MOLECULAR AND CELLULAR BIOLOGY, May 1997, p. 2944–2953 Vol. 17, No. 5
0270-7306/97/$04.00+0

A Minimal Spliceosomal Complex A Recognizes the Branch Site and Polypyrimidine Tract

CHARLES C. QUERY,† PATRICK S. MCCAW, AND PHILLIP A. SHARP*

Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139-4307

Received 16 December 1996/Returned for modification 22 January 1997/Accepted 24 February 1997

The association of U2 snRNP with the pre-mRNA branch region is a critical step in the assembly of spliceosomal complexes. We describe an assembly process that reveals both minimal requirements for formation of a U2 snRNP-substrate RNA complex, here designated the Amin complex, and specific interactions with the branch site adenosine. The substrate is a minimal RNA oligonucleotide, containing only a branch sequence and polypyrimidine tract. Interactions at the branch site adenosine and requirements for polypyrimidine tract-binding proteins for the Amin complex are the same as those of authentic prespliceosome complex A. Surprisingly, Amin complex formation does not require U1 snRNP or ATP, suggesting that these factors are not necessary for stable binding of U2 snRNP per se, but rather are necessary for accessibility of components on longer RNA substrates. Furthermore, there is an ATP-dependent activity that releases or destabilizes U2 snRNP from branch sequences. The simplicity of the Amin complex will facilitate a detailed understanding of the assembly of prespliceosomes.

The removal of introns from precursors to mRNA molecules (pre-mRNA) is catalyzed by the spliceosome, a dynamic 50S-60S complex composed of small nuclear RNAs (snRNAs) U1, U2, U5, and U4/6, as well as protein components (for review, see references 34, 39, 43, and 46). Such intron excision proceeds by way of two sequential transesterification reactions. The spliceosome assembles de novo on each substrate pre-mRNA, and several distinct intermediates in an assembly pathway can be observed in vitro. The E (early) or commitment complex contains U1 snRNP and non-snRNP protein factors (28, 42, 58). 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 U4/5/6 tri-snRNP with complex A. Complex C follows B after significant rearrangements and contains splicing intermediates (29, 30, 43).

The branch region contains the nucleophile for the first chemical step of splicing, and its recognition is required early in splicing complex assembly. U2 snRNP binds the pre-mRNA, in part, through U2 snRNA · branch region base pairing (48, 69, 73), and the first-step nucleophile is selected, in part, by virtue of being bulged from this duplex (51). Early branch site recognition in yeast requires U1 snRNP and a non-snRNP splicing factor, a component of which may be MUD2 (2, 55, 59). In mammals, factors SF3a and SF3b (both of which join 12S U2 to form 17S U2 snRNP), SF1, $U2AF^{65}$, $U2AF^{35}$, U1 snRNP, and members of a family of proteins containing arginine-serine dipeptide repeats (SR proteins; for review, see references 23, 40, and 66) are important for the stable association of U2 snRNP with the pre-mRNA (3, 6, 7, 9, 10, 33, 74). $U2AF^{65}$ binds specifically to polypyrimidine tracts (PPTs) in early complexes (24, 42, 56, 70). Another factor, poly(U)-binding factor 2 (PUF-2), which contains two more polypyrimidine-binding proteins, a p54 SR protein (14, 71) and p130, is also important for efficient complex A formation (41). Some of the components of SF3 have been shown to cross-link to the pre-mRNA upstream of the branch region and are suggested to tether or stabilize U2 snRNP binding to the pre-mRNA (13, 25). Within the branch region, but not at the adenosine, two proteins, BPS^{72} and BPS^{70}, have been cross-linked in E and A complexes, respectively (16, 52). At the branch site adenosine itself, three proteins have been detected in complex A within 15 Å: p14, p35, and p150 (38). One of these proteins, p14, can be directly photo-cross-linked to the branch site adenosine (52).

ATP is required at numerous points during the splicing process and probably for multiple distinct functions, although it is not involved directly in either of the two transesterification reactions (45). Phosphorylation and dephosphorylation of SR proteins are believed to occur, as well as structural rearrangements of the snRNAs (reviewed in references 23, 46, and 65). The earliest detected requirement for ATP is during the transition from complex E to complex A, when U2 snRNP joins the pre-mRNA (15, 29, 32, 37, 42, 50). Although this has been generally interpreted to indicate that U2 snRNP binding requires ATP, the exact mechanism is unclear. By analogy to known systems operative in the ribosome for the fidelity of translation, there have been many suggested steps of proofreading during the splicing process (11). The yeast protein PRP16 may be part of a proofreading-discard pathway that examines the branched nucleotide after chemical step 1, because mutant *prp16* alleles increase the rate of progression to the second step of splicing of certain nonadenine branches (12, 17).

In the present study, we have determined the minimal substrate requirements for formation of complexes containing U2 snRNP. A variety of criteria indicate that this minimal complex, the Amin complex, represents an accurate model for interactions with many factors influencing assembly of prespliceosome complex A. Amin complex formation is a more sensitive system, because it is more affected by subtle modifications than is complex A. Surprisingly, formation of the Amin complex does not require ATP, but the complex is subject to an ATP-dependent dissociation, which may reflect a fidelity

* Corresponding author. Mailing address: E17-529, Center for Cancer Research, Massachusetts Institute of Technology, 40 Ames St., Cambridge, MA 02139-4307. Phone: (617) 253-6421. Fax: (617) 253-3867. E-mail: sharppa@mit.edu.

† Present address: Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461.

mechanism normally operative at the time of prespliceosome assembly.

MATERIALS AND METHODS

RNA transcription and synthesis of substrates. pPIP85.B is a modification of pPIP85.A (44) that has only one adenosine in the branch region and that encodes the following 234-nucleotide sequence: 5′-GGGCGAAUUCGAGCUCACUCU CUUCCGCAUCGCUGUCUGCGAGGUACCCUACCAG ↓ GUGAGUAU GGAUCCCUCUAAAAGCGGGCAUGACUUCUAGAGUAGUCCAGGGU UUCCGAGGGUUUCCGUCGACGAUGUCAGCUCGUCUCGA**GGGUGC UGACUGGCUUCUUCUCUCUUUUUCCCUC**AG ↓ GUCCUACACAACAUA CUGCAGGACAAACUCUUCGCGGUCUCUGCAUGCAAGCU-3′. Arrows indicate the 5′ and 3′ splice sites, and underlining indicates the branch site. The boldface sequence represents RNA from positions 146 to 179 [RNA(146–179)], i.e., RNA containing a branch sequence and a PPT (BS-PPT). Transcription of this full-length pre-mRNA and of other RNAs was performed under standard conditions (51).

Two-way RNA ligation reactions and gel purification of products were performed as described previously (44, 51). Briefly, BS-PPT oligoribonucleotides [RNA(146–179)] were prepared by joining a branch region decamer [RNA(146–155); 5′-GGGUGCUGAC-3′] and a 5′-^{32}P-phosphorylated PPT [RNA(156–179); 5′-UGGCUUCUUCUCUCUUUUUCCCUC-3′] by using T4 DNA ligase (U.S. Biochemical Corp.) and a bridging oligonucleotide [cDNA(169–136); 5′-GAGAGAAGAAGCCAGTCAGCACCCTCGAGACGAG-3′]. PPT-BS RNA [RNA(156–179, 145–155)] was prepared by joining RNA(156–179) and a 5′-^{32}P-phosphorylated branch region decamer [RNA(146–155)] by using cDNA (5′-G TCAGCACCCGAGGGAAAAAGAGAGAAGAAGCC-3′). Ligation products were purified on 15% polyacrylamide (acrylamide-bisacrylamide, 29:1)–8 M urea gels run in 1× TBE (89 mM Tris-borate, 2 mM EDTA). All-RNA and 2,6-diaminopurine-containing branch region decamers were prepared by chemical synthesis as described previously (62). 2′-H-substituted branch region decamers and PPT-containing RNA(156–179) were prepared by chemical synthesis on an Expedite 8909 oligonucleotide synthesizer (by M. J. Moore) and were purified similarly. A branch region decamer containing a convertible adenosine for cross-linking experiments was described in reference 38.

Formation and native gel analysis of splicing complexes. To form splicing complexes, RNAs were incubated under standard splicing conditions (26) with nuclear extracts as described below; or, for ATP-depleted reactions, ATP and creatine phosphate were omitted from the mixes, which were preincubated for 15 min at 30°C to deplete endogenous ATP and, in some cases, then were adjusted to 10 mM EDTA. RNAs were then added and incubated at 30°C for the times indicated. Reactions were adjusted to 0.5 mg of heparin per ml and separated by electrophoresis in 50 mM Tris-glycine through nondenaturing 4% (80:1) polyacrylamide gels.

Nuclear extracts and purification of splicing factors. Nuclear extracts were prepared from HeLa cells as described previously (21). Extracts depleted of individual snRNPs were generous gifts from John Crispino, were prepared as described previously (6, 8), and were characterized in reference 18. Extracts depleted of poly(U)-binding proteins and U2AF^{65} were prepared by dialyzing nuclear extract directly into 1 M KCl–buffer D (20 mM HEPES [pH 7.9], 20% glycerol, 0.2 mM EDTA, 0.05% Nonidet P-40, 0.5 mM dithiothreitol). 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 this column had been washed 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, or PUF-2, which contains the poly(U)-binding proteins p54 and p130 (41). Mock-depleted extract was prepared in parallel to depleted extract by dialyzing nuclear extract into 1 M KCl–buffer D and subsequently dialyzing it against 0.1 M KCl–buffer D. Recombinant His$_6$-tagged U2AF^{65} was prepared from a 60% ammonium sulfate precipitate of a soluble *Escherichia coli* lysate. This was loaded onto a Ni^{2+}-nitrilotriacetic acid 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 substrate (10^7 cpm/reaction) containing an N_6-ethylthiol-modified adenosine was reduced by treatment with 5 mM dithiothreitol in 20 mM $NaHCO_3$ at 30°C for 1 h and then derivatized by reaction with 20 mM benzophenone maleimide (Molecular Probes) in 50% dimethyl formamide at room temperature for 1 h (38). Reactions were extracted with phenol-chloroform and chloroform and then ethanol precipitated. The RNA was incubated in HeLa nuclear extract as described above, except that RNasin was omitted, and was adjusted to 0.5 mg of heparin per ml prior to UV irradiation with a 302-nm-wavelength lamp (0.12 W/cm^2 at 1 cm; Ultraviolet Products) for 20 min on ice.

Alternatively, cross-linking of 2,6-diaminopurine-containing RNA was performed on ice by irradiation with a 254-nm-wavelength lamp (0.12 W/cm^2 at 1 cm; Ultraviolet Products) for 60 min. After either photo-cross-linking technique, reaction mixtures were separated on 4% (80:1) native polyacrylamide gels (29) and frozen; the individual complexes, visualized by autoradiography, were excised. These were incubated with 0.32 mg of RNase A/ml of gel in 125 mM Tris-HCl (pH 6.8) at 37°C overnight, incubated with sodium dodecyl sulfate (SDS) loading buffer at 37°C for 2 h and at 65°C for 5 min, and then placed directly onto the stacking gel of a disassembled SDS–16% polyacrylamide (acrylamide-bisacrylamide, 200:1) gel, which was reassembled and electrophoresed in 0.25 M Tris (pH 8.3)–0.192 M glycine–0.1% SDS.

RESULTS

A short oligonucleotide can form complexes with U2 snRNP. U2 snRNP complexes form on full-length pre-mRNAs (complex A) as well as on 3′ half RNAs that lack a 5′ splice site (A3′ complex [29]). These RNAs contain a number of elements, illustrated in Fig. 1A, believed to contribute to complex A formation and stability. 5′ to the branch site is a region that interacts with SF3a and SF3b components, which are believed to stabilize complex A (25). Surrounding the branch site is a region of U2 snRNA complementarity important for efficient complex formation (48, 69, 72, 73). The PPT interacts with several factors, including U2AF^{65} and PUF-2 (41, 56, 70), and exon enhancer sequences or downstream 5′ splice sites interact with SR and other proteins or U1 snRNP to promote U2AF^{65} binding and complex formation (27, 36, 61, 67). In addition, binding of U1 snRNP and other factors to the 5′ splice site probably stimulates complexes, and in a role that is not understood, U1 snRNP is also required for complex A formation independently of 5′ splice site interaction (6). To establish minimal requirements for this process, shorter RNAs, made by deletion from both ends of a 234-nucleotide model pre-mRNA, PIP85.B RNA (which contains a well-defined branch site with only one adenosine in the region [51]), were tested for formation of A-like U2 snRNP complexes (data not shown). The shortest RNA efficiently forming a complex that comigrated with complex A on native gels was RNA(146–179), a 34-nucleotide BS-PPT RNA (Fig. 1A, lower, and B). This complex is designated the Amin complex, since it represents an A-like complex on a minimal substrate. This substrate notably lacks several elements discussed above that presumably contribute to efficient complex A formation. It does not contain the region thought to be the binding site for SF3a and SF3b (25), nor does it contain any sequence 3′ to the 3′ splice site that could act as an exon enhancer element. In addition, it does not contain the 3′ splice site AG: comparison of RNAs either containing the 3′ splice site AG or with it deleted or containing a mutated 3′ splice site region did not show detectable differences in complex formation (data not shown). As indicated in Fig. 1B and discussed in depth below, formation of the Amin complex does not require ATP. In the analysis of truncated pre-mRNAs, RNAs containing additional sequences 3′ to the BS-PPT region also formed A-like complexes in the absence of ATP (e.g., Fig. 4C, lane 9), but RNAs containing additional sequences 5′ to the BS-PPT region did not [RNA(1–234), RNA(64–179), and RNA(104–179); Fig. 1B, lane 2, and data not shown].

Northern blot analysis of the Amin complex separated by native gel electrophoresis showed no detectable U1, U4, U5, or U6 snRNA in the complex; however, free 17S U2 snRNP (in the absence of BS-PPT RNA) migrates close to the Amin complex in this gel system, making evaluation of the U2 snRNA content of the Amin complex indeterminate (data not shown). To verify that the Amin complex contained U2 snRNP, the snRNA composition was analyzed by Northern blotting after streptavidin-agarose affinity selection with BS-PPT RNA containing 3′-terminal biotin [RNA(146–179, bio); Fig. 1C]. The Amin complex was highly enriched for U2 snRNA (lane 2) compared to all five snRNAs in spliceosomes formed on full-length pre-mRNA (lane 3). A small amount of U4, U5, and U6 snRNAs was selected (<5 to 10% of the level of U2 snRNA relative to full-length pre-mRNA); this may be related to

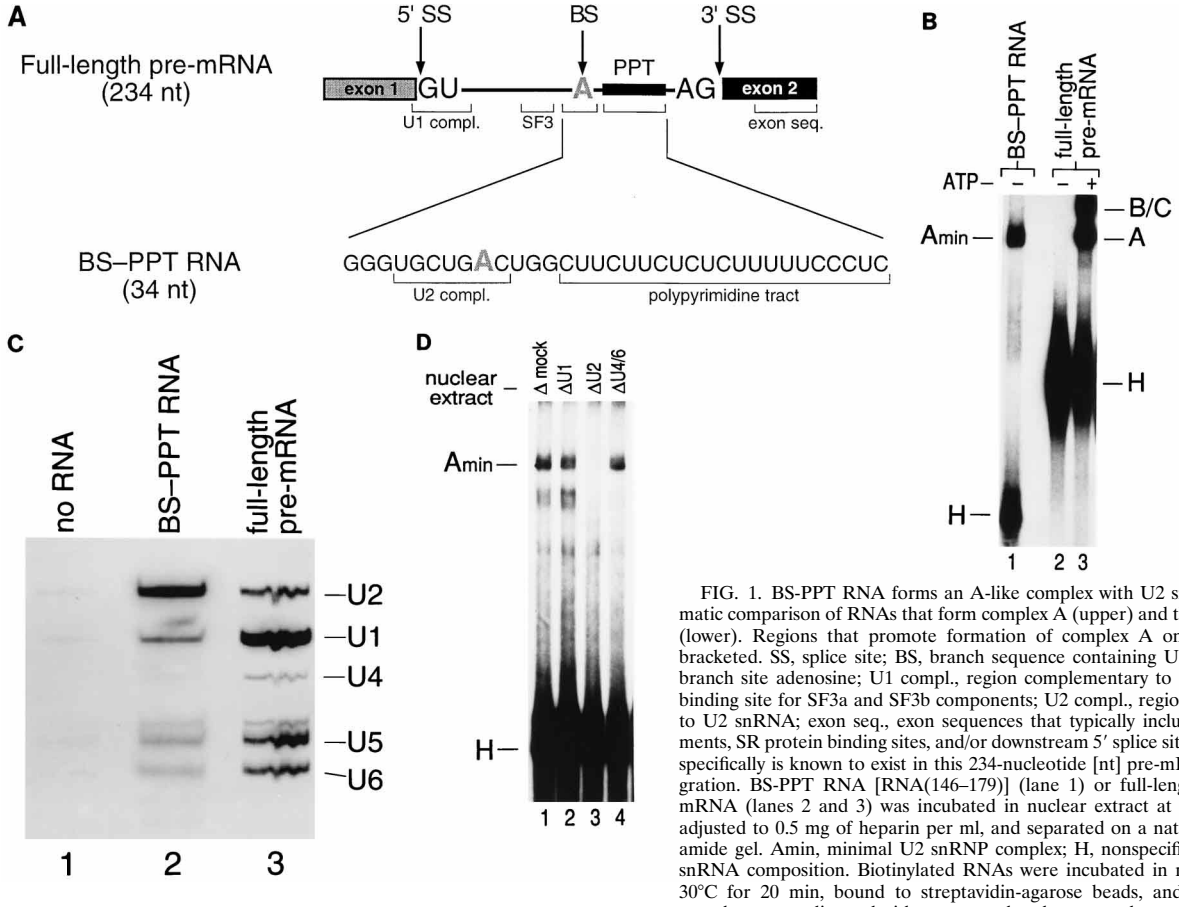

FIG. 1. BS-PPT RNA forms an A-like complex with U2 snRNP. (A) Schematic comparison of RNAs that form complex A (upper) and the Amin complex (lower). Regions that promote formation of complex A on pre-mRNA are bracketed. SS, splice site; BS, branch sequence containing U2 compl. and the branch site adenosine; U1 compl., region complementary to U1 snRNA; SF3, binding site for SF3a and SF3b components; U2 compl., region complementary to U2 snRNA; exon seq., exon sequences that typically include enhancer elements, SR protein binding sites, and/or downstream 5′ splice sites (none of which specifically is known to exist in this 234-nucleotide [nt] pre-mRNA). (B) Comigration. BS-PPT RNA [RNA(146–179)] (lane 1) or full-length PIP85.B pre-mRNA (lanes 2 and 3) was incubated in nuclear extract at 30°C for 20 min, adjusted to 0.5 mg of heparin per ml, and separated on a native 4% polyacrylamide gel. Amin, minimal U2 snRNP complex; H, nonspecific complexes. (C) snRNA composition. Biotinylated RNAs were incubated in nuclear extract at 30°C for 20 min, bound to streptavidin-agarose beads, and washed. Bound complexes were digested with protease, eluted, separated on a 10% (acrylamide-bisacrylamide, 19:1) polyacrylamide gel, transferred to Nytran, and probed with antisense RNA probes for U1, U2, U4, U5, and U6 snRNAs (30). Lane 1, beads alone; lane 2, biotinylated BS-PPT RNA [RNA(146–179, bio)]; lane 3, full-length PIP85.B pre-mRNA with biotin incorporated at random positions. (D) Dependence on snRNPs. BS-PPT RNA was incubated in mock-depleted extract (lane 1) or extracts depleted of U1 snRNPs (lane 2), U2 snRNPs (lane 3), or U4/6 snRNPs (lane 4) and analyzed as described for panel A.

larger, as yet uncharacterized complexes sometimes observed after long incubations (e.g., Fig. 3B, lanes 6 and 7, or 4B, lane 7).

U1 snRNA was also selected with biotin-tagged BS-PPT RNA; this was not unexpected, since formation of complex A on full-length pre-mRNA, as well as complex A3′ on 3′ partial RNA substrates, is dependent on both U1 and U2 snRNPs (6, 58). However, since the U1 snRNP association was not stoichiometric with U2 snRNP, the snRNP requirements for Amin complex formation were tested in extracts depleted of various snRNPs (6, 8). These extracts alone did not form mature spliceosomes on pre-mRNA, but when mixed, they complement for spliceosome formation and for splicing (data not shown; for an analysis of these specific extracts, see reference 18). In particular, the extracts depleted of either U1 or U2 snRNP did not form complex A on pre-mRNA (see Fig. 1 in reference 20). As expected, extracts depleted of U2 snRNP did not form the Amin complex (Fig. 1D, lane 3), and extracts depleted of U4/6 snRNP formed complexes just as well as mock-depleted extract (cf. lanes 4 and 1). Surprisingly, however, U1-depleted extracts also formed Amin complexes efficiently (cf. lanes 2 and 1); thus, the binding of U2 snRNP to the branch region per se does not require U1 snRNP.

Both BS and PPT are required. BS-PPT RNA contains two sequence elements—a BS (i.e., U2 complementarity region; 5′-UGCUGAC-3′, where the underlining represents the branch site adenosine) and a PPT (5′-CUUCUUCUCUCUUUUUCCCUC-3′) (Fig. 1A, lower). To investigate the individual contributions of each of these elements, we tested RNAs containing mutations in each element (Fig. 2). RNAs containing a mutated (double underlining) branch sequence (5′-. . .UGCUGAC. . .-3′→5′-. . .GUCGUAC. . .-3′) did not form the Amin complex (Fig. 2A, lane 3). Similarly, RNAs in which the PPT was replaced by 5′-. . .GACGGACAUGCAAUGCAACUC-3′ did not form the Amin complex (lane 2). Furthermore, RNAs containing shorter PPTs did not form complexes with U2 snRNP as efficiently. For example, removal of 7 or 14 pyrimidines from the 3′ end [RNA(146–172) or RNA(146–165), respectively] or an internal deletion [RNA(146–155, 169–179)] in the PPT resulted in significantly less complex (data not shown). These data suggest that both sequence elements make specific contributions to Amin complex formation, as expected for an analog of complex A.

Both elements, the BS and the PPT, were required in *cis*. As expected from the mutations tested above, neither sequence alone formed A-like complexes (Fig. 2B, lanes 1 and 6). When added in *trans*, they also could not form complexes: labeled BS RNA mixed with unlabeled PPT RNA did not form detectable complexes (Fig. 2B, lanes 2 to 5); similarly, unlabeled BS RNA mixed with labeled PPT RNA also did not form detectable complexes (lanes 7 to 10). We next tested the ability of each of the two RNAs to compete with BS-PPT RNA in complex formation. Although neither BS RNA nor PPT RNA formed a stable complex alone, PPT RNA did compete with BS-PPT

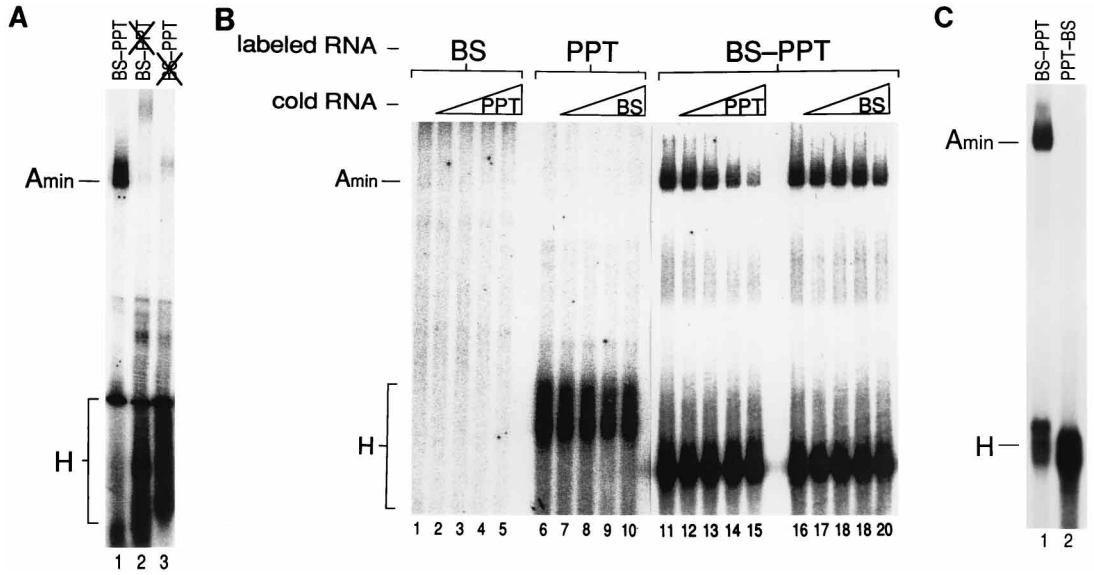

FIG. 2. Both BS and PPT are required in *cis*. (A) RNA(146–179) with wild-type BS and PPT (BS-PPT RNA; lane 1), with a mutated PPT (5′-. . .CUUCUUCU CUCUUUUUCCCUC-3′→5′-. . .GACGGACAUGCAAUGCAACUC-3′; lane 2), or with scrambled BS (double underlining) (5′-. . .UGCUGAC. . .-3′→5′-. . .GU CGUAC. . .-3′; lane 3) were incubated in nuclear extract at 30°C for 20 min and analyzed as described for Fig. 1B. (B) Labeled BS RNA (5′-GGGUGCUGAC-3′; lanes 1 to 5), labeled PPT RNA (5′-UGGCUUCUUCUCUCUUUUUCCCUC-3′; lanes 6 to 10), or labeled BS-PPT RNA (lanes 11 to 20) was incubated in nuclear extract at 30°C for 20 min in the presence of 0 μM (lanes 1, 6, 11, and 16), 0.001 μM (lanes 2, 7, 12, and 17), 0.01 μM (lanes 3, 8, 13, and 18), 0.1 μM (lanes 4, 9, 14, and 19), or 1 μM (lanes 5, 10, 15, and 20) cold competitor RNA. Competitor RNAs were either PPT RNA (lanes 1 to 5 and 11 to 15) or BS RNA (lanes 6 to 10 and 16 to 20). Reactions were adjusted to 0.5 mg of heparin per ml and analyzed as described above. (C) BS-PPT RNA [RNA(146–179)] (lane 1) and PPT-BS RNA [RNA(156–179, 146–155)] (lane 2) were incubated in nuclear extract at 30°C for 20 min and analyzed as described above.

RNA (lanes 11 to 15). BS RNA competed with BS-PPT RNA only at the highest concentrations tested (1 μM; lanes 16 to 20). Therefore, although each element may interact with the required factors independently at high concentrations, both elements are required in *cis* to form a stable complex. Furthermore, factors recognizing the PPT are either more limiting, required earlier in the binding process, or more critical than factors recognizing the branch region.

In addition, the Amin complex will form only on RNAs in which the BS and PPT elements are in the correct orientation. When the PPT was placed 5′ of the BS [PPT-BS RNA; RNA(156–179, 145–155)], no A-like complexes were detected (Fig. 2C). Thus, interactions between factors binding to these two elements are sensitive to their relative positions, and the branch sequence must be 5′ of the PPT in order to form the correct interactions in making the Amin complex.

Similarities of the Amin complex to complex A containing U2 snRNP. In addition to the data presented above, several lines of evidence suggest that the Amin complex reflects many aspects of authentic complex A. For example, the ionic strength dependence of Amin complex formation corresponds to that required for splicing (Fig. 3A). When assayed across a series of KCl concentrations, the optimum was 60 mM, as is found for splicing conditions (reviewed in reference 47). No complexes were observed at high ionic strength (>200 mM), which was previously found to stabilize the formation of pseudospliceosomes (31). It should be noted that these high ionic strengths would be expected to stabilize simple duplexes, so destabilization of the Amin complex suggests that the latter is not simply due to RNA-RNA base pairing. This is also supported by several other lines of evidence. When RNA-RNA pairing was enhanced by making the BS perfectly complementary to U2 snRNA (5′-. . .UGCUGC. . .-3′), Amin complexes were reduced 96% (52); this contrasts with the stable binding of oligonucleotides for tagging or depletion that is via a much longer sequence complementarity to U2 snRNP (5, 35). Also, unlike the stabilizing effect of 2′-*O*-methyl sugars on simple hybridization, 2′-O methylation across the branch region of BS-PPT RNA abrogated formation of Amin complexes (data not shown). Finally, when the melting temperatures of several RNA-RNA duplexes were measured in the absence of proteins, a BS-U2 RNA duplex was not stable under these conditions (51). In contrast, after formation, the Amin complex was stable enough to be chased with excess cold competitor for greater than 4 h at 30°C (in the absence of ATP, see below [Fig. 3B, lanes 1 to 7]). If added first, this level of competitor completely saturated the Amin complex-forming components (lanes 8 to 14), demonstrating that the maintenance of complexes in lanes 1 to 7 was not due to release and reformation. These data, together with the requirement for both the BS and the PPT sequence, argues strongly that the Amin complex is not based principally on base pairing interactions.

The factor requirements for the Amin complex are similar to those for complex A. Assembly of U2 complexes on full-length pre-mRNA requires the presence of $U2AF^{65}$ (56, 70) and is strongly stimulated by the presence of a factor, PUF-2, which elutes from a poly(U) column at 2 M KCl. This factor contains primarily two polypyrimidine binding proteins, p54 and p130 (41). Extracts depleted of these factors did not support Amin complex formation (Fig. 3C, lane 2), whereas a mock-treated extract did form the Amin complex (lane 1). Addition of the PUF-2 fraction alone did not significantly restore activity (lane 3), and addition of recombinant $U2AF^{65}$ restored only a low level of activity (lane 4). Addition of both PUF-2 and $U2AF^{65}$ restored the ability to form the Amin complex (lane 5), in keeping with the requirement of these protein factors for efficient formation of complex A and for splicing (41).

Previously, three proteins—p14, p35, and p150—were photo-cross-linked to the branch site as components of both complexes A and A3′ by using a linker and photoactive agent that

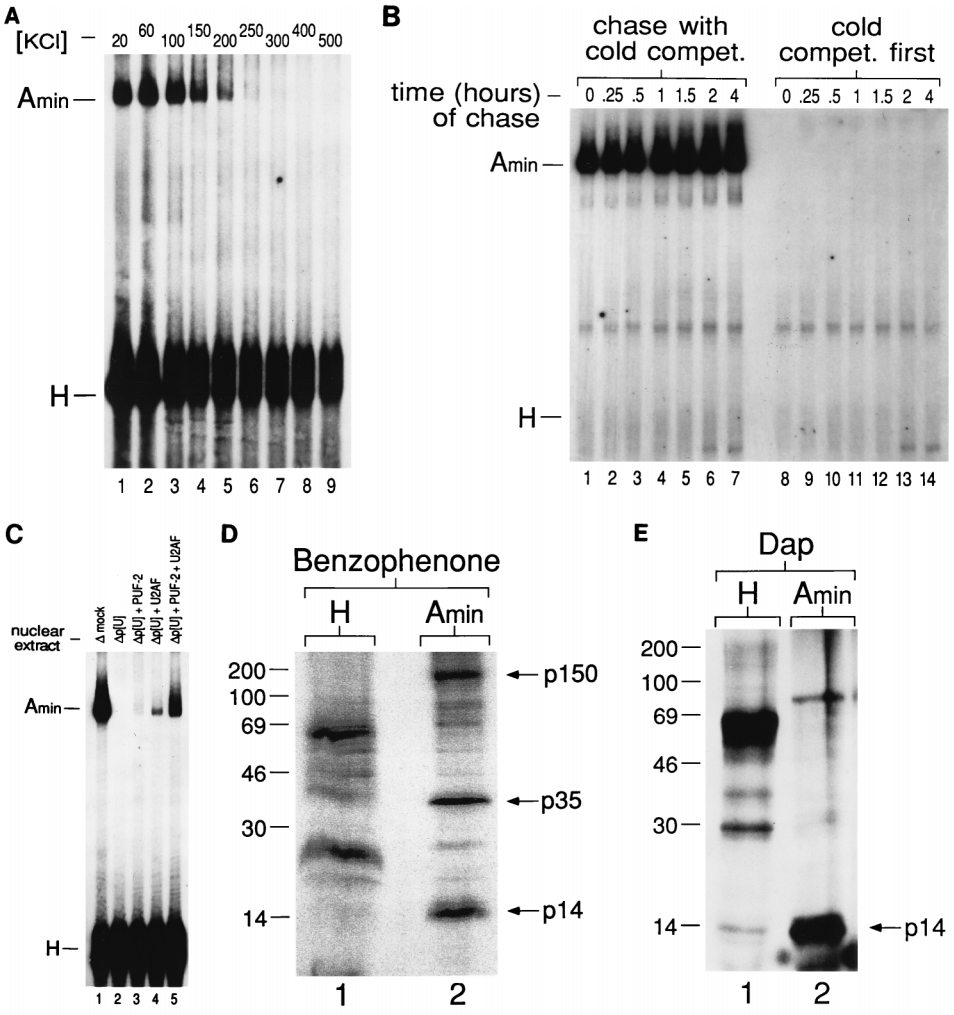

FIG. 3. Characteristics of the Amin complex. (A) Dependence on ionic strength. BS-PPT RNA was incubated in extracts adjusted to the KCl concentration indicated (millimolar), adjusted to 0.5 mg of heparin per ml, and separated on a native 4% polyacrylamide gel. (B) Stability. BS-PPT RNA was incubated in nuclear extracts depleted of ATP for 20 min to form Amin complexes and then challenged with 1 nmol of cold competitor RNA(146–179) per ml, reincubated for the time course indicated, and analyzed as described above (lanes 1 to 7). Alternatively, the cold competitor BS-PPT RNA was incubated first for 20 min, and labeled BS-PPT RNA was then added and reincubated for the times indicated (lanes 8 to 14). (C) Dependence on $U2AF^{65}$ and PUF-2. BS-PPT RNA was incubated in mock-depleted extract (lane 1) or extract depleted of poly(U)-binding proteins (lane 2) or poly(U)-depleted extract supplemented with a PUF-2 fraction (lane 3), supplemented with recombinant $U2AF^{65}$ (lane 4), or supplemented with both recombinant $U2AF^{65}$ and the PUF-2 fraction (lane 5) and analyzed as described above for panel A. (D) Proteins that cross-link in Amin complexes as determined with a 15-Å probe. BS-PPT RNA modified at the N6 position of the branch-site adenosine to contain benzophenone (38) was incubated to form Amin complexes, UV irradiated at 302 nm, and separated on a native polyacrylamide gel. Complexes were isolated and treated with RNase, and the proteins were subsequently separated on a 16% (acrylamide-bisacrylamide, 200:1) polyacrylamide–SDS gel. (E) Proteins that cross-link in Amin complexes within 2 Å of the branch site. BS-PPT RNA modified at the branch site to contain 2,6-diaminopurine (Dap) (52) was incubated to form Amin complexes, UV irradiated at 254 nm, and analyzed as described above for panel D.

could sample distances up to 15 Å (38). The same benzophenone photoreagent was placed site-specifically on the branch site adenosine of BS-PPT RNA (Fig. 3D). This modified RNA was incubated to form the Amin complex and UV irradiated; the complexes were separated on a native gel, the Amin complexes were excised and digested with RNase A, and the proteins were cross-linked to the labeled RNA fragment analyzed on an SDS gel. The same three molecular weight proteins, p14, p35, and p150, were labeled within the Amin complex as those observed within the full complex A. With direct UV irradiation, one of these three proteins, p14, cross-linked directly to the branch site nucleotide in the Amin complex as it does in complex A (Fig. 3E) (52). The other protein detected in this assay, p70, is cross-linked at another site within the branch region (52) and likely corresponds to BPS^{70}, previously observed to cross-link in complex A (16, 52). Thus, the Amin complex contains components and interactions proximal to the branch site adenosine similar to those detected in authentic complex A.

Amin complex formation is ATP independent and undergoes an ATP-dependent dissociation. Formation of complex A on full-length pre-mRNA, as well as that of A3′ complexes on 3′ partial RNAs, requires ATP (29). Surprisingly, as was suggested in Fig. 1B and 2, assembly of Amin complexes does not require ATP. The Amin complex formed more efficiently (see below) in the absence of ATP (i.e., in extracts depleted of ATP; see Materials and Methods) than in the presence of ATP (Fig. 4A, cf. lanes 8 to 14 with 1 to 7). As expected, A-type complexes did not form on full-length pre-mRNA in the absence of ATP (cf. lanes 24 and 25 to 22 and 23). Also, Amin

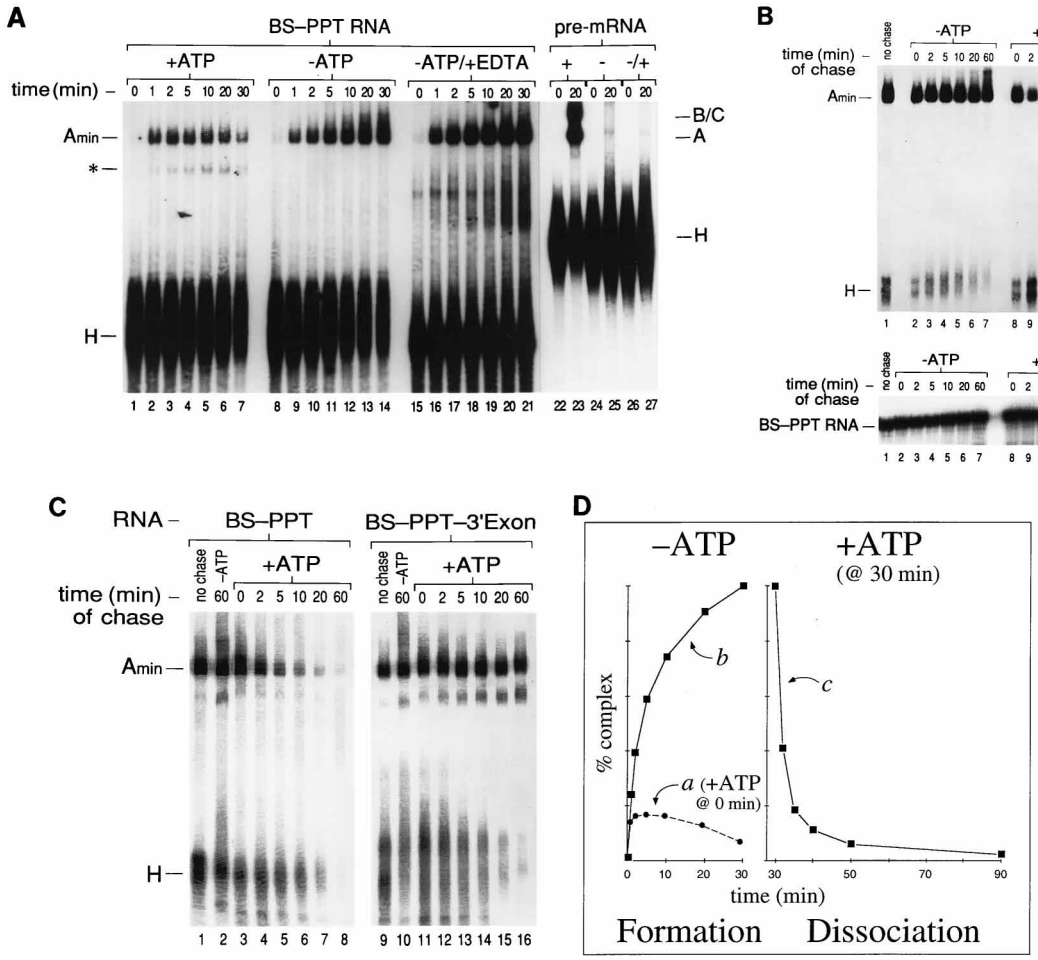

FIG. 4. Independence of Amin complex formation on the presence of ATP and dissociation of Amin complex in the presence of ATP. (A) Time course of complex assembly for BS-PPT RNA (lanes 1 to 21) or for full-length PIP85.B pre-mRNA (lanes 22 to 27) in the presence of ATP (lanes 1 to 7 and 22 and 23), absence of ATP (lanes 8 to 14 and 24 and 25), or absence of ATP and presence of EDTA (lanes 15 to 21 and 26 and 27). RNAs were incubated for the times indicated as described in Materials and Methods, adjusted to 0.5 mg of heparin per ml, and separated on a native 4% polyacrylamide gel. Amin, minimal U2 snRNP complex; B/C, spliceosomal complexes B and C containing U2/4/5/6 snRNPs and pre-mRNA; A, prespliceosomal complex A containing U2 snRNP and pre-mRNA; H, nonspecific complexes; *, a faster-migrating complex observed at low levels in the presence of ATP. (B [upper]) BS-PPT RNA was incubated in nuclear extract depleted of ATP for 20 min to form the Amin complex (lane 1) and then was chased with 1 nmol of cold competitor RNA per ml and reincubated for the time courses shown in either the absence (lanes 2 to 7) or presence (lanes 8 to 13) of ATP. Reaction mixtures then were adjusted to 0.5 mg of heparin per ml and loaded onto a native 4% polyacrylamide gel. (Lower) The RNA in samples from the reactions described above was analyzed on a 15% (19:1) 8 M urea gel. (C) BS-PPT RNA (lanes 1 to 8) and BS-PPT-3′Exon RNA [RNA(146–234); lanes 9 to 16] were incubated in nuclear extract depleted of ATP for 20 min to form Amin or Amin-like complexes, respectively. Cold competitor RNA was added [cold BS-PPT RNA for lanes 1 to 8 or cold RNA(146–234) for lanes 9 to 16], and reaction mixtures were reincubated at 30°C in the presence of ATP for the time courses indicated (lanes 3 to 8 and 11 to 16) or in the absence of ATP for 60 min (lanes 2 and 10). Lanes 1 and 9, no reincubation. Reactions were then analyzed as described above. (D) Kinetics of formation and dissociation of Amin complexes. For formation, complexes were assembled on BS-PPT RNA in the presence of ATP (● [curve *a*; as in panel A, lanes 1 to 7]) or in the absence of ATP (■ [curve *b*; as in panel A, lanes 15 to 21]). For dissociation, Amin complexes were first assembled by incubation for 30 min in nuclear extract depleted of ATP and then were reincubated in the presence of ATP (■ [curve *c*; as in panel B, lanes 8 to 13]). Relative complex formation was determined as the fraction of Amin complex relative to the input RNA. Polyacrylamide gels were quantitated with a Molecular Dynamics PhosphorImager and ImageQuant software, version 3.22.

complex formation was even more efficient, or stabilized, in the presence of EDTA (lanes 15 to 21); this increase may be due to many effects, including stabilization of the RNA from degradation or chelation of Mg^{2+} from trace levels of contaminating ATP (data not shown). Other studies have suggested that the presence of EDTA does not inhibit the formation of functional splicing complexes (1, 15). Furthermore, Amin complexes, but not A or A3′ complexes, form at 4°C, albeit with slower kinetics than at 30°C, also suggesting that ATP hydrolysis is not required (data not shown).

The increase and subsequent decrease in Amin complexes in the presence of ATP (Fig. 4A, lanes 1 to 7, and D, curve *a*) suggest that two distinct processes are at work: both formation and dissociation. The increased level of Amin complexes observed in the absence of ATP (Fig. 4A, lanes 8 to 14 or 15 to 21, and 4D, cf. curves *b* and *a*) suggested that the dissociation process was ATP dependent. To test whether this represented an active process, complexes were formed in the absence of ATP, challenged with excess cold competitor BS-PPT RNA, and reincubated either with or without the addition of ATP. During this reincubation, Amin complexes were dissociated in the presence of ATP, but not in the absence of ATP (Fig. 4B, upper panel; cf. lanes 8 to 13 with 2 to 7). This was not due to degradation of the RNA, which remained at similar levels throughout the incubations (Fig. 4B, lower panel; cf. lanes 8 to 13 with 2 to 7). The dissociation of complexes required both

TABLE 1. Relative yields for Amin complex formation of modified substrates

Modification	Branch sequence[a]	Relative Amin complex formation[b]
All ribose	. . .UGCUGAC. . .	1.0
2′-Deoxyribose	. . .UGCUGA^{H}C. . .	0.62
	. . .UGCUG^{H}AC. . .	0.80
	. . .UG^{H}CUGAC. . .	0.88
Double 2′-deoxyribose	. . .UGCUG^{H}A^{H}C. . .	0.03
	. . .UG^{H}C^{H}UGAC. . .	0.30
	. . .UG^{H}CUG^{H}AC. . .	0.59

[a] Site-specific 2′-deoxyribose modifications at or near the branch site are indicated by the superscripted letters.

[b] Relative complex formation was determined as the fraction of Amin complex formed during a time course relative to the input RNA and normalized to the respective all-ribose-containing RNA. Polyacrylamide gels were quantitated with a Molecular Dynamics PhosphorImager and ImageQuant software, version 3.22. For each band in every lane, an individual background value was determined from the area in the same lane immediately above or below that band.

magnesium cation and hydrolyzable NTP. For example, AMP-PcP, AMP-cPP, or AMP-PnP could not replace ATP, although other NTPs or deoxynucleoside triphosphates (dNTPs) could (data not shown). Thus, the Amin complex is a substrate for an NTP-dependent dissociation activity that results in rapid disassembly of U2 snRNP-containing complexes (Fig. 4D, curve *c*). The level of complexes formed in the presence of ATP (curve *a*) is probably the sum of the two processes of complex formation without ATP (curve *b*) and of dissociation with ATP (curve *c*), indicating a dynamic assembly and disassembly of U2 snRNP complexes.

To test whether the presence of additional sequences would alter the susceptibility of the complex to the dissociation activity, the stability of Amin complexes was compared to that of complexes formed on RNA additionally containing a 3′ splice site and 3′ exon [RNA(146–234); Fig. 4C]. Although this RNA ostensibly is similar to 3′ half RNAs used to form A3′ complex, it does not contain sequences 5′ to the branch region and forms complexes in the absence of ATP, making this comparison possible. As before, preformed Amin complexes dissociated rapidly when challenged with ATP and competitor BS-PPT RNA (lanes 2 to 8) compared to no chase (lane 1) or chase without added ATP (lane 2). In contrast, complexes containing the RNA with additional 3′ sequences were relatively stable to this challenge with ATP and competitor RNA(146–234) (lanes 11 to 16) compared to no chase (lane 9) or chase without ATP (lane 10). Thus, the presence of additional 3′ sequences stabilizes the Amin complex from disassembly in the presence of ATP.

Effects of 2′-H substitutions. Formation of the Amin complex is exquisitely sensitive to branch site modifications. In contrast, formation of complexes at the branch site of full-length pre-mRNAs is only minimally affected by branch site modifications (51, 52). For example, a double 2′-deoxynucleotide (2′-H) substitution at the branch site adenosine and immediately 5′ to it (5′-. . .UGCUG^{H}A^{H}C. . .-3′ [superscripted letters indicate the 2′ moiety]) only slightly reduced U2 snRNP complex formation on a full-length pre-mRNA; rather, there was an accumulation of later complexes unable to undergo the first chemical step of splicing (51). In contrast, the same double substitution in BS-PPT RNA resulted in a 97% decrease in Amin complex formation relative to the all-ribose RNA (Fig. 5, cf. lanes 8 to 14 to 1 to 7; Table 1).

To test whether the great effects of the double 2′-H substitutions at the branch site and adjacent nucleotide were specific to these positions, a similar double 2′-H substitution was prepared three and four residues 5′ to the branch site (. . .UG^{H}C^{H}UGAC. . .; Fig. 5, lanes 15 to 21). This resulted in a 70% decrease in the level of Amin complexes—significant but much less than the effect at the two positions above. Single 2′-deoxynucleotide substitutions at the branch site (. . .UGCUGA^{H}C. . .) or at the immediately 5′ residue (. . .UGCUG^{H}AC. . .) resulted in approximately 40 and 20% decreases in Amin complex formation, respectively (Table 1). These effects are comparable to that of a 2′-H placed four nucleotides 5′ distal to the branch site (. . .UG^{H}CUGAC. . .), which decreased complex formation by approximately 12%. The modest effects of single substitutions compared to the dramatic effect of two 2′-H substitutions are consistent with either position contributing an important contact (see Discussion). The strong effect of double substitutions at the branch site and 5′ to it (97% decrease) is not due just to cumulative effects of individual substitutions, because two separated 2′-H substitutions (. . .UG^{H}CUG^{H}AC. . .) resulted in only a 40% decrease (which is roughly cumulative of the individual effects), and the two adjacent substitutions discussed above (. . .UG^{H}C^{H}UGAC. . .) were inhibited only 70%. Nor are one or two 2′-H substitutions likely to alter the conformation of the BS-U2 helix (4, 22, 49). Thus, the simplified Amin complex system revealed an important 2′-OH contact at the branch site.

DISCUSSION

Interactions of the pre-mRNA branch site with U2 snRNP have been studied by using a minimal RNA sequence containing only the branch region and PPT. The Amin complex formed under these conditions is an accurate reflection of many interactions in the generation of complex A, because both are critically dependent upon the sequences of the branch region and PPT and both require U2AF^{65} and PUF-2 factors.

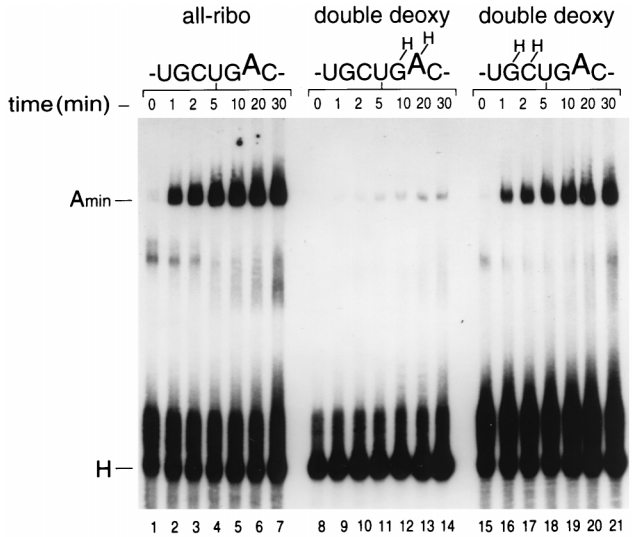

FIG. 5. Time course of complex assembly for RNA oligonucleotides containing 2′-H substitutions. BS-PPT RNAs containing an all-ribose branch sequence (lanes 1 to 7), two 2′-deoxynucleotides at the branch site and immediately 5′ adjacent (. . .UGCUG^{H}A^{H}C. . .; lanes 8 to 14), or two 2′-deoxynucleotides 5′ distal to the branch position (. . .UG^{H}C^{H}UGAC. . .; lanes 15 to 21) were incubated for the times indicated as described in Materials and Methods, adjusted to 0.5 mg of heparin per ml, and loaded onto a native 4% polyacrylamide gel. See Table 1 for quantitations of the effects of these and other substitutions.

Furthermore, the adenine base and 2′-OH constituents of the branch site are important for formation of the Amin complex. Finally, the protein-RNA contacts around the branch site in the Amin complex are identical to those of complex A, as shown by two photo-cross-linking methods. These characteristics indicate that the engagement of Amin complex components with the branch site is the same as that within complex A. Surprisingly, formation of the Amin complex does not require ATP or U1 snRNP, indicating that these factors are not necessary for stable association of U2 snRNP with a branch sequence per se. In the absence of ATP, Amin-type complexes do not form with RNAs containing sequences upstream of the branch region, suggesting that accessibility of the branch site in these RNAs might be ATP dependent. Finally, the association of U2 snRNP with the branch region on a minimal substrate is dynamic—rapid ATP-dependent turnover indicates the presence of an active mechanism that releases or destabilizes U2 snRNP from branch sequences.

A more sensitive system—2′-OH and adenine interactions. The dramatic effects of 2′-deoxynucleotide and branch site base substitutions on the formation of the Amin complex demonstrate that this complex is more sensitive to subtle atomic changes than is prespliceosomal complex A. If multiple interactions contribute to overall stability of complex A, then the absence of some of these interactions should result in complex formation being more critically dependent upon the remaining ones. In the case of the branch region, multiple weak interactions almost certainly contribute to the formation and stability of complex A (reviewed in reference 54). When some of these are absent—e.g., interactions at the 5′ splice site, at the U2 anchoring site 5′ to the branch region, and 3′ to the PPT at the 3′ splice site and exon enhancer sequences (25, 36, 53, 60, 63, 66, 68, 74)—recognition of the branch site and PPT becomes more important. This increased sensitivity to the precise nature of chemical groups at the branch site has revealed 2′-OH and adenine contacts in the Amin complex. The great effect of double 2′-H substitutions at the branch and the 5′ adjacent sites, unlike the modest effects of single substitutions, indicates that contacts with these two positions are critical and, in some way, cooperative. This may relate to the alternative bulging of these two positions described previously (51), which would allow either 2′-OH present to fill a similar position, or interaction with either 2′-OH might be adequate for formation of a stable complex.

Recognition of the adenine base at the branch site is critical for Amin complex formation and previously was shown to contribute to complex A stability (52). Relative to complex A, the Amin complex has enhanced dependency upon an exocyclic C6-NH_2 group at the branch site, which contributes a significant positive effect; a C2-NH_2 group has a significant negative effect, and a C6-oxo/N1-H of guanine is strongly inhibitory. Thus, even at the time of initial U2 snRNP addition, and without any ATP-dependent transitions, there are specific contacts both with the adenine base and with 2′-OH groups in the ribose phosphate backbone in the branch region. Interestingly, recognition of these specific contacts strongly correlates with the direct cross-linking of a p14 protein. This protein is thus a good candidate for the component of complex A that directly recognizes the branch site adenosine.

Amin complex forms independently of U1 snRNP and ATP. Formation of complexes in nuclear extracts with individual snRNPs depleted demonstrated that U2 snRNP, but neither the U1 nor U4/6 snRNPs, is required for Amin complex formation. The U1 snRNP independence of the Amin complex is surprising, since this snRNP is generally required for assembly of complexes on full-length pre-mRNAs (6). However, this requirement is not absolute, since both complex formation and splicing can sometimes occur in the absence of U1 snRNP (18–20, 64). Such U1 snRNP-independent splicing has been observed for a subset of pre-mRNAs exemplified by *fushi tarazu* (ftz) and with certain substrates in the presence of elevated concentrations of SR proteins; other substrates do not show U1-independent activity under any condition tested. The conditions reported here for Amin complex formation do not have elevated levels of SR proteins, and the BS-PPT RNA is derived from a pre-mRNA that does not exhibit U1-independent splicing even with added SR proteins (18). Since the Amin complex readily forms on the isolated BS-PPT in the absence of U1 snRNP, the general requirement of U1 snRNP for complex A must be due to sequences external to these elements.

The mechanism requiring ATP during the formation of complex A on pre-mRNA is not known. The independence of Amin complex formation from this ATP requirement implies that ATP is not needed for U2 binding per se. This is consistent with previous results that suggested that U2 addition could occur without ATP in the background of a weakened U1-5′ splice site interaction (37). The Amin complex does not form on RNA substrates with sequences 5′ of the branch site; formation of complex A or A3′ on these substrates requires ATP. This suggests that there may be an ATP-dependent step required for exposure of the longer substrate RNA for the binding to U2 snRNP. For example, a helicase-type activity might unfold the substrate RNA for the subsequent binding of U2 snRNP; alternatively, a conformational change in U2 snRNP or another complex A factor might be necessary to allow interaction with sequences 5′ to the branch region.

In the presence of ATP, the binding of U2 snRNP in complex A is probably stabilized by interactions with both U1 snRNP and SR proteins in a dynamic equilibrium. The strength of interactions with U1 snRNP and SR proteins summed with recognition of the branch region and PPT would determine the level of complex A. If the other interactions were weak, then the determinants for stable formation of the Amin complex—a consensus branch region and extended PPT—would be critical. Thus, the sequence requirements of the Amin complex probably reflect those of complex A at introns containing other weak splice site elements. That formation of the Amin complex is not as dependent upon the dynamic processes described above as the formation of complex A is likely due to the simplicity of the short consensus substrate RNA. At the moment, it is conjectured that $U2AF^{65}$ and the PUF-2 complex of proteins bind the short substrate RNA, since these proteins tightly bind poly(U) tracts and are required for complex formation. The other components that subsequently bind the substrate are the 17S U2 snRNP complex and perhaps other factors (Fig. 6). The simplicity of the Amin complex will facilitate a full analysis of the assembly of prespliceosomes.

An active mechanism of U2 snRNP removal. The Amin complex rapidly dissociates in the presence of ATP. This represents an active mechanism that removes or destabilizes U2 snRNP from branch sequences. There are several points in the spliceosome cycle at which such a mechanism may be required. This process might reflect the pathway of U2 snRNP removal from excised lariat introns, although this seems unlikely because both the chemical nature of the RNA substrate (no branched nucleotide) and the snRNP complement of the complex (U2 versus U2/6/5) are different. More likely, it could represent a destabilization of complex A interactions that normally occurs during formation of spliceosomes; this destabilization process could be a weakening of interactions needed to

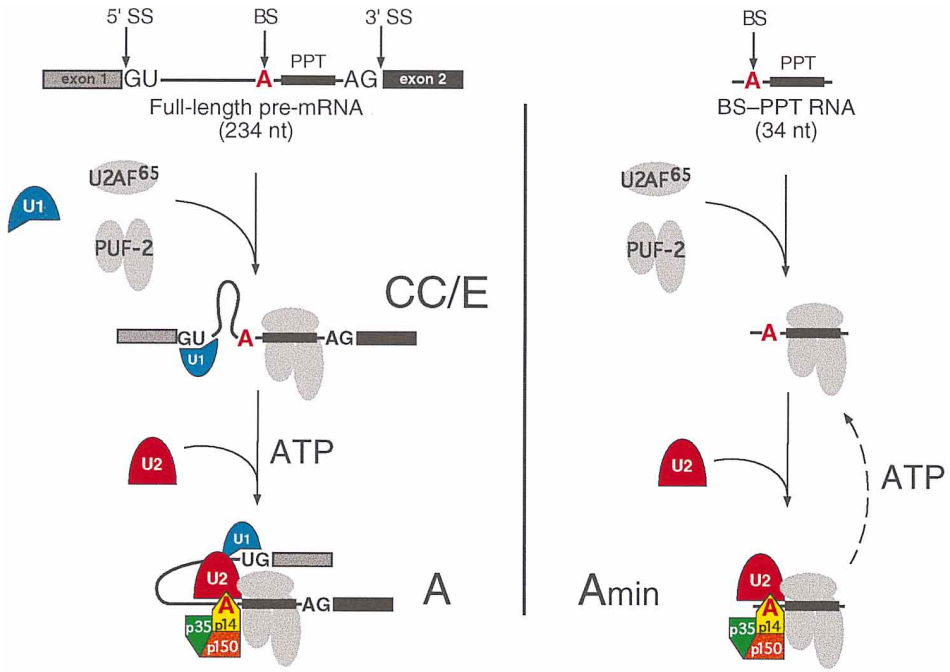

FIG. 6. Summary of formation of complex A (left) and the Amin complex (right). A schematic comparison of the assemblies of complex A and the Amin complex is shown. At the top of the panels are shown diagrams of full-length pre-mRNA and the minimal BS-PPT RNA substrates. Both complexes require branch sequence and PPT RNA elements, U2 snRNP, and protein factors U2AF^{65} and PUF-2. Complex A requires U1 snRNP and ATP, which are not necessary for Amin complex formation. In both complex A and the Amin complex, three proteins are detected within 15 Å of the branch site adenosine—p14, p35, and p150; the p14 cross-links directly to the adenosine in both complexes. In the presence of ATP, U2 snRNP is released from Amin complexes (dashed arrow). Other components not shown, such as SR proteins (23, 40, 66) and SF1 (3, 31), are important for complex A formation in other systems and may also be required for the Amin complex. The arrangement of proteins is illustrative only and is not meant to imply a known spatial order. SS, splice site; BS, branch sequence containing the branch site adenosine; PUF-2, poly(U)-binding factor-2; U1, U1 snRNP; U2, U2 snRNP; CC/E, commitment or early complex; A, prespliceosomal complex A containing U2 snRNP and pre-mRNA; Amin, minimal substrate RNA-U2 snRNP complex.

progress beyond complex A, or, alternatively, this dynamic process could act as a proofreading step. We propose that the ATP-dependent step may test the fidelity or total stability of the U2 snRNP-RNA complex. If the U2 snRNP complex is not stabilized by interactions with components recognizing other splicing signals, such as U1 snRNP and SR proteins bound to nearby sequences, then U2 snRNP would dissociate (Fig. 6, dashed arrow). This mechanism would preclude stable formation of an A-type complex on sequences fortuitously resembling branch regions and PPTs (57) found within introns and exons of nuclear precursor mRNAs. The biochemical assay demonstrated here will allow characterization of this active mechanism and its role in the splicing process.

ACKNOWLEDGMENTS

We are grateful to J. Crispino for generous sharing of reagents and M. Moore for synthesis of oligoribonucleotides. We thank D. Bartel, B. Blencowe, J. Crispino, L. Lim, M. Moore, and R. Pulak for critical review of the manuscript; M. Siafaca for her patience and ever-present assistance; and the members of the Sharp lab for their continued interest and support.

C.C.Q. is supported by Leukemia Society of America postdoctoral fellowship (3075-94). This work was supported by United States Public Health Service grant RO1-GM34277 and RO1-AI32486 from the National Institutes of Health to P.A.S. and was partially supported by a Cancer Center Support (core) grant, P30-CA14051, from the National Cancer Institute.

REFERENCES

1. **Abmayr, S. M., R. Reed, and T. Maniatis.** 1988. Identification of a functional mammalian spliceosome containing unspliced pre-mRNA. Proc. Natl. Acad. Sci. USA **85:**7216–7220.
2. **Abovich, N., X. C. Liao, and M. Rosbash.** 1994. The yeast MUD2 protein: an interaction with PRP11 defines a bridge between commitment complexes and U2 snRNP addition. Genes Dev. **8:**843–854.
3. **Arning, S., P. Grüter, G. Bilbe, and A. Krämer.** 1996. Mammalian splicing factor SF1 is encoded by variant cDNAs and binds to RNA. RNA **2:**794–810.
4. **Ban, C., B. Ramakrishnan, and M. Sundaralingam.** 1994. A single 2′-hydroxyl group converts B-DNA to A-DNA. Crystal structure of the DNA-RNA chimeric decamer duplex d(CCGGC)r(G)d(CCGG) with a novel intermolecular G · C base-paired quadruplet. J. Mol. Biol. **236:**275–285.
5. **Barabino, S. M., B. S. Sproat, U. Ryder, B. J. Blencowe, and A. I. Lamond.** 1989. Mapping U2 snRNP-pre-mRNA interactions using biotinylated oligonucleotides made of 2′-OMe RNA. EMBO J. **8:**4171–4178.
6. **Barabino, S. M. L., B. J. Blencowe, U. Ryder, B. S. Sproat, and A. I. Lamond.** 1990. Targeted snRNP depletion reveals an additional role for mammalian U1 snRNP in spliceosome assembly. Cell **63:**293–302.
7. **Behrens, S.-E., F. Galisson, P. Legrain, and R. Lührmann.** 1993. Evidence that the 60-kDa protein of 17S U2 small nuclear ribonucleoprotein is immunologically and functionally related to the yeast PRP9 splicing factor and is required for the efficient formation of prespliceosomes. Proc. Natl. Acad. Sci. USA **90:**8229–8233.
8. **Blencowe, B. J., and S. M. L. Barabino.** 1995. Antisense affinity depletion of RNP particles: application to spliceosomal snRNPs. Methods Mol. Biol. **37:**67–76.
9. **Brosi, R., K. Gröning, S.-E. Behrens, R. Lührmann, and A. Krämer.** 1993. Interaction of mammalian splicing factor SF3a with U2 snRNP and relation of its 60-kD subunit to yeast PRP9. Science **262:**102–105.
10. **Brosi, R., H.-P. Hauri, and A. Krämer.** 1993. Separation of splicing factor SF3 into two components and purification of SF3a activity. J. Biol. Chem. **268:**17640–17646.
11. **Burgess, S. M., and C. Guthrie.** 1993. Beat the clock: paradigms in the maintenance of biological fidelity. Trends Biochem. Sci. **18:**381–384.
12. **Burgess, S. M., and C. Guthrie.** 1993. A mechanism to enhance mRNA splicing fidelity: the RNA-dependent ATPase Prp16 governs usage of a discard pathway for aberrant lariat intermediates. Cell **73:**1377–1391.
13. **Champion-Arnaud, P., and R. Reed.** 1994. The prespliceosome components SAP 49 and SAP 145 interact in a complex implicated in tethering U2 snRNP to the branch site. Genes Dev. **8:**1974–1983.
14. **Chaudhary, N., C. McMahon, and G. Blobel.** 1991. Primary structure of a

human arginine-rich nuclear protein that colocalizes with spliceosome components. Proc. Natl. Acad. Sci. USA **88:**8189–8193.
15. **Cheng, S. C., and J. Abelson.** 1987. Spliceosome assembly in yeast. Genes Dev. **1:**1014–1027.
16. **Chiara, M. D., O. Gozani, M. Bennett, P. Champion-Arnaud, L. Palandjian, and R. Reed.** 1996. Identification of proteins that interact with exon sequences, splice sites, and the branchpoint sequence during each stage of spliceosome assembly. Mol. Cell. Biol. **16:**3317–3326.
17. **Couto, J. R., J. Tamm, R. Parker, and C. Guthrie.** 1987. A *trans*-acting suppressor restores splicing of a yeast intron with a branch point mutation. Genes Dev. **1:**445–455.
18. **Crispino, J. D., B. J. Blencowe, and P. A. Sharp.** 1994. Complementation by SR proteins of pre-mRNA splicing reactions depleted of U1 snRNP. Science **265:**1866–1869.
19. **Crispino, J. D., J. E. Mermoud, A. I. Lamond, and P. A. Sharp.** 1996. *cis*-acting elements distinct from the 5′ splice site promote U1-independent pre-mRNA splicing. RNA **2:**664–673.
20. **Crispino, J. D., and P. A. Sharp.** 1995. A U6 snRNA:pre-mRNA interaction can be rate-limiting for U1-independent splicing. Genes Dev. **9:**2314–2323.
21. **Dignam, J. D., R. M. Lebovitz, and R. D. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
22. **Egli, M., N. Usman, and A. Rich.** 1993. Conformational influence of the ribose 2′-hydroxyl group: crystal structures of DNA-RNA chimeric duplexes. Biochemistry **32:**3221–3237.
23. **Fu, X. D.** 1995. The superfamily of arginine/serine-rich splicing factors. RNA **1:**663–680.
24. **Gaur, R. K., J. Valcárcel, and M. R. Green.** 1995. Sequential recognition of the pre-mRNA branch point by $U2AF^{65}$ and a novel spliceosome-associated 28-kDa protein. RNA **1:**407–417.
25. **Gozani, O., R. Feld, and R. Reed.** 1996. Evidence that sequence-independent binding of highly conserved U2 snRNP proteins upstream of the branch site is required for assembly of spliceosomal complex A. Genes Dev. **10:**233–243.
26. **Grabowski, P. J., R. A. Padgett, and P. A. Sharp.** 1984. Messenger RNA splicing in vitro: an excised intervening sequence and a potential intermediate. Cell **37:**415–427.
27. **Hoffman, B. E., and P. J. Grabowski.** 1992. U1 snRNP targets an essential splicing factor, U2AF65, to the 3′ splice site by a network of interactions spanning the exon. Genes Dev. **6:**2554–2568.
28. **Jamison, S. F., and M. A. García-Blanco.** 1992. An ATP-independent U2 small nuclear ribonucleoprotein particle/precursor mRNA complex requires both splice sites and the polypyrimidine tract. Proc. Natl. Acad. Sci. USA **89:**5482–5486.
29. **Konarska, M. M., and P. A. Sharp.** 1986. Electrophoretic separation of complexes involved in the splicing of precursors to mRNAs. Cell **46:**845–855.
30. **Konarska, M. M., and P. A. Sharp.** 1987. Interactions between small nuclear ribonucleoprotein particles in formation of spliceosomes. Cell **49:**763–774.
31. **Konarska, M. M., and P. A. Sharp.** 1988. Association of U2, U4, U5 and U6 small nuclear ribonucleoproteins in a spliceosome-type complex in absence of precursor RNA. Proc. Natl. Acad. Sci. USA **85:**5459–5462.
32. **Krämer, A.** 1988. Presplicing complex formation requires two proteins and U2 snRNP. Genes Dev. **2:**1155–1167.
33. **Krämer, A.** 1995. The biochemistry of pre-mRNA splicing, p. 35–64. *In* A. I. Lamond (ed.), Pre-mRNA processing. R. G. Landes Company, Austin, Tex.
34. **Krämer, A.** 1996. The structure and function of proteins involved in mammalian pre-mRNA splicing. Annu. Rev. Biochem. **65:**367–409.
35. **Lamond, A. I., B. Sproat, U. Ryder, and J. Hamm.** 1989. Probing the structure and function of U2 snRNP with antisense oligonucleotides made of 2′-OMe RNA. Cell **58:**383–390.
36. **Lavigueur, A., H. LaBranche, A. R. Kornblihtt, and B. Chabot.** 1993. A splicing enhancer in the human fibronectin alternate ED1 exon interacts with SR proteins and stimulates U2 snRNP binding. Genes Dev. **7:**2405–2417.
37. **Liao, X. C., H. V. Colot, Y. Wang, and M. Rosbash.** 1992. Requirements for U2 snRNP addition to yeast pre-mRNA. Nucleic Acids Res. **20:**4237–4245.
38. **MacMillan, A. M., C. C. Query, C. R. Allerson, S. Chen, G. L. Verdine, and P. A. Sharp.** 1994. Dynamic association of proteins with the pre-mRNA branch region. Genes Dev. **8:**3008–3020.
39. **Madhani, H. D., and C. Guthrie.** 1994. Dynamic RNA-RNA interactions in the spliceosome. Annu. Rev. Genet. **28:**1–26.
40. **Manley, J. L., and R. Tacke.** 1996. SR proteins and splicing control. Genes Dev. **10:**1569–1579.
41. **McCaw, P. S., and P. A. Sharp.** Unpublished data.
42. **Michaud, S., and R. Reed.** 1991. An ATP-independent complex commits pre-mRNA to the mammalian spliceosome assembly pathway. Genes Dev. **5:**2534–2546.
43. **Moore, M. J., C. C. Query, and P. A. Sharp.** 1993. Splicing of precursors to mRNA by the spliceosome, p. 303–357. *In* R. Gesteland and J. Atkins (ed.), The RNA world. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Moore, M. J., and P. A. Sharp.** 1992. Site-specific modification of pre-mRNA: the 2′ hydroxyl groups at the splice sites. Science **256:**992–997.
45. **Moore, M. J., and P. A. Sharp.** 1993. The stereochemistry of pre-mRNA splicing: evidence for two active sites in the spliceosome. Nature **365:**364–368.
46. **Nilsen, T. W.** 1994. RNA-RNA interactions in the spliceosome: unraveling the ties that bind. Cell **78:**1–4.
47. **Padgett, R. A., P. J. Grabowski, M. M. Konarska, S. Seiler, and P. A. Sharp.** 1986. Splicing of messenger RNA precursors. Annu. Rev. Biochem. **55:**1119–1150.
48. **Parker, R., P. G. Siliciano, and C. Guthrie.** 1987. Recognition of the TAC TAAC box during mRNA splicing in yeast involves base pairing to the U2-like snRNA. Cell **49:**229–239.
49. **Portmann, S., S. Grimm, C. Workman, N. Usman, and M. Egli.** 1996. Crystal structures of an A-form duplex with single-adenosine bulges and a conformational basis for site-specific RNA self-cleavage. Chem. Biol. **3:**173–184.
50. **Pruzan, R., H. Furneaux, P. Lassota, G. Y. Hong, and J. Hurwitz.** 1990. Assemblage of the prespliceosome complex with separated fractions isolated from HeLa cells. J. Biol. Chem. **265:**2804–2813.
51. **Query, C. C., M. J. Moore, and P. A. Sharp.** 1994. Branch nucleophile selection in pre-mRNA splicing: evidence for the bulged duplex model. Genes Dev. **8:**587–597.
52. **Query, C. C., S. A. Strobel, and P. A. Sharp.** 1996. Three recognition events at the branch site adenine. EMBO J. **15:**1392–1402.
53. **Ramchatesingh, J., A. M. Zahler, K. M. Neugebauer, M. B. Roth, and T. A. Cooper.** 1995. A subset of SR proteins activates splicing of the cardiac troponin T alternative exon by direct interactions with an exonic enhancer. Mol. Cell. Biol. **15:**4898–4907.
54. **Reed, R.** 1996. Initial splice-site recognition and pairing during pre-mRNA splicing. Curr. Opin. Genet. Dev. **6:**215–220.
55. **Ruby, S. W., and J. Abelson.** 1988. An early hierarchic role of U1 small nuclear ribonucleoprotein in spliceosome assembly. Science **242:**1028–1035.
56. **Ruskin, B., P. D. Zamore, and M. R. Green.** 1988. A factor, U2AF, is required for U2 snRNP binding and splicing complex assembly. Cell **52:**207–219.
57. **Senapathy, P., M. B. Shapiro, and N. L. Harris.** 1990. Splice junctions, branch point sites, and exons: sequence statistics, identification, and applications to genome project. Methods Enzymol. **183:**252–278.
58. **Séraphin, B., and M. Rosbash.** 1989. Identification of functional U1 snRNP-pre-mRNA complexes committed to spliceosome assembly and splicing. Cell **59:**349–358.
59. **Séraphin, B., and M. Rosbash.** 1991. The yeast branchpoint sequence is not required for the formation of a stable U1 snRNP pre-mRNA complex and is recognized in the absence of U2 snRNA. EMBO J. **10:**1209–1216.
60. **Staknis, D., and R. Reed.** 1994. Direct interactions between pre-mRNA and six U2 small nuclear ribonucleoproteins during spliceosome assembly. Mol. Cell. Biol. **14:**2994–3005.
61. **Staknis, D., and R. Reed.** 1994. SR proteins promote the first specific recognition of pre-mRNA and are present together with the U1 small nuclear ribonucleoprotein particle in a general splicing enhancer complex. Mol. Cell. Biol. **14:**7670–7682.
62. **Strobel, S. A., T. R. Cech, N. Usman, and L. Beigelman.** 1994. The 2,6-diaminopurine riboside · 5-methylisocytidine wobble base pair: an isoenergetic substitution for the study of G · U pairs in RNA. Biochemistry **33:**13824–13835.
63. **Sun, Q., R. K. Hampson, A. T. Krainer, and F. M. Rottman.** 1993. General splicing factor SF2/ASF promotes alternative splicing by binding to an exonic splicing enhancer. Genes Dev. **7:**2598–2608.
64. **Tarn, W. Y., and J. A. Steitz.** 1994. SR proteins can compensate for the loss of U1 snRNP functions in vitro. Genes Dev. **8:**2704–2717.
65. **Umen, J. G., and C. Guthrie.** 1995. The second catalytic step of pre-mRNA splicing. RNA **1:**869–885.
66. **Valcárcel, J., and M. R. Green.** 1996. The SR protein family: pleiotropic functions in pre-mRNA splicing. Trends Biol. Sci. **21:**296–301.
67. **Wang, Z., H. M. Hoffmann, and P. J. Grabowski.** 1995. Intrinsic U2AF binding is modulated by exon enhancer signals in parallel with changes in splicing activity. RNA **1:**21–35.
68. **Watakabe, A., K. Tanaka, and Y. Shimura.** 1993. The role of exon sequences in splice site selection. Genes Dev. **7:**407–418.
69. **Wu, J., and J. Manley.** 1989. Mammalian pre-mRNA branch site selection by U2 snRNP involves base pairing. Genes Dev. **3:**1553–1561.
70. **Zamore, P. D., J. G. Patton, and M. R. Green.** 1992. Cloning and domain structure of the mammalian splicing factor U2AF. Nature **355:**609–614.
71. **Zhang, W.-J., and J. Y. Wu.** 1996. Functional properties of p54, a novel SR protein active in constitutive and alternative splicing. Mol. Cell. Biol. **16:**5400–5408.
72. **Zhuang, Y., A. M. Goldstein, and A. M. Weiner.** 1989. UACUAAC is the preferred branch site for mammalian mRNA splicing. Proc. Natl. Acad. Sci. USA **86:**2752–2756.
73. **Zhuang, Y., and A. M. Weiner.** 1989. A compensatory base change in human U2 snRNA can suppress a branch site mutation. Genes Dev. **3:**1545–1552.
74. **Zuo, P., and T. Maniatis.** 1996. The splicing factor $U2AF^{35}$ mediates critical protein-protein interactions in constitutive and enhancer-dependent splicing. Genes Dev. **10:**1356–1368.