An Early Hierarchic Role of U1 Small Nuclear Ribonucleoprotein in Spliceosome Assembly

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Splicing of nuclear precursor messenger RNA (pre-mRNA) occurs on a large ribonucleoprotein complex, the spliceosome. Several small nuclear ribonucleoproteins (snRNP's) are subunits of this complex that assembles on the pre-mRNA. Although the U1 snRNP is known to recognize the 5′ splice site, its roles in spliceosome formation and splice site alignment have been unclear. A new affinity purification method for the spliceosome is described which has provided insight into the very early stages of spliceosome formation in a yeast in vitro splicing system. Surprisingly, the U1 snRNP initially recognizes sequences at or near both splice junctions in the intron. This interaction must occur before the other snRNP's (U2, U4, U5, and U6) can join the complex. The results suggest that interaction of the two splice site regions occurs at an early stage of spliceosome formation and is probably mediated by U1 snRNP and perhaps other factors.

MESSENGER RNA SPLICING REQUIRES PRECISE RECOGNITION and joining of two splice sites on the pre-mRNA. The 5′ and 3′ splice sites are recognized during spliceosome formation and, at some point in this assembly, they must be brought together before splicing begins. How the two sites are recognized and juxtaposed depends on both the sequences of the pre-mRNA and transacting factors. At least three sequences of the pre-mRNA, the 5′ and 3′ splice sites and the branch-point region, are required for spliceosome formation and splicing (*1, 2*). These sequences are both physically and functionally conserved among eukaryotes, with a few notable differences. The consensus 5′ splice site in yeast, GUAPyGU (Py, any pyrimidine), and the branch-point sequence, UACUAAC near the 3′ splice site, are much more highly conserved than in mammals (*3, 4*). Mammalian introns contain an essential polypyrimidine stretch upstream of the 3′ splice site that is not always present in yeast introns (*5*). The 3′ splice site, PyAg, is the same in both organisms but it is not required for spliceosome formation in yeast (*1, 2, 6*). Mutations in the highly conserved yeast sequences usually inactivate an intron (*6, 7*), whereas in a mammalian intron similar mutations often activate nearby cryptic sites (*1*).

Five snRNP's and several proteins are transacting factors essential for splicing (*2, 8*). Two of the snRNP's, U1 and U2, interact with the intron via complementary base pairing. The 5′ end of U1 RNA base pairs with the 5′ splice site (*9–11*). A portion of U2 RNA near its 5′ end pairs with the branch point sequence of the pre-mRNA in yeast (*12*). The mammalian U2 snRNP also binds to sequences at the branch point, and a protein factor U2AF may mediate this interaction (*13*); it is not yet known whether base pairing is also required. A third snRNP, U5, may interact with the 3′ splice site by means of a protein-RNA contact (*14*). Although the U4 and U6 snRNP's are required for splicing (*11, 15*), there is no evidence that they base pair with the precursor.

Most aspects of splicing including the small nuclear RNA's (snRNA's), have been conserved from yeast to man. The five snRNA's in the yeast *Saccharomyces cerevisiae* are homologs of the mammalian ones (*16–21*) and are now referred to by the mammalian names. Although a part of each yeast snRNA is similar to its mammalian homolog, some of the yeast snRNA's contain extra sequences. The function of these extra sequences is not known, but in the case of U2 snRNA, most of the extra sequences are not essential for splicing (*22*).

In previous investigations of the in vitro assembly of the spliceosome, gradient sedimentation or nondenaturing gel electrophoresis was used to resolve the spliceosome and any intermediate complexes (*23–29*). Pathways of snRNP binding to the pre-mRNA have been proposed on the basis of the kinetics of formation of these complexes (*24–27*). In most of these pathways, U2 snRNP binds in an early step requiring adenosine triphosphate (ATP). In the next step, U4, U5, and U6 join the complex. Before an active spliceosome forms, U4 leaves the complex (*25–27*). In most studies, U1 snRNP could

Table 1. snRNP binding to mutant pre-mRNA's.

Pre-mRNA's*	N†	snRNP binding‡			
		U1		U2	
		−ATP	+ATP	−ATP	+ATP
Wild type	3	79 ± 20	100	3.0 ± 5.2	100
Mutants					
5′ splice site					
A1	2	44 ± 2.8§	25 ± 13§	4.6 ± 6.6	7.7 ± 1.6‖
C1	3	33 ± 33§	33 ± 19§	0.6 ± 1.1	0.93 ± 0.9‖
A5	1	85	49	6	11
Branch-point sequence					
A256	1	47	41	0	15
A257	4	16 ± 18§	10 ± 6.1§	0 ± 0	0.1 ± 0.2‖
C259	3	31 ± 13	17 ± 9.5§	0 ± 0	13 ± 18 ‖
3′ splice site					
C303/305	2	105 ± 45	91 ± 13	5.0 ± 7.1	117 ± 23

*Mutant pre-mRNA's (Fig. 4) were immobilized and used in assays as described in Fig. 9. †The data were combined from the number of experiments indicated. ‡The levels of snRNP's bound to the immobilized pre-mRNA's were determined by either densitometry of autoradiograms or liquid scintillation counting excised portions of the RNA blots (*37*). Each value is expressed as the mean percent (± SD) of wild-type level bound in the presence of ATP. §Significantly different ($P < 0.05$) from that for wild-type pre-mRNA in the presence or absence of ATP. ‖Significantly different ($P < 0.01$) from that for wild-type pre-mRNA in the presence of ATP.

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not be detected in the complexes or in the spliceosome, probably because conditions required to resolve the complexes dissociate U1 from the complexes (*29*). Thus, although U1 is essential for splicing and recognizes the 5′ splice site, its role in spliceosome assembly and splice site alignment was not clear.

Affinity purification of splicing complexes. To study the early events of spliceosome formation and the role of U1 snRNP in these events, we developed an affinity purification method (Fig. 1) (*30*) to rapidly purify components bound to pre-mRNA in a yeast in vitro splicing system. The yeast actin pre-mRNA is synthesized in vitro (Fig. 1, step 1) and is bound to a solid support via a small complementary "anchor" RNA hybridized to its 3′ end. The anchor RNA is synthesized in two steps (Fig. 2) and has multiple biotin molecules, each linked to C5 of a uridine residue via a linker containing a disulfide bond. It is hybridized to the pre-mRNA (Fig. 1, step 2) before the RNA hybrid is immobilized on the support (step 3). The anchor RNA attaches to a solid support, biotin-agarose, via avidin molecules. To purify components involved in splicing, we add an active yeast splicing extract to the immobilized pre-mRNA (step 4). During the ensuing incubation period, the spliceosome forms on the immobilized RNA and some of the RNA is spliced. After incubation, the agarose beads are washed quickly and extensively to remove extraneous extract components. The pre-mRNA, RNA intermediates, the mRNA product, and any components bound to them are eluted by the addition of 150 m*M* dithiothreitol (DTT) to cleave the disulfide bonds linking biotin to the anchor RNA (step 5) (*31*).

Routinely, about 90 percent of the input pre-mRNA anchor

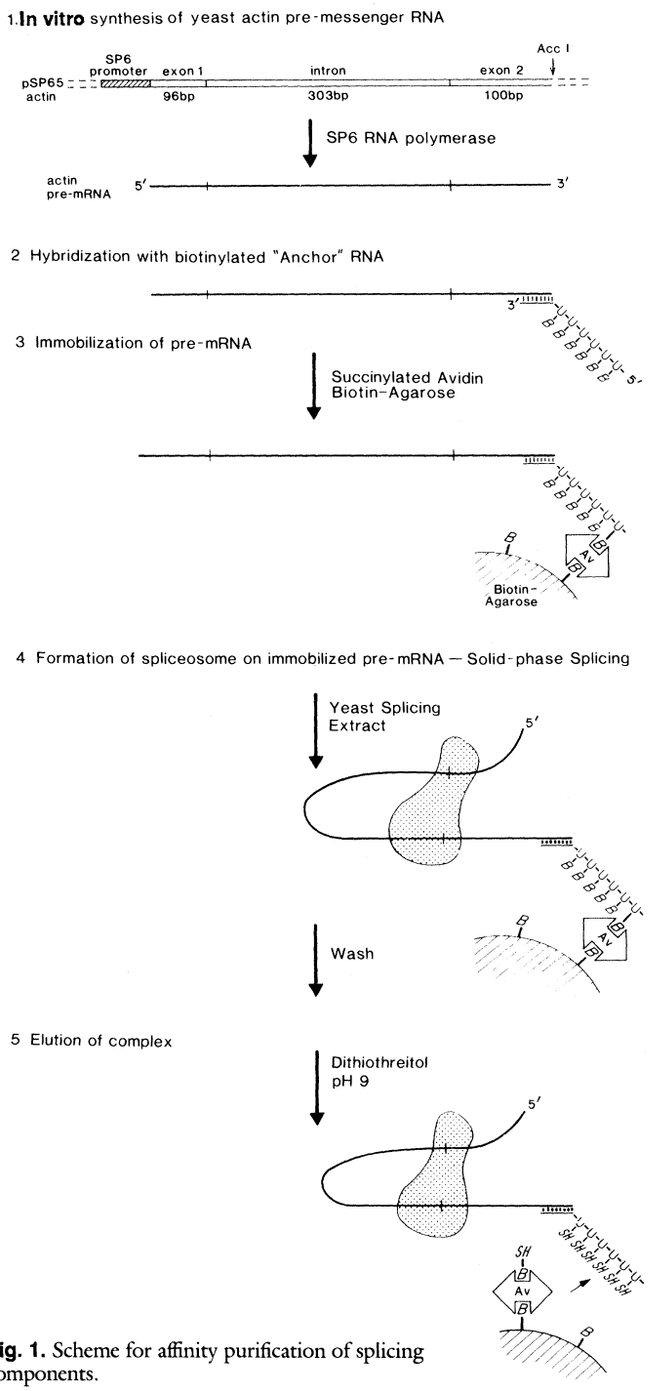

Fig. 1. Scheme for affinity purification of splicing components.

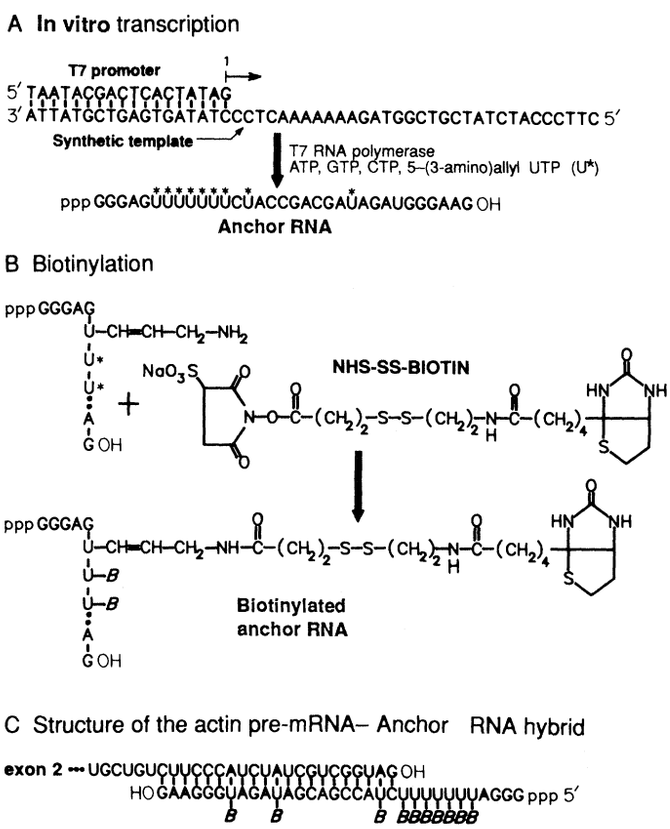

Fig. 2. Synthesis of biotinylated "anchor" RNA. The small, biotinylated anchor RNA was synthesized in two steps to obtain large amounts of RNA and to maintain the disulfide bond in the linker between the uridine and biotin moieties. (**A**) The RNA was transcribed in vitro from two synthetic oligodeoxynucleotides encoding the T7 promoter and the anchor RNA (*44*). The oligodeoxynucleotides were annealed together in solution (22.5 μ*M* each oligodeoxynucleotide, 100 m*M* NaCl, 10 m*M* tris-HCl, *p*H 7.6, and 1 m*M* EDTA). Anchor RNA was synthesized in a reaction (for 3 to 4 hours at 37°C) containing 50 m*M* tris-HCL, *p*H 8.1, 25 m*M* $MgCl_2$, 12.5 m*M* DTT, 2 m*M* spermidine, T7 polymerase (U.S. Biochemical) at 5 U/μl, RNasin (Promega) at 1 U/μl, 4 m*M* each of ATP, GTP, and CTP, 270 n*M* [α^{32}P]UTP (3000 Ci/mmol), 0.8 m*M* 5-(3-amino)allyl-UTP (allylamine-UTP; BRL) (*45*), and 250 n*M* duplex DNA. Full-length RNA was purified after resolution on a denaturing 15 percent polyacrylamide gel. (**B**) Anchor RNA was biotinylated by a chemical reaction with sulfosuccinimyl 2-(biotinamido)ethyl-1,3′-dithiopropionate (NHS-SS-biotin; Pierce Chemicals) (*45*). RNA from (A) was resuspended in 0.1*M* $NaHCO_3$, *p*H 8.5, to 40 μ*M* and dry NHS-SS-biotin was added to a final concentration of 2.5 m*M*. After 30 minutes at 23°C, the RNA was precipitated by the addition of one-tenth volumes each of 3*M* sodium acetate, *p*H 5, and dimethylformamide, and three volumes of ethanol, washed twice with 70 percent ethanol, and resuspended in water (*46*). (**C**) The biotinylated anchor RNA hybridizes to the 3′ end of the actin pre-mRNA via 21 complementary nucleotides.

RNA duplex is bound to the biotin-agarose via avidin (*32*), and 45 to 75 percent of it remains bound after incubation in the presence of extract, depending on the batch of extract used and the length of incubation in extract. Typically 70 to 90 percent of the remaining RNA is eluted.

Splicing of the pre-mRNA takes place on the solid support (Fig. 3). The splicing intermediate, lariat intron–exon 2, remains bound to the solid support whereas excised lariat intron product is freed and accumulates in the extract during the assay (compare lanes 2 and 4). [During a typical splicing assay with soluble pre-mRNA, lariat intermediate and excised lariat product accumulate in a ratio of 1:4 (*6*).] The other splicing reaction intermediate, free exon 1, and the other product, mature RNA, remain bound to the solid support as shown below. Some pre-mRNA is also released into the extract during the assay. Splicing of this freed pre-mRNA cannot account for the relative amounts of intermediates and products formed. We also find that RNA immobilized on biotin-agarose is spliced about five times less efficiently than free RNA (*33*) as a result, perhaps, of some pre-mRNA being inaccessible to the splicing components or to decreased rates of diffusion of some or all of these components to the pre-mRNA. The latter possibility would lead to a decreased rate of splicing and could explain why we can resolve early steps in the formation of the spliceosome.

We examined the RNA components bound to immobilized pre-mRNAs during incubation with splicing extract (*34*) in the presence or absence of ATP. After the bound material was eluted, the RNA's in the material were extracted and analyzed for total composition, for the snRNA's known to be involved in splicing, and for trimethylguanosine (m_3G) caps, as shown below. Many yeast snRNA's including U1, U2, U4, and U5 have such caps at their 5′ ends (*35*). For the splicing reactions, we used two pre-mRNA's that form active spliceosomes: wild type and the 3′ splice site mutant C303/305 (Fig. 4), which undergoes the first splicing reaction and accumulates spliceosomes (*6*). We also tested the A257 pre-mRNA that is mutated in the UACUAAC sequence (Fig. 4) and inactive for spliceosome formation and splicing. As a control for nonspecific binding of any factors, we used anchor RNA without pre-mRNA.

Specific RNA's identified by size (Fig. 5A), by immunoprecipitation with antibody to m_3G cap (Fig. 5B), and by RNA blot analysis (Fig. 5C) bind the pre-mRNA's known to form spliceosomes. They are purified substantially relative to ribosomal RNA's and other RNA's. The U1 snRNP binds equally well in both the absence and presence of ATP. The U2, U4, U5 S, and U5 L snRNA's bind in an ATP-dependent manner. Although U6 RNA is not observed in Fig. 5A [because of its inefficient labeling by RNA ligase (*36*)], it is detected by RNA hybridization analysis (Fig. 6).

Two other RNA species are retained by the wild-type and C303/305 pre-mRNA's. One of 180 nucleotides (nt) has an m_3G cap (Fig. 5B) and is enriched in the +ATP reactions with immobilized wild-type or C303/305 pre-mRNA's. It is either less abundant or less efficiently labeled than the other snRNA's. An snRNA of this size has not previously been observed with spliceosomes. The second RNA (650 nt) (Fig. 5A) lacks an m_3G cap and is part of the 35*S* ribosomal precursor (*33*). A trace of a 530-nt RNA in lanes 2 to 6 in Fig. 5B is probably pre-mRNA.

The reaction conditions used promote splicing. The splicing intermediates of wild-type (Fig. 5A, lane 2) and C303/305 (Fig. 5A, lane 4) pre-mRNA's and some mature mRNA from wild-type pre-mRNA (Fig. 5A, lane 2) can be seen. Because the pre-mRNA's were ^{32}P-labeled, these species were also identified on the RNA blot before it was probed for the snRNA's. Densitometric measurements of the autoradiogram showed that 0.5 percent of the wild-type pre-mRNA appeared as mature product and 1 percent of C303/305 formed intermediates.

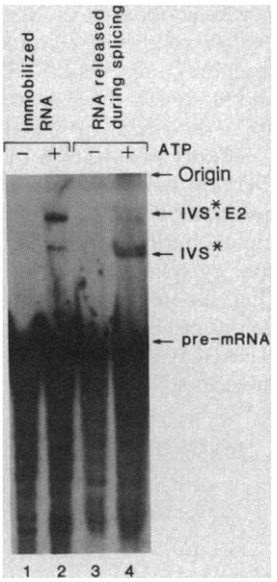

Fig. 3. Immobilized actin pre-mRNA is spliced. A ^{32}P-labeled actin pre-mRNA immobilized on the biotin agarose was incubated with splicing extract with or without ATP for 30 minutes. Both the RNA released (released) into the extract and the RNA remaining bound (bound) to the matrix during the reactions were analyzed as follows. At the end of the reactions, the splicing extract and first washing of the solid-phase splicing reactions were saved, and their RNA's were extracted for analysis. The RNA-agarose was further washed, and the bound RNA was eluted as described (*30*). The RNA's were analyzed by electrophoresis in 7.5 percent polyacrylamide (acrylamide:bisacrylamide, 29:1), 8*M* urea denaturing gels (*47*). The positions of the intermediate, lariat intron–exon 2 (IVS*E2), and lariat intron (IVS*), which characteristically migrate more slowly than pre-mRNA in the gel, are indicated.

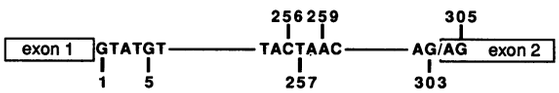

Fig. 4. Mutations in the cloned actin gene. The positions of the intron mutations used here are shown (*6*); number one is the first 5′ nucleotide of the intron. Each mutation is named for its base substitution and position; for example, T257 is mutated to A257. In wild-type pre-mRNA, A259 is the branch nucleotide.

A surprising result is that the A257 pre-mRNA binds almost no U1 snRNP or any other snRNP. We thought that this mutant pre-mRNA would bind only a subset of the snRNP's in that the A257 mutation inhibits pairing with U2 snRNP (*12*). However, because U1 snRNA pairs with the 5′ splice site, we did not expect this mutation to affect U1 snRNP binding. The average binding of U1 snRNP in this experiment is not significantly above background [less than 6 percent of the wild type (*37*)], an indication that U1 snRNP binding may depend on the UACUAAC sequence as well as on the 5′ splice site.

U1 is the first snRNP to bind to pre-mRNA. To analyze this effect of the A257 mutation, we first determined the kinetic order in which the splicing snRNP's bind to a functional pre-mRNA. The snRNP's binding to immobilized C303/305 pre-mRNA at 15°C was monitored by RNA blot analysis (Fig. 6A). The amounts of each snRNA in the autoradiograms were measured by densitometry (Fig. 6B). U1 is clearly the first snRNP to bind. There is a threefold increase in U1 snRNA at 2 minutes, a time when no other RNA can be detected above the background. The other snRNP's bind more slowly and with indistinguishable kinetics (*38*).

We also examined the snRNP's binding to immobilized pre-mRNA in the presence or absence of ATP at 0°C. The mammalian U1 snRNP, both from a partially purified fraction (*39*) and from nuclear splicing extracts (*10*) binds in vitro to the 5′ splice site of pre-mRNA's rapidly [50 percent of the maximum bound within 5 minutes (*39*)] at 0°C in the presence of Mg^{2+} and without ATP. If a similar interaction of U1 snRNP to the 5′ splice site occurs in yeast, we did not detect it since no yeast U1 snRNP bound at 0°C during a 20-minute incubation (Fig. 6A, lanes 9 to 11).

U1 snRNP binding is required for the binding of other snRNP's. The effect of the A257 mutation suggests that U1 snRNP binding depends on both the 5′ splice site and the UACUAAC

sequence. This is further suggested by the phenotypes conferred by other mutations in the UACUAAC sequence as well (see below). We considered that the A257 mutation could affect U1 snRNP binding in at least three possible ways. (i) If the U1 and U2 snRNP's bind cooperatively to the intron, the mutation could mediate its effect through U2 snRNP binding. (ii) The U1 snRNP could recognize the UACUAAC sequence and require that interaction for binding. (iii) A factor, other than U1 and U2 snRNP, binding to the UACUAAC sequence could be required for the U1 snRNP to bind. To test the first possibility, we inactivated either the U1 snRNP or the U2 snRNP in whole cell splicing extracts by oligodeoxynucleotide-directed cleavage catalyzed by the extract's endogenous ribonuclease (RNase) H activity. We determined that cleavage of the snRNP's resulted in the inhibition of splicing. The treated extracts were then assayed for snRNP binding activity in solid-phase splicing reactions. Binding of the snRNP's in these reactions was detected by RNA blot analyses (Fig. 7).

Incubating whole cell extract with an oligodeoxynucleotide complementary to the region of U2 snRNA known to base pair with the intron UACUAAC sequence results in nearly total degradation of the entire U2 snRNA; less than 1 percent and 6 percent of the initial amount of U2 snRNA remain in the extract as intact and truncated forms, respectively (Fig. 7, lane 13). In contrast, the U2 snRNA remains intact during control treatments of extract (lanes 11, 12, and 14). When such an extract with inactivated U2 snRNP is assayed, U2 binding is, as expected, undetectable whereas U1 binding is only moderately affected. No U5 binding above background is observed, however.

Incubating whole cell splicing extract with an oligodeoxynucleotide complementary to the first 12 nt of the 5′ end of U1 snRNA containing the region that base pairs with the pre-mRNA 5′ splice site results in specific cleavage of the 5′ end (*18*) of more than 99 percent of the U1 snRNA (Fig. 7, lane 12). Unlike the situation with the cleaved U2 snRNA, there is no other detectable degradation of the truncated U1 snRNA during prolonged incubation. When such a treated extract is assayed for snRNP binding activity, we find that no U1 binds [less than 1.0 percent of the control—compare also Fig. 8, lanes 11 to 14 (*37*)]. Nor do detectable amounts of U2, U5L, or U5S snRNP's bind in the presence or absence of ATP.

To show that the U2 snRNP in a U1-inactivated extract is still active, we tested its ability to bind to pre-mRNA in a complementation assay. In this two-step binding assay, U1 snRNP was first bound to immobilized pre-mRNA by incubating the pre-mRNA with splicing extract in the absence of ATP. After extensive washing in splicing buffer lacking both ATP and extract to remove any unbound components including U2 snRNP, the splicing extract with inactive U1 snRNP and ATP was added to the immobilized pre-mRNA. U2 snRNP binding was subsequently assayed by RNA blot analysis. We find that U2 snRNP in an extract with inactivated

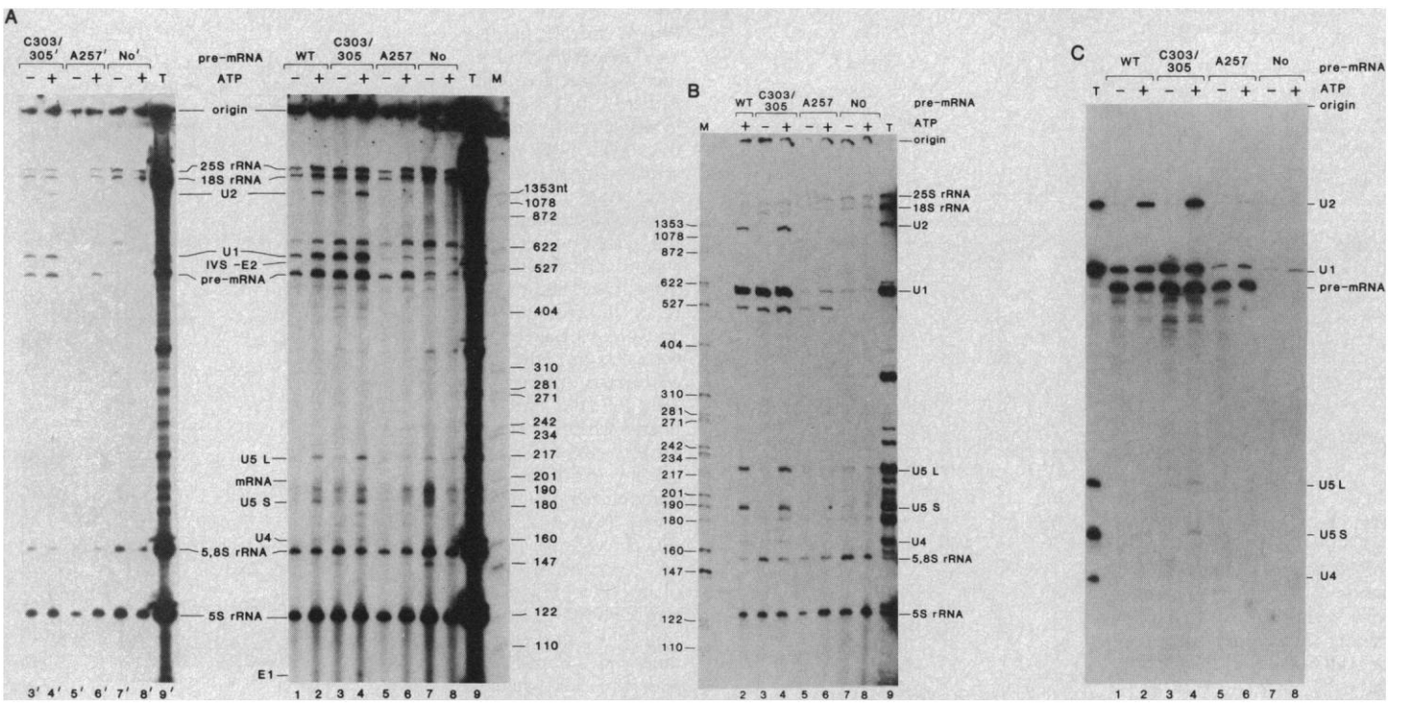

Fig. 5. Specific snRNA's associate with pre-mRNA's during splicing. Three immobilized actin pre-mRNA's were used in splicing reactions: wild type (WT) and two mutants, C303/305 and A257 (see Fig. 4). Anchor RNA without any pre-mRNA (No) was a control. The reactions with (+) or without (−) ATP were incubated for 30 minutes at 23°C. The RNA's eluted from each reaction were analyzed (i) by 3′ end-labeling with [^{32}P]pCp and T4 RNA ligase (*36*) so that any RNA with a free 3′ hydroxyl is labeled, and (ii) by RNA blot analysis. The RNA's from splicing reactions (lanes 1 to 8) and from extract (lane 9) were visualized by autoradiography after gel electrophoresis (A and B) or hybridization (C). (**A**) Total RNA eluted from splicing reactions. One-half of the 3′ end-labeled RNA's were size-fractionated by electrophoresis in denaturing 4 percent polyacrylamide gels. The positions of ribosomal RNA's, snRNA's (U1, U2, U4, U5 L, and U5 S), pre-mRNA, splicing intermediates (lariat intron–exon 2, IVS-E2; and free exon, E1), and spliced product (mRNA) are indicated. The other spliced product, lariat intron, cannot be seen as it comigrates with pre-mRNA. Single-stranded DNA fragments from phage ϕX174 and plasmid pBR322 digested with restriction endonucleases Hae III and Hpa II, respectively, were ^{32}P-labeled at their 5′ ends and used as markers (lane M); their sizes are indicated at the far right. The left panel (lanes 3′ to 9′) is a lighter exposure of a portion of the right panel. (**B**) RNA's with m_3G caps. One-half of the 3′ end-labeled RNA's were incubated with antibody to m_3G cap (*48*). The immunoprecipitated RNA's were resolved by gel electrophoresis. The markers (M) are described in (A). (**C**) RNA blot analysis. One-third of the RNA's from the reactions and from extract were resolved by gel electrophoresis, transferred to a nylon membrane, and hybridized simultaneously with ^{32}P-labeled probes for four snRNA's [U1, U2, U5 L and S, and U4 (*49*)]. By comparing the amount of each snRNA recovered in these reactions with that in the splicing extract in this and other experiments, we estimate that 5 to 15 percent of each snRNP in the splicing extract is bound in our assays.

U1 snRNP is still able to bind to pre-mRNA when exogenous U1 snRNP is first bound to the pre-mRNA (Fig. 8). Although it appears that less U2 snRNP is bound in U1-inactivated extract compared to the control extracts (compare lanes 4, 6, and 10), the ratios of U2 to intact U1 snRNP's bound in the reactions are equivalent. Therefore, the U1-inactivating oligodeoxynucleotide does not bind some factor other than U1 snRNA to prevent U2 binding. Furthermore, the inactivated U1 snRNP does not inhibit U2 binding. This experiment also shows that U1 snRNP bound in the absence of ATP can subsequently promote the binding of U2 snRNP in the presence of ATP.

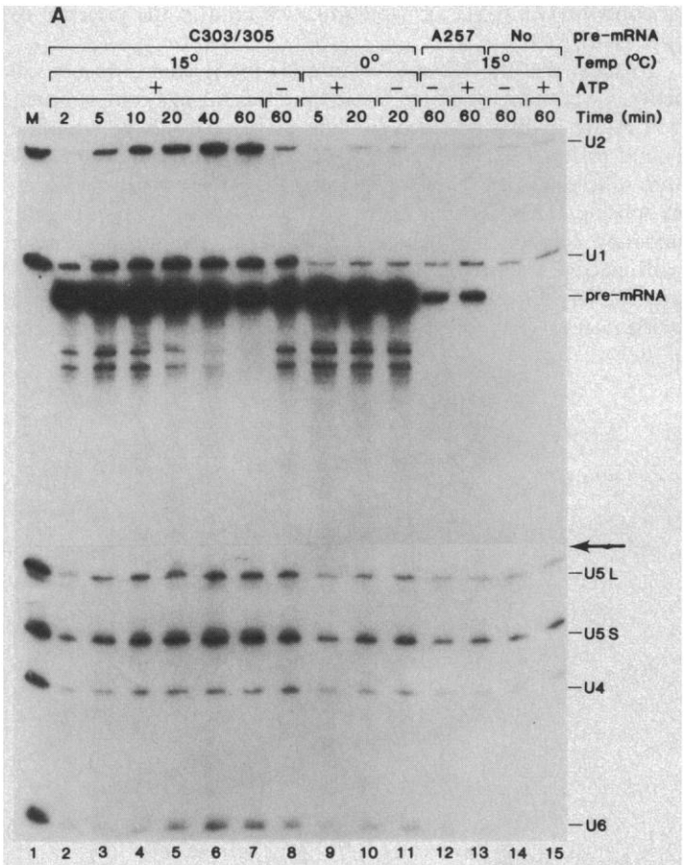

Fig. 6. Time course of snRNP's binding to pre-mRNA's during splicing reactions. (**A**) Splicing reactions (lanes 2 to 15) containing either immobilized pre-mRNA's, C303/305 (lanes 2 to 11) or A257 (lanes 12 and 13), or anchor RNA only (lanes 14 and 15) were incubated either with (+) or without (−) ATP at 15°C or 0°C for the times indicated. Splicing at 15°C is normal although slower than at 23°C whereas it is undetecable at 0°C (*50*). The RNA's were eluted, fractionated by size by gel electrophoresis, transferred to a nylon membrane, hybridized to the five snRNA probes indicated (*49*), and visualized by autoradiography as shown here. A darker exposure of the lower portion of the blot (below the arrow) replaces the original exposure. RNA's extracted from 35P cell extract were markers (lane 1). (**B**). The amounts of each snRNA present in (A) were determined by scanning autoradiograms with an LKB densitometer. The values (*37*) are expressed as the percent of each snRNA bound at 40 minutes in the presence of ATP (lane 8).

We conclude that U1 snRNP binding is required for subsequent binding of the U2 and U5 snRNP's to pre-mRNA and that U2 snRNP binding is required for U5 snRNP binding. However, neither the presence of intact U2 snRNP nor its binding to the pre-mRNA is required for U1 snRNP binding. Furthermore, the initial U1 snRNP binding requires the first 12 nt at the 5′ end of its snRNA since we see no significant binding to the pre-mRNA when this sequence is removed.

Both 5′ splice site and UACUAAC mutations affect snRNP binding to pre-mRNA. To further ascertain which pre-mRNA sequences are required for snRNP binding, we assayed the snRNP's binding to several mutant pre-mRNA's (Fig. 4). Two mutations, A1 and C1, in the 5′ splice site of actin pre-mRNA, are known to severely depress splicing both in vivo and in vitro and to inhibit spliceosome formation (*6, 7, 25, 26*). A third 5′ splice site mutation, A5, lowers splicing efficiency in vivo and blocks splicing and spliceosome formation in vitro. Two mutations, A256 and C259, in the UACUAAC sequence, like the A257 mutation, decrease splicing efficiency both in vivo and in vitro (*6, 12*) and inhibit spliceosome formation (*6, 26*). Pre-mRNA's with these mutations were used as immobilized splicing substrates and the amounts of the snRNP's bound were determined by RNA blot analysis (Fig. 9 and Table 1).

The 5′ splice site mutations, A1 and C1, exert two effects on snRNP binding. They reduce U1 snRNP binding both in the presence and absence of ATP. They also block both U2 and U5

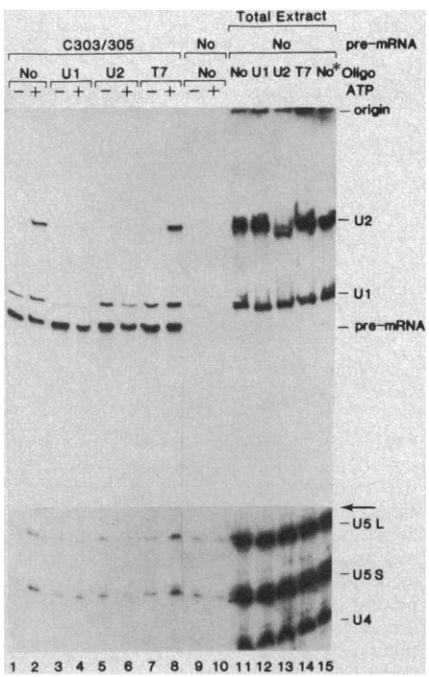

Fig. 7. snRNP binding after inactivation of either U1 or U2 snRNP. Either U1 or U2 snRNP in whole cell splicing extract (WCE) (*47*) was inactivated by oligodeoxylnucleotide-directed RNase H cleavage, and the treated extracts were assayed for snRNP binding activity. Shown here is an autoradiogram of an RNA blot analysis of the snRNA's in the treated extracts (lanes 11 to 15) and the snRNA's bound when the treated extracts were used in splicing reactions with immobilized pre-mRNA (lanes 1 to 10). WCE (50 percent v/v) in 100 m*M* potassium phosphate buffer *p*H 7, 5 percent PEG-8000, 4.5 m*M* $MgCl_2$, 1.3 m*M* spermidine, and 0.5 m*M* DTT was first incubated at 32°C for 30 minutes with an oligodeoxynucleotide complementary to either nt 1 to 12 of U1 snRNA (lane 12) or nt 29 to 43 of U2 snRNA (lane 13) as described (*18*). As controls, WCE was incubated with the oligodeoxynucleotide encoding the phage T7 promoter (Fig. 2) (lane 14, oligo T7) or without any oligodeoxynucleotide (lane 11, No oligo). Some WCE was not incubated (lane 15, No* oligo). The extracts were then diluted with water to a concentration of 40 percent extract and incubated at 23°C for 30 minutes with either immobilized C303/305 pre-mRNA (lanes 1 to 8) or anchor RNA (lanes 9 and 10) either with (+) ATP or without (−) ATP as indicated: extracts with inactivated U1 (lanes 3 and 4) or inactivated U2 (lanes 5 and 6) and control extracts (No oligo, lanes 1 and 2; T7 oligo, lanes 7 and 8). The U1 (5′-TGCCAGGTAAGTAT-3′) and U2 (5′-CAGATACTACACTTG-3′) oligodeoxynucleotides were synthesized chemically. A darker exposure of the lower portion of the blot (below the arrow) replaces the original exposure.

snRNP binding, with C1 having the stronger effect of the two mutations; A1 reduces U2 binding to less than 8 percent of the control whereas C1 reduces it to the background (Table 1). The A5 mutation has no effect on U1 snRNP binding in the absence of ATP, but in the presence of ATP the amounts of U1 and U2 snRNP bound are reduced. The two UACUAAC mutations, A256 and C259, reduce U1 binding either in the presence or absence of ATP and U2 snRNP binding in the presence of ATP. The effects of C259 are slightly stronger than those of A256. In whole cell extract or 35P fraction supplemented with whole cell extract, however, the effects of the C259 mutation are less severe. The effects of the third UACUAAC mutation, A257, in contrast, do not change substantially with the type of extract used (*33*).

As ordered by the severity of the decrease in U1 snRNP binding, C1>A1>A5 and A257>C259>A256, these mutations rank the same as in previous in vivo and in vitro assays. The results with the 5′ splice site mutations further support our conclusion that U1 snRNP binding is required for subsequent binding of U2 and U5 snRNP's. All three mutations cause a decrease in U1 snRNP binding but they have a more drastic effect on the binding of U2 snRNP. Thus, even though some U1 is bound, the complex is not an appropriate substrate for U2 binding.

Early role of the U1 snRNP in spliceosome assembly. In discussing the relevance of these results, it is important to establish that the early complexes observed in this study are intermediates in the formation of an active spliceosome. Formation of these complexes is correlated with splicing by the following criteria: (i) pre-mRNA tethered to the solid support can be spliced in the presence of ATP and an active extract; (ii) all snRNA's known to be required for mRNA splicing are selectively enriched by our procedure; and (iii) the relative efficiencies of snRNP binding to mutant precursors correlate well with the known phenotypes of these mutants in vivo and in vitro. Thus, among the UACUAAC mutants, A257 which is most impaired in U1 binding, is also the most defective in splicing. The same is true of C1 among the 5′ splice site mutants. The most convincing evidence that a complex is an intermediate in spliceosome assembly is the demonstration that pre-mRNA in the complex can be spliced to form product. However, because of the low efficiency of splicing of immobilized pre-mRNA, we have not yet been able to do this.

Our results suggest that U1 snRNP binding is an early and essential step in the formation of the spliceosome (Fig. 10). In the absence of ATP, only U1 binds to the precursor. In the presence of ATP, it is the first to bind. If U1 is inactivated, other snRNP's cannot bind to the precursor. Although U1 can bind to 5′ splice site mutant precursors, that binding does not support addition of other snRNP's. Therefore, U1 snRNP binding is a necessary, but not sufficient, condition for the binding of the other snRNP's. Apparently there are two modes of U1 snRNP binding to the precursor. Only when the correct interactions have been formed with both the

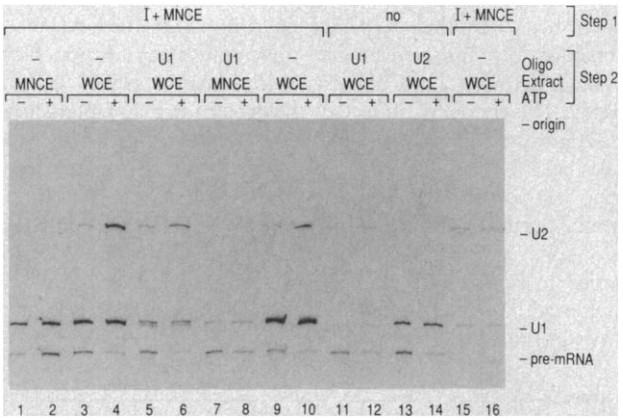

Fig. 8. snRNP binding complementation assay. The binding activity of U2 snRNP in an extract with inactivated U1 snRNP was determined in a two-step complementation assay. In the first step active U1 snRNP from an extract fraction enriched for U1 snRNP (fraction I) (*49*) was bound to immobilized C303/305 pre-mRNA in the absence of ATP (lanes 1 to 10) or was added to anchor RNA only (lanes 15 and 16); the RNA's were incubated for 20 minutes at 23°C in splicing buffer supplemented with 10 percent (v/v) each of fraction I and micrococcal nuclease–treated whole cell extract (MNCE) (*48*). The immobilized RNA's were then washed three times with 30 volumes of splicing buffer supplemented with 40 percent (v/v) buffer D [(20 m*M* Hepes, *p*H 7.6, 0.2 m*M* EDTA, 0.5 m*M* DTT, 50 m*M* KCL, and 20 percent glycerol (*47*)] to remove unbound components. In the second step, the immobilized RNA's were incubated for 30 minutes at 23°C in various extracts with ATP (even-numbered lanes) or without ATP (odd-numbered lanes). The extracts contained splicing buffer supplemented with the following: lanes 1 and 2, 40 percent (v/v) MNCE; lanes 3 and 4, 40 percent (v/v) WCE incubated previously at 32°C for 30 minutes as in Fig. 7; lanes 5 and 6, 40 percent (v/v) WCE incubated with U1-inactivating oligodeoxynucleotide as in Fig. 7; lanes 7 and 8, 40 percent v/v MNCE and U1-inactivating oligodeoxynucleotide; lanes 9 and 10, 40 percent (v/v) WCE; and lanes 15 and 16 (anchor RNA only), 40 percent (v/v) WCE. The amounts of snRNP's bound were determined by RNA blot analysis as in Fig. 5C. As controls, immobilized pre-mRNA's were also incubated in either the U1-inactivated extract (lanes 11 and 12) or U2-inactivated extract (lanes 13 and 14) as in Fig. 7. The levels of nonspecific binding of the snRNP's in lanes 15 and 16 are equivalent to those seen when anchor RNA is incubated in splicing buffer supplemented with 40 percent (v/v) WCE (*33*).

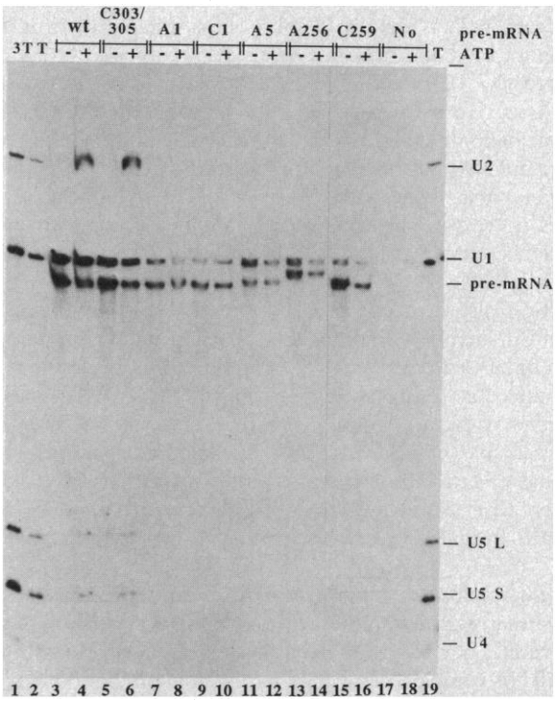

Fig. 9. snRNP binding to mutant pre-mRNA's. Several mutant actin pre-mRNA's (Fig. 4) were immobilized and tested for snRNP binding activity when incubated for 30 minutes with 35P splicing extract with (+) or without (−) ATP: 5′ splice site mutants, A1 (lanes 7 and 8), C1 (lanes 9 and 10), and A5 (lanes 11 and 12); UACUAAC mutants, A256 (lanes 13 and 14) and C259 (lanes 15 and 16); and 3′ splice site mutant C303/305 (lanes 5 and 6). Wild-type pre-mRNA (lanes 3 and 4) and no pre-mRNA (lanes 17 and 18) were controls. (The A256 pre-mRNA is 32 nt longer than the other pre-mRNA's because it was transcribed from a template linearized with Sty I enzyme.) The snRNP's bound to the pre-mRNA's were determined by RNA blot analysis as in Fig. 5C. Samples (0.1 μl, lanes 2 and 19; and 0.3 μl, lane 1) of RNA extracted from 35P splicing extract provided snRNA markers. The amounts of snRNA's were measured by liquid scintillation counting excised portions of the blot (*37*). These data were combined with those from additional experiments and are summarized in Table 1.

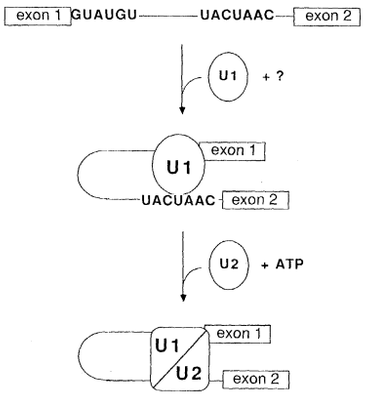

Fig. 10. A model for the early interaction of U1 snRNP with pre-mRNA in the formation of the spliceosome. The binding of U1 and U2 snRNP's to the pre-mRNA is schematically shown as the first two steps in a pathway for spliceosome assembly. Our data (Figs, 3 to 9) show that U1 is the first snRNP to bind to the pre-mRNA. This binding depends on both the 5′ splice site and UACUAAC sequence, which may or may not interact before U1 binds. Some factor or factors (?) may or may not be involved in this step. U2 is the second snRNP to bind. It requires ATP and the correct association of U1 with the 5′ splice site and UACUAAC. The contact between U1 and U2 is arbitrarily drawn.

5′ splice site and the branch-point sequence can complex formation proceed.

There is a good deal of evidence to support the early and hierarchic role of the U1 snRNP in spliceosome formation. The purified mammalian U1 snRNP can bind to the 5′ splice site, indicating that no factors are required for this binding (*39*). Bindereif and Green (*29*) have shown that only the U1 snRNP binds when mammalian extracts are incubated with precursor in the absence of ATP. When the 5′ splice site of the precursor is deleted, U1 cannot bind and only low levels of the other snRNP's bind to this precursor (*29*). In the absence of active U2 snRNA, yeast pre-mRNA can form a complex that is committed to forming a subsequent complex (as assayed by resistance to unlabeled competitor pre-mRNA). In order to form the first complex the precursor must have an active 5′ splice site as well as a UACUAAC sequence. ATP is not required (*40*). These results nicely complement those reported here if we assume that the early commitment step requires the U1 snRNP.

There is also evidence that supports a direct interaction of the human U1 snRNP with the 3′ splice site region. By immunoprecipitation of labeled precursor with antibodies to U1-specific proteins, Zillmann *et al.* (*28*) detected binding of U1 snRNP proteins to precursors without a 5′ splice site. This interaction is reduced, but still observable, with U1 snRNP's in which the 5′ terminal nucleotides have been removed by RNase H cleavage; the difference between their result and that of others (*29* and ours) may be explained by differences in experimental approach and precursors used.

Recent results also suggest an interaction between the 5′ and 3′ splice sites in early complex formation. Mutations in both the 3′ and 5′ splice sites of a β-globin precursor decrease formation of the early U2 snRNP complex more than either single mutation alone (*41*). Electron micrographs of *Drosophila* chromosome spreads show the splicing process coupled to transcription (*42*). A 10-nm particle appears occasionally at the 5′ splice site before 3′ splice site transcription. After 3′ splice site synthesis, two 25-nm particles and then one 40-nm particle quickly appear as the two splice sites coalesce and a circular form is removed from the growing transcript. The two splice sites apparently associate very soon after synthesis. Whether the 5′ 25-nm complex forms as an intermediate in the process or results from the 40-nm particle separating during sample preparation is not known. However, it has been suggested (*8, 42*) that two states of U1 binding may occur: a relatively weak association to the 5′ splice site and a more stable interaction requiring the 3′ splice site and other snRNPs.

As shown in our study, the binding of U1 to the pre-mRNA depends in part on the branch-point sequence. In fact, it is more strongly affected in either the presence or the absence of ATP by the A257 mutation in the UACUAAC sequence than it is by 5′ splice site changes. This result is important because it suggests that U1 in the absence of other snRNP's mediates the crucial interaction between the 5′ splice site and the branch point. Our result appears to contradict a previous result that a compensatory mutation in U2 snRNA partially suppresses the A257 mutation in vivo (*12*). We think that these results do not conflict for two reasons. (i) The effects of intron mutations on splicing are usually more severe in vitro than in vivo (*6, 43*). (ii) While splicing of the A257 mutant pre-mRNA is enhanced by the U2 suppressor, a significant amount of pre-mRNA remains, an indication that splicing of A257 pre-mRNA is not restored to wild-type levels. Our result could explain in part the inability of the suppressor U2 snRNA to fully restore splicing of the A257 mutant pre-mRNA in vivo.

The interaction between U1 snRNP, the 5′ splice site, and the branch-point sequence could occur in at least two ways—by RNA-RNA or RNA-protein associations. There is no obvious base pairing possible between U1 snRNA and UACUAAC. A weak complementarity, noted by others (*3*), does exist between the 5′ splice site and UACUAAC. If RNA-RNA interactions alone mediate the binding of U1 and the juxtaposition of 5′ splice site and branch point, then this potential base pairing may be important. The U257 base has been shown to pair with U2 snRNA (*12*); however, its pairing with U2 is not required for U1 binding to pre-mRNA as shown here. Another sequence in U2 could mediate the U1 interaction, but this seems unlikely given our data. It is possible that a protein recognizes the branch-point sequences as well as the U1 snRNP. This model requires that the protein strongly interacts with U257 and that this interaction stabilizes U1 binding. This protein could be a U1 or U2 snRNP protein or a non-snRNP splicing factor. A non-snRNP protein, U2AF, has been detected and partially purified from HeLa cell extracts (*13*). This protein binds to the 3′ splice site region and is required for spliceosome formation. Studies in which our affinity purification method is used may provide additional insights into the role of U1 snRNP and any factors in the 5′ splice site and branch-point interaction.

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30. Step 1. Synthetic actin pre-mRNA was transcribed in vitro by SP6 polymerase to a specific activity of 3.2×10^3 Cerenkov cpm/fmol with [α-^{32}P]UTP and purified by gel electrophoresis (*47*). The actin gene templates were previously cloned (*6*) in plasmid vectors pSP65 or pSP64 and had the cryptic branch-point sequence deleted from the intron. They were linearized with the Acc I or Sty I restriction endonucleases. Step 2. Anchor RNA and pre-mRNA were hybridized in solution (70 m*M* tris-HCl, *p*H 7.6, 8 m*M* NaCl, 1.5 m*M* EDTA, 25 to 500 n*M* anchor RNA, and 25 n*M* actin pre-mRNA) by heating at 100°C for 1 minute and 65°C for 1 minute and then gradually cooling from 37°C to 15°C for 40 minutes. Step 3. Succinylated avidin (EY Laboratories) was incubated for at least 1 hour at 0°C with biotin-agarose (12-atom biotin-linker arm; Sigma) in a ratio of 2 mg of avidin to 1 ml of agarose. The coupled agarose was washed three times with five volumes of binding buffer (150 m*M* potassium phosphate buffer, *p*H 7, 1 m*M* EDTA, 15 percent glycerol, and 0.05 percent NP-40) and resuspended in binding buffer to the original volume of agarose. The RNA duplex was then incubated with the agarose (either 0.6×10^4 or 1.2×10^4 Cerenkov cpm RNA hybrid per microliter of agarose) for at least 40 minutes. A second portion of succinylated avidin (0.5 μg per microliter of agarose) was added and incubated for 30 minutes. The RNA-agarose was washed three times with binding buffer, distributed into Eppendorf tubes, and washed once with four volumes of splicing buffer [60 m*M* potassium phosphate buffer, *p*H 7, 1 m*M* sperimidine, 3 m*M* $MgCl_2$, 3 percent PEG8000, 0.5 m*M* DTT, and RNAsin at 800 U/ml) with or without 2 m*M* ATP. The splicing buffer was removed, and ATP (2 nmol per microliter of splicing extract) was added as indicated. Step 4. Typically, 60 μl of splicing extract [splicing buffer supplemented with 40 percent yeast 35P cell extract (*6*) or whole cell extract (*47*)] were incubated at 23°C for 10 minutes to deplete any endogenous ATP by adenosine triphosphatase in the extract and then added to 40 μl of RNA-agarose with or without ATP. The reactions were incubated at 23°C for the indicated times during which they were gently mixed by hand at 5-minute intervals. They were stopped by the addition of a tenfold excess of washing buffer (150 m*M* NaCl, 20 m*M* Hepes, *p*H 7.8, 3 m*M* $MgCl_2$, 0.5 m*M* DTT, 15 percent glycerol, and 0.05 percent NP-40) at 0°C and by sedimentation for 7 seconds in an Eppendorf centrifuge. The RNA-agarose was washed twice again with washing buffer at 0°C. Step 5. The RNA, and the factors bound to it, were eluted twice from the agarose at 0°C for 5 minutes with five times the volume of elution buffer (40 m*M* tris-HCl, *p*H 9, 150 m*M* NaCl, 3 m*M* $MgCl_2$, and 150 m*M* DTT) (*30*). RNA in the combined eluents was extracted as described (*47*).
31. Avidin rather than streptavidin must be used for the disulfide bond in the 12-atom linker arm to be reduced [T. M. Herman, E. Lefever, M. Shimikus, *Anal. Biochem.* **156**, 48 (1986)]. Although splicing reactions contain 0.5 m*M* DTT, this concentration is not high enough to elute the anchor RNA with multiple biotin residues during the incubation periods used here.
32. Less than 10 percent of a truncated pre-mRNA lacking the region complementary to the anchor RNA attached to the matrix either with or without anchor RNA.
33. S. W. Ruby and J. Abelson, unpublished results.
34. Unless otherwise stated, the extract we used is a fraction (35P) prepared from a 35 percent ammonium sulfate precipitate of whole cell splicing extract (*6*). This fraction is active for splicing but has less nuclease activity than whole cell extract.
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37. To calculate the amount of an snRNA bound in each reaction, we first subtracted the background (the amount of the snRNA binding to anchor RNA) to get a net value. This value was then divided by the amount of pre-mRNA on the blot in the lane for that reaction. The value is usually expressed as the percentage of the amount of snRNA bound to the wild-type pre-mRNA in the presence of ATP as determined in each experiment.
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49. After electrophoresis in 4 percent denaturing polyacrylamide gels, RNA was transferred to GeneScreen membrane (NEN) by electroblotting at 4°C in 2 m*M* tris borate (*p*H 8), and 0.5 m*M* EDTA at 120 V as described by G. M. Church and W. Gilbert [*ibid.* **81**, 1991 (1984)]. RNA was cross-linked with ultraviolet light from three GE G1578 bulbs at a distance of 35 cm for 10 minutes. Hybridization was as described (*26*). For probes, DNA fragments containing the yeast snR6 (*21*), snR7 (*19*), snR14 (*20*), snR19 (*17*), and the 5′ two-thirds of the snR20 (*16*) genes were ^{32}P-labeled by random oligodeoxynucleotide-primed extension [A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* **132**, 6 (1983)].
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