The EMBO Journal vol.14 no.11 pp.2602–2612, 1995

Extensive interactions of PRP8 protein with the 5′ and 3′ splice sites during splicing suggest a role in stabilization of exon alignment by U5 snRNA

Stefan Teigelkamp[1], Andrew J.Newman[2] and Jean D.Beggs[3]

Institute of Cell and Molecular Biology, University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh EH9 3JR and [2]MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK
[1]Present address: Institut für Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Emil-Mannkopff-Strasse 2, 35037 Marburg, Germany

[3]Corresponding author

Communicated by J.D.Beggs

Precursor RNAs containing 4-thiouridine at specific sites were used with UV-crosslinking to map the binding sites of the yeast protein splicing factor PRP8. PRP8 protein interacts with a region of at least eight exon nucleotides at the 5′ splice site and a minimum of 13 exon nucleotides and part of the polypyrimidine tract in the 3′ splice site region. Crosslinking of PRP8 to mutant and duplicated 3′ splice sites indicated that the interaction is not sequence specific, nor does it depend on the splice site being functional. Binding of PRP8 to the 5′ exon was established before step 1 and to the 3′ splice site region after step 1 of splicing. These interactions place PRP8 close to the proposed catalytic core of the spliceosome during both transesterification reactions. To date, this represents the most extensive mapping of the binding site(s) of a splicing factor on the substrate RNA. We propose that the large binding sites of PRP8 stabilize the intrinsically weaker interactions of U5 snRNA with both exons at the splice sites for exon alignment by the U5 snRNP.

Key words: photo-crosslinking/protein–RNA interactions/RNA splicing/snRNP protein/yeast

Introduction

Nuclear pre-mRNA splicing involves two transesterification reactions which take place in a large ribonucleoprotein complex, the spliceosome. Spliceosomes are formed by consecutive assembly of the U1, U2, U4/U6 and U5 small nuclear ribonucleoprotein particles (snRNPs) plus various non-snRNP proteins onto the pre-mRNA in a highly ordered series of events, many of which are ATP dependent (reviewed in Green, 1991; Rymond and Rosbash, 1992; Moore *et al.*, 1993; Madhani and Guthrie, 1994; Newman, 1994; Nilsen, 1994).

The first steps in spliceosome assembly are association of U1 snRNP with the 5′ splice site and U2 snRNP with the branchpoint sequence of the substrate RNA, in which basepairing interactions form between the substrate and the respective snRNAs. The U4/U6 snRNP interacts with the U5 snRNP to form a U4/U6.U5 triple snRNP which then associates with the U1–U2–pre-mRNA complex to form the spliceosome. Prior to the first transesterification reaction (step 1) the extensive U4/U6 basepairing appears to be destabilized and U6 snRNA anneals with U2 snRNA to form two helices, one of them (helix I) immediately upstream of the branchpoint recognition domain of U2 (Hausner *et al.*, 1990; Datta and Weiner, 1991; Wu and Manley, 1991; Madhani and Guthrie, 1992). A conserved sequence, ACAGAG, in U6 immediately adjacent to the helix I region interacts with a conserved intron sequence at the 5′ splice site, bringing the branchpoint adenosine into close proximity with the scissile phosphate for the first transesterification reaction (Sawa and Abelson, 1992; Sawa and Shimura, 1992; Sontheimer and Steitz, 1993; Kandels-Lewis and Seraphin, 1993; Lesser and Guthrie, 1993). A uridine-rich, phylogenetically conserved loop of U5 snRNA interacts with the last three nucleotides of exon 1 and the first two nucleotides of exon 2, maintaining contact with free exon 1 after the first transesterification reaction and possibly aligning it with exon 2 for the second transesterification reaction (Newman and Norman, 1991, 1992; Cortes *et al.*, 1993; Sontheimer and Steitz, 1993). Since exon sequences at the splice sites are not conserved, the predominance of uridine residues in the U5 loop can be explained by their capacity for promiscuous basepairing. The question arises as to what provides the necessary stability for such a fragile, yet crucial, interaction.

The primary sequences required to characterize an intron in precursor mRNA of *Saccharomyces cerevisiae* are a conserved GUAUGU sequence at the 5′ end of the intron, the UACUAAC sequence at the branchpoint (the adenosine used for branch formation is in bold type), a polypyrimidine tract between the branchpoint and the 3′ splice site and PyAG at the 3′ end of the intron. RNA–RNA interactions involving these rather short conserved motifs are unlikely to be sufficiently stable by themselves to build up and hold the complex and dynamic spliceosomal structure. There is mounting evidence that protein components of the spliceosome contribute to the stability and specificity of these interactions (for review, see Ruby and Abelson, 1991; Moore *et al.*, 1993). For example, members of the SR protein family (serine/arginine-rich splicing factors) in higher eukaryotes can direct and stabilize binding of the U1 snRNP to the 5′ splice site and influence 5′ splice site selection in alternative splicing (Mayeda and Krainer, 1992; Kohtz *et al.*, 1994; Zuo and Manley, 1994). SC35 and ASF/SF2 proteins in mammals (Wu and Maniatis, 1993) and MUD2 protein in *S.cerevisiae* (Abovich *et al.*, 1994) interact simultaneously with components bound to the 5′ and 3′ splice sites, bridging the ends of the intron (reviewed in Hodges and Beggs, 1994).

Thus, protein splicing factors have an essential impact on the formation, fidelity and stability of RNA–RNA interactions in early spliceosome formation. The activities of putative RNA helicases of the DEAD/H-box protein family

are required for several steps in yeast pre-mRNA splicing and might influence RNA–RNA interactions (Wassarman and Steitz, 1991). Based on genetic experiments, it has been suggested that the DEAD box protein PRP28 destabilizes the U4/U6 snRNA interaction prior to step 1 of splicing, counterbalanced by a stabilizing effect of PRP8 protein (Strauss and Guthrie, 1991). A mutation affecting another DEAD box protein, DED1, has been isolated as a cold-sensitive suppressor of a temperature-sensitive *prp8* allele (Jamieson *et al.*, 1991); however, no direct role has been identified for DED1 in splicing. *In vitro*, PRP8 protein appears to play a role in the formation of U4/U6.U5 tri-snRNP complexes and their assembly into spliceosomes. Furthermore, *in vivo* depletion of PRP8 protein results in degradation of U4, U5 and U6 snRNAs (Brown and Beggs, 1992). Thus, PRP8 protein might be responsible for the stability of RNA–RNA interactions in the spliceosome.

PRP8 protein shows an extraordinarily high degree of conservation among eukaryotes, with no obvious homology to other proteins (Hodges *et al.*, 1995), suggesting a critical role in the splicing process. Since the high molecular weight (>200 kDa) is also conserved among homologues (Jackson *et al.*, 1988; Anderson *et al.*, 1989; Paterson *et al.*, 1991; Kulesza *et al.*, 1993), the large size might be essential for the function of PRP8 protein. Biochemical studies revealed that PRP8 protein is a component of the U5 snRNP and of spliceosomes (Lossky *et al.*, 1987; Whittaker *et al.*, 1990). Similar results have been obtained for p220, the human homologue of PRP8 (Anderson *et al.*, 1989; Pinto and Steitz, 1989). Following incorporation of the U4/U6.U5 triple snRNP into spliceosomes, PRP8 protein as well as the human homologue p220 interact directly with substrate RNA during both steps of splicing, shown by UV-crosslinking (Garcia-Blanco *et al.*, 1990; Whittaker and Beggs, 1991; Teigelkamp *et al.*, 1995). In contrast to many characterized RNA-binding proteins which can be studied in purified systems, PRP8 protein interacts with substrate RNA only in assembled spliceosomes. Thus, studies of PRP8 interactions with the substrate RNA are complicated by the complex composition of the spliceosome. UV-crosslinking to photo-activatable 4-thiouridine incorporated at specific sites in the precursor mRNA was therefore used both to enhance the efficiency of crosslinking and to detect binding of PRP8 protein at precise positions on the substrate RNA. We demonstrate here that PRP8 protein interacts extensively with exon residues at the 5′ splice site during step 1 and with the 3′ splice site region (defined as extending from the branchpoint to the downstream exon) during step 2 of splicing, indicating its close proximity to the putative catalytic centres of the spliceosome. We propose that PRP8 protein stabilizes the fragile interactions of U5 snRNA with the non-conserved sequences at the ends of both exons, to anchor and align them in the active sites of the spliceosome.

Results

The strategy used: site-specific incorporation of label into CYH2 pre-mRNA and dissecting splicing into pre- and post-step 1 events

UV-crosslinking experiments using randomly ^{32}P-labelled precursor RNA have shown previously that the yeast PRP8 protein splicing factor interacts directly with substrate RNA in a splicing-dependent manner (Whittaker and Beggs, 1991). To identify binding sites of the PRP8 protein on substrate RNA precisely, 4-thiouridine was incorporated at specific sites in a CYH2 precursor RNA for UV-crosslinking experiments.

High efficiency crosslinking of 4-thiouridine to contacting amino acids or nucleotides (usually in non-Watson–Crick interactions) can be induced by relatively low energy and brief duration UV irradiation (Favre, 1990; Sontheimer, 1994). Since the replacement of oxygen with sulfur at position 4 on the pyrimidine ring does not cause a major change in the size or conformation of uracil, one would expect no steric perturbances of the spliceosomal structure when 4-thiouracil is incorporated into precursor RNA (Favre, 1990). Thus, crosslinking of RNA or proteins to 4-thiouridine is expected to indicate normal molecular interactions. This approach has been successfully used to detect RNA–RNA interactions and protein–RNA interactions in mammalian spliceosomes (Wyatt *et al.*, 1992; Sontheimer and Steitz, 1993).

For site-specific incorporation of 4-thiouridine into precursor RNA, the 3′ portion of the pre-mRNA, starting at the test site, was produced by T7 transcription primed with the dinucleotide 4-thioUpG. The 5′ end of this RNA was labelled with ^{32}P and then ligated to the 5′ portion of the pre-mRNA by the method of Moore and Sharp (1992). As a control, for each experiment an identical RNA was generated using UpG instead of 4-thioUpG to prime the 3′ half. This RNA, containing only unmodified uracil, is not stimulated by irradiation with 360 nm UV light and serves as a negative control to confirm the site specificity of any detected crosslink. 4-ThioUpG was incorporated in a CYH2 precursor RNA in the upstream exon at positions –8, –2 and –1 relative to the 5′ splice site, at position +4 in the intron, at position +2 relative to the branch point adenosine, and in the downstream exon at position +1 relative to the 3′ splice site (Figure 1). To investigate the specificity of the PRP8 interaction with the 3′ splice site, experiments were carried out with a mutant 3′ splice site (CAG/UG to CUC/UG) and by duplicating either the pyrimidine-rich last 12 residues of the intron including the 3′ splice site (CYH2-py) or the purine-rich first 12 residues of exon 2 followed by a CAG consensus sequence, which results in a polypurine spacer between two 3′ splice site consensus sequences (CYH2-pu; Figure 1). Although the residues tested in this study were all close to the most crucial positions in the precursor RNA, in no case did incorporation of 4-thioUpG interfere with *in vitro* splicing activity (data not shown).

To allow a precise separation of the detected interactions into pre- and post-step 1 events, splicing extracts containing a temperature-sensitive PRP2 protein were used. As the activity of PRP2 protein is required to promote step 1 of splicing, heat inactivation of these extracts provided spliceosomes that are stalled prior to the first transesterification reaction (PRP2Δ spliceosomes). These spliceosomes can be chased through both steps of splicing by addition of purified PRP2 protein (Kim and Lin, 1993; Teigelkamp *et al.*, 1994), thus providing a useful tool to study the kinetics of protein–pre-mRNA interactions.

PRP8 protein can be UV-crosslinked to exon nucleotides at the 5′ splice site in wild-type splicing reactions and in PRP2Δ spliceosomes

An *in vitro* splicing reaction with a wild-type yeast splicing extract and CYH2 pre-mRNA labelled with 4-thioUpG

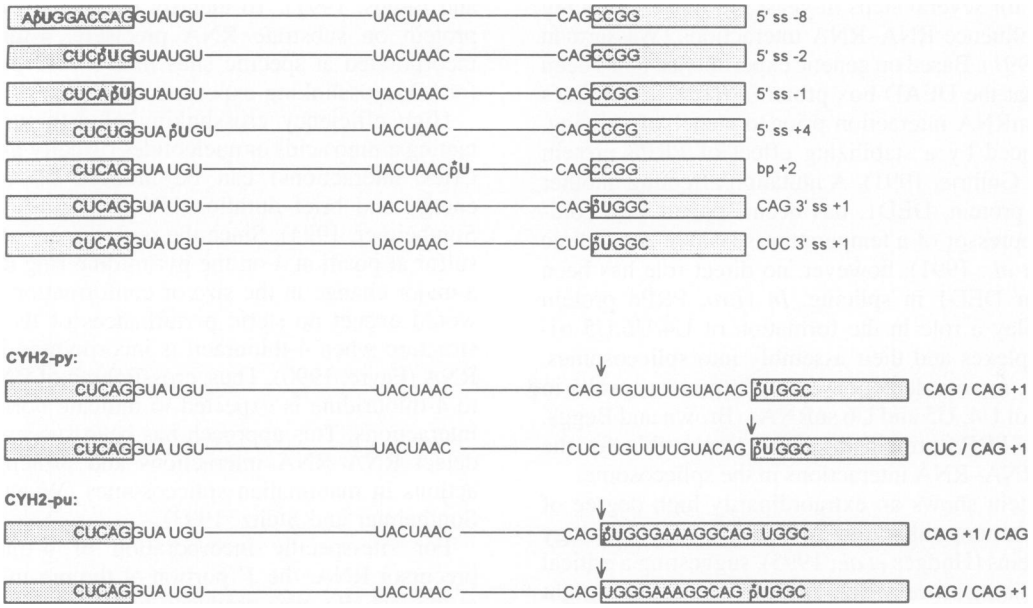

Fig. 1. Positions of 4-thiouridine incorporation into CYH2 precursor RNA. CYH2 RNA used in this study is shown with sequences (including all changes) at the 5′ and 3′ splice sites and the branchpoint consensus sequence; exons are shown as shaded boxes and the intron as a single line. The labelled position is indicated on the right of each pre-mRNA. 4-thiouridine is shown as a boldface **U** and the preceding ^{32}P is indicated by an asterisk on top of a p. CYH-py RNA contains a duplication of 12 nucleotides of the polypyrimidine tract (as shown) and CYH2-pu contains a duplication of 12 nucleotides of exon 2 (as shown). The arrow indicates the position of the 3′ splice site used for the second transesterification reaction. The combination of proximal and distal 3′ splice site sequences and the position of the incorporated 4-thiuouridine are shown on the right.

and ^{32}P at position –1 upstream of the 5′ splice site was incubated under standard splicing conditions (see Materials and methods) followed by long wave UV irradiation and RNase T1 digestion. Proteins that had become radiolabelled through crosslinking to the ^{32}P-4-thiouridine residue were visualized by autoradiography following fractionation by SDS–PAGE. Several proteins (mainly between 50 and 150 kDa) became radiolabelled in extracts incubated without ATP, conditions that do not support splicing or spliceosome assembly (Figure 2A, lane 1). Splicing conditions (with ATP) produced a subset of these bands and an additional radiolabelled protein of ~280 kDa was detected (Figure 2A, lane 2). Immunoprecipitation using PRP8-specific antibodies demonstrated that this was PRP8 protein (Figure 2A, lanes 5 and 6). The site specificity of the crosslink was confirmed by analysing splicing reactions containing CYH2 pre-mRNA labelled with ^{32}P and UpG instead of 4-thioUpG. No significant signal could be detected in these samples (Figure 2A, lanes 3, 4, 7 and 8). Thus, PRP8 protein was UV-crosslinked to position –1 at the 5′ splice site.

To determine the kinetics of this interaction PRP2Δ spliceosomes were UV irradiated and analysed as described above. The crosslink of PRP8 protein to position –1 at the 5′ splice site was detected in PRP2Δ spliceosomes with or without the addition of purified PRP2 protein (Figure 2B, lanes 3 and 4). Thus, the contact is established before the action of PRP2 protein, the addition of which initiates step 1 of splicing in this experimental system. The crosslink was less efficient than in a wild-type splicing extract (for comparison, see Figure 2B, lanes 2–4), because of decreased splicing activity in PRP2Δ extracts supplemented with PRP2 protein. The absence of radiolabelled PRP8 protein in samples with substrate RNA lacking 4-thiouridine confirmed the site specificity of the crosslink (Figure 2B, lanes 5–8).

Similar results were obtained for an interaction of PRP8 protein with position –2 relative to the 5′ splice site (Figure 3). As for the interaction at position –1, the crosslink at position –2 was detected in a wild-type splicing extract and in a PRP2Δ extract with and without added PRP2 protein (Figure 3, lanes 2–5). Immunoprecipitation under denaturing conditions resulted in the loss of a signal migrating faster than PRP8 protein, which was because of non-specific precipitation, whereas the specific PRP8 signal remained unchanged (Figure 3, lane 3).

UV-crosslinking of PRP8 protein to positions –8 and +4 relative to the 5′ splice site was investigated to map the extent of the PRP8 –5′ splice site interaction. Crosslinking of PRP8 to position –8 was detected in a wild-type splicing extract and in a PRP2Δ extract with and without supplementation with purified PRP2 protein (Figure 4, lanes 2–4), similar to the interactions at positions –1 and –2. No PRP8-specific signal was observed from splicing reactions with CYH2 pre-mRNA containing uridine instead of 4-thiouridine (Figure 4, lanes 5–8). In analogous experiments, carried out with CYH2 pre-mRNA labelled at position +4 relative to the 5′ splice site, no crosslinking of PRP8 protein was detected (data not shown).

PRP8 protein can be UV-crosslinked to intron and exon nucleotides in the 3′ splice site region in a wild-type splicing reaction but not in PRP2Δ spliceosomes

For analysis of PRP8 binding in the 3′ splice site region, 4-thiouridine was incorporated at position +1 relative to

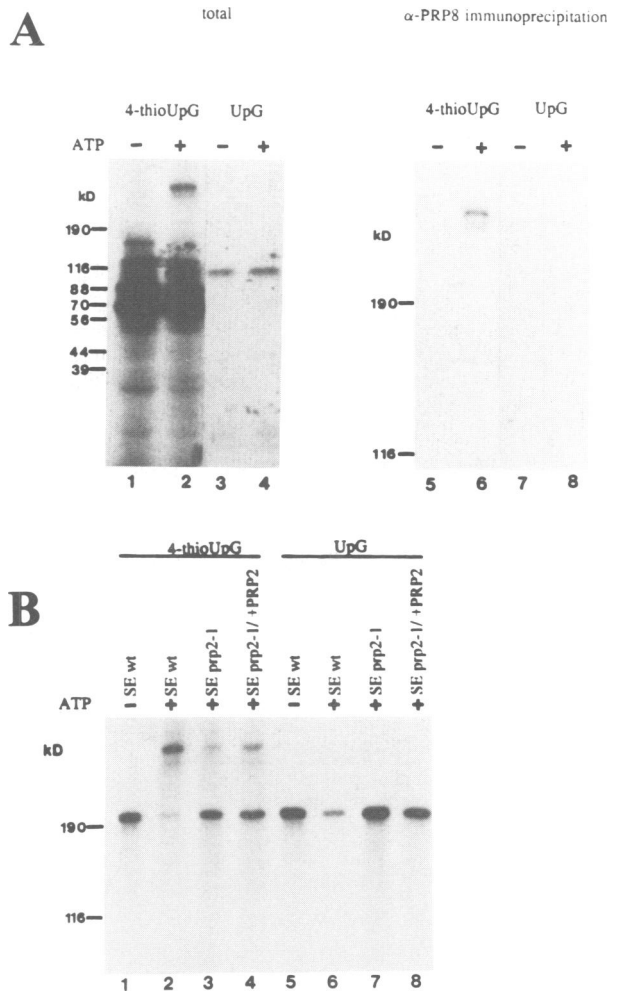

Fig. 2. Crosslinking of PRP8 protein to position –1 at the 5′ splice site. A wild-type splicing extract containing CYH2 pre-mRNA labelled at position –1 at the 5′ splice site as shown in Figure 1 was incubated under splicing conditions, followed by UV irradiation and RNase T1 digestion. Aliquots of the reactions were immunoprecipitated with PRP8-specific antibodies. Samples were separated by SDS–PAGE on 5–20% gradient gels (total) or 6% gels (immunoprecipitation), followed by autoradiography to detect crosslinked protein. (**A**) Proteins crosslinked to 4-thiouridine of CYH2 substrate RNA in a splicing reaction assembled in the absence (lane 1) and presence (lane 2) of ATP and to a CYH2 substrate RNA without 4-thiouridine in the absence (lane 3) and presence (lane 4) of ATP are shown. Immunoprecipitations with PRP8-specific antibodies of aliquots of these samples are shown in lanes 5–8. (**B**) Crosslinking to 4-thiouridine of CYH2 substrate RNA and immunoprecipitation of PRP8 protein was carried out as described in (A) with a wild-type splicing extract in the absence (lane 1) and presence (lane 2) of ATP and with PRP2Δ spliceosomes in the absence (lane 3) and presence (lane 4) of purified PRP2 protein. Samples containing crosslinking reactions to CYH2 substrate RNA without 4-thiouridine, but otherwise treated identically as described above, are shown in lanes 5–8. The migration of protein molecular weight markers is indicated on the left. The band at ~200 kDa that is also present in the UpG controls is probably undigested RNA that adheres to the protein A–Sepharose beads.

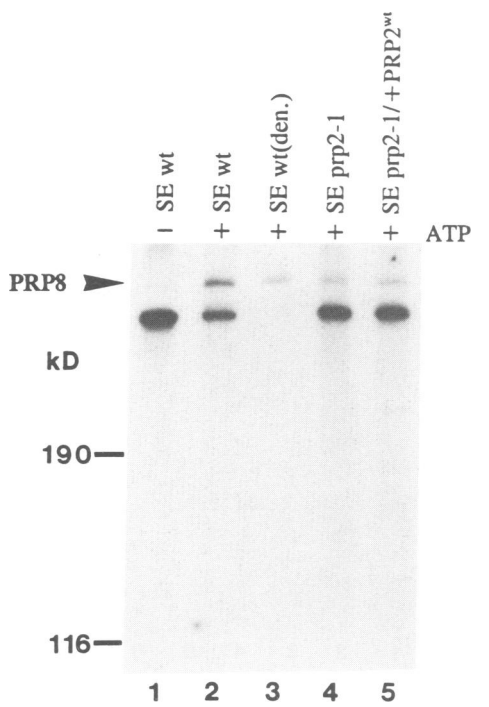

Fig. 3. Crosslinking of PRP8 protein to position –2 at the 5′ splice site. Crosslinking to 4-thiouridine (position –2 relative to the 5′ splice site) of CYH2 substrate RNA and immunoprecipitation of PRP8 protein was carried out as described for Figure 2 with a wild-type splicing extract in the absence (lane 1) and presence of ATP under non-denaturing (lane 2) and denaturing conditions (lane 3) and with PRP2Δ spliceosomes in the absence (lane 4) and presence (lane 5) of purified PRP2 protein. The migration of protein molecular weight markers is indicated on the left, as is the position of PRP8 protein. The strong signal migrating ahead of PRP8 is probably undigested RNA that adheres to the protein A–Sepharose beads unless denaturing conditions are used (lane 3).

the 3′ splice site. Using PRP8-specific antibodies for identification, crosslinking of PRP8 protein to this position was detected under splicing conditions (in the presence of ATP; Figure 5A, lane 2) but not in the absence of ATP (Figure 5A, lane 1) in a splicing reaction with a wild-type extract. Crosslinking of PRP8 protein to the 3′ splice site was not detected in a splicing reaction using a PRP2Δ extract (Figure 5A, lane 3). However, when the reaction containing the PRP2Δ extract was supplemented with purified PRP2 protein, crosslinking of PRP8 protein to position +1 at the 3′ splice site was detected (Figure 5A, lane 4), indicating that this interaction is established after the initiation of step 1 of splicing. Negative controls using CYH2 pre-mRNA lacking 4-thiouridine confirmed the site specificity of this interaction (Figure 5A, lanes 5–8).

To test a possible recognition of the PyAG consensus sequence at the 3′ splice site by PRP8 protein, 4-thiouridine was incorporated at the start of the 3′ exon of a CYH2 pre-mRNA containing a CAG to CUC mutation at the end of the intron. PRP8 protein was crosslinked to the first nucleotide of the 3′ exon under splicing conditions (Figure 5B, lane 3) with similar efficiency to PRP8 protein crosslinked to the same position preceded by a CAG sequence (Figure 5B, lane 2). As before, the site specificity was monitored by negative controls with CYH-2 RNA lacking 4-thiouridine (Figure 5B, lanes 4–6). Thus, the PyAG consensus sequence was not required for the interaction of PRP8 protein with the downstream exon. Since the CAG to CUC mutation prevents the second transesterification reaction (Vijayraghavan *et al.*, 1986; Newman and Norman, 1992; A.J.Newman, data not shown), this crosslink of PRP8 must be established prior to step 2 of splicing but after initiation of step 1 (caused by PRP2 action, see above).

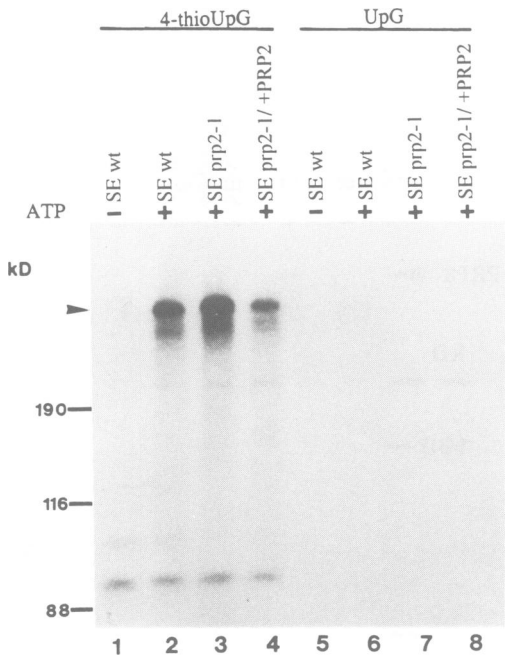

Fig. 4. Crosslinking of PRP8 protein to position –8 at the 5′ splice site. Crosslinking to 4-thiouridine (position –8 relative to the 5′ splice site) of CYH2 substrate RNA and immunoprecipitation of PRP8 protein were carried out as described for Figure 2 with a wild-type splicing extract in the absence (lane 1) and presence (lane 2) of ATP and with PRP2Δ spliceosomes in the absence (lane 3) and presence (lane 4) of purified PRP2 protein. Samples containing crosslinking reactions to CYH2 substrate RNA without 4-thiouridine, but otherwise treated identically as described above, are shown in lanes 5–8. The migration of protein molecular weight markers is indicated on the left and the position of PRP8 protein is indicated by an arrow.

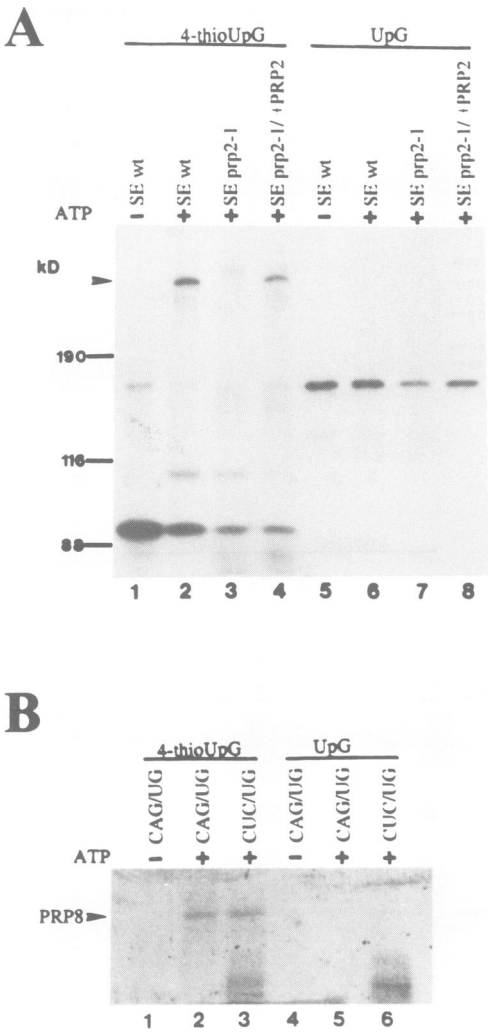

Fig. 5. Crosslinking of PRP8 protein to position +1 at a wild-type and mutant 3′ splice site. (**A**) Crosslinking to 4-thiouridine (position +1 relative to a 3′ splice site preceded by the CAG consensus sequence) of CYH2 substrate RNA and immunoprecipitation of PRP8 protein was carried out as described for Figure 2 with a wild-type splicing extract in the absence (lane 1) and presence (lane 2) of ATP and with PRP2Δ spliceosomes in the absence (lane 3) and presence (lane 4) of purified PRP2 protein. Samples containing crosslinking reactions to CYH2 substrate RNA without 4-thiouridine, but otherwise treated identically as described above, are shown in lanes 5–8. The migration of protein molecular weight markers is indicated on the left and the position of PRP8 protein is indicated by an arrow. The signal at ~150 kDa is probably undigested RNA that adheres to the protein A–Sepharose beads. The crosslink-dependent, ATP-independent band at ~90 kDa is most likely a crosslinked protein that adheres non-specifically to the antibody–protein A–Sepharose complexes under non-denaturing conditions. (**B**) Crosslinking of PRP8 protein to 4-thiouridine at position +1 relative to the 3′ splice site preceded by the CAG consensus sequence in the absence (lane 1) and presence (lane 2) of ATP and to 4-thiouridine in the same position, but preceded by a CUC sequence (lane 3). Samples containing crosslinking reactions to CYH2 substrate RNA without 4-thiouridine, but otherwise treated identically as described above, are shown in lanes 4–6. The position of PRP8 protein is indicated on the left.

To extend the characterization of this interaction to intron nucleotides upstream of the 3′ splice site, 4-thiouridine was incorporated at position +2 downstream of the branchpoint adenosine. PRP8 protein could be UV-crosslinked to this position in a wild-type splicing extract only in the presence of ATP (Figure 6, lanes 1 and 2). However, this crosslink was much less efficient than that at the 5′ splice site (autoradiographs were exposed up to seven times longer for crosslinks at the 3′ splice site than for crosslinks at the 5′ splice site). The interaction with the branchpoint region was detected in PRP2Δ spliceosomes only after addition of purified PRP2 protein (Figure 6, lanes 3 and 4), indicating that it occurs only subsequent to the initiation of the first transesterification reaction. Several other radiolabelled species detected in this experiment owing to the long exposure were most likely non-specifically precipitated proteins, as the immunoprecipitation was carried out under non-denaturing conditions. The absence of radiolabelled PRP8 protein in samples with substrate RNA lacking 4-thiouridine (Figure 6, lanes 5–8) confirmed the site specificity of this crosslink.

To determine the effect of a 3′ splice site duplication on the interactions of PRP8 protein with the downstream exon, CYH2 precursor RNAs containing a duplication of the 3′ splice site were produced: CYH2-py RNA contained a duplication of the last 12 nucleotides of the intron, thereby extending the polypyrimidine tract and introducing a second 3′ splice site. 4-Thiouridine was incorporated at position +1 relative to the distal 3′ splice site of CYH2-py RNA containing either a wild-type (CAG/UG) or mutant (CUC/UG) proximal 3′ splice site (Figures 1 and 7A). For comparison, CYH2-pu RNA was constructed, in which the purine-rich sequence from position +1 to +12 downstream of the 3′ splice site was duplicated but with two remaining pyrimidines changed to purines (C_4 to G,

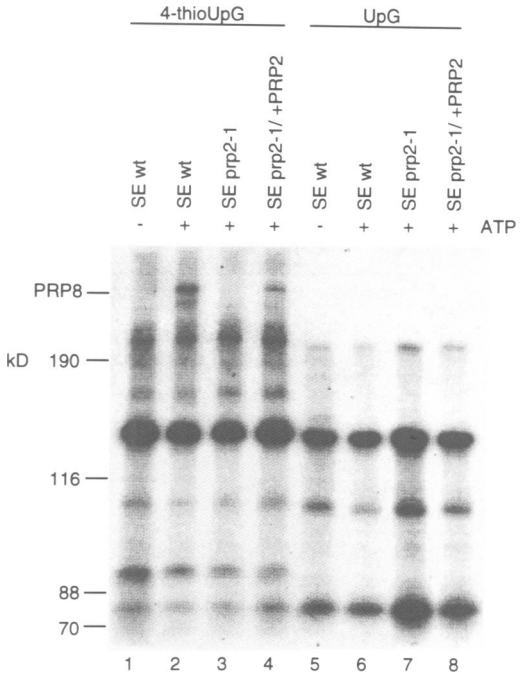

Fig. 6. Crosslinking of PRP8 protein to position +2 downstream of the branchpoint adenosine. Crosslinking to 4-thiouridine (position +2 relative to the branchpoint adenosine) of CYH2 substrate RNA and immunoprecipitation of PRP8 protein was carried out as described for Figure 2 with a wild-type splicing extract in the absence (lane 1) and presence (lane 2) of ATP and with PRP2Δ spliceosomes in the absence (lane 3) and presence (lane 4) of purified PRP2 protein. Samples containing crosslinking reactions to CYH2 substrate RNA without 4-thiouridine, but otherwise treated identically as described above, are shown in lanes 5–8. The migration of protein molecular weight markers is indicated on the left, as is the position of PRP8 protein. The PRP8 crosslink at this position is weaker than at other positions, and the longer exposure has resulted in a higher background.

U_5 to A) and incorporation of a CAG consensus sequence at the 3′ end of the duplication, resulting in a polypurine tract preceding the distal 3′ splice site. 4-ThioUpG was incorporated into position +1 at either the proximal or distal 3′ splice site for UV-crosslinking analysis (Figures 1 and 7B).

With these substrate RNAs that contain two potential 3′ splice sites, the proximal position was used as the sole acceptor site in the second transesterification reaction, independent of whether the duplicated sequence was a polypyrimidine or polypurine tract; however, a CAG to CUC mutation at the proximal 3′ splice site of CYH-py RNA activated the distal 3′ splice site (Figure 1; data not shown).

UV irradiation of splicing reactions and immunoprecipitation with PRP8-specific antibodies indicated that PRP8 protein could be crosslinked to the labelled position at the distal 3′ splice site of a CYH2-py substrate RNA in the presence of a wild-type or mutant proximal 3′ splice site, i.e. whether or not the distal sequence acted as an acceptor site in the second transesterification reaction (Figure 7A, lanes 1, 2, 5 and 6). Unfortunately, attempts to incorporate 4-thiouridine at the upstream 3′ splice site and into the polypyrimidine tract failed due to the inability of T7 polymerase to initiate transcription with uridine-rich sequences. Therefore, binding of PRP8 protein to the proximal sites could not be tested.

PRP8 protein could be crosslinked both to the distal 3′ splice site which was preceded by a polypurine tract in the CYH2-pu substrate RNA (Figure 7B, lanes 5 and 6) and to the proximal 3′ splice site which was preceded by a normal polypyrimidine tract (Figure 7B, lanes 1 and 2). No signal was observed in control samples with pre-mRNAs lacking 4-thiouridine (Figure 7A and B, lanes 3, 4, 7 and 8). Since the distal 3′ splice site was not used in the CYH2-pu RNA, crosslinking of PRP8 protein to this position is equivalent to an interaction with position +13 of the downstream exon (Figure 8B), indicating that the binding site for PRP8 protein contains at least 13 exon nucleotides adjacent to the 3′ splice site.

A summary of all positions in the CYH2 RNA that are crosslinked to PRP8 protein is shown in Figure 8.

Discussion

It was shown previously that the U5 snRNP protein PRP8 interacts directly with substrate RNA in spliceosomes during both steps of splicing (Whittaker and Beggs, 1991; Teigelkamp *et al.*, 1995). From the results presented here, the precise identification of binding sites for PRP8 protein on the substrate RNA and the kinetics of these interactions is a significant step towards an understanding of the function of PRP8 protein.

Site-specific UV-crosslinking of protein or RNA to a single photoactivatable 4-thiouridine incorporated into RNA molecules has proved to be a powerful technique for mapping intermolecular interactions precisely (Moore and Sharp, 1992; Wyatt *et al.*, 1992; Sontheimer and Steitz, 1993). Using this approach we detected an interaction of PRP8 protein with exon nucleotides –1, –2 and –8 relative to the 5′ splice site in a wild-type splicing extract only under splicing conditions (Figure 8). These data indicate that a region of at least eight exon nucleotides adjacent to the 5′ splice site is contacted by PRP8 protein. Considering that only ~25 nucleotides of the exon at the 5′ splice site in a precursor RNA are protected from RNase T1 degradation by spliceosomal complexes containing PRP8 protein (Teigelkamp *et al.*, 1995), the PRP8 binding site does not extend beyond a maximum length of ~25 residues upstream of the 5′ splice site. Nucleotide +4 was the intron position closest to the 5′ splice site which could be examined without altering the 5′ splice site consensus sequence GUAUGU with the 4-thioUpG dinucleotide primer. No interaction of PRP8 protein with this position was detected by this method. Although binding of PRP8 protein to the 5′ end of the intron in the pre-mRNA cannot be ruled out, PRP8 protein interacts predominantly with exon residues, and the 5′ splice site might be the downstream boundary of the PRP8–pre-mRNA interaction. The interactions with the 5′ exon are established prior to initiation of the first transesterification reaction and are most likely maintained because the efficiency of the crosslinks did not decrease after initiation of step 1 of splicing.

UV-crosslinking to the first nucleotide of the downstream exon and position +2 relative to the conserved branchpoint adenosine after initiation of step 1 of splicing revealed an additional interaction of PRP8 protein in a wild-type splicing extract. Mutation of the 3′ splice site sequence CAG to CUC did not abolish the interaction of

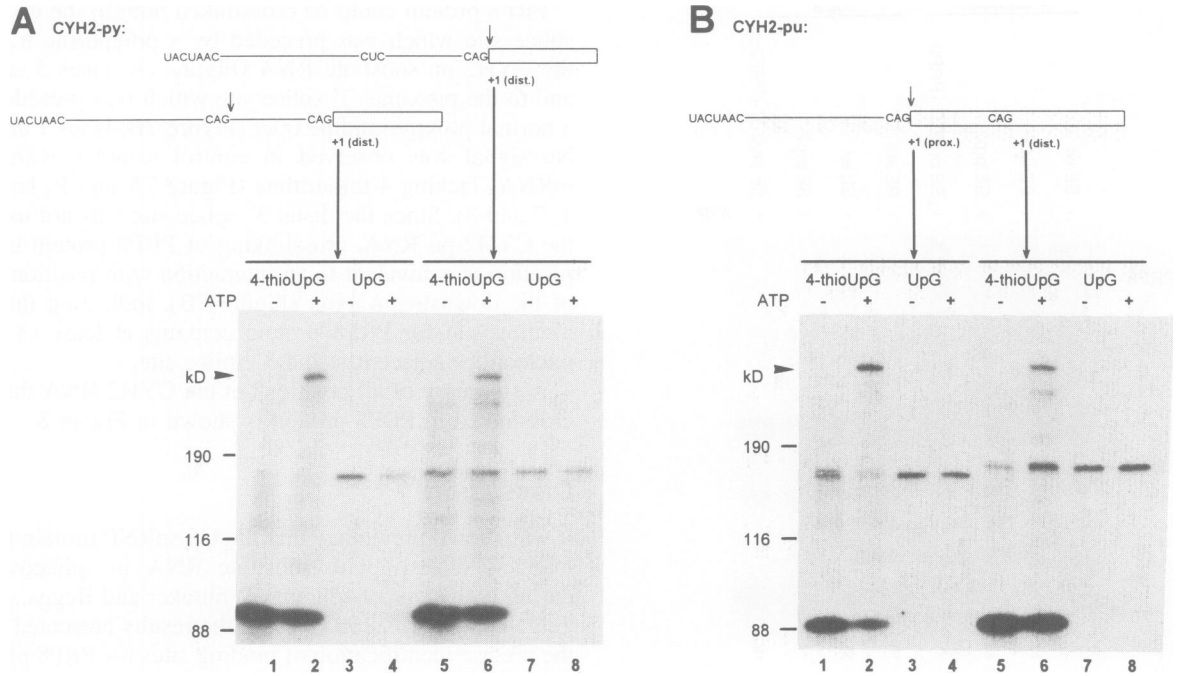

Fig. 7. Crosslinking of PRP8 protein to duplicated 3′ splice sites. Crosslinking to 4-thiouridine of CYH2 substrate RNA in a wild-type splicing extract and immunoprecipitation of PRP8 protein was carried out as described for Figure 2. (**A**) Crosslinking of PRP8 protein to CYH2-py RNA (CAG/CAG +1) in the absence (lane 1) and presence (lane 2) of ATP and to CYH2-py RNA (CUC/CAG +1) in the absence (lane 5) and presence (lane 6) of ATP is shown. Samples containing crosslinking reactions to CYH2 substrate RNA without 4-thiouridine, but otherwise treated identically as described above, are shown in lanes 3, 4, 7 and 8. (**B**) Crosslinking of PRP8 protein to CYH2-pu RNA (CAG +1/CAG) in the absence (lane 1) and presence (lane 2) of ATP and to CYH2-pu RNA (CAG/CAG +1) in the absence (lane 5) and presence (lane 6) of ATP is shown. Samples containing crosslinking reactions to CYH2 substrate RNA without 4-thiouridine, but otherwise treated identically as described above, are shown in lanes 3, 4, 7 and 8. The migration of protein molecular weight markers is indicated on the left and the position of PRP8 protein is indicated by an arrow. The crosslink-dependent, ATP-independent band at ~90 kDa is most likely a crosslinked protein that adheres non-specifically to the antibody–protein A–Sepharose complexes under non-denaturing conditions. Details of the 3′ end of the CYH2 substrate RNAs shown on the top are explained in Figure 8.

PRP8 protein with the first nucleotide of the 3′ exon, indicating that the CAG sequence is not a recognition motif for PRP8 protein. With a substrate RNA containing a duplication of 12 nucleotides of the polypyrimidine tract and the 3′ splice site, PRP8 protein was crosslinked to position +1 relative to the distal 3′ splice site sequence even when splicing occurred solely at the proximal 3′ splice site. Thus, it appears that the interaction of PRP8 protein with this region is not restricted to the immediate vicinity of the functional 3′ splice site. Also, given the choice between two 3′ splice sites separated by 12 nucleotides of downstream exon sequence (purines only), PRP8 protein was crosslinked to both positions although the proximal position was the only functional 3′ splice site. Thus, following the first transesterification reaction PRP8 protein binds to a region of at least 13 residues of the exon adjacent to the 3′ splice site. Furthermore, binding is not selective for polypyrimidine, indicated by the interaction of PRP8 protein with a region rich in purines. However, the polypyrimidine tract upstream of the proximal 3′ splice site may still direct the interaction of PRP8 protein with downstream exon sequence, either directly or indirectly via other factors. Since PRP8 protein interacts with position +1 with respect to a CUC mutant 3′ splice site, a mutation which causes a complete block of the second transesterification reaction (Vijayraghavan *et al.*, 1986; Newman and Norman, 1992; our own observations), the binding is established before step 2. Interaction with position +2 downstream of the branchpoint occurred with similar kinetics, and indicates that PRP8 protein also contacts a part of the polypyrimidine tract of the intron. Binding to the branchpoint UACUAAC sequence and the entire polypyrimidine tract cannot be ruled out. The binding of PRP8 protein to the 3′ splice site region is distinct from the 5′ splice site interaction, with clearly different kinetics.

Although PRP8 protein is present in spliceosomes before step 1 of splicing (indicated by its interaction with the 5′ splice site), the fact that it cannot be crosslinked to 4-thiouridine in the 3′ splice site region at this stage indicates that the contacts observed are indeed highly specific, and that binding of PRP8 protein to the 3′ splice site region subsequent to step 1 may require a conformational change in PRP8 protein and/or other components of the spliceosome. The inability to detect a crosslink of PRP8 protein to 4-thiouridine in position +4 relative to the 5′ splice site confirms the specificity of the PRP8–RNA interactions.

Binding of PRP8 protein to the 5′ splice site at the time of the first transesterification reaction, followed by an additional interaction with the 3′ splice site at the time of the second transesterification reaction, places it in the catalytic centre of the spliceosome during both steps of splicing. As the catalytic centre appears to undergo

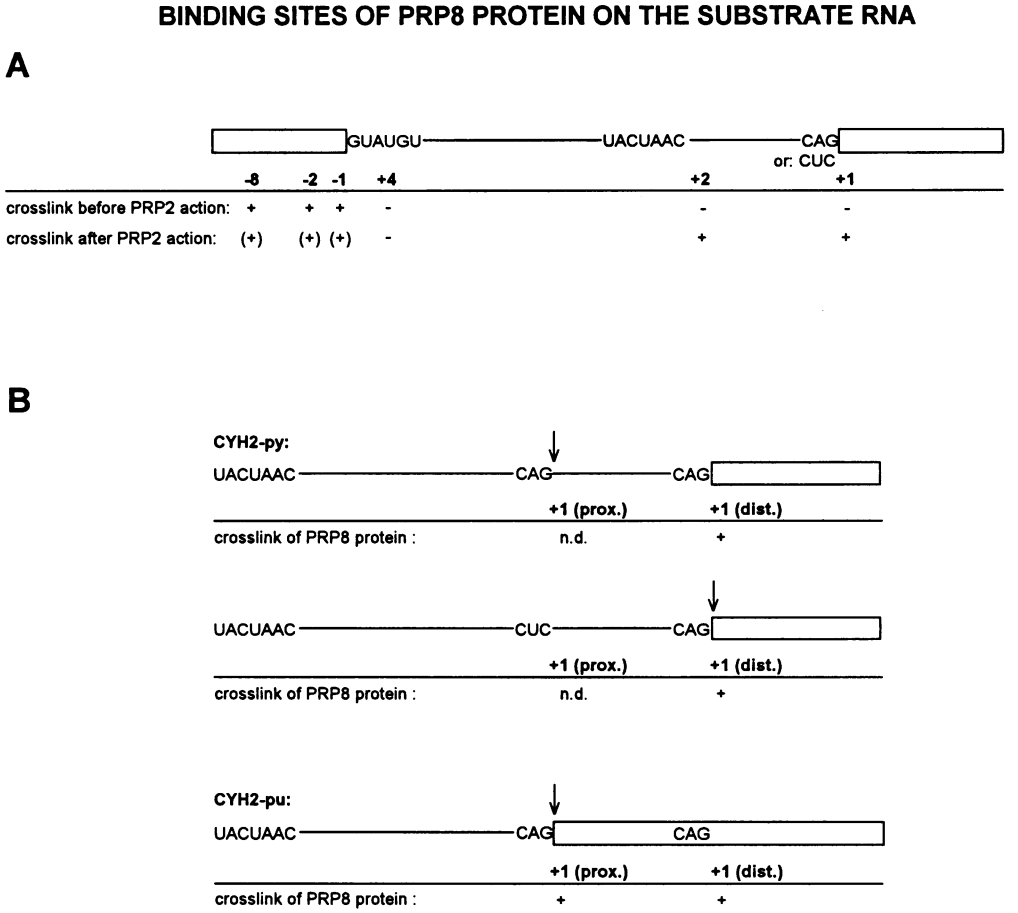

Fig. 8. Summary of the positions on the CYH2 precursor RNA that can be crosslinked to PRP8 protein. (**A**) CYH2 RNA is shown with the conserved sequences at both splice sites and the branchpoint consensus sequence, with the positions investigated for crosslinking of PRP8 protein shown underneath. Discrimination between crosslinks before and after PRP2 action is derived from experiments using PRP2Δ spliceosomes. + indicates a crosslink of PRP8 protein, – indicates no detectable crosslink of PRP8 protein and (+) refers to the fact that these crosslinks are most likely maintained after PRP2 action. (**B**) The 3′ splice site region of CYH2 RNA is shown, composed of the branchpoint consensus sequence, the polypyrimidine tract (single line), exon 2 (box) and a duplicated 3′ splice site consensus sequence, spaced by the last 12 residues of the intron (CYH2-py) or the first 12 residues of exon 2 (CYH2-pu). The arrow indicates the position of the 3′ splice site used for the second transesterification reaction, and + indicates a crosslink of PRP8 protein.

conformational changes between the two steps (Moore and Sharp, 1993; Steitz and Steitz, 1993), PRP8 protein might be actively involved in this process. In the HeLa system a protein of ~220 kDa, considered to correspond to the human homologue of PRP8, has been UV-crosslinked to position –2 relative to the 5′ splice site (Wyatt *et al.*, 1992; MacMillan *et al.*, 1994) and to the branchpoint nucleotide with similar kinetics to those reported here for PRP8 (MacMillan *et al.*, 1994). It is predictable now that human p220 could interact with equally large regions of the exons adjacent to the splice sites as does the yeast PRP8 protein.

Molecular contacts of yeast U5 snRNA with positions –2 and –3 in the 5′ exon were predicted by previous genetic experiments (Newman and Norman, 1991, 1992). Similarly, UV-crosslinking of mammalian U5 snRNA to 4-thiouridine incorporated at positions –1 and –2 of exon 1 was detected prior to step 1 of splicing in HeLa cells, but not to position +2 in the intron (Wyatt *et al.*, 1992; Sontheimer and Steitz, 1993). The extent of the U5 snRNA interaction with the 5′ exon has not been determined; however, PRP8 protein is most likely a major contributor to the stability of the U5 snRNP interaction with the 5′ splice site, as the U5 snRNA appears to make a comparatively fragile interaction with non-conserved exon sequences.

The situation appears to be very similar at the 3′ splice site. Interaction of loop I of the mammalian and yeast U5 snRNA with position +1 of the 3′ exon after step 1 has been detected by site-specific UV-crosslinking (Sontheimer and Steitz, 1993, A.J.Newman, unpublished results) and genetic experiments indicate an interaction of nucleotides of the yeast U5 snRNA loop with the first two bases of the 3′ exon. PRP8 protein interacts with a minimum of 13 exon nucleotides at the 3′ splice site and at least a part of the branchpoint–polypyrimidine tract region. Since the kinetics of both the branchpoint +2 and the 3′ splice site +1 interactions with PRP8 protein are identical, this may be one binding site rather than two, and could include the entire polypyrimidine tract. Again, U5 snRNA interactions with the 3′ exon may be stabilized by extensive binding of PRP8 protein in this region.

In the model represented in Figure 9 it is proposed that PRP8 protein stabilizes the interactions between U5

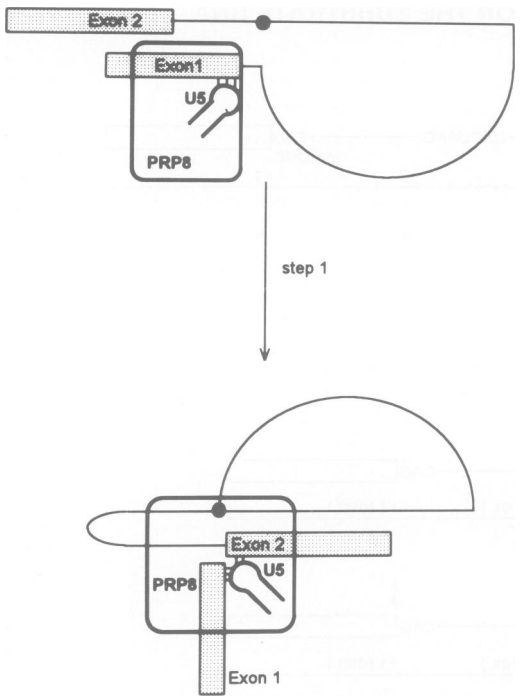

Fig. 9. Interactions of PRP8 protein with substrate RNA during splicing. In the model of PRP8 protein binding to substrate RNA before and after step 1 of splicing, exons are shown as shaded boxes, the intron as a single line and the branchpoint as a dot. Interaction of the conserved loop I of U5 snRNA with exon residues at the splice sites is indicated by straight lines. PRP8 protein is shown as a square box drawn with a thick line, covering the regions of interaction on the substrate RNA before (top) and after (bottom) step 1 of splicing. For reasons of simplicity, interactions of U2 and U6 snRNA with each other and with the substrate RNA are not drawn into this model.

snRNA and exon sequences at the 5′ and 3′ splice sites, tethering the 5′ exon prior to step 1 and then maintaining the precise positioning of both exons after step 1, which is critical for the catalytic structure for step 2 of splicing.

Although PRP8 protein binds specifically to regions at the 5′ and 3′ splice sites, the interaction with a mutant 3′ splice site indicates that the binding may lack sequence specificity, as no other conserved sequences occur in the bound regions. Rather than binding to a sequence motif, PRP8 protein might recognize particular RNA structures (e.g. substrate RNA–snRNA interactions) which in return could be modified by PRP8 binding. Perturbation of RNA structures as a result of protein binding has been described (Rould *et al.*, 1989; Oubridge *et al.*, 1994), which resembles an 'induced fit' mechanism and could explain why PRP8 protein binds substrate RNA only in assembled spliceosomes (Whittaker and Beggs, 1991). PRP8 protein does not contain any of the characterized RNA binding consensus motifs (Kenan *et al.*, 1991; Mattaj, 1993; Burd and Dreyfuss, 1994; Hodges *et al.*, 1995) that might indicate whether it uses one or more binding site(s) to interact with the distinct regions on a substrate RNA. Conceivably, the RNA binding site(s) may be complex, requiring contributions from other spliceosomal proteins. Mapping the RNA binding site(s) on the PRP8 protein will be the next step towards an understanding of the mechanism and function of protein–RNA interactions in the spliceosome.

Materials and methods

Oligonucleotides and DNA templates for in vitro transcription

DNA templates for *in vitro* transcriptions were made synthetically (short 5′ RNA fragments for labelling positions –8, –2, –1 and +4 relative to the 5′ splice site) or generated by polymerase chain reaction (PCR; all other templates) from r243(T7CYH2Δ200) plasmid. r243(T7CYH2Δ200) contains a T7 promoter upstream of a *CYH2* gene that has a deletion of 200 nucleotides between the 5′ splice site and the branchpoint to reduce the size of the intron. PCR products were made full-length by incubation with T4 DNA–polymerase (0.03 U/μl final conc.; New England Biolabs) for 30 min at 37°C. The DNA was purified by centrifugation in centricon cartridges having a 30 kDa molecular weight cut-off. DNA templates contained a bacteriophage T7 promoter upstream of the coding sequence.

The following oligonucleotides were used as DNA templates for 5′-transcripts.

Synthetic templates. 1529, 5′-TAA TAC GAC TCA CTA TA-3′ (T7 promoter top strand); 4155, 5′-TGG TAG TAG CGC TTT CTA GTC TTA GTG AAT CTG GAA GGC ATT CCT ATA GTG AGT CGT ATT A-3′ (with 1529 for label at position –8 of 5′ splice site); 1744, 5′-GAG ACG TGA CCT CTG TGC TTT CTA GTC TTA GTG AAT CTG GAA GGC ATT CCT ATA GTG AGT CGT ATT A-3′ (with 1529 for label at position –2 of 5′ splice site); 2596, 5′-TGA GAC GTG ACC TCT GTG CTT TCT AGT CTT AGT GAA TCT GGA AGG CAT TCC TAT AGT GAG TCG TAT TA-3′ (with 1529 for label at position –1 of 5′ splice site); 1748, 5′-TAC CAG AGA CGT GAC CTC TGT GCT TTC TAG TCT TAG TGA ATC TGG AAG GCA TTC CTA TAG TGA GTC GTA TTA-3′ (with 1529 for label at position +4 of 5′ splice site).

Oligonucleotides for PCR templates. 163, 5′-GCG CTA ATA CGA CTC ACT ATA GAC TAG AAA GCA CAG AGG TC-3′ (PCR-forward primer for 5′-fragments); G7095, 5′-GTT AGT AAA ACA GGT TTT CA-3′ (with 163 for label at position +2 of branchpoint); 142, 5′-CTG TAC AAA AAA AAT ATT GTA ATG AAA TAC-3′ (with 163 for label at position +1 of CAG-3′ splice site and label at position +1 at proximal 3′ splice site of CYH2-pu CAG/CAG); H4985, 5′-GAG TAC AAA AAA AAT ATT GTA ATG AAA TAC-3′ (with 163 for label at position +1 of CUC-3′ splice site); M0351, 5′-CTG TAC AAA ACA CTG TAC AAA AAA AAT ATT GTA ATG AAA TAC-3′ (with 163 for label at position +1 at distal 3′ splice site of CYH2-py CAG/CAG); H4984, 5′-CTG TAC AAA ACA GAG TAC AAA AAA AAT ATT GTA ATG AAA TAC-3′ (with 163 for label at position +1 at distal 3′ splice site of CYH2-py CUC/CAG); M4007, 5′-CTG CCT TTC CCA CTG TAC AAA AAA AAT ATT-3′ (with 163 for label at position +1 at distal 3′ splice site of CYH2-pu CAG/CAG).

The following oligonucleotides were used as DNA templates of 3′-fragments (all PCR): 451, 5′-AAG TAT CTC ATA CCA ACC TTA CCG-3′ (PCR-reverse primer for 3′-fragments); 4156, 5′-GCT AAT ACG ACT CAC TAT AGG ACC AGG TAT GTA GTT CCA-3′ (with 451 for label at position –8 of 5′ splice site); 1745, 5′-GCG CTA ATA CGA CTC ACT ATA GGT ATG TAG TTC CAT TTG GAA G-3′ (with 451 for label at position –2 of 5′ splice site); 2597, 5′-GCG CTA ATA CGA CTC ACT ATA GTA TGT AGT TCC ATT TGG AAG-3′ (with 451 for label at position –1 of 5′ splice site); 1749, 5′-GCG CTA ATA CGA CTC ACT ATA GTA GTT CCA TTT GGA AGA G-3′ (with 451 for label at position +4 of 5′ splice site); G7096, 5′-TAA TAC GAC TCA CTA TAG ATT TAA AAA TTG TAT TTC A-3′ (with 451 for label at position +2 of branchpoint); 174, 5′-GCG CTA ATA CGA CTC ACT ATA GGC TAA GGG TAG AAT CGG TA-3′ (with 451 for label at position +1 of CAG-3′ splice site, CUC-3′ splice site and all duplicated 3′ splice sites being labelled at the distal position); M4008, 5′-GCG CTA ATA CGA CTC ACT ATA GGG AAA GGC AGT GGC TAA GGG TAG AAT CGG TAA GCA CAG AA-3′ (with 451 for label at position +1 at proximal 3′ splice site of CYH2-pu CAG/CAG).

Oligodeoxynucleotides were synthesized by Oswel DNA Service (Edinburgh) or by Terry Smith, MRC Laboratory of Molecular Biology (Cambridge).

In vitro transcription, site-specific labelling and ligation of RNA substrates

RNAs were synthesized by *in vitro* transcription for 3 h at 37°C in a total volume of 250 μl. Transcription reactions for 5′ fragments contained 40 mM Tris–HCl (pH 8), 1 mM spermidine, 0.01% Triton X-100, 5 mM DTT, 0.5 U/μl RNasin (Promega), 20 mM $MgCl_2$, ATP, GTP, UTP, CTP (2 mM each; Pharmacia); 2.5 mM m_7G cap analogue (New England Biolabs), 8% (w/v) PEG 8000, 1–1.5 μM (synthetic template) or 0.4 μM

(PCR template) DNA and 16 U/μl T7 RNA polymerase. Transcription reactions for 3′ fragments contained 40 mM Tris–HCl (pH 8), 2 mM spermidine, 10 mM DTT, 0.5 U/μl RNasin (Promega), 20 mM $MgCl_2$, ATP, UTP, CTP (1 mM each), GTP (0.5 mM), 2mM 4-thioUpG or UpG (both Sigma), 8% (w/v) PEG 8000, 0.4 μM DNA template and 16 U/μl T7 RNA polymerase. Following transcription, reactions were stopped with EDTA (50 mM final conc.) and extracted with phenol and chloroform; the RNA was precipitated with ethanol. Transcripts were purified by electrophoresis on denaturing gels (4 or 10% acrylamide–8% urea), followed by elution from the gel and extraction with phenol and chloroform and were kept at –80°C in ethanol.

To synthesize full-length CYH2 precursor RNA, 5′ and 3′ RNA-fragments were ligated as described previously (Moore and Sharp, 1992; Wyatt *et al.*, 1992): 3′ transcripts were 5′-^{32}P-labelled by incubation with T4 polynucleotide kinase for 30 min at 37°C in a total volume of 30 μl. Kinase reactions contained 50 mM Tris–HCl (pH 8), 10 mM Mg Cl_2, 5 mM DTT, 2.5 U/μl RNasin, 7 μCi/μl [γ-^{32}P]ATP, 50 pmol RNA and 1 U/μl T4 polynucleotide kinase (New England Biolabs). The radiolabelled RNA was extracted with phenol, precipitated in ethanol and redissolved in H_2O.

The ^{32}P-radiolabelled 3′ fragment (50 pmol) was redissolved in a total volume of 19.2 μl, containing 200 pmol of 5′ fragment, 100 pmol of bridging oligodeoxynucleotide (see below), 0.5 mM ATP, 31 mM Tris–HCl (pH 8), 6 mM $MgCl_2$ and 3 mM DTT. The reaction mix was incubated at 90°C for 2 min and cooled to room temperature during 10 min to allow efficient annealing of the RNA fragments with the bridging oligodeoxynucleotide. Subsequently, 1 μl ligase buffer (0.5 M Tris–HCl pH 7.4, 10 mM ATP, 10 mM $MgCl_2$, 1 μg/μl acetyl BSA), 1 μl DTT (400 mM), 50 U RNasin and 4000 U of DNA ligase (New England Biolabs) were added, followed by incubation for 1 h at 37°C to ligate the fragments. Finally, the RNA was extracted with phenol, precipitated with ethanol, gel purified as described above and stored in TE-buffer at –80°C.

The following bridging oligodeoxynucleotides were used for RNA ligations: 4157, 5′-TGG AAC TAC ATA CCT GGT CCA TGG TAG TAG CGC TTT CTA G-3′ (position –8 at the 5′ splice site); 1747, 5′-CTC TTC CAA ATG GAA CTA CAT ACC AGA GAC GTG ACC TCT GTG CTT-3′ (positions –2 and +4 at the 5′ splice site); 2598, 5′-CTC TTC CAA ATG GAA CTA CAT ACA TGA GAC GTG ACC TCT GTG CTT-3′ (position –1 at the 5′ splice site); G7097, 5′-AAT GAA ATA CAA TTT TTA AAT CAG TTA GTA AAA CAG GTT TTC AAC-3′ (position +2 at the branchpoint); 1526, 5′-TTA CCG ATT CTA CCC TTA GCC ACT GTA CAA AAA AAA TAT TGT AA-3′ (position +1 at the CAG-3′ splice site); H1009, 5′-TTA CCG ATT CTA CCC TTA GCC AGA CTA CAA AAA AAA TAT TGT AA-3′ (position +1 at the CUC -3′ splice site); M0352, 5′-TTC TAC CCT TAG CCA CTG TAC AAA ACA CTG TAC AAA AAA AAT-3′ (position +1 at both 3′ splice sites of CYH2-py CAG/CAG); H4983, 5′-TTC TAC CCT TAG CCA CTG TAC AAA ACA GAG TAC AAA AAA AAT-3′ (position +1 at the CUC 3′ splice site and at both 3′ splice sites of CYH2-py CUC/CAG); M4001, 5′-TTC CAT CCT TAG CCA CTG CCT TTC CCA CTG TAC AAA AAA AAT-3′ (position +1 at both 3′ splice sites of CYH2-pu CAG/CAG).

Yeast strains, splicing extract preparation and in vitro splicing reactions

Saccharomyces cerevisiae strains: BJ2412 was described previously (Lossky *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.

Yeast whole cell extracts were prepared and splicing reactions were performed as described (Lin *et al.*, 1985). Extracts from strain DJY85 were heat-inactivated by incubation at 36°C for 40 min. For analysis of reaction products, samples were treated with proteinase K (0.3 μg/μl), extracted with phenol and chloroform, precipitated with ethanol, fractionated on 6% (w/v) polyacrylamide–8 M urea gels and visualized by autoradiography.

PRP2Δ spliceosomes were assembled at 25°C for 20 min in a total volume of 60 μl containing 50% (v/v) heat-inactivated splicing extract from DJY85 cells, 0.5 pmol site specifically 4-thiouridine/^{32}P-labelled pre-mRNA, 1.5 mM ATP and 50% (v/v) splicing buffer (Lin *et al.*, 1985). 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 were added to 60 μl of PRP2Δ spliceosomes and incubated for 12 min at 25°C. Following the incubation, reactions 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 60 μl aliquots, except that samples were UV-irradiated for 5 min at 360 nm on ice. Following digestion with RNase T1 (5 U/μl final conc.; Boehringer Mannheim, UK) or RNase A (70 ng/μl final conc.; Sigma, UK), samples were diluted 3-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).

Anti-8.6 antibodies were raised against a peptide matching an amino-terminal sequence of PRP8 protein (G.J.Anderson and J.D.Beggs, unpublished). 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 electrophoresis through 6% polyacrylamide–SDS gels. Gels were analysed by autoradiography.

Acknowledgements

We are grateful to Reinhard Lührmann for reading the manuscript and making helpful suggestions. Bacteriophage T7 RNA polymerase was a generous gift from Kiyoshi Nagai; Margaret McGarvey kindly provided purified PRP2 protein. This work was supported by the Cancer Research Campaign and the Medical Research Council. J.D.B. holds a Royal Society EPA Cephalosporin Fund Senior Research Fellowship.

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Received on January 20, 1995; revised on March 1, 1995