RNA (1998), *4*:709–718. Cambridge University Press. Printed in the USA.

Base pairing with U6atac snRNA is required for 5′ splice site activation of U12-dependent introns in vivo

ROBERT INCORVAIA and RICHARD A. PADGETT

Department of Molecular Biology, The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195, USA

ABSTRACT

The minor U12-dependent class of eukaryotic nuclear pre-mRNA introns is spliced by a distinct spliceosomal mechanism that requires the function of U11, U12, U5, U4atac, and U6atac snRNAs. Previous work has shown that U11 snRNA plays a role similar to U1 snRNA in the major class spliceosome by base pairing to the conserved 5′ splice site sequence. Here we show that U6atac snRNA also base pairs to the 5′ splice site in a manner analogous to that of U6 snRNA in the major class spliceosome. We show that splicing defective mutants of the 5′ splice site can be activated for splicing in vivo by the coexpression of compensatory U6atac snRNA mutants. In some cases, maximal restoration of splicing required the coexpression of compensatory U11 snRNA mutants. The allelic specificity of mutant phenotype suppression is consistent with Watson–Crick base pairing between the pre-mRNA and the snRNAs. These results provide support for a model of the RNA–RNA interactions at the core of the U12-dependent spliceosome that is strikingly similar to that of the major class U2-dependent spliceosome.

Keywords: base pairing; genetic suppression; snRNP; splicing

INTRODUCTION

Two distinct spliceosomal systems have co-existed in eukaryotic cells since at least the divergence of the plant and animal kingdoms (reviewed in Tarn & Steitz, 1997). These two systems act on pairs of mutually incompatible splice sites flanking pre-mRNA introns in eukaryotic nuclear genomes. The large majority of introns in all known organisms are spliced by a well-studied pathway requiring the function of the small nuclear RNAs U1, U2, U4, U5, and U6, as well as a large number of additional proteins. In this pathway, multiple RNA–RNA interactions have been demonstrated to form between the splice site sequences and the snRNAs and between various snRNAs in the spliceosome (reviewed in Nilsen, 1998).

One of the earliest interactions takes place between the 5′ end of U1 snRNA and the 5′ splice site via base pairing (Zhuang & Weiner, 1986; Seraphin et al., 1988; Siliciano & Guthrie, 1988). A second base pairing interaction takes place between the sequence in the intron surrounding the site of branching and a region of U2 snRNA (Parker et al., 1987; Wu & Manley, 1989; Zhuang et al., 1989; Zhuang & Weiner, 1989). Following these initial recognition events, a complex of U4, U5, and U6 snRNPs joins the nascent spliceosome and the combined assemblage undergoes several structural rearrangements. During this portion of the spliceosome assembly process, the extensive base pairing between U4 snRNA and U6 snRNA is disrupted so that U6 snRNA can participate in base pairing to U2 snRNA (Hausner et al., 1990; Datta & Weiner, 1991; Wu & Manley, 1991; Madhani & Guthrie, 1992). In addition, an adjacent sequence in U6 snRNA forms base pairs to the 5′ splice site, which displaces U1 snRNA from the complex (Kandels-Lewis & Seraphin, 1993; Lesser & Guthrie, 1993; Hwang & Cohen, 1996). U5 snRNP interacts with exon sequences near the 5′ and 3′ splice sites, but apparently without substantial sequence specificity (Wyatt et al., 1992; Sontheimer & Steitz, 1993; Newman, 1997). Thus, 5′ splice site activation appears to be at least a two-step process in which U1 snRNP, probably in cooperation with additional factors, specifies the 5′ splice site followed by U5 and U6 snRNP interactions that activate the site for reaction. A striking feature of these RNA–RNA interactions is their apparent high degree of conservation throughout eukaryotic phylogeny.

Reprint requests to: Richard A. Padgett, Department of Molecular Biology, NC-20, The Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, Ohio 44195, USA; e-mail: padgetr@cesmtp.ccf.org

These interactions have been studied in both yeast and human systems in vivo and in vitro by a variety of techniques (see Moore et al., 1993 and Nilsen, 1998 for reviews). These studies have demonstrated the stepwise assembly of the spliceosome and the roles of sequence elements in the pre-mRNA and the snRNAs in various interactions. Biochemical crosslinking studies, in particular, have provided direct evidence for the physical association of RNAs, which nicely complement the genetic studies (Sawa & Abelson, 1992; Wassarman & Steitz, 1992; Wyatt et al., 1992; Sontheimer & Steitz, 1993). However, a limitation of crosslinking data is that it does not show that a given close contact between RNAs is functional or necessary for splicing.

The recent identification and characterization of a second spliceosomal system in cells has provided evidence that aspects of these RNA–RNA interactions are also conserved between the two systems. This is particularly striking in light of evidence that the two systems have both been present for a substantial fraction of the history of life (Wu et al., 1996). This recently described system is responsible for splicing a relatively small set of introns with distinctive splice site signals (Jackson, 1991; Hall & Padgett, 1994; Dietrich et al., 1997; Sharp & Burge, 1997). Functional and structural studies of this new spliceosomal system have revealed that it uses a distinct set of snRNAs (Hall & Padgett, 1996; Tarn & Steitz, 1996a, 1996b; Kolossova & Padgett, 1997).

The minor snRNA species U11, U12, U4atac, and U6atac appear to be the functional analogues of U1, U2, U4, and U6, respectively, at least to a first approximation. U5 snRNA appears to be common to both systems. Both in vivo and in vitro data support the idea that U12 snRNA interacts by base pairing with the highly conserved branch site sequence (Hall & Padgett, 1996; Tarn & Steitz, 1996a) and U11 snRNA interacts similarly with at least a portion of the 5′ splice site sequence (Kolossova & Padgett, 1997; Yu & Steitz, 1997). Because these distinctive biochemical requirements define the two spliceosomal systems rather than any one feature of the intronic splice sites, we refer to the two classes of introns as U2-dependent or U12-dependent (Dietrich et al., 1997).

Recently, a novel U6-like snRNA called U6atac snRNA was identified that appears to be the functional analogue of U6 snRNA in the U12-dependent splicing system (Tarn & Steitz, 1996b). This finding has provided an opportunity to examine the conservation of some of the RNA–RNA interactions believed to be central to the splicing process. One candidate interaction that emerged directly from the comparison of the U6 and U6atac snRNA sequences was the potential for base pairing between the U12-dependent 5′ splice site and U6atac. U6 snRNA contains a phylogenetically highly conserved sequence of ACAGA located just 5′ of a region known to base pair with U2 snRNA in the spliceosome (Fig. 1A). This sequence has been shown to interact, at least partially by base pairing, with the 5′ splice site and, in particular, with the conserved G at position +5 (Kandels-Lewis & Seraphin, 1993; Lesser & Guthrie, 1993; Hwang & Cohen, 1996). In U6atac snRNA, this sequence is replaced by AAGGAGA, which is also located 5′ of a region that can potentially form base pairs with U12 snRNA and where a U12-U6atac in vitro crosslink has been mapped (Tarn & Steitz, 1996b). As shown in the shaded region of Figure 1B, this U6atac sequence has the potential to form several base pairs with the 5′ splice site sequence of U12-dependent introns by displacing U11 snRNA. In support of this idea, a recent crosslinking study detected an interaction between the 5′ splice site of a U12-dependent intron and U6atac (Yu & Steitz, 1997). However, the site of crosslinking on U6atac was not determined and the functional significance of this crosslink remains to be shown.

To examine this potential U6atac snRNA–5′ splice site interaction in an in vivo functional context, we examined the ability of specifically mutated U6atac snRNAs to rescue splicing of a U12-dependent intron containing splicing-defective mutations in the highly conserved 5′ splice site sequence. Our previous experiments looking for suppression of these intron mutants by mutant U11 snRNAs showed that splicing of some but not all 5′ splice site mutants could be rescued (Kolossova & Padgett, 1997). We proposed that a possible reason for the inability of U11 snRNA mutations to suppress other 5′ splice site mutations might be due to a requirement for these sequences to also base pair to U6atac snRNA.

Here we show that this is indeed the case. At least in terms of the in vivo suppression of mutations, the 5′ splice site sequence can be divided into regions that appear to interact predominantly with U11 snRNA, with U6atac snRNA, or with both. Analysis of the allelic specificity of suppression supports the idea that these interactions are mediated by Watson–Crick base pairing.

RESULTS

Mutants of U6atac suppress the splicing defect of 5′ splice site mutants

To examine the possible base pairing of snRNAs to the 5′ splice site sequence of U12-dependent introns, a series of mutations of this sequence was constructed in the human nucleolar protein P120 intron F expression system. This minigene construct contains exons 5–8 and introns E, F, and G of the gene driven from a CMV promoter (Hall & Padgett, 1996). Figure 2A shows the sequences of the 5′ splice site of P120 intron F and the portions of U11 and U6atac snRNAs that are proposed to base pair to the 5′ splice site. Figure 2B

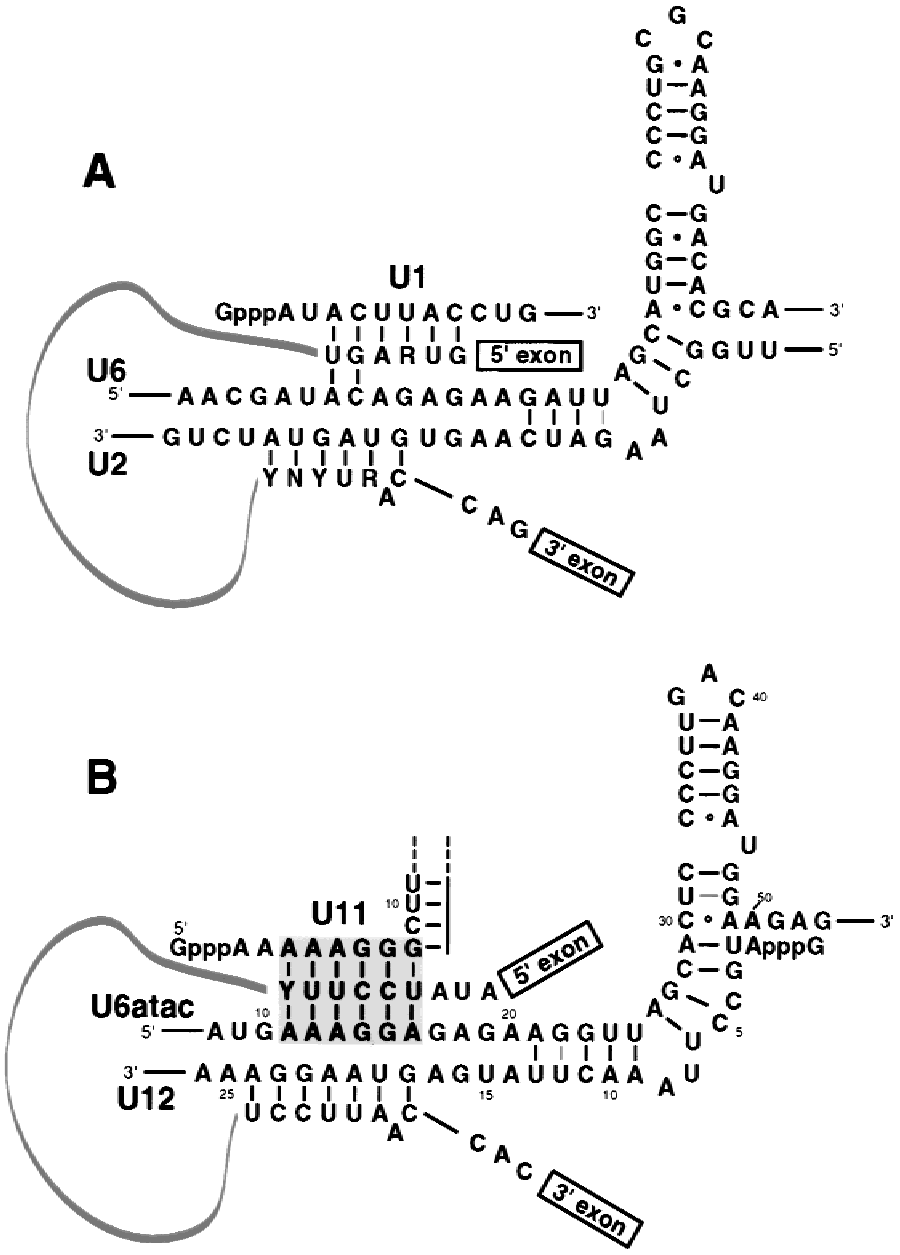


FIGURE 1. Comparison of RNA–RNA interactions in the U2- and U12-dependent spliceosomes. **A:** Base pairing interactions that can be formed between a U2-dependent intron and U1, U2, and U6 snRNAs. **B:** Base pairing interactions that can be formed between a U12-dependent intron and U11, U12, and U6atac snRNAs. Diagrams adapted from Tarn and Steitz (1996b).

shows the sequences of the P120 5′ splice mutants and the compensating mutant snRNAs used in this work. The numbering of the P120 and snRNA mutants shown in Figure 2B corresponds to the numbering of nucleotide positions in Figure 2A. To test the effects of specific U6atac mutations on the in vivo activity of these 5′ splice site mutants, a U6atac expression gene was constructed by inserting the coding sequence of U6atac (Tarn & Steitz, 1996b) into a U6 gene that had been previously shown to be active when transfected into cells (Wu & Manley, 1991). In vitro transcription of this construct in the presence of α-amanitin produced a band of approximately 126 nt, which is consistent with the production of U6atac RNA by RNA polymerase III (data not shown). Mutations were introduced into this construct that were designed to restore base pairing to the 5′ splice site mutations (Fig. 2A,B). The U11 snRNA mutants were described previously (Kolossova & Padgett, 1997) or were constructed as described in Materials and Methods.

The wild-type and mutant P120 constructs were transfected into cultured CHO cells with or without cotransfection of U11 and/or U6atac expression constructs containing various mutations. Total RNA was prepared from the cells after 48 h and reverse transcribed using a minigene-specific primer. The cDNA was amplified by PCR using primers in the exons flanking the U12-dependent intron F to assess the efficiency and accuracy of splicing of the mutant introns (Kolossova & Padgett, 1997). In this assay system, three predomi-

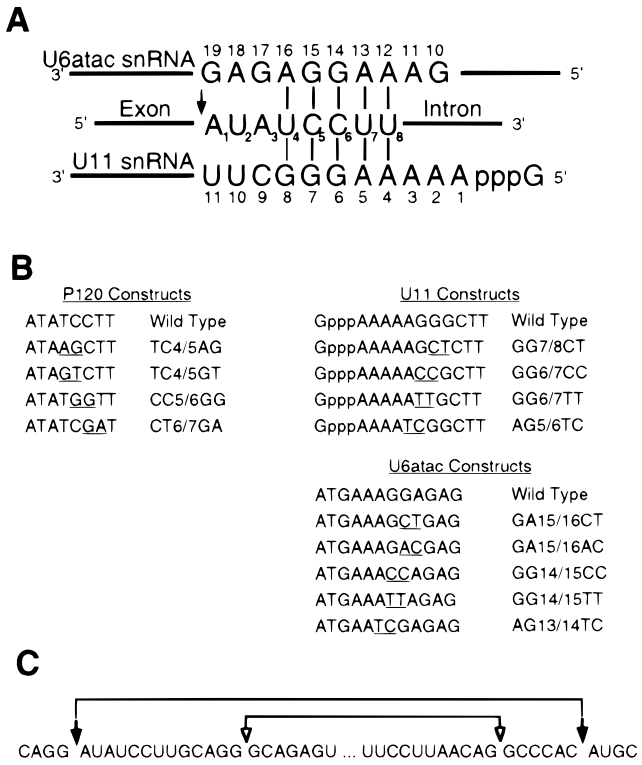


FIGURE 2. A: Diagram of the potential base pairs that could form between the highly conserved 5′ splice site sequence of the human P120 gene U12-dependent intron F (center line), U6atac snRNA (top line), and U11 snRNA (bottom line). The location of the 5′ splice junction is indicated by the arrow. Numbering is from the 5′ ends of U11 and U6atac snRNAs and the 5′ intron/exon junction of intron F. **B:** Sequences of the P120 intron F 5′ splice site mutant constructs, the U11 snRNA mutant constructs, and the U6atac snRNA mutant constructs used in this study. Altered nucleotides are underlined. Mutants are named according to the numbering of nucleotide positions shown in A. **C:** Sequence of the P120 gene around the splice sites of the U12-dependent intron F used in this work. Solid arrows denote the normal 5′ and 3′ splice sites used by this intron. Open arrows indicate the splice sites used in the U2-dependent cryptic splice activated by 5′ splice site mutations.

nant RNA species can be observed and quantitated: unspliced RNA, properly spliced RNA, and RNA from a cryptic, internal U2-dependent intron that is activated in several 5′ splice site mutants. The normal U12-dependent splice sites and the cryptic U2-dependent splice sites activated in the mutants are diagrammed in Figure 2C.

In the experiment shown in Figure 3, three P120 intron F 5′ splice site mutants were tested for suppression of their splicing defects by co-transfection of expression constructs carrying compensating mutations of U11 and U6atac singly and in combination. Figure 3, lane 1, shows that the wild-type P120 construct was spliced almost quantitatively at the U12-dependent sites. As we observed previously (Kolossova & Padgett, 1997), mutations of the conserved 5′ splice site sequence reduced or abolished correct splicing and, in addition to accumulating unspliced RNA, led to the activation of the internal U2-dependent cryptic splice sites.

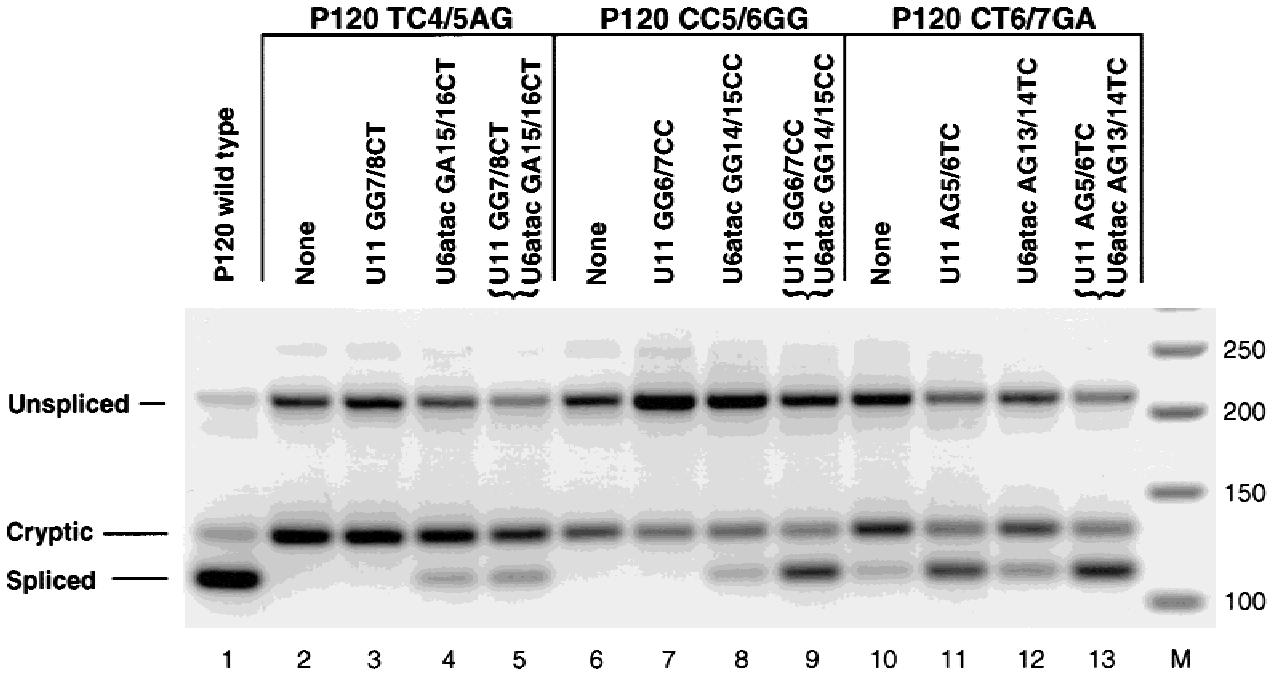
Cotransfection with U11 snRNA mutants that were designed to restore the potential base pairs to the 5′ splice site mutants gave the results we observed before (Kolossova & Padgett, 1997). Of the mutants tested here, only P120 CT6/7GA could be suppressed by a compensating U11 mutation alone (Fig. 3, lane 11). Concomitant with the activation of correct splicing, the amounts of both unspliced and cryptic spliced products were reduced. In contrast, neither P120 TC4/5AG (Fig. 3, lane 3) nor P120 CC5/6GG (Fig. 3, lane 7) was detectably suppressed by the appropriate U11 mutants.

A different result was obtained when compensating U6atac snRNA mutants were cotransfected with the P120 mutants. Correctly spliced products from both P120 TC4/5AG (Fig. 3, lane 4) and P120 CC5/6GG (Fig. 3, lane 8) were produced following cotransfection of the compensating U6atac mutants. Correct splicing of P120 CT6/7GA, which by itself produced some properly spliced RNA, was slightly enhanced by the U6atac suppressor mutant (Fig. 3, lane 12). When both the U11 and U6atac mutants were cotransfected with the P120 mutants, the P120 CC5/6GG mutant was suppressed substantially more efficiently than with either snRNA alone (Fig. 3, lane 9), the P120 CT6/7GA mutant was suppressed by the sum of the effects of the two snRNAs alone (Fig. 3, lane 13), whereas suppression of the P120 TC4/5AG mutant was only slightly improved over that seen using U6atac alone (Fig. 3, lane 5).

Allelic specificity of suppression of the 5′ splice site mutants

These results show that both U11 and U6atac functionally interact with the 5′ splice site sequence of the U12-dependent intron to promote correct U12-dependent splicing in vivo. In our previous work, we demonstrated that the suppression activity of U11 snRNA on mutations at positions 6 and 7 of the 5′ splice site was due to restoration of Watson–Crick base pairing between the snRNA and the pre-mRNA (Kolossova & Padgett, 1997). To demonstrate that the suppression effects of U6atac mutants on the 5′ splice site mutants are also mediated by Watson–Crick base pairing between the pre-mRNA and the snRNA, the allelic specificity of the suppression effects was investigated.

The specificity of suppression of the P120 TC4/5AG mutation by the U6atac GA15/16CT mutation was tested by constructing a new P120 mutant, TC4/5GT, and a new U6atac mutant, GA15/16AC (Fig. 2B). If U6atac interacts with the 5′ splice site by Watson–Crick base pairing, the suppression of the splicing defect should be allele specific, i.e., P120 TC4/5GT would be suppressed by U6atac GA15/16AC, but not by U6atac GA15/16CT, whereas P120 TC4/5AG would be suppressed by U6atac GA15/16CT, but not by GA15/16AC. This is exactly what was observed. Figure 4



% Unspliced	3	30	32	19	17	43	64	48	28	35	19	26	13
% Cryptic	16	70	68	63	59	57	36	36	25	51	32	49	27
% Spliced	81	0	0	18	24	0	0	16	47	14	49	25	60

FIGURE 3. Suppression of splicing defects in P120 intron F 5′ splice site mutants by coexpression of compensating U11 and U6atac snRNA mutant constructs. The wild-type P120 minigene (lane 1) or three different 5′ splice site mutants were transfected into CHO cells along with expression constructs for compensating U11 and/or U6atac snRNAs as indicated. RNA from the transfected cells was isolated and the splicing pattern of the P120 intron F in the minigenes was analyzed by RT-PCR. Amplified DNA products were separated by agarose gel electrophoresis, stained with ethidium bromide, and photographed with a digital video camera system. Bands corresponding to unspliced RNA (Unspliced), RNA spliced using a pair of cryptic U2-dependent splice sites (Cryptic), and RNA spliced by the U12-dependent system (Spliced) are indicated. The fraction of the total products found in each of the three bands was determined by quantitation of the digital image, corrected for fragment length, and expressed as a percentage in the table below the image.

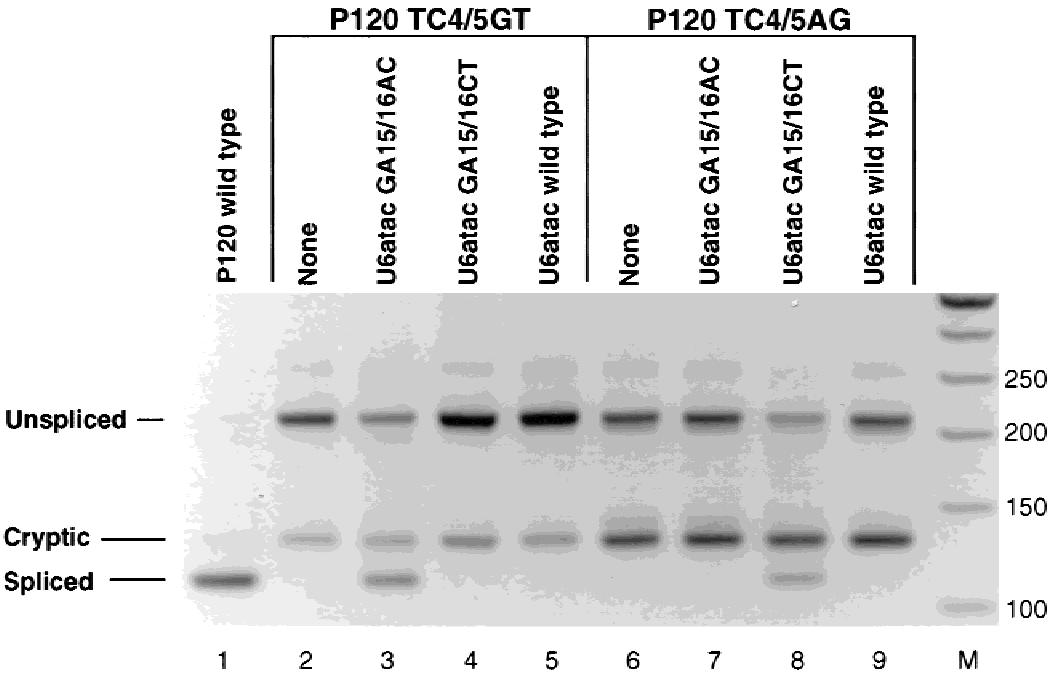
shows that only the complementary pairs of P120 and U6atac mutants restore correct splicing, whereas the noncomplementary pairs are inactive. The reciprocal suppression effects observed in this experiment demonstrate that both U6atac alleles can be expressed in an active form in transfected cells. Note that the two P120 mutants differ in their extents of activation of the cryptic U2-dependent splice sites and that the effect of the suppressor U6atac snRNAs is to produce correctly spliced RNA at the expense of unspliced RNA. The amount of cryptic spliced product is reduced only slightly.

To demonstrate the allele specificity of suppression of the P120 CC5/6GG mutant by both U11 and U6atac snRNA mutants, an additional U11 mutant, GG6/7TT, and an additional U6atac mutant, GG14/15TT, were constructed (Fig. 2B). Figure 5 shows that suppression of the splicing defect was only observed in the presence of the compensating U6atac GG14/15CC mutant and not with the U6atac GG14/15TT mutant. Maximal suppression also required the presence of the compensating U11 GG6/7CC mutant (Fig. 5, lane 5). The U11 GG6/7TT mutant also stimulated suppression, but by a lesser amount (Fig. 5, lane 10), presumably due to the formation of two G-U base pairs with the 5′ splice site. Thus, both the U6atac and U11 sequences must be complementary to the 5′ splice site sequence to produce maximal levels of properly spliced RNA. This supports the idea that the effects are due to the formation of Watson–Crick base pairs between these RNA sequences. Note that, as in Figure 4, the predominant effect of U6atac suppressor function is to activate correct splicing at the expense of unspliced RNA. The amount of cryptic spliced product is virtually unaffected (Fig. 5, lanes 4 and 10).

DISCUSSION

Both U11 and U6atac snRNAs interact with the 5′ splice site

U12-dependent introns are present in both higher plants and animals (Wu et al., 1996), which implies that the



% Unspliced	1	66	27	63	70	37	39	20	40
% Cryptic	8	32	29	37	28	62	60	55	60
% Spliced	91	2	44	0	2	1	1	25	0

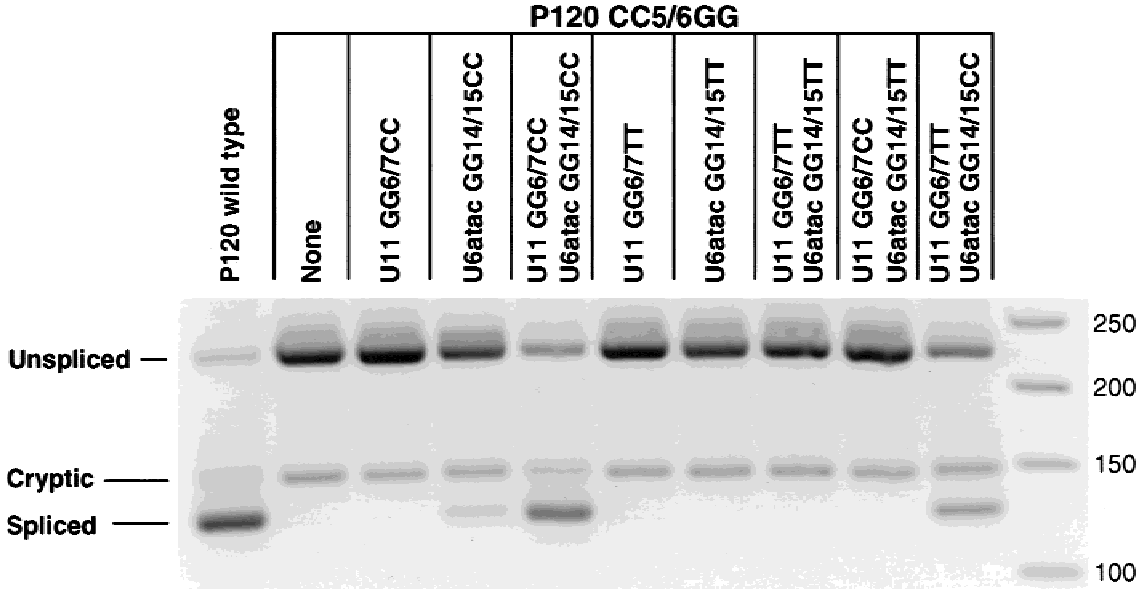
FIGURE 4. Allelic specificity of suppression of P120 mutations at positions 4 and 5 of the 5′ splice site sequence by U6atac snRNA mutants. The P120 5′ splice site mutants indicated at the top were cotransfected with the U6atac expression alleles indicated above each lane. Splicing of the wild-type P120 minigene is shown in lane 1. The analysis and presentation is the same as in Figure 3.

U12- and U2-dependent spliceosomal systems have existed side by side for at least a billion years, possibly much longer. In light of this long period of separate evolution, it is remarkable how similar in structure and function the two systems appear to be. Whether these similarities reflect conservation of a common ancestral mechanism, the constraints of chemical determinism (Weiner, 1993), or a need to use common snRNP and non-snRNP factors for both types of splicing is unclear. The current investigations are directed toward determining if the apparent similarities are functional or coincidental.

The 5′ splice site of U2-dependent introns interacts sequentially with U1 snRNA and U6 snRNA (reviewed in Nilsen, 1998). The U6 snRNA interaction alone appears to be sufficient for proper splice site activation under special conditions (Crispino et al., 1994; Tarn & Steitz, 1994; Konforti & Konarska, 1995) or in organisms that *trans*-splice. When U6atac snRNA was identified as the probable U6 analogue in the U12-dependent spliceosome, its sequence immediately suggested that it made a similar base pairing interaction with the U12-dependent 5′ splice site sequence (Tarn & Steitz, 1996b). Subsequently, a crosslink has been detected between one residue of the U12-dependent 5′ splice site and an undetermined site on U6atac snRNA (Yu & Steitz, 1997).

Here we have taken a functional approach to investigating this issue by asking if splicing-defective 5′ splice site mutants could be rescued in vivo by coexpression of U6atac snRNAs bearing compensating nucleotide changes in the putatively interacting nucleotides. Our results demonstrate that U6atac snRNA functionally interacts with the 5′ splice site sequence of a U12-dependent intron. Coexpression of U6atac snRNAs containing compensatory mutations can rescue the splicing defect of mutant 5′ splice sites either alone or in combination with suppressor U11 snRNAs. The allelic specificity of the in vivo suppression effects by both U11 and U6atac snRNAs supports the idea that these snRNAs interact with the 5′ splice site sequence by Watson–Crick base pairing.

The sensitivity of the U12-dependent 5′ splice site to inactivation by mutation is striking. In addition to the double mutations described here, we have shown that a single point mutation of position C_5 to G in the context of mutation of the terminal intron nucleotides to G was sufficient to convert the U12-dependent 5′ splice site into a U2-dependent 5′ splice site (Dietrich et al., 1997). An equally striking finding is the very high degree of



	1	2	3	4	5	6	7	8	9	10
% Unspliced	9	70	76	57	24	67	63	63	67	35
% Cryptic	14	29	23	28	20	31	34	33	29	29
% Spliced	77	1	1	15	55	2	3	4	4	36

FIGURE 5. Allelic specificity of suppression of the 5′ splice site mutant P120 CC5/6GG by U11 and U6atac snRNA mutants. The wild-type P120 minigene (lane 1) or the P120 CC5/6GG mutant (lanes 2–10) were cotransfected with the U11 and U6atac expression constructs indicated above each lane. The analysis and presentation is the same as in Figure 3.

conservation of these nucleotides in all known examples of U12-dependent introns (Sharp & Burge, 1997; Tarn & Steitz, 1997; Wu & Krainer, 1997). The results described here show that the major role of these nucleotides is to base pair with the appropriate snRNAs. Thus, the splicing of U12-dependent introns appears to be substantially more dependent on specific RNA–RNA base pairing than does U2-dependent splicing, where much more variation in splice site sequences is tolerated.

An interesting aspect of these results is that, although it is possible to draw potential base pairs between the 5′ splice site and both U11 and U6atac throughout the same region of the 5′ splice site (Figs. 1B, 2A), there appear to be subregions of this sequence where interactions with one or the other snRNA are more important in the in vivo rescue assay. For example, toward the 5′ end of the region, U6atac can suppress mutations alone, whereas, toward the 3′ end, U11 snRNA can suppress mutations alone. In the middle of this region, when the C_5C_6 dinucleotide of the 5′ splice site is changed to GG, suppressor alleles of both U11 and U6atac snRNAs are required to maximally rescue the P120 mutation.

It is difficult in the context of the in vivo assay to determine the mechanistic causes of this result. As discussed in more detail below, the activation of the U12-dependent 5′ splice site is likely to involve the sequential interaction of U11 and U6atac snRNAs. There is no a priori reason to believe that all the base pairs drawn in Figure 2A are of equal weight or that all of them are even present. Some of the snRNA sequences may not be available for base pairing in the native snRNP structure. For example, the lack of significant U11 suppression of mutations at positions 4 and 5 of the 5′ splice site may reflect the inaccessibility of U11 positions 7 and 8 due to the proximity of these residues to the 5′ stem-loop structure of U11 snRNA (see Fig. 1B). Alternatively, mispairing with wild-type U6atac may be better tolerated in cases where the mutations are farther away from the U6atac–U12 interaction region than when they are closer. Because in vivo suppression of the 5′ splice site mutations is likely to be the result of both selection and activation of the 5′ splice site, these issues may need to be investigated at the biochemical level.

Differential effects of U11 and U6atac suggest an ordered pathway of in vivo splicing

There is significant evidence of an ordered and sequential pathway of 5′ splice site–snRNA interactions in the U2-dependent spliceosome assembly process in which base pairing to U1 snRNA occurs at an early

step and is subsequently displaced by base pairing to U6 snRNA (reviewed in Nilsen, 1998). Similarly, in vitro data support an analogous ordered pathway for U12-dependent spliceosome formation. U11 snRNA can be detected in complexes with the pre-mRNA that form at early times, but not in later-forming and larger complexes, which include U6atac snRNA (Tarn & Steitz, 1996a, 1996b). In addition, crosslinks that can be induced between the 5′ splice site and U11 snRNA can be detected prior to crosslinks between the 5′ splice site and U6atac snRNA (Yu & Steitz, 1997).

Suggestive evidence that such an ordered pathway is operating in vivo can be seen in the data presented here. In several cases, it appears that the effects of suppressor snRNA mutants on the distribution of the various RNAs produced by the P120 5′ splice site mutants differ, suggesting that the two snRNAs affect different steps. In these in vivo assays, it seems reasonable to propose that the unspliced RNA signal represents the proportion of RNA molecules that are not committed to either splicing pathway plus the proportion that are committed to either the U2- or U12-dependent splicing pathway but have not completed splicing. The cryptic spliced RNA signal, of course, represents the proportion of the RNA molecules that have completed splicing via the U2-dependent system. By this model, a suppressor snRNA that decreases the proportion of RNA that becomes spliced at the cryptic site must be acting early in competition with the activation of the U2-dependent cryptic site. A suppressor snRNA that acts later can only affect the distribution of RNA between the unspliced and correctly spliced products.

In the data shown here for the P120 CT6/7GA mutant, where a compensating U11 mutation has the largest affect by itself, both the unspliced and the cryptic spliced signals are reduced concomitant with the activation of the U12-dependent splicing pathway. In contrast, the activation of U12-dependent splicing in P120 mutants at positions 4–6 by U6atac alone is almost entirely at the expense of the unspliced RNA component. The cryptic spliced signal remains nearly unchanged. When both U11 and U6atac suppressors are cotransfected with the P120 CC5/6GG mutant, both the unspliced and cryptic signals are reduced. These results are consistent with a model in which U11 competes with U1 or other components to select which 5′ splice site will be active, whereas U6atac acts at a later step to complete the splicing reaction.

How are the U2- and U12-dependent splicing systems related?

These results provide additional confirmation of the striking parallels between the U2- and U12-dependent splicing systems. Experimental support for most of the RNA–RNA interactions diagramed in Figure 1B for the U12-dependent splicing pathway has now been obtained by in vivo genetic and/or in vitro biochemical investigations (Hall & Padgett, 1996; Tarn & Steitz, 1996a, 1996b; Kolossova & Padgett, 1997; Yu & Steitz, 1997; this work). All of these interactions have clear analogues in the U2-dependent splicing pathway (Fig. 1A). These structural and functional similarities reinforce the notion that these interactions are central to the assembly and function of both types of spliceosome.

An intriguing question is whether the similarities between the U2- and U12-dependent splicing systems are homologies due to descent from a common ancestral splicing mechanism or are due to convergence caused, perhaps, by a need to use factors common to both systems. It appears that, of the snRNAs, only U5 snRNA is used by both systems (Tarn & Steitz, 1996a). Assuming that U5 plays a similar functional role in both systems, it is likely that the U5 snRNP proteins (Bach et al., 1989) will also be involved in both systems. Beyond this, the extent of shared use of snRNP and non-snRNP proteins has yet to be explored. However, if the common use of proteins is significant, a requirement for similar RNA sequences and structures within the spliceosome might be a predictable consequence, thus supporting a convergence model. If, on the other hand, it turns out that many or most protein factors involved in U2-dependent splicing have variant homologues that are specific for the U12-dependent splicing system, a stronger case can be made for common descent of the two systems.

MATERIALS AND METHODS

Construction of U6atac expression plasmid

The U6 coding region of a U6 snRNA gene (Wu & Manley, 1991; obtained from J. Manley) was replaced with the U6atac sequence by PCR techniques. First, the U6atac cDNA sequence from a U6atac plasmid (Tarn & Steitz, 1996b; obtained from J. Steitz) was amplified using the primers TGTGGAAAGGACGAAACACCGTGTTGTATGAAAGGAG AGA (primer 1) and GCTCTAGAAAAACAACCTGATGTAA AAACAGAAAAACAACCTGATGTAAAAACGATGGTTAGAT GCCA (primer 2). This produced a DNA fragment with the 5′ end of U6atac joined to the promoter-proximal region of the U6 snRNA gene and the 3′ end of U6atac joined to the 3′ downstream region of the U6 gene terminating in an *Xba* I restriction site. Next, the upstream portion of the U6 snRNA gene from −328 to −1 was amplified using the primers CGGAATTCCCCAGTGGAAAGACGCGCA (primer 3) and TCTCTCCTTTCATACAACACGGTGTTTCGTCCTTTCC ACA (primer 4). This produced a DNA fragment with a 3′ end complementary to the U6atac fragment and a 5′ end with an *Eco*R 1 restriction site. The two fragments were then combined and amplified with primers 2 and 3 to join the U6 snRNA upstream sequences to the U6atac cDNA sequences. Finally, the resulting fragment was digested with *Eco*R 1 and *Xba* I and ligated into pALTER-1 (Promega). The correct structure was confirmed by sequencing.

Construction of mutant DNA constructs

The construction of the P120 intron F minigene and mutants derived from it were as described (Kolossova & Padgett, 1997). The construction of the wild-type and mutant U11 snRNA expression plasmids was also as described (Kolossova & Padgett, 1997). The U6atac snRNA mutants were made in the expression plasmid described above using the Altered Sites II system from Promega and single mutagenic oligonucleotides. All mutations were confirmed by DNA sequencing.

Analysis of in vivo splicing

Transient transfection of the P120 minigene and snRNA expression plasmids into cultured CHO cells was as described (Hall & Padgett, 1996; Kolossova & Padgett, 1997). For these experiments, 0.5 μg of P120 plasmid, 5 μg of U11 snRNA expression plasmid, and 5 μg of U6atac expression plasmid were added to 1×10^6 cells. Where one or both snRNA plasmids were omitted, a corresponding amount of pUC19 plasmid DNA was substituted. Total RNA was isolated from cells 48 h after transfection, reverse transcribed using a vector specific primer, and amplified by PCR using P120 exon specific primers as described (Kolossova & Padgett, 1997). The products were analyzed by agarose gel electrophoresis. The DNA bands were visualized using ethidium bromide and photographed using a digital video camera (BioPhotonics). Band intensities were quantitated using ImageQuant software (Molecular Dynamics), adjusted to reflect the size of the DNA fragments, and expressed as a percentage of the total signal from the three DNA products. Independent transfections and analyses gave substantially similar results. Relative band intensities were not sensitive to alterations in the number of amplification cycles between 20 and 30. The data presented here were from reactions amplified for 30 cycles.

ACKNOWLEDGMENTS

We thank A. Seyboldt for technical assistance and J. Manley and J. Steitz for kindly supplying snRNA constructs. This work was supported by grant GM55105 from the National Institutes of Health.

Received February 4, 1998; returned for revision March 3, 1998; revised manuscript received March 10, 1998

REFERENCES

Bach M, Winkelmann G, Lührmann R. 1989. 20S small nuclear ribonucleoprotein U5 shows a surprisingly complex protein composition. *Proc Natl Acad Sci USA 86*:6038–6042.

Crispino JD, Blencowe BJ, Sharp PA. 1994. Complementation by SR proteins of pre-mRNA splicing reactions depleted of U1 snRNP. *Science 265*:1866–1869.

Datta B, Weiner AM. 1991. Genetic evidence for base pairing between U2 and U6 snRNA in mammalian mRNA splicing. *Nature 352*:821–824.

Dietrich RC, Incorvaia R, Padgett RA. 1997. Terminal intron dinucleotide sequences do not distinguish between U2- and U12-dependent introns. *Molecular Cell 1*:151–160.

Hall SL, Padgett RA. 1994. Conserved sequences in a class of rare eukaryotic introns with non-consensus splice sites. *J Mol Biol 239*:357–365.

Hall SL, Padgett RA. 1996. Requirement of U12 snRNA for the in vivo splicing of a minor class of eukaryotic nuclear pre-mRNA introns. *Science 271*:1716–1718.

Hausner TP, Giglio LM, Weiner AM. 1990. Evidence for base-pairing between mammalian U2 and U6 small nuclear ribonucleoprotein particles. *Genes & Dev 4*:2146–2156.

Hwang DY, Cohen JB. 1996. U1 snRNA promotes the selection of nearby 5′ splice sites by U6 snRNA in mammalian cells. *Genes & Dev 10*:338–350.

Jackson IJ. 1991. A reappraisal of non-consensus mRNA splice sites. *Nucleic Acids Res 19*:3795–3798.

Kandels-Lewis S, Seraphin B. 1993. Involvement of U6 snRNA in 5′ splice site selection. *Science 262*:2035–2039.

Kolossova I, Padgett RA. 1997. U11 snRNA interacts in vivo with the 5′ splice site of U12-dependent (AU-AC) introns. *RNA 3*:227–233.

Konforti BB, Konarska MM. 1995. A short 5′ splice site RNA oligo can participate in both steps of splicing in mammalian cell extracts. *RNA 1*:815–827.

Lesser CF, Guthrie C. 1993. Mutations in U6 snRNA that alter splice site specificity: Implications for the active site. *Science 262*:1982–1988.

Madhani HD, Guthrie C. 1992. A novel base-pairing interaction between U2 and U6 snRNAs suggests a mechanism for the catalytic activation of the spliceosome. *Cell 71*:803–817.

Moore MJ, Query CC, Sharp PA. 1993. Splicing of precursors to mRNAs by the spliceosome. In: Gestland RF, Atkins JF, eds. *The RNA world*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 303–358.

Newman AJ. 1997. The role of U5 snRNP in pre-mRNA splicing. *EMBO J 16*:5797–5800.

Nilsen TW. 1998. RNA–RNA interactions in nuclear pre-mRNA splicing. In: Simon RW, Grunberg-Manago M, eds. *RNA structure and function*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 279–307.

Parker R, Siliciano PG, Guthrie C. 1987. Recognition of the TAC TAAC box during mRNA splicing in yeast involves base pairing to the U2-like snRNA. *Cell 49*:229–239.

Sawa H, Abelson J. 1992. Evidence for a base-pairing interaction between U6 small nuclear RNA and 5′ splice site during the splicing reaction in yeast. *Proc Natl Acad Sci USA 89*:11269–11273.

Seraphin B, Kretzner L, Rosbash M. 1988. A U1 snRNA:pre-mRNA base pairing interaction is required early in yeast spliceosome assembly but does not uniquely define the 5′ cleavage site. *EMBO J 7*:2533–2538.

Sharp PA, Burge CB. 1997. Classification of introns: U2-type or U12-type. *Cell 91*:875–879.

Siliciano PG, Guthrie C. 1988. 5′ splice site selection in yeast: Genetic alterations in base pairing with U1 reveal additional requirements. *Genes & Dev 2*:1258–1267.

Sontheimer EJ, Steitz JA. 1993. The U5 and U6 small nuclear RNAs as active components of the spliceosome. *Science 262*:1898–1996.

Tarn WY, Steitz JA. 1994. SR proteins can compensate for the loss of U1 snRNP functions in vitro. *Genes & Dev 8*:2704–2717.

Tarn WY, Steitz JA. 1996a. A novel spliceosome containing U11, U12 and U5 snRNPs excises a minor class (AT-AC) intron in vitro. *Cell 84*:801–811.

Tarn WY, Steitz JA. 1996b. Highly diverged U4 and U6 small nuclear RNAs required for splicing rare AT-AC introns. *Science 273*:1824–1832.

Tarn WY, Steitz JA. 1997. Pre-mRNA splicing: The discovery of a new spliceosome doubles the challenge. *Trends Biochem Sci 22*:132–137.

Wassarman DA, Steitz JA. 1992. Interactions of small nuclear RNA's with precursor messenger RNA during in vitro splicing. *Science 257*:1918–1925.

Weiner AM. 1993. mRNA splicing and autocatalytic introns: Distant cousins or the products of chemical determinism? *Cell 72*:161–164.

Wu HJ, Gaubier-Comella P, Delseny M, Grellet F, Van Montagu M, Rouzé P. 1996. Non-canonical introns are at least 10^9 years old. *Nature Genetics 14*:383–384.

Wu J, Manley JL. 1989. Mammalian pre-mRNA branch site selection by U2 snRNP involves base pairing. *Genes & Dev 3*:1553–1561.

Wu J, Manley JL. 1991. Base pairing between U2 and U6 snRNAs is necessary for splicing of a mammalian pre-mRNA. *Nature 352*: 818–821.

Wu Q, Krainer AR. 1997. Splicing of a divergent subclass of AT-AC introns requires the major spliceosomal snRNAs. *RNA 3*:586–601.

Wyatt JR, Sontheimer EJ, Steitz JA. 1992. Site-specific crosslinking of mammalian U5 snRNP to the 5′ splice site before the first step of pre-mRNA splicing. *Genes & Dev 6*:2542–2553.

Yu YT, Steitz JA. 1997. Site-specific crosslinking of mammalian U11 and U6atac to the 5′ splice site of an AT-AC intron. *Proc Natl Acad Sci USA 94*:6030–6035.

Zhuang Y, Goldstein AM, Weiner AM. 1989. UACUAAC is the preferred branch site for mammalian mRNA splicing. *Proc Natl Acad Sci USA 86*:2752–2756.

Zhuang Y, Weiner AM. 1986. A compensatory base change in U1 snRNA suppresses a 5′ splice site mutation. *Cell 46*:827–835.

Zhuang Y, Weiner AM. 1989. A compensatory base change in human U2 snRNA can suppress a branch site mutation. *Genes & Dev 3*:1545–1552.