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The splicing factor PRP2, a putative RNA helicase, interacts directly with pre-mRNA

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The RNA helicase-like splicing factor PRP2 interacts only transiently with spliceosomes. To facilitate analysis of interactions of PRP2 with spliceosomal components, PRP2 protein was stalled in splicing complexes by two different methods. A dominant negative mutant form of PRP2 protein, which associates stably with spliceosomes, was found to interact directly with pre-mRNAs, as demonstrated by UV-crosslinking experiments. The use of various mutant and truncated pre-mRNAs revealed that this interaction requires a spliceable pre-mRNA and an assembled spliceosome; a 3′ splice site is not required. To extend these observations to the wild-type PRP2 protein, spliceosomes were depleted of ATP; PRP2 protein interacts with pre-mRNA in these spliceosomes in an ATP-independent fashion. Comparison of RNA binding by PRP2 protein in the presence of ATP or γS-ATP showed that ATP hydrolysis rather than mere ATP binding is required to release PRP2 protein from pre-mRNA. As PRP2 is an RNA-stimulated ATPase, these experiments strongly suggest that the pre-mRNA is the native co-factor stimulating ATP hydrolysis by PRP2 protein in spliceosomes. Since PRP2 is a putative RNA helicase, we propose that the pre-mRNA is the target of RNA displacement activity of PRP2 protein, promoting the first step of splicing.

Key words: pre-mRNA splicing/RNA helicase/RNA-protein interaction/UV-crosslinking/yeast

Introduction

The removal of introns from eukaryotic RNA transcripts involves two transesterification reactions that occur in a large ribonucleoprotein complex, the spliceosome, consisting of the small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U5 and U4/U6 and non-snRNP proteins (reviewed in for example Green, 1991; Guthrie, 1991; Moore *et al.*, 1993).

This process involves multiple RNA-RNA interactions that change in a specific temporal order as splicing progresses, indicating that the spliceosome is a highly dynamic structure. Interactions of the U1 RNA with the 5′ splice site (Zhuang and Weiner, 1986; Siliciano and Guthrie, 1988) and of the U2 RNA with the branchpoint sequence (Parker *et al.*, 1987; Wu and Manley, 1989; Zhuang and Weiner, 1989) have been extensively characterized in *Saccaromyces cerevisiae* and in mammalian systems. In *Schizosaccaromyces pombe*, but not in *S.cerevisiae*, U1 RNA interacts with both the 5′ splice site and the conserved AG at the 3′ splice site (Reich *et al.*, 1992; Seraphin and Kandels-Lewis, 1993). Newman and Norman (1991, 1992) demonstrated in elegant genetic experiments in *S.cerevisiae* that a phylogenetically conserved U5 RNA loop interacts with exon sequences both at the 5′ and 3′ splice sites. Their experiments suggest that the U5 snRNA might be involved in the fine tuning of 5′ splice site definition. The U5 RNA-5′ splice site interaction was confirmed by UV-crosslinking experiments in a HeLa nuclear extract (Wyatt *et al.*, 1992). Furthermore, this interaction appears to shift to intron sequences after U1 RNA has dissociated from the 5′ splice site region to give way to the U5 RNA (Wassarman and Steitz, 1992). U6 RNA has been shown to interact with 5′ splice site intron sequences in yeast (Sawa and Abelson, 1992) and mammalian splicing reactions *in vitro* (Sawa and Shimura, 1992). Most of these interactions seem to occur before step 1 of splicing. The extensive base-pairing between the U4 and U6 RNAs is probably the best characterized snRNA-snRNA interaction (Bringmann *et al.*, 1984; Hashimoto and Steitz, 1984; Siliciano *et al.*, 1987). In *S.cerevisiae*, destabilization of the U4/U6 helix makes a region of the U6 RNA, that was previously base-paired to U4 RNA, available to interact with a sequence in U2 RNA immediately upstream of the U2-branchpoint base-pairing region (Madhani and Guthrie, 1992). Another base-pairing of U6 RNA with the 5′ terminal sequence of U2 RNA was detected in the mammalian system (Hausner *et al.*, 1990; Datta and Weiner, 1991; Wu and Manley, 1991). Thus the U6 RNA is in close proximity to both pre-mRNA sequences partaking in the first transesterification reaction. It has been suggested that the U6 RNA might contribute to the catalytic core of the spliceosome (Guthrie, 1989), activated by the dissociation of U4 RNA.

Although the two cleavage-ligation reactions in theory do not require the utilization of energy, nuclear pre-mRNA splicing is an ATP-consuming process. Irrespective of the fact that the cleavage-ligation reactions might be RNA catalysed, the assistance and enzymatic activity of *trans*-acting protein factors is required to accomplish splicing (reviewed in e.g. Green, 1991; Moore *et al.*, 1993). Some of these factors interact directly with the pre-mRNA, and many protein splicing factors containing conserved RNA binding domains have been described (reviewed in Kenan *et al.*, 1991; Mattaj, 1993; Moore *et al.*, 1993).

Five of the yeast PRP (**p**recursor **R**NA **p**rocessing) protein splicing factors are members of a rapidly expanding family of putative RNA helicases termed DEAD/H-box proteins (reviewed in Fuller-Pace and Lane, 1992; Schmid and Linder, 1992): PRP5 and PRP28 are DEAD-box proteins; PRP2, PRP16 and PRP22 form the subgroup of yeast

DEAH-box proteins (Company *et al.*, 1991; Wassarman and Steitz, 1991). The prototype of the DEAD superfamily is the translation initiation factor eIF-4A (reviewed in Rhoads, 1988; Merrick, 1992). It contains eight conserved primary sequence motifs that define this protein family. Since all DEAD/H proteins share these conserved primary sequence motifs it is likely that they are functionally related, that is they might be associated with RNA displacement activity. Several of the DEAD/H-box proteins, including PRP2 and PRP16, exhibit RNA-stimulated ATPase activity *in vitro* (Schwer and Guthrie, 1991; Fuller-Pace and Lane, 1992; Kim *et al.*, 1992).

Although no RNA helicase-like activity has been reported so far for any of the five DEAD/H splicing factors, they are excellent candidates to promote RNA conformational shifts and/or RNA displacement events during eukaryotic pre-mRNA splicing. PRP5 protein is involved in the interaction of U2 snRNP with the pre-mRNA (Dalbadie-McFarland and Abelson, 1990), the first ATP-requiring step in spliceosome assembly. The detection of a genetic interaction of *PRP28* with *PRP24*, which encodes a U6 RNA binding protein, led to the suggestion that PRP28 protein may destabilize the U4–U6 interaction (Strauss and Guthrie, 1991).

The DEAH-box protein splicing factors PRP2, PRP16 and PRP22 are involved in three consecutive steps occuring after spliceosome assembly. PRP2 is a 100 kDa protein (Lee *et al.*, 1986) required for promotion of the first cleavage–ligation reaction (Lin *et al.*, 1987), consistent with the fact that PRP2 interacts transiently with spliceosomes prior to and during step 1 of splicing (King and Beggs, 1990). In addition, ATP hydrolysis is required at this step (Kim and Lin, 1993). The fact that PRP2 is an RNA-stimulated ATPase (Kim *et al.*, 1992) suggests that PRP2 is one of the ATP-consuming factors at this step of splicing. Like PRP2, PRP16 is an RNA-stimulated ATPase; the ATP hydrolysis activity of PRP16 is required to promote step 2 of splicing (Schwer and Guthrie, 1991) and a conformational rearrangement in the spliceosome (Schwer and Guthrie, 1992a,b). Alleles of *prp16* have been identified as suppressors of an A to C branchpoint mutation (Couto *et al.*, 1987). It was speculated that the ATPase activity of PRP16 protein may be linked to a putative proofreading function of this protein in the fidelity of branchpoint recognition (Burgess *et al.*, 1990; Burgess and Guthrie, 1993). Finally, PRP22 protein is responsible for the release of the spliced RNA from spliceosomes after completion of the splicing reaction (Company *et al.*, 1991).

Since two of the three DEAH-box splicing factors are demonstrated RNA-stimulated ATPases (PRP2 and PRP16), it is conceivable that they require direct RNA contact within the spliceosome to perform their function in splicing. However, as the association of PRP2 and PRP16 with spliceosomes is highly transient, it is difficult to detect their interactions with spliceosomal components. To study PRP2–pre-mRNA interactions, we used (i) a dominant negative mutant (Herskowitz, 1987) form of the PRP2 protein which stably (i.e. non-transiently) associates with spliceosomes but does not support RNA splicing (Plumpton *et al.*, 1994) and (ii) wild-type PRP2 protein stalled in spliceosomes due to the absence of ATP. With both systems, we show that PRP2 protein interacts directly with the pre-mRNA before step 1 of splicing. This interaction is highly specific in that it requires a spliceable pre-mRNA and an assembled spliceosome. The initial PRP2–pre-mRNA contact is ATP-independent, but ATP hydrolysis is required to release the PRP2 protein from the pre-mRNA. The interaction of PRP2 with the pre-mRNA together with ATP hydrolysis promote the first transesterification reaction.

Results

A dominant negative mutant form of the PRP2 protein can be UV-crosslinked to pre-mRNA

The dominant negative PRP2 protein ($PRP2^{dn1}$) prevents the function of wild-type PRP2 protein ($PRP2^{wt}$) and results in the accumulation of stalled spliceosomes (Plumpton *et al.*, 1994). The gel system used for separation of splicing complexes (Pikielny *et al.*, 1986) resolves three discrete complexes termed I, II and III according to their increasing electrophoretic mobility. Complexes assemble in the order III, I and II, with complex II containing the intermediates and products of a splicing reaction. Titration experiments with splicing extract containing overproduced $PRP2^{dn1}$ protein showed that a 1:8 dilution was sufficient to stall RNA processing in a wild-type splicing extract (data not shown), causing splicing complex I to accumulate but no complex II (Figure 1A, lane 1).

Samples were irradiated with short wavelength UV light and digested with RNase T1. Following immunoprecipitation with rabbit anti-PRP2 polyclonal antibodies under non-denaturing conditions the immunoprecipitate was analysed by SDS–PAGE. Several radiolabelled bands were recovered (Figure 1B, lane 4), corresponding to proteins that had been UV-crosslinked to the pre-mRNA. Among these bands was one of the size of PRP2. To confirm the identity of this protein, immunoprecipitations were carried out under denaturing conditions (Figure 1B, lane 3). Denaturation fully disrupted non-covalent interactions of spliceosomal components, indicated by the breakdown of splicing complexes analysed by native gel electrophoresis (Figure 1A, lane 2). Analysis of the immunoprecipitate by SDS–PAGE, Western blotting and immunostaining with anti-PRP2 antiserum demonstrated efficient immunoprecipitation of $PRP2^{dn1}$ protein under both denaturing and non-denaturing conditions (Figure 1C, lanes 3 and 4). Immunoprecipitation with pre-immune serum did not recover any PRP2 protein (Figure 1C, lane 5), demonstrating the specificity of the anti-PRP2 antibodies.

After denaturation of the sample, the only radiolabelled protein immunoprecipitated was PRP2 (Figure 1B, lane 3). Thus we conclude that the $PRP2^{dn1}$ protein was UV-crosslinked to the pre-mRNA. Additional radiolabelled bands immunoprecipitated under non-denaturing conditions most likely represented RNA-crosslinked proteins non-covalently associated, either directly or indirectly, with PRP2. PRP2 protein was efficiently precipitated from a non-UV-irradiated sample (Figure 1C, lane 2). In this case the protein was not radiolabelled (Figure 1B lane 2), indicating that the 100 kDa immunoprecipitated radiolabelled protein in lanes 3 and 4 (Figure 1B) was the $PRP2^{dn1}$ protein UV-crosslinked to the pre-mRNA. These results demonstrated that the $PRP2^{dn1}$ protein was in direct contact with the pre-mRNA.

Similar experiments were carried out using a different pre-mRNA (rp51A) and produced identical results (Figure 2),

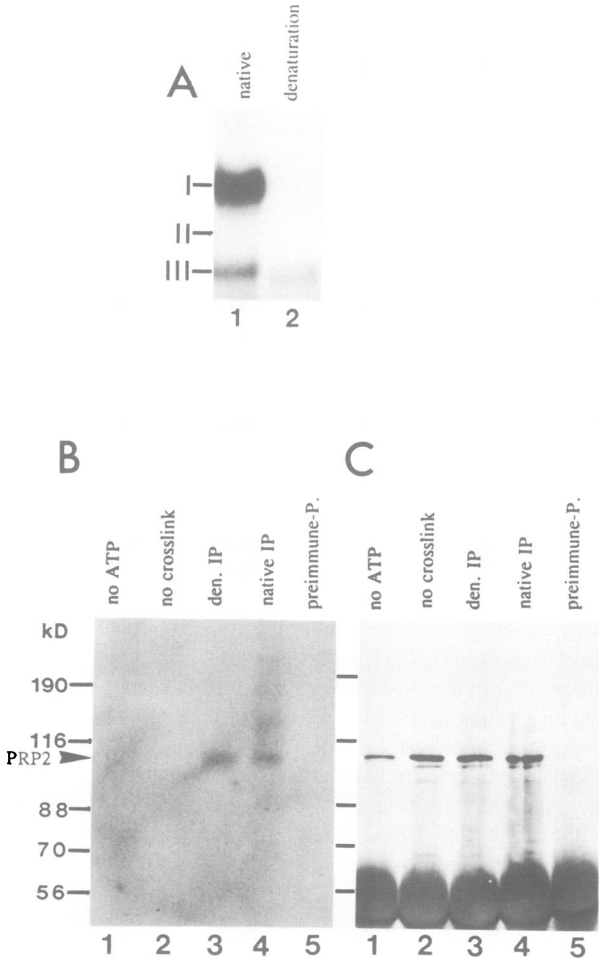

Fig. 1. UV-crosslinking of dominant negative PRP2 protein to rp28 pre-mRNA. (**A**) A 1:8 mixture of dominant negative PRP2 splicing extract – wild-type splicing extract containing uniformly ^{32}P-labelled rp28 pre-mRNA was incubated under splicing conditions. Subsequently, splicing complex formation (lane 1) and disruption of complexes by denaturation (lane 2) were analysed by native gel electrophoresis. The position of splicing complexes I, II and III are indicated on the left. (**B**) Identically assembled splicing reactions were irradiated with short wavelength UV light, digested with RNase T1 and subjected to immunoprecipitation with anti-PRP2-specific antibodies. The immunoprecipitates were analysed by SDS–PAGE followed by autoradiography to detect UV-crosslinked protein. Immunoprecipitation of UV-crosslinked dominant negative PRP2 protein is shown in a splicing reaction assembled without ATP (lane 1), without UV-crosslinking (lane 2), under denaturing (lane 3) and non-denaturing (lane 4) conditions and with preimmune serum (lane 5). The migration of protein molecular weight markers is indicated on the left, as is the position of the PRP2 protein. (**C**) Aliquots of these samples were analysed by western blotting and detection with anti-PRP2-specific antibodies. The numbering of the samples is as in panel B.

indicating that the detected interaction was not particular to the rp28 pre-mRNA.

The PRP2–pre-mRNA interaction requires a spliceable RNA and an assembled spliceosome

The interaction of PRP2^{dn1} protein with the pre-mRNA was not detected in control samples lacking ATP (Figures 1B and 2, lane 1). This indicated either a direct ATP requirement for the PRP2–RNA interaction or the need for an assembled spliceosome, as spliceosome assembly itself is ATP-dependent. In order to test these possibilities, mutant pre-

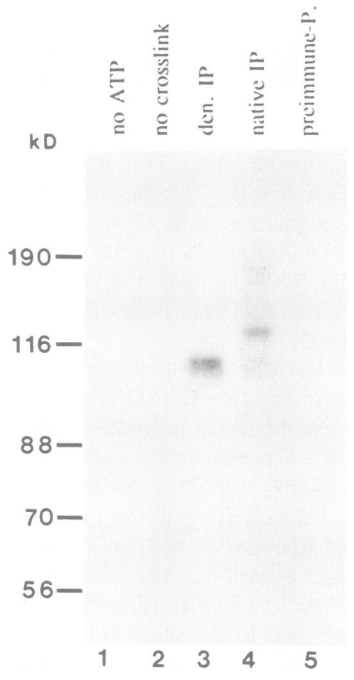

Fig. 2. UV-crosslinking of dominant negative PRP2 protein to rp51A pre-mRNA. Treatment of the samples and numbering is as described in Figure 1B, except that uniformly ^{32}P-labelled rp51A pre-mRNA was used in this experiment.

mRNAs were used in an *in vitro* splicing reaction containing the PRP2^{dn1} protein: (i) rp28 G>A (at the 5′ splice site, the conserved G at position 1 of the intron is changed to A), (ii) rp28 A>C (in the branchpoint consensus sequence UACUAAC, the branch-nucleotide (bold) is changed from A to C), (iii) rp51A(5′-0) (contains a 5′ splice site deletion), (iv) rp51A(Δ3b) (contains a branchpoint deletion). These mutant substrate RNAs are incapable of forming splicing complexes and therefore are not spliced *in vitro* (our observations; Pikielny and Rosbash, 1986). None of the mutant pre-mRNAs could be UV-crosslinked to PRP2 protein (Figure 3, lanes 2, 3, 5 and 6), indicating that a spliceable RNA and assembled spliceosomes were required for the interaction of PRP2 protein with the pre-mRNA.

The PRP2–pre-mRNA interaction does not require a 3′ -splice site

To determine the requirements for the PRP2–pre-mRNA interaction in more detail, UV-crosslinking of PRP2^{dn1} protein to truncated rp51A substrate RNAs was carried out. Full-length rp51A RNA was truncated by targeting RNase H cleavage with DNA oligonucleotides complementary to two specific regions between the branchpoint and the 3′ splice site (Figure 4A) as described previously (Rymond *et al.*, 1987; Whittaker and Beggs, 1991). Due to an insufficient RNA length downstream of the branchpoint region, oligoA-cleaved RNA is not spliced *in vitro* and assembles only splicing complex I, whereas oligoC-cleaved rp51A RNA proceeds efficiently through step 1 of splicing *in vitro*, producing splicing complex II (Rymond *et al.*, 1987; Whittaker and Beggs, 1991). OligoA-truncated rp51A pre-mRNA was clearly co-immunoprecipitated with anti-PRP2-specific antibodies from an *in vitro* splicing reaction containing PRP2^{dn1} protein (Figure 4B, lane 3), although

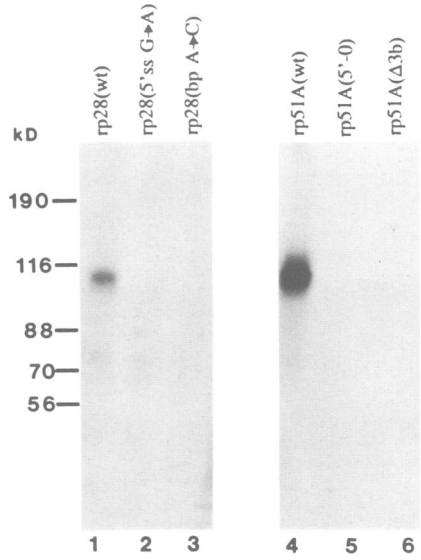

Fig. 3. UV-crosslinking of dominant negative PRP2 protein to mutant pre-RNAs. Treatment of the samples was as described in Figure 1B. Dominant negative PRP2 protein was UV-crosslinked to ^{32}P-labelled wild-type pre-mRNAs rp28 (lane 1) and rp51A (lane 4), to rp28 RNA containing a 5′ splice site G to A substitution (lane 2) and to rp28 RNA containing a branchpoint A to C substitution (lane 3), to rp51A(5′-0) RNA containing a 5′ splice site deletion (lane 5) and to rp51A(Δ3b) RNA containing a branchpoint deletion (lane 6).

less efficiently than oligoC-truncated rp51A pre-mRNA (0.5-fold, as estimated by dilution experiments; Figure 4B, lanes 4 and 5). This indicated that PRP2 protein was associated with spliceosomes assembled on both types of RNA. However, analysis of PRP2^{dn1}–pre-mRNA interactions by UV-crosslinking revealed that PRP2^{dn1} did not detectably interact with an oligoA-truncated rp51A RNA (Figure 4C, lane 3). Thus, in splicing complexes containing this truncated rp51A pre-mRNA, PRP2^{dn1} protein was associated with spliceosomal factors other than the pre-mRNA. In contrast, PRP2^{dn1} was efficiently crosslinked to oligoC-cleaved rp51A RNA, detectable even at a 1:8 dilution (Figure 4C, lanes 4–7), indicating that a 3′ splice site was not required for this interaction.

The wild-type PRP2 protein can be UV-crosslinked to pre-mRNA in an ATP-independent fashion

It was important to determine whether the results obtained with the dominant negative PRP2^{dn1} protein reflected the behaviour of the wild-type PRP2^{wt} protein. To investigate this, we used a system similar to that developed by Kim and Lin (1993) to stall PRP2^{wt} protein in assembled spliceosomes. *In vitro* splicing extract containing a temperature-sensitive mutant form of the PRP2 protein was heat-inactivated and then allowed to form spliceosomes upon the addition of ATP and pre-mRNA. Due to the heat-inactivation, the temperature-sensitive prp2 protein does not interact with spliceosomes at all (King and Beggs, 1990; PRP2Δ spliceosomes). Subsequently, incubation with glucose depleted the extract of ATP due to the endogenous hexokinase activity, and gel filtration removed residual glucose and nucleotides. This provided assembled spliceosomes depleted of PRP2 and of ATP.

Analysis of the splicing activity of these spliceosomes demonstrated that they could not process pre-mRNA into spliced mRNA (Figure 5A, lane 1), consistent with the fact

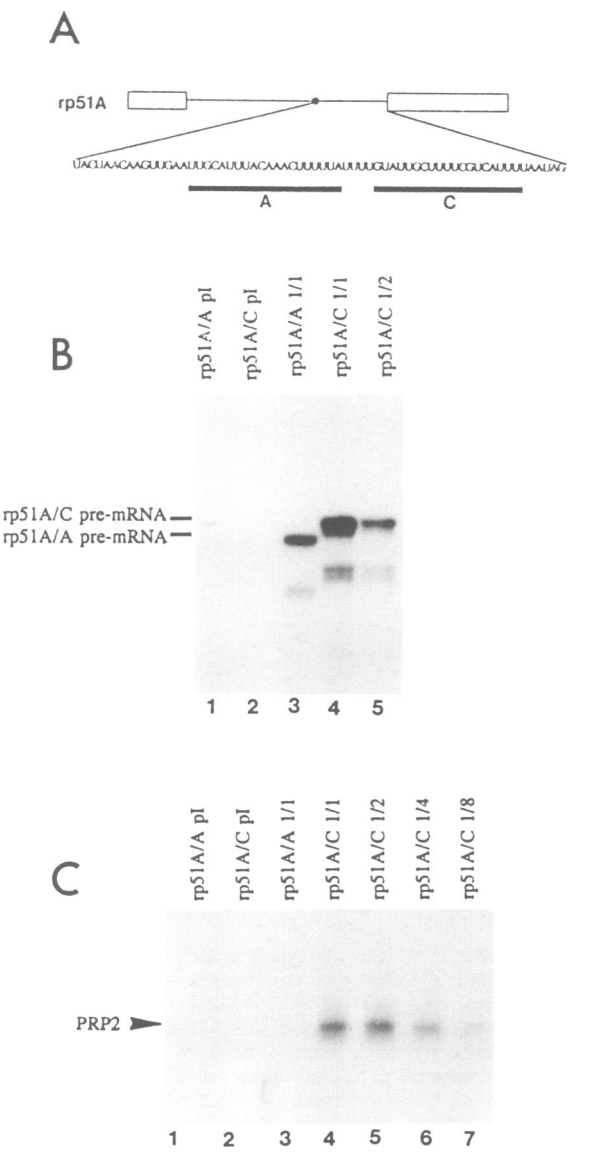

Fig. 4. UV-crosslinking of dominant negative PRP2 protein to 3′-truncated ^{32}P-labelled rp51A pre-mRNA. (**A**) Schematic presentation of the rp51A pre-mRNA; the sequence between the branchpoint and the 3′ splice site is enlarged. Thick lines indicate the region of hybridization of the DNA oligonucleotides A and C to target sequence-specific cleavage by RNase H. (**B**) A 1:8 mixture of dominant negative PRP2 splicing extract–wild-type splicing extract containing oligonucleotide A or C was incubated under splicing conditions, but without ATP, to allow sequence-specific cleavage of the rp51A pre-mRNA by endogenous RNase H. Spliceosome formation was initiated by addition of ATP and followed by immunoprecipitation with anti-PRP2 antibodies. Co-immunoprecipitated rp51A RNA was analysed on a 6% denaturing polyacrylamide gel. Precipitation with preimmune serum of oligoA-truncated (lane 1) or oligoC-truncated (lane 2) rp51A pre-mRNA and co-immunoprecipitation with anti-PRP2 antibodies of oligoA-truncated rp51A pre-mRNA (lane 3) and oligoC-truncated pre-mRNA (lane 4) is shown. Lane 5 shows a 1:2 dilution of an immunoprecipitate identical to lane 4 for comparison. The positions of oligoA-truncated (rp51A/A) and oligoC-truncated (rp51A/C) pre-mRNAs are indicated on the left. (**C**) For UV-crosslinking, aliquots of the samples in panel B were treated after spliceosome formation as described in Figure 1B. Precipitation with preimmune serum (lanes 1 and 2) or anti-PRP2 antibodies (lanes 3–7) following UV-crosslinking in the presence of oligoA-truncated rp51A RNA (lanes 1 and 3) or oligoC-truncated rp51A RNA (lanes 2 and 4) is shown, with dilutions of reactions identical to lane 4, diluted 1:2 (lane 5), 1:4 (lane 6) and 1:8 (lane 7) for comparison. The position of PRP2 protein is indicated on the left.

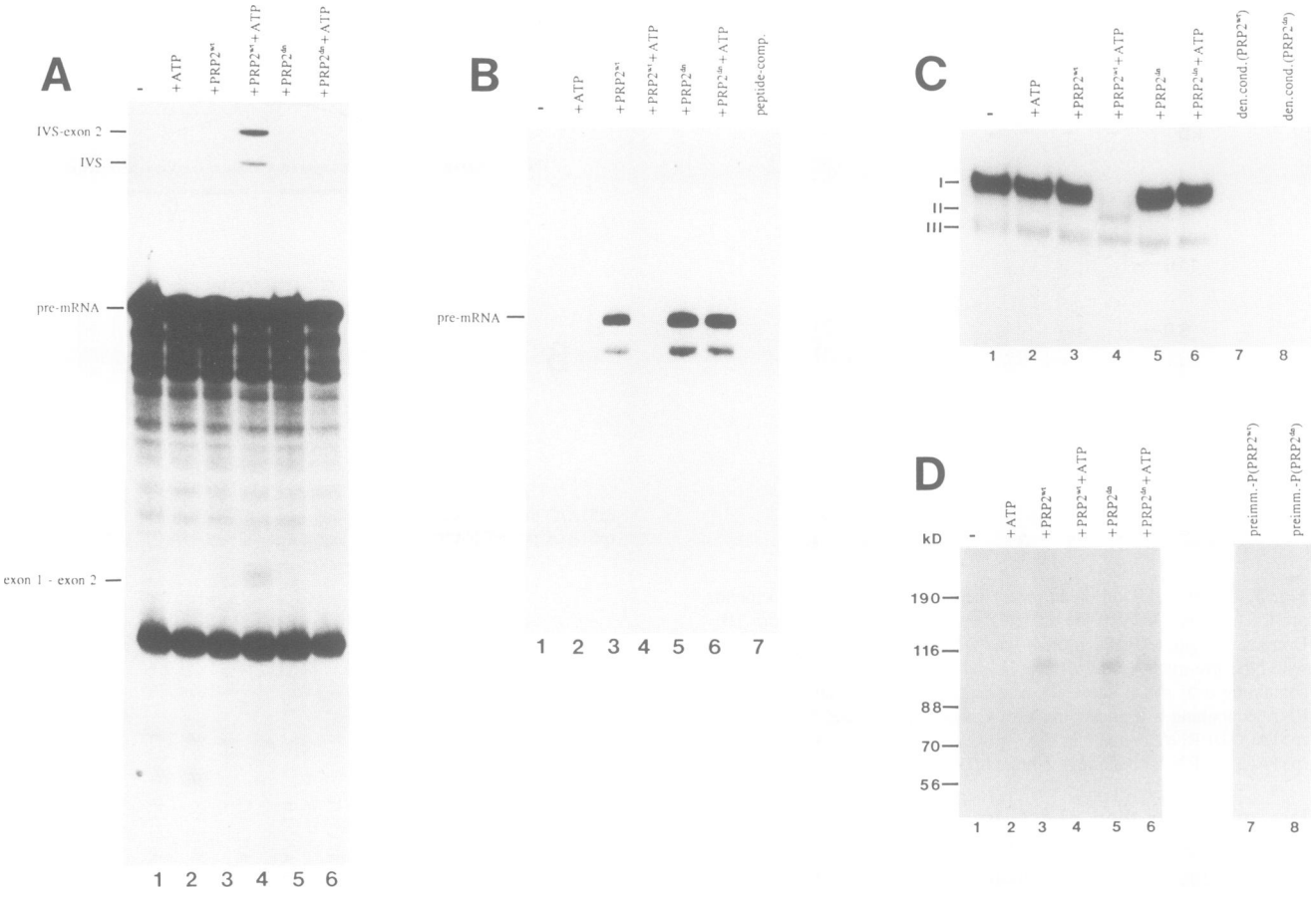

Fig. 5. Interaction of purified wild-type and dominant negative PRP2 protein with PRP2Δ spliceosomes and UV-crosslinking to rp28 pre-mRNA. (**A**) ATP-depleted PRP2Δ spliceosomes containing uniformly ^{32}P-labelled rp28 pre-mRNA were incubated alone (lane 1) and in the presence of ATP (lane 2), purified wild-type PRP2 protein (lane 3), purified wild-type PRP2 protein plus ATP (lane 4), purified dominant negative protein (lane 5) and purified dominant negative protein plus ATP (lane 6). The RNA was fractionated on a 6% denaturing polyacrylamide gel. The positions of the pre-mRNA, the intervening sequence (IVS)−exon 2 intermediate and the IVS and exon 1−exon 2 products are indicated. The free exon 1 intermediate is too small to be seen on this gel. (**B**) Aliquots of the samples in panel A were subjected to immunoprecipitation with anti-PRP2 specific antibodies. Co-immunoprecipitated rp28 pre-mRNA was analysed on a 6% denaturing polyacrylamide gel. In the negative control, prior to immunoprecipitation the PRP2 antibodies were blocked with the fusion peptide to which the antibodies were raised (lane 7). (**C**) Aliquots of the samples in panel A were analysed for splicing complex formation by non-denaturing PAGE. Disruption of the complexes formed in the presence of wild-type PRP2 protein (lane 7) and dominant negative PRP2 protein (lane 8) prior to denaturing immunoprecipitation of UV-crosslinked PRP2 (see Figure 5D) was assessed. (**D**) PRP2 protein was UV-crosslinked to rp28 pre-mRNA in aliquots of the samples in panel A and analysed as described in Figure 1B. Precipitation with preimmune serum was carried out in the presence of wild-type PRP2 protein (lane 7) and dominant negative PRP2 protein (lane 8).

that they formed splicing complex I but no splicing complex II (Figure 5C, lane 1). Addition of ATP or purified PRP2^{wt} protein alone was not sufficient to initiate splicing and complex II formation (Figure 5A and C, lanes 2 and 3), and due to the lack of ATP, PRP2^{wt} protein remained bound to spliceosomes, as indicated by co-immunoprecipitation of pre-mRNA with anti-PRP2-specific antibodies (Figure 5B, lane 3). Both ATP and PRP2^{wt} protein were required to promote splicing (Figure 5A, lane 4) and the formation of splicing complex II (Figure 5C, lane 4). Following initiation of splicing, PRP2^{wt} protein dissociated from spliceosomes, as demonstrated by the failure to co-immunoprecipitate pre-mRNA with anti-PRP2 specific antibodies (Figure 5B, lane 4). In contrast, the purified PRP2^{dn1} protein, as a consequence of its mutational defect, was unable to promote step 1 of splicing even in the presence of ATP (Figure 5A and C, lanes 6) and remained bound to spliceosomes (Figure 5B, lanes 5 and 6).

Analysis of a direct interaction of the PRP2 protein with the pre-mRNA by UV-crosslinking and subsequent immunoprecipitation revealed that both the PRP2^{wt} and the PRP2^{dn1} proteins could be UV-crosslinked to the pre-mRNA in the absence of ATP (Figure 5D, lanes 3 and 5). In the presence of ATP, PRP2^{wt} protein dissociated from the substrate RNA as expected (Figure 5D, lane 4), whereas the PRP2^{dn1} protein remained bound to the pre-mRNA (Figure 5D, lane 6).

The specificity of the immunoprecipitation of PRP2 protein was confirmed by antigen competition (i.e. pre-incubation of the anti-PRP2 antibodies with the fusion peptide to which the antibodies were raised; Figure 5B, lane 7) or by using the appropriate preimmune serum (Figure 5D, lanes 7 and 8).

Again, identical results were obtained using rp51A pre-mRNA (Figure 6), indicating that the interaction with the pre-mRNA is a general property of PRP2 rather than being a phenomenon specific to a particular pre-mRNA.

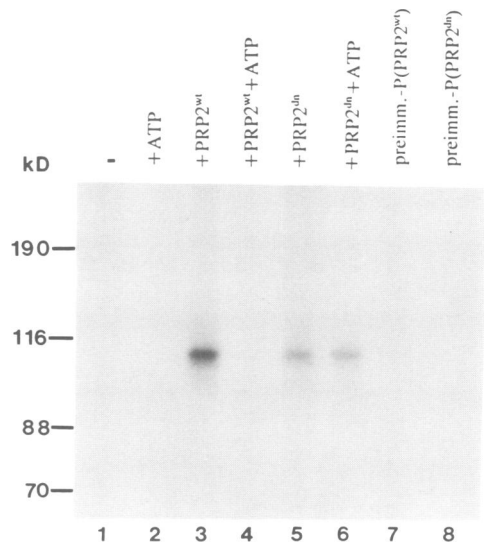

Fig. 6. UV-crosslinking of purified wild-type and dominant negative PRP2 protein to rp51A pre-mRNA in PRP2Δ spliceosomes. Treatment of the samples and numbering is as described in Figure 5D, except that uniformly ^{32}P-labelled rp51A pre-mRNA was used in this experiment.

ATP hydrolysis rather than ATP binding is required to disrupt the PRP2–pre-mRNA contact and to release PRP2 from spliceosomes

The previous experiments demonstrated that ATP was required to release PRP2 from the pre-mRNA, however, they did not distinguish whether mere ATP binding to spliceosomes and/or to PRP2 or ATP hydrolysis was required to disrupt the interaction of PRP2 protein with the pre-mRNA. To address this question the effects of ATP and of γS-ATP on the splicing efficiency, splicing complex formation, association of PRP2 with spliceosomes and, most importantly, the interaction of PRP2 protein with the pre-mRNA were compared. γS-ATP is a slowly hydrolysed ATP analogue and therefore remains bound to ATP-hydrolysing enzymes longer than normal ATP (Eckstein, 1985). It was reported that PRP2 protein binds ATP and γS-ATP with equal affinity, as judged by inhibition of ATP hydrolysing activity of PRP2 protein by γS-ATP (Kim and Lin, 1993).

Consistent with the result of others (Kim and Lin, 1993), γS-ATP supported splicing in the presence of PRP2^{wt} protein much less efficiently than ATP (Figure 7A). In addition, there was a similar effect on the conformational shift seen in splicing complexes (Figure 7B). Co-immunoprecipitation of pre-mRNA with anti-PRP2-specific antibodies revealed that the dissociation of PRP2^{wt} protein from spliceosomes was less effective in the presence of γS-ATP than in the presence of ATP (Figure 7C). UV-crosslinking experiments under identical conditions demonstrated that the release of PRP2^{wt} protein from the pre-mRNA was also less effective with γS-ATP, compared with ATP (Figure 7D). The extent of crosslinking was up to 3-fold greater in the presence of γS-ATP than in the presence of ATP. The difference increased with the amount of the respective nucleotides present in the reaction (0–240 μM ATP/γS-ATP; Figure 7E)

As PRP2 protein apparently binds ATP and γS-ATP with equal affinity, the release from pre-mRNA should be equally efficient with ATP or γS-ATP if ATP binding alone was sufficient to release PRP2 from the pre-mRNA. However, the observed difference in UV-crosslinking efficiency suggested that ATP hydrolysis rather than mere ATP binding was required to release PRP2 protein from the pre-mRNA and from spliceosomes.

Discussion

The association of PRP2 protein with the spliceosomal machinery is highly transient (King and Beggs, 1990). In order to study interactions of PRP2 protein with spliceosomal components it was therefore required to stall any further progress in splicing as soon as PRP2 had entered the spliceosome and thus render its interactions non-transient.

In the present study, we used two different approaches to achieve this. (i) Plumpton *et al.* (1994) have identified a mutant PRP2 protein which, when overproduced in yeast cells, competes with the endogenous wild-type protein. This dominant negative (Herskowitz, 1987) form of PRP2 protein (PRP2^{dn1}) stably associates with spliceosomes, thereby preventing processing of the pre-mRNA, because of a functional defect. The mutation produces a single serine to leucine change in the highly conserved 'SAT' motif (Plumpton *et al.*, 1994) common to (putative) RNA helicases of the DEAD/H-box family. This motif might be specifically involved in RNA helicase activity of DEAD/H-box proteins (Pause and Sonenberg, 1992). (ii) Considering that the PRP2^{dn1} protein is a mutated form of PRP2 that has altered spliceosome binding properties, it was important to extend our studies to the wild-type protein (PRP2^{wt}). PRP2-mediated ATP hydrolysis within the spliceosome is apparently required to initiate step 1 of splicing, but not for the initial interaction of PRP2 protein with spliceosomes (Kim and Lin, 1993; this study). Consequently, addition of purified wild-type PRP2 protein to assembled spliceosomes which have been previously depleted of endogenous PRP2 (PRP2Δ spliceosomes) and of ATP results in permanent binding of PRP2 protein to spliceosomes and in cessation of any further processing, due to the lack of ATP.

Since PRP2 is an RNA-stimulated ATPase and a putative RNA helicase (Kim *et al.*, 1992; Plumpton *et al.*, 1994), it is possible that this splicing factor interacts with the pre-mRNA and/or snRNAs in spliceosomes. Here, we demonstrate by a combination of UV-crosslinking and immunoprecipitation that the wild-type PRP2 protein and the dominant negative PRP2 protein interact directly with pre-mRNA with equal efficiency, indicating that the SAT to LAT mutation did not decrease the RNA binding capability of PRP2^{dn1} protein. The use of two different pre-mRNAs in both experimental systems emphasized that the detected interaction is a general and characteristic part of the function(s) that PRP2 protein performs while in contact with the spliceosome.

PRP2 protein could not be UV-crosslinked to mutant RNAs which were unable to form spliceosomes, nor to spliceable pre-mRNAs if splicing complexes were not formed due to a lack of ATP. Considering that ATP was not necessary for the immediate contact of PRP2 with the pre-mRNA (see below), we conclude that this interaction requires a spliceable RNA and, more importantly, an assembled spliceosome. These prerequisites for RNA binding by PRP2 protein in the *in vitro* splicing reaction seem to contrast with the ability of non-specific RNAs to interact with

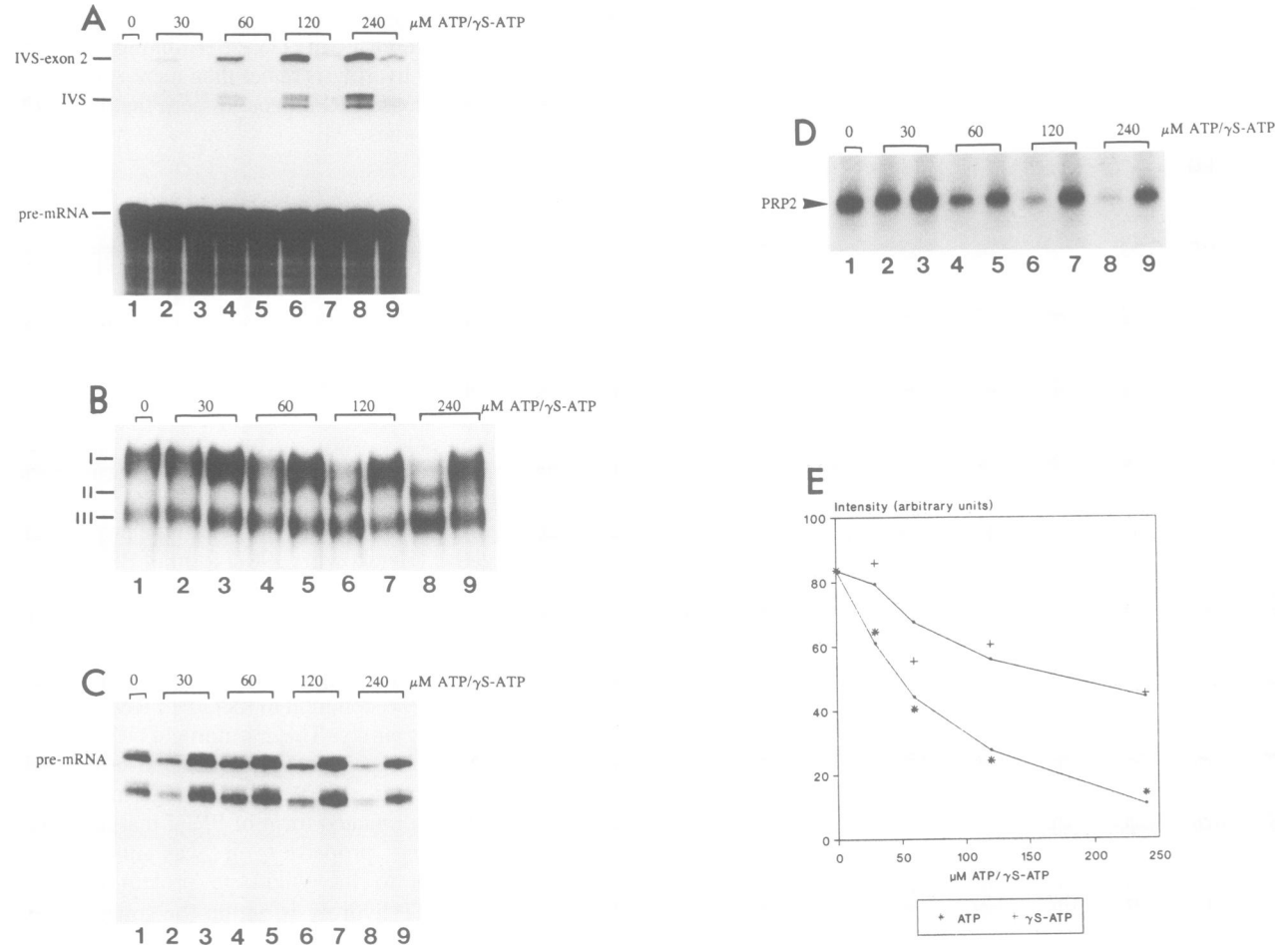

Fig. 7. Effect of γS-ATP on the interaction of purified wild-type and dominant negative PRP2 protein with PRP2Δ spliceosomes and on UV-crosslinking to rp51A pre-mRNA. (**A**) ATP-depleted PRP2Δ spliceosomes containing uniformly ^{32}P-labelled rp51A pre-mRNA were incubated under splicing conditions in the presence of wild-type PRP2 protein alone (lane 1) and with 30 μM ATP (lane 2) or γS-ATP (lane 3), 60 μM ATP (lane 4) or γS-ATP (lane 5), 120 μM ATP (lane 6) or γS-ATP (lane 7) and 240 μM ATP (lane 8) or γS-ATP (lane 9). The RNA was fractionated on a 6% denaturing polyacrylamide gel. (**B**) Splicing complex formation of aliquots of the samples in panel A was analysed by non-denaturing gel electrophoresis. (**C**) Aliquots of the samples in panel A were subjected to immunoprecipitation with anti-PRP2 antibodies. Co-immunoprecipitated rp51A pre-mRNA was analysed on a 6% denaturing polyacrylamide gel. (**D**) PRP2 protein was UV-crosslinked to rp51A pre-mRNA in aliquots of the samples in panel A and analysed as described in Figure 1B by autoradiography. (**E**) The extent of UV-crosslinking of PRP2 protein to rp51A pre-mRNA, indicated by the intensity of the bands shown in Figure 7D, was quantitated by Phosphorimager analysis. Integration of the bands revealed the extent of crosslinking in arbitrary units (*y*-axis), dependent on the concentration (*x*-axis) of ATP (*, lower curve) and γS-ATP (+, upper curve).

PRP2 as judged by binding of the purified protein to various homopolymer RNAs in ATPase assays (Kim *et al.*, 1992; Plumpton *et al.*, 1994). However, in the ATPase assay the RNA concentration is substantially greater (~1000-fold) than that of substrate RNA in an *in vitro* splicing reaction. In the more physiological environment of an *in vitro* splicing extract which contains many RNA binding proteins competing with each other, it appears that PRP2 requires the assistance of a spliceosome to interact with the pre-mRNA.

Similar results have been obtained for PRP8 protein, the only other yeast splicing factor which has been demonstrated to interact directly with pre-mRNA (Whittaker and Beggs, 1991). In a splicing assay, spliceosome assembly was required for PRP8 protein to contact the substrate RNA.

PRP2 was efficiently UV-crosslinked to an oligoC-truncated rp51A RNA, demonstrating that a 3′ splice site was not required for this interaction. Considering that PRP2 functions before the first step of splicing this result is not surprising. The failure to detect any interaction of PRP2 protein with an oligoA-truncated RNA demonstrates that the same minimum sequence downstream of the UACUAAC box which is required for step 1 of splicing (Rymond *et al.*, 1987) was also necessary for the PRP2–pre-mRNA interaction. The simplest explanation of this result would be that PRP2 protein binds directly to this particular RNA sequence. However, as PRP8 protein also fails to interact with an oligoA-truncated rp51A RNA (Whittaker and Beggs, 1991), in this respect at least, complex I is aberrantly assembled. Thus, the inability of PRP2 protein to interact with an oligoA-truncated rp51A RNA could reflect the requirement for correctly assembled spliceosomes to permit the PRP2–pre-mRNA contact. Certainly, PRP2 also interacts with spliceosomal components other than the pre-mRNA as indicated by co-immunoprecipitation with anti-PRP2 specific antibodies of splicing complexes formed on oligoA-cleaved rp51A RNA. The fact that PRP2 protein failed to contact an oligoA-truncated RNA, but did associate with splicing complexes assembled on this RNA suggests that these are independent interactions.

Spliceosomes might present a particular RNA sequence or more likely, an appropriate secondary and tertiary structure of the pre-mRNA and thus create a high affinity binding site specific for the PRP2 protein. A spliceosome may even put the two molecules in juxtaposition, with a particular spliceosomal factor assisting the binding of PRP2 protein to the pre-mRNA. A similar cooperative effect on RNA binding has been postulated for the prototype of this protein family, the translation initiation factor eIF-4A, which alone is very inefficient in hydrolysing ATP and in binding to and displacement of RNA, but these activities are stimulated by the association of eIF-4B (Abramson *et al.*, 1987; Lawson *et al.*, 1989; Rozen *et al.*, 1990).

In ATP-depleted PRP2Δ spliceosomes, $PRP2^{wt}$ protein interacted with the pre-mRNA in an ATP-independent fashion, but required ATP for release from the pre-mRNA and from spliceosomes. As the amount of $PRP2^{wt}$ protein UV-crosslinked to pre-mRNA was significantly higher in the presence of γS-ATP than in the presence of ATP, it appears that ATP hydrolysis rather than merely ATP binding was necessary to disrupt the interaction of the PRP2 protein with the pre-mRNA and the spliceosome.

In contrast, $PRP2^{dn1}$ protein remained bound to the pre-mRNA in the presence of ATP, indicating that the mutation causes the failure of $PRP2^{dn1}$ protein to release the bound RNA, thus conferring the observed hyperstabilization of the $PRP2^{dn1}$–spliceosome complex. This could be due to loss of ATPase activity or to uncoupling of ATP hydrolysis from another activity such as an RNA displacement. Keeping in mind that in an *in vitro* assay with purified protein this mutation does not result in a major defect in ATP hydrolysis by $PRP2^{dn1}$ protein (only 2.5-fold reduction relative to the $PRP2^{wt}$ protein; Plumpton *et al.*, 1994), these results support the idea that the SAT motif in proteins of the DEAD/H-box family could be important for an RNA helicase-like activity, as has been demonstrated for the translation initiation factor eIF-4A (Pause and Sonenberg, 1992).

These observations allow the development of a rather detailed model of the interactions of PRP2 protein with the spliceosome and the pre-mRNA. After spliceosome complex I has assembled, PRP2 interacts with the pre-mRNA and (an)other spliceosomal factor(s) in an ATP-independent fashion. ATP hydrolysis occurs after this initial interaction of PRP2 protein with the pre-mRNA. As PRP2 has been shown to be an RNA-stimulated ATPase (Kim *et al.*, 1992; Plumpton *et al.*, 1994) we propose that the pre-mRNA is the native co-factor in the spliceosome which stimulates ATP hydrolysis by PRP2 protein. PRP2 protein may cause a change in the bound region of the pre-mRNA, thereby promoting step 1 of splicing. Considering that PRP2 is a putative RNA helicase (Chen and Lin, 1990; Wassarman and Steitz, 1991; Fuller-Pace and Lane, 1992; Schmid and Linder, 1992), this might be an RNA conformational change/displacement event. PRP2 protein is able to release the substrate RNA after ATP hydrolysis. These events are required to permit step 1 of splicing and formation of splicing complex II.

This model of the PRP2 activity during splicing contains interesting similarities to the action of the prototype DEAD-box protein eIF-4A in the initiation of translation. The activity of eIF-4A appears to be linked to an ATP hydrolysis-dependent RNA displacement event near the 5′ cap structure, thereby permitting the association of the initiation factor–mRNA complex with the 40S ribosomal subunit (Ray *et al.*, 1985; Lawson *et al.*, 1989; Rozen *et al.*, 1990; Jackson, 1991; Thach, 1992). Similarly, PRP2 may promote an RNA conformational change/displacement in the spliceosome which could correspond to or result in the conformational shift of splicing complex I to complex II. Evidence has been presented in both yeast and mammalian splicing that the spliceosome is a highly dynamic structure with multiple changes in RNA–RNA interactions (Newman and Norman, 1992; Steitz, 1992; Wassarman and Steitz, 1992; Wyatt *et al.*, 1992) and DEAD/H-box proteins could play fundamental roles in regulating such changes by promoting conformational shifts and/or displacement of RNAs (Wassarman and Steitz, 1991). They may also provide a molecular proofreading device for steps in the splicing pathway that involve changes in RNA–RNA interactions. This has been suggested for PRP16 (Burgess *et al.*, 1990; Burgess and Guthrie, 1993), the DEAH-box protein promoting the second step of pre-mRNA splicing. Like PRP2, PRP16 interacts with spliceosomes in an ATP-independent fashion, being released upon addition of ATP (Schwer and Guthrie, 1991, 1992). So far, however, no data are available for interactions of PRP16 with substrate RNA.

The search for putative interaction(s) of PRP2 protein with snRNAs will be extremely interesting, as the pre-mRNA might be only one half of the RNA substrate for PRP2 protein. Finally, the mapping of the binding site(s) on both the RNA(s) and the PRP2 protein could provide evidence for the direct involvement of PRP2 protein in changing spliceosomal RNA–RNA interactions. Considering the close relationship of the DEAH-box protein splicing factors PRP2, PRP16 and PRP22 (Company *et al.*, 1991; Wassarman and Steitz, 1991; Schmid and Linder, 1992), a general picture might emerge of how these factors perform their function in eukaryotic pre-mRNA splicing.

Materials and methods

Yeast strains, plasmids and RNA substrates

S.cerevisiae strains: BJ2412 was described previously (Lossky *et al.*, 1987). S150-2B (*MATa ura3-52 leu2-3,-11, trp1-289 his3-Δ1*) was described by Baldari *et al.* (1987) and DJY85 (*MATa/α prp2-1/prp2-1 ura3/ura3 ade1/ADE1 ade2/ade2 trp1/TRP1 his3/HIS3 tyr1/TYR1 lys2-801/LYS2-801 can1/CAN1*) was obtained from D.J.Jamieson (Dundee). Plasmid pBM-$PRP2^{dn1}$ contains the *PRP2–dn1* gene fused to the *GAL1* inducible promoter (Plumpton *et al.*, 1994). The *PRP2–dn1* allele harbours a single transition mutation causing a serine to leucine substitution at amino acid 378 within the conserved SAT motif (Plumpton *et al.*, 1994).

The following plasmid DNAs were linearized by digestion with *Eco*RI to provide templates for *in vitro* transcription. Plasmids pT7rp28 (Lossky *et al.*, 1987), p(T7)GEM284 and p(T7)GEM287 were transcribed *in vitro* with T7 RNA polymerase, and plasmids pSPrp51A, pSPrp51A(5′-0) and pSPrp51A(Δ3b) (Pikielny and Rosbash, 1986) were transcribed *in vitro* with SP6 RNA polymerase to generate wild-type and mutant pre-mRNAs. The transcripts were uniformly ^{32}P-labelled by using 60 μCi [α-^{32}P]UTP (800 Ci/mmol, Amersham UK) in a 18 μl transcription reaction containing a total concentration of 8 μM UTP, according to the protocol of the manufacturer (Pharmacia LKB). Oligodeoxynucleotide-directed cleavage of rp51A pre-mRNA utilising the RNase H activity endogenous to yeast splicing extracts, was performed as described (Rymond and Rosbash, 1985). Oligodeoxynucleotides A and C (Rymond *et al.*, 1987) were synthesized by OSWEL DNA Service (Edinburgh).

Splicing extract preparation and in vitro splicing reactions

Growth of strain S150-2B carrying pBM-$PRP2^{dn1}$ and galactose induction for overproduction of $PRP2^{dn1}$ protein was carried out according to the protocol of Plumpton *et al.* (1994). Yeast whole cell extracts were prepared

as described (Lin *et al.*, 1985). Extracts from strain DJY85 were heat-inactivated by incubation at 36°C for 1 h. *In vitro* splicing reactions were performed as described (Lin *et al.*, 1985), containing $PRP2^{dn1}$ extract and wild-type extract in a 1:8 ratio. The reaction products were fractionated on 6% (w/v) polyacrylamide−8 M urea gels and visualized by autoradiography.

Native gel electrophoresis of splicing reactions was performed as described (Pikielny *et al.*, 1986), except that 10 mM EDTA was present in both the electrophoresis buffer and the acrylamide−agarose gel.

Generation and complementation of ATP-depleted PRPΔ spliceosomes

PRP2Δ spliceosomes were assembled at 25°C for 25 min in a total volume of 100 μl containing 50% (v/v) heat-inactivated splicing extract from DJY85 cells, 2 pmol ^{32}P-labelled pre-mRNA, 1.5 mM ATP and 50 % (v/v) splicing buffer (Lin *et al.*, 1985). After spliceosome assembly, residual ATP was depleted by incubation with 1.5 mM glucose for 5 min at 25°C, utilizing the hexokinase activity endogenous to yeast splicing extracts, followed by gel filtration on Sephadex G25 columns (NAP-5, Pharmacia LKB) pre-equilibrated with splicing buffer lacking ATP.

PRP2 protein bearing a histidine$_6$ repeat at the N-terminus was purified in a two step procedure by Ni^{2+} chelate chromatography and RNA affinity chromatography according to Plumpton *et al.* (1994). For complementation, 10 ng of purified PRP2 protein, 2 mM $MgCl_2$ and 2 mM ATP were added to 70 μl of ATP-depleted PRP2Δ spliceosomes and incubated for 25 min at 25°C. To compare the effect of ATP and γS-ATP, 25 ng of purified PRP2 protein were allowed to bind to ATP-depleted PRP2Δ spliceosomes for 5 min on ice; splicing was initiated by addition of varied amounts of ATP or γS-ATP and a further incubation for 3 min at 25°C. Following the incubation, samples were quenched on ice.

UV-crosslinking assay and immunoprecipitations

UV light-induced crosslinking of RNA to protein was performed essentially as described (Whittaker and Beggs, 1991) in 50 μl aliquots, except that samples were UV-irradiated for 25 min on ice. Following digestion with RNase T1 (5 U/μl final conc.; Boehringer Mannheim, UK), some of the samples were subjected to denaturation by addition of SDS to 2% (w/v), Triton X-100 to 1% (v/v) and DTT to 0.9% (w/v) and heated to 90°C for 1.5 min. To reduce the concentration of denaturants before immunoprecipitation, these samples were diluted 10-fold with immunoprecipitation buffer, containing 150 mM NaCl, 2.5 mM $MgCl_2$, 6 mM HEPES (pH 7.5), 0.05 % (v/v) Nonidet P-40 and protease inhibitors PMSF (200 μg/ml), bestatin (40 μg/ml), pepstatin (1.4 μg/ml) and leupeptin (1 μg/ml).

Antibodies to PRP2−β-galactosidase fusion protein 2.5 have been described (King and Beggs, 1990). Anti-2.3 antibodies were raised against a β-galactosidase fusion protein containing an N-terminal 205 amino acid fragment of PRP2. These antibodies gave identical results when used in immunoprecipitation experiments. Antibodies were bound to protein A−Sepharose beads (PAS; Sigma) in TBSN buffer (145 mM NaCl, 50 mM Tris−HCl (pH 7.6), 0.05 % (v/v) Nonidet P-40) and washed four times with the same buffer. UV-irradiated splicing reactions were incubated at 4°C with PAS-bound antibodies for 2 h with rotation. The antibody complexes were washed twice with TBSN and once with TBS (TBSN without Nonidet P-40). Immunoprecipitates were subjected to SDS gel electrophoresis through 7.5% polyacrylamide gels. Gels were analysed by autoradiography and on a Phosphorimager (Molecular Dynamics, UK) for quantification of the extent of UV-crosslinking. For immunological detection of PRP2 protein, the samples fractionated on SDS−polyacrylamide gels were electrophoretically transferred to PVDF membranes (Immobilon P; Millipore) and the blots were probed with anti-PRP2 antibodies. Co-immunoprecipitation of pre-mRNA with anti-PRP2 antibodies was performed according to Whittaker and Beggs (1991).

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