pre-mRNA, assembly of the active sites for the chemical reactions and transfer of the products into their respective downstream pathways.

Of the proteins required for pre-mRNA splicing in yeast identified to date, a growing subset possess a set of sequence motifs common to proteins that hydrolyze nucleoside triphosphates as a component of their respective functions (NTPases, Staley and Guthrie, 1998). Most of these proteins possess variants of a so-called 'helicase' domain characterized by seven blocks of conserved amino acids (Gorbalenya *et al.*, 1989; Schmid and Linder, 1992). Four of these proteins, PRP2, PRP16, PRP22 and PRP43 (JA1), have been designated 'DEAH-box' proteins (Company *et al.*, 1991; Arenas and Abelson, 1997). These proteins exhibit extensive homology within the central 'helicase' domain, and the homology extends into an additional region located in the C-terminal portion of the protein (Chen and Lin, 1990; Company *et al.*, 1991; Schwer and Guthrie, 1991). Interestingly, each of the four characterized yeast DEAH-box proteins are required for pre-mRNA splicing at specific steps, PRP2 prior to the first chemical step, PRP16 prior to the second chemical step, PRP22 for release of the mRNA following the second chemical step, and PRP43 for spliceosome dissociation after the PRP22 step (Staley and Guthrie, 1998). The activity of PRP2, PRP16 and PRP22 is correlated with gross morphological alterations in spliceosome structure, as detected for PRP2 and PRP22 by gel electrophoretic analysis of spliceosome complexes, and, for PRP16, in an altered sensitivity of the 3' splice site to oligonucleotide-mediated RNase H cleavage (Cheng and Abelson, 1987; Company *et al.*, 1991; Schwer and Guthrie, 1992a; Kim and Lin, 1996). PRP16 and PRP2 have been shown to be RNA-dependent ATPases (Schwer and Guthrie, 1991; Kim *et al.*, 1992). Both bind to spliceosomes in the absence of

ATP and appear to be released from specific spliceosomal interactions concomitant with or following ATP hydrolysis, correlating the hydrolysis to function within the spliceosome (Schwer and Guthrie, 1991; Kim and Lin, 1993; Teigelkamp *et al.*, 1994). In sum, it seems likely that these DEAH-box proteins are effectors of spliceosomal structural rearrangements, in reactions that are RNA dependent and ATP driven.

A major unresolved issue relating to the DEAH-box proteins, as well as all identified ATPases required for pre-mRNA splicing, is how these proteins effect the observed conformational rearrangements. It has been hypothesized that these proteins act directly on RNA substrates in the spliceosome to disrupt or establish RNA base pairing interactions, thereby altering RNA conformation or intermolecular interactions (Company *et al.*, 1991; Schwer and Guthrie, 1991; Wassarman and Steitz, 1991). This hypothesis is attractive because several mutually exclusive sets of alternative RNA–RNA base pairing interactions have been identified within the spliceosome (for a discussion of these, see Staley and Guthrie, 1998). However, equally tenable hypotheses for the activity of these proteins are that they modify interactions between RNA and other proteins, as has been suggested for Mot1, a member of the SWI1/SNF1 family of DNA-dependent ATPases (Auble *et al.*, 1997), or that their direct activity involves ATP-dependent modulation of protein–protein interactions. To date, the demonstration that any one of these proteins can modify RNA–RNA base pairing interactions in a system with purified components is lacking.

PRP22 was isolated originally in a screen for temperature-sensitive (ts) mutants in pre-mRNA splicing (Vijayraghavan *et al.*, 1989). In yeast, at the restrictive temperature, *prp22-1* mutants accumulate pre-mRNA and excised intron lariat, a phenotype that suggests a block late in the splicing pathway that also blocks factor recycling. In heat-inactivated *prp22-1* extract *in vitro*, efficient splicing is observed, but the products (mRNA and intron lariat) accumulate in large complexes that have similar mobility to spliceosomes in non-denaturing gel electrophoresis (Company *et al.*, 1991). These results suggested that PRP22 is required for spliceosome dissociation and mRNA release following the second step of splicing. More recently, HRH1, an apparent PRP22 human homolog, has been identified and this protein also appears to be required for the dissociation of spliceosomal complexes following the second step (Ohno and Shimura, 1996). Based on sequence homology, putative PRP22/HRH1 homologs have also been identified in *Caenorhabditis elegans* and *Arabidopsis thaliana*, suggesting that PRP22 is highly conserved throughout the eukaryotes (J. Wagner, unpublished).

We have investigated the biochemical properties of PRP22 using recombinant purified protein. We show that the spliceosome dissociation block observed in heat-inactivated *prp22-1* extracts can be alleviated by addition of the recombinant protein, and that this complementation requires ATP. We also show that the PRP22 protein is an ATPase, and that the ATPase activity is stimulated by RNA. Most importantly, we show that PRP22 can unwind partial duplex RNA molecules in a reaction that probably requires ATP hydrolysis. Thus, in overall requirements

and features, the reaction resembles that which occurs in the spliceosome. Therefore, this provides evidence that a DEAH-box protein can actively alter the duplex structure of RNA molecules, and we propose that it is highly likely that the direct alteration of RNA duplex structures is central to the catalytic function of the protein *in vivo*.

Results

To facilitate biochemical characterization of the yeast PRP22 protein, a plasmid was constructed for overexpression in *Escherichia coli* of recombinant PRP22 protein modified by C-terminal His 8 and Flag epitope tags (Trotta *et al.*, 1997). Since the presence of the affinity tags on the C-terminus of the recombinant protein could be deleterious to protein function, we also tested the functionality of the modified protein in budding yeast. The modified PRP22 protein was expressed as the sole form of PRP22 in a chromosomal PRP22 null yeast strain. Western blot analysis using a Flag epitope-specific antibody verified expression of the modified protein (data not shown). Normal growth was observed at a variety of temperatures, suggesting that the modified PRP22 we have characterized is a functional form of PRP22 *in vivo* (data not shown).

Recombinant PRP22 complements the *prp22-1* mutant phenotype *in vitro*

Following overexpression in *E. coli*, although most of the PRP22 polypeptide was insoluble, an appreciable portion was found in the soluble fraction of cell extracts. Recombinant PRP22 was partially purified from this soluble fraction by a combination of Ni-NTA and Flag epitope affinity chromatography (method 1). As a control, a mock purification was performed from extracts of *E. coli* containing the expression vector without the PRP22 insert (protein control). We tested the activity of the recombinant PRP22 protein in splicing *in vitro*. Reactions were conducted using heat- or mock-treated extract from the *prp22-1* strain and radiolabeled actin precursor, either alone or together with the recombinant PRP22 or protein control. Comparison of the distribution of radiolabeled RNA in the heat- versus mock-treated *prp22-1* extract following non-denaturing (complex) gel electrophoresis revealed that heat treatment led to the accumulation of the radiolabeled RNA in a large complex corresponding in mobility to the A2 complex (spliceosomes) together with the disappearance of a more rapidly migrating complex termed the 'D' complex (Figure 1A, lanes 3–4 versus lanes 1–2), as reported previously (Company *et al.*, 1991). Addition of PRP22 at all concentrations tested reversed the effect of the heat treatment (Figure 1A, lanes 5–7). In contrast, addition of a preparation derived from an *E. coli* strain harboring the expression vector minus PRP22 had no observable effect on the accumulation of the A2 complexes or on the absence of the D complex in the heat-treated *prp22-1* extract (Figure 1A, lanes 8–10). Using this protocol, similar complementation was observed with each recombinant PRP22 preparation used in this communication, whereas addition of the mutated recombinant PRP22 preparation [prp22(K512E), see below] gave results similar to the protein control (Figure 1A, and data not shown).

The composition of the radiolabeled RNA present in the A2 and D complexes was examined by denaturing gel electrophoresis. In the A2 complex, using the precursor RNA as a reference, a dramatic increase in the relative amount of the products of splicing (the intron lariat and mRNA) was observed in the heat-treated compared with the mock-treated extract, as expected (Figure 1B, compare lanes 3–4 and 1–2). Addition of the purified PRP22 to the heat-inactivated *prp22-1* extract led to an obvious and specific reduction in the amount of the mRNA and intron located in the A2 complex to levels equal to or below that of the precursor (Figure 1B, lane 5). In contrast, addition of PRP22 minus extract did not alter the composition of the pre-mRNA, intermediates or products found in the A2 complex (Figure 1B, lane 6). The D complex in the mock-treated extract was composed exclusively of

splicing products; the amount of these RNAs, in particular the mRNA, was greatly reduced in the heat-treated extract (Figure 1C, lanes 2–3). Addition of recombinant PRP22 to the heat-treated extract fully and specifically reversed the effect of the heat treatment, such that the radiolabeled RNA in this D complex region of the gel was indistinguishable in composition and amount from that of the mock-treated control (Figure 1C, compare lane 4 with lane 3).

The observed effect of recombinant PRP22 addition was specific for the *prp22-1* splicing lesion. The composition of the radiolabeled RNA extracted from the unfractionated extracts from all treatments was examined following denaturing gel electrophoresis (Figure 1D). Addition of the protein had little effect on the overall accumulation of precursor, intermediates and splicing products, other than reversing the previously described modest increase in the amount of intron lariat and lariat intermediate in the heat-treated temperature-sensitive extract (Figure 1D; Company *et al.*, 1991; Arenas and Abelson, 1997). Furthermore, the addition of either the recombinant PRP22 or the mock protein preparation had little effect on mock-treated *prp22* extract, or heat- or mock-treated PRP22 extract, establishing that the observed effect was specific to the heat-inactivated *prp22-1* extract (not shown). Overall, these results demonstrate that the recombinant protein is active in the splicing extract, and that this protein alone is sufficient to reverse the lesion observed in heat-treated *prp22-1* extracts under the conditions of the assay.

PRP22 and ATP are necessary and sufficient for release of accumulated mRNA from gradient-isolated spliceosomes

Using glycerol gradient centrifugation of heat-treated *prp22-1* extracts, we identified and partially purified a complex corresponding to the mRNA- and intron-containing native gel A2 complex ('blocked spliceosomes'). For these experiments, heat- and mock-treated extract was

Fig. 1. Recombinant PRP22 complements heat-inactivated *prp22-1* extract splicing *in vitro*. (A) Non-denaturing gel electrophoresis analysis of *in vitro* splicing reactions conducted with radiolabeled actin precursor and heat-inactivated *prp22-1* extract (lanes 1 and 2 and 5–10) or mock-treated *prp22-1* extract (lanes 3 and 4). Prior to incubation, various reactions were supplemented with recombinant PRP22 protein (6, 0.6 and 0.06 nM, lanes 5–7, respectively) or an equal volume of control protein (lanes 8–10). The spliceosome A2 complex and the D complex are identified to the left of the gel. (B) Radiolabeled RNA composition of specific A2 complexes shown in (A), resolved by denaturing gel electrophoresis. Specific RNA species are identified to the left of the gel, including, from top to bottom, the 2/3 intron–exon lariat intermediate, the intron lariat product, the precursor RNA, the spliced exon mRNA product and the exon 1 intermediate. The species marked with an asterisk has been identified previously to be linearized intron (Company *et al.*, 1991). Lanes 1–4 correspond to the A2 complexes in lanes 1–4, lane 5 to the A2 complex in lane 6, lane 6 to the A2 complex in lane 9 in (A), respectively, and lane 7 corresponds to the D complex from lanes 1 and 2 in (A) shown for reference. Lanes 1 and 2 are overexposed with respect to the remaining lanes to facilitate comparison of the ratio of products to the precursor. (C) Radiolabeled RNA composition in the specific D complexes shown in (A). Lane 1 is a reference which corresponds to the A2 complex in (A) lane 10, and lanes 2–4 correspond to D complexes in lanes 1 and 2, lanes 3 and 4, and lanes 6 and 7 in (A), respectively. (D) Denaturing gel electrophoresis analysis of the splicing reactions (unfractionated). Lane designations are as in (A), except that lanes 1 and 2, and 3 and 4 are inverted, so that the mock-inactivated controls are located in lanes 3 and 4 and the heat-treated controls in lanes 1 and 2.

Fig. 2. Actin mRNA and intron lariat accumulate in 40S spliceosomes in heat-treated *prp22-1* extracts. (A) The total radioactivity in each glycerol gradient fraction (shown in kc.p.m. on the y-axis) of the fractions of gradients loaded with *prp22-1* extract that was heat-treated or mock-treated prior to the splicing reactions. (B) Radiolabeled RNA recovered from 40 μ l of fractions 11 and 20 of each gradient shown in (A), resolved by denaturing gel electrophoresis. Individual species are identified using the symbols as in Figure 1.

incubated in 1 h splicing reactions using radiolabeled actin precursor, then centrifuged into glycerol gradients (Yean and Lin, 1991). A splicing reaction using PRP22 extract incubated in the presence of EDTA and centrifuged in parallel indicated the location in the gradients of the previously characterized 40S spliceosomes and 30S pre-spliceosomes (arrows in Figure 2A; Brody and Abelson, 1985). As shown in Figure 2A, the spliceosome peak (fractions 11–13) was modest in the mock-treated control, reflecting the advanced stage of the *in vitro* splicing reaction after the 1 h incubation. In contrast, in the heat-treated *prp22-1* extract, a peak of radiolabeled RNA in fractions 8–13 was more pronounced. Additionally, the mock-treated control exhibited a clear shoulder of radioactivity in fractions 18–21 that was absent in the heat-treated *prp22-1* extract.

The composition of the radiolabeled RNA in the spliceosome peak (fraction 11) and the region of the shoulder (fraction 20) in each of the gradients was examined by denaturing gel electrophoresis. Fraction 11 in the control extract contained precursor, intermediates and some

products of the splicing reaction, corresponding to the spliceosome (Figure 2B, lane 1). In contrast, heat-treated *prp22-1* extract exhibited a marked accumulation of the mRNA and intron lariat products in this fraction (Figure 2B, lane 3). The distribution of splicing products, intermediates and precursor was similar in this fraction to that identified in the A2 complex resolved by non-denaturing gel electrophoresis (Figure 1B), suggesting a correspondence between the two. Conversely, in fraction 20, a clear peak of mRNA accumulation was detected in the control but not in the heat-treated *prp22-1* extract (Figure 2B, compare lanes 2 and 4). Interestingly, the intron lariat did not appear to co-migrate with this peak of mRNA in any of several experiments, a result that may suggest that the co-migration of the intron with the native gel D complex is fortuitous (Figure 2B, lane 2, and data not shown). Nonetheless, these results provide independent support for the hypothesis that PRP22 is required for release of mRNA from the spliceosome, since heat inactivation of the protein leads to accumulation of mRNA in spliceosome complexes in the gradient and to the concomitant disappearance of more slowly sedimenting mRNA containing complexes.

A precursor-product relationship may exist between the A2 complex observed in heat-treated *prp22-1* extracts and the D complex that accumulates in wild-type extract. If so, it should be possible to release the splicing products from the blocked spliceosomes by adding the necessary factors. We attempted to complement aliquots of the spliceosome peak fractions shown in Figure 2 (heat-treated extract), using non-denaturing gel electrophoresis to analyze the products. Incubation under splicing reaction conditions without addition of PRP22 revealed that the vast majority of the radiolabeled RNA migrated as A2 spliceosome complexes (data not shown; and Figure 3A, lane 4). Addition of recombinant PRP22 protein or of wild-type PRP22 extract to the reaction led to a reduction in the amount of the A2 band together with the appearance of a more rapidly migrating band of radioactive RNA, corresponding to the D complex (Figure 3A). In clear contrast, addition of the protein control (derived from *E.coli* harboring the vector) or of heat-treated *prp22-1* extract had no detectable effect on the accumulated A2 complexes (Figure 3A). D complex formation was not detected in the reaction containing the DEAD-box protein PRP5 (Figure 3C, lane 10). Thus, the function supplied by PRP22 in the observed D complex formation appears to be specific to the protein, rather than to the broader class of related proteins. Taken together, these data suggest that the complex isolated from the glycerol gradient is, at least in part, a blocked intermediate that can be chased into a dissociated product following addition of PRP22.

The requirement for nucleoside triphosphates as cofactors in the dissociation reaction was also examined. The accumulated A2 complexes were stable when either no cofactor, ADP or AMP-PNP (adenyl-*imidodiphosphate*, a non-hydrolyzable ATP analog) was added to the reaction, regardless of whether PRP22 was present or not (Figure 3B and C). In contrast, D complex formation was efficient in reactions augmented with 2 mM ATP, 20 μ M ATP and 2 mM GTP (Figure 3C). In each case, PRP22 was required, as no D complex formation was detected when the control protein preparation was added (Figure 3C). Note that the

Fig. 3. Gradient-purified spliceosomes from heat-treated *prp22-1* extracts require exogenous PRP22 and NTP for conversion of complex A2 to D. Complementation reactions were conducted as described in Materials and methods and complexes were resolved using non-denaturing gel electrophoresis. Reactions were conducted with portions of fraction 11 (A), fraction 10 (B) or fraction 9 (C) of the heat-treated *prp22-1* extract gradient shown in Figure 2A. (A) Additions included heat-treated PRP22 extract (lane 1), heat-treated *prp22-1* extract (lane 2), PRP22 protein (lane 3) or control protein (lane 4). All reactions were supplemented with ATP (2 mM). (B) Complementation reactions containing 2 mM ATP (lanes 1 and 2), no cofactor (lanes 3 and 4) or 2 mM ADP (lanes 5 and 6) and PRP22 protein (odd-numbered lanes) or control protein alone (even-numbered lanes). (C) Reactions with 20 μ M ATP (lanes 1 and 2), 2 mM ATP (lanes 3, 4, 9 and 10), 2 mM AMP-PNP (lanes 5 and 6) or 2 mM GTP (lanes 7 and 8) and PRP22 protein (lanes 1, 3, 5, 7 and 9), control protein alone (lanes 2, 4, 6 and 8) or PRP5 protein (lane 10). The PRP22 was diluted 1:100 in control protein prior to use, yielding a concentration of 0.06 nM.

length of electrophoresis differed between the experiments presented in Figure 3, a fact that is sufficient to explain the slightly different location of the D complex in each panel. These results indicate that nucleoside triphosphate is required for the dissociation reaction. Furthermore, as the non-hydrolyzable ATP analog AMP-PNP cannot substitute for ATP, NTP hydrolysis appears to be required as a component of the observed reaction. Thus, taken together, these results define a novel concurrent requirement for ATP and PRP22 after the second chemical step of pre-mRNA splicing. Since we show below that PRP22 is an ATPase, it is likely that ATP hydrolysis by PRP22 is a component of the spliceosome dissociation reaction.

PRP22 is an RNA stimulated ATPase

The presence of the DEAH-box motifs in PRP22 leads to the prediction that this protein is an ATPase, and that the ATPase activity is likely to be stimulated by the presence of RNA. As shown in Figure 4A, the recombinant PRP22 was indeed found to be an active ATPase in the presence of RNA *in vitro* (shown is protein purified by method 2). Surprisingly, however, multiple recombinant protein preparations exhibited extensive ATPase activity in the absence of added RNA (Figure 4A, and data not shown). As a control for potential *E.coli*-derived contaminants, we constructed an expression plasmid with a mutation in PRP22 that alters a predicted ATP-binding motif of the recombinant protein (Subramanya *et al.*, 1996), the conserved Walker A site, also known as the 'GKT motif' (Walker *et al.*, 1982), by changing a conserved lysine to a glutamic acid (K512E, 'GET'). Following overexpression in *E.coli*, preparations of this mutated polypeptide [prp22(K512E)] were obtained that were similar in both yield and purity to those obtained with tagged PRP22 preparations prepared by the same protocol (not shown).

However, the prp22(K512E) preparations did not exhibit ATPase activity above background over a 2 h period, either in the presence or the absence of added RNA (Figure 4A). These results strongly suggest that both the RNA-dependent and RNA-independent ATPase activities are linked directly to the recombinant PRP22. Moreover, the results indicate that the lysine at position 512 (in the GKT motif) is essential for both forms of ATPase activity, suggesting that these activities may not be distinct.

RNA cofactor specificity in ATP hydrolysis has been reported for two ATPases involved in pre-mRNA splicing, PRP5 and SLT22 (O'Day *et al.*, 1996; Xu *et al.*, 1996). Therefore, we compared a variety of RNAs in the ATPase assay, including several of the yeast snRNAs required for pre-mRNA splicing (T7 polymerase transcribed forms). Overall, the RNAs examined stimulated the ATPase similarly (2.5- to 4-fold) based on initial rates of multiple-turnover reactions at subsaturating concentrations of ATP (Figure 4B). The highest stimulation was detected with the homopolymers, poly(U) and poly(A). Either singly or in various combinations, none of the snRNAs were found to stimulate Prp22 ATPase activity to an extent significantly greater than homopolymeric RNAs. DNA did not stimulate the ATPase activity.

In order to understand the mechanistic basis for the RNA stimulation of ATP hydrolysis, kinetic parameters for the reaction were estimated by determining the initial rate, r_0 , at saturation (proportional to k_{cat} , as described in Materials and methods), and at subsaturation (proportional to k_{cat}/K_m) of ATP. This analysis resulted in a k_{cat} value of $102 \pm 30/\text{min}$ and a K_m value of $103 \pm 30 \mu\text{M}$ for the reaction without added RNA. By comparison, a k_{cat} value of $399 \pm 56/\text{min}$ and a K_m value of $95 \pm 12 \mu\text{M}$ were observed for ATP hydrolysis in the presence of poly(U) RNA. Similar trends were observed in the

Fig. 4. PRP22, but not the GKT to GET mutant, is an RNA-stimulated ATPase. (A) ATP hydrolysis over time in reactions with wild-type GKT PRP22 or mutant GET prp22(K512E), either with or without 0.1 $\mu\text{g/ml}$ poly(U) RNA, as indicated. All reactions were conducted with 500 μM ATP and 22 nM recombinant protein. (B) Stimulation of PRP22 ATPase activity by various RNA substrates. The y-axis reflects the fold increase in the initial rate, r_0 , with RNA at a saturating ATP concentration (500 μM) and 22 nM PRP22, normalized to the r_0 of reactions without RNA. Poly(U), poly(A) and rRNA (16S and 23S rRNA) were at 0.1 $\mu\text{g}/\mu\text{l}$. snRNAs U2, U4, U5 and U6 were used at 2 μM each, either alone or in combination. Oligonucleotides (2 μM) included a poly(U) 35mer (rU-35) and 20mer (rU-20), and 18mer and 35mer DNA oligonucleotides. Amounts of the RNAs employed were determined independently to be sufficient for maximal ATPase stimulation. The initial reaction rate r_0 without RNA was $r_0 = 2.6 \pm 0.2$ $\mu\text{M}/\text{min}$. The error bars represent the mean standard deviation of the fold increase between at least three independent time courses.

presence of the other RNAs tested. Thus, RNA appears to stimulate the rate by increasing the k_{cat} rather than by improving the binding of ATP.

PRP22 unwinds U4–U6 duplexes in vitro

In the presence of purified PRP22 and ATP, highly reproducible disruption of intermolecular RNA–RNA duplexes was detected. The model substrate employed in the initial characterization was a duplex of yeast U4 and U6 RNA (bacteriophage T7 polymerase synthesized). The U4–U6 partial duplex is expected to have 21 canonical base pairs, with the longest contiguous region predicted to have 10 bp interactions. The presence of extended single-stranded regions located both 5' and 3' to the duplex regions, and a relatively low melting temperature despite extensive base-paired regions (53°C, Brow and Guthrie, 1988) are consistent with the RNA being a sensitive and relatively universal substrate for use with RNA-unwinding enzymes (e.g. Claude *et al.*, 1991). As shown in Figure 5A, in a 10 min reaction at 30°C in the presence of PRP22 and ATP, the input U4–U6 hybrid was converted efficiently into the individual RNAs. The level of conversion of U4–U6 into the individual monomers

Fig. 5. Recombinant PRP22 unwinds U4–U6 heteroduplexes. (A) RNA unwinding is PRP22 concentration dependent. Lane 1, no PRP22; lane 2, no PRP22, RNA was denatured prior to the SDS/proteinase K incubation; lanes 3–8, 34, 10, 3.4, 0.1, 0.34 and 0.01 nM PRP22, respectively (prepared by method 2). Reactions were conducted under standard conditions with ~ 0.23 nM U4–U6 duplex for 10 min, except that 40 mM Tris (pH 8.0 at 23°C) and 50 mM KCl were substituted for the HEPES and KAc, respectively. (B) Time course of U4–U6 unwinding or U4 to U6 annealing with or without PRP22 (27 nM), as indicated, shown as the percentage of U4 and U6 monomers, calculated as described in Materials and methods. The total amount of duplex or individual monomers at time zero was 2.5 nM. U4 and U6 were prepared from the U4–U6 duplex sample by denaturation prior to the experiment.

was correlated with the amount of PRP22 preparation in the sample (Figure 5, lanes 3–8). At an ~ 10 -fold molar excess of PRP22 to the U4–U6 duplex, nearly quantitative conversion into monomers was observed over a 2 h period (Figures 5B and 6A). In contrast, little or no annealing of the U4 and U6 monomers (separated by boiling prior to the experiment) was detected in the presence of PRP22 and ATP (Figure 5B), suggesting that PRP22 is more active in disrupting the U4–U6 duplex than in annealing the monomers under these conditions.

The RNA-unwinding activity we have detected is novel, and another group did not detect similar activity with PRP2, a closely related protein (Kim *et al.*, 1992). Therefore, it was important to demonstrate convincingly that the recombinant PRP22, and not a contaminating *E. coli*-derived activity, catalyzed the RNA unwinding. At a 10-fold molar excess of prp22(K512E) to the U4–U6 substrate, no appreciable unwinding activity was detected over a 90 min period, in sharp contrast to the results with the purified PRP22 polypeptide (Figure 6A). Titration of mutant polypeptide preparations in single time point experiments revealed that no U4–U6 unwinding activity

Fig. 6. U4-U6 unwinding activity co-purifies with recombinant PRP22. **(A)** A time course experiment of RNA resolved by electrophoresis after unwinding reactions with 2.5 nM U4-U6. Reactions without recombinant protein, with PRP22 or with prp22(K512E) (27 nM each, indicated by a plus above the gel) were conducted in parallel, and portions of each were terminated at the indicated times. The RNA in the reaction shown in lanes 1 and 20 was denatured prior to the experiment. The prp22(K512E) protein was purified by method 3 up to the gel filtration step. **(B)** Silver-stained SDS-PAGE gel of the elution profile of purified PRP22 from an anion exchange column. PRP22 was purified by method 3. Twenty μ l of fractions in the range 160–230 mM KCl, shown numbered left to right, were applied to the gel. **(C)** MonoQ column elution profile of U4-U6 heteroduplex-unwinding activity in 10 min reactions using 10 μ l of each fraction (numbering corresponds to A). The portion of U4-U6 unwound in each 10 min reaction is shown (filled circles, left y-axis), as well as the theoretical concentration of KCl (dotted line, right y-axis). The concentration of PRP22 in the unwinding reaction using the peak fraction (fraction 6) was estimated to be $\sim$ 9 nM, the concentration of U4-U6 duplex at time zero was 0.47 nM.

was detected over a wide range of concentrations [100–10 nM prp22(K512E), data not shown]. Note that although the PRP22 and prp22(512K) proteins compared were purified by different protocols, PRP22 purified by the same protocol as used with the prp22(512K) preparation was active in RNA unwinding, both after the gel chromatography step (not shown) and after further puri-

fication (Figure 6C). Furthermore, a clear positive correlation was established between the elution of highly purified PRP22 (shown in a silver-stained SDS-PAGE gel of the fractions, Figure 6B) and U4-U6 RNA-unwinding activity (Figure 6C). Similarly, a strong correlation was found between the PRP22 concentration and the observed U4-U6 unwinding activity in the elution profile from an independent chromatographic separation procedure, gel filtration (not shown). Taken together, these results provide compelling evidence that the unwinding activity is linked directly to the PRP22 polypeptide.

RNA unwinding requires ATP hydrolysis

PRP22 and related proteins have been shown to be ATPases, and the ATPase-deficient prp22(K512E) protein is also deficient in RNA-unwinding activity (Figures 4A and 6A). Therefore, we next asked whether the observed unwinding reaction was linked to ATP hydrolysis. U4-U6 unwinding was not detected when nucleotides were omitted, or in the presence of ADP or the ATP analog AMP-PNP, in contrast to the results using ATP (Figure 7A). Furthermore, using the ATP analog, adenosine-5'-*O*-(3-thiotriphosphate) (ATP- γ -S), which is modified on the γ -phosphate, only a low, reproducible level of unwinding was detected (Figure 7A, bottom). A control reaction with the Mg^{2+} concentration increased from 1 to 3 mM above the ATP concentration demonstrated that the lack of unwinding observed in the presence of the ATP analogs was not due to potentially inefficient divalent cation chelation efficiency (Figure 7A, top). These results are consistent with the notion that the hydrolysis of γ -phosphate of ATP is required for the unwinding reaction.

An alternative interpretation of these results, however, is that ATP binding but not hydrolysis is required for the unwinding reaction and that the ATP analogs are deficient in PRP22 binding. If the latter hypothesis were correct, the prediction would be that the non-hydrolyzable analog would not compete with ATP in the unwinding reaction. To test this hypothesis, we lowered the ATP concentration in the reaction to subsaturation (50 μ M, 2-fold below the determined K_m) then tested the unwinding activity, which was measured by estimation of the initial rate of unwinding, r_0 . Compared with the reaction at 3 mM ATP (Figure 7A), the unwinding reaction was reduced in efficiency, but was easily detectable under these conditions. Reactions were also conducted in the presence of 50 μ M ATP and equimolar ATP- γ -S or AMP-PNP, a 10-fold molar excess of each ATP analog and a 100-fold molar excess of each analog. A concentration-dependent inhibitory effect was apparent with both AMP-PNP and ATP- γ -S, and this effect was, at least in part, due to a reduction of the initial reaction rate (Figure 7B). From these results, we conclude that both AMP-PNP and ATP- γ -S can compete with ATP as a cofactor in the unwinding reaction, and are thus able to bind to PRP22 in a manner that is competitive with ATP binding. Therefore, the results strongly suggest that the unwinding reaction requires ATP hydrolysis, rather than ATP binding *per se*, suggesting further that the PRP22 ATPase activity is linked directly to the unwinding reaction.

PRP22 unwinds a variety of RNA heteroduplexes

In addition to U4-U6, two RNA heteroduplexes were tested as potential substrates for PRP22-induced

Fig. 7. PRP22-induced RNA unwinding requires ATP hydrolysis. (A) Time course comparing U4–U6 unwinding in reactions with no cofactor or 2 mM ATP, AMP-PNP, ADP or ATP-γ-S, as indicated. Reactions were otherwise standard, except in a control reaction (above, Δ), where the MgCl₂ concentration was 5 mM; 27 nM PRP22 and 2.5 nM U4/U6 duplex were added to each reaction. (B) Effect of ATP analogs on the initial rate of ATP-dependent PRP22-induced U4–U6 unwinding. The initial rate of unwinding in the presence of 50 μ M ATP and 50 (1:1), 500 (1:10) or 5000 μ M (1:100) ATP-γ-S or AMP-PNP is shown as a percentage of the initial rate determined in the presence of 50 μ M ATP without ATP analogs (set at 100%). Initial rates were determined from at least four data points between 1 and 4 min after initiation of the reaction for the rapid reactions, and up to 30 min for the least rapid unwinding reactions.

unwinding. The first, substrate 2, has been used previously as a model substrate for RNA-unwinding proteins (Lee and Hurwitz, 1992; Gross and Shuman, 1996). Substrate 2 is predicted to form a structure with a 29 bp intermolecular duplex, surrounded by single-stranded regions on both the 3' and 5' sides. The second, substrate 3, is predicted to form an intermolecular duplex of 9 bp, with identical 33 nucleotide single-stranded regions 3' to the duplex on each molecule. Substrate 3 is a modified form of an

Fig. 8. PRP22-induced unwinding of two intermolecular RNA duplexes. (A) A time course experiment of substrate 2 RNA duplex (2.5 nM) unwinding (conducted as in Figure 6A, except that a reaction minus ATP, identified above the gel, was also included). (B) Unwinding experiment using 2.5 nM substrate 3 comparing five parallel reactions; 27 nM PRP22 or prp22(K512E) was added and the ATP omitted or the RNA denatured prior to the experiment, as indicated.

unwinding substrate used previously (Rozen *et al.*, 1990). Using either substrate, appreciable unwinding was detected in reactions containing ATP and PRP22, but not in reactions without PRP22, without ATP or with prp22(K512E) instead of PRP22 (Figure 8 and data not shown). The capacity to unwind substrate 3, that lacks single-stranded RNA regions located 5' to the intermolecular duplex, demonstrates that such regions are not a prerequisite for the unwinding reaction. Overall, these results demonstrate that PRP22 can unwind a variety of RNA duplex substrates.

Discussion

mRNA release from the spliceosome is PRP22 and ATP dependent

Our results establish that the release of mRNA from the spliceosome requires both active PRP22 and hydrolyzable nucleoside triphosphates. Thus, these experiments clearly link an ATP-dependent step in splicing, i.e. the release of mRNA following splicing catalysis (Brys and Schwer, 1996), with a protein now demonstrated to be an ATPase, PRP22. Taken together, these results suggest that the modulation of one or more intermolecular interactions is a consequence of ATP hydrolysis by PRP22 within the spliceosome. Therefore, the overall consequence of PRP22 ATPase activity is akin to that of the PRP2 and PRP16 ATPases in that ATP hydrolysis leads to a rearrangement of the spliceosome (Cheng and Abelson, 1987; Schwer and Guthrie, 1992a; Kim and Lin, 1996). Since these proteins share a remarkable degree of sequence homology, beginning in the central 'helicase' domain and continuing through the C-terminal portion of the protein (Chen and Lin, 1990; Company *et al.*, 1991; Schwer and Guthrie, 1991), it is likely that these structural rearrangements are catalyzed by a common mechanism. Formally, the rearrangements could involve RNA–RNA, RNA–protein or protein–protein interactions. We have now shown, however, that PRP22 exhibits the capacity to alter RNA–RNA interactions directly, thus suggesting that RNA unwinding is an intrinsic element of the function of this group of homologous spliceosomal proteins *in vivo*.

In eukaryotic cells, mRNA splicing is an integral component of gene expression that precedes the export of mature mRNA from the nucleus. In the effort to delineate the link between these processes, characterization of mRNA-containing complexes formed subsequent to splicing are of interest. Our experiments suggest a precursor–product relationship between the spliceosome and the mRNA-containing D complex identified previously (Company *et al.*, 1991). The procedure we have employed for partial purification of the mRNA containing 'blocked spliceosomes', followed by addition of a single protein (PRP22) and ATP to release mRNA into the electrophoretically distinct D complex should facilitate future characterization of this complex. Such characterization could shed light on the events of gene expression just downstream of pre-mRNA splicing.

PRP22 is an RNA-stimulated ATPase

Our demonstration that PRP22 exhibits the capacity to hydrolyze ATP is not surprising, as many proteins that possess the superfamily II 'helicase' domain have been shown previously to be ATPases (Gorbalenya *et al.*, 1989; Schmid and Linder, 1992; Kadare and Haenni, 1997). However, PRP22 ATPase activity unexpectedly displayed only a modest stimulation (2.5- to 4-fold) in the presence of RNA, in apparent contrast to the other spliceosomal DEAH-box proteins PRP16 and PRP2 (Schwer and Guthrie, 1991, 1992b; Kim *et al.*, 1992; Kim and Lin, 1993). To investigate this apparent difference, comparative quantitative data regarding the ATPase activity of PRP2, PRP16 and PRP22 would be required. In any event, although most of the investigated DEAD/H-box proteins show a higher ATPase stimulation by nucleic acids than

PRP22, there are several viral DEXH proteins whose ATPase activity is stimulated only weakly by nucleic acids (Kadare and Haenni, 1997). It is noteworthy that the RNA stimulation of the ATPase activity is due primarily to effects on k_{cat} , and not on K_m , indicating that RNA apparently stimulates ATP turnover rather than ATP affinity. This is particularly interesting in light of the fact that the K_m with respect to ATP for PRP22 is within the range of magnitude similar to that reported for related superfamily II helicases (e.g. see Lamm *et al.*, 1996 and references therein).

PRP22 unwinds RNA duplexes

A particularly novel result of our studies is that recombinant PRP22 exhibits RNA duplex-unwinding activity. That RNA-unwinding activity is likely to be a general feature of the DEAH-box proteins is supported by the recent observation that recombinant PRP16 also displays RNA duplex-unwinding activity (Wang *et al.*, 1998). In contrast, purified recombinant PRP2 failed to exhibit unwinding activity using a substrate that we show can be unwound by PRP22 (Kim *et al.*, 1992). However, the two sets of experiments are not directly comparable, since the experiments were conducted under different conditions. Although we show that PRP22 can modulate the structure of RNA, the mechanism of the observed RNA unwinding remains unclear. RNA duplex disruption could be the result of processive unwinding of the duplex involving translocation along the RNA strands of a protein complex akin to the DNA helicases (Lohman and Bjornson, 1996), or non-processive duplex destabilization through interaction of the protein with the RNA, or possibly another mechanism, such as an intramolecular RNA annealing reaction that disrupts intermolecular duplexes. Therefore, we have avoided the use of the term 'helicase', even though the activity we detect is indistinguishable presently from that of a number of the 'RNA helicases' (Schmid and Linder, 1992; Kadare and Haenni, 1997).

PRP22 is a member of the superfamily II 'helicases' (Gorbalenya *et al.*, 1989). Possession of this conserved set of amino acid motifs has had a predictive value for certain attributes, such as a capacity to bind nucleic acids and nucleoside triphosphates as well as nucleoside triphosphatase activity (Schmid and Linder, 1992; Kadare and Haenni, 1997). However, the predictive value has not extended to include RNA-unwinding activity, or more generally to predictions regarding the function of these proteins *in vivo*. The biological function of the RNA-unwinding proteins best characterized biochemically, most notably the translation initiation factor eIF4A (Rozen *et al.*, 1990) and the vaccinia virus protein NPH-II (Gross and Shuman, 1996), remains unclear. On the other hand, for RhlB, a component of the degradosome in *E.coli*, the one superfamily II 'helicase' protein where a clear function in cellular RNA metabolism has been demonstrated (Py *et al.*, 1996), RNA-unwinding activity has not been demonstrated directly. With PRP22, both RNA-unwinding activity and an ATP-dependent function in a cellular process (mRNA splicing) have now been shown. Thus, in the continuing effort to understand the specific functions of superfamily II 'helicases', PRP22 has now become an attractive model.

To date, we have not identified PRP22 RNA specificity,

either as a substrate for unwinding or as a cofactor for ATP hydrolysis. What then lends specificity to PRP22 activity in the spliceosome? One possibility is that specific RNA substrates exist that remain to be identified. Potential substrates are expected to be present in the spliceosome following the second chemical step. Since it is known that PRP22 activity is required for release of the mRNA, the simplest idea is that PRP22 unwinds an mRNA–snRNA interaction. In this respect, it will be of interest to determine the snRNA composition of the D complex. Second, the specificity of the postulated ATP-dependent RNA-unwinding reaction may be conferred by the context of the reaction, i.e. that the RNA substrate only comes in contact with the PRP22-unwinding domain at a specific time point during splicing. A third possibility is that the unwinding activity in the cell is regulated through inhibition, so that PRP22 is only derepressed at the appropriate time point in splicing. A better understanding of the direct interaction between PRP22 and other components of the spliceosome could help to distinguish between these alternatives.

What is the function of PRP22 *in vivo*? Several models are compatible with the available data. First, it is possible that the protein functions in a specific ‘isomerization’ of spliceosome structure. This type of function has been postulated in general for the group of DEAH-, DEAD- and DExH-box proteins known to be required for pre-mRNA splicing in budding yeast (Staley and Guthrie, 1998). A number of mutually exclusive base pairing interactions between the RNA components of the spliceosome have been identified and, therefore, it is attractive to hypothesize that the conversion of one set of RNA–RNA base pairing interactions into another is catalyzed by a specific RNA unwindase. Implicit in this model is the assumption that the ATP-driven activity of these proteins is highly specific, either to a specific ‘substrate’ or to a specific temporal event.

Second, a very different model is that PRP22 might have an essentially non-specific clearing function. This model posits that the PRP22-unwinding activity *in vivo* removes non-specific, inhibitory RNA–RNA base pairing. For example, PRP22 may be the catalytic component of a complex that ‘untangles’ the mRNA from other RNAs to effect the release of mRNA from the spliceosome. PRP2 and PRP16 each appear to serve an ‘activation’ role, since, in each case, the ATP-dependent function is clearly separable from the subsequent transesterification reaction (Horowitz and Abelson, 1993; Kim and Lin, 1996). By extension, each of these proteins could function to clear specific splicing signals on the pre-mRNA, so that they are available for binding by the temporally appropriate factors (either RNA or protein). This second model differs from the ‘isomerization’ model in that the specificity of the RNA duplexes that serve as substrates for unwinding *in vivo* is expected to be lower. A prediction of the clearance model is that the specificity of the unwinding reaction is governed by spatial context, suggesting that the unwindase is tethered to a specific location on the pre-mRNA through interaction with a specific sequence of RNA or a specific protein.

The observation that PRP22 can modify RNA duplexes directly is especially significant given the importance of RNAs in the spliceosome both as the substrate (pre-

mRNA) and as potential catalysts of the transesterification reactions (snRNA) (Moore *et al.*, 1993). Interestingly, the models presented lead to very different explanations for the presence of DEAH-box proteins in the spliceosome. In the ‘isomerization’ model, these proteins play a regulatory or guide function and therefore play a role in facilitating the progression of splicing or increasing splicing fidelity, as proposed for PRP16 (Staley and Guthrie, 1998). In contrast, the ‘blockage unwinding’ model suggests that these proteins are present in response to the vast sequence diversity of pre-mRNA substrates, as a ‘lubricant’ to dissociate non-productive RNA duplexes.

Materials and methods

Plasmids and nucleotides

The plasmid pCHF22, that contains an in-frame fusion of the entire open reading frame (ORF) of PRP22 to tandem octahistidine and Flag epitope tags (Trotta *et al.*, 1997), was constructed in the plasmid pET11a (Studier *et al.*, 1990). The plasmid pCHF22(K512E) was constructed using two-step PCR amplification with the appropriate mutagenic oligonucleotides and subsequent cloning of an *EcoRI*–*MscI* fragment from the amplification into pCHF22. The sequence of the entire region amplified by PCR was verified. NTPs (Ultrapure) were from Pharmacia; ADP, AMP-PNP and ATP- γ -S from Boehringer Mannheim.

RNA preparation

Poly(U) and poly(A) (from Aldrich) and 16S and 23S RNAs (Boehringer Mannheim) were used without further purification. Unlabeled RNAs were quantified spectrophotometrically at 260 nM. snRNAs were prepared by *in vitro* transcription with T7 RNA polymerase from linearized plasmids containing T7 promoters (Ghetti *et al.*, 1995). Substrate 2 monomers were synthesized from the plasmids pSP65, linearized with *XbaI*, and pGem1, linearized with *PvuII*, using SP6 RNA polymerase (Lee and Hurwitz, 1992). Transcripts were purified by gel electrophoresis in 7 M urea/polyacrylamide. Radiolabeled transcripts were labeled internally using [α - 32 P]UTP to a specific activity of ~5000 c.p.m./fmol. Concentrations were calculated by comparing the radioactivity of a portion of the transcription reactions with that of the purified RNA. Substrate 3 was composed of two RNAs synthesized using phosphoramidite chemistry, and end-labeled using polynucleotide kinase and [γ - 32 P]ATP. The sequence of the top strand of substrate 3 was AGCGUGCCGUGCAA-CAACAUUACAAUUUACAAUCCACCAUGG, and of the bottom strand was GCACGGCACGCUAACAACAAUUUACAAUUUACAAUCCACCAUGG. Substrate 3 duplex concentrations were determined using the specific activity of the single radiolabeled monomer, determined prior to duplex formation by comparison of the concentration (determined spectrophotometrically at 260 nM using the extinction coefficient $\epsilon = 4.17 \times 10^5$ l/mol*cm) and the radioactivity/unit volume. RNA duplexes were prepared by mixing the monomers in 10 mM Tris (pH 8.0 at 25°C), 1 mM EDTA and 100 mM KCl, heating to 95°C for 2 min, and slow cooling to 30°C. RNA duplexes were purified using non-denaturing polyacrylamide gel electrophoresis in 0.5× TBE. Following crushing, RNAs were eluted from polyacrylamide gel slices in 300 mM sodium acetate (pH 5.3 at room temperature), 5 mM EDTA. Duplex RNAs purified by this method typically contained monomers at 5–8% (U4–U6) or <5% (substrate 2 and substrate 3) of the total.

In vitro splicing

Yeast extracts were prepared from the strain SS330 and the *prp22-1* mutant strain derived from SS330 by chemical mutagenesis (Vijayaghavan *et al.*, 1989), essentially as described (Lin *et al.*, 1987). For heat treatment, extracts were adjusted with 2.5 μ l of HTM/4.0 μ l extract and incubated for 10 min at 37°C. HTM consisted of 5.6 mM MgCl₂, 2.0 mM dithiothreitol (DTT) and 5.2 U/ μ l RNasin (Promega). Radiolabeled actin precursor was synthesized as described (Cheng and Abelson, 1987). Splicing reactions were conducted for 1 h at 16°C using standard conditions (Cheng and Abelson, 1987), except for the presence of RNasin and the alterations described in the individual experiments. Native gel electrophoresis and recovery of RNA from the native gels was as described (Cheng and Abelson, 1987).

Gradient centrifugation and complementation assays

In vitro splicing was conducted in 50 µl reactions with 20 µl of splicing extract. Each reaction was layered onto a 4.6 ml 15–27% linear glycerol gradient, prepared in 40 mM KPO₄ (pH 7.3), 1.5 mM MgCl₂, 0.2 mM EDTA and 0.05% Triton X-100 (Yean and Lin, 1991). Centrifugation was for 2 h at 50 000 r.p.m. (250 000 g at r_{av}) in a SW 55Ti rotor (Beckman instruments). Fractions (~200 µl) were collected from the bottom of the tubes. Complementation assays from the gradient fractions were performed in a volume of 20 µl using 14 µl of the gradient fraction and 1 µl of yeast extract + HTM or protein sample in 28 mM KPO₄ (pH 7.3), 5.0 mM MgCl₂, 2.5% PEG 8000, 1 mM spermidine, 1 mM DTT, 0.14 mM EDTA and 0.035% Triton X-100 for 30 min at 23°C.

Protein purification

Recombinant protein was purified from BLR cells transformed with pET vector-based plasmids (Novagen). Cells were grown in 6 l of Luria broth supplemented with ampicillin (100 µg/ml) at 37°C to an OD₆₀₀ of 0.4–0.5. Isopropyl β-D-thiogalactopyranoside was added to 0.4 mM, and the cultures were shifted to ambient temperature (~23°C) for a 12–14 h incubation. Cells were collected by centrifugation, washed in 100 mM KCl, 10 mM Tris (pH 8.0) and 1 mM EDTA, resuspended in 50 ml of buffer A + 300 mM KCl and 5 mM imidazole (pH 7.3 at room temperature), and sonicated (3 × 30 s). Buffer A consists of 20 mM Tris (pH 7.3 at room temperature), 10% glycerol, 5 mM β-mercaptoethanol, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 20 µM leupeptin, 1 µM pepstatin and 2 µg/ml aprotinin. The lysate was centrifuged for 30 min at 40 000 r.p.m. in a 45Ti rotor (Beckman Instruments) and the resulting supernatant was applied to a 2 ml Ni-NTA column. The column was washed with 30 ml of buffer A + 300 mM KCl and 25 mM imidazole, and the recombinant protein was eluted in buffer A + 300 mM KCl and 120 mM imidazole. The protein was purified further using one of three methods. Method 1: the eluate from the Ni-NTA column was diluted with an equal volume of buffer A and applied to a 1 ml M2-agarose affinity column. The column was washed with 20 ml of buffer A + 150 mM KCl, and the bound protein was eluted with 5 ml of buffer A + 150 mM KCl and 342 µg/ml Flag peptide. The fractions containing the recombinant protein were pooled, dialyzed against three changes of storage buffer [100 mM KCl, 20 mM Tris (pH 8.0, at room temperature), 40% glycerol, 2 mM DTT] and stored at –20°C. The resulting PRP22 was judged to be >50% pure, with truncated versions of the protein constituting the major contaminants. Method 2: protein was purified by Ni-NTA and Flag epitope affinity chromatography as in method 1. The eluate from the Flag affinity column was then concentrated ~20-fold using a Centricon 100 concentrator (Amicon), and was applied directly to a 25 ml Superose 6 column (Pharmacia) equilibrated in buffer A + 200 mM KCl. The column was developed with buffer A + 200 mM KCl, and the eluate fractions containing the peak of PRP22 were pooled, dialyzed and stored as in method 1. Method 3: the affinity-tagged protein was partially purified by Ni-NTA chromatography as in method 1. The eluate fractions containing maximal PRP22 concentrations were pooled to yield a mix with >1 mg/ml total protein. Then a 0.727 vol. of 95% saturated ammonium sulfate solution (650 mg in 1 ml ddH₂O) was added, followed by stirring for an additional 30 min in an ice-water bath. The precipitate was collected by centrifugation and resuspended in 200 µl of buffer A. This sample was resolved on a Superdex 200 HR column (Pharmacia) equilibrated with buffer A + 200 mM KCl, and the fractions containing the PRP22 polypeptide peak were pooled. This pool was diluted 5-fold with buffer A, and applied to a 1 ml MonoQ column (Pharmacia) equilibrated with buffer A + 50 mM KCl. The column was washed with 30 ml of buffer A + 150 mM KCl, and developed with a 40 ml 150–550 mM linear KCl gradient (in buffer A). Individual fractions were dialyzed extensively against storage buffer and stored at –20°C. SDS-PAGE was conducted on 4–15% gradient minigels (Bio-Rad). PRP22 protein concentrations were determined by comparison with dilution series of bovine serum albumin (BSA) and other PRP22 preparations, and are believed to be accurate within a range ±30% of the given value.

ATPase assays

ATPase activity was determined using 10–20 µl reactions in 40 mM Tris (pH 7.5 at room temperature), 50 mM KCl, 3 mM MgCl₂, 1 mM DTT, 4 U/10 µl RNasin, and 0.05 mg/ml BSA at 23°C. ATP concentrations were either 2.5 or 500 µM; the recombinant PRP22 concentration was 22 nM. Reactions were assembled without ATP, pre-incubated for 2 min then initiated by adding the ATP. Aliquots were withdrawn at appropriate times and spotted onto a polyethylene imine-cellulose TLC plate

(20 × 10 cm). The TLC was developed in 0.75 M LiCl/1 M formic acid for 20 min (O'Day *et al.*, 1996). The radioactivity in the spots of ATP and P_i was quantified by radioanalytic scanning with a Packard Instant Imager. The amount of ATP hydrolysis was calculated by determining the fraction of inorganic phosphate at every time point. Time courses for multiple turnover reactions were constructed out of six time points, and measured in triplicate. Initial rates (r_0 values) were determined from a plot of the fraction of ATP hydrolyzed versus time using only early time points in the linear portion of the curve. Values for k_{cat} and K_m were approximated by using the following two relationships: $r_0 = E \cdot S_0 \cdot k_{cat} / K_m$ at subsaturating ATP concentrations (i.e. 2.5 µM ATP) and $r_0 = k_{cat} \cdot E$ at saturating substrate concentrations (i.e. 500 µM ATP), where E is enzyme concentration, S_0 is the initial ATP concentration and r_0 is the initial reaction rate. Saturation was checked by measuring r_0 at two different ATP concentrations (500 µM, as shown in Figure 4B, and 1 mM ATP) under four different reaction conditions [no RNA, U-35, U4–U6 and poly(U)]. For each reaction condition, r_0 values were indistinguishable within error. Subsaturating was evaluated by measuring r_0 at three low ATP concentrations (2.5, 7.5 and 30 µM) under the four reactions conditions described above. For a given reaction, r_0 values at subsaturation increased steeply and linearly with increasing [ATP].

Unwinding assays

Unwinding assays were conducted at 30°C in 20–30 µl of 40 mM HEPES (pH 8.0 at room temperature), 50 mM potassium acetate (pH 8.0 at room temperature), 3 mM MgCl₂, 2 mM ATP, 1 mM DTT, 4 U/10 µl RNasin (Promega), 0.05 mg/ml BSA and 2.5 nM duplex RNA, unless stated otherwise (Wang *et al.*, 1998). These conditions do not include the additional contributions from the protein storage buffer (see above), which comprised <4% of the final mix, except where noted. Within individual experiments, the amount of storage buffer added to each tube was equalized. As indicated in the figure legends, the RNA included in some reactions was separated into monomers by denaturation through incubation in boiling water for 2 min, followed by rapid cooling in ice-water. Reactions were set up on ice, pre-incubated without ATP (or other nucleotide controls) at 30°C for 2 min and then initiated by nucleotide addition (time zero). Reactions were terminated with an equal volume 2 × stop buffer (0.4% SDS, 20 mM EDTA, 2 mM DTT, 100 µg/ml proteinase K, 8% glycerol, bromophenol blue and xylene cyanol), and the tubes were incubated for 15–30 min at 37°C. In most experiments, the samples were then loaded directly onto the polyacrylamide gels, whereas in some U4–U6 time courses, the samples were frozen on dry ice and, following the completion of the time course, were incubated briefly at 37°C (~2 min), mixed and loaded onto the gel as a group. The results using these two methods were indistinguishable. Duplex and monomer RNAs were separated by gel electrophoresis in 0.5 × TBE, using polyacrylamide (30:1 polyacrylamide:bis ratio). Results were quantified using Imagequant software and a Molecular Dynamics PhosphorImager. The percentage unwinding was calculated using the formula: $\{[(\text{Monomers}/\text{Total})_t - (\text{Monomers}/\text{Total})_{t_0}] / [1 - (\text{Monomers}/\text{Total})_{t_0}]\} \times 100$ where 't' is the amount at time t, 't₀' the amount at time 0 and 'Total' the amount of monomers plus duplexes (Amaratunga and Lohman, 1993).

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