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U11 snRNA interacts in vivo with the 5′ splice site of U12-dependent (AU-AC) pre-mRNA introns

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ABSTRACT

A notable feature of the newly described U12 snRNA-dependent class of eukaryotic nuclear pre-mRNA introns is the highly conserved 8-nt 5′ splice site sequence. This sequence is virtually invariant in all known members of this class from plants to mammals. Based on sequence complementarity between this sequence and the 5′ end of the U11 snRNA, we proposed that U11 snRNP may play a role in identifying and/or activating the 5′ splice site for splicing. Here we show that mutations of the conserved 5′ splice site sequence of a U12-dependent intron severely reduce correct splicing in vivo and that compensatory mutations in U11 snRNA can suppress the effects of the 5′ splice site mutations to varying extents. This provides evidence for a required interaction between U11 snRNA and the 5′ splice site sequence involving Watson–Crick base pairing. This data, in addition to a report that U11 snRNP is bound transiently to the U12-dependent spliceosome, suggests that U11 snRNP is the analogue of U1 snRNP in splicing this rare class of introns.

Keywords: base pairing; genetic suppression; nonconsensus splice sites; snRNP; splicing

INTRODUCTION

A new class of eukaryotic nuclear spliceosomal introns has been described that utilizes a distinct set of snRNP factors for splicing. These introns are characterized by highly conserved sequences at and near the splice sites that differ from the standard intron class splice sites (Hall & Padgett, 1994). Based on complementarity between these conserved sequences and known snRNA sequences, we proposed that U12 snRNA interacted by base pairing with the 8-nt branch site sequence and that U11 snRNA base paired with a portion of the 8-nt conserved 5′ splice site sequence of this new class of introns (Hall & Padgett, 1994). Subsequently, U12 snRNP has been shown to function in splicing these introns both in vivo (Hall & Padgett, 1996) and in vitro (Tarn & Steitz, 1996a) by base pairing to the branch site. In the standard intron class, U2 snRNP interacts with the branch site in an early and essential step in the spliceosome formation pathway. Thus, this difference in snRNP requirements allows us to functionally distinguish two classes of introns: the U2-dependent class, comprising the bulk of introns, and the U12-dependent class of rare introns. In this paper, we examine another potential functional difference between these classes of introns by investigating the proposed interaction of U11 snRNA with the 5′ splice site.

U11 snRNP has no established function in the cell, although the U11 snRNA clearly belongs to the Sm class of small nuclear RNAs, most of which are involved in pre-mRNA processing (Montzka & Steitz, 1988; Baserga & Steitz, 1993). U11 snRNP has been shown to exist in nuclear extracts in a complex with U12 snRNP, probably associating via protein–protein interactions (Montzka Wassarman & Steitz, 1992). In vitro investigations of the snRNAs associated with spliceosomes formed on a U12-dependent intron RNA detected U11 snRNA in the initial A complex, but not in more mature spliceosomal complexes (Tarn & Steitz, 1996a). This pattern also resembles the weak and transient association of U1 snRNA with the U2-dependent spliceosome (Ruskin & Green, 1985; Konarska & Sharp, 1987; Kuo et al., 1991). Attempts to show a function for U11 snRNA in the in vitro splicing reaction were unsuccessful (Tarn & Steitz, 1996a), possibly due to the resistance of native U11 snRNP to disruption or degradation by antisense oligonucleotides (Montzka Wassarman & Steitz, 1992). Here we show, through in vivo suppression of 5′ splice site mutations by compensatory mutations in U11 snRNA, that U11 snRNP functions in splicing these introns via base pairing interactions with the highly conserved 5′ splice site sequence.

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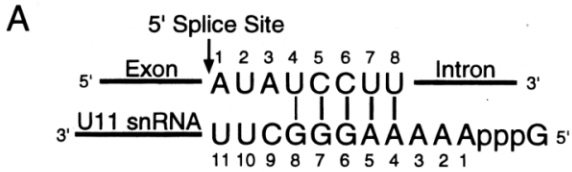
RESULTS

To test the hypothesis that U11 snRNA interacts with the 5′ splice site sequence, we used the in vivo splice site mutation suppression assay. In this assay, we ask for restoration of splicing of a mutated intron by co-expression of an interacting snRNA containing a compensatory mutation that restores base pairing between the intron and the snRNA. The target intron was the U12-dependent human P120 gene intron F, which was cloned into a mammalian cell expression vector. The construct contained exons 5, 6, 7, and 8 and introns E, F, and G of the P120 gene. We used this construct previously to demonstrate the requirement for base pairing between U12 snRNA and the branch site of intron F (Hall & Padgett, 1996). For the current investigation, we produced several mutations of the conserved 5′ splice site sequence of intron F in the region proposed to base pair with U11 snRNA (Fig. 1A and B). We also constructed a U11 snRNA expression vector by replacing the U1 snRNA sequence of a functional U1 snRNA gene with the sequence of U11 snRNA. The variants of this construct designed to restore base pairing with the 5′ splice site mutants are also shown in Figure 1B.

The extraordinary conservation of the 5′ splice site sequence of U12-dependent introns (Hall & Padgett, 1994) suggests that this sequence plays a critical role in some step or steps of the splicing reaction and that most mutations of this sequence would thus likely disrupt splicing. As shown in Figure 2, this is indeed the case. Although the wild-type construct produced correctly spliced RNA almost exclusively (lane 2), of the four mutants tested, only one (P120 TT78AA, lane 6) produced an appreciable amount of accurately spliced RNA. The other mutants led to increased levels of unspliced RNA and to the activation of a pair of cryptic splice sites within the intron. These cryptic splice sites are almost certainly spliced via the U2-dependent pathway. They are the same sites observed previously to be used in vitro under conditions in which the U12-dependent pathway is inactive (Tarn & Steitz, 1996a) and the splice site sequences clearly resemble those of U2- rather than U12-dependent introns (Fig. 1C, open arrows).



B

P120 Constructs		U11 Constructs	
AUAUCCUU	Wild Type	GpppAAAAAGGGCUU	Wild Type
AUAAGCUU	TC45AG	GpppAAAAAGCUCUU	GG78CT
AUAUGGUU	CC56GG	GpppAAAAACCGCUU	GG67CC
AUAUCGAU	CT67GA	GpppAAAATCGGCUU	AG56TC
AUAUCCAA	TT78AA	GpppAAATTGGGCUU	AA45TT
		GpppAAAGGGGGCUU	AA45GG

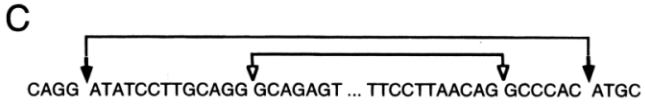


FIGURE 1. A: Diagram of the potential base pairs that could form between the highly conserved 5′ splice site sequence of U12-dependent introns (top line) and the 5′ end of U11 snRNA (bottom line). **B:** Sequences of the P120 intron 5′ splice site mutants and of the U11 snRNA mutants used in this study. 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.

To determine if compensatory mutations in the U11 snRNA could restore correct splicing to the 5′ splice site mutants, cells were cotransfected with each of the four 5′ splice site mutants along with the appropriate U11 snRNA expression construct containing mutations that would be predicted to restore Watson–Crick base pairing. Of the three 5′ splice site mutants that showed defective splicing, only the defect in the P120 CT67GA mutant was suppressed by the compensating U11 snRNA mutant AG56TC (Fig. 2, lane 9). To ensure that this suppression was due to restoration of base pairing and not simply due to an increase in the amount of U11 snRNA in the cell, the effect of cotransfection of the 5′ splice site CT67GA mutant with the wild-type and four mutant U11 snRNA constructs was examined.

As shown in Figure 3, full suppression of the splicing defect of P120 CT67GA is only seen in the presence of the fully compensating U11 AG56TC mutant (lane 5). Partial suppression of the splicing defect occurred with cotransfection of the U11 AA45TT mutant, which restores one of the two base pairs lost in the P120 CT67GA mutant (lane 4). However, neither wild-type U11 snRNA nor the other two U11 snRNA mutants could suppress the P120 mutation. In addition, none of the mutant U11 snRNAs affected the splicing of the wild-type P120 intron (lanes 8–13). These results argue strongly that the suppression of the splicing defect in the P120 5′ splice site mutant is due to restoration of base pairing with U11 snRNA.

The nearly wild-type splicing seen with the P120 TT78AA mutant (Fig. 2, lane 6) was surprising in light of the high degree of conservation of these residues in the known examples of U12-dependent introns (Hall & Padgett, 1994). To provide a more sensitive assay for the effect of this mutation, we prepared constructs in which a wild-type 5′ splice site was placed upstream in *cis* with either the wild-type 5′ splice site (5′ SS Dup WT-WT) or the TT78AA mutant 5′ splice site (5′ SS Dup WT-Mut) (Fig. 4A). We expected that the two 5′ splice sites would compete with each other and that

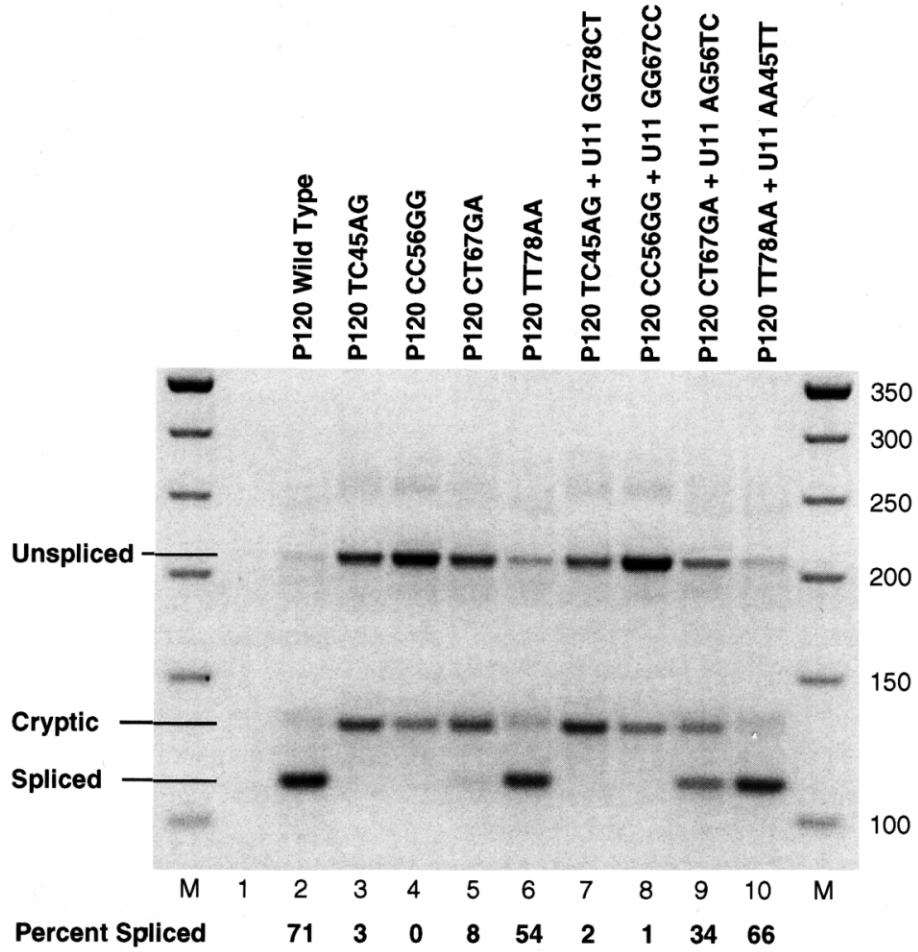


FIGURE 2. In vivo splicing patterns of 5′ splice site mutants and effects of cotransfection with compensatory U11 mutants. RNA was prepared from CHO cells transfected with the indicated DNAs and analyzed by reverse transcription followed by PCR amplification of the exons flanking the U12-dependent intron F. Positions of properly spliced, cryptic spliced, and unspliced products are indicated. The percentage of PCR products from properly spliced RNA is listed below lanes 2–10. Lane 1 is from cells transfected with the pCB6 vector alone. Marker (M) sizes are in base pairs.

the mutant 5′ splice site would be used poorly, if at all, unless a suppressor U11 snRNA was supplied.

When these double 5′ splice site constructs were transfected into cells and the splicing pattern of the P120 intron was examined (Fig. 4B), both 5′ splice sites were used when both were wild-type, although the upstream site was used about twice as efficiently as the downstream 5′ splice site (lane 2). In contrast, when the downstream site contained the TT78AA mutation, the upstream wild-type 5′ splice site was used almost exclusively (lane 6). Thus, as expected, the TT78AA mutation impairs the function of the 5′ splice site. Quantitation suggests that the mutation reduces the efficiency of the splice site by a factor of 6–10.

To determine if this impairment could be relieved by a compensating U11 mutation, the effect of cotransfection of either the wild-type or two mutant U11 snRNA expression constructs was tested. As seen before, none of the U11 constructs affected the splicing pattern of the double wild-type 5′ splice site construct (Fig. 4B, lanes 3–5). In contrast, the compensatory U11 snRNA (U11 AA45TT, lane 8) promoted splicing to the downstream P120 TT78AA mutant 5′ splice site. Cotransfection of a U11 snRNA gene containing a different mutation of the same residues (U11 AA45GG, lane 9), which should not restore base pairing, had no effect beyond that seen with cotransfection of the wild-type U11 snRNA construct (lane 7). Thus, the suppression of the P120 TT78AA 5′ splice site mutation is allele specific, further implicating a Watson–Crick base pairing interaction between the intron 5′ splice site and U11 snRNA.

DISCUSSION

The data presented here show that mutation of the highly conserved 5′ splice site sequence of U12-dependent introns inhibits splicing of such an intron in vivo. This result is, perhaps, not surprising given that this sequence occurs in all known examples of this class of introns from plants to humans, with the only

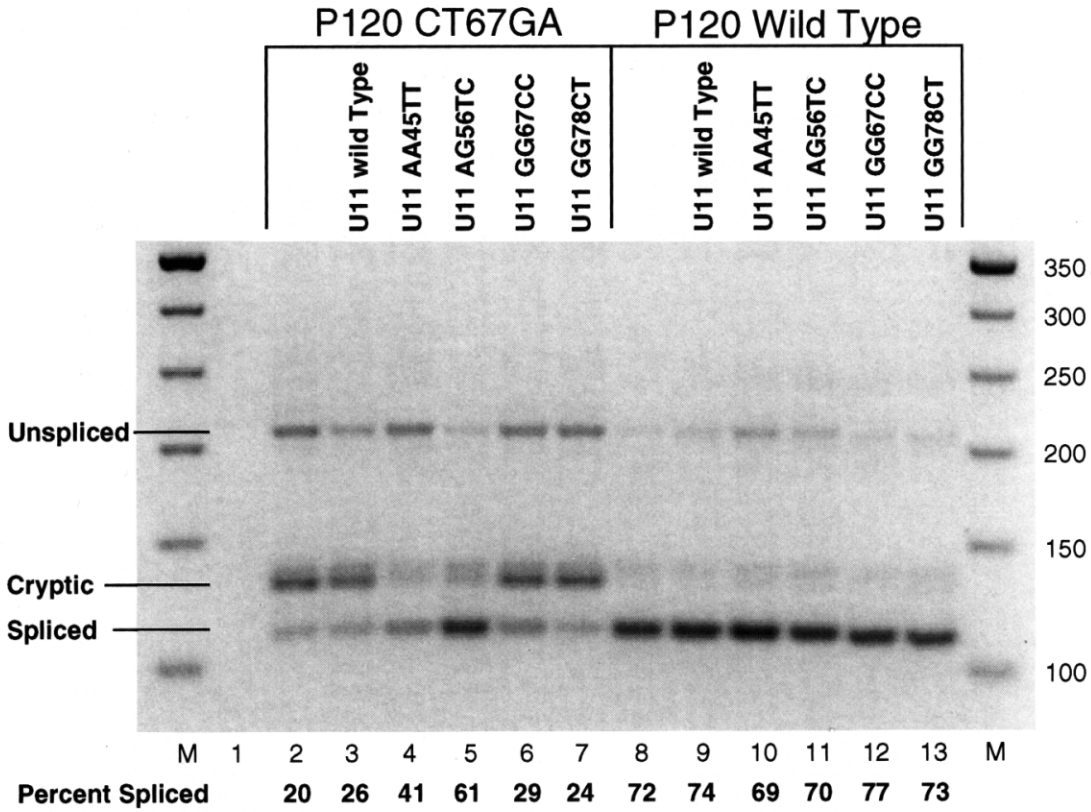


FIGURE 3. Specificity of suppression of the 5′ splice site mutant CT67GA by U11 snRNA mutants. P120 mutant CT67GA (lanes 2–7) or wild-type P120 (lanes 8–13) constructs were cotransfected with the indicated U11 wild-type or mutant constructs and the RNA was analyzed for splicing of the U12-dependent P120 intron F as in Figure 1. The percentage of PCR products from properly spliced RNA is listed below lanes 2–13. Lane 1 is from cells transfected with the pCB6 vector alone.

deviation occurring in the 3′-terminal residue. Thus, whatever interactions are mediated by this sequence, they must be highly specific to have maintained this degree of conservation over such a long time. The strengths of these interactions can be estimated from the results shown here. For the TT78AA mutation, which changed one completely conserved and one partially conserved residue, splicing was diminished by about a factor of 10 in the dual splice site assay shown in Figure 4. However, this same mutation had only a small effect on splicing in the single splice site assay (Fig. 2). The other three mutations had much more severe effects on splicing in the single site assay, suggesting that they were debilitated by another one or more orders of magnitude. These results confirm the impression derived from the phylogenetic conservation of the 5′ splice site that the splicing apparatus is very sensitive to variations in this sequence. It is not clear why this class of introns should require a longer and more highly conserved 5′ splice site than the major class of introns. Perhaps these 5′ splice sites are more autonomous such that they require fewer enhancer signals or other elements of the splice site context that appear to contribute to the strength of standard splice sites.

In the splicing of the major U2-dependent class of introns, the 5′ splice site interacts with three snRNPs at various steps in the process [reviewed by Moore et al. (1993) and Nilsen (1994)]. In an early step, U1 snRNP base pairs to the 5′ splice site as part of a process that selects or activates the 5′ splice site. Biochemical evidence suggests that this interaction is destabilized as the splicing process proceeds in favor of interactions between the 5′ splice site and U5 and U6 snRNPs. Even during the early stages of spliceosome formation, the binding of U1 snRNP to the 5′ splice site is difficult to detect in contrast to the highly stable binding of U2 snRNP to the branch site. U1 snRNP seems to be even less firmly bound to more mature forms of the spliceosome. Both by physical association with the spliceosome and by crosslinking analysis, the U5 and U6 snRNP interactions are more important in the mature spliceosome than the U1 interaction.

Because U12 snRNA appears to be the functional analogue in minor class intron splicing of U2 snRNA (Hall & Padgett, 1996; Tarn & Steitz, 1996a), it seemed likely that there might be a functional analogue of U1 snRNA for minor class introns. We have suggested that U11 snRNA might be one of the components that interacts with the 5′ splice site via Watson–Crick base

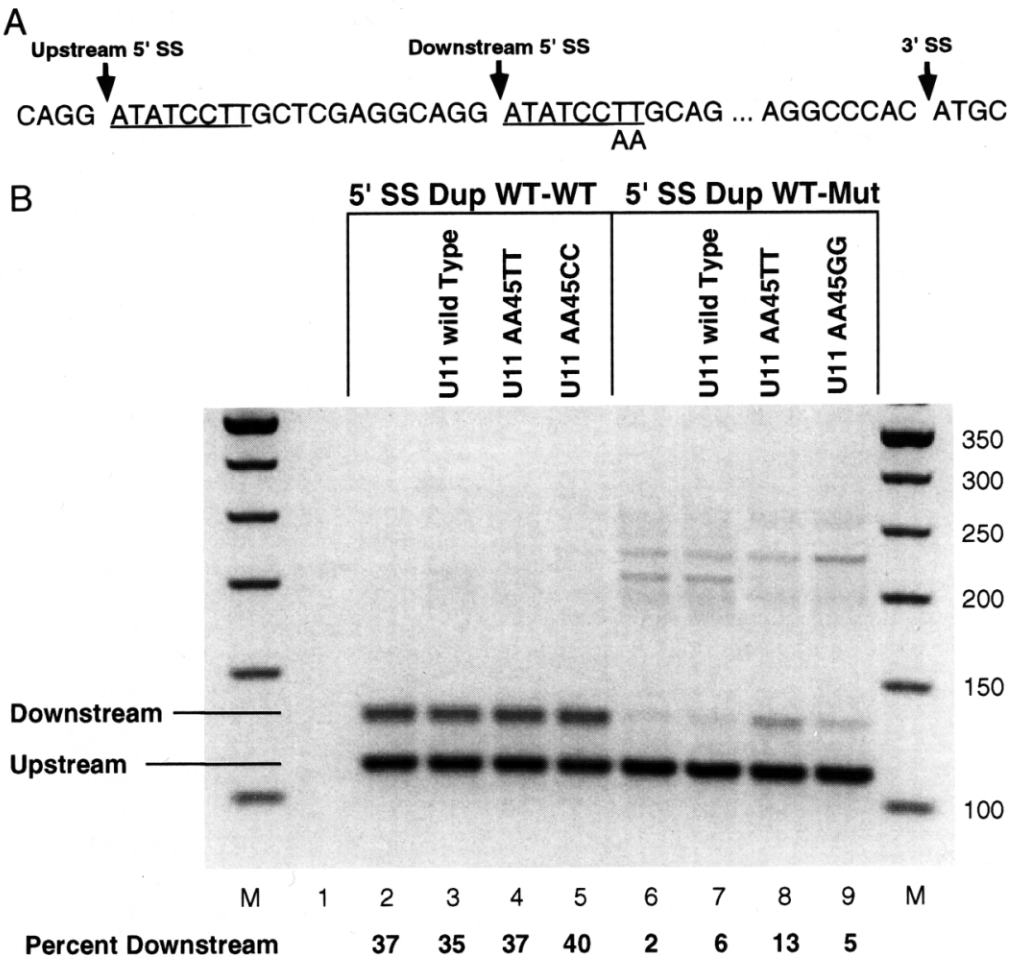


FIGURE 4. In vivo splicing patterns of duplicated 5′ splice sites. **A:** Sequence around the splice sites of the double 5′ splice site constructs. The sequence of the 5′ SS Dup WT-WT construct is shown with the mutations present in the 5′ SS Dup WT-Mut construct shown below. The 5′ splice site consensus sequences are underlined. **B:** Reverse transcription-PCR amplification analysis of the splicing of these constructs in the presence of various U11 snRNA constructs as indicated. The percentage of PCR products from RNA spliced to the downstream 5′ splice site is listed below lanes 2–9. Lane 1 is from cells transfected with the pCB6 vector alone.

pairing (Hall & Padgett, 1994). In addition to this potential base pairing interaction, there are other common features between U1 and U11 snRNAs. The secondary structure of U11 is similar to U1 (Montzka Wassarman & Steitz, 1992), as is the location of the potential 5′ splice site pairing region. Recent in vitro data suggests that U11 snRNA interacts with a U12-dependent intron in the early stages of spliceosome assembly, but not at later stages (Tarn & Steitz, 1996a). It should be noted, however, that U11 and U12 snRNAs are known to be associated in nuclear extracts (Montzka Wassarman & Steitz, 1992), so that the observed association of U11 with U12-containing early splicing complexes might be adventitious. The resistance of the U11 snRNP to oligonucleotide-mediated cleavage precluded a test of its requirement for splicing in vitro (Tarn & Steitz, 1996a). The analogy of U11 and U1 snRNAs is not complete, however. Although U1 snRNA can form base pairs to the entire conserved 5′ splice site of U2-dependent introns, the potential base pairing between the U12-dependent intron 5′ splice site and U11 snRNA only involves nt 4–8 (Fig. 1). The conservation of nt 1–3 suggests that some component of the splicing machinery recognizes these bases specifically, and thus the possibility exists that some as yet undetected RNA or protein interacts with these nucleotides.

In this paper, we have confirmed the proposed functional interaction of U11 snRNA with the 5′ splice site of a U12-dependent intron by the in vivo suppression of two mutations in the 5′ splice site. The allelic specificity of this suppression suggests that the interaction is via canonical base pairing as diagramed in Figure 1, although, because not all 5′ splice site mutants could be suppressed, only a subset of the possible base pairs between U11 snRNA and the 5′ splice site may actually form. This result suggests that the interaction observed in vitro between U11 snRNP and a U12-dependent intron (Tarn & Steitz, 1996a) is likely to be functional, and

further suggests that the role of U11 snRNP is at an early step in the spliceosome formation pathway.

If the analogy of U11 and U1 snRNPs is correct, the U11 snRNP interaction should be displaced by interactions of the 5′ splice site with other snRNAs. U5 snRNA was shown previously to be present in a U12-dependent spliceosome (Tarn & Steitz, 1996a), suggesting that it might interact with sequences at the splice sites as it does in U2-dependent introns. Recently, a second snRNA has been described that appears to be the U6 analogue for U12-dependent introns (Tarn & Steitz, 1996b). This RNA, called U6atac, has a high degree of sequence identity to a region of U6 snRNA that makes several important RNA–RNA interactions in the spliceosome. U6atac snRNA has been shown to be essential for splicing in vitro and can be crosslinked to U12 snRNA (Tarn & Steitz, 1996b), suggesting that it plays a functional role similar to that of U6 snRNA in U2-dependent splicing. A notable feature of U6atac snRNA is that it contains the sequence AAGGA positioned in the analogous location to the ACA sequence of U6 snRNA, which has been shown to base pair to the 5′ splice site of U2-dependent introns (Kandels-Lewis & Seraphin, 1993; Lesser & Guthrie, 1993; Sontheimer & Steitz, 1993). This U6atac snRNA sequence could base pair to the TCCTT sequence of the U12-dependent intron 5′ splice site, displacing U11 snRNA. In support of this, U6atac snRNA was found only in the larger and later-forming spliceosomal complexes, whereas U11 snRNA was not detected (Tarn & Steitz, 1996a, 1996b). Thus, the incomplete suppression of the P120 mutants CT67GA and TT78AA and the lack of suppression of the CC56GG and TC45AG mutants by the compensating U11 snRNAs could reflect an additional or separate requirement that these sequences engage in base pairing to U6atac snRNA. This could be tested easily with the appropriate U6atac mutants coexpressed in vivo.

From recent in vitro and in vivo investigations of the splicing mechanism of U12-dependent introns, there has emerged a striking overall similarity between the U2- and U12-dependent splicing pathways. At least to a first approximation, the functional analogues of U1, U2, U4, and U6 snRNAs in U2-dependent splicing are U11, U12, U4atac, and U6atac in U12-dependent splicing. There are clear correspondences between many of the pre-mRNA–snRNA and snRNA–snRNA interactions in the two systems. With the recent finding of two members of the U12-dependent intron class in the plant *Arabidopsis thaliana*, it appears that the two spliceosomal mechanisms have existed side-by-side for more than one billion years (Wu et al., 1996). The similarity of the functional features of the splicing pathways given this interval of independent evolution is truly remarkable. At this point, it is not clear if the two pathways arose independently or from a common source. In either event, the similarities in the functional interactions provides support for our current models of the critical interactions in the spliceosomes.

MATERIALS AND METHODS

DNA constructs

The P120 gene construct contains exons 5, 6, 7, and 8 and introns E, F, and G of the human nucleolar protein P120 gene inserted into the mammalian cell expression vector pCB6 (Andersson et al., 1989) as described previously (Hall & Padgett, 1996). The 5′ splice site mutants of the U12-dependent intron F were constructed by PCR amplification of fragments of the construct using mutagenic primers. Mutated fragments were then inserted back into the pCB6 expression vector. The U11 snRNA wild-type and mutant expression constructs were made by PCR amplifying the U11 coding region from a human genomic clone (Suter-Crazzolara & Keller, 1991) using oligonucleotides containing the wild-type or mutant sequences and cloning into the pUC/U1 vector (Zhuang & Weiner, 1986) such that the U11 sequences replace the U1 sequences as described (Bond et al., 1991; Hall & Padgett, 1996). For the double 5′ splice site constructions, a double-stranded oligonucleotide containing a wild-type 5′ splice site sequence was ligated into the *Eco*R V site of either the wild-type or P120 TT78AA mutant construct. All constructs were verified by DNA sequencing.

Transfections and RT-PCR analysis of RNA

Equal masses of P120 wild-type or mutant construct and U11 snRNA construct DNAs (20 μg total DNA per 100-mm plate) were transfected into CHO cells and total cell RNA was prepared after 48 h as described (Hall & Padgett, 1996). Following DNase I treatment, 200 ng of total cell RNA was reverse transcribed with rTth polymerase (Perkin Elmer) in 10 mM Tris-HCl, pH 8.3, 90 mM KCl, 1 mM $MnCl_2$, 0.2 mM of each dNTP, and 0.75 μM of a pCB6 vector specific oligonucleotide (TGGGGAGGGGTCACAGGGATGCCACC) at 70 °C for 15 min. A sample of this reaction was then PCR amplified using oligonucleotides from exons 6 (TTGTGCT GCCCCCTGCTGGGGAGATG) and 7 (TGAGCCCCAAAAT CACGCAGAATTCC) of the P120 gene, displayed on a 3% NuSieve (FMC Corp.) agarose gel and visualized by ethidium bromide staining. Images were captured using a Bio-Photonics Gel Print 2000i system. Quantitation of band intensities was performed using ImageQuant software (Molecular Dynamics). The identities of the PCR products were confirmed by DNA sequencing of the gel band-isolated DNAs. All transfections were performed independently at least twice and each PCR analysis was repeated at least three times with substantially similar results. The relative band intensities shown are not sensitive to alterations in the number of PCR cycles.

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