Identification of a human protein that recognizes the 3' splice site during the second step of pre-mRNA splicing

Shaoping Wu and Michael R.Green

Howard Hughes Medical Institute, Program in Molecular Medicine,
University of Massachusetts Medical Center, Worcester, MA 01605,
USA

Accurate splicing of precursor mRNAs (pre-mRNAs) requires recognition of the 5' and 3' splice sites at the intron boundaries. Interactions between several splicing factors and the 5' splice site, which occur prior to the first step of splicing, have been well described. In contrast, recognition of the 3' splice site, which is cleaved during the second catalytic step, is poorly understood, particularly in higher eukaryotes. Here, using site-specific photo-crosslinking, we find that the conserved AG dinucleotide at the 3' splice site is contacted specifically by a 70 kDa polypeptide (p70). The p70–3' splice site crosslink has kinetics and biochemical requirements similar to those of splicing, was detected only in the mature spliceosome and occurs subsequent to the first step. Thus, p70 has all the properties expected of a factor that functionally interacts with the 3' splice site during the second step of splicing. Using antisera to various known splicing factors, we find that p70 corresponds to a previously reported 69 kDa protein of unknown function associated with the Sm core domain of spliceosomal small nuclear ribonucleoproteins.

Keywords: mammalian pre-mRNA splicing/protein splicing factors/second step of splicing/3' splice site/UV crosslinking

Introduction

Splicing of precursor mRNAs (pre-mRNAs) involves the assembly of a multicomponent complex, the spliceosome, which contains four small nuclear ribonucleoprotein (snRNP) particles (U1, U2, U4/U6 and U5) and many non-snRNP proteins (Green, 1991; Guthrie, 1991; Bennett *et al.*, 1992; Lamm and Lamond, 1993; Moore *et al.*, 1993; Madhani and Guthrie, 1994; Neilsen, 1994; Kramer, 1996). An important function of the spliceosome is to bring into proximity the chemical groups on the pre-mRNA that participate in the two trans-esterification reactions that result in intron excision and exon ligation.

In higher eukaryotes, three major signals direct accurate splicing: the 5' splice site, the branch point and the 3' splice site. The 5' splice site is recognized early during spliceosome assembly by intermolecular base-pairing with U1 snRNA (Zhuang and Weiner, 1986; Seraphin *et al.*, 1988; Siliciano and Guthrie, 1988). At later times, this base-pairing is disrupted and the 5' splice site is bound by U6 snRNA (Sawa and Abelson, 1992; Sawa and Shimura, 1992; Wassarman and Steitz, 1992; Kandel-

Lewis and Seraphin, 1993; Konforti *et al.*, 1993; Lesser and Guthrie, 1993; Sontheimer and Steitz, 1993). The branch point region is also recognized by intermolecular base-pairing, in this case by the U2 snRNA component of U2 snRNP (Parker *et al.*, 1987; Zhuang and Weiner, 1989; Wu and Manley, 1989).

In higher eukaryotes, the 3' end of the intron comprises a polypyrimidine (Py) tract followed by an AG dinucleotide at which cleavage occurs (for review, see Umen and Guthrie, 1995c). Several factors are known to interact with the Py tract early during spliceosome assembly, including U2AF⁶⁵, IBP, PTB/hnRNP I, PSF and a 200 kDa U5 snRNP protein (reviewed in Lamm and Lamond, 1993; Moore *et al.*, 1993; Umen and Guthrie, 1995c; Kramer, 1996). One such Py tract-binding protein, PSF, is thought to be a second step splicing factor (Gozani *et al.*, 1994). However, in higher eukaryotes, little is known about splicing components that interact with the highly conserved AG dinucleotide during the second step of splicing. The AG dinucleotide is essential for the second step of splicing, and 3' splice site cleavage occurs, in general, at the first AG downstream of the branch point (Smith *et al.*, 1989; Zhuang and Weiner, 1990). These observations imply that during the second step of splicing an as yet to be identified factor functionally interacts with the AG dinucleotide.

Here we use site-directed photo-crosslinking procedures to identify a 70 kDa polypeptide that interacts with the conserved AG dinucleotide at the 3' splice site and whose properties strongly suggest a role in the second step of the splicing reaction. We go on to show that p70 corresponds to a previously identified 69 kDa of unknown function that reversibly interacts with Sm core domain of snRNPs (Hackl *et al.*, 1994).

Results

Identification of polypeptides that interact with the AG dinucleotide at the 3' splice site

To identify polypeptides that interact with the AG dinucleotide at the 3' splice site, we prepared an adenovirus major late pre-mRNA derivative (MINX) containing a single radioactive phosphate within the AG dinucleotide (A³²pG) (see Figure 1A). The splicing assay of Figure 1B shows that in a HeLa cell nuclear extract this pre-mRNA substrate was spliced with typical kinetics: the lariat–exon 2 and excised intron appeared after ~15 and 20 min, respectively. Because the single radioactive phosphate lies within the intron, the 5' exon and the ligated exons were not visualized.

At various times, aliquots of the splicing reaction mixture shown in Figure 1B were irradiated with UV light and treated with RNase A. ³²P-Tagged polypeptides were detected by SDS–PAGE and autoradiography (see Figure 1A). The crosslinking assay of Figure 1B shows that four

A

B

Fig. 1. Crosslinking of polypeptides to the AG dinucleotide. (A) Experimental strategy. ^{32}P was incorporated between the AG dinucleotide of an adenovirus major late pre-mRNA substrate (MINX) based on the procedure of Moore and Sharp (1992). The substrate was incubated in a HeLa cell nuclear extract under standard splicing conditions. The reaction mixture was irradiated with UV light followed by treatment with RNase A. ^{32}P -tagged polypeptides were detected by electrophoresis on a 10% SDS-polyacrylamide gel followed by autoradiography. (B) Splicing time course and UV crosslinking assay. Splicing reactions (top) were performed for the times indicated. The lariat, lariat-exon 2 intermediate and the pre-mRNA substrate are marked. The exon 1 and the exon 1-exon 2 ligated product were not detected because the ^{32}P -label was located within the intron. Aliquots were removed from the splicing reactions and subjected to the UV crosslinking assay (bottom) described in (A). Crosslinked polypeptides are marked on the right and molecular weight standards on the left.

groups of polypeptides were detected. A polypeptide of ~50 kDa was evident at time zero but disappeared upon incubation at 30°C. A 35–37 kDa doublet (p35/37) was observed at time zero and remained throughout the time-course, although its intensity was diminished at later times. In some experiments, a weak band of ~200 kDa could be detected as early as 15 min and reached a maximal level at 20–30 min. Based upon our data and previous studies (Teigelkamp *et al.*, 1995a,b; Umen and Guthrie, 1995a,b; Chiara *et al.*, 1996), it is likely that this band corresponds to a 200 kDa U5 snRNP polypeptide that is the probable homolog of yeast Prp8. Most importantly, a 70 kDa polypeptide (p70) was first detected at 15 min, concomitant with the appearance of splicing intermediates and products (compare splicing and crosslinking in Figure 1B). Crosslinking of this polypeptide reached a maximal level between 20 and 45 min, and its intensity diminished at later times.

Figure 2 presents several controls to verify the authenticity of the crosslinked products and their relevance to splicing. In the absence of UV irradiation, no crosslinked polypeptides were detected (crosslinking, lane 1). As predicted from the kinetics of Figure 1B, crosslinking of p70 was ATP dependent, whereas crosslinking of p35/37 was not (compare lanes 2 and 3). To determine whether crosslinking of p70 was specific to RNAs that contained authentic splicing signals, we performed competition experiments. The homologous competitor, unlabeled MINX pre-mRNA, competed with crosslinking of p70 to the 3' splice site (lanes 4–6), whereas tRNA had little effect (lanes 7–9). Pre-treatment of the nuclear extract with micrococcal nuclease, which inactivates splicing factors containing essential RNA components, abolished crosslinking of p70 but not of p35/37 (lane 11), whereas a control reaction containing both micrococcal nuclease and EGTA had no effect (lane 12). The splicing assay of

Fig. 2. Requirements for crosslinking of polypeptides to the 3' splice site. UV crosslinking assays were performed as described in Figure 1A. Lane 1, no UV irradiation (No UV); lane 2, in the absence of ATP and creatine phosphate (-ATP); lane 3, in the presence of ATP and creatine phosphate (+ATP); lanes 4-6, 1000-, 500- and 300-fold molar excess of unlabeled pre-mRNA substrate (Unlabeled MINX); lanes 7-9, 3000-, 1500- and 300-fold molar excess of unlabeled tRNA (tRNA); lane 10, with a HeLa cell nuclear extract (NE) under standard splicing conditions; lane 11, in the presence of micrococcal nuclease (MN/NE); lane 12, in the presence of micrococcal nuclease and EGTA (MN/NE/EGTA). Crosslinked polypeptides are marked on the right. The splicing assay analyzes the identical reaction mixtures shown in the crosslinking experiment.

Figure 2 analyzes the identical reaction mixtures shown in the crosslinking experiment.

p70 is bound to pre-mRNA only in the catalytically active spliceosome

The ATP dependence and micrococcal nuclease sensitivity of the p70 crosslink suggested that interaction of p70 with the 3' splice site would occur only under conditions that supported spliceosome assembly. The experiment shown in Figure 3A addresses this issue in greater detail. Addition of EDTA to the splicing reaction mixture allows formation of the pre-spliceosome (complex A) and spliceosome (complex B) but blocks formation of the catalytically active spliceosome (complex C) (Abmayr *et al.*, 1988; our unpublished data). This block can be reversed by addition of Mg^{2+} . Figure 3A shows that 4 mM EDTA blocked crosslinking of p70, which was restored by addition of 7.5 mM Mg^{2+} . These results indicate that

p70 contacts the AG dinucleotide following spliceosome assembly, and that this binding is associated with formation of the catalytically active spliceosome (C complex).

Next, we asked at what point of spliceosome assembly do these polypeptides contact the pre-mRNA. In Figure 3B, a reaction mixture was irradiated with UV light, splicing complexes were separated by native gel electrophoresis, digested *in situ* with RNase A, and crosslinked polypeptides were detected by SDS-PAGE and autoradiography. The results indicate that crosslinking of p70 was observed only in the mature spliceosomal complex C. In contrast, the p35/37 crosslink was present in the pre-spliceosome (complex A) and the B complex but was absent from complex C.

Interaction of p70 with the AG dinucleotide occurs during the second step of splicing

To delineate further the role of p70, we analyzed several previously characterized pre-mRNA mutants. Pre-mRNA derivatives bearing specific mutations within the GU dinucleotide at the 5' splice site (Newman *et al.*, 1985; Aebi *et al.*, 1986, 1987; Seraphin and Rosbash, 1990) or the AG dinucleotide at the 3' splice site (Reed and Maniatis, 1985) can undergo the first step of splicing, giving rise to a lariat intermediate that is blocked for the second step (reviewed in Green, 1991).

We constructed a pre-mRNA substrate containing a GU→AU substitution at the 5' splice site and a single radioactive phosphate within the AG dinucleotide at the 3' splice site (Figure 4A). As expected from previous results (Aebi *et al.*, 1986; Lamond *et al.*, 1987), the first step of splicing was decreased and the second step was almost completely blocked (Figure 4B). The results of Figure 4C show that crosslinking of p70 was significantly impaired by the GU→AU substitution (lanes 1-5). Similar results were obtained with a GU→GA substitution mutant (data not shown). Collectively, these data suggest that binding of p70 to the 3' splice site requires a normal RNA branch.

To characterize further the interaction between p70 and the AG dinucleotide, we constructed a pre-mRNA containing an AG→GG substitution and a radioactive phosphate at this position (Figure 5A). Consistent with previous results (Reed and Maniatis, 1985), splicing of this mutant pre-mRNA proceeded normally through the first step of splicing, but was completely blocked in the second step and thus accumulated high levels of the lariat-containing intermediate (Figure 5B). Significantly, this mutation also abolished crosslinking of p70 (Figure 5C, lanes 1-5). Taken together, these results suggest that interaction of p70 with the 3' splice site requires both a normal RNA branch (Figure 4) and an intact AG dinucleotide (Figure 5). The fact that these mutant pre-mRNAs can undergo the first step of splicing without binding p70 implies that the p70-3' splice site interaction occurs after the first step.

To confirm this conclusion, we took advantage of the fact that a nuclear extract can be heat treated to inhibit the second step selectively (Krainer and Maniatis, 1985; Reed *et al.*, 1988; Sawa and Shimura, 1991; Figure 6, Splicing, lane 4). Complete splicing can be restored by addition of an S100 extract, which by itself is inactive but contains the heat-labile second step splicing factor

Fig. 3. Presence of the crosslinked polypeptides in spliceosomal complexes. (A) Crosslinking of p70 is blocked by EDTA. UV crosslinking time course in the absence of MgCl_2 plus 4 mM EDTA (top). UV crosslinking time course in which 7.2 mM MgCl_2 was added following a 20 min pre-incubation with 4 mM EDTA (bottom). Control, standard splicing conditions. (B) Spliceosomal complexes were separated by native gel electrophoresis, the gel slice was irradiated with UV light, treated with RNase A *in situ* and then placed on top of a 10% SDS-polyacrylamide gel for analysis in the second dimension. The positions of the A, B and C spliceosomal complexes are marked. The polypeptides present in the various spliceosomal complexes are indicated. Molecular weight markers are shown.

(Sawa and Shimura, 1991; Figure 6, Splicing, lanes 2 and 3).

Crosslinking assays were performed with a wild-type pre-mRNA substrate in either mock- or heat-treated nuclear extract. Crosslinking of p70 was severely reduced in the heat-treated nuclear extract (lane 4), and was restored by addition of S100 (lane 3). These results confirm that p70 is not crosslinked to the 3' splice site under conditions in which only the first step of splicing occurs.

Delineating the boundaries of p70 interaction

To map more precisely the p70–3' splice site interaction, we analyzed a series of pre-mRNAs containing a single ^{32}P -label at either four or six nucleotides upstream (–4 or –6), or four or seven nucleotides downstream (+4 or +7), from the 3' splice site (Figure 7A). Because T7 and SP6 RNA polymerase efficiently initiate transcription only with a guanosine (Milligan *et al.*, 1987), the adenosine at –4 and cytosine at –6 were substituted with a guanosine (Figure 7A). Pre-mRNAs carrying these single guanosine substitutions were spliced normally (see Figure 7D, lanes 9–12). Figure 7B shows that p70 was not crosslinked to

RNA substrates site-specifically labeled at +7 (lane 2), +4 (lane 4) or –6 (lane 6), but a 70 kDa polypeptide was crosslinked to the –4 position (lane 3). We note that an ~100 kDa polypeptide was detected with the RNA substrate labeled at the –6 position, and may correspond to a 100 kDa polypeptide that crosslinked to the 3' splice site reported by Chiara *et al.* (1996).

To determine whether the 70 kDa polypeptide that crosslinked to the –4 position was in fact p70, we analyzed the requirements for its interaction with the pre-mRNA. First, the results of Figure 7C show that crosslinking of the 70 kDa polypeptide to position –4 required ATP and Mg^{2+} (lanes 4–6) as does crosslinking of p70 to the AG dinucleotide (lanes 1–3). Second, heat treatment of the nuclear extract abolished crosslinking of the 70 kDa polypeptide to position –4 (lanes 9 and 10) and crosslinking of p70 to the AG dinucleotide (lanes 7 and 8). Finally, immunoprecipitation analysis also indicated that the 70 kDa polypeptide that crosslinked to –4 was p70 (see below and data not shown). On the basis of these data, we conclude, first, that crosslinking of p70 to the 3' splice site is specific and, second, that the maximal p70-binding site extends from five nucleotides upstream (–5) to three

Fig. 4. Crosslinking of p70 to the AG dinucleotide in a 5' splice site mutant pre-mRNA. (A) Diagram of wild-type (wt) and mutant (5' ss mutant) pre-mRNA substrates. (B) Splicing time course with the wild-type and 5' ss mutant substrate. (C) Crosslinking assay with the wild-type and the mutant substrate.

nucleotides downstream (+3) of the 3' cleavage site (see Figure 7E).

Identification of p70

To determine whether p70 corresponded to a known splicing factor, we tested whether ^{32}P -tagged p70 could be immunoprecipitated by previously characterized antisera directed against various splicing factors of size ~70 kDa [U1 70K (Query *et al.*, 1989), the SF3A 66 kDa subunit (Brosi *et al.*, 1993) and a 69 kDa protein (Hackl *et al.*, 1994)], previously identified Py tract-binding proteins [PTB (Garcia-Blanco *et al.*, 1989), PSF (Patton *et al.*, 1993) and IBP (Gerke and Steiz, 1986; Tazi *et al.*, 1986)] or proteins otherwise implicated in the second step of splicing [PRP17 (Frank and Guthrie, 1992) and PSF]. Figure 8A shows that the ^{32}P -tagged p70 was immunoprecipitated by a monoclonal antibody against U1 snRNP 70 kDa protein (2.73; Billings *et al.*, 1982) and by a polyclonal antibody directed against a 69 kDa protein reversibly associated with Sm core proteins (Hackl *et al.*, 1994), but not by antibodies directed against PTB, PSF, U1A, the SF3A 66 kDa subunit, Sm or PRP17.

It has been demonstrated previously that U1 70K and the 69 kDa protein are distinct (Hackl *et al.*, 1994). We

Fig. 5. Crosslinking of p70 to the AG dinucleotide in a 3' splice site mutant pre-mRNA. (A) Diagram of wild-type (wt) and mutant (3' ss mutant) pre-mRNA substrates. (B) Splicing time course with the wild-type and the 3' ss mutant substrate. (C) Crosslinking assay with the wild-type and the mutant substrate.

Fig. 6. Crosslinking of p70 in heat-treated nuclear extract. Left: an *in vitro* splicing complementation assay. Splicing reaction with HeLa nuclear extract (lane 1, NE); cytoplasmic S100 (lane 2, S100); ΔNE and S100 (lane 3, ΔNE/S100); and heat-treated nuclear extract (lane 4, ΔNE). Right: crosslinking assay. Aliquots were removed from the splicing reactions performed on the left and subjected to the UV crosslinking assay. Crosslinked polypeptides are indicated on the right.

performed four experiments to determine whether p70 corresponded to the 69 kDa protein or to U1 70K. First, we carried out immunoprecipitation analysis using another antibody to U1 70K. Figure 8B shows that the U1 70K-specific HIII monoclonal antibody (Reuter and Luhrmann,

1986) failed to immunoprecipitate ³²P-tagged p70 (lane 3), suggesting that p70 was not U1 70K.

Second, we carefully compared the electrophoretic mobility of the polypeptides. In Figure 9A a reaction mixture containing ³²P-tagged p70 was fractionated by

Fig. 8. Immunoprecipitation analysis. (A) Immunoprecipitation of 32 P-tagged p70 with antibodies to splicing factors. Lane 1, total input of UV-irradiated, RNase A-treated splicing reaction mixture (Total Input); lane 2, 2.73 monoclonal antibody against human U1 70K [U1-70K(2.73)]; lane 3, polyclonal antiserum against human PTB (PTB); lane 4, polyclonal antiserum against human PSF (PSF); lane 5, polyclonal antiserum against human U1-A protein (U1A); lane 6, polyclonal antiserum against human 69 kDa Sm core-associated protein (69kD); lane 7, total input of UV-irradiated, RNase A-treated splicing reaction mixture (Total Input); lane 8, 2.73 monoclonal antibody against human U1 70K [U1-70K(2.73)]; lane 9, monoclonal antibody against human SF3a66 (mAb66); lane 10, monoclonal antibody against human Sm epitope, Y12 (Sm); lane 11, polyclonal antiserum against yeast PRP17 protein (PRP17). (B) Immunoprecipitation of 32 P-tagged p70 with U1 70K monoclonal antibodies. Lane 1, total input of reaction mixture (Total Input); lane 2, 2.73 U1 70K monoclonal antibody [U1-70K(2.73)]; lane 3, H111 U1 70K monoclonal antibody [U1-70K(H111)].

Fig. 9. Electrophoretic mobilities of p70, 69 kDa and U1 70K. (A) One-dimensional SDS-PAGE. The UV-irradiated, RNase A-treated splicing reaction mixture was separated on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose and the strips of the filter were either autoradiographed (lane 1) or immunoblotted with H111 U1 70K monoclonal antibody (lane 2), with antiserum against 69 kDa (lane 3) and with 2.73 U1 70K monoclonal antibody (lane 4). (B) Two-dimensional gel analysis. A crosslinking reaction mixture containing 32 P-tagged p70 and p35/37 was separated by two-dimensional gel electrophoresis, transferred to a nitrocellulose filter and visualized by autoradiography (a). The identities of the crosslinked polypeptides are indicated. The same nitrocellulose filter was immunoblotted with the α -69 kDa protein antibody (b). The position of 69 kDa protein is indicated.

SDS-PAGE, transferred to nitrocellulose and analyzed by immunoblotting. Comparison of the autoradiogram (lane 1) and immunoblots (lanes 2–4) indicate that 32 P-tagged p70 co-migrated with the 69 kDa protein (compare lanes 1 and 3) and not U1 70K (compare lanes 1 and 2). In agreement with previous studies (Staknis and Reed, 1995), the 2.73 U1 70K monoclonal antibody cross-reacted with many polypeptides, including a major polypeptide co-migrating with U1 70K and the 69 kDa protein (lane 4).

Third, to rule out the unlikely possibility that 32 P-tagged p70 was not the 69 kDa protein but instead was immunoprecipitated by virtue of association with it, we

carried out the experiment shown in Figure 9B. A reaction mixture containing 32 P-tagged p70 was fractionated by two-dimensional gel electrophoresis and the polypeptides were transferred to nitrocellulose. The nitrocellulose filters were analyzed by autoradiography (i) or were probed with α -69 kDa antibody (ii). The results show that 32 P-tagged p70 migrated almost exactly with immunoreactive 69 kDa protein. However, upon careful inspection, 32 P-tagged p70 was slightly more acidic than immunoreactive 69 kDa, presumably as a result of the negatively charged RNA tag (see, for example, Gozani *et al.*, 1994; Staknis and Reed, 1994).

Fig. 10. *N*-chlorosuccinimide (NCS) cleavage patterns of p70, 69 kDa protein and U1 70K. Left: undigested ^{32}P -tagged p70 (lane 1), NCS-digested ^{32}P -tagged p70 (lane 2), immunoblot of undigested 69 kDa protein (lane 3), immunoblot of NCS-digested 69 kDa protein (lane 4). Right upper: immunoblot of undigested and NCS-digested U1 70K by 2.73 α U1-70K antibody. Right lower: diagram of U1 70K and NCS partial cleavage products of U1 70K. The two tryptophans and the epitope of mAb 2.73 are indicated (Spritz *et al.*, 1987; Query *et al.*, 1989).

Finally, we compared the chemical digestion patterns of ^{32}P -tagged p70 with the 69 kDa protein (Figure 10). ^{32}P -Tagged p70 was treated with *N*-chlorosuccinimide (NCS), which cleaves at tryptophan residues (Mirfakhrai and Weiner, 1993), fractionated by SDS-PAGE and transferred to nitrocellulose strips followed by autoradiography (lane 2). HeLa nuclear extract was fractionated by SDS-PAGE as in Figure 9A, and regions corresponding to the 69 kDa protein were excised, treated with NCS, fractionated by SDS-PAGE and transferred to nitrocellulose strips followed by immunoblotting. The NCS partial digestion of ^{32}P -tagged p70 generated products of ~62 and ~52 kDa. NCS digestion gave rise to a 62 kDa digestion product that was detected by the α -69 kDa antibody and which corresponded precisely to the 62 kDa fragment derived from digestion of ^{32}P -tagged p70 (compare lanes 2 and 4). The 52 kDa NCS digestion product of ^{32}P -tagged p70 was not detected, most likely because it lacked the epitope recognized by the α -69 kDa antibody. In contrast, the NCS digestion pattern of U1 70K was clearly distinct from that of ^{32}P -tagged p70: the 2.73 monoclonal antibody detected NCS digestion products of ~55 and ~40 kDa, resulting from cleavage at two tryptophans within U1 70K (see Figure 10, right). These data provide independent evidence that p70 is the 69 kDa protein.

Discussion

Higher eukaryotic pre-mRNAs contain several sequence elements that are involved in splicing, including the 5'

splice site, the branch point and the Py tract/3' splice site. These serve as recognition signals for splicing factors and participate in the two trans-esterification reactions that occur during the first and second steps of splicing. Methods for preparing pre-mRNAs containing site-specific modifications provide a powerful approach to identify splicing components that contact these sequence elements (Moore and Sharp, 1992; Wyatt *et al.*, 1992; Gaur *et al.*, 1995; Teigelkamp *et al.*, 1995a,b; Umen and Guthrie, 1995a,b; Chiara *et al.*, 1996). Here we have performed UV crosslinking experiments with pre-mRNAs containing a single radioactive phosphate between the A and G residues of the conserved AG dinucleotide. This approach has enabled us to identify p70, and several other polypeptides, that specifically contact the 3' splice site.

Evidence that p70 is a second step splicing factor

Several lines of evidence argue strongly that the p70-3' splice site interaction is functionally relevant: crosslinking of p70 is highly specific for the conserved AG dinucleotide (Figure 5); crosslinking of p70 and splicing have similar biochemical requirements (Figures 2 and 3A); the kinetics of p70 crosslinking parallel those of the second step of splicing (Figures 1B); both the second step of splicing and crosslinking of p70 require a normal RNA branch structure (Figure 4); p70 is bound to the 3' splice site in the mature spliceosomal complex (Figure 3); certain *cis*-acting mutations impair both the binding of p70 and the second step of splicing (Figures 4 and 5); and interaction

of p70 with the 3' splice site is severely reduced in a heat-treated nuclear extract that can only support the first step of splicing (Figure 6). Taken together, the most straightforward interpretation of these results is that the conserved AG dinucleotide at the 3' splice site is recognized by p70 during the second step of splicing, and that this interaction is involved in the second step. However, isolation of a p70 cDNA clone and subsequent biochemical complementation experiments will be required to confirm this interpretation.

Sequential recognition of the AG dinucleotide

In several instances, pre-mRNA splicing signals appear to be recognized many times during a single splicing reaction. For example, the 5' splice site is first bound by U1 snRNA, and subsequently by U6 snRNA (reviewed in Moore *et al.*, 1993; Madhani and Guthrie, 1994; Nielsen, 1994). The interpretation of previous analyses of pre-mRNA mutants was that the conserved AG dinucleotide is recognized more than once, and may also participate in the first step of splicing (Reed, 1989; Zhuang and Weiner, 1990). A number of factors, in addition to p70, have been reported to bind at or near the 3' splice site (reviewed in Umen and Guthrie, 1995c). Although the precise role of these factors is not yet clear, one or more of them may be involved in sequential 3' splice site recognition. Such factors include: a human 35/37 kDa protein that we have described (see Figure 1); a 100 kDa human polypeptide reported by Chiara *et al.* (1996); a 200 kDa U5 snRNP polypeptide that is the probable homolog of yeast Prp8 (Chiara *et al.*, 1996); U6 snRNA (Lesser and Guthrie, 1993); and several yeast proteins including Prp8, Prp16 and Slu7 (Teigelkamp *et al.*, 1995a,b; Umen and Guthrie, 1995a,b). Finally, at some point during the splicing process, there is probably an interaction between the AG dinucleotide and the 5' splice site (Parker and Siliciano, 1993).

We have shown that under several different circumstances in which splicing failed to progress to the second step, p70 was not crosslinked to the AG dinucleotide. Thus, during spliceosome assembly, crosslinking of p70 to the AG dinucleotide must be regulated temporally. There are several possible mechanisms by which the p70–3' splice site interaction may be controlled. For example, at early times, the AG dinucleotide may be occupied by another factor, such as p35/37, which blocks binding of p70. Alternatively, interaction of p70 with the AG dinucleotide could be facilitated by conformational changes or by other spliceosomal components that enter the complex at later times. For example, p70 binds poorly to an abnormal RNA intermediate that contains an A(2'–5')A branch and is blocked in the second step (Figure 4). Thus, an additional factor could selectively bind to a normal RNA branch and promote binding of p70 to the AG dinucleotide. Finally, p70 is a phosphoprotein (data not shown) and its phosphorylation state could regulate the 3' splice site interaction.

In the transition from the first to second step, the spliceosome undergoes a major conformational change (Sawa and Shimura, 1991; Schwer and Guthrie, 1992). Our results suggest that this conformational change may involve binding of p70 to the 3' splice site. The splicing components that recognize the splice site and comprise the catalytic site are primarily snRNAs (reviewed in Lesser

and Guthrie, 1993; Madhani and Guthrie, 1994; Nielsen, 1994), but this does not preclude participation of protein factors. Further experiments will be required to determine the role of p70 in splice site recognition and catalysis.

p70 is the 69 kDa protein

We have provided several lines of evidence that p70 corresponds to a previously reported 69 kDa protein of unknown function. First, p70 was specifically immunoprecipitated by an α -69 kDa antibody (Figure 8). Second, the equivalence of p70 and the 69 kDa protein is supported by their electrophoretic mobilities in both one- and two-dimensional protein gels (Figure 9). Third, the chemical digestion patterns of p70 and the 69 kDa protein support the conclusion that the two proteins are the same (Figure 10).

Conversely, our results allow us to conclude that several other proteins implicated in 3' splice site recognition and splicing are not p70. For example, unlike p70, IBP and PTB bind to the Py tract, not the AG dinucleotide, in a manner that does not require an AG dinucleotide or ATP (Gerke and Steitz, 1986; Tazi *et al.*, 1986; Ruskin *et al.*, 1988; Garcia-Blanco *et al.*, 1989; Singh *et al.*, 1995). The Py tract-binding protein PSF is a second step splicing factor, but our immunoprecipitation experiments indicate that p70 is not PSF (Figure 8). Moreover, binding of PSF to the Py tract does not require the AG dinucleotide (Patton *et al.*, 1993; Gozani *et al.*, 1994), again distinguishing PSF from p70. In similar UV crosslinking assays, Chiara *et al.* (1996) detected binding of 75 and 100 kDa polypeptides to the 3' splice site. The properties of the 75 kDa polypeptide were similar to those of our p70, suggesting that the two proteins may be the same. Although we did not detect a 100 kDa polypeptide binding to the AG dinucleotide, a polypeptide of this size was crosslinked to a pre-mRNA labeled at the –6 position (Figure 7B, lane 6).

The 69 kDa protein was identified originally in 12S U1 snRNP (Hackl *et al.*, 1994), but was also found to associate reversibly with the Sm core domains present in all spliceosomal snRNPs (Hackl *et al.*, 1994). The function of the 69 kDa protein was completely obscure and in fact was speculated to involve assembly and/or nucleocytoplasmic transport of snRNP particles. Here, we show that this protein specifically recognizes the 3' splice site during the second step of splicing. These results provide a rationale for isolating a p70 cDNA clone to study the role of this protein in 3' splice site recognition and splicing.

Materials and methods

Pre-mRNA substrates

Plasmid SpP65-AdML (MINX) derived from the adenovirus major late promoter (Zillmann *et al.*, 1988) was used to transcribe pre-mRNA substrates *in vitro*. To prepare a pre-mRNA substrate containing a radioactive phosphate between the A and G residues of the AG dinucleotide, two DNA templates were synthesized by PCR from the MINX plasmid: one of the DNA templates (5' DNA template) was designed to encompass the 5' exon and the entire intron except for the last G residue, and the other DNA template (3' DNA template) to encode the last residue of the intron and the 3' exon. The 5' primer for making the 5' DNA template was 5'-TAATACGACTCACTATAGAATTTCG-AGCTCGC3', and the 3' primer was 5'-GGATGACCGCGAGCTGTGG-AAAAAAG3'. The resulting PCR product contained a T7 RNA polymerase promoter, the 5' exon, the intron and a *FokI* restriction enzyme site at the 3' end. Digestion of the PCR product with *FokI* created the 5' DNA template containing a T7 RNA polymerase promoter,

the 5' exon and the intron except for the last G residue. The 5' primer for making the 3' DNA template was 5'GGAATTCATAACGACTCACTATAGCTCGCCCTTGAGGACAAA3', and the 3' primer was 5'CTTGGGCTGCAGGTGCACTCTAGAGGATCCCCACTGG3'. The resulting PCR product contained a T7 RNA polymerase promoter, the last nucleotide of the intron (the G residue) and the 3' exon, and was used as the 3' DNA template.

To prepare a pre-mRNA substrate containing a GU→AU mutation at the 5' splice site, the 5' primer for the 5' DNA template was 5'CGATTAGGTGACACTATAGGAATCTTGGATCGGAAACCCGTCGGCTCCGAACGATAAGAGCTAGC3', a GT→AT change was introduced in the primer; the 3' primer was the same as that for the wild-type substrate.

To prepare a substrate containing an AG→GG mutation at the 3' splice site, the 3' primer for the 5' DNA template was 5'CGCGGATCCGGATGACCGCGAGCCGTGGAAGG3', and the 5' primer was the same as that for the wild-type substrate. The *FokI*-digested PCR product contained a T7 RNA polymerase promoter, the 5' exon and the intron with the second last A residue changed to G and not containing the last guanosine residue, and was used as the 5' DNA template.

Pre-mRNA substrates carrying the ³²P-label at the position several nucleotides downstream or upstream of the 3' splice site were constructed similarly to the substrate side-specifically labeled at the AG dinucleotide described above. Sequences of oligonucleotides for synthesis of DNA templates of these substrates by PCR are available upon request.

RNA was synthesized *in vitro* by T7 or SP6 RNA polymerase at 37°C for 2 h. Transcription reactions for the 5' RNA fragment contained 1× transcription buffer (Promega Inc.), 10 mM dithiothreitol (DTT), 0.05 mM GTP, 0.5 mM of each nucleotide except GTP, 0.5 mM G(5')ppp(5')G (Promega Inc.), 0.1 mg/ml of the DNA template and 10 U/ml SP6 or T7 RNA polymerase. The 3' RNA fragment was synthesized in a reaction mixture containing 1× transcription buffer (Promega, Inc.), 10 mM DTT, 0.5 mM of each of the four NTPs and 0.1 mg/ml of the DNA template. The 3' RNA fragment was treated with alkaline phosphatase (1 U/ml) (Boehringer Mannheim Biochemicals) in 1× phosphatase buffer for 30 min at 37°C, followed by addition of 2 µl of 500 mM EGTA and incubated at 62°C for 20 min. The 3' RNA fragment was then 5'-end-labeled with [γ-³²P]ATP and polynucleotide kinase. The two RNAs were annealed to a DNA bridging oligonucleotide complementary to the 20 nucleotides at the 3' end of the 5' RNA fragment and the 20 nucleotides at the 5' end of the 3' RNA fragment and ligated using T4 DNA ligase as described (Moore and Sharp, 1992; Wyatt *et al.*, 1992). The ligation product was purified following electrophoresis on an 8% denaturing polyacrylamide gel.

UV crosslinking assay

Splicing reactions (12.5 µl) were performed with ~7000–70 000 c.p.m. (sp. act. ~2×10⁶ to ~4×10⁶ c.p.m./µg) of pre-mRNA substrate containing single radioactive phosphate at the 3' splice site in 50% HeLa nuclear extract for various times at 30°C (Zamore and Green, 1991). For –ATP reactions, ATP, creatine phosphate and creatine phosphate kinase were omitted, and the nuclear extract was pre-incubated at 30°C for 20 min. For micrococcal nuclease-treated reactions, micrococcal nuclease was added to nuclear extract with 0.02 volumes of 0.05 M CaCl₂ and 0.02 volumes of micrococcal nuclease (5000 U/ml), and the splicing reaction was incubated for 30 min at 30°C. For the micrococcal nuclease/EGTA control reaction, 0.02 volumes of 0.1 M EGTA/K⁺ (pH 8.0) was added together with micrococcal nuclease. For +EDTA reactions, MgCl₂ was replaced by 4 mM EDTA. To restore the splicing activity in the +EDTA reaction, 7.5 mM of MgCl₂ was added and the reaction mixtures were incubated for the times indicated. To analyze splicing products, proteinase K was added to the reaction mixture and RNA fractionated on a 13% sequencing gel.

To analyze proteins that interact with the 3' splice site, the reaction mixtures were irradiated with UV light (wavelength 254 nm) in a Stratilinker (Stratagene) at 800 000 µJ/cm² (~5 min) on ice at a distance of 10 cm from the source. RNase A was added to 2 mg/ml and the reaction mixtures were incubated for 30 min at 37°C. SDS gel loading buffer was added and the reaction mixtures were boiled for 5 min and fractionated on 10% SDS–polyacrylamide gels. The gel was dried and the ³²P-tagged polypeptides were detected by autoradiography.

Native gel analysis

After incubation at 30°C for various times, the splicing reaction mixtures were loaded on a 4% polyacrylamide (80:1 acrylamide/bis-acrylamide)/0.5% agarose composite gel containing 50 mM glycine/50 mM Tris

base. Electrophoresis was carried out at 250 V for 5 h at 4°C. ³²P-labeled RNP complexes were detected by autoradiography. A gel slice containing the complexes from a 30 min splicing reaction was then excised, UV irradiated on ice and incubated with 2 mg/ml RNase A at 37°C for 20 min, followed by incubation in 4× SDS gel loading buffer at 37°C for 20 min. The gel slice was embedded in the stacking portion of an SDS–polyacrylamide gel and subjected to electrophoresis. Crosslinked polypeptides were visualized by autoradiography.

Splicing complementation in heat-treated nuclear extract

The heat-treated nuclear extract (ΔNE) was prepared by warming at 43°C for 20 min (Krainer and Maniatis, 1985). Splicing reactions were performed with 50% nuclear extract (NE), 50% heat-treated nuclear extract (ΔNE) or 50% cytoplasmic S100 extract (S100) at 30°C for 30 min. In the splicing complementation assay with ΔNE and S100, 25% ΔNE and 25% S100 were used and incubated at 30°C for 30 min.

Immunoprecipitation and immunoblotting analysis

Splicing and UV crosslinking assays were performed as described above in a reaction mixture containing 30% nuclear extract. The reaction mixtures were treated with RNase A (2 mg/ml) for 30 min at 37°C. Antibody (12 µl) was added to 12 µl aliquots of the reaction mixtures followed by incubation at 4°C for 1 h. Protein A–Sepharose 4B (fast flow, Sigma) beads were then added and incubated for another 1 h at 4°C. The beads were washed four times with 1 ml of NET-2 (150 mM NaCl, 0.05% NP-40, and 50 mM Tris–HCl, pH 7.5). SDS gel loading buffer was added and the reaction mixtures were boiled for 10 min, fractionated by SDS–PAGE, and ³²P-labeled polypeptides detected by autoradiography.

The following immunological reagents were used: the monoclonal antibody against U1 70K (2.73, described by Billings *et al.*, 1982); the monoclonal antibody against U1 70K (H111, provided by W.-Y.Tarn and J.A.Steitz, Yale University, described by Reuter and Luhrmann, 1986); the polyclonal antiserum against human U1-A protein (pAB856, provided by I.W.Mattaj, EMBL, Heidelberg, Germany); the polyclonal antiserum against 69 kDa Sm core-associated protein (provided by W.Hackl, Philipps Universität Marburg, Hackl *et al.*, 1994); the polyclonal antiserum against PSF and PTB (provided by J.G.Patton, Vanderbilt University, Patton *et al.*, 1993); the monoclonal antibody against SF3a66 (mAB66, provided by A.Kramer, Université de Genève, described by Bennett *et al.*, 1992; Brosi *et al.*, 1993); the monoclonal antibody against Sm epitope (Y12, provided by J.A.Steitz, Yale University, described by Gerke and Steitz, 1986); and the polyclonal antiserum against yeast PRP17 protein (provided by D.Frank and C.Guthrie, University of California, San Francisco, described by Frank and Guthrie, 1992).

For immunoblotting analysis, the UV-irradiated, RNase A-treated splicing reaction mixture was fractionated in SDS–PAGE or by two-dimensional gel electrophoresis [Bio-Lyte 3/10 Ampholyte (Bio-Rad) was used for the isoelectrofocusing in the first dimension], transferred to nitrocellulose filter (Millipore) and subjected to autoradiography. The same membrane was then probed with the primary antibody at room temperature for 1 h. Secondary antibody was conjugated to horseradish peroxidase and detection was with the ECL system (Amersham).

N-chlorosuccinimide cleavage

The crosslink mixture was separated by SDS–PAGE. The gel slides containing ³²P-tagged p70 and proteins of size ~70 kDa were treated with NCS as described (Mirfakhrai and Weiner, 1993). Briefly, the labeled crosslinked p70 band was identified by autoradiography, excised and equilibrated with H₂O, followed by a solution containing 6.6 M urea and 40% acetic acid. After chemical cleavage in the same buffer with 15 mM NCS, the gel slides were washed with H₂O, equilibrated in a stacking buffer (10% glycerol, 15% β-mercaptoethanol, 3% SDS, 0.0625 M Tris–HCl, pH 6.8) and loaded into 12 or 15% SDS–polyacrylamide gels. Proteins in the gels were transferred to nitrocellulose filter (Millipore), and subjected to autoradiography or immunoblotting.

Acknowledgements

We are particularly grateful to J.Valcarcel for advice during the course of the work and on the manuscript. We thank R.Singh, M.L.Zapp, J.Umen, C.Guthrie, and M.Rosbash for critical comments; J.L.Patton, I.W.Mattaj, A.Kramer, W.Hackl, W.-Y.Tarn, J.A.Steitz, D.Frank and C.Guthrie for antibodies; T.O'Toole for secretarial assistance; and L.Chiang for technical assistance. This work was supported by a postdoctoral fellowship from the American Cancer Society to S.W. and

by a grant from the National Institutes of Health to M.R.G. M.R.G. is an investigator of the Howard Hughes Medical Institute.

References

- Abmayr, S.M., Reed, R. and Manatis, T. (1988) Identification of a functional mammalian spliceosome containing unspliced pre-mRNA. *Proc. Natl Acad. Sci. USA*, **85**, 7216–7220.
- Aebi, M., Hornig, H., Padgett, R.A., Reiser, J. and Weissmann, C. (1986) Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA. *Cell*, **47**, 555–565.
- Aebi, M., Hornig, M. and Weissmann, C. (1987) 5' Cleavage site in eukaryotic pre-mRNA splicing is determined by the overall 5' splice region, not by the conserved 5' GU. *Cell*, **50**, 237–246.
- Bennett, M., Michaud, S., Kingston, J. and Reed, R. (1992) Protein components specifically associated with prespliceosome and spliceosome complexes. *Genes Dev.*, **6**, 1986–2000.
- Billings, P.B., Allen, R.W., Jensen, F.C. and Hoch, S.O. (1982) Anti-RNP monoclonal antibodies derived from a mouse strain with lupus-like autoimmunity. *J. Immunol.*, **128**, 1176–1180.
- Brosi, R., Groning, K., Behrens, S.-E., Luhrmann, R. and Kramer, A. (1993) Interaction of mammalian splicing factor SF3a with U2 snRNP and relation of its 60-kD subunit to yeast PRP9. *Science*, **262**, 102–104.
- Chiara, D., Gozani, O., Bennett, M., Champion-Arnaud, P., Palandjian, L. and Reed, R. (1996) Identification of proteins that interact with exon sequences, splice site, and the branchpoint sequence during each stage of spliceosome assembly. *Mol. Cell. Biol.*, **16**, 3317–3326.
- Frank, D. and Guthrie, C. (1992) An essential splicing factor, SLU7, mediates 3' splice site choice in yeast. *Genes Dev.*, **6**, 2112–2124.
- Garcia-Blanco, M.A., Jamison, S.F. and Sharp, P.A. (1989) Identification and purification of a 62,000-dalton protein that binds specifically to the polypyrimidine tract of introns. *Genes Dev.*, **3**, 1874–1886.
- Gaur, R.K., Valcarcel, J. and Green, M.R. (1995) Sequential recognition of the pre-mRNA branch point by U2AF65 and a novel spliceosome-associated 28-kDa protein. *RNA*, **1**, 407–417.
- Gerke, V. and Steitz, J.A. (1986) A protein associated with small nuclear ribonucleoprotein particles recognizes the 3' splice site of premessenger RNA. *Cell*, **47**, 973–984.
- Gozani, O., Patton, J.G. and Reed, R. (1994) A novel set of spliceosome-associated proteins and the essential splicing factor PSF bind stably to pre-mRNA prior to catalytic step II of the splicing reaction. *EMBO J.*, **13**, 3356–3367.
- Green, M.R. (1991) Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. *Annu. Rev. Cell Biol.*, **7**, 559–599.
- Guthrie, C. (1991) Messenger RNA splicing in yeast: clues to why the spliceosome is a ribonucleoprotein. *Science*, **253**, 157–163.
- Hackl, W., Fischer, U. and Luhrmann, R. (1994) A 69-kD protein that associates reversibly with the Sm core domain of several spliceosomal snRNP species. *J. Cell Biol.*, **124**, 261–272.
- Kandel-Lewis, S. and Seraphin, B. (1993) Role of U6 snRNA in 5' splice site selection. *Science*, **262**, 2035–2039.
- Konforti, B.B., Koziolkiewicz, M.J. and Konarska, M.M. (1993) Disruption of base pairing between the 5' splice site and the 5' end of U1 snRNA is required for spliceosome assembly. *Cell*, **75**, 863–873.
- Krainer, A.R. and Maniatis, T. (1985) Multiple factors including the small nuclear ribonucleoproteins U1 and U2 are necessary for pre-mRNA splicing *in vitro*. *Cell*, **42**, 725–736.
- Kramer, A. (1996) The structure and function of proteins involved in mammalian pre-mRNA splicing. *Annu. Rev. Biochem.*, **65**, 367–409.
- Lamm, G.M. and Lamond, A.I. (1993) Non-snRNP protein splicing factors. *Biochim. Biophys. Acta*, **1173**, 247–265.
- Lamond, A.I., Konarska, M.M. and Sharp, P.A. (1987) A mutational analysis of spliceosome assembly: evidence for splice site collaboration during spliceosome formation. *Genes Dev.*, **1**, 532–543.
- Lesser, C.F. and Guthrie, C. (1993) Mutations in U6 snRNA that alter splice site specificity: implications for the active site. *Science*, **262**, 1982–1988.
- Madhani, H.D. and Guthrie, C. (1994) Dynamic RNA–RNA interactions in the spliceosome. *Annu. Rev. Genet.*, **28**, 1–26.
- Milligan, J.F., Groebe, D.R., Witherell, G.W. and Uhlenbeck, O.C. (1987) Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.*, **15**, 8783–8798.
- Mirfakhrai, M. and Weiner, A.M. (1993) Chemical cleavage mapping: a rapid technique for characterization of crosslinked nucleic acid–protein complexes. *Nucleic Acids Res.*, **21**, 3591–3592.
- Moore, M.J. and Sharp, P.A. (1992) Site-specific modification of pre-mRNA: the 2' hydroxyl groups at the splice sites. *Science*, **256**, 992–997.
- Moore, M.J., Query, C.C. and Sharp, P.A. (1993) Splicing of precursors to messenger RNAs by the spliceosome. In Gestland, R.I. and Atkins, J.F. (eds), *The RNA World*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp.1–30.
- Neilsen, T.W. (1994) RNA–RNA interactions in the spliceosome: unraveling the ties that bind. *Cell*, **78**, 1–4.
- Newman, A.J., Lin, R., Cheng, S. and Abelson, J. (1985) Molecular consequence of specific intron mutations on yeast mRNA splicing *in vivo* and *in vitro*. *Cell*, **42**, 335–344.
- Parker, R. and Siliciano, P.G. (1993) Evidence for an essential non-Watson–Crick interaction between the first and last nucleotides of a nuclear pre-mRNA intron. *Nature*, **361**, 660–662.
- Parker, R., Siliciano, P.G. and Guthrie, C. (1987) Recognition of the TACTAAC box during mRNA splicing in yeast involves base pairing to the U2-like snRNA. *Cell*, **49**, 229–239.
- Patton, J.G., Porro, E.B., Galceran, J., Tempst, P. and Nadal-Ginord, B. (1993) Cloning and characterization of PSF, a novel pre-mRNA splicing factor. *Genes Dev.*, **7**, 393–406.
- Query, C.C., Bentley, R.C. and Keene, J.D. (1989) A common RNA recognition motif identified within a defined U1 RNA binding domain of the 70K U1 snRNP protein. *Cell*, **57**, 89–101.
- Reed, R. (1989) The organization of 3' splice-site sequences in mammalian introns. *Genes Dev.*, **3**, 2113–2123.
- Reed, R. and Maniatis, T. (1985) Intron sequences involved in lariat formation during pre-mRNA splicing. *Cell*, **41**, 95–105.
- Reed, R., Griffith, J. and Maniatis, T. (1988) Purification and visualization of native spliceosomes. *Cell*, **53**, 949–961.
- Reuter, R. and Luhrmann, R. (1986) Immunization of mice with purified U1 small nuclear ribonucleoprotein (RNP) induces a pattern of antibody specificities characteristic of the anti-Sm and anti-RNP autoimmune response of patients with lupus erythematosus, as measured by monoclonal antibodies. *Proc. Natl Acad. Sci. USA*, **83**, 8689–8693.
- Ruskin, B., Zamore, P.D. and Green, M.R. (1988) A factor, U2AF, is required for U2 snRNP binding and splicing complex assembly. *Cell*, **52**, 207–219.
- Sawa, H. and Abelson, J. (1992) Evidence for a base-pairing interaction between U6 small nuclear RNA and the 5' splice site during the splicing reaction in yeast. *Proc. Natl Acad. Sci. USA*, **89**, 11269–11273.
- Sawa, H. and Shimura, Y. (1991) Alterations of RNase H sensitivity of the 3' splice site region during the *in vitro* splicing reaction. *Nucleic Acids Res.*, **19**, 3953–3958.
- Sawa, H. and Shimura, Y. (1992) Association of U6 snRNA with the 5'-splice site region of pre-mRNA in the spliceosome. *Genes Dev.*, **6**, 244–254.
- Schwer, B. and Guthrie, C. (1992) A conformational rearrangement in the spliceosome is dependent on PRP16 and ATP hydrolysis. *EMBO J.*, **11**, 5033–5039.
- Seraphin, B. and Rosbash, M. (1990) Exon mutations uncouple 5' splice site selection from U1 snRNA pairing. *Cell*, **63**, 619–629.
- Seraphin, B., Kretzner, L. and Rosbash, M. (1988) A U1 snRNA:pre-mRNA base-pairing interaction is required early in yeast spliceosome assembly but does not uniquely define the 5' cleavage site. *EMBO J.*, **7**, 2533–2538.
- Siliciano, P.G. and Guthrie, C. (1988) 5' Splice site selection in yeast: genetic alterations in base-pairing with U1 reveal additional requirements. *Genes Dev.*, **2**, 1258–1267.
- Singh, R., Valcarcel, J. and Green, M.R. (1995) Distinct binding specificities and functions of higher eukaryotic polypyrimidine tract-binding proteins. *Science*, **268**, 1173–1176.
- Smith, C.W.J., Porro, E.B., Patton, J.G. and Nadal-Ginard, B. (1989) Scanning from an independently specified branch point defines the 3' splice site of mammalian introns. *Nature*, **342**, 243–247.
- Sontheimer, E. and Steitz, J.A. (1993) The U5 and U6 small nuclear RNAs as active site components of the spliceosome. *Science*, **262**, 1989–1995.
- Spritz, R.A., Strunk, K., Surowy, C.S., Hoch, S.O., Barton, D.E. and Franke, U. (1987) The human U1-70K snRNP protein: cDNA cloning, chromosomal localization, expression, alternative splicing and RNA-binding. *Nucleic Acids Res.*, **15**, 10373–10391.
- Staknis, D. and Reed, R. (1994) Direct interactions between pre-mRNA and six U2 small ribonucleoproteins during spliceosome assembly. *Mol. Cell. Biol.*, **14**, 7670–7682.

- Staknis,D. and Reed,R. (1995) Members of a family of proteins (the RD family) detected by a U1-70K monoclonal antibody are present in spliceosomal complexes. *Nucleic Acids Res.*, **23**, 4081–4086.
- Tazi,J., Alibert,C., Temsamani,J., Reveilland,I., Cathala,G., Brunel,C. and Jeanteur,P. (1986) A protein that specifically recognizes the 3' splice site of mammalian pre-mRNA introns is associated with a small nuclear ribonucleoprotein. *Cell*, **47**, 755–766.
- Teigelkamp,S., Newman,A.J. and Beggs,J.D. (1995a) 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. *EMBO J.*, **14**, 2602–2612.
- Teigelkamp,S., Whittaker,E. and Beggs,J.D. (1995b) Interaction of the yeast splicing factor PRP8 with substrate RNA during both steps of splicing. *Nucleic Acids Res.*, **23**, 320–326.
- Umen,J.G. and Guthrie,C. (1995a) Prp16p, Slu7p, and Prp8p interact with the 3' splice site in two distinct stages during the second catalytic step of pre-mRNA splicing. *RNA*, **1**, 584–597.
- Umen,J.G. and Guthrie,C. (1995b) A novel role for a U5 snRNP protein in 3' splice site selection. *Genes Dev.*, **9**, 855–868.
- Umen,J.G. and Guthrie,C. (1995c) The second catalytic step of pre-mRNA splicing. *RNA*, **1**, 869–885.
- Wassarman,D.A. and Steitz,J.A. (1992) Interactions of small nuclear RNA's with precursor messenger RNA during *in vitro* splicing. *Science*, **257**, 1918–1925.
- Wu,J. and Manley,J.L. (1989) Mammalian pre-mRNA branch site selection by U2 snRNP involves base-pairing. *Genes Dev.*, **3**, 1553–1561.
- Wyatt,J.R., Sontheimer,E.J. and Steitz,J.A. (1992) Site-specific cross-linking of mammalian U5 snRNP to the 5' splice site before the first step of pre-mRNA splicing. *Genes Dev.*, **6**, 2542–2553.
- Zamore,P.D. and Green,M.R. (1991) Biochemical characterization of U2 snRNP auxiliary factor: an essential pre-mRNA splicing factor with a novel intranuclear distribution. *EMBO J.*, **10**, 207–214.
- Zhuang,Y. and Weiner,A.M. (1986) A compensatory base change in U1 snRNA suppresses a 5' splice site mutation. *Cell*, **46**, 827–835.
- Zhuang,Y. and Weiner,A.M. (1989) A compensatory base change in human U2 snRNA can suppress a branch site mutation. *Genes Dev.*, **3**, 1545.
- Zhuang,Y. and Weiner,A.M. (1990) The conserved dinucleotide AG of the 3' splice site may be recognized twice during *in vitro* splicing of mammalian mRNA precursors. *Gene*, **90**, 263–269.
- Zillmann,M., Zapp,M.L. and Berget,S.M. (1988) Gel electrophoretic isolation of splicing complexes containing U1 small nuclear ribonucleoprotein particles. *Mol. Cell. Biol.*, **8**, 814–821.

Received on November 5, 1996; revised on March 26, 1997