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# 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

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**We have isolated and determined the protein composition of the spliceosomal complex C. The pre-mRNA in this complex has undergone catalytic step I, but not step II, of the splicing reaction. We show that a novel set of 14 spliceosome-associated proteins (SAPs) and the essential splicing factor PSF are specifically associated with the C complex, implicating these proteins in catalytic step II. Significantly, immunodepletion and biochemical complementation studies demonstrate directly that PSF is essential for catalytic step II. Purified PSF is known to UV crosslink to pyrimidine tracts, and our data show that PSF UV crosslinks to pre-mRNA in purified C complex. Thus, PSF may replace the 3′ splice site binding factor U2AF$^{65}$ which is destabilized during spliceosome assembly. Finally, we show that SAPs 60 and 90, which are present in both the B and C complexes, are specifically associated with U4 and U6 snRNPs, and thus may have important roles in the functioning of these snRNPs during the splicing reaction.**

*Key words:* spliceosomal complexes/splicing reaction

## Introduction

Pre-mRNA splicing takes place in a multi-component complex designated the spliceosome. Spliceosomes assemble on pre-mRNA in a stepwise manner, and four discrete complexes, which assemble in the order E → A → B → C, are functional intermediates in the pathway (see for reviews Rymond and Rosbash, 1992; Lamm and Lamond, 1993; Moore *et al.*, 1993). The E and A complexes are designated prespliceosomes, and the B and C complexes are referred to as spliceosomes. The E, A and B complexes contain unspliced pre-mRNA, whereas the C complex contains the products of catalytic step I of the splicing reaction (exon 1 and lariat-exon 2). The C complex is a short-lived intermediate due to the rapid conversion of the splicing intermediates into the spliced products (the ligated exons and lariat intron). The spliced mRNA is in turn rapidly released from the C complex, such that no discrete spliceosomal complex has been identified that contains both the ligated exons and the excised lariat intron. The spliced mRNA alone is detected in a complex that migrates much faster than any of the spliceosomal complexes on a native gel, and most likely contains non-snRNP RNA binding proteins, whereas the snRNPs remain associated with the lariat intron in a larger complex (Konarska and Sharp, 1987; Lamond *et al.*, 1987). Conversion of the B to the C complex, as well as conversion of the splicing intermediates into the spliced products, requires ATP and incubation at 30°C (Cheng and Abelson, 1987; Lin *et al.*, 1987; Abmayr *et al.*, 1988; Reed *et al.*, 1988).

Affinity-purified E complex contains U2AF$^{35}$, U2AF$^{65}$, U1 snRNP components, and several spliceosome-associated proteins (SAPs) (Bennett *et al.*, 1992; D.Staknis, M.Bennett and R.Reed, unpublished observations). The 5′ and 3′ splice sites are functionally associated with one another in the E complex, and this interaction may be mediated by U1 snRNP bound to the 5′ splice site and U2AF bound to the 3′ splice site with SR proteins bridging these factors (Michaud and Reed, 1993; Wu and Maniatis, 1993). Although affinity-purified E complex lacks SR proteins (Bennett *et al.*, 1992), these proteins are bound to pre-mRNA in the functional gel filtration-purified E and B complexes (D.Staknis and R.Reed, unpublished observations). U1 snRNP, U2AF$^{35}$ and U2AF$^{65}$ appear to be destabilized at some point during the E to B complex transition, possibly as early as A complex assembly (Michaud and Reed, 1991, 1993; Staknis and Reed, 1994). Ten new proteins, most of which are U2 snRNP components, then become stably bound to the pre-mRNA in the A complex (Bennett *et al.*, 1992; Staknis and Reed, 1994). A subset of the U2 snRNP components UV crosslink to pre-mRNA in affinity-purified A complex and may mediate interactions between U2 snRNP and the pre-mRNA (Staknis and Reed, 1994).

The B complex contains U2, U4, U5 and U6 snRNAs (Grabowski and Sharp, 1986; Konarska and Sharp, 1986, 1987; Pikeilny *et al.*, 1986; Cheng and Abelson, 1987). In addition, this complex contains all of the proteins present in the A complex plus 12 additional proteins, five of which are 20S U5 snRNP components (Bennett *et al.*, 1992). One of the U5 snRNP proteins (200 kDa) UV crosslinks to exon 1 adjacent to the 5′ splice junction and may mediate U5 snRNA–pre-mRNA interactions (Wyatt *et al.*, 1992). Prior to catalytic step I of the splicing reaction, U4 snRNA dissociates from the spliceosome (Pikeilny *et al.*, 1986; Cheng and Abelson, 1987; Lamond *et al.*, 1988) and is not required for the catalytic steps (Yean and Lin, 1991). In contrast, U6 snRNA, which forms an essential base-pairing interaction with the 5′ splice site, is thought to be involved directly in the catalytic steps of the reaction (Sawa and Abelson, 1992; Sawa and Shimura, 1992; Wassarman and Steitz, 1992; Kandels-Lewis and Seraphin, 1993; Lesser and Guthrie, 1993; Sontheimer and Steitz, 1993). Although proteins associated with U1, U2 and U5 snRNAs have been



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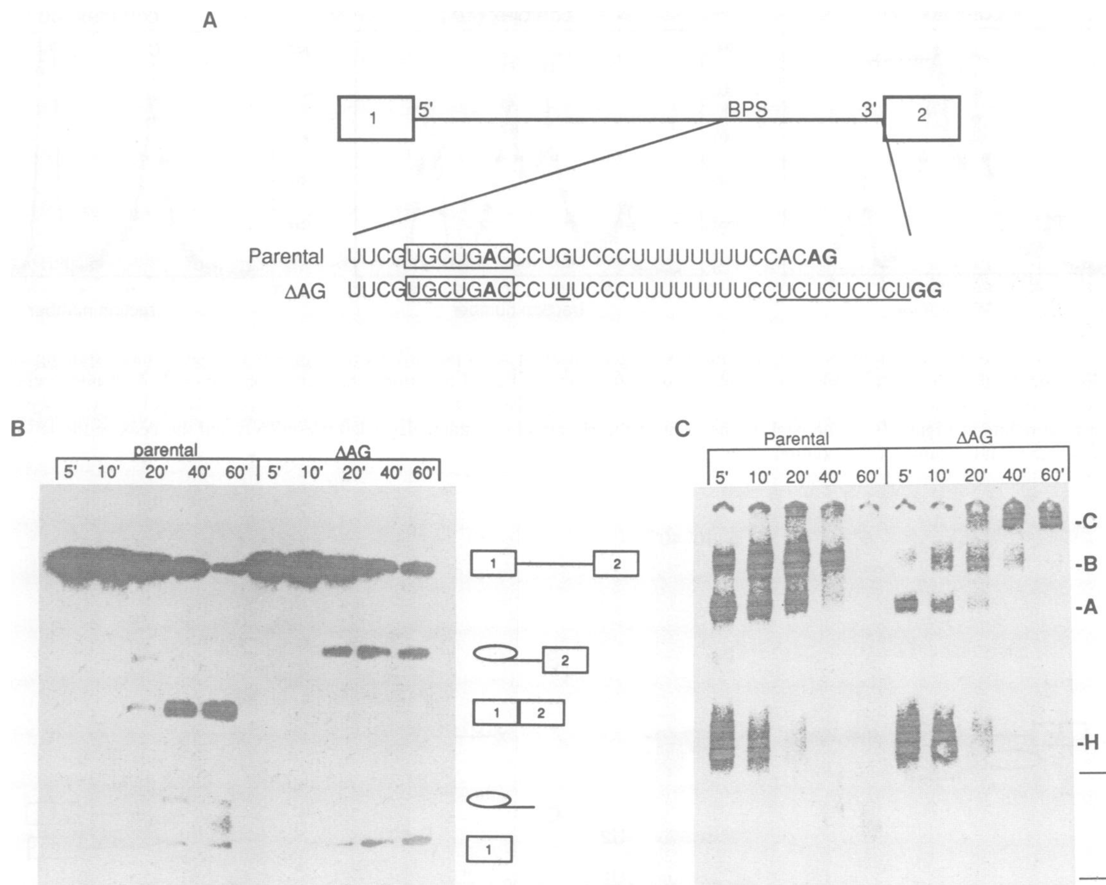


**Fig. 1.** Accumulation of C complex on ΔAG pre-mRNA. (**A**) Structure of pre-mRNAs. The exons and intron are represented by the boxes and line, respectively. The 5′ and 3′ splice sites and branchpoint sequence (BPS) are indicated. The nucleotide sequence of the 3′ portion of the intron is shown. The BPS is boxed (branch-site A in bold) and the additional pyrimidine residues present in the ΔAG pre-mRNA are underlined. The AG dinucleotide in the parental pre-mRNA was changed to GG in ΔAG pre-mRNA (indicated in bold). (**B**) Splicing time course with parental or ΔAG pre-mRNAs. Parental or ΔAG pre-mRNAs (20 ng) were incubated under splicing conditions (25 μl reaction) for the times indicated, and total RNA was fractionated on an 8% denaturing polyacrylamide gel. The bands corresponding to intermediates and spliced products are indicated. (**C**) Time course of complex assembly on parental or ΔAG pre-mRNAs. Parental or ΔAG pre-mRNAs (20 ng) were incubated under splicing conditions (25 μl reaction) for the times indicated and then complexes were fractionated on a 4% non-denaturing polyacrylamide gel. The bands corresponding to H, A, B and C complexes are indicated. The bracket indicates a band that was observed only with the parental pre-mRNA at 40 and 60 min. This band most likely contains spliced mRNA.

identified in purified spliceosomal complexes (Bennett *et al.*, 1992; Staknis and Reed, 1994), no spliceosomal proteins have been identified that are specifically associated with U4 or U6 snRNPs.

Under normal splicing conditions, the C complex is detected at low levels as a discrete band that migrates more slowly than the A and B complexes on native gels (Lamond *et al.*, 1987). To characterize the C complex, it has been necessary to find conditions that result in its accumulation. Catalytic step II of the splicing reaction can be blocked by mild heat treatment (Krainer and Maniatis, 1985) and the exon 1 and lariat-exon 2 that accumulate in this C complex can be chased into spliced products (Reed *et al.*, 1988). Heat treatment of nuclear extracts, however, has not proven to be a reliable method for accumulating the C complex due to variability between extracts. Catalytic step II can also be blocked by assembling complexes on a pre-mRNA that lacks the AG dinucleotide at the 3′ splice site, but contains a long pyrimidine tract (Reed, 1989, 1990; Smith *et al.*, 1989). The complex assembled on such pre-mRNAs co-fractionates with spliceosomes on density gradients (Smith *et al.*, 1989) and contains U2, U5 and U6 snRNAs and a large number of unidentified proteins (Reed, 1990).

In this study we have carried out a detailed characterization of affinity-purified C complex assembled on a pre-mRNA lacking the AG dinucleotide. We show that this complex contains all of the proteins present in the B complex, as well as an additional 14 novel SAPs. The essential splicing factor PSF is abundant in and UV crosslinks to pre-mRNA in the C complex. Moreover, biochemical complementation of PSF-depleted extracts shows that PSF is essential for catalytic step II of the splicing reaction. Finally, we have identified SAPs 60 and 90 as the first U4/U6 snRNP-specific spliceosomal proteins.

## Results

To accumulate the C complex, we employed a derivative of AdML pre-mRNA designated ΔAG pre-mRNA, that has a long, uninterrupted pyrimidine tract and a GG

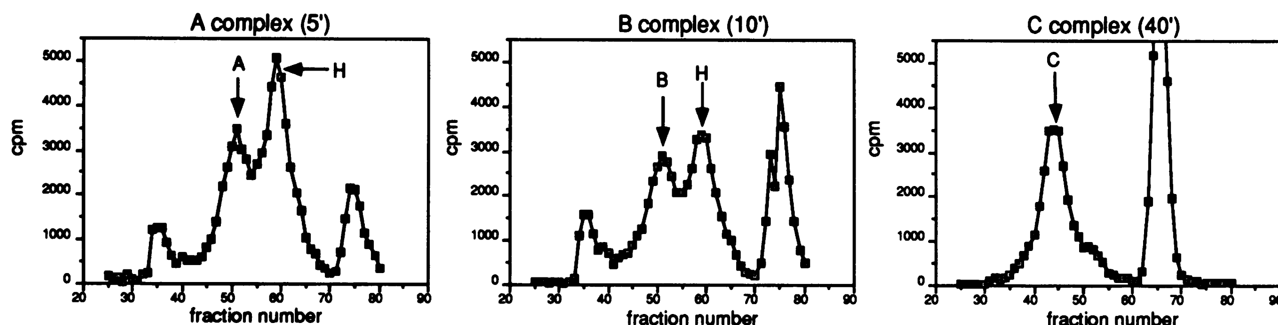


**Fig. 2.** Analysis of spliceosome assembly on ΔAG pre-mRNA by gel filtration. ΔAG pre-mRNA (80 ng) was incubated under splicing conditions (100 μl reaction) for the times indicated and then complexes were fractionated by gel filtration. The peaks containing H, A, B and C complexes are indicated; note that the A, B and C complexes are contaminated with one another to the extent indicated by the native gel analysis (see corresponding time points in Figure 1C). The peak to the right of the H complex contains degraded pre-mRNA and the peak to the left of the A, B or C complexes is the void volume of the column.

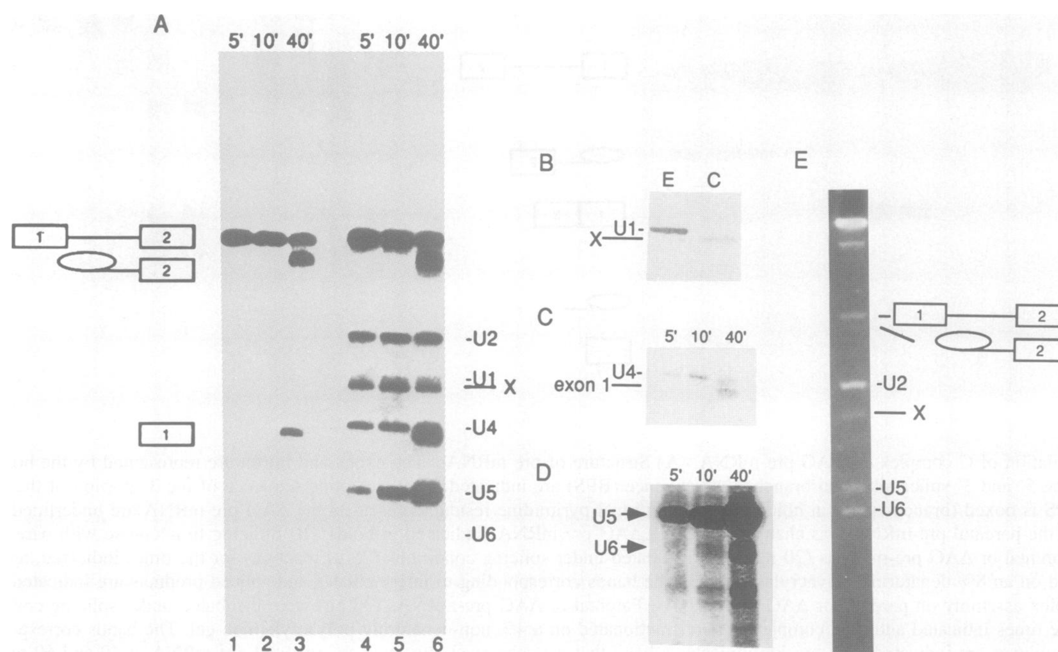


**Fig. 3.** The snRNA compositions of spliceosomal complexes assembled on ΔAG pre-mRNA. (**A**) ΔAG pre-mRNA (1.92 μg) containing biotin was incubated under splicing conditions (2.4 ml reaction) for the times indicated, complexes were fractionated by gel filtration and then affinity-purified in 250 mM salt by binding to avidin agarose (see Materials and methods). Total RNA was prepared, 3′ end-labeled with [$^{32}$P]pCp and then fractionated on an 8% denaturing polyacrylamide gel (lanes 4–6). Total RNA prior to end-labeling is shown in lanes 1–3. The bands corresponding to the snRNAs, pre-mRNA and intermediates are indicated. X designates a band that fractionates below U1 snRNA that is not seen in all nuclear extracts (Michaud and Reed, 1991, 1993). (**B**) Total RNA from the E and C complexes were 3′ end-labeled with [$^{32}$P]pCp and then fractionated on a 6% denaturing polyacrylamide gel. The portion of the gel containing U1 snRNA and the band X is shown. (**C**) Samples from (A), lanes 4–6, were fractionated on a 6% denaturing polyacrylamide gel. The portion of the gel containing exon 1 and U4 snRNA is shown. (**D**) A long exposure of the lower portion of (A), lanes 4–6. The band below U6 snRNA may correspond to a band detected previously in affinity-purified spliceosomes (Grabowski and Sharp, 1986; Lamond *et al.*, 1988). (**E**) Total RNA was prepared from 80 ng ΔAG pre-mRNA assembled into the C complex and fractionated on an 8% denaturing polyacrylamide gel. Bands were visualized by ethidium bromide staining.

substitution of the AG dinucleotide at the 3′ terminus of the intron (compare ΔAG and parental pre-mRNAs, Figure 1A). When ΔAG pre-mRNA is incubated in splicing extracts, the splicing intermediates account for ~50% of the total pre-mRNA detected at 40 and 60 min (Figure 1B, ΔAG, 40′, 60′). With the parental pre-mRNA, >50% conversion to the spliced products is detected at 60 min, and only low levels of the splicing intermediates are observed at the earlier time points (Figure 1B, parental). Analysis of the reactions shown in Figure 1B by non-denaturing gel electrophoresis shows that the A and B complexes assemble on the ΔAG pre-mRNA, though assembly is reproducibly less efficient than observed with the parental pre-mRNA (Figure 1C). In contrast to the parental pre-mRNA, the C complex accumulates on the ΔAG pre-mRNA and is the major complex detected at 40 and 60 min (Figure 1C, compare parental with ΔAG). We conclude that the C complex, containing the products of catalytic step I (see Figure 1B, ΔAG, 40′, 60′), accumulates on ΔAG pre-mRNA. In the case of the parental pre-mRNA, spliceosomal complexes are no longer detected at 60 min (Figure 1C, parental), but a complex that fractionates faster than the H complex (designated by the bracket) is detected and most likely contains spliced

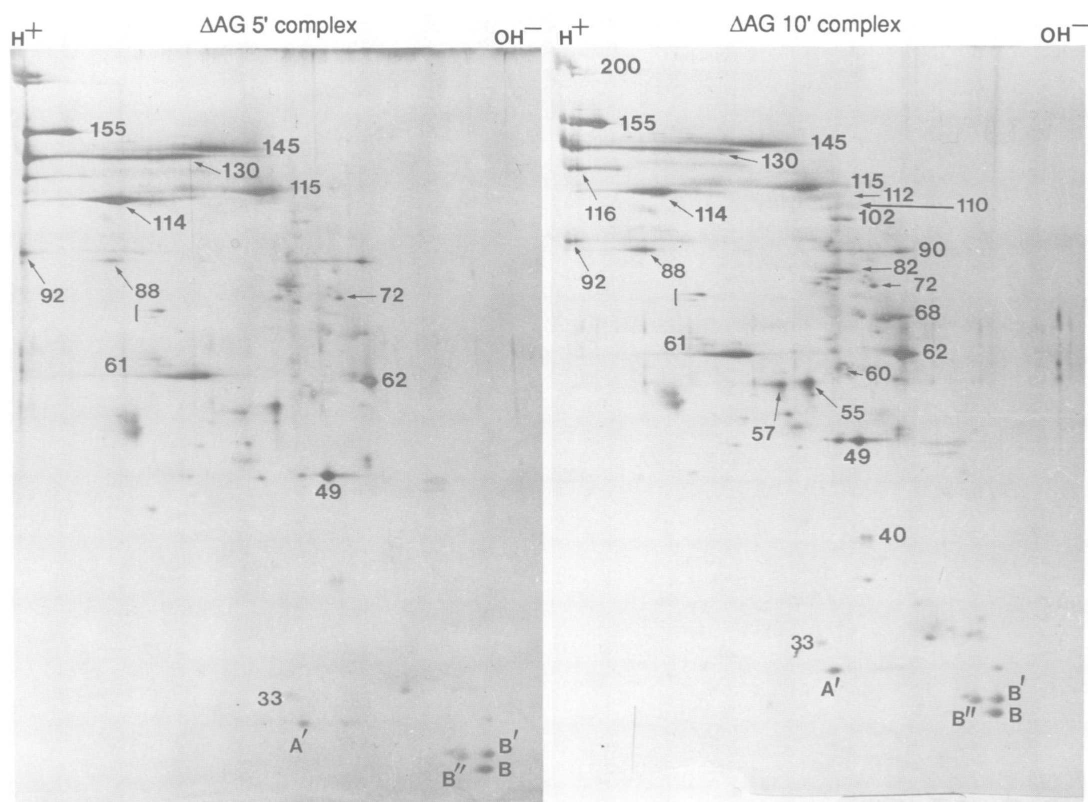


**Fig. 4.** Protein composition of 5 and 10 min spliceosomal complexes assembled on ΔAG pre-mRNA. Total protein was prepared from 150 ng ΔAG pre-mRNA present in affinity-purified 5 or 10 min spliceosomal complexes and fractionated by 2-D gel electrophoresis. Proteins were detected by silver staining. The proteins in the 5 and 10 min complexes are labeled according to the nomenclature in Bennett *et al.* (1992). The A complex proteins are labeled in both the 5 and 10 min complexes whereas the B complex-specific proteins are labeled only in the 10 min complex. The bracket indicates the heat-shock proteins (Bennett *et al.*, 1992).

mRNA released from the spliceosome (Konarska and Sharp, 1987; Lamond *et al.*, 1987).

The 40 min time point was used to analyze the composition of the C complex (Figure 1C, ΔAG, 40′). To ensure that the AG mutation did not result in assembly of aberrant complexes, we also analyzed the compositions of complexes assembled for 5 and 10 min on this pre-mRNA (see Figure 1C, ΔAG, 5′, 10′). The A and B, but not the C, complexes are detected at these times. For purification, complexes were assembled on biotinylated pre-mRNA, fractionated by gel filtration and then affinity-purified in 250 mM salt (Bennett *et al.*, 1992). We note that we used the same conditions (i.e. the same relative amounts of nuclear extract and pre-mRNA) to generate the complexes analyzed by gel filtration as were used to analyze complexes on the native gels. As expected from previous work (Reed *et al.*, 1988; Michaud and Reed, 1991), the complexes at all three time points co-fractionate by gel filtration (Figure 2; due to the inherent variability between different gel filtration columns, only the relative elution positions of each peak can be compared). Thus, the spliceosomal complexes are contaminated with one another to the extents indicated by the non-denaturing gel analysis (see Figure 1C, ΔAG). For example, both the A and B complexes are present at 10 min (Figure 1C, ΔAG, 10′), but only a single gel filtration peak containing these complexes is detected (Figure 2, B complex, 10′). Importantly for this study, the C complex detected at 40 min is contaminated at only a low level with the B complex (Figure 1C, ΔAG).

The snRNA compositions of the 5 and 10 min complexes assembled on ΔAG pre-mRNA (Figure 3A, lanes 4 and 5) are similar to those observed in the corresponding complexes assembled on normal pre-mRNAs (data not shown; Michaud and Reed, 1993). These compositions are consistent with the levels of A and B complex observed at these two times in Figure 1C (ΔAG). As expected, the C complex (40 min) contains the same level of U2 snRNA as observed in the 5 and 10 min complexes (Figure 3A, lanes 4–6). The levels of U5 and U6 snRNAs are higher in the C complex relative to the 5 and 10 min complexes (Figure 3A, lanes 4–6; see Figure 3D for a longer exposure of the portion of the gel containing U6 snRNA). The lower levels of U5 and U6 snRNAs in the B (10 min) complex (Figure 3A and D) are due, at least in part, to contamination with the A complex (see Figure 1C, ΔAG). Analysis of the snRNAs in the C complex by ethidium bromide staining shows that U2, U5 and U6 snRNAs are about equimolar in this complex (Figure 3E).

U1 snRNA is barely detected in the 5, 10 and 40 min complexes (Figure 3A, lanes 4–6; the band below U1 snRNA, designated X, is a background band as shown in Figure 3B; Michaud and Reed, 1993). This is consistent with previous work showing that U1 snRNP is tightly bound in the ATP-independent E complex and then becomes destabilized during the E to B complex transition (see Figure 3B; Michaud and Reed, 1991, 1993). U4 snRNA is not readily detectable in the C complex by ethidium bromide staining (Figure 3E; see also Figure 3C, which resolves exon 1 from U4 snRNA in the C complex).

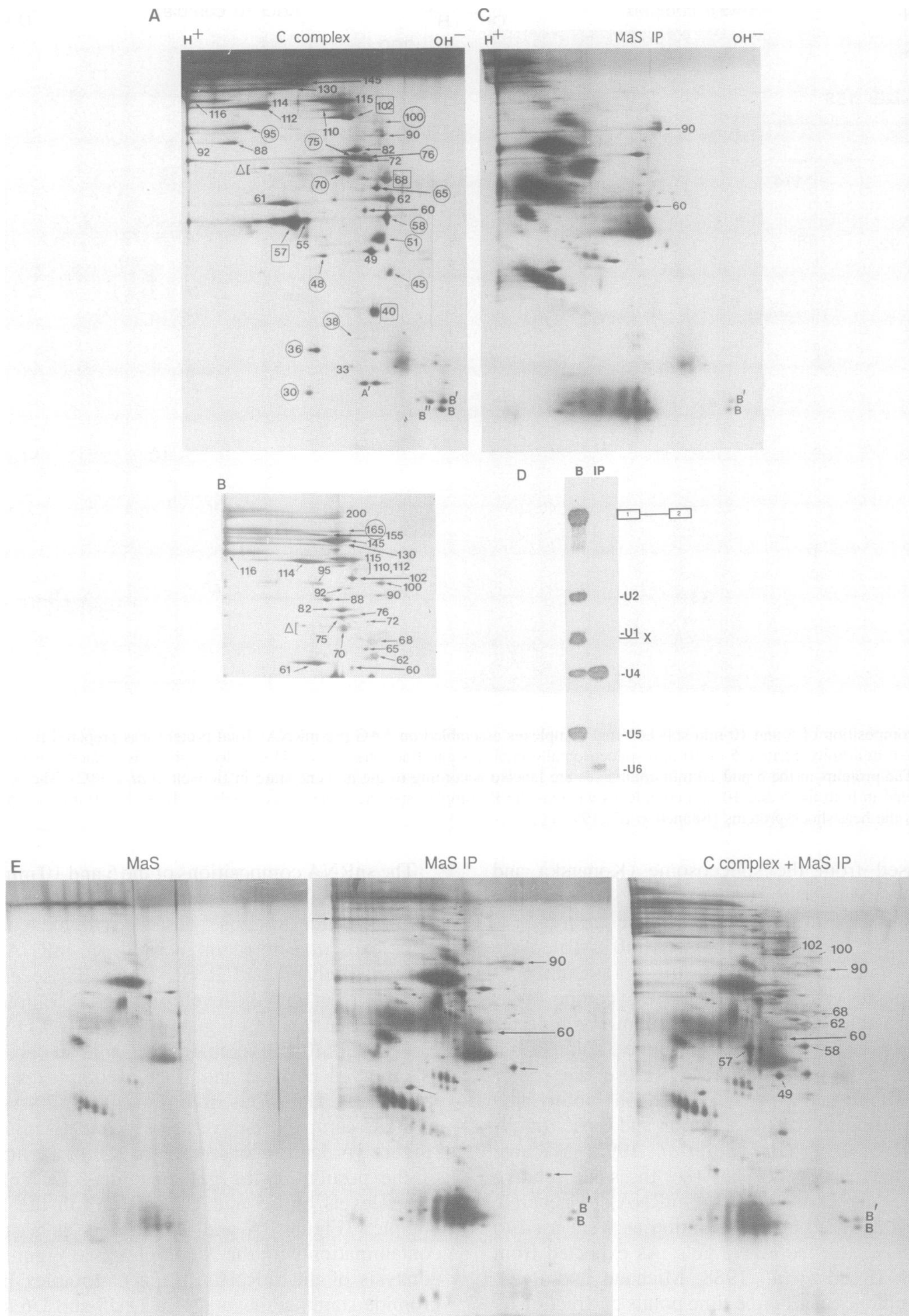


**Fig. 5.** Protein composition of the C complex and identification of U4/U6 snRNP components using MaS antiserum. (**A**) Affinity-purified C complex assembled on 200 ng ΔAG pre-mRNA was fractionated by 2-D gel electrophoresis, and proteins were detected by silver staining. The 14 novel SAPs in the C complex are indicated by circles [see (B) for SAP 165], while the four proteins highly enriched in the C versus the B complex are boxed. The heat-shock proteins are indicated (Δ). (**B**) A portion of a 2-D gel of C complex which shows SAPs 165 and 100 more clearly. The heat-shock proteins are indicated (Δ). (**C**) Proteins immunoprecipitated from gel filtration-isolated U4/U6 snRNPs using MaS patient antiserum were fractionated by 2-D gel electrophoresis and proteins were detected by silver staining. The proteins specifically immunoprecipitated by this antibody and that specifically comigrate with C complex proteins are indicated (SAPs 60, 90 and B and B′). (**D**) SnRNAs immunoprecipitated from gel filtration-isolated U4/U6 snRNPs using MaS patient antiserum. B, B complex. IP, MaS immunoprecipitation. The positions of the snRNAs and pre-mRNA are indicated. (**E**) MaS antiserum alone (MaS), immunoprecipitation of gel filtration fractions using MaS antiserum (MaS IP) or affinity-purified C complex mixed with the MaS IP (C complex + MaS IP) were fractionated on 2-D gels. Only B and B′ and the proteins in the vicinity of SAPs 60 and 90 are labeled. The arrows in the MaS IP panel indicate proteins that are present in the MaS immunoprecipitation and not in the MaS antiserum alone. The acidic and basic ends of the gels are as indicated in (A).

These data are consistent with the observation that U4 snRNP dissociates at some point prior to catalytic step I (Pikeilny *et al.*, 1986; Cheng and Abelson, 1987; Lamond *et al.*, 1988) and is no longer required for the splicing reaction (Yean and Lin, 1991). Note that the levels of U4 snRNA may be low in the 10 min complex due to its contamination with the A complex (see Figure 3A, lanes 4–6 and C).

The protein composition of the 5 and 10 min complexes assembled on ΔAG pre-mRNA (Figure 4) is similar to that observed previously in the B complex assembled on parental pre-mRNA (Bennett *et al.*, 1992). As predicted from the native gel analysis (Figure 1C, ΔAG, 5′, 10′), there are higher levels of the B complex-specific proteins at 10 min (B complex proteins are labeled only in the 10 min complex, Figure 4). No proteins other than those detected in the B complex assembled on normal pre-mRNAs are present in the 5 or 10 min complexes assembled on the ΔAG pre-mRNA. In addition, these 5 and 10 min complexes do not appear to be lacking any of the proteins found in the corresponding complexes assembled on normal pre-mRNAs. These data, together with the analysis of the snRNAs, indicate that the initial stages of spliceosome assembly occur normally on the ΔAG pre-mRNA, despite the presence of the AG mutation.

### Identification of C complex-specific SAPs

The protein composition of the C complex assembled on ΔAG pre-mRNA is shown in Figure 5A. Comparison of the 10 min B complex (Figure 4) with the C complex revealed the presence of 14 new proteins in the C complex (indicated by circles, Figure 5A and B). These are designated SAPs 165, 100, 95, 76, 75, 70, 65, 58, 51, 48, 45, 38, 36 and 30. SAPs 165 and 100 are seen more clearly in Figure 5B. In addition to the novel proteins that bind in the C complex, the 40 kDa U5 snRNP protein and SAPs 102, 68 and 57 (indicated by boxes) are present at much higher relative levels in the C than in the B complex (e.g. compare the levels of SAPs 40 and 49, Figures 4 and 5A). To be consistent with our previous nomenclature, in which spliceosomal proteins are designated according to the complex in which they are most abundant, SAPs 102, 68 and 57 and the 40 kDa U5 snRNP protein, as well as the 14 novel SAPs, are designated C complex-specific proteins. None of the these proteins were detected when the affinity-purification procedure was carried out using the ΔAG pre-mRNA lacking biotin (data not shown). In addition, analysis of the C complex on higher percentage gels (12 and 15%) did not reveal any additional C complex-specific proteins (data not shown). Finally, the C complex-specific proteins were detected reproducibly in >20 independent preparations of the C complex and in several different preparations of nuclear extracts. However, as pointed out with SAPs 165 and 100 above, we find that resolution of some of the proteins varies on different 2-D gels.

### SAPs 60 and 90 are U4/U6 snRNP proteins

U1, U2 and U5, but not U4/U6, snRNP-specific proteins (Bach *et al.*, 1989; Okano and Medsger, 1991; Behrens *et al.*, 1993a; see Luhrmann *et al.*, 1990 for a review) have been detected in purified spliceosomal complexes (Bennett *et al.*, 1992; Staknis and Reed, 1994). To determine whether any of the SAPs in purified B or C complexes correspond to U4/U6 snRNP proteins, we used the U4/U6 snRNP-specific MaS antiserum (Okano and Medsger, 1991) to immunoprecipitate this snRNP from gel filtration-fractionated nuclear extract (see Materials and methods). As expected, U4 and U6 snRNAs are immunoprecipitated by this antisera (Figure 5D; Okano and Medsger, 1991; Blencowe *et al.*, 1993). The immunoprecipitated proteins are shown in Figure 5C. [Most of the unlabeled proteins in the MaS immunoprecipitate are due to the antibody (see below).] Comparison of the C complex with the MaS immunoprecipitate indicated that SAPs 60 and 90 and the snRNP core proteins B and B′ are in common. To determine whether these proteins precisely comigrate we carried out a mixing experiment. Figure 5E shows a comparison of the background bands that elute from the resin containing the MaS antiserum alone (MaS), the proteins present in the MaS immunoprecipitation of the U4/U6 snRNP-containing gel filtration fractions (MaS IP) and a mixture of the MaS IP sample and the C complex (MaS IP + C complex). These data reveal that SAPs 60 and 90 comigrate as no extra bands in the 60 and 90 kDa region are detected. We conclude that SAPs 60 and 90 are associated with U4/U6 snRNAs.

We were unable to identify conclusively additional proteins in common between the C complex and the MaS immunoprecipitate due to the complexity of the pattern. The high background also obscures the region of the gel containing the 150 kDa MaS antigen making its identification difficult. Using the MaS antibody to probe a Western blot of spliceosomes, we were unable to detect the 150 kDa MaS antigen or any other proteins (M.Bennett and R.Reed, unpublished observation). The failure to detect SAPs 60 and 90 with the MaS antiserum, which is polyclonal, indicates that these SAPs cannot be breakdown products of the 150 kDa MaS antigen. In previous work, the MaS antisera were used to immunoprecipitate [$^{35}$S]-methionine-labeled proteins from HeLa whole cell extracts. This study identified proteins of 150 (the MaS antigen), 120, 80, 36 and 34 kDa (Okano and Medsger, 1991). Comparison of the 2-D gels containing the MaS IP (Figure 5E, MaS IP) and the MaS antiserum alone (Figure 5E, MaS) reveals several proteins that are specifically immunoprecipitated by the MaS antiserum (indicated by arrows; our estimated molecular weights from top to bottom for these proteins are 130, 50, 45 and 34 kDa). It is possible that some of these proteins correspond to those detected previously by Okano and Medsger (1991). For example, the 130 and 34 kDa proteins could correspond to their 120 and 34 kDa proteins. However, our MaS IP was from nuclear extract fractions whereas they used whole cell extracts. Thus, it is possible that some of their proteins are not associated with U4/U6 snRNP in the nucleus.

### SAPs 102 and 68 correspond to PSF and an apparent breakdown product of PSF

Comparison of a partially purified preparation of the essential splicing factor PSF (Patton *et al.*, 1993) to affinity-purified spliceosomes on a 2-D gel revealed that SAPs 102 and 68 co-fractionate with PSF and its apparent

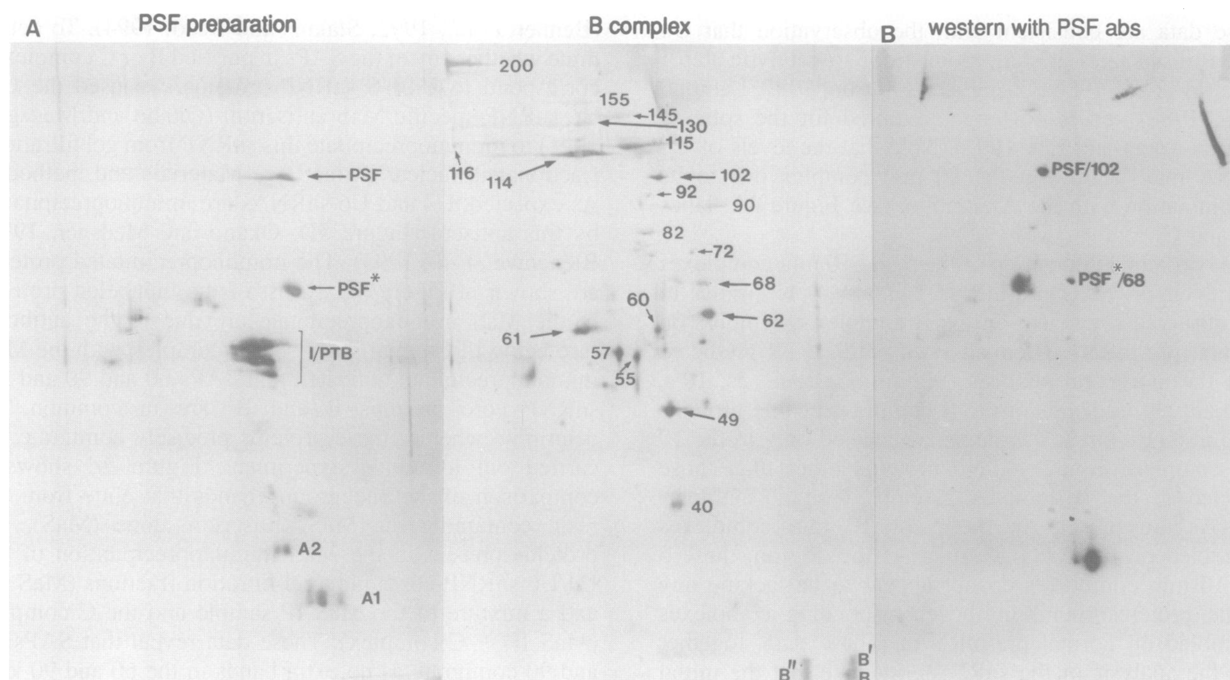


**Fig. 6.** SAPs 102 and 68 correspond to the essential splicing factor PSF and its apparent breakdown product PSF*. (**A**) 2-D gel comparison of PSF and spliceosomes. A partially purified preparation of PSF or affinity-purified B complex were fractionated by 2-D gel electrophoresis. Proteins were detected by silver staining. PSF, PSF*, hnRNPs I/PTB, A1 and A2 are indicated in the PSF preparation and the spliceosomal proteins are indicated in the B complex. (**B**) Western analysis. The C complex was fractionated on a 2-D gel, transferred to nitrocellulose and probed with polyclonal antibodies to PSF. Spots corresponding to PSF (SAP 102) and PSF* (SAP 68) are indicated. The spot to the left of PSF* and the streaks above PSF are not proteins and are not detected reproducibly. The identity of the low molecular weight protein detected by the PSF antibody is not known.

breakdown product PSF* (Figure 6A). PSF* is thought to be a breakdown product of PSF because in purified preparations of PSF, PSF* accumulates over time whereas PSF diminishes (J.G.Patton, unpublished observation). As shown previously, hnRNP I/PTB and a 33 kDa protein are present in the PSF preparation (Patton *et al.*, 1991, 1993). Peptide sequence analysis of the 33 kDa protein indicates that it is hnRNP A (J.G.Patton, unpublished observation). Consistent with this, our data show that the purified PSF preparation contains hnRNPs A1 and A2 (based on a 2-D gel comparison with purified hnRNP complexes; data not shown). Further evidence that SAPs 102 and 68 correspond to PSF and PSF* is the observation that antibodies to PSF specifically detect SAPs 102 and 68 on 2-D Western blots of affinity-purified C complex (Figure 6B). As noted above, SAPs 102 and 68 are present in the C complex at significantly higher relative levels than in the B complex (e.g. compare levels of these proteins in Figures 5A and 6A, B complex). We conclude that PSF is specifically enriched in the C complex.

### C complex-specific proteins UV crosslink to pre-mRNA

As observed previously, we find that the 200 kDa U5 snRNP protein (García-Blanco *et al.*, 1990; Whittaker and Beggs, 1991; Wyatt *et al.*, 1992; Staknis and Reed, 1994) and the U2 snRNP-specific SAPs 155, 145, 114, 62, 61 and 49 (Staknis and Reed, 1994) UV crosslink to parental or ΔAG pre-mRNA in affinity-purified B complex (Figure 7A; 'x' is a non-specific protein that also crosslinks to RNAs lacking splice sites; Staknis and Reed, 1994). The same U2 and U5 snRNP proteins that crosslink in the B complex also crosslink in the C complex (Figure 7A). Significantly, however, three additional proteins crosslink to pre-mRNA in the C complex. By superimposing the crosslinking and silver-stained patterns of the gel, we identified these as the C complex components SAP 36, and SAPs 102 and 68 (PSF and PSF*, respectively) (Figure 7B). We note that, as observed previously, the crosslinked proteins are shifted slightly above and to the acidic side of the silver-stained proteins (Figure 7B; Staknis and Reed, 1994). This shift is most likely due to the presence of the crosslinked RNase digestion product and, as expected, the shift is larger with smaller proteins. We detect no other likely candidates for these crosslinked proteins on our silver-stained 2-D gels (Figure 7B). Moreover, the correspondence between the crosslinked and silver-stained proteins was also seen on lower percentage gels which resolve the proteins differently (data not shown). Consistent with our data indicating that PSF crosslinks to pre-mRNA, this factor contains two RNA binding domains, and the purified protein crosslinks specifically to pyrimidine tracts in pre-mRNA (Patton *et al.*, 1993).

### PSF is required for catalytic step II of the splicing reaction

It was thought previously that PSF was required for A complex assembly (Patton *et al.*, 1993). This was based on the observation that A complex assembly was inefficient in splicing extracts immunodepleted of PSF. However, analysis of splicing reactions in PSF-depleted extracts typically showed that catalytic step I still occurred (Patton *et al.*, 1993). This observation, together with our observa-

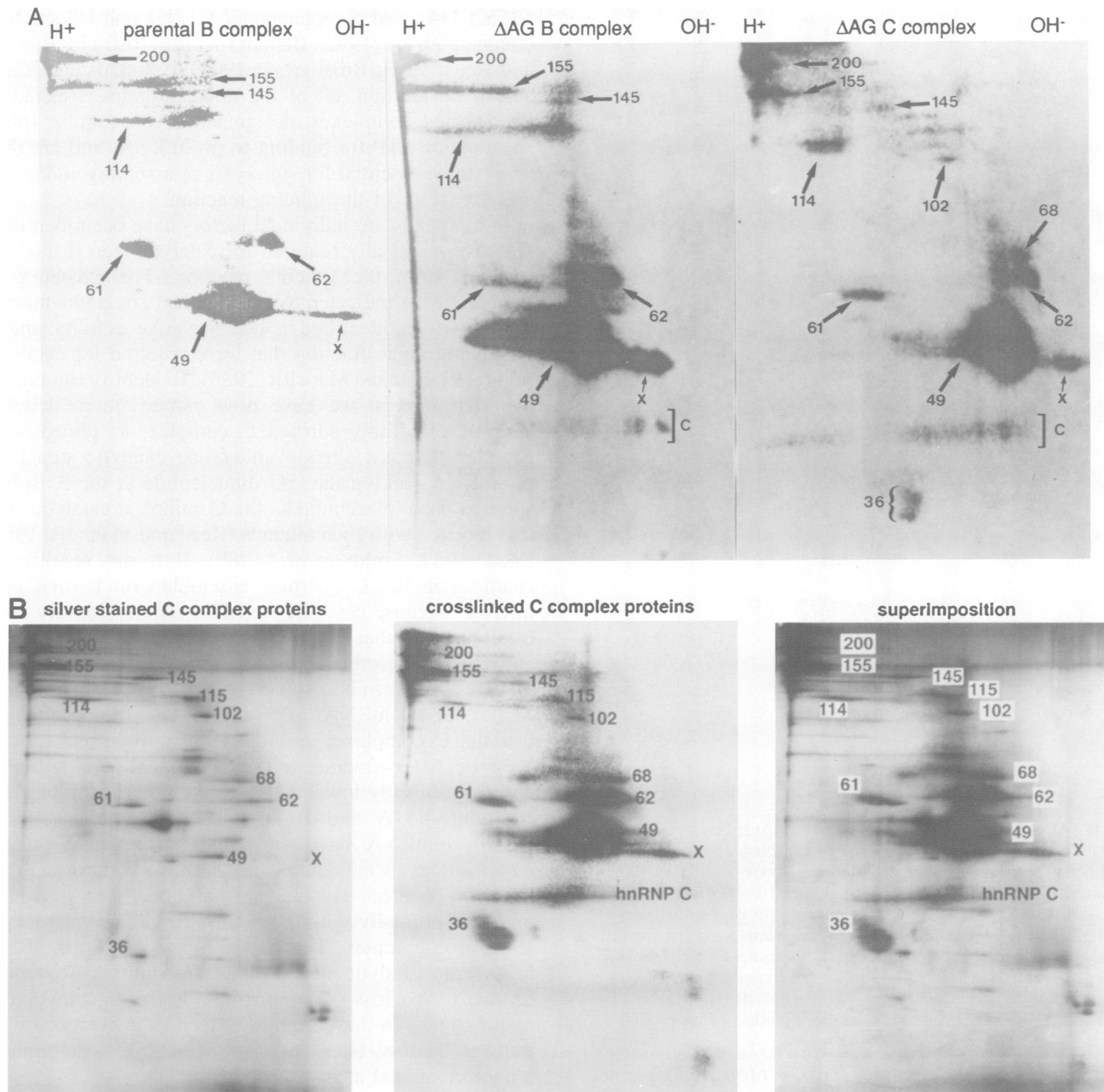


**Fig. 7.** SAPs 102, 68 and 36 UV crosslink to pre-mRNA in the C complex. (**A**) Affinity-purified B complex assembled on parental pre-mRNA or B and C complexes assembled on ΔAG pre-mRNA were UV crosslinked, treated with RNase and fractionated by 2-D gel electrophoresis (see Materials and methods). The crosslinked proteins were detected by phosphorimager analysis and identified as described in (B). Note that low levels of hnRNP C crosslink in the ΔAG B and C complexes; this protein crosslinks much more efficiently in the H complex and is most likely present in the C complex due to contamination with the H complex (Staknis and Reed, 1994). (**B**) A sample of affinity-purified C complex prepared as in (A) was fractionated on a 2-D gel, and silver-stained proteins (silver-stained C complex proteins) or crosslinked proteins (crosslinked C complex proteins) were detected. The crosslinked pattern superimposed on the silver-stained pattern is shown in the third panel (superimposition). The crosslinked proteins identified by the superimposition are indicated. SAP 115, which is better resolved in (B) than in (A), has only been found to crosslink on complexes assembled on AdmL pre-mRNA. The acidic and basic ends of the gels in (B) are the same as indicated in (A).

tions that PSF is specifically enriched in the C complex and also crosslinks in this complex, prompted us to re-evaluate the data using PSF-depleted extracts. Significantly, our data show that the levels of splicing intermediates (exon 1 and lariat-exon 2) in PSF-depleted extracts are similar to the levels of spliced products in the normal nuclear extract or in the mock-depleted extract (Figure 8, lanes 2–4). Notably, however, there is a complete block in catalytic step II only with PSF-depleted extracts (Figure 8, lane 4). Moreover, addition of a pET–PSF fusion protein fully restores catalytic step II activity to the PSF-depleted extracts (Figure 8, lane 5). These results are seen best by comparing the ratio of lariat-intron versus lariat-exon 2, due to the presence of a breakdown product of the pre-mRNA near the spliced mRNA (a darker exposure of the top portion of the gel (lanes 2–5) is presented in Figure 8, right panel). On the basis of these data and the observations that PSF is specifically enriched in and UV crosslinks in the C complex, we conclude that PSF is essential for catalytic step II of the splicing reaction. The

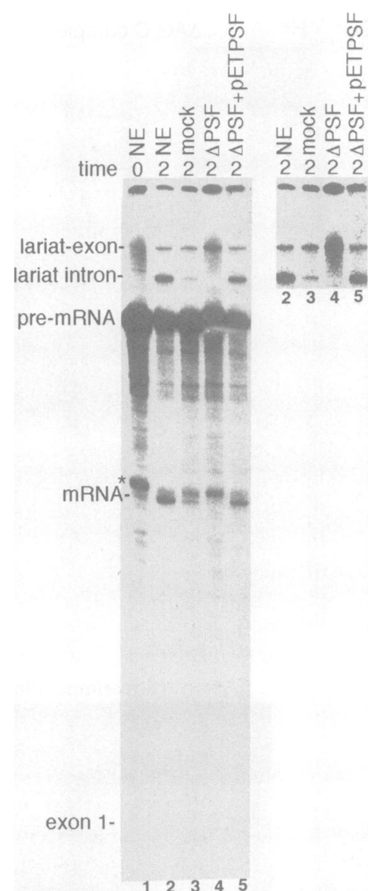


**Fig. 8.** PSF is required for catalytic step II of the splicing reaction. $^{32}$P-labeled α-tropomyosin pre-mRNA was incubated under splicing conditions for the times indicated in normal, mock-depleted or PSF-immunodepleted (ΔPSF) nuclear extracts (lanes 1–4). Bacterially synthesized PSF (pETPSF) was added to the PSF-depleted extracts (lane 5). Total RNA was prepared from each sample and fractionated on an 8% denaturing polyacrylamide gel. The panel on the right is a darker exposure of the top portion of the gel (lanes 2–5). The bands corresponding to intermediates and spliced products are indicated. The band indicated by the arrow is a breakdown product of the pre-mRNA.

discrepancy with the previous work is probably explained by the observation that significantly higher levels of PSF antibodies were required to block A complex assembly than were required to block catalytic step II of the splicing reaction (Patton *et al.*, 1993; J.G.Patton, unpublished observation). Thus, it is possible that the PSF antibodies deplete an A complex factor that has much lower affinity for the antibodies or non-specifically inactivate a factor required for A complex assembly.

## Discussion

As an approach for identifying, characterizing and cloning factors required for spliceosome assembly, we identified previously the proteins that are stably associated with affinity-purified pre-spliceosomal complexes E and A and the spliceosomal complex B (Bennett *et al.*, 1992; Bennett and Reed, 1993). Several factors that are known to be, or are likely to be, essential for spliceosome assembly are present in the purified complexes. These factors include U2AF$^{65}$, U2AF$^{35}$, the three subunits of SF3a (SAPs 61, 62 and 114), and components of U1, U2 and U5 snRNPs (Bennett *et al.*, 1992; Behrens *et al.*, 1993a,b; Bennett and Reed, 1993; Brosi *et al.*, 1993a,b; Staknis and Reed, 1994). In addition, all of the novel proteins detected in the purified complexes, designated SAPs, require splice sites and/or ATP for binding to pre-mRNA, and are thus likely to be essential for spliceosome assembly and/or the catalytic steps of the splicing reaction.

In mammals, no individual factors have been identified that are specifically required for catalytic step II, but not for step I, of the splicing reaction. The existence of such factors is indicated by biochemical complementation studies which identified a heat-sensitive activity and a chromatographic fraction that were required for catalytic step II (Krainer and Maniatis, 1985). To identify candidates for such factors, we have now carried out a detailed analysis of affinity-purified C complex, a spliceosomal complex that has already undergone catalytic step I. A pre-mRNA lacking the AG dinucleotide at the 3′ splice site was used to accumulate the C complex; catalytic step II is blocked with such mutants (Reed and Maniatis, 1985; Reed, 1989; Smith *et al.*, 1989). It is not possible to characterize the C complex assembled on normal pre-mRNAs because catalytic step II occurs so rapidly after catalytic step I that little C complex accumulates. Significantly, however, the C complex assembled on ΔAG pre-mRNA appears to be similar to the C complex assembled on normal pre-mRNAs. This is based on the observation that the C complexes assembled on normal and mutant pre-mRNAs co-fractionate on native gels, as a complex with significantly lower mobility than the B complex. In addition, the A and B complexes assembled on the ΔAG pre-mRNA have the same snRNA and protein compositions as the complexes assembled on normal pre-mRNAs. Thus, our data indicate that spliceosome assembly proceeds normally with the ΔAG pre-mRNA through the stage of C complex. The failure of the ΔAG pre-mRNA to undergo catalytic step II of the splicing reaction could be due to the loss of interactions that do not involve a major change in the C complex, such as interactions with transient factors, interactions with just a few proteins or a conformational change in the complex.

Proteins that are unique to the C complex are obvious candidates for factors involved in catalytic step II of the splicing reaction. However, it is also possible that some of the C complex-specific proteins are involved in catalytic step I. In this case, the proteins would have to bind to the spliceosome immediately before step I, and remain stably bound after step I. Such factors would not be detected in the B complex, but could remain in the C complex. Our analyses are unable to distinguish between this type of first-step factor and factors required for catalytic step II.

We detected 14 novel SAPs in the C complex (SAPs 165, 100, 95, 76, 75, 70, 65, 58, 51, 48, 45, 38, 36 and 30). In addition, we identified four proteins, previously detected in the B complex, that are highly enriched in the C complex. These are the 40 kDa U5 snRNP protein and SAPs 102, 68 and 57. Our data show that SAPs 102 and 68 correspond to the essential splicing factor PSF and its apparent breakdown product PSF*, respectively (see below). We do not yet know whether any of the other C complex-specific SAPs are breakdown products of SAP

102 or other spliceosomal proteins. However, the apparent high complexity of the C complex is consistent with the fact that this complex migrates as a significantly larger particle than the B complex on native gels (Lamond *et al.*, 1987).

SAP 57 is the most abundant protein detected in any of the spliceosomal complexes and appears to be present at much greater than a 1:1 stoichiometry in purified C complex. Further studies are needed to understand the significance of this observation. The 40 kDa U5 snRNP protein is specifically enriched in the C complex, even though U5 snRNP binds in the B complex. However, we have found that there is not, in general, a tight correlation between the presence of U5 snRNA and proteins classified as U5 snRNP components (Bach *et al.*, 1989) in the purified spliceosomal complexes. For example, U5 snRNA is detected at lower levels in the B than in the C complex, yet the levels of the 200 and 116 kDa U5 snRNP proteins are similar between these complexes. Similarly, the 200 kDa protein is detected in the A complex when little U5 snRNA is present. Thus, although these proteins can be detected in association with purified 20S U5 snRNP (Bach *et al.*, 1989), it is possible that they bind to the spliceosome at different times or with different stabilities than does U5 snRNP.

UV crosslinking studies of affinity-purified C complex showed that all of the same proteins that crosslink in the B complex crosslink in the C complex. These include six U2 snRNP proteins (SAPs 155, 145, 114, 62, 61 and 49) and the 200 kDa U5 snRNP protein (Staknis and Reed, 1994). In contrast, PSF and PSF* (SAPs 102 and 68) and SAP 36 were identified as proteins that crosslink only in the C complex, implicating a role for these proteins in catalytic step II of the splicing reaction.

The observation that PSF crosslinks to pre-mRNA in the C complex is consistent with the fact that PSF has two RNA recognition motifs; in addition, purified PSF crosslinks to the pyrimidine tract of pre-mRNAs (Patton *et al.*, 1993). $U2AF^{65}$ (Zamore and Green, 1989) binds to the pyrimidine tract in the E complex, but is detected in significantly lower levels in affinity-purified A, B and C complexes (Staknis and Reed, 1994; this study). Thus, $U2AF^{65}$ appears to be destabilized during the E to A complex transition (Staknis and Reed, 1994). It is not clear from these studies whether $U2AF^{65}$ remains in the A complex in a more loosely bound state or dissociates completely. However, the observations that PSF crosslinks in the C complex (this study) and crosslinks to pyrimidine tracts (Patton *et al.*, 1993) suggest that PSF could ultimately replace $U2AF^{65}$ on the pyrimidine tract. This possibility is supported by the observation that PSF is required for catalytic step II of the splicing reaction.

Significantly, the pyrimidine tract appears to have at least two roles in the splicing pathway (Reed, 1989; Smith *et al.*, 1989). This sequence element is first required for spliceosome assembly, most likely for U2AF binding in the E complex (Smith *et al.*, 1989; Zamore and Green, 1989; Michaud and Reed, 1993). The pyrimidine tract is then required again for efficient recognition of the AG dinucleotide during catalytic step II of the splicing reaction (Reed, 1989). A role for the pyrimidine tract in AG recognition for catalytic step II has also been observed in yeast (Patterson and Guthrie, 1991). Our data, together with the observation that purified PSF binds pyrimidine tracts, are consistent with the possibility that PSF recognizes the pyrimidine tract for catalytic step II.

As suggested previously (Patton *et al.*, 1993), it is possible that PSF corresponds to intron-binding protein (IBP), a factor that associates with U5 snRNP (Gerke and Steitz, 1986; Tazi *et al.*, 1986). Similar to PSF and PSF* (SAPs 102 and 68, respectively), IBP is a 100 kDa protein, and a 70 kDa protein is thought to be its breakdown product (Gerke and Steitz, 1986; Tazi *et al.*, 1986; Pinto and Steitz, 1989). Furthermore, IBP and PSF both bind to pyrimidine tracts in pre-mRNA (Gerke and Steitz, 1986; Tazi *et al.*, 1986; Patton *et al.*, 1993). In addition, the observation that IBP is associated with U5 snRNP is consistent with the late role in splicing observed for PSF. Although an association of PSF with U5 snRNP has not been detected, this could be for a variety of reasons, including that the PSF epitope may not always be accessible and that IBP may be associated with U5 snRNP only under certain conditions (Gerke and Steitz, 1986; Tazi *et al.*, 1986; Pinto and Steitz, 1989).

In contrast to mammals, several factors involved in catalytic step II of the splicing reaction have been identified in yeast [see Rymond and Rosbash (1992) for a review]. These include SLU7 (Frank and Guthrie, 1992; Frank *et al.*, 1992) and PRPs 16 (Couto *et al.*, 1987; Burgess *et al.*, 1990; Schwer and Guthrie, 1991, 1992), 17 (Vijayraghavan *et al.*, 1989; Ruby and Abelson, 1991; Frank and Guthrie, 1992; Frank *et al.*, 1992), 18 (Vijayraghavan and Abelson, 1990; Horowitz and Abelson, 1993b) and 29 (Ruby and Abelson, 1991). As appears to occur with PSF, PRP18 can bind to spliceosomes that have undergone catalytic step I (i.e. the C complex) (Horowitz and Abelson, 1993a). In the case of PRP18, the C complex is formed in the absence of functional PRP18 and the addition of PRP18 allows catalytic step II to occur (Horowitz and Abelson, 1993a). PRP18 is thought to be associated with U5 snRNP (Horowitz and Abelson, 1993b). Thus, data in both yeast and mammals show that U5 snRNP proteins (e.g. the mammalian 40 kDa U5 snRNP protein) can bind to pre-mRNA after U5 snRNP has bound.

Interestingly, antibodies to PRP18 detect a mammalian protein, designated $p54^{nrb}$, which bears a strong amino acid similarity to a portion of PSF (Dong *et al.*, 1993). Although there is no obvious similarity between PRP18 and $p54^{nrb}$ (or PSF) at the amino acid level (Dong *et al.*, 1993), the observations regarding PRP18, PSF, $p54^{nrb}$, IBP and U5 snRNP raise the possibility that there may be some relationship between these splicing components. Significantly, in both yeast and mammals, a pyrimidine tract preceding the AG dinucleotide increases the efficiency of catalytic step II (Reed, 1989; Patterson and Guthrie, 1991). The observation that PRP18 is a U5 snRNP component suggests that, as proposed for PSF, PRP18 could interact at the 3′ splice site.

### *SAPs 60 and 90 are U4/U6 snRNP proteins*

A large number of proteins that are specifically associated with purified mammalian U5 snRNP or U4/U5/U6 snRNP have been identified (Bach *et al.*, 1989; Behrens and Luhrmann, 1991). In contrast, no snRNP-specific proteins

have been detected in purified U4/U6 snRNP (see Luhrmann *et al.*, 1990). However, a rare patient antiserum, designated MaS, immunoprecipitates U4/U6 snRNAs from cell extracts (Okano and Medsger, 1991; Blencowe *et al.*, 1993; this study). The MaS antigen is a 150 kDa protein, and four proteins (120, 80, 36 and 34 kDa) in addition to the core snRNP proteins are co-immunoprecipitated from cell extracts along with the 150 kDa MaS antigen (Okano and Medsger, 1991). Substoichiometric amounts of the MaS antigen can be detected in purified U2, U5 and U4/U5/U6 snRNPs, but this protein does not correspond to any of the snRNP-specific proteins identified previously (Blencowe *et al.*, 1993).

We used the MaS antiserum to immunoprecipitate U4/U6 snRNPs from nuclear extracts and were able to identify proteins in the immunoprecipitate that precisely co-fractionated with SAPs 60 and 90 and the snRNP core proteins B and B′. SAPs 60 and 90 were not detected by Western analysis using the MaS antiserum. Thus, these data indicate that SAPs 60 and 90 are specifically associated with U4/U6 snRNPs. We were unable to detect the 150 kDa MaS antigen in our purified complexes due to the complexity of the high molecular weight proteins. In addition, further work is needed to determine whether there is a relationship between SAPs 60 and 90 and the four proteins shown previously to be co-immunoprecipitated by the MaS antiserum (120, 80, 36 and 34 kDa). In yeast, several proteins associated with U4/U6 snRNP, including PRPs 3 (Ruby and Abelson, 1991), 4 (Banroques and Abelson, 1989; Dalrymple *et al.*, 1989; Petersen-Bjorn *et al.*, 1989; Bordonne *et al.*, 1990; Xu *et al.*, 1990), 6 (Abovich *et al.*, 1990; Legrain and Choulika, 1990) and 24 (Shannon and Guthrie, 1991; Strauss and Guthrie, 1991), have been identified as essential splicing factors. Our analysis has identified SAPs 60 and 90 as the first mammalian U4/U6 snRNP proteins in the spliceosome. U4 and U6 snRNAs play critical roles in the catalytic steps of splicing, and SAPs 60 and 90 may be important factors involved in the functioning of these snRNAs.

## Materials and methods

### Plasmids

Plasmids pAdMLΔAG and pAdMLPar were constructed by inserting an oligonucleotide into the *Hin*dIII and *Pst*I sites in pAdMLΔ3′ss (Michaud and Reed, 1993). The sequences of the 3′ portions of the pre-mRNAs encoded by these plasmids are shown in Figure 1. The pre-mRNAs contain exon 1 (129 nucleotides), intron 1 (104 nucleotides in pAdMLΔAG and 97 nucleotides in pAdMLPar) and exon 2 (45 nucleotides) derived from the adenovirus 2 major late transcription unit. DNA was linearized with *Bam*HI for transcription and transcribed with T7 RNA polymerase.

### Splicing complex purification, [$^{32}$P]pCp end-labeling and UV crosslinking

Capped biotinylated pre-mRNAs (Grabowski and Sharp, 1986) were synthesized in standard transcription reactions (Melton *et al.*, 1984). For UV crosslinking, transcription reactions (100 μl) contained 50 μCi each of [$^{32}$P]ATP, [$^{32}$P]GTP, and [$^{32}$P]CTP (3000 Ci/mmol), 100 μM cold ATP, GTP, CTP and UTP, and 15–20 μM biotinylated UTP (Enzo Biochemicals). For all other transcriptions, reactions contained 10 μCi [$^{32}$P]UTP (800 Ci/mmol), 200 μM cold ATP, GTP, CTP and UTP, and 15–20 μM biotinylated UTP. Assembly of splicing complexes and splicing reactions were carried out under standard *in vitro* splicing conditions (Krainer *et al.*, 1984). To accumulate maximal levels of C complex, we optimized the levels of ΔAG pre-mRNA and the time of incubation (data not shown). Native gel electrophoresis of splicing complexes was carried out as described (Konarska and Sharp, 1987), except that 1 μl of 6.5 mg/ml heparin was added to 25 μl reactions, and 10 μl of each reaction was fractionated on the gel. For purification of complexes, splicing reactions (2.4 ml) containing 1.92 μg pre-mRNA were incubated at 30°C for the times indicated. Gel filtration and affinity purification of splicing complexes were carried out as described (Bennett *et al.*, 1992). For identification of spliceosomal snRNA composition, total RNA was prepared from equivalent amounts of each affinity-purified complex and end-labeled with [$^{32}$P]pCp and RNA ligase, as described (Reed, 1990). In UV crosslinking experiments, 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 (Staknis and Reed, 1994). To digest the $^{32}$P-labeled RNA after crosslinking, 1 μl 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 and acetone-precipitated (Bennett *et al.*, 1992). 2-D gel electrophoresis was carried out as described (O'Farrell *et al.*, 1977; Bennett *et al.*, 1992). The first dimension was non-equilibrium pH gradient gel electrophoresis [NEPHGE, ampholytes pH 3–10 (Bio-Rad)] and the second dimension was 9% SDS–PAGE. The total protein obtained from splicing complexes assembled on 200 ng of pre-mRNA was loaded on 2-D gels. Proteins were visualized by silver staining (Morrissey, 1981), and crosslinked proteins were detected by Phosphorimager analysis (Molecular Dynamics) and autoradiography.

### Immunoprecipitation of U4/U6 snRNP

Total nuclear extract (lacking pre-mRNA) was fractionated by gel filtration, and fractions containing U4/U6 snRNP were pooled. MaS patient antiserum (7.5 μl) was coupled to an AminoLink gel column as described (Pierce ImmunoPure Ag/Ab Immobilization Kit). 100 μl of column resin were removed and mixed overnight at 4°C with 30 ml of gel filtration fractions containing U4/U6 snRNP. After washing the immunoprecipitate with 125 mM NaCl, 20 mM Tris, pH 7.6, total RNA was prepared, end-labeled with [$^{32}$P]pCp and fractionated on an 8% denaturing polyacrylamide gel. Proteins were prepared and analyzed by 2-D gel electrophoresis. As a marker for the antibody proteins, a sample of the antibodies bound to the AminoLink gel column was analyzed by 2-D gel electrophoresis.

### Western analysis, comparison of B complex and PSF, and immunodepletion

For Western blots, affinity-purified B complex assembled on 250 ng pre-mRNA was fractionated by 2-D gel electrophoresis, transferred to nitrocellulose and probed with PSF polyclonal antibodies (Patton *et al.*, 1993). Anti-rabbit secondary antibodies were horseradish peroxidase-linked and the ECL detection system (Amersham) was used.

For comparison of PSF and B complex, PSF was partially purified as described (Patton *et al.*, 1993) and fractionated on a 2-D gel in parallel with affinity-purified B complex. Immunodepletion or mock depletion of nuclear extracts using PSF antibodies was carried out as described; depletion of PSF was estimated to be ~90% complete based on Western analysis (Patton *et al.*, 1993). The pET–PSF fusion protein was purified by affinity-chromatography on a nickel column. The amount needed for complement of the immunodepleted extracts was determined by titration (Patton *et al.*, 1993).

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