

# Dynamic association of proteins with the pre-mRNA branch region

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**The association of proteins with the branch site region during pre-mRNA splicing was probed using a novel methodology to site-specifically modify the pre-mRNA with the photo-reagent benzophenone. Three sets of proteins were distinguished by the kinetics of their associations with pre-mRNAs, by their association with discrete splicing complexes, and by their differing factor requirements. An early U1 snRNP-dependent cross-link of the branch region to a p80 species was followed by cross-links to p14, p35, and p150 polypeptides associated with the U2 snRNP-pre-mRNA complex. Concomitant with formation of the spliceosome, a rearrangement of protein factors about the branch region occurred, in which the p35 and p150 cross-links were replaced by p220 and p70 species. These results establish that the branch region is recognized in a dynamic fashion by multiple distinct proteins during the course of spliceosomal assembly.**

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Pre-mRNA splicing occurs via two sequential transesterification reactions in a complex known as the spliceosome, a 60S entity that assembles on the pre-mRNA substrate in an ordered fashion through several stable complexes. The spliceosome includes the small nuclear ribonucleoprotein (snRNP) particles U1, U2, U4/6, and U5, as well as non-snRNP-associated splicing factors (for review, see Guthrie 1991; Lamm and Lamond 1993; Moore et al. 1993). Commitment of a pre-mRNA substrate to the splicing pathway involves the ATP-independent formation of the early or commitment complex, which contains tightly bound U1 snRNP as well as non-snRNP protein factors, including U2AF bound near the branch region (S eraphin and Rosbash 1989; Michaud and Reed 1991; Jamison and Garcia-Blanco 1992). Native gel analysis allows the separation of three later splicing complexes, termed A, B, and C, from the nonspecific H complex (Konarska and Sharp 1986, 1987). Complex A is generated by the stable binding of U2 snRNP to the branch region of the pre-mRNA. A larger complex, B, is formed by association of complex A with U4/5/6 tri-snRNP. Complex C, which follows B, contains splicing intermediates but does not contain U1 or U4 snRNPs. The excised intron is part of complex I, which contains the same snRNPs as complex C.

Conserved sequence elements at the 5' and 3' splice sites and in the branch sequence and polypyrimidine tract of the pre-mRNA, probably in combination with other sequences, direct the formation of the spliceosome. In particular, recognition of the branch region is required early in splicing complex assembly and specifies the nucleophile for the first transesterification reaction (for review, see Moore et al. 1993). The branch sequence 5'-UACUAAC-3' is highly conserved in yeast (the underlined adenosine is the nucleophile for the first step); in mammals, the consensus sequence is YURAC (Y, pyrimidine; R, purine), but the yeast sequence remains optimal (Reed and Maniatis 1988; Zhuang et al. 1989). Recognition of this sequence as well as selection of the splice sites involves both RNA-RNA and RNA-protein interactions. A variety of experiments in yeast and mammalian systems have demonstrated snRNA-pre-mRNA interactions involved in recognition of the branch sequence. U2 snRNP interacts with the intron in part through U2 snRNA/branch region base-pairing (Parker et al. 1987; Wu and Manley 1989; Zhuang and Weiner 1989). Modifications within the branch region have recently provided biochemical evidence that the nucleophile for the first step is selected by virtue of being bulged from the pre-mRNA/U2 snRNA duplex (Query et al. 1994). The branch region is also involved after the first step, as a suitably positioned guanosine residue may function as the branch nucleophile in the first step but

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constitutes a strong block to the second step. This suggests that the branch site is important for recognition of the 3' splice site. The role of snRNAs in the chemistry of splicing is unclear, but mutations of U6 in the vicinity of the branch region (because of nearby U2/U6 base-pairings; see Fig. 9C, below) have been shown to affect both steps of splicing (Madhani and Guthrie 1992; 1994; McPheeters and Abelson 1992, and references therein). Thus, interactions both at and near to the branch region are critical for both spliceosomal assembly and the two transesterification steps.

The remarkable precision of the splicing process over introns thousands of nucleotides in length must depend on an extensive set of protein-RNA and protein-protein interactions. The isolation of temperature-sensitive mutants defective in pre-mRNA processing in yeast has suggested that a large number of RNA-binding proteins and RNA helicases interact with the snRNAs and pre-mRNA in the context of the spliceosome (for review, see Guthrie 1991). Few of these proteins, however, have been shown to interact directly with the pre-mRNA. A number of factors have been identified that interact with the pre-mRNA in the vicinity of the branch region. For example, U2AF<sup>65</sup> binds specifically to polypyrimidine tracts (Ruskin et al. 1988; Zamore et al. 1992) and is required for stable branch region-U2 snRNA interactions, although it may not interact directly with sequences of the branch region. Other polypeptides that bind the polypyrimidine tract include polypyrimidine tract-binding protein (PTB; Garcia-Blanco et al. 1989) and the PTB-associated splicing factor PSF, which is required at an early step of spliceosomal assembly (Patton et al. 1991). Additionally, U2 snRNP-associated proteins may interact in the region of the branch sequence (Staknis and Reed 1994). Some of these, separated in fractions SF3a and SF3b, are required for the stable association of U2 snRNP with the pre-mRNA (Behrens et al. 1993a; Brosi et al. 1993a,b). Spliceosome-associated protein (SAP) 49, thought to be a component of SF3b, cross-links to the pre-mRNA 5' to the branch region (Champion-Arnaud and Reed 1994). Finally, early branch site recognition is thought to be mediated by an as-yet-unidentified non-snRNP splicing factor in a manner dependent on U1 snRNP (Ruby and Abelson 1988; Séraphin and Rosbash 1991).

Because recognition of the branch site is an early step in the assembly of splicing complexes and because the identity of the branch nucleotide is important during both transesterification steps, it is important to identify and elucidate the factors that interact with this region of the pre-mRNA. We have developed a versatile technique for the introduction of a variety of photo-probes or other labeling reagents within a pre-mRNA substrate. Photo-probes were introduced site specifically and tested for cross-linking under splicing conditions. We have characterized a number of proteins closely associated with the branch site adenosine that appear in splicing complexes with different kinetics, providing a dynamic view of interactions at the branch region during the early steps of pre-mRNA splicing.

## Results

### *Synthesis and characterization of photo-active pre-mRNAs*

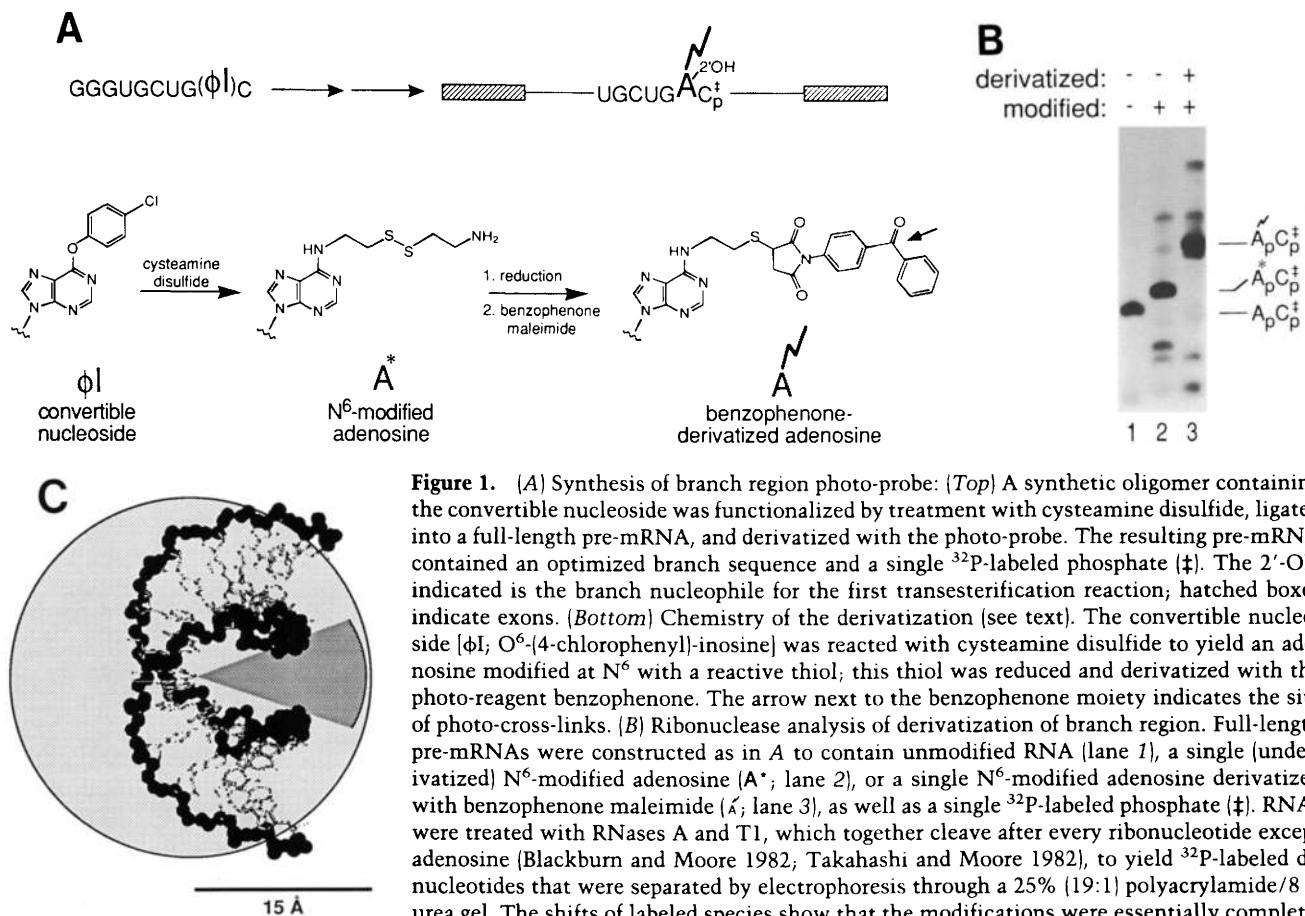
A two-step strategy was chosen to introduce a photo-cross-linking reagent into pre-mRNA substrates. First, a reactive thiol functionality was introduced into synthetic RNA oligomers. Second, following the incorporation of these oligomers into full-length pre-mRNA molecules via a three-way ligation procedure (Query et al. 1994), the thiol was derivatized with the photo-reagent.

Two oligoribonucleotide decamers were synthesized containing the convertible nucleoside O<sup>6</sup>-(4-chlorophenyl)-inosine ( $\phi$ I; C.R. Allerson, S. Chen, and G.L. Verdine, unpubl.): the branch sequence 5'-GGGUG-CUG( $\phi$ I)C-3' and the scrambled branch sequence 5'-GGUGUCGC( $\phi$ I)G-3'. These were deprotected with cysteamine disulfide to yield oligomers containing a single modified adenosine residue: 5'-GGGUGCUG(A\*)C-3' and 5'-GGUGUCGC(A\*)G-3' (where A\* denotes the N<sup>6</sup>-modified adenosine residue; see Fig. 1A). The modified RNAs were then ligated to RNAs containing a single <sup>32</sup>P-label (Moore and Sharp 1992; Query et al. 1994) to generate pre-mRNAs site-specifically modified with a reactive thiol within the branch region and bearing a unique <sup>32</sup>P-label one nucleotide 3' to the modification (Fig. 1A). The scrambled branch sequence was also used to create a novel 5' splice site containing the photo-active group two nucleotides 5' to the 5' splice site, as well as at a site within the intron midway between the 5' splice site and the branch region; both these constructs also contained a single <sup>32</sup>P-label one nucleotide 3' to the modification (see Materials and methods). Combinations of these sequences with a mutated polypyrimidine tract were also used.

Full-length RNAs were derivatized with the cross-linking reagent by reduction with dithiothreitol (DTT) followed by reaction with benzophenone maleimide. The maleimide derivatization is specific for the reactive thiol moiety and this reaction proceeded in high yield as indicated by electrophoretic mobility following digestion with ribonucleases A and T1 (Fig. 1B).

The benzophenone moiety is attached to the N<sup>6</sup> position of adenosine by a 15 Å tether. If the derivatized adenosine is base-paired within duplex RNA, the N<sup>6</sup> position faces the major groove and the space accessible to the photo-probe is quite limited (darker area in Fig. 1C). If the adenosine is bulged from the RNA, the photo-probe can, in theory, be cross-linked to any position within a sphere with a radius of 15 Å (lighter circle in Fig. 1C).

Both complex formation and splicing of the derivatized RNAs were examined. All derivatized pre-mRNAs of wild-type sequence formed A, B, and C complexes with similar kinetics as unmodified RNA as assayed by non-denaturing PAGE (Fig. 2B; data not shown). The pre-mRNA derivatized proximal to the 5' splice site was spliced in HeLa nuclear extracts to the same extent as unmodified pre-mRNA and with similar kinetics (data not shown). The substrate derivatized within the branch



**Figure 1.** (A) Synthesis of branch region photo-probe: (Top) A synthetic oligomer containing the convertible nucleoside was functionalized by treatment with cysteamine disulfide, ligated into a full-length pre-mRNA, and derivatized with the photo-probe. The resulting pre-mRNA contained an optimized branch sequence and a single  $^{32}\text{P}$ -labeled phosphate ( $\ddagger$ ). The 2'-OH indicated is the branch nucleophile for the first transesterification reaction; hatched boxes indicate exons. (Bottom) Chemistry of the derivatization (see text). The convertible nucleoside [ $\phi\text{I}$ ;  $\text{O}^6$ -(4-chlorophenyl)-inosine] was reacted with cysteamine disulfide to yield an adenosine modified at  $\text{N}^6$  with a reactive thiol; this thiol was reduced and derivatized with the photo-reactive benzophenone. The arrow next to the benzophenone moiety indicates the site of photo-cross-links. (B) Ribonuclease analysis of derivatization of branch region. Full-length pre-mRNAs were constructed as in A to contain unmodified RNA (lane 1), a single (underivatized)  $\text{N}^6$ -modified adenosine ( $\text{A}^*$ ; lane 2), or a single  $\text{N}^6$ -modified adenosine derivatized with benzophenone maleimide ( $\text{A}$ ; lane 3), as well as a single  $^{32}\text{P}$ -labeled phosphate ( $\ddagger$ ). RNAs were treated with RNases A and T1, which together cleave after every ribonucleotide except adenosine (Blackburn and Moore 1982; Takahashi and Moore 1982), to yield  $^{32}\text{P}$ -labeled dinucleotides that were separated by electrophoresis through a 25% (19:1) polyacrylamide/8 M urea gel. The shifts of labeled species show that the modifications were essentially complete.

(C) Schematic representation of the region accessible to the photo-reagent either from the floor of the major groove (dark wedge) or the surface (light circle) of an A-form helix rotated  $\sim 30^\circ$  with respect to the horizontal. The phosphodiester backbone is shown in black. The sweep of the reagent assumed is 15 Å from the site of attachment to the adenosine residue. The helix shown is typical A-form RNA-RNA duplex and not intended to represent the branch region per se (after the coordinates in Dock-Bregeon et al. 1988).

region displayed a reduced level of step one (a decrease of two- to fourfold) and a threefold reduction in step two (the reduction in step 1 is stated with reference to the unmodified substrate and the reduction in step 2 represents a comparison with unmodified substrate normalized to the amount of material that underwent the first step; see Fig. 2A). The electrophoretic mobility of the lariat species and ribonuclease analyses of the lariats showed that branch formation was at either the modified adenosine or the nucleotide immediately adjacent (data not shown). These results are consistent with the modification interfering with the second chemical step or with branch formation occurring at the immediately 5' guanosine (which is a strong block to step 2; e.g., see Query et al. 1994). Treatment of unmodified control RNA under derivatizing conditions did not affect splicing of that RNA (data not shown). Neither complex formation nor splicing was detectable with constructs containing the scrambled branch sequence or a mutated polypyrimidine tract.

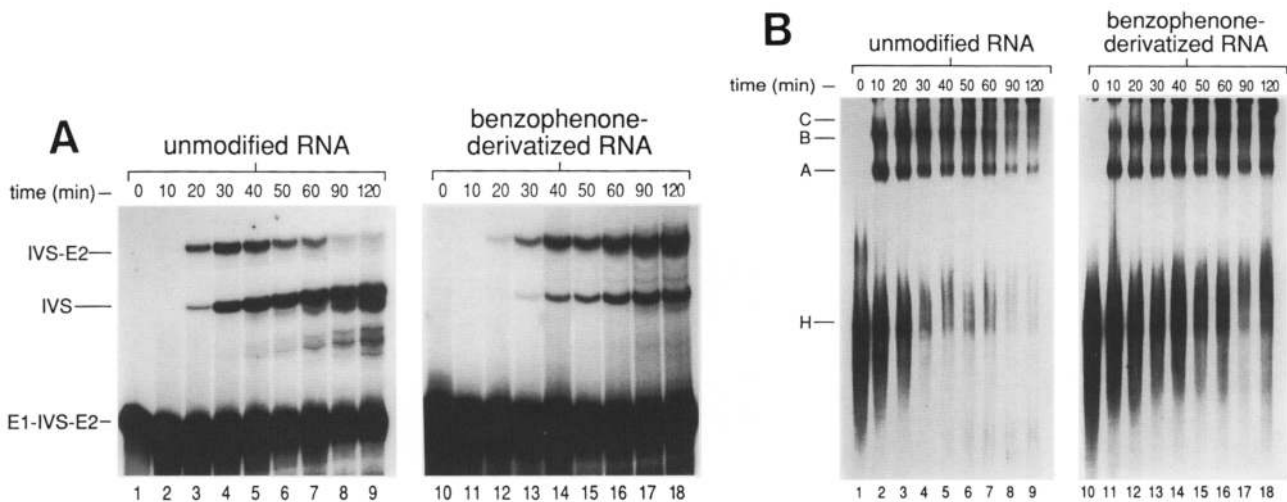
*Detection of proteins cross-linking to the branch region*  
Derivatized pre-mRNAs were incubated in HeLa nuclear

extract under standard splicing conditions and cross-linked on ice by irradiation with a 302-nm lamp. Following cross-linking, the reactions were treated with RNase A and/or RNase T1, which would yield a  $^{32}\text{P}$ -labeled di- or trinucleotide after complete digestion, and analyzed by SDS-PAGE.

The site specificity and efficiency of the cross-linking reagent were tested by comparing four pre-mRNAs in cross-linking reactions: a control pre-mRNA substrate singly labeled with  $^{32}\text{P}$  but otherwise unmodified, the same control treated with benzophenone (but underivatized), a pre-mRNA containing the underivatized  $\text{N}^6$ -alkyl thiol adenosine, and a pre-mRNA derivatized in the branch region with benzophenone. Analysis of the cross-linked proteins indicated that cross-linking was highly efficient ( $\sim 30\%$ ) and solely a function of derivatization of the modified nucleotide with benzophenone (Fig. 3).

Proteins interacting specifically near the branch site sequence were detected when benzophenone-derivatized wild-type or mutant RNAs were incubated for varying times in the presence and absence of ATP, irradiated, treated with RNase, and analyzed by SDS-PAGE. The





**Figure 2.** Time courses for splicing and complex assembly for pre-mRNA containing a single benzophenone modification within the branch region. (A) Splicing time course of unmodified RNA (lanes 1–9) or benzophenone-derivatized RNA (lanes 10–18). RNAs prepared via a three-part ligation and containing a single  $^{32}\text{P}$ -label within the intron (as in Fig. 1A) were incubated under splicing conditions for the times indicated and separated on a 15% polyacrylamide (29:1) gel. Only the portion of the gel containing the pre-mRNA and lariat species is shown as these were the only splicing species containing the  $^{32}\text{P}$ -label. (IVS–E2) The lariat form of the intron–3' exon intermediate; (IVS) the excised lariat intron; (E1–IVS–E2) the pre-mRNA. To compare relative rates for the first and second steps of splicing, polyacrylamide gels were quantitated using a Molecular Dynamics PhosphorImager and ImageQuant Software v. 3.22. The relative amount of RNA in each band was expressed as a percentage of the total obtained by summing the values of E1–IVS–E2, IVS–E2, and IVS at that time point; for each band in every lane an individual background value was determined for the area in the same lane immediately above or below that band. Initial rates were chosen from the linear portion of each curve (three time points). (B) Time course of splicing complex assembly for unmodified (lanes 1–9) and benzophenone-derivatized RNA (lanes 10–18). A portion of the reactions analyzed in A were adjusted to 0.5 mg/ml of heparin and analyzed by electrophoresis in a native 4% (80:1) polyacrylamide gel.

benzophenone-derivatized branch substrate cross-linked specifically to two polypeptides of apparent molecular mass 14 and 220 kD; both cross-links were ATP dependent and increased with time (Fig. 4A; also see Fig. 3, lanes 10–12). All the photo-labeled bands were proteinase K sensitive, indicating that none were attributable to RNA alone (data not shown). Both the p220 and p14 cross-links were dependent on the presence of a functional branch sequence (Fig. 4, cf. A and B) and polypyrimidine tract (data not shown). Other specific species are probably present but cannot be visualized because of the background of nonsplicing-specific cross-links.

Cross-linking to the pre-mRNA derivatized within the branch region was compared with cross-linking to pre-mRNAs derivatized two nucleotides 5' to the 5' splice site or at a position within the intron midway between the branch region and 5' splice site. The substrate derivatized at the 5' splice site formed ATP-dependent cross-links to a p220 species with faster kinetics than the labeling of a similar p220 species using the branch region photo-probe, perhaps because the 5' splice site-derivatized substrate splices with faster kinetics. In addition, a strong ATP-independent cross-link to a p16 species was generated with pre-mRNA modified at the 5' splice site. This band was observed if the reaction was incubated either on ice or at 30°C (Fig. 4C). The substrate derivatized midway between the branch region and 5' splice site did not exhibit any ATP-dependent or incubation-

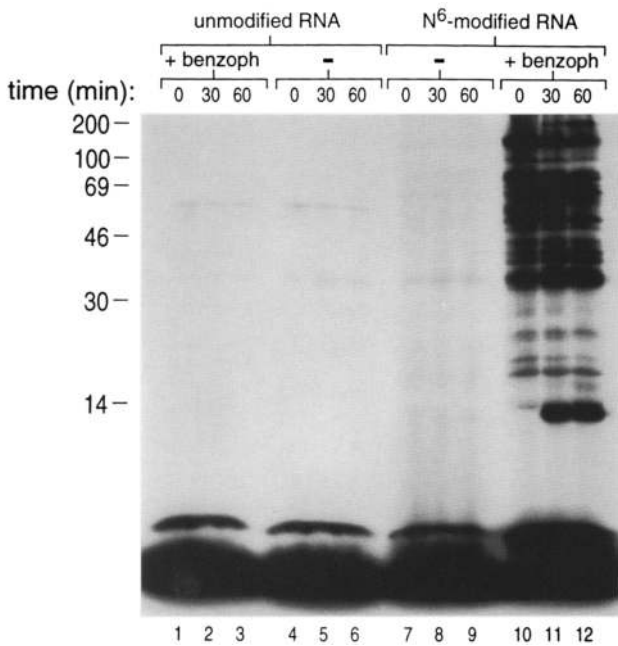
dependent bands demonstrating that proteins with such characteristics were specific (data not shown).

Finally, a 3'-half substrate from which the 5' splice site and part of the 5' intron had been deleted was used in similar reactions (Fig. 4D). This substrate cross-linked in an ATP- and time-dependent fashion to the p14 but not to the p220 species.

#### *Immunoprecipitation of spliceosomal cross-linked RNAs*

Most of the proteins cross-linked to the branch substrate were not specific to the splicing process, as they were generated in reactions containing mutant substrates (Fig. 4; data not shown). To distinguish factors that may be related to splicing, the reactions with the branch region-derivatized substrate were immunoprecipitated with the monoclonal antibodies Y12 (Lerner and Steitz 1979) and B1C8 (Wan et al. 1994). mAb Y12 reacts with epitopes on the core Sm proteins of the snRNP particles and thereby immunoprecipitates RNAs bound to U1 snRNP and other snRNPs. mAb B1C8 reacts with epitopes on proteins related to members of the SR family of polypeptides and was selected because it immunoprecipitates spliceosomal complexes efficiently, specifically enriching for exon-containing species (Blencowe et al. 1994).

Immunoprecipitations performed with either mAb Y12 or B1C8 significantly reduced background because



**Figure 3.** Specificity of cross-linking to benzophenone-derivatized pre-mRNA. RNAs were incubated in HeLa nuclear extract under splicing conditions, irradiated using a 302-nm lamp on ice, and digested with RNase A. The reaction mixtures were analyzed on a 16% (200:1) SDS-PAGE gel. (Lanes 1–3) Unmodified RNA, containing a single  $^{32}\text{P}$ -label, treated under derivatization conditions with benzophenone maleimide; (lanes 4–6) unmodified control RNA; (lanes 7–9) branch region  $\text{N}^6$ -modified adenosine underivatized with photo-reagent; (lanes 10–12) branch region  $\text{N}^6$ -modified adenosine derivatized with benzophenone maleimide. Molecular mass standards at left are given in kilodaltons. The efficiency of cross-linking to proteins ( $\sim 30\%$ ) was determined using a Molecular Dynamics PhosphorImager by comparing the counts in the  $>12$ -kD range of the gel to those near the bottom of the gel, which represent the non-cross-linked RNA after digestion with RNase.

of cross-links not associated with the splicing process (cf. Figs. 4A and 5). The pattern observed in the B1C8 immunoprecipitation was greatly simplified compared with that observed in the Y12 immunoprecipitation. Specifically, in experiments involving B1C8, five cross-linked polypeptides were precipitated after a 50-min incubation in nuclear extract in the presence of ATP: p14, p35, p70, p150, and p220 (Fig. 5, lane 8). In addition, a p80 species was cross-linked and immunoprecipitated at early times (see below) in an apparently ATP-independent fashion (Fig. 5, lane 7). Three of the cross-links (p14, p35, p220) were clearly ATP dependent (cf. lanes 7 and 8). In addition, the appearance of both the p150 and p70 species in ATP-dependent splicing complexes (see below) means that cross-linking to these proteins is ATP dependent. All of the cross-linked proteins immunoprecipitated by mAb B1C8 were constituents of complexes to which the antigen recognized by the monoclonal was bound. Immunoprecipitation of reactions with B1C8 af-

ter treatment with RNase A did not yield photo-labeled species (data not shown).

The time course of cross-linking to proteins immunoprecipitated with B1C8 (Fig. 5, lanes 9–21) demonstrated that these species were dependent on incubation at  $30^\circ\text{C}$ : p14, p35, and p150 all demonstrated similar kinetics, appearing early and increasing with time. The p35 and p150 cross-links decreased at later times while the p14 cross-link persisted. The p220 cross-link appeared later and increased with time. The kinetics of the cross-linking to p70 were difficult to assess from this time course because of comigrating background bands. Interestingly, at early time points a strong cross-link to a polypeptide, p80, was noted: This cross-link diminished in intensity after incubation for 2–20 min.

#### *Analysis of proteins within splicing complexes*

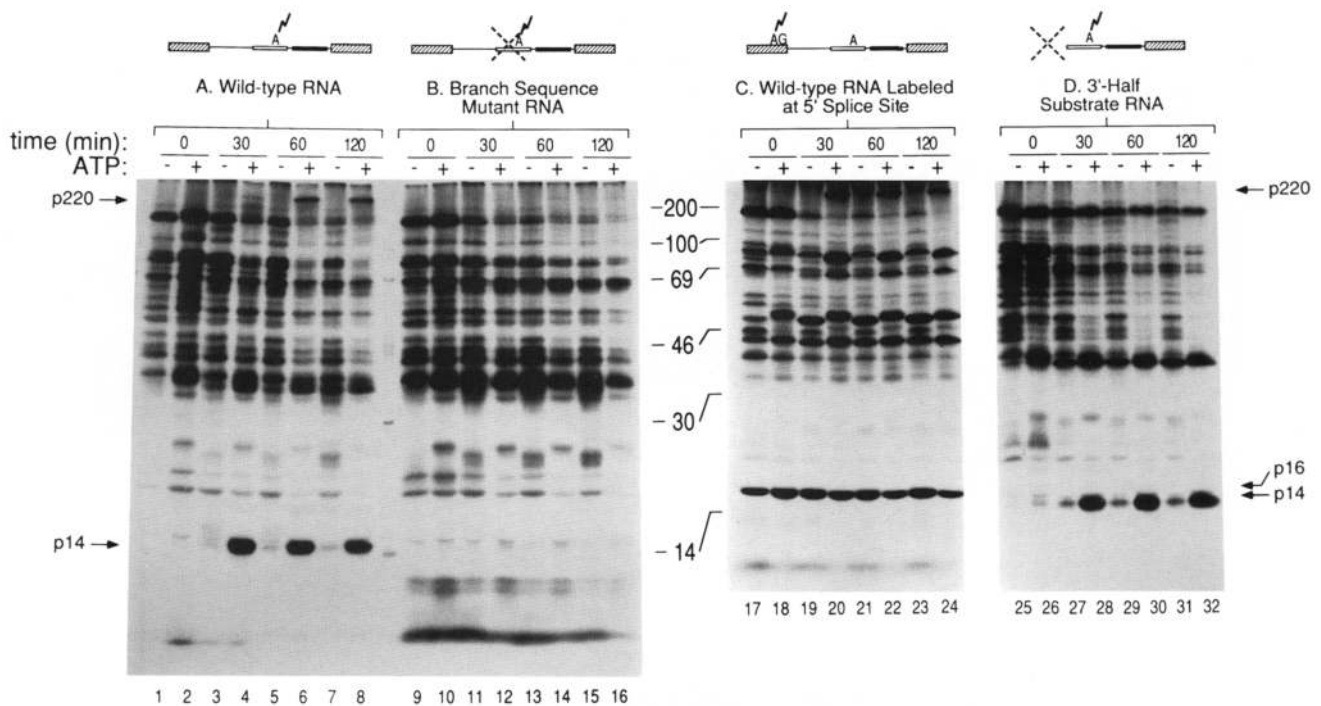
To address which of the splicing complexes, A, B, or C, contain particular cross-linked proteins, branch site-modified pre-mRNA was incubated under splicing conditions and cross-linked as above, and then individual splicing complexes were isolated from native gels. These were RNase digested, and the cross-linked proteins were separated by SDS-PAGE (Fig. 6).

First, the majority of proteins not selected by B1C8 immunoprecipitation (Fig. 5) remained in H complex. Second, the p14, p35, p70, p150, and p220 proteins discussed above were all cross-linked to the branch region in splicing complexes (Fig. 6, lanes A,B/C). The p14, p35, and p150 species were present within complex A and were not detectable in complex H. The p14 species persisted in complexes B and C, whereas the efficiency of cross-linking to p35 and p150 diminished in B and C. The p220 and p70 species were cross-linked only in complexes B and C, and not in complex A. One other protein, p25, was cross-linked only within complex A; generation of this band was proteinase K sensitive (data not shown), confirming that it contains a polypeptide. However, this species was not detected in other assays and may represent a proteolysis product of a larger protein. The p80 species was not observed cross-linked in splicing complexes, consistent with its early time of cross-linking (see above).

Cross-linked proteins were also analyzed from isolated splicing complexes using branch site-modified RNA deleted of the 5' exon and 5' portion of the intron. Such RNAs form an A-like complex (Konarska and Sharp 1986), termed A3' complex. Cross-links to the same proteins, p14, p25, p35, and p150 were observed in A3' complex as in complex A from full-length RNA (Fig. 6, cf. lanes A3' and A). The remarkable similarity in the cross-links observed with the intact pre-mRNA substrate and the 3' half-substrate suggests that the A3' complex is analogous to the actual intermediate in the splicing pathway.

#### *snRNP and factor dependence of cross-linked proteins*

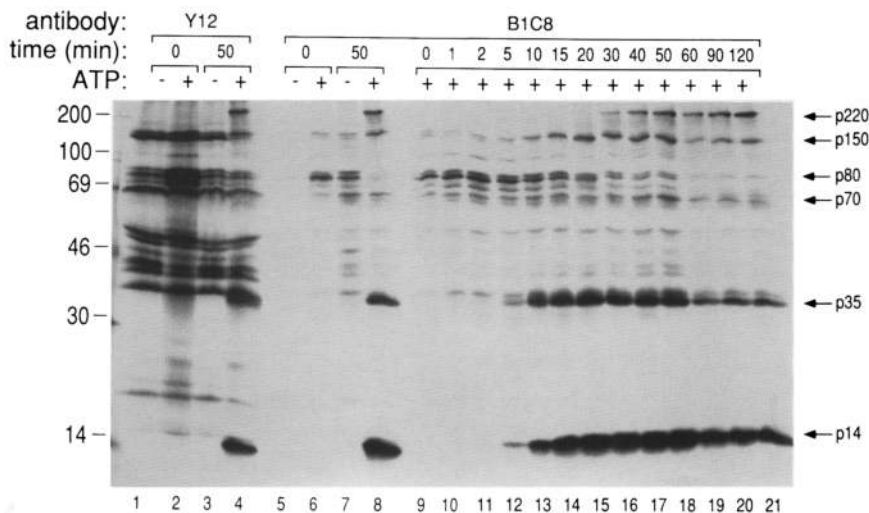
For additional evidence that the proteins observed cross-



**Figure 4.** Cross-linking of RNAs derivatized with benzophenone. (A, lanes 1–8) Branch site-modified pre-mRNA. (B, lanes 9–16) Substrate modified within a scrambled branch sequence. (C, lanes 17–24) Pre-mRNA derivatized two nucleotides 5' to the 5' splice site. (D, lanes 25–32) 3'-Half substrate derivatized within the branch sequence. Icons (*top*) indicate the RNA substrate; (hatched boxes) Exons; (open boxes) the branch regions; (solid bar) the polypyrimidine tract; (large X) a mutated or deleted region. ( $\zeta$ ) The N<sup>6</sup>-modified adenosine derivatized with benzophenone maleimide.

linked to the branch site are related to the splicing process, nuclear extracts were depleted of various snRNPs (Barabino et al. 1990; Blencowe and Barabino 1994) or of other known splicing factors and assayed in cross-linking assays as described above. These extracts did not form mature spliceosomes but, when mixed or supplemented with the depleted factors, complemented for spliceosome formation and for splicing (data not shown;

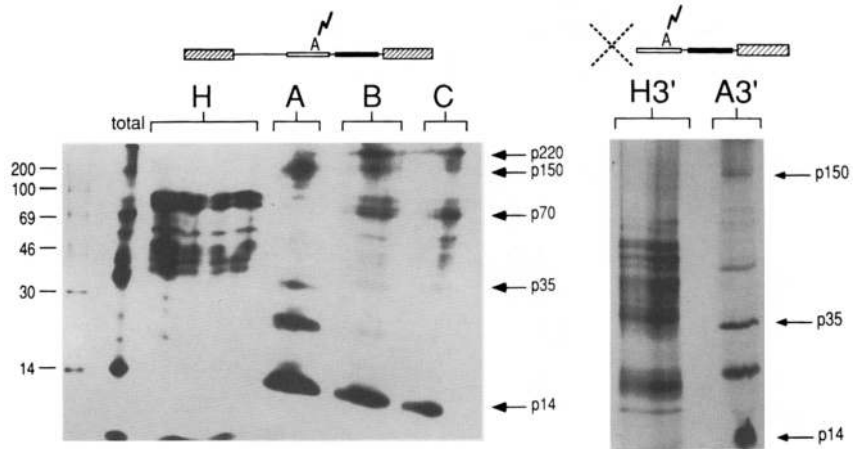
for an analysis of these specific extracts, see Crispino et al. 1994). Extracts depleted of U1 or U2 snRNPs (Fig. 7, lanes 4,6) were greatly reduced in cross-linking of p14 to the branch site relative to a mock-depleted extract (lane 2), whereas a mixture of these two extracts complemented for cross-linking to p14 (lane 10) as they complemented for splicing. Consistent with the appearance of the p14 cross-link in complex A, a U4/6 snRNP-de-



**Figure 5.** Proteins cross-linked to the branch region substrate within spliceosomal complexes immunoprecipitated with the anti-Sm mAb Y12 and mAb B1C8, which immunoprecipitates spliceosomal complexes. ATP and incubation dependence (lanes 1–4, Y12; lanes 5–8, B1C8) and time course of B1C8 immunoprecipitation in the presence of ATP (lanes 9–21).



**Figure 6.** Two-dimensional analysis of cross-linked proteins from splicing complexes. Pre-mRNAs modified at the branch site were incubated under splicing conditions, UV irradiated and separated on native gels. Isolated splicing complexes were subsequently treated with RNase and the proteins separated on 16% (200:1) polyacrylamide gels. Proteins were cross-linked at the branch site using either full-length RNA (*left*) or RNA deleted of the 5' exon and 5' splice site region (*right*).



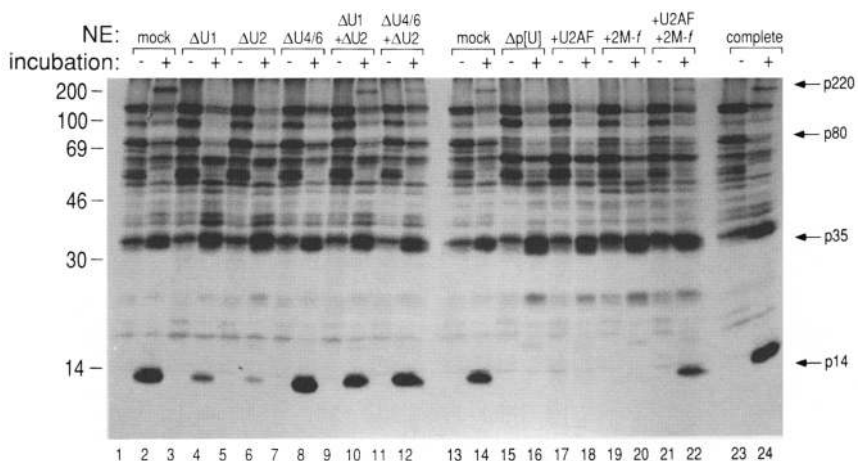
pleted nuclear extract generated the p14 cross-link (lane 8). The cross-link to p220 was not efficient in extracts depleted of U1, U2, or U4/6 snRNPs (lanes 4,6,8) but was efficient when any two of these were mixed (lanes 10,12), consistent with the appearance of this species in complexes B and C and not in complex A. Finally, the p80 cross-link observed at very early times in the B1C8 precipitations required U1 snRNP (cf. lanes 2 and 4; data not shown) but not U2 or U4/U6 snRNPs.

Nuclear extract depleted of polyuridylylate [poly(U)]-binding proteins requires, in order to restore splicing and formation of splicing complexes, both U2AF<sup>65</sup> (Ruskin et al. 1988; Zamore and Green 1991) and components of a 2 M KCl fraction (Patton et al. 1991; P.S. McCaw and P.A. Sharp, unpubl.). This depleted extract did not generate the p14 or p220 cross-links to the branch site (cf.

lanes 14 and 16). Addition of either recombinant U2AF<sup>65</sup> or the 2 M KCl fraction to the depleted extract was not sufficient to restore the cross-links (lanes 18,20). However, addition of both components to the depleted extract did restore both splicing and the p14 and p220 cross-links (lane 22).

Thus, requirements for cross-linking of p14 were the same as for complex A formation [i.e., U1 and U2 snRNPs, U2AF<sup>65</sup> and component(s) of the 2 M KCl fraction], whereas the requirements for the cross-linking of p220 correlated with complex B formation. The p80 cross-link required U1 snRNP but was not present in complex A (see above). These results are consistent with the kinetics of formation of the complexes and cross-links and suggest that binding of the p80 species precedes formation of complex A. Furthermore, the specific

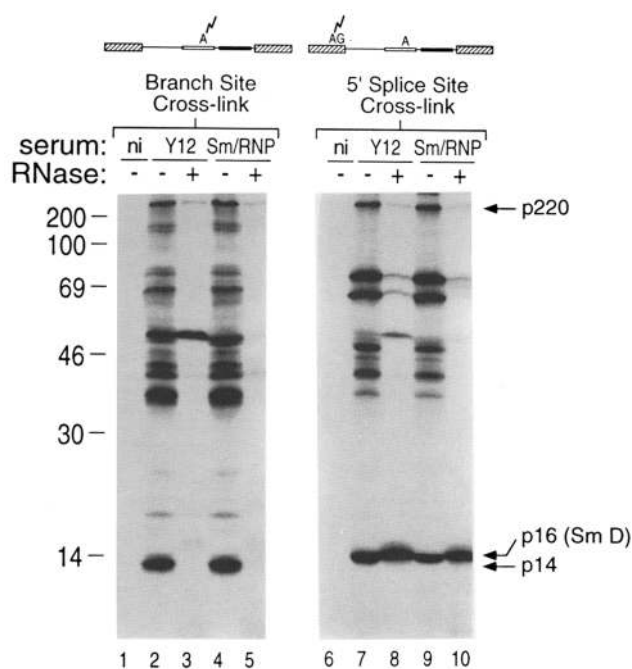
**Figure 7.** Cross-linking to the branch site-modified RNA in nuclear extracts depleted of various splicing factors. Branch site-modified RNA was incubated with depleted extracts at 30°C (+) or on ice (-) for 90 min, UV irradiated, treated with RNase, and separated on a 16% (200:1) polyacrylamide gel. Parallel analysis of the pre-mRNA demonstrated that the depleted extracts did not support splicing but that the appropriate mixtures of extracts and factors restored splicing (see also Crispino et al. 1994). (Lanes 1–12) Reactions contained nuclear extracts that were mock-depleted (lanes 1,2), depleted of U1 snRNP (lanes 3,4), depleted of U2 snRNP (lanes 5,6), depleted of U4/6 snRNP (lanes 7,8), or contained a mixture of U1-depleted and U2-depleted extracts (lanes 9,10), or a mixture of U2-depleted and U4/6-depleted extracts (lanes 11,12). (Lanes 13–22) Reactions contained nuclear extracts that were mock-depleted (lanes 13,14), depleted of poly(U)-binding proteins (lanes 15,16), or contained a mixture of poly(U)-depleted extract and recombinant U2AF<sup>65</sup> (lanes 17,18), a mixture of poly(U)-depleted extract and the 2 M KCl fraction (lanes 21,22), or poly(U)-depleted extract supplemented with mixture of recombinant U2AF<sup>65</sup> and the 2 M KCl fraction (lanes 21,22). (Complete) Nondepleted nuclear extract as in all other figures (lanes 23,24). p70 and p150 are not indicated, as they are not clearly distinguished from background cross-links.



dependence of cross-links on snRNPs or other splicing factors is strong evidence that these proteins are related to the splicing process.

#### Comparison of branch and 5' splice site cross-linking through immunoprecipitation

The pattern of cross-linking observed with the photo-probe proximal to the 5' splice site was similar to that observed previously with a 4-thiouridine probe, also placed two nucleotides 5' to the 5' splice site (Wyatt et al. 1992). This cross-linking pattern also resembled that observed with the photo-probe at the branch region, specifically with respect to labeling of a p220 and a small polypeptide in the vicinity of molecular mass 16 kD. To compare the cross-linking patterns at these two sites more closely, the two cross-linking reactions were immunoprecipitated with anti-Sm mAb Y12 and also with an Sm/U1 RNP-specific patient serum (Wilusz and Keene 1986). The p16 cross-link was precipitated efficiently by both Y12 and anti-Sm/U1 RNP serum even after RNase treatment of the cross-linking reaction mixture (Fig. 8, lanes 7–10), consistent with the identification of this species as the snRNP core protein Sm D



**Figure 8.** Comparison of cross-links to the 5' splice site and branch region by immunoprecipitation with anti-Sm antibody Y12 and anti-Sm/RNP patient serum. (Lanes 1–5) Immunoprecipitation of polypeptides cross-linked to the branch region with nonimmune serum (ni; lane 1), Y12 (lanes 2,3), and Sm/RNP serum (lanes 4,5) with RNase treatment after (–; lanes 1,2,4) and before (+; lanes 3,5) immunoprecipitation. (Lanes 6–10) Immunoprecipitation of polypeptides cross-linked two nucleotides 5' to the 5' splice site with nonimmune serum (lane 6), Y12 (lanes 7,8), and Sm/RNP serum (lanes 9,10) with RNase treatment after (–; lanes 6,7,9) and before (+; lanes 8,10) immunoprecipitation.

(Wyatt et al. 1992). In contrast, the branch site p14 cross-link could not be directly precipitated by either Y12 or anti-Sm/U1 RNP serum after RNase treatment (lanes 3,5), although it could be immunoprecipitated prior to treatment with RNase (lanes 2,4). These results suggest that whereas p14 is a component of the spliceosome, it is distinct from Sm D.

The p70 species photolabeled at late times in the reaction is similar in molecular weight to the U1–70K protein. Because the Sm/U1 RNP-specific patient serum immunoprecipitates U1–70K (Query 1990) the absence of a 70K band in reactions analyzed with this serum after RNase treatment indicates that the cross-linked p70 species is not U1–70K.

#### Discussion

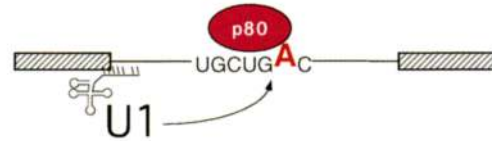
Although it is widely believed, in analogy to group II ribozymes, that the transesterification steps of pre-mRNA splicing are catalyzed by snRNA components (for review, see Nilsen 1994), protein factors are critical for both spliceosomal assembly and progression through the chemical steps. We have developed a general and powerful method for the introduction of photo-probes into the pre-mRNA and have identified a discrete set of proteins that interact with the branch region of the substrate through the complex process of spliceosomal assembly (Fig. 9).

#### General strategy for the synthesis of RNA-based affinity reagents

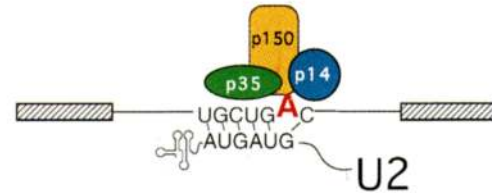
In this study short oligomers containing a single modified adenosine residue were synthesized, incorporated into full-length RNAs, and derivatized with the photo-probe benzophenone. This approach is highly versatile. The general methodology can be readily applied to the functionalization of A, C, or G residues; within the context of helical duplexes this provides access to both the major and minor grooves (Fig. 1C). Both the tether length and appended functionality may be varied easily. In the present case, a reagent was synthesized with sufficient reach to effectively probe the space within 15 Å of the branch site. Benzophenone derivatives have been used previously as cross-linking reagents to characterize both RNA–ligand (Barta et al. 1984) and RNA–protein interactions (Musier-Forsyth and Schimmel 1994; for a general review, see Dormán and Prestwich 1994). The reagent has several advantages over other classes of photo-active groups. It is chemically stable and not particularly sensitive to exposure to ambient light, in contrast to both aryl azides and thionucleosides. The excitation wavelength (>320 nm) results in relatively low background in experiments involving nucleic acids. Finally, efficiency of cross-linking is high, as the excited  $\pi^*$  triplet state prefers to react with C–H bonds over O–H; thus, in the absence of a suitable acceptor, the excited probe relaxes to its initial state rather than being quenched by water. In this study cross-linking efficiencies of 30% were routinely observed.



## Early

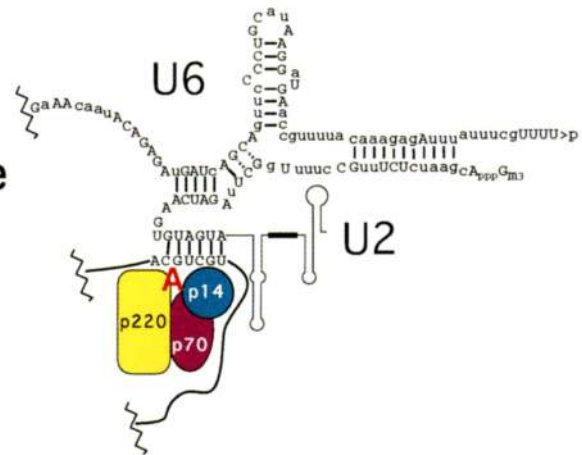


## Complex A



**Figure 9.** Schematic of cross-links to the branch region at early times (*top*), in the U2 snRNP-pre-mRNA complex A (*middle*), and within the context of the spliceosome (*bottom*), representing the rearrangement of proteins detectable during splicing complex assembly. (*Top*) At early times, a p80 species interacts with the branch region dependent on U1 snRNP. (*Middle*) In complex A, the p80 is displaced, and three new interactions appear: p14, p35, and p150. (*Bottom*) The addition of U4/5/6 tri-snRNP to form the spliceosome results in displacement of the p35 and p150 species by p220 and p70. The p14 remains through both complex A and the spliceosome. Only the regions of branch region/U2 snRNA and U2/U6 snRNA base-pairings are depicted; for review of other snRNA/snRNA interactions, see Moore et al. (1993) and Nilsen (1994). Interactions in complex A and in the spliceosome are also dependent on U1 snRNP, but these are not depicted for simplicity.

## Spliceosome



## Cross-linking to the branch site

Several proteins cross-linked to the branch region of pre-mRNA in an efficient, time-dependent, and ATP-dependent fashion. Furthermore, the proteins were only labeled in the context of a substrate capable of forming spliceosomal or prespliceosomal complexes. A time course of immunoprecipitation of spliceosome-associated proteins using mAb B1C8 revealed three distinct kinetic groups. The analysis of cross-linked proteins within splicing complexes, as well as in depleted extracts, placed these three groups within the context of discrete steps in spliceosomal assembly: an early complex, the U2 snRNP-containing complex A, and the spliceosomal complexes B and C (Fig. 9).

The cross-linked p80 species is remarkable for both its early ATP-independent appearance and subsequent disappearance in the 2- to 20-min time period. This cross-link was not detected in the analysis of splicing complexes, suggesting that it is associated with steps prior to the formation of the U2 snRNP-containing complex A. Furthermore, this cross-link was dependent on the pres-

ence of U1 snRNP but not U2 or U4/U6 snRNPs. Studies in the yeast system have suggested two U1 snRNP-dependent pre-A commitment complexes: CC1, which assembles in the presence of a 5' splice site alone, and CC2, which requires both a 5' splice site and a branch region (S eraphin and Rosbash 1989, 1991). An analogous complex may be represented by the E complex described in mammalian systems (Michaud and Reed 1991; Jamison and Garcia-Blanco 1992). The results in yeast suggest that in addition to an early U1-5' splice site interaction, there is an early U1 snRNP-mediated recognition of the branch region through unidentified factors (Ruby and Abelson 1988; S eraphin and Rosbash 1991). The interaction of the p80 species with the branch site may represent such an early step involving communication between U1 snRNP and the branch region.

Three of the branch cross-links, p14, p35, and p150, appear in complex A with similar kinetics between 20 and 60 min of incubation. These proteins further resolve into two groups, with p14 persisting through the B and C complexes while cross-linking to p35 and p150 decreases. The requirement of both U1 and U2 snRNPs but

not U4/U6 snRNP as well as the requirement for both U2AF<sup>65</sup> and the 2 M KCl fraction are consistent with the initial appearance of the p14 cross-link in complex A. Several protein factors have been reported to be important for formation of the U2 snRNP-branch region complex. Although U2AF<sup>65</sup> is required for the stable association of U2 snRNP with the pre-mRNA, the U2AF<sup>65</sup> protein may not remain with this complex (Staknis and Reed 1994). The p150 species may represent a protein of similar molecular mass in the 17S U2 particle (Behrens et al. 1993b) and is thought to be a component of SF3b (Brosi et al. 1993b; Staknis and Reed 1994; for review, see Hodges and Beggs 1994). The p35 cross-link is reminiscent of a factor recently reported to interact with the pre-mRNA at the 3' splice site in A and B complexes (S. Wu and M.R. Green, pers. comm.); thus, it is possible that the 3' splice site and branch region are close to each other in early steps of assembly. It is notable in this regard that the substrates used in both of these studies contained 3' splice site AG-dependent introns. The cross-link to p14 is remarkable both in its intensity (~10% of total cross-linking) and persistence through the A, B, and C complexes. This persistence suggests that p14 may be an RNA-binding protein with specificity for the structure of the branch region paired to U2 snRNA.

The recruitment of the U4/5/6 tri-snRNP completes the addition of snRNP components to the spliceosome in complex B; complex C is associated with the transesterification steps. Cross-links of p220 and p70 to the branch region were associated with the B and C complexes. The p220 is almost certainly the U5-associated factor, the largest identified factor in the spliceosome and a homolog of the yeast PRP8 (Anderson et al. 1989; Garcia-Blanco et al. 1990). In this and other studies, p220 has also been cross-linked to sites proximal to the 5' splice site (Wyatt et al. 1992). It is possible that p220 plays a role in conjunction with U5 snRNA in the displacement of U1 snRNP and in branch site-5' splice site juxtaposition. Finally, it has been suggested that a p35 interaction near the 3' splice site (see above) is followed by an interaction of this site with a p70 species, the kinetics of which correlate with formation of complex C after the first step (S. Wu and M.R. Green, pers. comm.); the p70 cross-link noted here displays similar characteristics.

#### *Rearrangements of factors about the branch nucleophile*

The interactions with the branch region described above are dynamic through all the detectable stages of spliceosome assembly (Fig. 9). First, a p80 species interacts with the pre-mRNA very early in a manner dependent on U1 snRNP. Second, concomitant with the stable binding of U2 snRNP, this p80 is displaced and three new cross-links appear: p14, p35, and p150. These constitute the interacting species within complex A, in which the branch nucleophile has presumably been selected. Third, in the transition from complex A to the mature spliceosome, the addition of U4/5/6 tri-snRNP results in a re-

assortment of protein factors about the branch region: p35 and p150 are replaced by p220 and p70 while the strongest cross-linking p14 species remains. These interactions occur just before or after the first transesterification reaction.

A number of key spliceosomal RNA rearrangements occur within the context of complex B, notably the disruption of a U4-U6 snRNA interaction followed by the establishment of a U2-U6 snRNA interaction in the vicinity of the branch region/U2 snRNA duplex. Mutations affecting this U2-U6 interaction affect both steps of splicing, implicating the U2-U6 structure in the catalysis of the transesterifications (Madhani and Guthrie 1992, 1994; McPheeters and Abelson 1992). Clearly, protein rearrangements about the branch region parallel the snRNA-snRNA rearrangements that occur during splicing complex assembly. Such rearrangements of proteins and RNAs are likely processes that build the catalytic core of the spliceosome.

## Materials and methods

### *RNA transcription*

RNA(1-145) was transcribed from plasmid pPIP85.B (Query et al. 1994) cleaved with *Xho*I. DNA templates for transcription of RNA(1-55), RNA(1-103), and RNA(1-156) were generated by PCR with an M13 forward primer and another primer whose 5' end corresponded to the transcriptional runoff site using pPIP85.B as PCR template. Templates for RNA(56-234), RNA(104-234), RNA(146-234), and RNA(157-234) were generated by PCR with one deoxyoligonucleotide primer that inserted a T7 promoter immediately 5' to the desired transcriptional start site and another whose 5' end corresponded to the transcriptional runoff site. Template for RNA(mutPPT,180-234) was generated by PCR to replace the pPIP85.B polypyrimidine tract (157-179) with 5'-GGGACGGACATGCAATGCAACTC-3'. Transcriptions were performed under standard conditions (see Moore and Sharp 1992; Query et al. 1994).

### *Synthesis of substrates*

The oligoribonucleotides containing the convertible nucleoside were synthesized according to standard procedures on an ABI instrument utilizing a 0.15 M solution of the O<sup>6</sup>-(4-chlorophenyl)-inosine phosphoramidite (C.R. Allerson, S. Chen, and G.L. Verdine, unpubl.). The RNAs were deprotected with a 4 M solution of methanolic cysteamine disulfide for 4 hr at room temperature followed by 12 hr at 55°C. The deprotection reactions were neutralized with glacial acetic acid, lyophilized, desilylated with 1 M tetrabutylammonium fluoride (room temperature, 24 hr), desalted (Waters C-18 Sep Pak), and then gel purified.

Two- and three-way RNA ligation reactions and gel purification of products were performed as described previously (Moore and Sharp 1992; Query et al. 1994); in addition, following the ligation reaction the mixture was treated with 3.5 volumes of Ellman's reagent, 5,5'-dithio-bis(nitrobenzoic acid), (5 mM; 40% DMSO) at 30°C for 30 min before gel purification to insure protection of the reactive sulfur group from oxidation.

Branch site-modified RNA was prepared by joining RNA(1-145), the branch decamer [5'-GGGUGCUG(A\*)C-3'], and 5'-<sup>32</sup>P-phosphorylated RNA(157-234) using a complementary deoxy-oligonucleotide [cDNA(169-136): 5'-GAGAGAAGAAGCCAGTCAGCACCCTCGAGACGAG-3']. Branch site-labeled

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(but unmodified) RNA was prepared by joining RNA(1–156) and 5'-<sup>32</sup>P-phosphorylated RNA(157–234) using cDNA(169–136). Mutant branch sequence RNA was prepared by joining RNA(1–145), the scrambled decamer [5'-GGUGUCGC(A\*)G-3'], and 5'-<sup>32</sup>P-phosphorylated RNA(157–234) using cDNA(5'-GAGA-GAAGAAGCCACTGCGACACCTCGAGACGAG-3'). Branch (-50)-modified RNA (a pre-mRNA modified midway between the branch region and 5' splice site) was prepared by joining RNA(1–103), the scrambled decamer, and 5'-<sup>32</sup>P-phosphorylated RNA(104–234) using cDNA(5'-CCCTCGGAAACCCCT-GCGACACCTGGACTACTCTAGAAG-3'). Pre-mRNA modified at the 5' splice site was prepared by joining RNA(1–55), the scrambled decamer, and 5'-<sup>32</sup>P-phosphorylated RNA(56–234) using cDNA(5'-GATCCATACTCACCTGCGACACCTGG-TAGGGTAC-3'). RNA deleted of the 5' exon and 5' splice site region was prepared by joining the branch decamer, and 5'-<sup>32</sup>P-phosphorylated RNA(157–234) using cDNA(169–136). The RNA containing a mutant polypyrimidine tract was prepared by joining RNA(1–145), the branch decamer, and 5'-<sup>32</sup>P-phosphorylated RNA(mutPPT, 180–234) using cDNA(5'-GCATGTC-CGTCCCAGTCAGCACCCCTCGAGACGAG-3').

#### Derivatization of full-length RNAs

The gel-purified full-length pre-mRNA substrates were reduced by treatment with 5 mM DTT (20 mM NaHCO<sub>3</sub>, 30°C, 1 hr) and then derivatized by reaction with 20 mM benzophenone maleimide (Molecular Probes) in 50% dimethyl formamide at room temperature for 1 hr. Reactions were extracted with phenol/chloroform and chloroform and then ethanol precipitated.

#### Nuclear extracts

Nuclear extracts were prepared from HeLa cells as described by Dignam et al. (1983). Extracts depleted of individual snRNPs were generous gifts from John Crispino and were prepared as described (Barabino et al. 1990; Blencowe and Barabino 1994). Extracts depleted of poly(U)-binding proteins and U2AF<sup>65</sup> were a generous gift from Patrick McCaw and were prepared by dialyzing nuclear extract directly into 1 M KCl/buffer D (20 mM HEPES at pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.05% NP-40, 0.5 mM DTT). The resulting extract was passed over a poly(U)-Sepharose 4B column (Pharmacia) at 0.1 ml/min and subsequently dialyzed against 0.1 M KCl/buffer D. After washing this column with 1 M KCl/buffer D, the column was eluted with buffer D containing 2 M KCl; the eluate was dialyzed against 0.1 M KCl to obtain the "2 M KCl fraction." Recombinant His<sub>6</sub>-tagged U2AF<sup>65</sup> was prepared from a 60% ammonium sulfate precipitate of a soluble *Escherichia coli* lysate. This was loaded onto a Ni<sup>2+</sup>-NTA-agarose column (Qiagen), eluted with 250 mM imidazole/buffer D, and dialyzed into 0.1 M KCl/buffer D.

#### Photo-cross-linking assays

High specific activity photo-active substrates (10<sup>5</sup> cpm/reaction) were incubated in HeLa nuclear extract under splicing conditions as described previously (Grabowski et al. 1984) except that RNasin was omitted. Cross-linking was performed on ice by irradiation with a 302-nm lamp (0.12 W/cm<sup>2</sup> at 1 cm; Ultraviolet Products) for 12 min. Reactions were then digested with 20 µg of RNase A (Boehringer) and/or 20 units of RNase T1 (Calbiochem) for 30 min at 30°C and analyzed by SDS-PAGE (16%; 200:1).

#### Immunoprecipitations

Ten microliters of rabbit anti-mouse IgG + IgM (Pierce) was incubated with 40 µl of protein A-Sepharose (PAS; Pharmacia) in 300 µl of Ip buffer (10 mM Tris at pH 7.6, 150 mM KCl, 0.1% NP-40) at 4°C for 2 hr and washed three times with Ip buffer. The beads were then incubated with 20 µl of primary antibody or serum at 4°C for 2 hr and washed three times with Ip buffer. Splicing reactions and cross-linking were carried out as described above, and the reactions were added to suspensions of PAS-bound antibodies. After 3 hr of mixing at 4°C, samples were washed three times and then treated with RNase A and/or RNase T1 as described above, heated in loading buffer, and analyzed by SDS-PAGE. Alternatively, RNase was added after cross-linking but before immunoprecipitation.

#### Analysis of proteins from splicing complexes

Photo-cross-linked splicing reactions were prepared as described above except that the reactions were adjusted to 0.5 mg/ml of heparin prior to UV irradiation. Reactions were separated on 4% native polyacrylamide gels (Konarska and Sharp 1986) and frozen; the individual complexes, visualized by autoradiography, were excised. These were incubated with 320 µg of RNase A/ml of gel at 37°C overnight, incubated with SDS loading buffer at 37°C for 2 hr and 65°C for 5 min, and placed directly onto the stacking gel of a disassembled SDS 16% (200:1) polyacrylamide gel.

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