Prp22, a DExH-box RNA helicase, plays two distinct roles in yeast pre-mRNA splicing

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In order to assess the role of Prp22 in yeast pre-mRNA splicing, we have purified the 130 kDa Prp22 protein and developed an *in vitro* depletion/reconstitution assay. We show that Prp22 is required for the second step of actin pre-mRNA splicing. Prp22 can act on pre-assembled spliceosomes that are arrested after step 1 in an ATP-independent fashion. The requirement for Prp22 during step 2 depends on the distance between the branchpoint and the 3' splice site, suggesting a previously unrecognized role for Prp22 in splice site selection. We characterize the biochemical activities of Prp22, a member of the DExH-box family of proteins, and we show that purified recombinant Prp22 protein is an RNA-dependent ATPase and an ATP-dependent RNA helicase. Prp22 uses the energy of ATP hydrolysis to effect the release of mRNA from the spliceosome. Thus, Prp22 has two distinct functions in yeast pre-mRNA splicing: an ATP-independent role during the second catalytic step and an ATP-requiring function in disassembly of the spliceosome.

Keywords: ATPase/DExH-box/Prp22/RNA helicase/spliceosome

Introduction

Pre-mRNAs are spliced by two successive transesterification reactions. In the first step, the 2' OH of the branchpoint adenosine residue attacks the phosphodiester bond at the 5' splice site. This results in a 2'–5' branched lariat intermediate and a 3' OH terminated 5' exon. In step 2, the 3' hydroxyl of the 5' exon attacks the phosphodiester bond at the 3' splice site, resulting in exon ligation and expulsion of the lariat intron. Intron removal is catalyzed by the spliceosome, a complex particle formed by the ordered assembly of snRNPs (U1, U2, U4/6/5) and non-snRNP proteins onto the precursor RNA (Guthrie, 1991; Moore *et al.*, 1993). Conformational rearrangements of RNA–RNA and RNA–protein interactions are essential for faithful spliceosome assembly and for the catalytic activation of the splice sites (Madhani and Guthrie, 1994; Nilsen, 1994). Protein components of the splicing machinery, in particular those with associated enzymatic activities, coordinate these rearrangements.

The yeast splicing factors Prp2, Prp16, Prp22 and Prp43 are related DExH-box proteins that act at distinct stages during the splicing reaction (Lin *et al.*, 1987; Burgess

et al., 1990; Chen and Lin, 1990; Company *et al.*, 1991; Schwer and Guthrie, 1991; Arenas and Abelson, 1997). ATP hydrolysis by Prp2 is required for activation of the spliceosome prior to step 1 transesterification (Kim and Lin, 1993, 1996). Prp16 is a 121 kDa RNA-dependent NTPase that is essential for the second step (Schwer and Guthrie, 1991). Prior to step 2 transesterification, the 3' splice site needs to be identified and positioned for attack by the 3' terminal hydroxyl of the 5' exon. ATP hydrolysis by Prp16 elicits a conformational change in the spliceosome that results in protection of the 3' splice site from oligonucleotide-directed RNaseH cleavage (Schwer and Guthrie, 1992). This protection probably reflects the masking of the 3' splice site by protein(s) and/or RNPs prior to the second transesterification reaction. The Prp16-induced conformational change requires the recruitment of splicing factors Slu7, Prp18 and Ssf1 to the spliceosome (Ansari and Schwer, 1995; Brys and Schwer, 1996).

Slu7 and Prp18 function during step 2 in an ATP-independent fashion after Prp16 (Horowitz and Abelson, 1993a,b; Ansari and Schwer, 1995; Jones *et al.*, 1995). Although not essential for transesterification chemistry, the 29 kDa Prp18 protein cooperates with Slu7 in 3' splice site selection. Slu7 and Prp18 are dispensable for splicing of precursor RNAs *in vitro* when the 3' splice site (PyAG) is in close proximity to the branchpoint (<12 nucleotides), but are important when the branchpoint to 3' splice site distance is ≥ 12 nucleotides (Brys and Schwer, 1996; Zhang and Schwer, 1997). *In vivo*, when there is a choice of two competing 3' splice sites, a mutation in *SLU7* (*slu7-1*) reduces splicing to the branch-distal site without affecting the use of the branch-proximal site (Frank and Guthrie, 1992).

Prp16 interacts transiently with the spliceosome and dissociates upon ATP hydrolysis (Schwer and Guthrie, 1991; Brys and Schwer, 1996). In contrast, Slu7 remains in the spliceosome even after step 2 is completed. Spliceosome disassembly is an energy-dependent process (Sawa and Shimura, 1991; Brys and Schwer, 1996). Prp22 and Prp43 are implicated at this stage of the reaction, insofar as extracts from *prp22-1* and *prp43-1* strains catalyze pre-mRNA splicing, but mature mRNA or excised lariat intron, respectively, are retained in the spliceosomes (Company *et al.*, 1991; Arenas and Abelson, 1997).

In order to assess directly the function of Prp22 in yeast pre-mRNA splicing, we purified the 130 kDa Prp22 protein and characterized its biochemical activities. Prp22 exhibits RNA-stimulated ATPase and ATP-dependent RNA helicase activity. Prp22 uses the energy of ATP hydrolysis to effect the release of mRNA from the spliceosomes. *In vitro* reconstitution experiments revealed that Prp22 is a bona fide second step factor, i.e. the products of the first step accumulate in extracts that are immunodepleted of Prp22. This defect can be reversed by the addition of

Fig. 1. Prp22 is required for step 2 of splicing *in vitro*. Yeast whole cell extract was immunodepleted of Prp22 or mock-depleted and incubated with ^{32}P -labeled actin pre-mRNA. The reaction mixtures (60 μl) were supplemented with Prp22 protein (0.35 μg of the phosphocellulose preparation) where indicated by (+). Aliquots (10 μl) were withdrawn and halted after incubation for 0, 5, 10, 15 or 20 min at 22°C. The RNA products were analyzed by PAGE and autoradiography. The positions of the pre-mRNA and the products of steps 1 and 2 are indicated by the symbols on the left.

recombinant Prp22 protein. Prp22 functions during step 2 in an ATP-independent fashion. We show that Prp22 is necessary for step 2 only when the distance between the branchpoint and the 3' splice site is ≥ 21 nucleotides. We propose that Prp22 acts in concert with Slu7 and Prp18 to position the 3' splice site PyAG and the 3' OH terminus of the 5' exon for catalysis.

Results

Prp22 is required for the second step of splicing in vitro

In order to assess the role of Prp22 in yeast pre-mRNA splicing we established a depletion/complementation assay. Polyclonal antibodies against the N-terminal portion of the Prp22 polypeptide were affinity-purified and used to deplete Prp22 from yeast whole cell extract. Control extracts were mock-depleted in parallel. We compared the ability of these extracts to catalyze splicing of ^{32}P -labeled actin pre-mRNA (Lin *et al.*, 1985; Ansari and Schwer, 1995). Splicing occurred in mock-depleted extracts within 5 min and the amount of mRNA increased with time up to 20 min (Figure 1, lanes 11–15). Addition of recombinant Prp22 protein to the control extract did not affect the reaction kinetics (Figure 1, lanes 16–20). In extract depleted of Prp22, the products of step 1 (lariat intermediate and exon 1) accumulated and only a small amount of mature RNA was formed during the 20 min incubation

(Figure 1, lanes 1–5). The yield of mature RNA did not increase with longer incubations. However, when the ΔPrp22 extract was supplemented with purified recombinant Prp22 protein, the rate and extent of splicing were restored to wild type levels (Figure 1, lanes 6–10).

Prp22 acted on assembled spliceosomes, insofar as intermediates formed in ΔPrp22 extracts could be chased into mRNA upon addition of recombinant Prp22 (data not shown and Figure 5). The requirement for Prp22 could not be bypassed by the addition of Prp16 protein, nor could Prp18 or Slu7, added individually or in combination, restore the splicing activity of ΔPrp22 extract (not shown). These results demonstrate that the second step block is attributable to Prp22 depletion *per se*.

Prp22 is required for 3' splice site protection

At which stage during step 2 does Prp22 function? Preceding the cleavage/ligation reaction is a change in the accessibility of the 3' splice site RNA to oligo-directed RNaseH cleavage (Schwer and Guthrie, 1992). Normally lariat intermediates are rapidly converted to mRNA. In order to detect the conformational change, one uses a mutant actin precursor C303/305 (PyAG changed to PyAC) which cannot undergo the catalytic step (Vijayraghavan *et al.*, 1986). C303/305 precursor RNA was incubated in control extract (Δmock) or in extracts depleted of Prp22 (ΔPrp22) and lariat intermediates accumulated. A DNA oligonucleotide complementary to the

Fig. 2. Prp22 is required for protection of the 3' splice site. Mutant C303/305 precursor RNA (3' splice site UAG is changed to UAC; Vijayraghavan *et al.*, 1986) was spliced for 20 min at 22°C using mock-depleted or Δ Prp22 extract in 20 μ l reactions. To half of the reaction mixtures, an oligonucleotide complementary to the 3' splice site (5'-AGCAACGTGTAAACATAT) was added and the incubation continued for 10 min to allow cleavage of the RNA in the hybrid by endogenous RNaseH. The RNA products were analyzed by denaturing PAGE and visualized by autoradiographic exposure. The symbols on the left indicate the positions of the RNA species.

3' splice site segment was then added to the reaction mixtures (Figure 2, lanes 2 and 4). Endogenous RNaseH cleaved the RNA in the RNA-DNA hybrid. In the precursor RNA, the 3' splice site region was accessible to oligo-directed RNaseH cleavage, resulting in quantitative conversion of the pre-mRNA to a shorter RNA species (lanes 2 and 4). In control extracts, 54% of lariat intermediates were protected from targeted RNaseH cleavage (lane 2). In contrast, lariat intermediates formed in Δ Prp22 extract were cleaved completely upon the addition of oligo (lane 4). We surmise that Prp22 is required for protection of the 3' splice site.

The requirement for Prp22 in step 2 is dictated by the distance between the branchpoint (BP) and 3' splice site (3' SS)

We prepared a series of 32 P-labeled actin pre-mRNAs in which the length of the RNA segment between the BP and the 3' SS varied from 7 to 38 nucleotides. These pre-mRNAs were incubated in extracts depleted of Prp22 and the products analyzed (Figure 3). Act7 and Act9 precursors, in which the BP to 3' SS segments were 7 and 9 nucleotides long, respectively, were spliced efficiently. Act12 and Act15 showed intermediate levels of splicing; i.e. the amount of mRNA was reduced and intermediates accumulated. Splicing of precursor RNAs with longer BP to 3' SS intervals (21, 24, 27 and 38 nt) was blocked after the first step and lariat intermediates accumulated. The same pre-mRNAs were tested in extract depleted of Slu7 (Δ Slu7). Mature RNA was formed only when the BP to 3' SS distance was <12 nucleotides (Figure 3). We conclude that increasing the distance between the BP and 3' SS obligates the participation of Prp22 and Slu7. Slu7 is important when the distance is ≥ 12 nucleotides and Prp22 is necessary when the distance is ≥ 21 nucleotides. All the precursor RNAs were spliced efficiently in mock-depleted extracts and splicing was in all cases dependent on Prp16 (not shown).

Fig. 3. Prp22 requirement is distance-dependent. The length of the segment between the branchpoint (UACUAACA) and 3' splice site (UAG) sequences is given above each lane. Two fmoles each of Act7, Act9, Act12, Act15, Act21, Act24, Act27 and Act38 (wild type) actin precursor RNA were spliced in Δ Prp22 or Δ Slu7 extracts and the products were analyzed by denaturing PAGE and autoradiography. The symbols on the left indicate the position of the corresponding RNA species. Note that the size of the excised lariat varies according to the difference in the branchpoint to 3' splice site distance.

Mature RNA formed in Δ Prp22 extract is retained in spliceosomes

Previous studies suggested a role for Prp22 in the release of mRNA from the spliceosome (Company *et al.*, 1991). Does Prp22 directly mediate the release reaction and if so, is this function independent of its action during the second step? We asked whether mature Act7 mRNA formed in the absence of Prp22 was retained in spliceosomes (Figure 4). Act7 pre-mRNA was spliced in either mock-depleted extract or in Δ Prp22 extract. An aliquot of each reaction was withdrawn to assess splicing (Figure 4, I). The remaining reaction mixtures were sedimented by glycerol gradient centrifugation to separate splicing complexes. Aliquots of the gradient fractions were extracted and the RNA species analyzed (Figure 4). In mock-depleted extract, 96% of the mature mRNA was released and spliceosomal fractions contained only 4% of the total mRNA (Figure 4, upper panel). In contrast, 46% of Act7 mRNA formed in Δ Prp22 extract (Figure 4, lower panel) was present in spliceosomes. We attribute the 54% of 'released' mRNA to dissociation of spliceosomes during the extended period of centrifugation. These results show that although Prp22 is not required for step 2 of splicing of Act7, it is nonetheless important for release of mRNA from the spliceosome. The excised lariat intron also accumulated when the spliceosomes did not disassemble (compare lanes I in Figure 4).

Fig. 4. Glycerol gradient sedimentation of splicing complexes. Uniformly ^{32}P -labeled Act7 precursor RNA was spliced in control extract (upper panel) or ΔPrp22 extract (lower panel) for 25 min at 22°C (200 μl reaction). Aliquots (10 μl) were withdrawn (I) and the remaining 190 μl were sedimented in a 15–40% glycerol gradient for 12 h at 35 000 r.p.m. and 4°C . Fractions (0.4 ml) were collected from the top of the gradient. Aliquots (100 μl) of the even numbered fractions were extracted and analyzed by denaturing PAGE. The labeled RNA was visualized by autoradiography. The amount of mRNA in the released and spliceosome fractions (indicated by the bars) was quantitated by scanning of the dried gels using a STORMER PhosphorImager (Molecular Dynamics).

The role of Prp22 in step 2 is ATP-independent

Are the two functions of Prp22 in splicing—its role in step 2 and in the release of mRNA—dependent on ATP? We addressed this question by: (i) depletion of ATP from the splicing reaction and (ii) complementation by a mutant version of Prp22 (K512A) in which the lysine residue in the conserved ATP-binding motif (GxGKT) was replaced by alanine. The mutant allele *prp22(K512A)* could not sustain growth of a Δprp22 null strain (not shown). Actin pre-mRNA was incubated in ΔPrp22 extract to allow the formation of lariat intermediate and exon 1. Then, glucose was added and ATP was depleted by endogenous hexokinase (Horowitz and Abelson, 1993a). The reaction mixture was split and supplemented with ATP, recombinant wild type Prp22 or K512A proteins. After 5 min of incubation, aliquots were withdrawn in order to (i) assess the RNA species in the reaction mixtures (Figure 5, Input), (ii) test if Prp22 protein was associated with spliceosomes (Figure 5, IP) and (iii) assay release of mRNA from the spliceosome (Figure 5, non-denaturing gel). RNA intermediates were converted to mRNA in the absence and presence of ATP, when either Prp22 or K512A protein was added (Figure 5, lanes 2, 3, 5 and 6). We conclude that neither ATP nor the ability of Prp22 to hydrolyze ATP efficiently (see below) is required to relieve the second step block incurred by Prp22 depletion.

Aliquots of the reconstituted reactions were subjected

to immunoprecipitation by $\alpha\text{-Prp22}$ antibodies (Figure 5, lanes 7–12). In the absence of ATP, splicing products were precipitated by $\alpha\text{-Prp22}$ when either wild type or mutant protein had been added (Figure 5, lanes 8 and 9). When the reactions were supplemented with ATP, wild type Prp22 was no longer associated with the products of the splicing reaction, whereas the K512A protein remained bound (Figure 5, compare lanes 11 and 12). These results indicate that Prp22 binds to the spliceosome to promote step 2 and remains bound until it hydrolyzes ATP, at which point it dissociates. (The fact that pre-mRNA was not precipitated as well as the products of the reaction attests to the specificity of the IPs.)

Does dissociation of Prp22 from the spliceosome coincide with the release of mRNA from the spliceosomes? Aliquots of the reconstituted reactions were resolved by native gel electrophoresis (Figure 5, lanes 13–18). Splicing complexes of low electrophoretic mobility (complex A), were formed (Brys and Schwer, 1996). The faster migrating complex C2 (which contained mature mRNA) was detected when the reactions were supplemented with ATP plus wild type Prp22 protein (Figure 5, lane 17). We conclude that ATP and enzymatically active Prp22 protein were necessary to release mature mRNA (and Prp22 protein) from the spliceosome. Given this strict correlation, we surmise that Prp22 itself is responsible for the ATP-requirement in mRNA release.

Prp22 has ATPase and RNA unwinding activity

Prp22 is a member of the DEXH-box family and most closely related to Prp16 and Prp2 (Company *et al.*, 1991). Prp16 and Prp2 both have RNA-dependent NTPase activities (Schwer and Guthrie, 1991; Kim *et al.*, 1992). We investigated the enzymatic activities of wild type Prp22 and the mutant version K512A. The recombinant proteins, which were purified from bacterial extracts by Ni-agarose and phosphocellulose chromatography, were further purified by glycerol gradient sedimentation. This yielded homogeneous preparations of Prp22 and K512A proteins (Figure 6A and B). Aliquots of the gradient fractions were tested for the ability to catalyze the hydrolysis of ATP in the presence of an RNA cofactor, poly(C). A single peak of ATPase activity was detected (Figure 6C) that coincided with the abundance of the Prp22 protein (Figure 6A and B, fractions 14 and 16). In the linear range of enzyme dependence, 1 pmol of Prp22 protein hydrolyzed 28 pmol of ATP per second. Activity in the absence of an RNA cofactor was 25–33% of the RNA-stimulated level. The specific activity of Prp22-K512A was 15% that of wild type Prp22.

DEXH-box proteins are often referred to as helicases or putative helicases (Schmid and Linder, 1992). In order to assess whether Prp22 has RNA unwinding activity, glycerol gradient fractions were incubated with a partially duplex RNA substrate (a 29 bp duplex RNA with 3' and 5' single-stranded tails) in the presence of ATP (Lee and Hurwitz, 1992; Gross and Shuman, 1995). Gradient fractions containing wild type Prp22 (fractions 14 and 16) displayed helicase activity (Figure 6D). This was manifest as the displacement of the labeled RNA strand from the tailed duplex structure to generate a more rapidly migrating free strand. No unwinding activity was detected in glycerol gradient fractions containing K512A protein (not shown).

Fig. 5. Wild type actin pre-mRNA was spliced in ΔPrp22 extract for 20 min at 22°C (400 μl reaction). Then glucose (to final 2.5 mM) was added to allow depletion of ATP by endogenous hexokinase for 7 min (Horowitz and Abelson, 1993a). To 200 μl of the reaction mixture, ATP (plus Mg^{2+}) was added to 4 mM and aliquots of 60 μl each were supplemented with wild type Prp22 or K512A proteins (0.35 μg each) as indicated by +. The incubation was continued for 5 min. After this time 10 μl aliquots were analyzed by denaturing PAGE (Input, lanes 1–6), 40 μl were used for immunoprecipitation using anti-Prp22 antibodies bound to ProteinA-Sepharose beads (IP $\alpha\text{-prp22}$, lanes 7–12) and 10 μl aliquots were adjusted with heparin and analyzed on a native agarose-acrylamide composite gel (non-denaturing gel, lanes 13–18) (Goodwin and Dahlberg, 1982; Brys and Schwer, 1996). (The identity of the RNA species in complex C2—mature mRNA—was confirmed by denaturing PAGE upon elution of complex C2.)

The vaccinia RNA helicase NPH-II served as a positive control (Shuman, 1992; Gross and Shuman, 1995). Heat-denaturation of the duplex indicates the position of the liberated strand. In the linear range of enzyme dependence, 1 fmol of RNA was unwound by 14 fmol of Prp22 protein. At comparable protein concentrations, K512A did not unwind the duplex substrate (Figure 6E). The unwinding activity of Prp22 was strictly ATP-dependent (Figure 6E).

Discussion

This study describes the characterization of the 130 kDa DEXH-box splicing factor Prp22 and provides evidence for two separate functions of this protein in the splicing reaction. Earlier experiments using extracts from the mutant *prp22-1* strain had implicated Prp22 in the release of mRNA from the spliceosome (Company *et al.*, 1991). We now find that immunodepletion of Prp22 arrests splicing after step 1, resulting in the accumulation of lariat intermediate and 5' exon. Purified recombinant Prp22 protein restores full splicing activity. Several lines of evidence suggest that the observed requirement for Prp22 during step 2 *in vitro* is not a consequence of the inability to recycle second step components when mRNA release is blocked: (i) depletion of ATP, which prevents disassembly of spliceosomes, does not interfere with the formation of mature mRNA in ΔPrp22 extracts; (ii) the K512A protein satisfies the requirement for Prp22 in step 2, but is non-functional in mRNA release; (iii) purified Prp16, Prp18 and Slu7 proteins do not complement ΔPrp22 extracts, indicating that these factors are not rate-limiting in these extracts; (iv) addition of twice as much ΔPrp22 extract to arrested ΔPrp22 spliceosomes does not result in conversion of the intermediates to mature mRNA. How-

ever, it remains possible that Prp22 contributes to step 2 by releasing a limiting factor in an ATP-independent fashion or by activating another step 2 factor.

prp22 alleles have been identified in genetic screens for temperature-sensitive splicing mutants (Vijayraghavan *et al.*, 1989; Noble and Guthrie, 1996). In mutant strains, lariat intermediates and lariat intron accumulate at non-permissive temperatures (Noble and Guthrie, 1996; Arenas and Abelson, 1997). Our finding that Prp22 plays a role in step 2 of splicing *in vitro* explains this aspect of the *in vivo* RNA phenotypes of *prp22* strains.

Prior to the second transesterification reaction, the 3' splice site needs to be identified and positioned for attack by the 3' terminal group of the 5' exon. *In vitro* studies have defined two stages during step 2, which are distinct in their requirement for ATP and protein factors. The first stage requires Prp16 and is ATP-dependent. The second stage, which is ATP-independent, involves Slu7, Prp18 and, as shown here, Prp22. Prp22, Slu7 and Prp18 are not essential for the transesterification chemistry, because precursor RNAs in which the 3' splice site is immediately adjacent to the branchpoint can be spliced efficiently in the absence of Prp22, Prp18 or Slu7. Increasing the BP to 3'SS spacing to ≥ 12 nucleotides mandates the participation of Slu7 and Prp18. Prp22 is required when the distance is ≥ 21 nucleotides. For those introns in which the 3' cleavage site is close to the active center of the spliceosome (presumed to reside at the branchpoint at step 1), the Prp16-induced conformational change is sufficient to rearrange the active site so that the reactants for step 2 are positioned correctly. Proximity to the branch site appears to be a major determinant for 3' splice site selection, at least *in vitro*. If presented with a choice of two potential 3' splice sites, the splicing machinery utilizes

Fig. 6. Glycerol gradient sedimentation of the wild type Prp22 and mutant K512A proteins. (A) and (B) Coomassie Blue-stained gels. Aliquots (20 μ l) of the indicated glycerol gradient fractions were analyzed by SDS-PAGE. The asterisk at the left marks the position of the wild type Prp22 (A) and mutant K512A (B) proteins. The positions and sizes (in kDa) of marker proteins are indicated on the right. (C) ATPase activity. Aliquots (2 μ l) of the indicated glycerol gradient fraction were assayed for ATPase activity in the presence of poly(C) cofactor. ATPase activity is expressed as nmoles of 32 Pi released from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ during 30 min incubation at 37°C. (WT: fractions 14 and 16 contained 142 and 88 ng Prp22 protein, respectively. K512A: fractions 14 and 16 contained 68 and 100 ng of mutant protein, respectively.) (D) RNA unwinding activity by wild type Prp22 protein. Aliquots of gradient fractions were incubated with the RNA substrate in the presence of ATP (Lee and Hurwitz, 1992; Gross and Shuman, 1995). Unwinding is measured as displacement of a 32 P-labeled 38 nt single-stranded RNA. Heat-denaturation (95°C for 5 min followed by cooling on ice) of the duplex RNA shows the position of the displaced ssRNA (Δ). The RNA helicase NPH-II from vaccinia virus was used as a positive control (Shuman, 1992; Gross and Shuman, 1995). Enzyme was omitted in reactions marked (-). (E) Unwinding depends on the amount of Prp22 protein and on the cofactor ATP. Decreasing amounts of wild type Prp22 (17.8, 8.9 and 4.4 ng in lanes 3–5) and mutant protein K512A (17, 8.5 and 4.3 ng in lanes 6–8) were assayed for RNA unwinding in the presence of ATP. Wild type Prp22 protein was incubated with the substrate in the presence (+, lane 9) and absence (-, lane 10) of ATP.

the first PyAG downstream of the branchpoint (Teigelkamp *et al.*, 1995).

Prp16 and ATP hydrolysis are essential for splicing of all precursors tested. If the distance between the branchpoint and the 3' splice site is short, the PyAG is located within the spliceosome and is therefore not accessible to targeted RNaseH cleavage (Rymond *et al.*, 1987; our unpublished results). If the distance is longer, a change in the protection of the 3' splice site can be measured in response to Prp16-catalyzed ATP hydrolysis and the

recruitment of additional protein factors. Because Slu7, Prp18 and Prp22 are all required for protection of the 3' splice site, we suggest that the protection is a consequence of binding of these factors to the spliceosome, thereby forming a molecular bridge between the branch and the 3' splice site. This may entail direct binding to the lariat intermediate RNA or binding to other spliceosomal components. Recently, a two-hybrid interaction between Prp22 and ScSF1/BBP was reported (Fromont-Racine *et al.*, 1997). ScSF1/BBP (*Saccharomyces cerevisiae* SF1/

branchpoint bridging protein) functions early in spliceosome assembly and is required for commitment to complex formation (Arning *et al.*, 1996; Abovich and Rosbash, 1997). It directly contacts the branchpoint sequence and bridges the 5' and 3' end of the intron by protein-protein interactions (Abovich and Rosbash, 1997; Berglund *et al.*, 1997). However, the finding that ScSF1/BBP could not be detected in splicing complexes other than the early commitment complex (Abovich and Rosbash, 1997) calls into question whether the ScSF1/BBP-Prp22 interaction is contributing to Prp22 action on spliceosomes arrested after step 1.

Genetic and biochemical evidence argues that Slu7, Prp18, Prp16 and the U5 snRNP cooperate in the process of 3' splice site selection (reviewed by Umen and Guthrie, 1995b). For example, Prp18, which interacts physically and genetically with Slu7, facilitates Slu7 function in splicing (Frank *et al.*, 1992; Jones *et al.*, 1995; Zhang and Schwer, 1997). Slu7, Prp16 and the U5 snRNP-specific Prp8 protein can be crosslinked to the 3' splice site segment of the RNA (Umen and Guthrie, 1995a). Prp22 has RNA-binding capacity, because ATP hydrolysis by Prp22 is stimulated by an RNA cofactor and Prp22 is an RNA helicase. Apart from the highly conserved motifs found in members of the DExH-box family and implicated in the enzymatic activities, Prp22 contains a putative RNA-binding motif originally identified in bacterial ribosomal protein S1 and *Escherichia coli* polynucleotide phosphorylase (Company *et al.*, 1991). This motif is conserved in the human homolog of Prp22, HRH1 (Ono *et al.*, 1994).

Splicing of pre-mRNAs is an energy-dependent process. ATP hydrolysis is required for spliceosome assembly, for the two catalytic steps, and for release of mature mRNA from the spliceosome (reviewed by Krämer, 1996; Ruby and Abelson, 1991). ATP hydrolysis by Prp2 and Prp16 is required for the first and second catalytic steps, respectively (Schwer and Guthrie, 1991; Kim *et al.*, 1992; Kim and Lin, 1993; Plumpton *et al.*, 1994). Prp22, like Prp2 and Prp16, is an ATPase whose activity is stimulated by an RNA cofactor. All three of these proteins act on the spliceosome and dissociate after hydrolyzing ATP (Schwer and Guthrie, 1991; Kim and Lin, 1993; Teigelkamp *et al.*, 1994). The energy of ATP hydrolysis elicits conformational changes in the spliceosome (Schwer and Guthrie, 1992; Kim and Lin, 1996). In the case of Prp22, the phosphohydrolase reaction drives the release of mRNA from the spliceosome. Disassembly of a residual complex after mRNA release may require Prp43 (Arenas and Abelson, 1997).

The DExH-box proteins *Drosophila* maleless (MLE), mammalian RNA helicase A and vaccinia virus NPH-II are well-characterized RNA helicases (Kuroda *et al.*, 1991; Lee and Hurwitz, 1992, 1993; Shuman, 1992; Lee *et al.*, 1997) that display sequence similarity to Prp2, Prp16 and Prp22 beyond the highly conserved ATP-binding motifs (Company *et al.*, 1991). While helicase activity has not been reported for Prp2 or Prp16 (Kim *et al.*, 1992), Prp22 can unwind duplex RNA in an ATP-dependent fashion. We speculate that the helicase activity of Prp22 disrupts RNA-RNA interactions that hold the spliceosome together. For example, Prp22 may break contacts made by U5 snRNA with exon sequences adjacent to the splice sites (Newman and Norman, 1992; Sontheimer and Steitz,

1993; Newman *et al.*, 1995; Newman, 1997). Spliceosome disassembly makes the mature mRNA available for export to the cytoplasm (Ohno and Shimura, 1996) and permits the recycling of factors that may be rate-limiting *in vivo*. The finding that the K512A mutant, which is compromised in its enzymatic activities, is lethal *in vivo* suggests that the ATPase and/or RNA unwinding functions of Prp22 are essential.

Materials and methods

Anti-Prp22 serum

Antiserum was raised against the N-terminal portion of Prp22 (amino acids 1–480). Immunization was performed at Pocono Hill Rabbit Farm Laboratories (Canadensis, PA). Polyclonal anti-Prp22 serum was affinity-purified on Affigel 10 resin (Bio-Rad) to which the Prp22(1–480) polypeptide had been coupled according to the manufacturer's protocol. Eluted antibodies were dialyzed against PBS, the final concentration was 0.8 mg/ml. Immunodepletions were performed as described (Ansari and Schwer, 1995), using 12 µg of antibodies to deplete 120 µl of yeast whole cell extract.

Expression of Prp22 in bacteria

pET16b-PRP22 was constructed by ligating the *PRP22* gene (+1 to +3438) into pET16b (Novagen), thus fusing Prp22 to an N-terminal leader peptide containing 10 histidines. The mutation in K512A was created by PCR mutagenesis and a 215 bp *AccI*-*BalI* fragment was replaced in the wild type gene. (The replaced fragment was sequenced in its entirety to confirm the mutation and ensure that no additional mutations were generated by PCR amplification.) pET16b-PRP22 and pET16b-Prp22(K512A) were transformed into *E.coli* strain BL21(DE3). Cultures (1.5 l) were grown at 37°C in LB medium containing 0.1 mg/ml ampicillin until the A600 reached 0.8. The cultures were chilled on ice for 30 min and then isopropyl β-D-thiogalactopyranoside (IPTG) was added to 0.4 mM. Cells were incubated overnight at 18°C and harvested by centrifugation. The cell pellets were stored at –80°C.

Purification of Prp22

All operations were performed at 4°C. The cell pellet was suspended in 420 ml of buffer A (50 mM Tris pH 7.5, 250 mM NaCl, 10% sucrose). Lysozyme was added to 0.2 mg/ml and the suspension gently stirred for 40 min. The suspension was adjusted to 0.1% Triton X-100. Insoluble material was removed by centrifugation for 40 min at 18 000 r.p.m. in a Sorvall SS-34 rotor. The supernatant (360 mg protein) was incubated with 4 ml of Ni-NTA-agarose resin (Qiagen) on a nutating platform mixer for 1 h. The resin was recovered by centrifugation and then subjected to repeated cycles of washing with buffer A. The resin was suspended in 10 ml of buffer E (50 mM Tris pH 7.5, 250 mM NaCl, 10% glycerol) containing 10 mM imidazole, poured into a column and washed with a total of 10 column volumes of the same buffer. Adsorbed material was eluted stepwise with buffer E containing 20, 50, 100 and 500 mM imidazole, and 1.5 ml fractions were collected. Elution of the Prp22 protein was monitored by SDS-PAGE analysis of the column fractions. Prp22 was recovered in the 100 mM imidazole eluate (9.5 mg protein). An aliquot (2.5 mg protein) was diluted with buffer D (50 mM Tris pH 7.5, 2 mM DTT, 1 mM EDTA, 10% glycerol) to adjust the NaCl concentration to 50 mM. The suspension was applied to 1 ml of phosphocellulose resin (equilibrated in buffer D containing 50 mM NaCl) and washed extensively with the same buffer. Adsorbed material was eluted stepwise (2 ml each) with buffer D containing 100, 200, 300 and 500 mM NaCl. Prp22 was recovered in the 300 mM NaCl eluate (1.5 mg protein). (The yield of K512A protein in a comparable purification was 0.8 mg.) An aliquot was dialyzed against buffer D containing 50 mM NaCl prior to use in the splicing reaction. The protein concentrations (wild type and mutant K512A) were adjusted to 0.35 mg/ml. Protein concentrations were determined using the Bradford dye reagent (Bio-Rad), with bovine serum albumin (BSA) as the standard.

Glycerol gradient

Aliquots (0.25 ml) of the phosphocellulose eluates (190 µg of wild type Prp22 and 100 µg of K512A) were applied to 4.8 ml 15–30% glycerol gradients containing 0.3 M NaCl in buffer B (50 mM Tris-HCl pH 8.0, 2 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100) and then centrifuged for 17 h at 50 000 r.p.m. in a Beckman SW50 rotor

at 4°C. Fractions (0.18 ml) were collected from the bottom of the tube. The concentrations of the Prp22 proteins were determined by SDS-PAGE analysis of a 2-fold dilution series of the protein in parallel with known amounts of BSA. The gels were stained with Coomassie Blue and the extent of dye binding to Prp22 and BSA was quantitated by densitometric scanning of the lanes. The concentrations of Prp22 and K512A were calculated by extrapolation to the BSA standard curve.

Glycerol gradient sedimentation of splicing complexes

Splicing of Act7 pre-mRNA was carried out using mock-depleted or Δ Prp22 extract in 200 μ l reactions. 10 μ l of each reaction were saved for analysis (Figure 4, I) and 190 μ l were layered onto a 11.5 ml 15–40% glycerol gradient containing 20 mM HEPES–KOH pH 6.5, 2 mM $MgCl_2$, 100 mM KCl. After centrifugation for 12 h at 35 000 r.p.m. in a Sorvall TH-641 rotor at 4°C, 0.4 ml fractions were collected from the top. Aliquots (100 μ l) of the even numbered fractions were extracted and the RNA species analyzed by denaturing PAGE and visualized by autoradiography. The amounts of mRNA in the fractions were quantitated by scanning the dried gels with a STORMER PhosphorImager (Molecular Dynamics).

ATPase assay

ATPase activity was assayed in the presence of poly(C) RNA cofactor as the release of ^{32}P i from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Conway and Lipmann, 1964). Reaction mixtures (50 μl) containing 40 mM Tris-HCl pH 8.0, 2 mM dithiothreitol, 4 mM MgCl_2 , 1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 0.2 mM poly(C) (expressed as concentration of nucleotide) and enzyme as specified were incubated for 30 min at 37°C.

RNA helicase substrate

Radiolabeled dsRNA helicase substrate was prepared as described (Gross and Shuman, 1995). In brief, the component RNA strands were transcribed *in vitro* from linear plasmid DNA templates using SP6 RNA polymerase. The 38-mer strand 5'-GAUACACGGAAUUCGAGCU-CGCCCCGGGAUCCUCUAG was radiolabeled to high specific activity with [α^{32} P]GTP. The partially complementary 98-mer strand 5'-GAAUA-CAAGCUUGGGCUGCAGGUCGACUCUAGAGGAUCCCCGGCGC-AGCUCGAAUUCGGGUCUCCCUAUAGUGAGUCGUAUUAUUU-UCGAUAAGCCAG was labeled with [α^{32} P]GTP at 300-fold lower specific activity. The 29 bp duplex region is underlined. The transcription reaction products were subjected to polyacrylamide-urea gel electrophoresis; radiolabeled transcripts were localized by autoradiography and recovered from excised gel slices. Strand annealing was performed at a 3:1 molar ratio of 98-mer to 38-mer. The tailed RNA duplexes were then purified by native gel electrophoresis (Lee and Hurwitz, 1992).

Helicase assay

Reaction mixtures (20 μ l) contained 40 mM Tris-HCl pH 8.0, 2 mM DTT, 2 mM $MgCl_2$, 2 mM ATP, and 25 fmol of [$\alpha^{32}P$]GMP-labeled standard dsRNA substrate. After incubation for 15 min at 37°C, the reactions were halted by addition of 5 μ l of 0.1 M Tris-HCl pH 7.4, 5 mM EDTA, 0.5% SDS, 50% glycerol, 0.1% xylene cyanol, 0.1% bromophenol blue. Aliquots (20 μ l) were electrophoresed at 15 mA constant current through an 8% polyacrylamide gel containing 0.5 $\times$ TBE. Labeled RNAs were visualized by autoradiographic exposure. The extent of unwinding (displaced RNA/total RNA) was quantitated by scanning the gel using a Fuji BAS1000 Phosphorimager.

Splicing substrates

We used pT7-ACT₉, pT7-ACT₁₂ and pT7-ACT₁₅, described previously (Brys and Schwer, 1996) to insert a linker of 12 nucleotides into the *Clal* site in the actin gene (Ng and Abelson, 1980). This yielded pT7-ACT₂₁, pT7-ACT₂₄ and pT7-ACT₂₇. The plasmids were cleaved with *Hind*III and run-off transcripts synthesized using T7 RNA polymerase (Boehringer). The RNA segments between the branch point UACUAACA and 3' splice site UAG were UCGUUCUUCUUCCGAUUUA in Act21, UCGUUCUUCUUCCGAUUUGUUA in Act24 and UCGUUCUUCUUCCGAUUUAUGUUUA in Act27.

Splicing extract preparation, splicing reactions, immunodepletions and immuno-precipitations were carried out as described (Ansari and Schwer, 1995; Brys and Schwer, 1996). The sequence of the oligonucleotide used in Figure 2 (targeted RNaseH cleavage) is : 5'-AGCAACG-TGTAACATAT.

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