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Identification of nuclear proteins that specifically bind to RNAs containing 5′ splice sites

(splicing factors/UV crosslinking)

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ABSTRACT **Two polypeptides of 26 and 37 kDa (designated SPP-1 and SPP-2) were identified in *in vitro* splicing extracts by UV crosslinking to splicing precursor RNAs. Crosslinking of both polypeptides required a functional 5′ splice site but was not dependent on sequences at the 3′ end of the intron. Centrifugation of extract separated the two polypeptides from major U small nuclear ribonucleoproteins (snRNPs), including U1 snRNPs. Both polypeptides crosslinked to precursor RNAs containing 5′ splice sites in the absence of U1 RNA. Complexes containing both polypeptides also contained U1 snRNPs, suggesting that SPP-1 and SPP-2 are a part of the functional spliceosome. We propose that SPP-1 and SPP-2 are factors that participate in the recognition of 5′ splice sites.**

Splicing of pre-mRNA requires the participation of a large number of factors. The active splicing machinery contains multiple small nuclear ribonucleoproteins (snRNPs) and polypeptides organized into a large complex known as the spliceosome, which is 60 S when assembled on a single intron *in vitro* (reviewed in refs. 1–3). The actual number of factors required to recognize and remove an intron is unknown. In yeast, multiple complementation groups have been detected that are involved in the splicing process (4–6). Only a handful of these have been correlated as to their respective protein or RNA (7–11). In vertebrates, a growing number of non-snRNP polypeptides have been implicated as involved in splicing (12–21). Several of these appear to be RNA binding proteins that interact with consensus sequences at the 3′ end of the intron during early spliceosome assembly (12–16). U2AF (U2 snRNP auxiliary factor), pPTB (polypyrimidine tract binding protein), and IBP (intron binding protein) have been reported to bind to the branch point, pyrimidine tract, and 3′ splice site, respectively (12–16). Several additional small nuclear proteins (SC35, SF2, and ASF) have recently been reported to be involved in processing and associate with RNA (17–19). And, finally, the heterogeneous RNP A and C polypeptides interact with splicing precursor RNAs *in vitro* (20, 21).

The consensus sequence defining 5′ splice sites is recognized by U1 snRNPs via complementarity between U1 RNA and 5′ splice site consensus sequences (1–3). This interaction is necessary for recognition (22). It may not be sufficient, however. Although isolated U1 snRNPs can bind 5′ splice sites (23, 24), soluble factors other than U1 snRNPs that facilitate recognition of 5′ splice sites have been detected during extract fractionation (25, 26). In addition, UV crosslinking has revealed a 55-kDa polypeptide that crosslinks to 5′ splice sites (15). Thus, factors other than U1 snRNPs may participate in recognition of 5′ splice sites.

In this report, we have used UV crosslinking to examine nuclear proteins that associate with splicing precursor RNAs. We have identified two polypeptides of 26 and 37 kDa that crosslink to precursor RNAs possessing a 5′ splice site. For maximal crosslinking, a functional CAG:GU(A/G)AG was required at the 5′ splice site. Both polypeptides associated with precursor RNAs very early during assembly and did not require U1 snRNA for association. These polypeptides, therefore, are candidates for splicing factors that function in the recognition of splice sites along with snRNPs and other components of the splicing machinery.

MATERIALS AND METHODS

Transcripts. The adenovirus precursor construct contains information from the human adenovirus late transcription unit and is based on the MINX transcript described by this laboratory (27). The first exon contains 16 nucleotides of pSP65 polylinker and the last 43 nucleotides of viral exon 2 including the 5′ splice site. The intron following this exon is 88 nucleotides and contains the first 22 nucleotides of intron 2 fused via a *Bgl* II site to the last 58 nucleotides of intron 1. The second exon contains the first 38 nucleotides of viral exon 2, and 3 nucleotides of polylinker when generated by *Bam*HI cleavage. When cut with *Bgl* II, this construct yields an RNA of 77 nucleotides lacking all known splicing signals from the 3′ end of the intron. *Bam*HI-cut DNA gives a 187-nucleotide transcript. Mutations were introduced into the 5′ splice site beginning with the first intron by polymerase chain reaction-mediated cloning using oligonucleotides altered to change GU to CG. The mouse metallothionein construct contains the last 63 nucleotides of exon 2, intact intron 2, and the first 76 nucleotides of exon 3 of the natural gene (provided by R. Palmiter, University of Washington). Cut with *Pvu* II, a 311-nucleotide two-exon transcript is generated. The cardiac troponin T construct contains 48 nucleotides of exon 4 and 267 nucleotides of intron 5 from the natural human gene and was provided by T. Cooper (Baylor College of Medicine). Cut with *Cla* I, a transcript of 315 nucleotides that contains the 5′ splice site from the fourth exon was produced. The nonspecific RNA was generated by transcription of *Pvu* II-truncated pSP65 vector to produce a 231-nucleotide transcript.

Splicing Reactions. Unless otherwise indicated, splicing reaction mixtures contained 1 mM creatine phosphate, 0.25 mM ATP, 1.5 mM $MgCl_2$, 0.37 mM dithiothreitol, 1% polyethylene glycol, 44 mM KCl, 8.8 mM Tris·HCl, 8.8% (vol/vol) glycerol, 0.2 mM EDTA, and 44% nuclear extract. RNAs were extracted after the reaction and analyzed as described (27).

UV Crosslinking. Polypeptides interacting with radiolabeled precursor RNAs were detected by UV crosslinking methods (28, 29) modified as described (30). Precursor RNAs labeled with [^{32}P]UTP were incubated in processing extract for 5 min at 30°C unless otherwise indicated. *Escherichia coli* tRNA was added to a final concentration of 200 μg/ml.

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Abbreviations: snRNP, small nuclear ribonucleoprotein; pPTB, polypyrimidine tract binding protein.

Samples were crosslinked with a Sylvania G15T8 lamp at a distance of 4 cm for 10 min at 4°C. RNA was digested for 15 min at 37°C in the presence of pancreatic RNase A (1 mg/ml). SDS sample buffer was added and the samples were heated to 100°C for 5 min. The proteins were subsequently displayed on SDS/12.5% PAGE. Molecular size markers were included to determine sizes of crosslinked proteins. The gels were stained with Coomassie brilliant blue and dried. Dried gels were exposed 1–2 days with Fuji RX 100 x-ray film.

Immunoprecipitations. Immunoprecipitations were performed with anti-Sm (Y12 monoclonal), anti-RNP (systemic lupus erythematosus patient sera specific for the U1 snRNP 70-kDa protein; ref. 31), and control human sera. After crosslinking, samples were digested with RNase A and then incubated on ice with antibody for 30 min. Prewashed pansorbin was added and the incubation was continued for 5 min on ice. Pellets were collected as described (31). Alternatively, immunoprecipitations were done directly after crosslinking, and the collected supernatant and pellet fractions were digested with pancreatic RNase A. Samples were then displayed on SDS/12.5% PAGE, and crosslinked proteins were detected by autoradiography.

Extract Fractionation. One milliliter of HeLa nuclear extract was spun at 30 psi (1 psi = 6.9 kPa) (134,000 × *g*) for 1 hr in a Beckman airfuge ultracentrifuge. The supernatant was collected and the pellet was resuspended in an equal volume of buffer D (32) by gentle pipetting. UV crosslinking was performed with equivalent volumes of total extract, supernatant, and pellet fractions.

RESULTS

A UV crosslinking assay was used to study nuclear proteins that interact with known splicing consensus sequences. Simple splicing precursor RNAs containing one or two exons were transcribed with SP6 polymerase, incorporating [^{32}P]UTP. The labeled RNAs were incubated with HeLa cell nuclear extract for 5 min, crosslinked with UV light, and treated with pancreatic RNase A to digest noncrosslinked RNA. Labeled proteins were resolved by SDS/PAGE and subsequently visualized by autoradiography. Several splicing polypeptides previously characterized by this approach include the 62-kDa pPTB and the 38- to 41-kDa heterogeneous RNP C polypeptides (15). This communication centers on two smaller polypeptides of 26 and 37 kDa that crosslink to precursor RNAs containing 5′ splice sites.

A 5′ Splice Site Is Required for Crosslinking of SPP-1 and SPP-2. In Fig. 1, precursor RNAs containing three different 5′ splice sites and a nonspecific RNA containing no splicing signals were crosslinked to proteins in HeLa cell nuclear splicing extract. A variety of crosslinked proteins were detected. In particular, two proteins designated splicing proteins 1 and 2 (SPP-1 and SPP-2) crosslinked to precursor RNAs containing a 5′ splice site. Crosslinking of both SPP-1 and SPP-2 was only observed with RNAs containing a 5′ splice site. A nonspecific RNA generated from pSP65 vector sequences did not crosslink either polypeptide. Precursor RNAs containing characterized consensus sequences from the 3′ end of the intron but lacking a 5′ splice site or precursor RNAs containing poly(A) sites but no splicing signals also did not crosslink to either the SPP-1 or SPP-2 polypeptide (data not shown).

Crosslinking of SPP-1 and SPP-2 appeared to require only a 5′ splice site. Two of the constructs in Fig. 1 also contained consensus sequences from the 3′ end of the intron. The presence of these sequences caused no detectable alteration in crosslinking of SPP-1 or SPP-2. These substrates did crosslink to additional polypeptides, including pPTB that interacts with consensus sequences at the 3′ end of the intron.

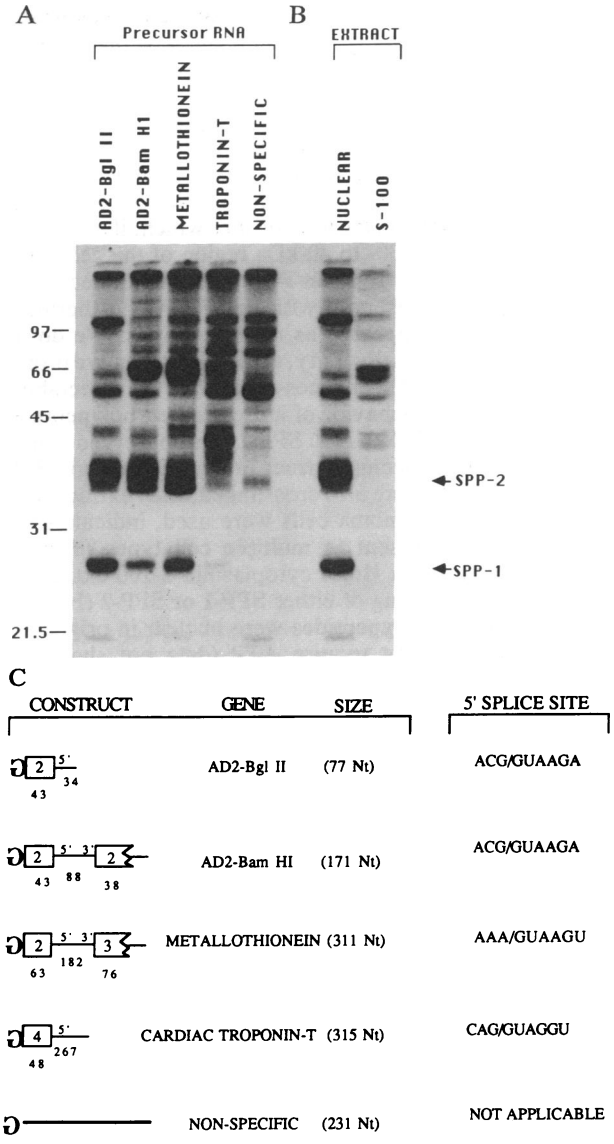

FIG. 1. UV crosslinking of polypeptides to ^{32}P-labeled precursor RNAs containing 5′ splice sites. (*A*) The diagrammed precursor RNAs were incubated with nuclear splicing extract for 5 min at 30°C prior to UV crosslinking. After crosslinking, samples were digested with RNase A and proteins were resolved by SDS/PAGE and subsequently visualized by autoradiography. Two crosslinked proteins, 26 (SPP-1) and 37 (SPP-2) kDa, associating with precursor RNAs containing 5′ splice sites are indicated. (*B*) Adenovirus 2 (AD2) *Bgl* II was incubated in either HeLa nuclear or cytoplasmic S-100 extract, as indicated, and crosslinked. Numbers on left are kDa. (*C*) Precursor RNAs used, exon numbers from the parental gene, exon lengths, and 5′ splice site sequences are diagrammed. Nt, nucleotides.

Although SPP-1 and SPP-2 crosslinked to all precursor RNAs containing a 5′ splice site, the intensity and gel migration of the observed polypeptide bands varied when different precursor RNAs were used. The adenovirus and metallothionein precursor RNAs repeatedly crosslinked both SPP-1 and SPP-2 at higher efficiency than troponin T precursor RNA. Both the adenovirus and metallothionein 5′ splice sites fit bases +1 to +5 of the consensus 5′ splice site sequence perfectly. The troponin T 5′ splice site diverges from the consensus sequence at position +4 (Fig. 1*C*). Both the adenovirus and metallothionein precursor RNAs spliced very efficiently in our HeLa extract, whereas the troponin T precursor RNA spliced poorly (data not shown). Thus, crosslinking efficiency paralleled splicing activity. It should

be noted that the troponin T 5′ splice site used was from a constitutive exon (exon 4) that immediately precedes an optional exon. It is possible that this 5′ splice site is recognized differently than standard ones.

The smaller polypeptide, SPP-1, exhibited the same molecular mass when crosslinked to each tested precursor. The larger peptide, SPP-2, crosslinked with variable efficiency and migrated at a slightly different gel position for each of the tested precursor RNAs. We are not sure which, if any, of the multiple bands in the 30- to 40-kDa region of the gel in Fig. 1 represents SPP-2 for troponin T precursor RNA because of the reduced signal observed with this RNA. Furthermore, SPP-2 consistently appeared as multiple bands. We do not know the source of this diversity, although alternative protein forms and crosslinked sequences could shift the detectability and electrophoretic behavior of a crosslinked polypeptide.

Crosslinking of SPP-1 and SPP-2 was observed with a variety of nuclear splicing extract preparations from HeLa cells. Both proteins were also crosslinked when extracts from murine F9 teratocarcinoma cells were used, indicating that the factors were present in multiple cell types (data not shown). In contrast, a HeLa cytoplasmic S-100 extract did not support crosslinking of either SPP-1 or SPP-2 (Fig. 1*B*), indicating that the polypeptides were nuclear in origin.

Crosslinking did not require ATP (data not shown). A similar property has previously been observed for the binding of non-snRNP polypeptides that interact with splicing consensus sequences at the 3′ end of the intron (15, 16).

Mutation of the 5′ Splice Site Depresses Crosslinking of SPP-1 and SPP-2. Our initial experiments suggested that both SPP-1 and SPP-2 crosslinked to RNAs containing 5′ splice site sequences. To determine whether the observed crosslinking was specifically due to the conserved CAG:GU(G/A)AG at the 5′ splice site or to other nearby sequences, RNAs were constructed that contain a double mutation at the 5′ splice site beginning intron 1, changing the +1 and +2 nucleotides from GU to CG (Fig. 2*C*). Precursor RNAs were prepared that contained one or two exons and were assayed for crosslinked polypeptides (Fig. 2*A*). Mutation diminished crosslinking of both SPP-1 and SPP-2 polypeptides. The crosslinking pattern of other polypeptides was not altered in the mutants. Crosslinked proteins such as pPTB were still displayed with the same intensity. These results indicated that crosslinking of SPP-1 and SPP-2 required the presence of a 5′ splice site.

Whereas mutation of the 5′ splice site drastically decreased crosslinking of SPP-1 and SPP-2, a residual low level of crosslinking was still observed (Fig. 2*A*). To determine whether the observed low level was dependent on the presence of a 5′ terminal cap, wild-type and mutant RNAs containing just a first exon (*Bgl* II cut) were transcribed capped and noncapped and crosslinked after a 5-min incubation (Fig. 2*B*). The wild-type transcript crosslinked at equal efficiency in the absence or presence of a 7-methylguanosine cap. However, the low-level binding of SPP-1 and SPP-2 of the capped mutant precursor RNAs was further depressed in the absence of a cap even though the crosslinking of background polypeptides remained the same. It is possible that although the presence of a cap is not necessary for binding and crosslinking of SPP-1 and SPP-2 to wild-type RNA, it does contribute to the basal level of crosslinking observed with mutant RNA.

Crosslinking of SPP-1 and SPP-2 Is Time Dependent. A two-exon adenovirus RNA active for splicing and containing only one 5′ splice site was incubated in splicing extract for 90 min. Samples were removed at various times to monitor UV crosslinking and splicing (Fig. 3). No crosslinking of either SPP-1 or SPP-2 was observed after a 5-min incubation on ice (Fig. 3*B*, time 0). The signal was detectable after 5 min of incubation at 30°C, indicating that these two polypeptides

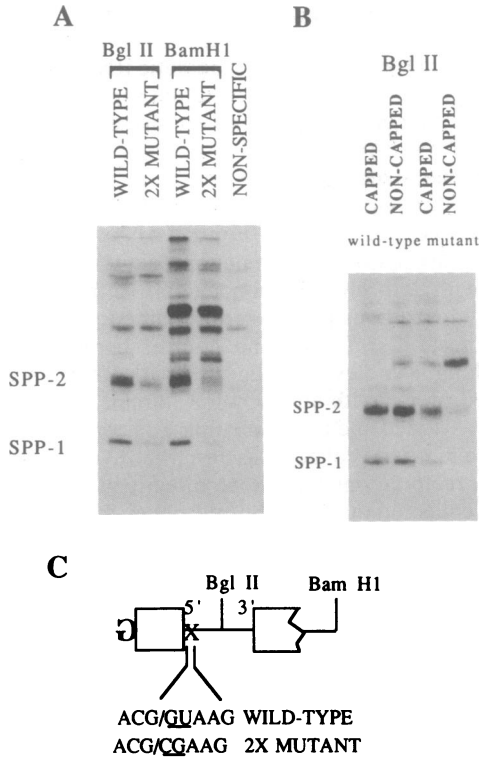

FIG. 2. Effect of 5′ splice site mutations on UV crosslinking of SPP-1 and SPP-2. Mutations were introduced at the 5′ splice site of the adenovirus construct changing ACG:GUAAG to ACG:CGAAG. Precursor RNA was prepared containing a single first exon with one 5′ splice site (*Bgl* II cut) or two exons with one 5′ and one 3′ splice site (*Bam*HI cut). Nonspecific RNA contains plasmid sequences. (*A*) Crosslinking of mutant and wild-type precursor RNAs. (*B*) Crosslinking of capped or uncapped mutant or wild-type precursor RNAs containing a single first exon (*Bgl* II cut). Incubation was for 5 min prior to crosslinking as in Fig. 1. Lack of a cap did not affect RNA stability during the short incubation used for this experiment; equal amounts of all four RNAs in *B* were detected after incubation.

quickly associated with 5′ splice sites. The intensity of the signal remained high until 20 min, after which time it decreased.

Splicing products began to accumulate by 20 min of incubation and increased thereafter (Fig. 3*A*) SPP-1 and SPP-2 interaction and crosslinking to precursor RNAs, therefore,

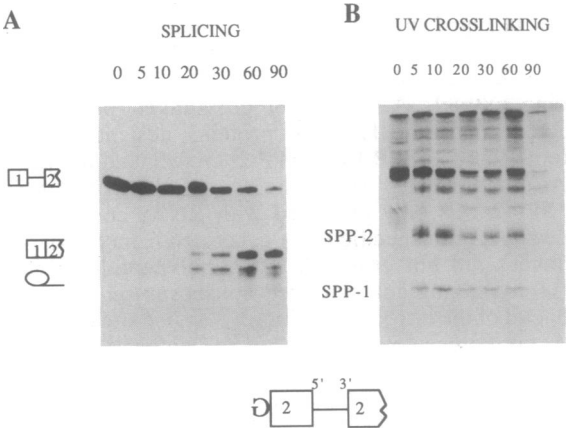

FIG. 3. Time course of crosslinking of SPP-1 and SPP-2. The diagrammed two-exon adenovirus precursor RNA was incubated in splicing extract for the indicated times and sampled for both reactivity (*A*) and UV crosslinked polypeptides (*B*). Reaction products are indicated for the RNA gel, and crosslinked proteins SPP-1 and SPP-2 are demarcated for the protein gel.

occurred during splicing complex formation. The ability to crosslink both factors during the time frame in which assembly of the active spliceosome occurred suggested that these factors may play a role in 5′ splice site selection and/or assembly. The alteration in crosslinking of SPP-1 and SPP-2 at 20 min coincided with both the appearance of reaction products and a decrease in the crosslinked signal of a 60- to 65-kDa protein that is likely pPTB and suggests changes in the way these factors associate with precursor RNA as the splicing reaction commences.

SPP-1 and SPP-2 Crosslink in the Absence of U1 snRNA. To determine whether SPP-1 and SPP-2 were part of the U1 snRNP complex, the HeLa cell nuclear extract was spun at 134,000 × *g* for 1 hr. Under these conditions, all of the U1 snRNA (as monitored by either silver or ethidium bromide staining) pelleted (Fig. 4). Supernatant and pellet fractions were assayed for their ability to support UV crosslinking. UV crosslinking of SPP-1 and SPP-2 was not detected in the pellet but was detected in the supernatant fraction at levels comparable to that observed with unfractionated extract. This result indicates that crosslinking of SPP-1 or SPP-2 did not require U1 snRNA and suggests that neither factor is a stable part of U1 snRNPs.

snRNP-Specific Antibodies Do Not Recognize SPP-1 or SPP-2. To determine whether SPP-1 or SPP-2 contained antigens recognized by anti-snRNP antibodies, UV crosslinked reaction mixtures were immunoprecipitated with anti-Sm and anti-RNP antibodies after RNase treatment of the crosslinked sample (Fig. 5 *Right*). Neither SPP-1 nor SPP-2 was immunoprecipitated by anti-Sm or U1-specific RNP (70 kDa) antibodies. This result indicates that these proteins do not contain the epitopes recognized by these antibodies although it is difficult to assess the effect crosslinking might have on the tested epitope. Both SPP-1 and SPP-2 have molecular masses in the range of the smaller snRNP polypeptides (33, 34). The lack of recognition of SPP-1 and SPP-2 with anti-Sm antibodies suggests that they are not core snRNP polypeptides.

SPP-1 and SPP-2 Reside in Precursor RNA Complexes That Also Contain snRNPs. Immunoprecipitations after RNase treatment indicated that SPP-1 and SPP-2 were not Sm-detectable snRNP polypeptides. To determine whether these polypeptides resided in splicing assemblies that also contained U1 snRNPs, crosslinked samples were immunoprecipitated before RNase treatment (Fig. 5 *Left*). The precursor RNA used in this experiment contained only one 5′ splice site. In this protocol, any factor whose association with both precursor RNA and a U snRNP is stable enough to survive immunoprecipitation will appear in the pellet. Under these conditions, both Sm- and U1-specific RNP antibodies immunoprecipitated ≈50% of the SPP-1 and SPP-2 that had been crosslinked. These results indicated that SPP-1 and SPP-2 resided in complexes assembled on precursor RNA that also contain U1 snRNPs. Therefore, SPP-1 and SPP-2 association with precursor RNA and 5′ splice sites can occur simultaneously with U1 snRNP association with 5′ splice sites.

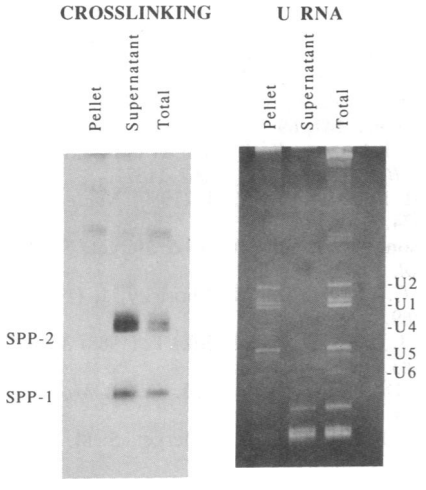

FIG. 4. Distribution of SPP-1 and SPP-2 and U1 snRNPs after extract centrifugation. HeLa nuclear extract was spun for 1 hr in a Beckman airfuge at 30 psi (134,000 × *g*). Supernatant and pellet fractions were examined for crosslinkable polypeptides and U RNA content. The pellet fraction was resuspended in an equal volume of buffer D (32) before use. Crosslinking as described in Fig. 1 utilized a single exon adenovirus precursor RNA. RNA was prepared from each fraction and displayed on an 8 M urea/10% polyacrylamide gel to identify U RNAs. Each lane utilized equivalent amounts of starting unfractionated extract, supernatant, and pellet.

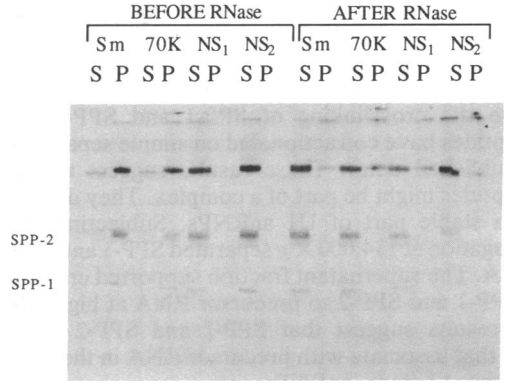

FIG. 5. Immunoprecipitation of SPP-1 and SPP-2 with snRNP-specific antibodies. Two-exon adenovirus precursor RNA was incubated for 5 min in HeLa nuclear extract and UV crosslinked. Each sample was split and subjected to immunoprecipitation either before (*Left*) or after (*Right*) RNase A treatment. Pairs of lanes show the supernatant (S) and pellet (P) from each precipitation. Sera used were Sm, monoclonal Y12 antibody; 70 K, patient RNP antibody specific for the U1 70-kDa protein; and NS_1, NS_2, two nonspecific patient sera.

DISCUSSION

UV crosslinking has been used to monitor polypeptides that interact with splicing precursor RNAs *in vitro*. We detected two polypeptides of 26 and 37 kDa that efficiently crosslinked to precursor RNAs containing 5′ splice sites. No crosslinking was observed to nonspecific RNA, to a polyadenylylation precursor RNA, or to a splicing RNA that contained all necessary splicing sequences at the 3′ end of the intron but lacked a first exon and its 5′ splice site. Furthermore, crosslinking was severely depressed when the 5′ splice site was mutated at nucleotides +1 and +2 of the intron. Thus, detection of association of SPP-1 and SPP-2 with precursor RNAs by UV crosslinking was dependent on the presence of a wild-type 5′ splice site.

Sequences other than the 5′ splice site within the precursor RNA had little effect on observation of crosslinking, nor did removal of the cap beginning the precursor RNA, suggesting that SPP-1 and SPP-2 recognized just the consensus sequence at the 5′ splice site. We did notice, however, that precursor RNAs containing only a first exon bearing a mutated 5′ splice site retained a low level of crosslinking to SPP-1 and SPP-2 despite the presence of the mutation. Removal of the 5′ cap beginning the mutant RNA inhibited this residual crosslinking but permitted the continual crosslinking of several background polypeptides. These results suggest that both the cap (or cap binding polypeptides) and the 5′ splice site play a role in crosslinking SPP-1 and SPP-2. It has been reported that the presence of a cap is required for the removal of intron 1 but

not intron 2 of a two-intron precursor RNA (35), suggesting that a cap plays a role in the mechanism whereby first exons and their 5′ splice sites are recognized.

We were unable to find substrates or conditions that could separate the crosslinking of SPP-1 and SPP-2. The two polypeptides have cofractionated on simple separations (Fig. 4; unpublished data). These results suggest that the two polypeptides might be part of a complex. They do not appear to be a stable part of U1 snRNPs. Subjecting extract to centrifugation at 134,000 × *g* separated SPP-1 and SPP-2 from U1 RNA. The supernatant fraction supported crosslinking of both SPP-1 and SPP-2 to precursor RNA at high efficiency. These results suggest that SPP-1 and SPP-2 are splicing factors that associate with precursor RNA in the absence of, and perhaps before, snRNPs.

We did, however, detect association of SPP-1 and SPP-2 with complexes containing U1 snRNPs. When crosslinking reaction mixtures were immunoprecipitated with anti-U1 snRNP antibodies prior to disruption of the bound complex with RNases, both SPP-1 and SPP-2 distributed at least partially into the immunoprecipitate. This association is occurring in the presence of precursor RNA. Therefore, we suggest that although SPP-1 and SPP-2 do not appear to be associated with U1 snRNPs in the extract, such association does occur via the assembly of precursor RNA. Furthermore, these results indicate that U1 snRNPs and SPP-1 and SPP-2 can simultaneously be associated with the same precursor RNA, suggesting that SPP-1 and SPP-2 are components of functional spliceosomes.

SPP-1 and SPP-2 are 26 and 37 kDa after crosslinking. We do not know their molecular mass before crosslinking. Although SPP-1 and SPP-2 have molecular masses similar to those of the common Sm-reactive snRNP polypeptides (33, 34), neither SPP-1 nor SPP-2 could be immunoprecipitated with anti-Sm antibodies. Two U1 snRNP polypeptides (A and C) have molecular masses in this range. We do not, however, think that either polypeptide is the U1-specific A or C protein because we have separated SPP-1 and SPP-2 from U1 RNA by centrifugation of the extract. The A and C polypeptides can be removed from U1 snRNPs, but chromatography at high temperatures is required (24). Furthermore, the A and C polypeptides released by this technique do not bind RNA. Therefore, we suggest that SPP-1 and SPP-2 may be new, previously uncharacterized splicing factors involved in 5′ splice site selection.

Recently, several reports have identified splicing factors the size of SPP-2 (17–19). The relationship between these factors and our polypeptide is unclear. None of these reports suggested association with a polypeptide of the size of SPP-1; nor did they report specific crosslinking to precursor RNAs containing 5′ splice sites. Two of the polypeptides, however, had an influence on 5′ splice site selection (18, 19), particularly selection of the 5′ splice site beginning the first intron. Our factors associate with the first 5′ splice site in a precursor RNA. Interestingly, when we examined the ability of SPP-1 and SPP-2 to crosslink to the same 5′ splice site placed at the beginning of an internal intron (i.e., downstream of a 3′ splice site), crosslinking was substantially depressed. Thus, it is not clear whether these polypeptides are involved in recognition of all 5′ splice sites or only those beginning the first intron.

Isolated U1 snRNPs have the capacity to bind to 5′ splice sites (24). This ability suggests that SPP-1 and SPP-2 are not obligate factors for recognition of 5′ splice sites. We have previously reported that recognition of the first 5′ splice site in an *in vitro* precursor RNA requires a soluble factor in addition to U1 snRNPs (26). In the absence of this factor, spliceosome assembly occurs at the 3′ end of the intron, and the resulting complexes are distinguishable on native RNP gels from wild-type complexes. SPP-1 and SPP-2 are candidates for these soluble factors.

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