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Direct Interactions between Pre-mRNA and Six U2 Small Nuclear Ribonucleoproteins during Spliceosome Assembly

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Highly purified mammalian spliceosomal complex B contains more than 30 specific protein components. We have carried out UV cross-linking studies to determine which of these components directly contacts pre-mRNA in purified prespliceosomal and spliceosomal complexes. We show that heterogeneous nuclear ribonucleoproteins cross-link in the nonspecific complex H but not in the B complex. U2AF^{65}, which binds to the 3′ splice site, is the only splicing factor that cross-links in purified prespliceosomal complex E. U2AF^{65} and the U1 small nuclear ribonucleoprotein particle (snRNP) are subsequently destabilized, and a set of six spliceosome-associated proteins (SAPs) cross-links to the pre-mRNA in the prespliceosomal complex A. These proteins require the 3′ splice site for binding and cross-link to an RNA containing only the branch site and 3′ splice site. Significantly, all six of these SAPs are specifically associated with U2 snRNP. These proteins and a U5 snRNP component cross-link in the fully assembled B complex. Previous work detected an ATP-dependent, U2 snRNP-associated factor that protects a 30- to 40-nucleotide region surrounding the branchpoint sequence from RNase digestion. Our data indicate that the six U2 snRNP-associated SAPs correspond to this branchpoint protection factor. Four of the snRNP proteins that are in intimate contact with the pre-mRNA are conserved between *Saccharomyces cerevisiae* and humans, consistent with the possibility that these factors play key roles in mediating snRNA–pre-mRNA interactions during the splicing reaction.

The excision of introns from pre-mRNA requires specific and highly ordered interactions between pre-mRNA, five spliceosomal small nuclear RNAs (snRNAs), and a large number of proteins (for reviews, see references 22, 23, 29, 36, 51, and 57). Genetic studies have defined several functional base-pairing interactions between pre-mRNA and the snRNAs (39, 41, 48, 54, 56, 62, 68, 69). These base-pairing interactions, as well as other potentially important snRNA–pre-mRNA associations, have been identified by UV cross-linking and shown to occur at specific stages of spliceosome assembly (52, 53, 60, 63). The numerous snRNA–pre-mRNA interactions, coupled with mechanistic similarities between the splicing of nuclear pre-mRNAs and group II introns, support the notion that RNA-RNA interactions play a central role in, and perhaps even catalyze, the splicing reaction (23, 36, 57). Thus, the large number of proteins that are essential for splicing may play critical roles in positioning, stabilizing, or disrupting RNA-RNA interactions in the spliceosome.

Spliceosome formation proceeds by means of an ordered assembly of discrete complexes. The assembly pathway is E (or commitment) prespliceosome → A prespliceosome → B spliceosome → C spliceosome (for review, see references 36 and 51). Prior to assembly of these functional intermediate complexes, the pre-mRNA assembles into a heterogeneous nuclear ribonucleoprotein (hnRNP) complex (H complex) which consists of the more than 20 abundant nuclear proteins that package newly transcribed pre-mRNA in vivo (8). The relationship between the H complex, which also assembles on RNAs lacking splice sites, and spliceosome assembly is not known. In addition to U1, U2, U4, U5, and U6 snRNAs, several protein factors are required for spliceosome formation. Among the purified factors required in mammals are the SR proteins, U2AF^{65}, PSF, SF1, and SF3a (13, 15, 19, 25, 27, 28, 43, 64, 65). U2AF^{65} and SF3a are detected in affinity-purified mammalian prespliceosomes and/or spliceosomes by two-dimensional (2D) gel electrophoresis (6, 7, 9, 12, 13). U1, U2, and U5 small nuclear ribonucleoprotein (snRNP) components and several additional proteins designated spliceosome-associated proteins (SAPs) are also detected in the purified complexes (7).

In contrast to the relatively detailed understanding of snRNA–pre-mRNA interactions during spliceosome assembly, little is known about the protein–pre-mRNA interactions. UV cross-linking has been used as a strategy to identify specific protein–pre-mRNA interactions in splicing extracts but has resulted primarily in the detection of hnRNPs (17, 18, 20, 42, 58). This is most likely because of their high abundance in nuclear extracts. hnRNP C and hnRNP I/polypyrimidine tract-binding protein (PTB) have been shown to cross-link specifically to the 3′ splice site (18, 42), while hnRNP A1 cross-links to the 5′ splice site (58). Other than the hnRNPs, a 200-kDa U5 snRNP protein and U2AF^{65} cross-link upstream of the 5′ splice junction and to the pyrimidine tract at the 3′ splice site, respectively (17, 61, 63, 65, 67).

In this study, we have carried out UV cross-linking in combination with 2D gel analysis to determine which of the proteins present in purified prespliceosomes and spliceosomes bind directly to pre-mRNA. This analysis identified a set of six SAPs (SAPs 155, 145, 114, 62, 61, and 49) that cross-link to the 3′ portion of the intron in the A complex. We show that these six SAPs are specifically associated with U2 snRNP. In addition, we show that the 200-kDa U5 snRNP component and the six U2 snRNP-associated SAPs cross-link in the B complex. The observation that the U2 and U5 snRNP proteins directly contact the pre-mRNA during spliceosome assembly suggests that these factors play important roles in mediating snRNA–pre-mRNA interactions.

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MATERIALS AND METHODS

Plasmids. The construction of plasmids pAdML and pAd3′ was described by Bennett et al. (7). pAdML contains exon 1 (129 nucleotides [nt]), intron 1 (97 nt), and exon 2 (45 nt) derived from the adenovirus type 2 major late (AdML) transcription unit. DNA was linearized with *Bam*HI (+271 in pAdML) for transcription. pAd3′, which contains the 3′ portion of the AdML intron and exon 2 (+165 to +271), was linearized with *Bam*HI for transcription. pFtz (a gift from D. Rio, University of California, Berkeley [55]) was linearized with *Xho*I to generate fushi tarazu (*ftz*) pre-mRNA (473 nt). pAdML, pAd3′, and pFtz were transcribed with T7 RNA polymerase.

Pre-mRNA synthesis and in vitro splicing reactions. Capped biotinylated pre-mRNAs (21) were synthesized in standard transcription reaction mixtures (33) containing 50 μCi each of [^{32}P]ATP, -GTP, -CTP, and -UTP (3,000 Ci/mmol), 100 μM unlabelled ATP, GTP, CTP, and UTP, and 15 to 20 μM biotinylated UTP (Enzo Biochemicals). Assembly of splicing complexes was carried out under in vitro splicing conditions (26). Reaction mixtures contained 30% nuclear extract and were incubated at 30°C for 20 or 60 min as indicated. For assembly of the E complex, nuclear extract was depleted of ATP as described previously (34). E complex assembly reactions lacked ATP, $MgCl_2$, and creatine phosphate. Reaction mixtures were incubated at 30°C for 15 min.

Purification of splicing complexes and UV cross-linking. Gel filtration and affinity purification of splicing complexes were carried out as described previously (7). Affinity-purified complexes were washed four times in 20 mM Tris (pH 7.6) containing either 125 or 250 mM NaCl as indicated in the figure legends. In either case, a final wash was carried out in 20 mM Tris (pH 7.6)–50 mM NaCl, and then the buffer above the avidin agarose beads was removed. Complexes bound to avidin agarose were immediately irradiated on ice with 254-nm UV light (Sylvania G15T8 lamp) for 5 min at a distance of 5.5 cm from the light source. The optimal time for cross-linking of all of the proteins was determined by carrying out a time course (data not shown).

Preparation and analysis of protein and RNA samples. To digest the ^{32}P-labeled RNA after cross-linking, 1 μl of 10-mg/ml protease-free RNase A (Pharmacia) was added per 10 μl of avidin agarose-bound splicing complexes and incubated at 37°C for 30 min. Proteins were then eluted from the avidin beads in a buffer consisting of 20 mM Tris (pH 7.6), 20 mM dithiothreitol, and 2% sodium dodecyl sulfate (SDS). Eluted proteins were heated at 65°C for 5 min. (This step was essential to reproducibly detect all proteins by 2D gel electrophoresis.) Glycogen (2 μl as a carrier) and 4 volumes of acetone were added; samples were left at room temperature for 10 min and then spun for 10 min at room temperature. Recovered proteins were immediately dissolved in SDS or 2D sample buffer (8 M urea, 1.7% Nonidet P-40, 1.7% pH 3 to 10 ampholytes [Bio-Rad]) and stored at −70°C or loaded directly onto gels.

2D gel electrophoresis was carried out as described previously (40). The first dimension was non-equilibrium pH gradient gel electrophoresis (ampholytes pH 3 to 10 [Bio-Rad]), and the second dimension was SDS–9% polyacrylamide gel electrophoresis as indicated. For Fig. 5, pH 7 to 9 ampholytes (Pharmacia) were used for the isofocusing dimension, and the second dimension was SDS–6.5% polyacrylamide gel electrophoresis. The total protein obtained from splicing complexes assembled on 200 ng of pre-mRNA was loaded on 2D gels. Proteins were visualized by silver staining (37), and cross-linked proteins were detected by PhosphorImager (Molecular Dynamics) analysis.

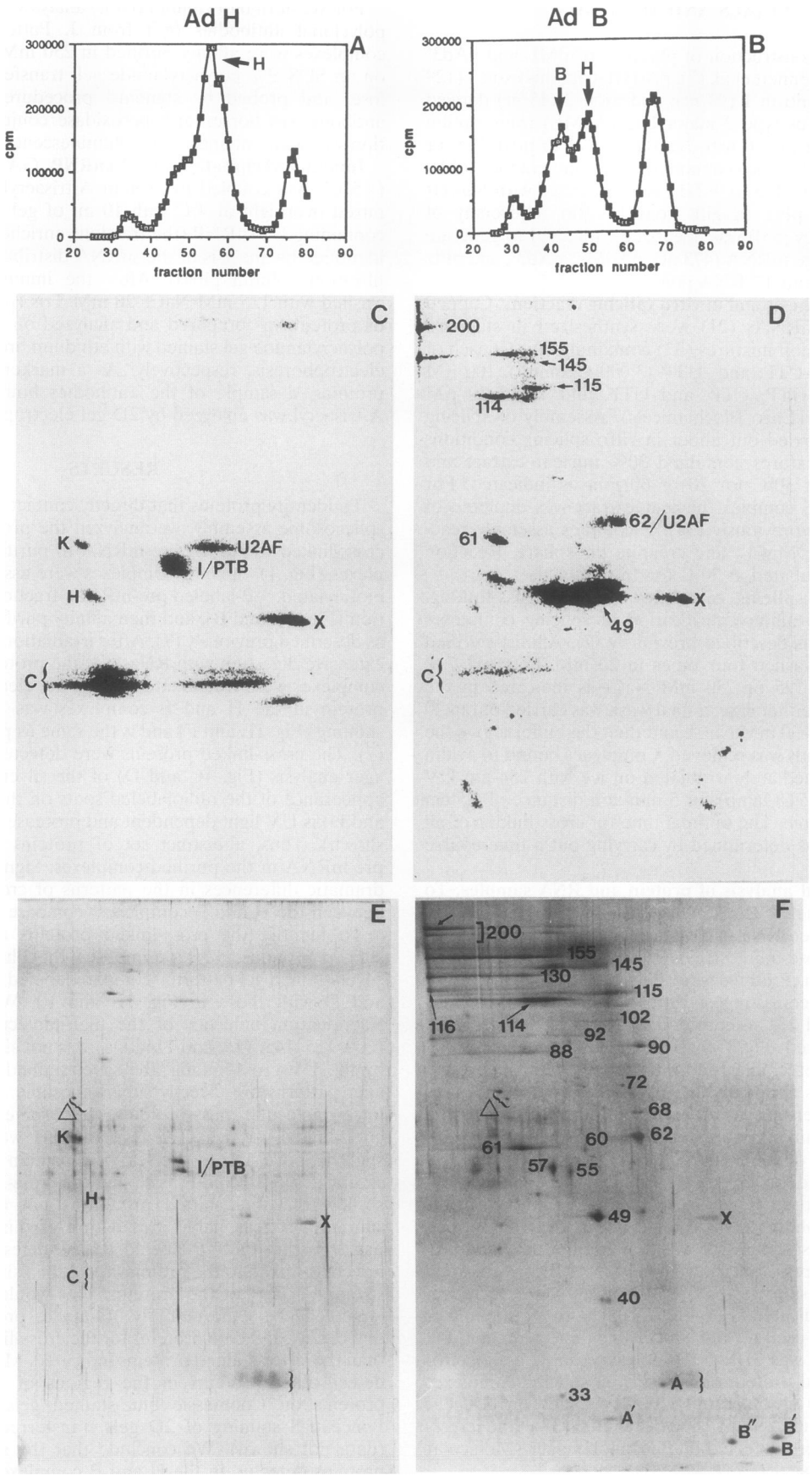
For Western blot (immunoblot) analysis with U2AF^{65} rabbit polyclonal antibodies (gift from J. Patton), E, A, and B complexes were affinity purified in 250 mM salt, fractionated on an SDS–9% polyacrylamide gel, transferred to nitrocellulose, and probed by standard procedures. The secondary antibody was horseradish peroxidase conjugated, and detection was with enhanced chemiluminescence (Amersham).

Immunoprecipitation of U2 snRNP. GA patient antiserum (7.5 μl) was coupled to protein A-trisacryl (25 μl) and then mixed overnight at 4°C with 10 ml of gel filtration fractions containing U2 snRNP (the fractions enriched in snRNPs were identified by analysis of the snRNA distribution across the gel filtration column [46a]). After the immunoprecipitate was washed with 125 mM NaCl–20 mM Tris (pH 7.6), total RNA or protein was prepared and analyzed on an 8% denaturing polyacrylamide gel stained with ethidium bromide or by 2D gel electrophoresis, respectively. As a marker for the antibody proteins, a sample of the antibodies bound to the protein A-trisacryl was analyzed by 2D gel electrophoresis.

RESULTS

To identify proteins that directly contact pre-mRNA during spliceosome assembly, we analyzed the proteins that are UV cross-linked to AdML pre-mRNA in purified H and B complexes (Fig. 1). Splicing complexes were assembled in vitro on biotinylated, ^{32}P-labeled pre-mRNA, fractionated by gel filtration (Fig. 1A and B), and then affinity purified in 250 mM salt as described previously (7). After irradiation with UV light and extensive digestion with RNase A, the proteins in the purified complexes were fractionated by 2D gel electrophoresis. Total protein in the H and B complexes was detected by silver staining (Fig. 1E and F) and is the same as previously reported (7). The cross-linked proteins were detected by PhosphorImager analysis (Fig. 1C and D) of the silver-stained gels. The appearance of the radiolabeled spots on the 2D gels (Fig. 1C and D) is UV light dependent and protease sensitive (data not shown). Thus, a distinct set of proteins cross-links to the pre-mRNA in the purified complexes. Significantly, there are dramatic differences in the patterns of cross-linked proteins between the H and B complexes (compare Fig. 1C and D).

To identify the cross-linked proteins among the silver-stained proteins, we superimposed the PhosphorImager and silver-stained gel patterns (compare labeled proteins in Fig. 1C and D with those in Fig. 1E and F). We found that the fractionation behavior of the high-molecular-mass proteins (200, 155, 145, 115, and 114 kDa) was not significantly affected by the UV cross-linking; the silver-stained and PhosphorImager patterns are directly superimposable. In the case of the lower-molecular-mass proteins, we observed that each of the cross-linked species (Fig. 1C and D) was usually located slightly above and to the left of a protein detected by silver staining (Fig. 1E and F; note that the cross-linking efficiency is so low that the shifted protein is not detected by silver staining). A reasonable explanation of this fractionation behavior is that the ^{32}P-labeled RNase digestion products that are cross-linked to the proteins result in a slight increase in the size and acidity of the proteins. This would obviously have a larger effect on the mobility of smaller proteins. We do not detect any candidate proteins for the cross-linked species other than the silver-stained proteins indicated. Moreover, we do not detect other proteins in the regions of these cross-linked proteins on Coomassie blue-stained gels, nor by gold or Ponceau S staining of 2D gels transferred to nitrocellulose (data not shown). We conclude that the set of cross-linked proteins detected in the H and B complexes correspond to a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subset of the silver-stained proteins identified previously in these complexes (7). In the H complex, the cross-linked proteins are hnRNPs C, I/PTB, K, and H (note that hnRNP C consists of two proteins, C1 and C2, which fractionate as streaks and appear faintly in the photographs of the silver-stained gels). In addition, a protein designated X cross-links in the H complex and all other complexes (Fig. 1C and D; see below) but to our knowledge has not been described as an hnRNP protein (44). We also observe that low levels of $U2AF^{65}$ cross-link in the H complex (compared with the E complex; see below), but this varies in different extracts, possibly as a function of the $U2AF^{65}$ levels in each extract.

We identified the spliceosome-specific proteins that cross-link in the B complex as the 115-kDa SAP, the top band of the 200-kDa doublet (arrow in Fig. 1F) which is a component of U5 snRNP (4, 7), and the 155-, 145-, 114-, 62-, 61-, and 49-kDa SAPs (see below for studies which resolve $U2AF^{65}$ and the 62-kDa SAP). The spot below SAP 61 in the B complex is not detected reproducibly. In some gels, it is difficult to detect cross-linking of the 145-kDa SAP (e.g., Fig. 1D), which sometimes fractionates as a streak and/or does not resolve well from the 155-kDa SAP. Other examples of gels which clearly demonstrate cross-linking of the 145-kDa SAP are shown below. No additional cross-linked proteins were detected by analysis of the H and B complexes on high-percentage gels (data not shown).

Our data show that hnRNP proteins, such as I/PTB, C, and K, do not cross-link, or cross-link much less efficiently, in the spliceosome relative to the H complex (compare Fig. 1C and D). However, this may be attributed, at least in part, to the fact that splicing complexes were purified in 250 mM salt, conditions under which many of the hnRNPs dissociate completely from splicing complexes (7, 46). Thus, we purified the H complex and the spliceosome under conditions in which the hnRNPs are known to remain bound to the pre-mRNA in the H complex (125 mM salt [7]). As expected (7), the types and levels of hnRNPs are significantly increased in the H complex under these conditions (compare Fig. 2C with Fig. 1E and Fig. 2A with Fig. 1C). Surprisingly, however, we observe that the levels of most of the cross-linked hnRNPs are dramatically reduced in the spliceosome relative to the H complex (compare Fig. 2A and B). For example, the levels of cross-linking of hnRNPs A, B, C, F, H, K, P, Q, R, and T are clearly lower in the spliceosome than in the H complex. The insets in Fig. 2A and B show a lighter exposure of hnRNP I/PTB revealing that the cross-linked levels of this protein are also significantly lower in the B than in the H complex. This difference in the levels of cross-linked hnRNPs between the H complex and the spliceosome is readily apparent when the cross-linking patterns are compared by SDS-gel electrophoresis (Fig. 2E and F; hnRNPs I/PTB and C are indicated). We conclude that most of the hnRNPs that cross-link in the H complex either do not crosslink or cross-link significantly less efficiently in the spliceosome.

In our previous study (7), we noted that the levels of hnRNPs detected by silver staining appeared to be lower in the B than in the H complex purified in high salt (250 mM). In the present study, in which cross-linking is used as an assay, we have found that the levels of cross-linked hnRNPs are dramatically lower in the B than in the H complex (also at 250 mM salt). Moreover, we find that the levels of cross-linked hnRNPs are also dramatically lower in the B than in the H complex when the complexes are purified in low salt. Thus, we conclude that hnRNPs are specifically destabilized in the B complex.

A longer exposure of Fig. 2B (Fig. 2G) indicates that the same set of spliceosome-specific proteins cross-links in spliceosomes purified in 125 mM as in 250 mM salt (see Fig. 1D). These are the 200-, 155-, 145-, 115-, and 114-kDa, 62/U2AF, and 61- and 49-kDa proteins. The relative levels of the cross-linked 115-kDa SAP are higher in spliceosomes purified in low versus high salt (compare Fig. 1D with Fig. 2B or G). However, the 115-kDa SAP is also present at high levels in AdML H complex purified in low salt (Fig. 2A and C). This protein was not detected previously in the H complex, perhaps because of differences in nuclear extracts (8). In any case, further studies are needed to understand the significance of the 115-kDa SAP which is abundant in AdML spliceosomes but lacking in tropomyosin and *ftz* spliceosomes (7) (see below). The 115-kDa protein will not be discussed further in this report.

From the studies with AdML, we conclude that most of the hnRNPs that cross-link in the H complex do not cross-link efficiently in the spliceosome and that a set of spliceosome-specific proteins cross-links to the pre-mRNA in the spliceosome. To determine whether these two observations can be generalized, we examined the cross-linking patterns of a different pre-mRNA (Fig. 3). *Drosophila ftz* pre-mRNA, which assembles spliceosomes and splices with very high efficiencies in HeLa nuclear extracts (47, 55) (data not shown), is about 200 nt longer than, and does not share any obvious sequence similarities to, AdML pre-mRNA (AdML, 271 nt; *ftz*, 473 nt). As predicted from previous studies (7, 8), *ftz* and AdML H complexes are distinct from one another (compare Fig. 3B with Fig. 1E and Fig. 3A with Fig. 1C). Significantly, however, cross-linking of the hnRNPs to *ftz* pre-mRNA is dramatically lower in the spliceosome than in the H complex (compare Fig. 3A with Fig. 3C and E), as was observed with AdML (Fig. 1C and D). The hnRNPs are also deficient in *ftz* spliceosomes purified in low salt (Fig. 3G and H). Although hnRNPs A and B cross-link in the low-salt *ftz* B complex, these proteins cross-link at much higher levels in the low-salt *ftz* H complex (data not shown). In contrast to H complex, the cross-linked

FIG. 1. Distinct sets of proteins cross-link in AdML H and B complexes purified in 250 mM salt. Splicing reaction mixtures (2.4 ml) containing 9.6 μg of ^{32}P-labeled, biotinylated AdML pre-mRNA were incubated under splicing conditions in the absence (A) or presence (B) of ATP and then fractionated by gel filtration (see Materials and Methods). The peaks containing the H and B complexes (spliceosomes) are indicated. The peak between fractions 30 and 40 is the void volume of the column, and the peak to the right of the H complex is degraded RNA. (C to F) Gel filtration fractions containing B or H complexes were pooled, affinity purified in 250 mM salt, and irradiated with UV light (see Materials and Methods). After extensive digestion with RNase A, proteins from equivalent amounts of AdML pre-mRNA (200 ng) assembled into B or H complexes were fractionated by 2D gel electrophoresis, silver stained (E and F), and subjected to PhosphorImager analysis (C and D). The abundant spliceosomal proteins and hnRNPs are designated by molecular weight (in thousands) or by name if known (nomenclature for spliceosomal proteins and hnRNPs is according to Bennett et al. [7] and Piñol-Roma et al. [44], respectively); Δ indicates the heat shock proteins. B, B′, B″, A, and A′ are snRNP proteins, and X indicates the unidentified protein that cross-links in all of the complexes (see text). The braces in panels E and F indicate a contaminant from RNase A treatment that is seen in all of the gels. The arrow in panel F indicates the 200-kDa protein detected by cross-linking in panel D. Note that the low levels of $U2AF^{65}$ detected by cross-linking in the H complex were not detected on the silver-stained gel (compare panels C and E). The 61-kDa SAP can be distinguished from hnRNP K, which cross-links in the H complex, because the 61-kDa SAP fractionates slightly below the $62/U2AF^{65}$ band while the hnRNP K fractionates above it (compare panels C and D).

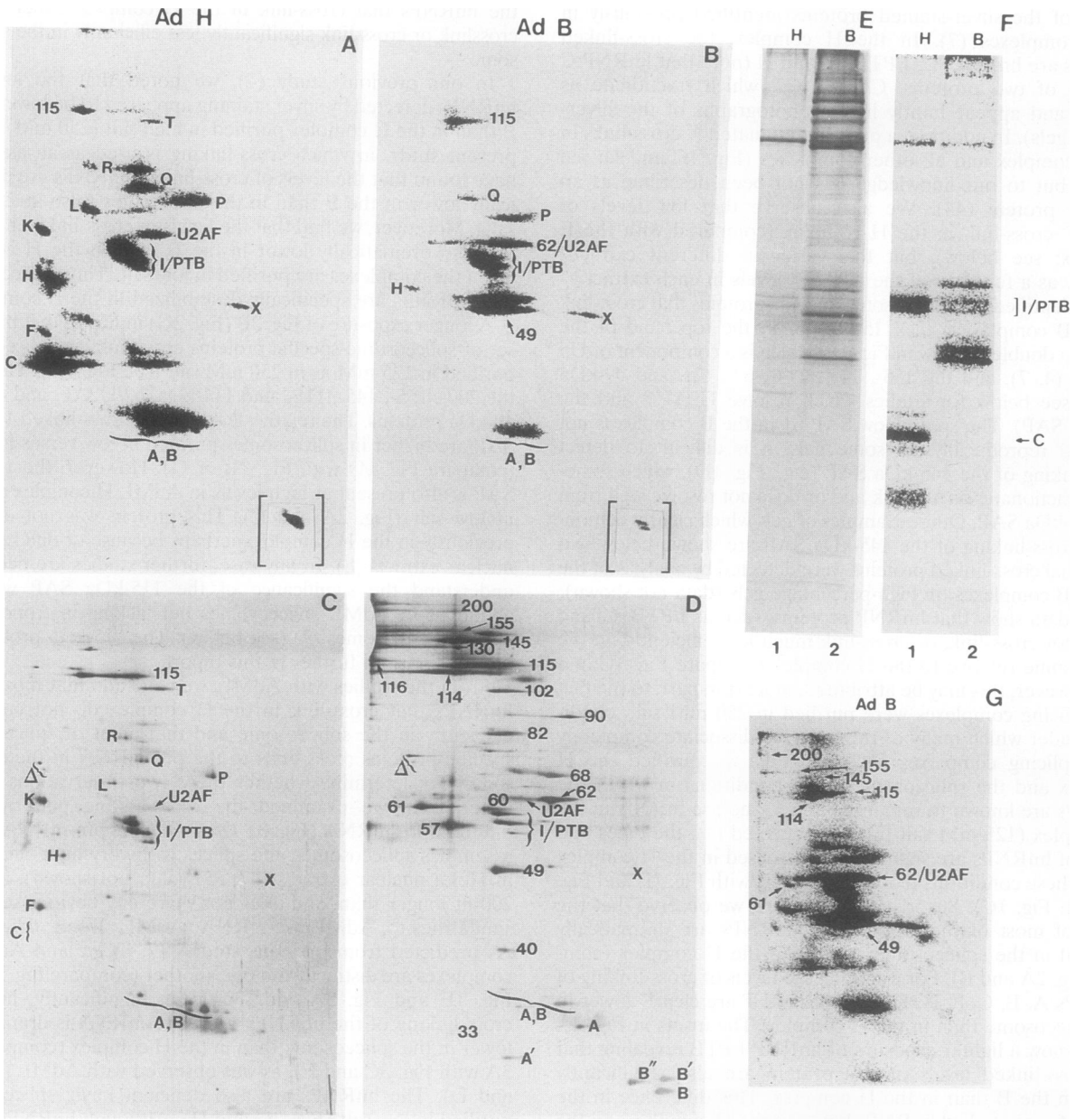

FIG. 2. Identification of cross-linked proteins in AdML H and B complexes purified in 125 mM salt. Complexes were assembled, purified, and cross-linked as described in the legend to Fig. 1 except that complexes were washed in 125 mM salt after affinity purification. Equivalent amounts of AdML pre-mRNA (200 ng) assembled into the B or H complexes were fractionated by 2D gel electrophoresis, silver stained (C, D, and E), and subjected to PhosphorImager analysis (A, B, F, and G). The insets in panels A and B show a lighter exposure of the portion of the gel containing hnRNP I/PTB. The abundant spliceosomal and hnRNPs are designated by molecular weight (in thousands) or by name if known; Δ indicates the heat shock proteins. In panels A to D, hnRNP A is in the braces, whereas the U1 snRNP component A is outside the brace in panel D. (E and F) H (lane 1) and B (lane 2) complexes were fractionated by SDS-gel electrophoresis followed by silver staining (E) and PhosphorImager analysis (F). hnRNP C and I/PTB are indicated. (G) A longer exposure of panel B.

spliceosome-specific proteins in AdML and *ftz* spliceosomes are strikingly similar to one another, both quantitatively and qualitatively (e.g., compare Fig. 1D with Fig. 3C, E, or G). We conclude that distinct patterns of hnRNPs cross-link in AdML and *ftz* H complexes. These proteins do not cross-link or cross-link much less efficiently in the spliceosome, whether complexes are purified in low or high salt. In contrast, a common set of spliceosome-specific proteins cross-links to *ftz* and AdML pre-mRNAs in the spliceosome purified in low or high salt.

Temporal order of cross-linking. To determine the specific stage of spliceosome assembly at which the individual proteins cross-link, we carried out UV cross-linking of AdML prespliceosomes and spliceosomes affinity purified in 250 mM salt (Fig. 4). The profiles of the gel filtration columns used to isolate each complex are shown in Fig. 4A. Previous studies showed that hnRNP I/PTB and U2AF^{65} cross-link to the pyrimidine tract at the 3′ splice site (21, 42, 65, 66). We observe that U2AF^{65} cross-links significantly more efficiently in the prespliceosome complex E than in the H complex, while the reverse is observed with hnRNP I/PTB (compare Fig. 1C and

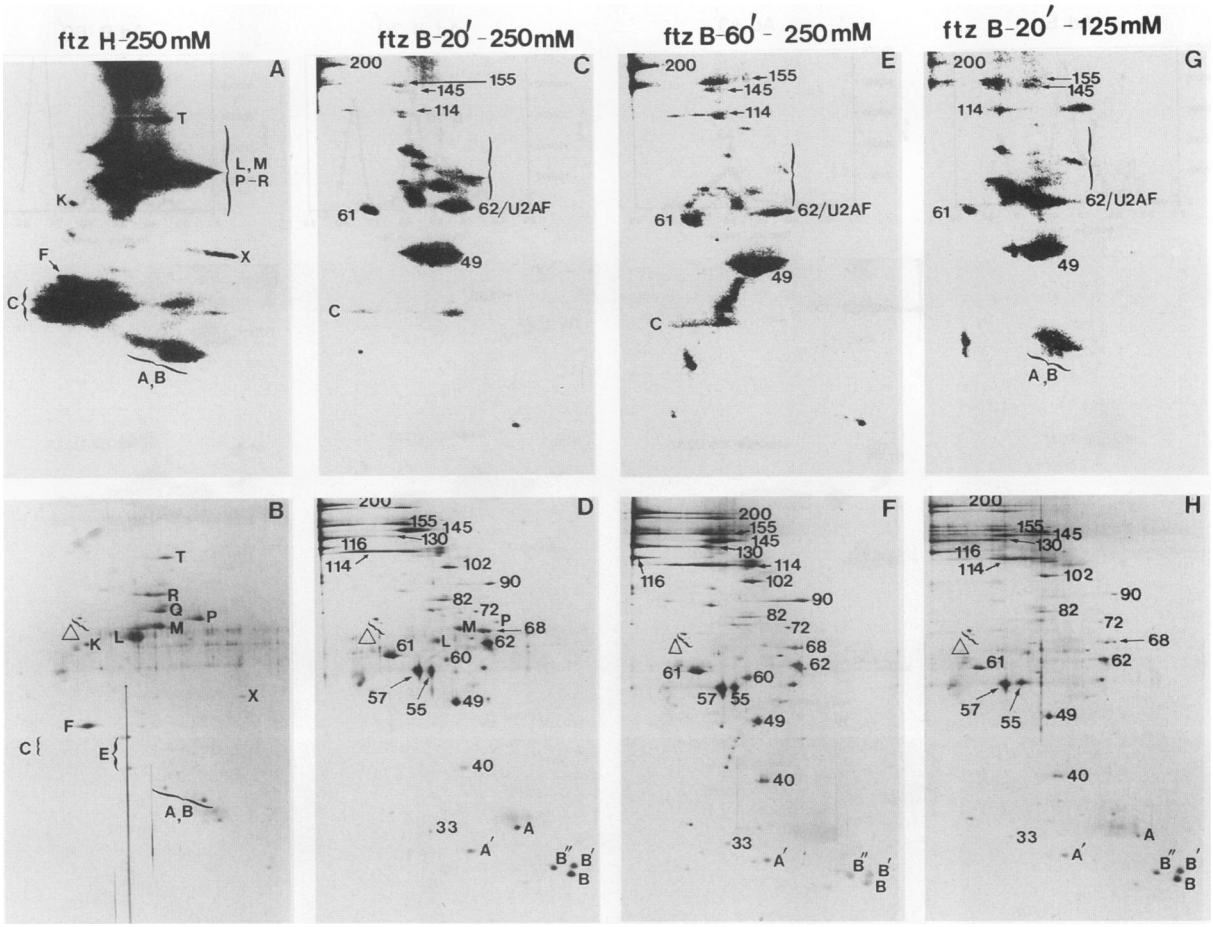

FIG. 3. Analysis of the cross-linked proteins in the H complex and spliceosomes assembled on *ftz* pre-mRNA. Complexes assembled on *ftz* pre-mRNA were purified in 250 mM (A to F) or 125 mM (G and H) salt, cross-linked, and fractionated by 2D gel electrophoresis. The silver-stained (B, D, F, and H) and PhosphorImager (A, C, E, and G) patterns are shown. Proteins are designated as described in the legend to Fig. 1. In panels A, B, and G, hnRNP A is in the braces. In panels D and H, the A outside the braces indicates the U1 snRNP component A. Note that the high-salt and low-salt samples were prepared independently, and thus the PhosphorImager exposures are not directly comparable. 20′ and 60′, 20 min and 60 min.

4B [Ad E]). This observation is consistent with the fact that U2AF^{65} is an essential splicing factor whereas hnRNP I/PTB is dispensable (43, 66). hnRNP C, which also binds to the 3′ splice site (59), cross-links with similar efficiencies in the E and H complexes, but then the levels drop significantly in the spliceosome (compare Fig. 1C and 4B [Ad E] and 4B [Ad B-20′]; note that there is some variability in the levels of hnRNP C cross-linking in the spliceosome (e.g., compare Fig. 1C and D, 2A and B, 3A and C, 4B [Ad B-20′ and Ad B-60′], and 5B). Subsequent studies have shown that this is due to variable levels of contamination of the spliceosome with the H complex which fractionates very near the spliceosome (see Fig. 4A). At present, we do not understand why there are differences in the cross-linking behavior of hnRNPs I and C, both of which have been shown previously to bind to 3′ splice site sequences (21, 42, 59).

To identify proteins that cross-link to the pre-mRNA at the time of A complex assembly, we analyzed the A3′ complex, which assembles on pre-mRNAs lacking the 5′ splice site. This was necessary because the A complex is so short-lived when assembled on intact pre-mRNA that it is difficult to purify reproducibly. The A3′ complex contains U2 snRNA and fractionates similarly to the A complex on native gels (24). The 155-, 145 (see inset)-, 114-, 62-, 61-, and 49-kDa SAPs cross-link to the pre-mRNA in the A3′ complex (Fig. 4B [Ad A3′]; see below for studies in which the 62-kDa SAP and U2AF^{65} are resolved). These six SAPs, as well as the 200-kDa U5 snRNP protein, cross-link in the B complex (Fig. 4B [Ad B-20′ and Ad B-60′]). The observation that only the top band of the 200-kDa doublet cross-links suggests either that the two proteins in the doublet are distinct from one another or that the proteins are related, and the smaller of the two proteins lacks the RNA binding domain. The observation that cross-linking of the 200-kDa protein requires the 5′ splice site is consistent with data showing that this protein cross-links in the spliceosome to a thiouridine residue which was inserted just upstream of the 5′ splice junction (63).

With both *ftz* and AdML, we do not observe significant differences in either the cross-linking or silver-stained patterns of spliceosomes assembled for 20 min versus 60 min (Fig. 4B and C [Ad B-20′ and Ad B-60′]; Fig. 3). However, denaturing gel analysis of a splicing time course shows that the second step of the splicing reaction occurs so rapidly with *ftz* and AdML pre-mRNAs that the splicing intermediates (exon 1 and lariat-exon) are present at very low levels at all times during the time course (data not shown). Thus, it is possible that we are not detecting changes in the cross-linking pattern that occur at the time of lariat formation. We are currently establishing conditions that result in efficient accumulation of the lariat intermediate so that this question can be addressed.

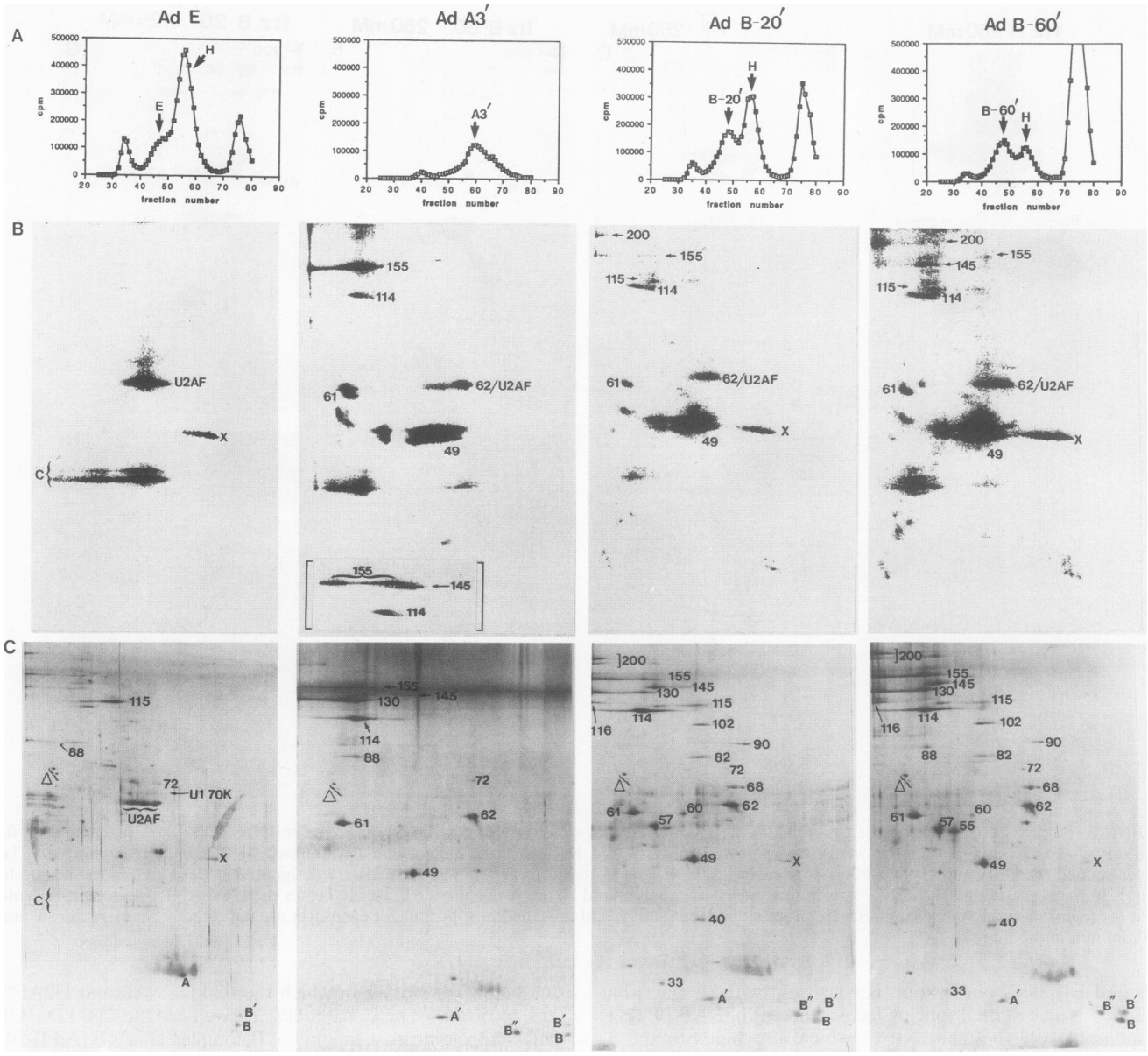

FIG. 4. Cross-linked proteins in prespliceosomes and spliceosomes purified in 250 mM salt. (A) AdML pre-mRNA was incubated in splicing extracts in the absence of ATP for 20 min (E) or in the presence of ATP for 20 min (B-20′) or 60 min (B-60′) and then fractionated by gel filtration. The A3′ complex was obtained by incubating Ad3′ RNA, which lacks the 5′ splice site, under splicing conditions for 20 min (A3′). The gel filtration profile of each column is shown, and the complexes are indicated. The unlabeled peaks to the left and right in each column profile correspond to the void volume and the degraded RNA, respectively. (B and C) Gel filtration-isolated complexes were affinity purified in 250 mM salt and cross-linked, and equivalent amounts of complexes (assembled on 200 ng of pre-mRNA) were fractionated by 2D gel electrophoresis. Panel B shows the PhosphorImager pattern of the silver-stained gel shown in panel C. Proteins are designated as in the legend to Fig. 1.

U2AF^{65} cross-links efficiently in the E but not in the B complex. The levels of U2AF^{65} detected by silver staining appear to be significantly lower in the A3′ and B complexes than in the E complex (e.g., Fig. 4C) (7). However, a prominent cross-linked band is present in both A3′ and B complexes which could correspond either to U2AF^{65} or to SAP 62, as these proteins fractionate similarly to one another (Fig. 1 to 4). We therefore used a narrower range of ampholytes for the isofocusing dimension and a lower-percentage gel for the second dimension to resolve these proteins. Comparison of the E and B complexes under these electrophoretic conditions revealed that the loss in the silver-stained levels of U2AF^{65} between the E and B complexes parallels the loss in cross-linking (Fig. 5). A similar loss in U2AF^{65} cross-linking was observed with both AdML and *ftz* pre-mRNAs (Fig. 5). These data indicate that SAP 62 cross-links in the spliceosome, whereas U2AF^{65} is displaced from the pre-mRNA in the spliceosome. The latter conclusion is further supported by Western analysis using U2AF^{65} antibodies (Fig. 5I; compare lanes E, A, and B). U2AF^{65} is abundant in the E complex relative to the A or B complex. The protein that is detected in B complex (arrow, Fig. 5I, lane B) was identified as SAP 68 by Western analysis of 2D gels (data not shown). SAP 68 is a breakdown product of SAP 102 which corresponds to the essential splicing factor PSF (43), and it may share an epitope with U2AF^{65}.

Correspondence between prespliceosomal SAPs and U2 snRNPs. Recently, a 17S form of U2 snRNP was isolated and found to contain nine proteins (160, 150, 120, 110, 92, 66, 60, 53, and 35 kDa) in addition to those previously identified as 12S U2 snRNP-specific proteins (A′ and B″ [5]). Several observations prompted us to examine whether these newly identified U2 snRNP proteins correspond to the SAPs that first bind in the A complex (SAPs 155, 145, 130, 114, 62, 61, 49, and 33 [7]). Like U2 snRNP, the prespliceosomal SAPs require ATP and the 3′, but not the 5′, splice site for binding to pre-mRNA. In addition, the prespliceosomal SAPs bind to pre-mRNA at same time as U2 snRNP and have molecular sizes similar to those reported for the 17S U2 snRNP proteins. To determine whether the prespliceosomal SAPs correspond to U2 snRNP proteins, we carried out an immunoprecipitation of U2 snRNP by using GA patient antiserum, which was previously shown to be specific for U2 snRNP and to detect U1 snRNP very weakly (14). Total nuclear extract (lacking pre-mRNA) fractionated on a gel filtration column was used as the source of U2 snRNP (see Materials and Methods). As shown in Fig. 6A (lane IP), U2 snRNA is immunoprecipitated by the GA patient antiserum from gel filtration fractions containing U2 snRNP. In addition, the U2 snRNP-specific A′ and B″ and the snRNP core proteins B and B′ are immunoprecipitated by this antibody (Fig. 6B, GA IP). These proteins are also detected in the A complex, as expected (Fig. 6B, A complex) (7). Note that the proteins in common between panels GA and GA IP are due to the antibody alone (Fig. 6B; see Materials and Methods). Proteins that cofractionate with SAPs 155, 145, 130, 114, 62, 61, and 49 are also present in the GA immunoprecipitate at levels similar to those of A′, B, B′, and B″ (Fig. 6B, GA IP). We conclude that these SAPs correspond to U2 snRNP proteins and hence that the six prespliceosomal SAPs that cross-link to the pre-mRNA in the A3′ and B complexes are U2 snRNP components.

DISCUSSION

Base pairing between U2 snRNA and the branchpoint sequence (BPS) plays an essential role in splicing (41, 62, 69). However, it appears that only six base pairs, and often even fewer, are involved in this interaction. Thus, a critical unanswered question is how this base-pairing interaction is first established and how it is maintained, given such a short base-paired region. We show here that six U2 snRNP proteins cross-link to the pre-mRNA in purified prespliceosomal complex A and in the spliceosomal complex B. On the basis of a number of considerations (see below), we propose that these U2 snRNP proteins play a role in mediating U2 snRNA–pre-mRNA interactions during spliceosome assembly. In addition to the U2 snRNP proteins, a 200-kDa U5 snRNP protein cross-links in the spliceosome and may function to facilitate 5′ splice site-U5 snRNA interactions, as proposed previously (63).

The prespliceosomal SAPs are U2 snRNP proteins. Previously, eight SAPs were shown to first bind in the A complex (7). Seven of these SAPs have now been identified as components of U2 snRNP (SAPs 155, 145, 130, 114, 62, 61, and 49), and all but SAP 130 cross-link to the pre-mRNA. All seven U2 snRNP-associated SAPs are likely to be the same as the 17S U2 snRNP-specific proteins of corresponding molecular sizes (5). Direct evidence for this in the case of SAPs 114, 62, and 61 is the observation that these three proteins constitute the essential heterotrimeric splicing factor SF3a (6, 12, 13), and all three subunits of SF3a correspond to 17S U2 snRNP components (12). Moreover, SAP 61, SF3a^{60}, and the 60-kDa component of the 17S U2 snRNP are all detected by antibodies to the yeast splicing factor PRP9 (3, 9, 12).

In addition to the relationship between PRP9 and the mammalian splicing factor, PRP11 and PRP21 appear to be the homologs of SAPs 62 and 114 (SF3a^{66} and SF3a^{120}), respectively (3, 9, 12, 30, 49). Consistent with our finding that SAP 62 crosslinks to pre-mRNA, both PRP11 and SAP 62 contain a conserved zinc finger motif (9). SF3a in mammalian cells and PRP9, PRP11, and PRP21 in yeast cells are required for U2 snRNP binding and are present in the A complex (1, 12, 30, 31, 49). Thus, the RNA-protein interactions that we have detected with SF3a and the other U2 snRNP components are likely to be functionally significant.

A model for the early steps of spliceosome assembly, based on our work together with earlier studies, is shown in Fig. 7. Previously, it was shown that the E complex contains U1 snRNP, U2AF^{65}, U2AF^{35}, and several SAPs (7, 9a). The 5′ and 3′ splice sites are functionally associated with one another at the time of E complex assembly, and this association is likely to be mediated, either directly or indirectly, by interactions between U2AF bound to the 3′ splice site and U1 snRNP bound to the 5′ splice site (35). We show here that U2AF^{65} cross-links to pre-mRNA in the E complex but is destabilized in subsequent complexes. Although further studies are needed to determine the precise timing, the loss of U2AF^{65} cross-linking may be concurrent with the loss of stable U1 snRNP and U2AF^{35} binding which also occurs between E and B complex assembly (9a, 35). These alterations in U1 snRNP and U2AF binding may be due to conformational changes that destabilize the interactions of these factors with the pre-mRNA. Alternatively, these factors may be released entirely from the complexes. In either case, such destabilizations may be a prerequisite for U2 snRNP binding to the branch site in the A complex and/or for subsequent U5 snRNP-5′ splice site interactions in the B complex (39, 57, 60, 63).

Concomitant with the destabilization of U1 snRNP and U2AF, we find that six U2 snRNP-specific SAPs, SAPs 155, 145, 114, 62, 61, and 49, can be specifically UV cross-linked to the pre-mRNA in A3′ complex. The 3′, but not the 5′, splice site is required for the association of these proteins with the A3′ complex (7). Previous studies identified a U2 snRNP-associated branchpoint protection factor that protects a 30- to 40-nt region of the intron spanning that BPS from RNase digestion (10, 11, 50). The pyrimidine tract and AG dinucleotide at the 3′ splice site are not part of this protected region but are required for its formation. U2AF and ATP are also required for the protection (50). As depicted in the model (Fig. 7), our data are consistent with the possibility that the six SAPs that cross-link in the A3′ and B complexes correspond to the previously identified branchpoint protection factor. This is supported by the observations that, as observed for the branchpoint protection factor, the cross-linking SAPs interact stably with the pre-mRNA, are associated with U2 snRNP, bind to the 3′ portion of the intron, and require ATP and the 3′, but not the 5′, splice site for binding. It could be argued that there are other components of the A complex that we have not detected that mediate this protection. However, we believe that this possibility is unlikely because the branchpoint protection factor binds stably to the pre-mRNA (50), and all of the stably bound components of the A complex have now been identified. The same RNase protection pattern is observed in both the A and B complexes (10). Consistent with the idea that this protection is due to the cross-linking SAPs, our data show that the cross-linking pattern of these proteins is the same in the A and B complexes. Further studies are needed to precisely map the sites of cross-linking of each of the U2 snRNP-associated SAPs.

The 5′ portion of U2 snRNA contains both the sequence that forms the essential base pairing interaction with U6 snRNA (so far detected only in *Saccharomyces cerevisiae*) and

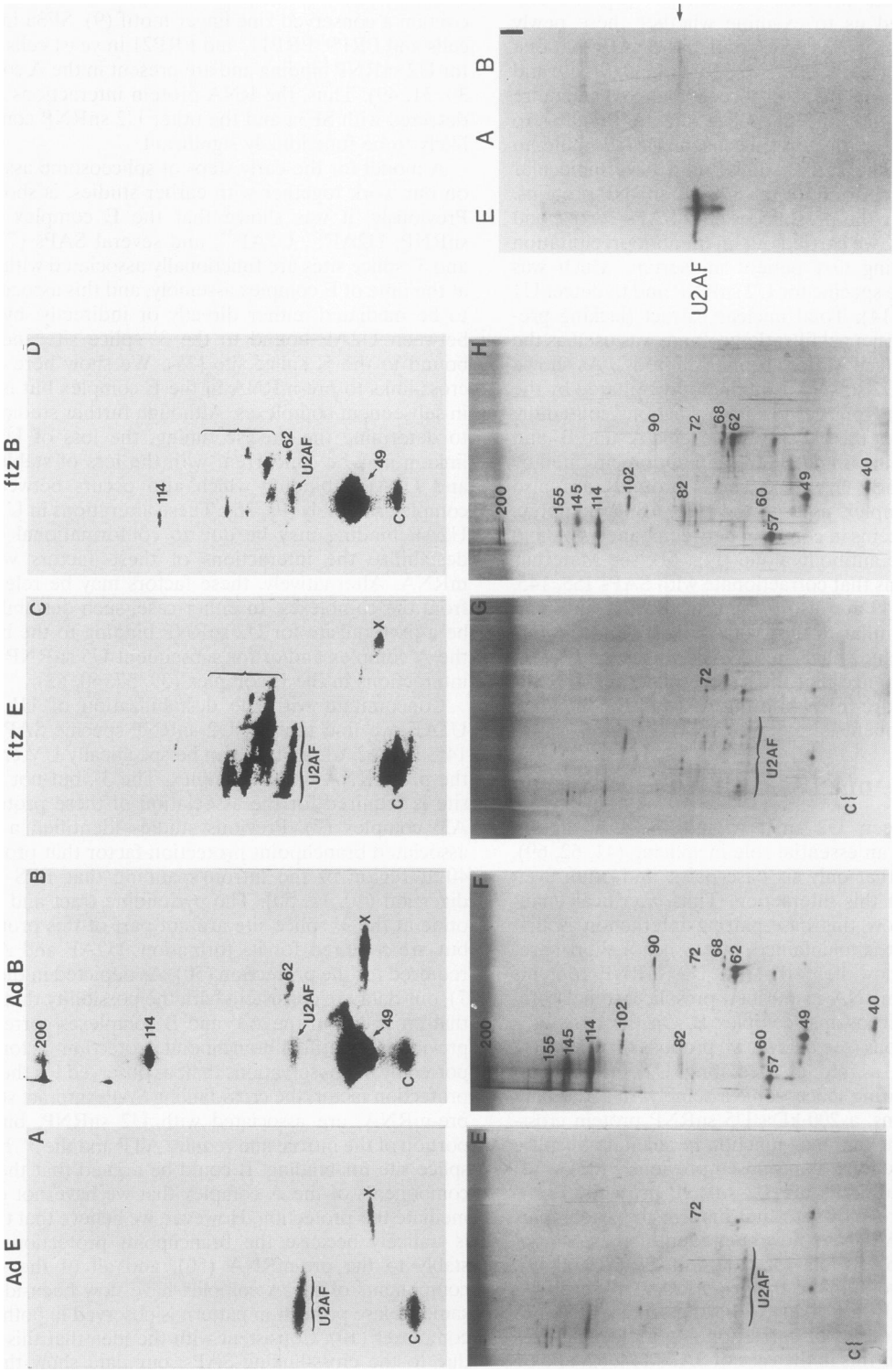

FIG. 5. U2AF^{65} cross-links to pre-mRNA in the E complex but is destabilized in the B complex. E and B complexes assembled on AdML (A, B, E, and F) or *ftz* (C, D, G, and H) pre-mRNAs were affinity purified in 250 mM salt and cross-linked. Complexes assembled on 200 ng of pre-mRNA were fractionated by 2D gel electrophoresis, using conditions modified to resolve U2AF^{65} and the 62-kDa SAP (see text). Panels A to D show the PhosphorImager patterns of the silver-stained gels shown in panels E to H. Proteins are designated as in the legend to Fig. 1. The braces in panels C and D indicate hnRNPs. (I) Western analysis of E, A, and B complexes, using antibodies to U2AF^{65} as a probe. U2AF^{65} is labeled, and the arrow indicates SAP 68 (see text).

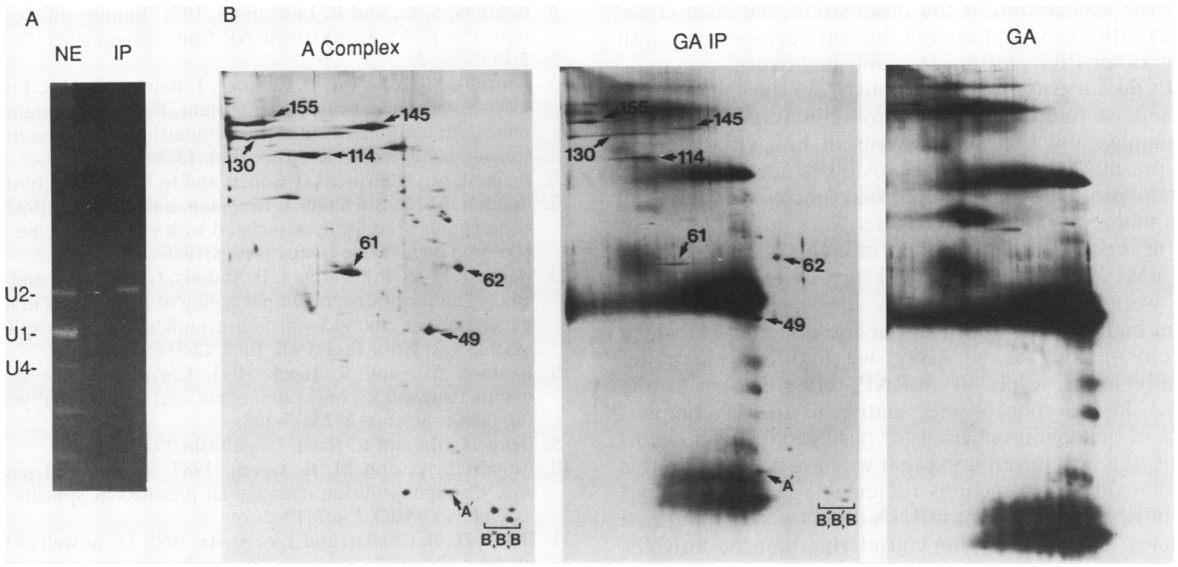

FIG. 6. Correspondence between prespliceosomal SAPs and U2 snRNP proteins. GA patient antiserum coupled to protein A-trisacryl was incubated with gel filtration fractions containing U2 snRNP (see Materials and Methods). (A) Total RNA was prepared from the immunoprecipitate, fractionated on an 8% denaturing polyacrylamide gel, and stained with ethidium bromide. NE, nuclear extract; IP, immunoprecipitate. The bands corresponding to U1, U2, and U4 snRNAs are indicated. (B) Protein was analyzed by 2D gel electrophoresis. A complex, affinity-purified A complex; GA IP, immunoprecipitation of gel filtration-isolated U2 snRNP with GA antiserum; GA, GA antiserum alone bound to the beads. The prespliceosomal SAPs which correspond to the U2 snRNP proteins are indicated. The 17S U2 snRNP contains two additional proteins of 92 and 35 kDa. We did not identify these proteins in our preparation of the U2 snRNP.

the sequence that base pairs with the branch site; these two regions of U2 snRNA are within a few nucleotides of one another (32). It has been proposed that U6 snRNA base pairs with the 5′ splice site concomitant with the U2-U6 and U2-BPS interactions; this results in the close juxtapositioning of the 5′ splice site, the branch site, and the putative catalytic domain of U6 snRNA (32, 52, 53, 60). The six cross-linking SAPs are thought to interact with the 5′ portion of U2 snRNA (5). This would position these SAPs at the catalytic center of the spliceosome where all of the essential RNA-RNA interactions are occurring. Thus, it is possible that these factors play critical roles in mediating snRNA-snRNA and snRNA–pre-mRNA interactions. U5 snRNA also appears to be positioned at the catalytic center of the spliceosome, interacting with exon sequences immediately adjacent to both the 5′ and 3′ splice sites (38, 39, 60, 63). Our data show that the 200-kDa U5 snRNP protein cross-links to the pre-mRNA in the B complex, and site-specific cross-linking studies showed that this protein contacts the exon sequences next to the 5′ splice site in the B complex (63). Thus, this protein may serve a function for U5 snRNA that the U2 snRNP-associated SAPs serve for U2 snRNA (63).

The notion that the 200-kDa U5 snRNP protein and the U2 snRNP-specific SAPs, all of which directly contact the pre-mRNA, play such key roles in the splicing reaction is consistent with the observation that these proteins, and their functions, appear to have been highly conserved between *S. cerevisiae* and humans. For three of them, the U5 protein (PRP8) and two of the U2 proteins (PRP9 and PRP11), strong cross-reactivity between yeast antibodies and the corresponding mammalian protein has been detected, and similarity at the amino acid level was identified for PRP11 and SAP 62 (2, 3, 9, 12, 17, 45). A possible clue that these antibody cross-reactivities have revealed the key factors involved in forming the catalytic

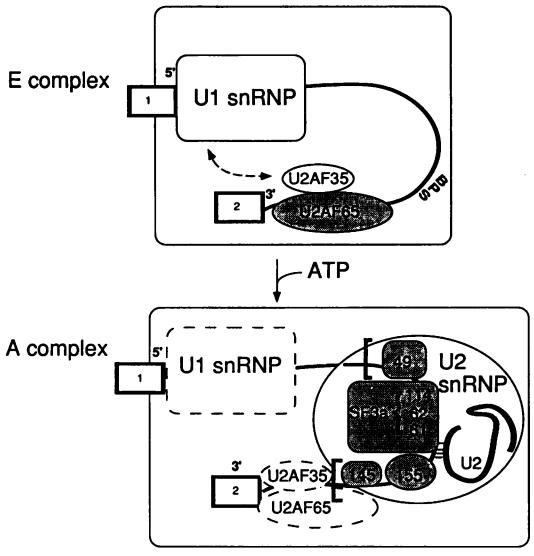

FIG. 7. Model for early steps in spliceosome assembly. The 5′ and 3′ splice sites and BPS are indicated. The functional interaction between the 5′ and 3′ splice sites in the E complex is indicated by the dashed arrow. The proteins that can be UV cross-linked to pre-mRNA are shaded, and components that either dissociate or become less tightly bound in the A complex are indicated by dashed lines. The proteins indicated only by molecular weight (in thousands) in the A complex are SAPs, and SAPs 114, 62, and 61 comprise the splicing factor SF3a. For simplicity, only a subset of the proteins present in each complex is shown. U2 snRNA is shown base paired to the BPS in the A complex. The braces on the pre-mRNA in the A complex designate the 30- to 40-nt RNase-protected region (see text).

center of the spliceosome is the observation that such cross-reactivities with the SAPs have not thus far been detected with any of the other PRP antisera (unpublished data).

hnRNPs do not cross-link to pre-mRNA in the spliceosome. As expected, we find that hnRNPs cross-link to pre-mRNA in the H complex and that the patterns of hnRNPs differ on different pre-mRNAs (7, 8). In a previous study, in which we analyzed the proteins present in purified splicing complexes by silver staining, we found that there were lower levels of hnRNPs in the H complex purified in high (250 mM) versus low (100 mM) salt. Thus, we were not surprised to find that the hnRNPs were also present at low levels in spliceosomes purified in high salt. However, in our present study, in which we use cross-linking as an assay, we find that there are dramatically lower levels of hnRNPs cross-linking to the pre-mRNA in the spliceosome relative to the H complex, regardless of the conditions used for purification (high or low salt). Thus, the new information that we have obtained in this study is that direct interactions (detectable by cross-linking) between hnRNPs and the pre-mRNA do not occur in purified spliceosomes. This is surprising considering that the hnRNPs avidly bind to RNA and are among the most abundant proteins in the nuclear extract. Moreover, the AdML and *ftz* pre-mRNAs used for this study are 271 and 473 nt long, respectively. Thus, although it could be argued that the hnRNPs are excluded from the pre-mRNA in the AdML spliceosome simply because of the presence of spliceosome components, the *ftz* pre-mRNA would be expected to have significant extra sequences available for hnRNP binding. It will be interesting to determine whether hnRNPs are also lacking from spliceosomes assembled on very long pre-mRNAs.

The relationship between H complex assembly and spliceosome assembly is not known. H complex assembles immediately when RNA is added to the nuclear extract, and analysis of the H complex by gel filtration suggests that the RNA is quantitatively packaged into this complex (34). If this is true, then the H complex is a temporal precursor to the spliceosome. However, it is possible that the H complex is not homogeneous and that hnRNPs are specifically excluded from the population of the H complex that is a substrate for spliceosome assembly. Alternatively, the hnRNPs may be bound to pre-mRNA initially and then stripped off during spliceosome assembly. In either case, these observations suggest that any role for direct pre-mRNA–hnRNP interactions is limited to the very early steps in spliceosome assembly.

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