The conserved 3' end domain of U6atac snRNA can direct U6 snRNA to the minor spliceosome

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ABSTRACT

U6 and U6atac snRNAs play analogous critical roles in the major U2-dependent and minor U12-dependent spliceosomes, respectively. Previous results have shown that most of the functional cores of these two snRNAs are either highly similar in sequence or functionally interchangeable. Thus, a mechanism must exist to restrict each snRNA to its own spliceosome. Here we show that a chimeric U6 snRNA containing the unique and highly conserved 3' end domain of U6atac snRNA is able to function *in vivo* in U12-dependent spliceosomal splicing. Function of this chimera required the coexpression of a modified U4atac snRNA; U4 snRNA could not substitute. Partial deletions of this element *in vivo*, as well as *in vitro* antisense experiments, showed that the 3' end domain of U6atac snRNA is necessary to direct the U4atac/U6atac.U5 tri-snRNP to the forming U12-dependent spliceosome. *In vitro* experiments also uncovered a role for U4atac snRNA in this targeting.

Keywords: RNA–RNA interactions; U2-dependent splicing; pre-mRNA

INTRODUCTION

The nuclear pre-mRNA introns of eukaryotes are removed by a large ribonucleoprotein complex known as the spliceosome (for review, see Tarn and Steitz 1997; Nilsen 1998; Burge et al. 1999; Wu and Krainer 1999; Will and Lührmann 2006). In addition to hundreds of distinct polypeptides (Jurica and Moore 2003; Will and Lührmann 2006; Chen et al. 2007; Bessonov et al. 2008), the spliceosome contains five small nuclear RNAs (snRNAs), which take part in a network of RNA–RNA interactions with the pre-mRNA splice sites and with each other. It is now recognized that there are two types of nuclear pre-mRNA introns widely distributed among higher eukaryotic species. The two types of introns are spliced via the action of two distinct types of spliceosome. These spliceosomes differ in their snRNA compositions, such that the more abundant U2-dependent type contains the snRNAs U1, U2, U4, U5, and U6, while the less abundant U12-dependent type contains the snRNAs U11, U12, U4atac, U5, and U6atac.

The four snRNAs that are unique to each spliceosome have been shown to be functional analogs. Thus, U11 snRNA appears to be the functional analog of U1 snRNA, U12 snRNA is the analog of U2 snRNA, U4atac snRNA is the analog of U4 snRNA, and U6atac snRNA is the analog of U6 snRNA. U5 snRNA appears to function similarly in both spliceosomes. These analogies are based, in large part, on the similarities in RNA–RNA interactions displayed by the respective snRNAs. Most of these interactions have been validated by genetic evidence or biochemical cross-linking or both.

The current model of pre-mRNA splicing involves the assembly of spliceosomes from smaller subunits around each intron of a gene (for review, see Burge et al. 1999; Will and Lührmann 2006). For U2-dependent introns, U1 and U2 snRNPs cooperate to form an early intermediate complex followed by addition of a preassembled tri-snRNP complex of U4, U5, and U6 snRNPs (abbreviated U4/U6.U5) in which U4 and U6 snRNAs are extensively base paired. Upon addition of this complex to the forming spliceosome, a sequence in U6 snRNA base pairs with the intron 5' splice site displacing U1 snRNA in the process. Further rearrangements lead to the unwinding of the U4/U6 duplex and the destabilization of U4 snRNA from the spliceosome.

An analogous sequence of events appears to lead to assembly of the U12-dependent spliceosome. U11 and U12,

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as a di-snRNP complex, base pair to the 5' splice site and branch site/3' splice site, respectively, to form an initial complex with the intron. A U4atac/U6atac.U5 tri-snRNP complex in which the U4atac and U6atac snRNAs are base paired, then joins the forming spliceosome followed by base pairing of U6atac with the 5' splice site, displacement of U11 snRNA from the 5' splice site, unwinding of the U4atac/U6atac duplex and destabilization of U4atac from the spliceosome.

The U2- and U12-dependent spliceosomes splice two different types of introns. The biochemical and bioinformatic evidence so far suggests that mixed introns, which are spliced by some combination of the two spliceosomes, do not exist. Although most of the snRNAs in the two spliceosomes are distinct at the sequence level, many of the RNA–RNA interactions required for spliceosome function are remarkably similar. In addition, there appears to be considerable overlap in the proteins used by the two spliceosomes. For example, all of the known proteins that are specific for the U4/U6.U5 tri-snRNP complex also appear to be part of the U4atac/U6atac.U5 complex (Schneider et al. 2002). Some of this overlap is likely to be due to the presence of the U5 snRNP in both tri-snRNP complexes. In addition to the protein similarities, we have shown that an important RNA element of U6 snRNA can functionally replace the analogous element of U6atac snRNA (Shukla and Padgett 2001). More recently, we have also shown that U4atac snRNA can be functionally replaced by U4 snRNA providing that it can base pair with U6atac snRNA (Shukla and Padgett 2004).

These results raise the question of what features and interactions maintain the specificity of spliceosome assembly. In other words, how is the U4atac/U6atac.U5 tri-snRNP recruited to the forming U12-dependent spliceosome in the presence of the vastly more abundant U4/U6.U5 tri-snRNP? Since the only remaining specific snRNA in the tri-snRNP complex is U6 or U6atac, we focused on the differences in these molecules. Here we show that a unique and highly conserved 3' substructure of U6atac, when transferred to U6 snRNA, allows the chimeric snRNA to function in U12-dependent splicing *in vivo*.

RESULTS

A modified U6 snRNA can function in U12-dependent splicing *in vivo*

We have previously described the *in vivo* mutational suppressor assay for the function of several of the snRNAs involved in U12-dependent splicing (Hall and Padgett 1996; Kolossova and Padgett 1997; Incorvaia and Padgett 1998; Shukla and Padgett 1999). This assay relies on the genetic suppression of splicing defects due to splice site mutations in a U12-dependent intron by coexpression of compensatory mutant snRNAs. The test U12-dependent

intron is contained within a four exon, three intron minigene construct derived from the human nucleolar P120 gene driven by a CMV promoter. The minigene contains exons 5–8, U2-dependent introns E and G and the U12-dependent intron F. To assay the *in vivo* function of U6atac snRNA, a mutation in the 5' splice site of intron F (CC5/6GG), which blocks splicing at the normal sites, is suppressed (i.e., splicing is restored at the normal sites) by cotransfection of expression constructs containing compensatory mutants of U11 and U6atac snRNAs (U11 GG6/7CC and U6atac GG14/15CC) (Incorvaia and Padgett 1998).

Since U6 and U6atac snRNAs have similarly placed 5' splice site binding regions, which differ in sequence, we first asked if altering the U6 snRNA sequence to match the U6atac sequence would suffice to permit U6 to function in the U12-dependent system. To that end, we altered the U6 5' splice site binding sequence to that of the U6atac GG14/15CC suppressor mutant and used a previously described U4atac snRNA mutant that can base pair with U6 snRNA. These constructs were combined with the 5' splice site mutant minigene and transfected into cells. This combination was inactive for U12-dependent splicing (data not shown). From this, we concluded that some additional component was necessary to direct the tri-snRNP to the U12-dependent spliceosome.

We next prepared chimeric snRNA constructs with portions of U6atac replacing equivalent portions of U6. As shown in Figure 1, the central regions of U6 and U6atac snRNA, which base pair to the U4 and U4atac snRNA, respectively, are quite similar. However, the 5' and 3' ends of the snRNAs differ in size, sequence, and predicted secondary structure. Note that the 5' end of U6 is longer than that of U6atac and contains an additional stem-loop structure. Similarly, the 3' end of U6atac snRNA is significantly longer than the 3' end of U6 snRNA and contains multiple secondary structure elements. We therefore generated the two constructs shown in Figure 1. In the U6 Mod-1 mutant, we exchanged the 3' end domain of U6 for that of U6atac in the background of the U6 snRNA with the U12-type suppressor mutations. The U6 Mod-2 mutant exchanged the 5' end domain of U6 for that of U6atac and included the U6atac GG14/15CC suppressor mutations. Also shown in Figure 1 is the sequence of a mutant U4atac snRNA that was designed to base pair with these chimeras.

These chimeric constructs were cotransfected into cells along with the minigene containing the 5' splice site mutant U12-dependent intron both in the presence and the absence of the modified U4atac snRNA. After 48 h, total RNA was prepared from the cells and splicing of the U12-dependent intron F was assayed by RT-PCR using minigene specific primers. Figure 2 shows the gel analysis of the RT-PCR products. This assay produces three RT-PCR products. The largest is the unspliced RNA, the smallest is the product of U12-dependent splicing at the correct in

FIGURE 1. Sequences and base-pairing interactions of the U6/U6atac snRNA chimeras Mod-1 and Mod-2 with the modified U4atac snRNA. (A) The U6 Mod-1 chimera consists of U6 nucleotides 1–79 joined to U6atac nucleotides 52–125 (3' stem-loop region shown within the box). The U6 nucleotides U40, A41, and C42 are also replaced by the sequence AACC, which base pairs to the mutant U12-dependent 5' splice site in the P120 intron F minigene. The modified U4atac snRNA is shown base paired to the U6 portion of the chimera due to the changes shown in bold in the stem II region. (B) The U6 Mod-2 chimera consists of U6atac nucleotides 1–22 joined to U6 nucleotides 51–108 (as shown in the box). The U6atac sequence includes the GG14/15CC mutation that suppresses the CC5/6GG mutation in the P120 minigene construct. Also shown is the modified U4atac snRNA base paired to the U6 snRNA region of the chimera.

vivo splice sites while the middle band is the product of cryptic U2-dependent splicing using a 5' splice site 13 nucleotides (nt) downstream of the U12-type 5' splice site and a 3' splice site 6 nt upstream of the U12-type 3' splice site (Tarn and Steitz 1996a; Dietrich et al. 1997). As shown in lane 3, the mutant minigene alone produces only unspliced and cryptic spliced products. When various combinations of snRNA constructs were cotransfected with the mutant minigene, only the U6 Mod-1 mutant could suppress the 5' splice site mutation and restore correct U12-dependent splicing. Somewhat surprisingly, since we had shown that U4 could function in place of U4atac in this assay, the U6 Mod-1 mutant was active only in the presence of the modified U4atac snRNA (Fig. 2, cf. lane 6 and lane 7).

The 3' end domain of U6atac snRNA contains critical RNA elements

These results show that the 3' domain of U6atac snRNA allows U6 snRNA to function in the U12-type spliceosome. This domain could be providing a positive function by directing the chimeric snRNA to the U12 spliceosome or the normal 3' end of U6 snRNA could have a negative function preventing its association with the U12 spliceosome. One way to resolve this question is to identify critical substructures of the U6atac 3' end domain whose deletion or mutation abrogates function. This will also serve to identify regions or substructures that are likely to be involved in functional interactions.

To this end, we deleted or mutated several regions of the U6atac domain of the U6 Mod-1 chimera. As shown in Figure 3A, this domain contains two stem-loop structures separated by a linker region. Our initial mutants included a deletion of the smaller 5' stem-loop, deletion of the linker region, deletion of the entire 3' stem-loop or the distal or proximal parts of the 3' stem-loop and replacement of the terminal loop region with its Watson Crick complement. Each of these mutants was tested for function by cotransfection as described above. The results are shown in Figure 3B and quantitation of splicing is shown in Figure 3C. Lane 3 is the mutant minigene alone, while lane 5 shows suppression by the U6 Mod-1 parent construct. Lanes 6 and 8 (Fig.

3B,C) show that a complete deletion of either of the stem-loop elements abolishes the function of U6 in this assay. Deletion of the proximal section of the 3' stem-loop also abolishes function (Fig. 3B,C, lane 10), while deletion of the distal part reduces, but does not abolish, suppressor activity (Fig. 3B,C, lane 9). The sequence of the terminal loop of this element does not appear to be important since the simultaneous mutation of all seven residues does not affect function (Fig. 3B,C, lane 11). Deletion of the linker region (Fig. 3B,C, lane 7) also significantly reduced splicing activity.

These results establish that the U6atac 3' domain is performing an active process in the targeting of the U6/U6atac chimera to the U12 spliceosome. They further show that the activity of this domain requires the presence of several substructures. Given that the chimeric construct

FIGURE 2. RT-PCR analysis of the *in vivo* splicing of the U12-dependent P120 intron F from the transiently transfected constructs shown. The region spanning the U12-dependent intron F was amplified using primers in the adjacent exons. The positions of bands corresponding to unspliced and correctly spliced products are shown, as well as the cryptic spliced product. The cryptic spliced band is due to splicing between a pair of U2-dependent splice sites located within the P120 F intron. The constructs used for the various lanes are: (lane 1) empty pCB6 expression vector and (lane 2) wild-type P120 minigene. Lanes 3–10 used the P120 5' splice site mutant CC5/6GG and the following snRNA expression constructs: (lane 3) none; (lane 4) U6atac GG14/15CC plus U11 GG6/7CC; (lane 5) U6 Mod-1 plus U11 GG6/7CC; (lane 6) U6 Mod-1 plus U11 GG6/7CC plus U4atac Hu U6 Supp.; (lane 7) U6 Mod-1 plus U11 GG6/7CC plus wild-type U4; (lane 8) U6 Mod-2 plus U11 GG6/7CC; (lane 9) U6 Mod-2 plus U11 GG6/7CC plus U4atac Hu U6 Supp.; and (lane 10) U6 Mod-2 plus U11 GG6/7CC plus wild-type U4. Lane M contains molecular size markers.

used to identify these important substructures is highly artificial, we asked if any of these mutations affected the function of intact U6atac snRNA. We constructed the same set of mutants in the 3' domain of our starting GG14/15CC suppressor U6atac snRNA and tested these in the *in vivo* splicing suppressor assay. Figure 4 shows that the complete deletion of the 3' stem-loop (Fig. 4A, lane 8) had the most deleterious effect on suppressor function. Deletion of the proximal part of this stem-loop reduced suppressor activity (Fig. 4A, lane 10), while the deletion of the distal section (Fig. 4A, lane 9) or mutation of the loop residues (Fig. 4A, lane 11) had no significant effect. Deletion of the 5' stem-loop element or the linker region showed modest, but reproducible, reductions in splicing activity.

The 3' end domain of U6atac snRNA is required for U12-dependent splicing in vitro

Another strategy to identify important regions of snRNAs is to use antisense oligonucleotides to block function in vitro. To this end, we tested the effects on U12- and U2-dependent splicing in vitro of addition of 2'-O-methyl (Me) oligonucleotides complementary to various regions of U6atac snRNA as diagrammed in Figure 5A. For U12-dependent splicing, a radio-labeled in vitro RNA transcript containing the P120 intron F was spliced in HeLa cell

nuclear extract in the presence or absence of the various 2'-O-Me oligonucleotides and the RNA products were resolved on a denaturing polyacrylamide gel (Fig. 5B). Control splicing reactions (Fig. 5B, lanes 1,10,11) showed the production of exon and intron splicing products. As a positive control, an oligo against the U6atac snRNA 5' end (Fig. 5B, lane 12, U6atac 1–20) that covers the region of U6atac that interacts with the 5' splice site, completely blocked *in vitro* splicing as shown previously (Dietrich et al. 1997). As a negative control, an oligo directed against U6 snRNA (Fig. 5B, lane 13) had only a small effect at four times the concentration used in the other reactions.

FIGURE 3. Effects on *in vivo* splicing of mutations of the U6 Mod-1 3' end domain. (A) Diagram of the U6atac 3' end domain. The numbering is relative to human U6atac snRNA. (B) RT-PCR analysis of the *in vivo* splicing of the U12-dependent P120 intron F from the transiently transfected constructs shown. Refer to Figure 2 for details of the assay. (Lane 1) Empty pCB6 expression vector and (lane 2) wild-type P120 minigene. Lanes 3–11 used the P120 5' splice site mutant CC5/6GG. Lanes 4–11 included the U11 GG6/7CC mutant. Lanes 5–11 included the U4atac Hu U6 Supp. plus the U6 Mod-1 construct with the following mutations: (lane 5), none; (lane 6), deletion of the 5' stem-loop element (nucleotides 53–64); (lane 7) deletion of the single stranded region between nucleotides 65 and 79; (lane 8) deletion of the entire 3' stem-loop element (nucleotides 80–116); (lane 9) deletion of the distal section of the 3' stem-loop element (nucleotides 91–109); (lane 10) deletion of the proximal section of the 3' stem-loop element (nucleotides 80–90 and 110–116); and (lane 11) substitution mutation of the terminal loop nucleotides 97–103 with the sequence GAUGAAG. (C) Quantitative analysis of the spliced product in panel B, lanes 2–11, showing the means and standard deviations from three experiments.

FIGURE 4. Effects on in vivo splicing of mutations of the U6atac 3' end domain. (A) RT-PCR analysis of the in vivo splicing of the U12-dependent P120 intron F from the transiently transfected constructs shown. Refer to Figure 2 for details of the assay. (Lane 1) Mock transfection; (lane 2) empty pCB6 expression vector; and (lane 3) wild-type P120 minigene. Lanes 4–11 used the P120 5' splice site mutant CC5/6GG. Lanes 5–11 included the U11 GG6/7CC mutant. Lanes 5–11 included the U6atac GG14/15CC construct with the following mutations (see Fig. 3A): (lane 5) none; (lane 6), deletion of the 5' stem-loop element (nucleotides 53–64); (lane 7) deletion of the single stranded region between nucleotides 65 and 79; (lane 8) deletion of the entire 3' stem-loop element (nucleotides 80–116); (lane 9) deletion of the distal section of the 3' stem-loop element (nucleotides 91–109); (lane 10) deletion of the proximal section of the 3' stem-loop element (nucleotides 80–90 and 110–116); and (lane 11) substitution mutation of the terminal loop nucleotides 97–103 with the sequence GAUGAAG. (B) Quantitative analysis of the spliced product in panel A, lanes 3–11, showing the means and standard deviations from three experiments.

Four antisense oligos were designed against the 3' end domain of U6atac snRNA. These covered the 5' stem-loop and the linker region (U6atac 3' A), the linker region alone (U6atac 3' B), the proximal section of the 3' stem-loop (U6atac 3' C), and the distal section of the 3' stem-loop (U6atac 3' D). As shown in Figure 5B, oligo A strongly inhibited in vitro U12-dependent splicing, oligo C inhibited splicing, but less completely, while oligos B and D had little effect on splicing. The absence of inhibition by oligo B suggests that the inhibition caused by oligos A and C is due to disruption of the stem-loop elements, rather than by binding to the linker region. An alternative, but less likely, possibility is that oligos B and D are unable to bind to U6atac. These results show that at least two regions of the U6atac 3' domain are required for splicing in vitro. To rule out nonspecific inhibitory effects from addition of the oligonucleotides, they were also tested at the maximum concentration for effects on U2-dependent splicing in vitro. None of these oligos significantly inhibited in vitro splicing of an RNA substrate containing a previously studied U2-

dependent intron derived from Adenovirus 2 (Supplemental Fig. S1; Dietrich et al. 1997).

To determine the point at which the antisense oligos blocked U12-dependent spliceosome formation or function,

FIGURE 5. Effects on in vitro splicing of 2'-O-methyl antisense oligonucleotides against U6atac and U4atac. (A) Schematic of U6atac (top strand) and U4atac (bottom strand) showing the regions covered by the 2'-O-Me oligos. (B) In vitro splicing of the U12-dependent P120 intron F in the presence of the indicated final concentrations of the antisense oligos. All reactions also contained an anti-U2 2'-O-Me oligo as described in Methods. The bands corresponding to the precursor RNA, the spliced exon product, the 5' exon intermediate and the lariat intron product are indicated from top to bottom. Lanes 1,10,11 are from reactions containing only the anti-U2 oligo. (C) Splicing complexes formed in vitro on radiolabeled U12-dependent P120 intron F RNA in the presence of the indicated antisense 2'-O-Me oligos. All reactions also contained an anti-U2 2'-O-Me oligo. Complex H is a nonspecific complex, Complex A is the initial splicing-specific complex formed by addition of the U11/U12 snRNP, Complex B is formed by the addition of the U4atac/U6atac.U5 tri-snRNP to Complex A.

parallel *in vitro* splicing reactions were analyzed on native gels to resolve intermediates in the spliceosome formation pathway (Fig. 5C). Control reactions (Fig. 5C, lanes 1,10) showed production of the nonspecific H complex and the spliceosomal A and B complexes (Tarn and Steitz 1996b). Complex A forms early in the splicing reaction and contains U11 and U12 snRNPs. The subsequent addition of the U4atac/U6atac.U5 tri-snRNP to Complex A yields Complex B. The requirement of U12 for both Complexes A and B is demonstrated by addition of an oligo antisense to the branch site interaction region of U12 snRNA (Fig. 5C, lane 9). The requirement of U6atac for formation of Complex B is shown by addition of an oligo antisense to the 5' end of this snRNA (Fig. 5B, lane 7, U6atac 1–20). As shown in lanes 2–5, the effects on Complex B formation of the oligos antisense to the 3' end domain of U6atac largely mirror their effects on the overall splicing reaction shown in Figure 5B. These results show that oligos A and C inhibit a step in the process by which the U4atac/U6atac.U5 triple snRNP associates with the U11 and U12-containing Complex A to form Complex B.

A region of U4atac snRNA is also required for splicing *in vitro*

In addition to the anti-U6atac snRNA oligos analyzed above, we asked if an anti-U4atac snRNA oligo would inhibit splicing. This possibility was suggested by the results shown above for *in vivo* splicing using the U6 Mod-1 snRNA, where the U4atac, but not U4 snRNA, was required to show splicing suppressor activity. This finding is in contrast to earlier results showing that U4 snRNA could function in conjunction with U6atac snRNA in U12-dependent splicing *in vivo* (Shukla and Padgett 2004).

The question then becomes what region of U4atac snRNA might play a specific role in triple snRNP targeting? As shown in Figure 1, the 5' region of U4atac snRNA is involved in the stem I and stem II interactions with U6atac snRNA with a stem-loop structure between them. This stem-loop structure has been shown to bind the same 15.5 kD protein and to adopt the same structure as U4 snRNA (Nottrott et al. 1999; Schultz et al. 2006). Thus, these elements did not appear to be likely targeting sequences. Turning to the 3' region of U4atac snRNA, the terminal section contains the essential Sm protein binding site centered on residue 121, the adjacent stem-loop structure and the unpaired region between residues 66 and 83. This latter region differs in sequence from U4 snRNA and has no previously defined function. We therefore tested a 2'-O-Me oligo complementary to this region of U4atac snRNA (Fig. 5A, U4atac 3') in the *in vitro* U12-dependent splicing assay. As shown in Figure 5B, lanes 14 and 15, this oligo effectively blocked splicing. In addition, as shown in Figure 5C, lane 6, the oligo-blocked formation of spliceosomal Complex B but not Complex A. These results suggest that

this region of U4atac snRNA also contains a sequence element that is involved in the addition of the U4atac/U6atac.U5 tri-snRNP to the forming spliceosome.

The U6atac snRNA 3' end domain is phylogenetically conserved

In addition to the functional evidence presented above for the importance of the 3' domain of U6atac and its subdomains, there is substantial evolutionary conservation of this region. This was first apparent from the sequence of the U6atac snRNA from the plant *Arabidopsis thaliana*, which is, overall, 65% identical to mammalian U6atac (Shukla and Padgett 1999). More recent work has identified U12-dependent introns and/or spliceosomal components in several organisms from deep branches of eukaryotic phylogeny (Russell et al. 2006). Sequence analysis of a large number of genomes for spliceosomal snRNAs has identified putative U6atac snRNA homologs in many of these lineages (Lopez et al. 2008). This analysis showed that, within the 3' domain, the 5' stem-loop and the 3' stem-loop structures are well conserved, while there is little conservation of the region between these elements. A notable exception is seen in insects where all species appear to lack the 5' stem-loop, while retaining the 3' stem-loop element.

Figure 6 shows a comparison of the predicted secondary structures of the 3' domains of human, plant (*Arabidopsis*), and protist (*Phytophthora*) U6atac snRNAs. These organisms encompass a large swath of eukaryotic phylogeny having diverged near the root of eukaryota. This comparison highlights the conservation of the structural features with similar sizes and placements of the stem-loop elements. There is also substantial sequence conservation within the proximal region of the 3' stem-loop element, as highlighted in Figure 6. This conservation extends to almost all of the U6atac sequences collected by Lopez et al. (2008). The distal part of this element is less conserved in sequence, but shows several compensatory base-pair changes in the stem, while the loop sequences are not conserved. The spacer region between the two stem-loops shows little sequence conservation, but is conserved in length. The evident conservation of structure and sequence despite billions of years of separate evolution suggests that this region plays an important role in U12-dependent splicing. In fact, as shown above, the structurally conserved 5' stem-loop and the proximal portion of the 3' stem-loop are precisely the regions that are required for the function of this element in spliceosome targeting and *in vitro* splicing.

DISCUSSION

The discovery that higher eukaryotic genomes harbored two distinct types of introns acted upon by two distinct spliceosomes raised a number of interesting questions. Among these questions is how is the correct spliceosome

FIGURE 6. Comparison of sequences and predicted secondary structures of the 3' end domains of human, plant (*Arabidopsis thaliana*), and protist (*Phytophthora infestans*) U6atac snRNAs (Lopez et al. 2008). Conserved sequence elements are highlighted.

assembled on the different types of introns? The interspersal of different intron types within genes implies that both types of spliceosomes must assemble in roughly the same place and at roughly the same time. Indeed, there is evidence that adjacent U2-dependent splice sites promote U12-dependent intron splicing (Wu and Krainer 1996). Thus, the two splicing systems appear to cooperate in order to accurately process many primary transcripts. Yet, the evidence so far suggests that each intron is removed by either one type of spliceosome or the other. That is, there is no evidence of “mixed” spliceosomes that can join a splice site of one type to a splice site of the other type. This implies that mechanisms likely exist for the specific recruitment of type-specific spliceosomal components. This would be particularly important in the case of the U12-

dependent spliceosome, where the snRNP components of this system are outnumbered by the components of the U2-dependent spliceosome by about 100 to 1 (Patel and Steitz 2003).

This need for specificity must also contend with the striking similarities between the two spliceosomes. Not only do they share one of the five snRNAs (U5), as well as a significant number of proteins (Will and Lührmann 2005), but RNA substructures and entire snRNAs can be functionally transplanted between the two splicing systems (Shukla and Padgett 1999, 2001, 2002, 2004). These considerations led us to investigate how the U4atac/U6atac.U5 tri-snRNP complex is specifically recruited to U12-dependent introns. The addition of this complex is a critical step in the formation of an active spliceosome (Will and Lührmann 2006). However, most of the components of this complex are or can be shared with the analogous U4/U6.U5 tri-snRNP complex including two of the three snRNAs (Shukla and Padgett 2004) and many, if not most, of the proteins (Schneider et al. 2002).

We therefore focused on the remaining snRNA components that differentiated the two spliceosomal tri-snRNPs, U6 and U6atac snRNAs, and asked if U6 could substitute for U6atac in U12-dependent splicing. U6 and U6atac snRNAs participate in three known RNA–RNA interactions that are important for splicing (Patel and Steitz 2003). They base pair extensively with U4 and U4atac snRNAs, respectively; they base pair with U2 and U12 snRNAs, respectively, in the spliceosome to form structures called helix 1a and helix 1b; and they base pair to nucleotides within the 5' splice site region of the target intron. U6 snRNA also participates in additional interactions with U2 snRNA called helix 2 and helix 3, which do not appear to occur between U6atac and U12 snRNAs.

We have previously shown that U4 snRNA can substitute for U4atac snRNA in U12-dependent splicing (Shukla and Padgett 2004). In addition, the sequences that form the helix 1a and helix 1b interactions are almost identical in the two splicing systems. This left the 5' splice site interaction as the most obvious component of specificity in this complex. However, attempts to show that a U6 snRNA carrying the 5' splice site interaction sequence of U6atac snRNA could function in U12-dependent splicing in vivo were unsuccessful. A further modification to allow this U6 RNA to base pair to U4atac snRNA also proved to be nonfunctional.

We then asked what additional features of U6 or U6atac snRNAs might be involved in targeting the complex to the spliceosome. Comparison of U6 and U6atac snRNAs showed that the central regions were either highly conserved or interchangeable based on our previous studies (Shukla and Padgett 2001). We therefore focused on the 5' and 3' ends of the RNAs, which are quite different (see Fig. 1). Using the U6 snRNA construct with the U6atac 5' splice site interaction sequence, we switched the 5' and 3' ends of

U6 and U6atac to create chimeric molecules and then tested these for *in vivo* splicing activity on a U12-dependent intron. As shown above, the 3' domain of U6atac snRNA is both necessary and sufficient to allow U6 snRNA to function in U12-dependent splicing. This domain is providing a positive function since small internal deletions of this element abolish activity and antisense oligonucleotides against parts of this region block U12-dependent spliceosome formation and splicing. Further evidence for its importance is the conservation of its sequence and secondary structure among the U6atac snRNAs of mammals, plants, protists, and fungi (Fig. 6; Lopez et al. 2008).

A surprising requirement in these experiments was that only U4atac could function, after appropriate modification of the interacting nucleotides, with the modified U6 snRNA. Our earlier work led us to predict that U4 snRNA would be able to serve this function as well (Shukla and Padgett 2004). This finding suggests that U4atac snRNA may also be somehow involved in the targeting of the tri-snRNP complex in addition to the U6atac snRNA 3' element discussed here. While U4 and U4atac snRNAs appear quite similar in both structure and function, there are also unique regions that could contain this function. In fact, an antisense oligonucleotide against one of these regions in U4atac was highly effective in blocking U12-dependent spliceosome formation and splicing *in vitro* (Fig. 5).

The 3' domain of U6atac snRNA contains two conserved secondary structural features, a small 5' stem-loop located next to the stem II interaction with U4atac snRNA and a larger and more complex stem-loop element located near the 3' end of the snRNA. Both of these features appear to be important for the function of the chimeric snRNA, as well as for the intact U6atac snRNA. Comparison of the small stem-loop element among humans, plants, and protists shows compensatory changes in the sequence of the stem region that retains base-pairing potential (Fig. 6; Lopez et al. 2008). This provides evidence for the functional conservation of this secondary structure element. Similarly, the sequence and structure of the proximal section of the 3' stem-loop element is also conserved. The distal region of this element, however, differs significantly among sequences from various organisms (Lopez et al. 2008). These conserved regions agree well with the functional effects of deletions and mutations of the various parts of this element. In addition, the ability of antisense oligos against these two stem-loop elements to inhibit U12-dependent spliceosome formation and splicing also show that they are functionally significant.

The role of U4atac snRNA uncovered in this investigation is also unexpected. We have shown previously that U4 snRNA when modified to base pair to U6atac snRNA, can function in U12-dependent splicing *in vivo* (Shukla and Padgett 2004). Yet only U4atac snRNA was able to functionally interact with the U6 Mod-1 snRNA *in vivo*. In addition, an antisense oligo against a portion of the 3'

region of U4atac snRNA could block spliceosome formation *in vitro*. The region targeted by this oligo was previously tested by mutation in an *in vivo* assay where the mutants had little or no effect on function (Shukla et al. 2002). To reconcile these findings, we suggest that the targeting of the triple snRNP to the forming U12-dependent spliceosome requires the function of multiple, partially redundant snRNA regions. These would be composed of two elements in the U6atac 3' domain (the 5' stem-loop and the proximal part of the 3' stem-loop) and an element in the 3' region of U4atac snRNA. None of these elements is absolutely required in the native triple snRNP so that individual deletions or mutations have only modest effects *in vivo*. However, in the nonnative contexts of the U6 Mod-1 snRNA or the *in vitro* splicing assay, the importance of each of these elements is increased.

With the demonstration that these U6atac and U4atac elements are required for U12-dependent spliceosomal function, the question becomes how this function is carried out. There are no known RNA–RNA interactions that involve these regions of U4atac and U6atac snRNAs. The analogous 3' region of U6 snRNA base pairs to U2 snRNA in the spliceosome to form the helix 2 interaction (Nilsen 1998). U12 snRNA cannot form an analogous helix with U6atac because the 5' end of U12 is located immediately after the helix 1b region. As for U4 snRNA, the analogous region has been shown to be sensitive to deletion or mutation *in vitro* and in *Xenopus* oocytes (Wersig and Bindereif 1990; Vankan et al. 1992), to crosslink to the 5' splice site region in a model yeast splicing system (Johnson and Abelson 2001) and its digestion was shown to eliminate an ATP-dependent crosslink between the 5' splice site and the prp8 protein (Maroney et al. 2000). We have also tested an antisense oligonucleotide directed against the region of U4 analogous to that of U4atac shown in Figure 5A. This oligo inhibited U2-dependent splicing *in vitro*, but not U12-dependent splicing (R.C. Dietrich and R.A. Padgett, unpubl.). All these results suggest that this region of both U4 and U4atac plays an essential, but undefined role in the respective splicing systems. It is possible that the function of the U6atac and the U4/U4atac regions is to bind specific protein(s) which could contribute to spliceosome specificity by protein–protein or RNA–protein interactions with other components of the spliceosome. The report of Schneider et al. (2002) that all known U4/U6.U5 tri-snRNP-specific proteins were also found on the U4atac/U6atac.U5 tri-snRNP complex does not rule out this idea. These authors did not examine the complete protein composition of the minor tri-snRNP and so would not have detected proteins that are specific for this complex.

The nature of the step that is targeted by the *in vivo* deletions and the *in vitro* antisense oligos is of significant interest. Several reports have demonstrated that there are specific interactions between the U4/U6.U5 tri-snRNP and the forming spliceosome around the 5' splice site region in

the U2-dependent system (Kim and Abelson 1996; Maroney et al. 2000; Ryan et al. 2004). A potentially analogous interaction in the U12-dependent pathway has also been reported (Frilander and Steitz 2001). Previous evidence suggests that these interactions are initially guided by U5 snRNA and its associated Prp8/220 protein via recognition of the 5' splice site region and U1 or U11 snRNA (Abovich and Rosbash 1997; Ast and Weiner 1997; Reyes et al. 1999; Maroney et al. 2000). However, as both spliceosomes contain U5 snRNA and Prp8/220 (Will and Lührmann 2005), specificity must reside elsewhere. The results presented here suggest that, in the case of the U12-dependent spliceosome, the 3' end domains of U6atac and U4atac snRNAs contain critical RNA elements that contribute to this specificity. At this point it is not clear what component(s) of the forming spliceosome interacts with these RNA elements to dock the correct tri-snRNP particle. Sequence specific RNA–RNA base-pairing interactions are not apparent and have not been described. However, protein–protein or protein–RNA interactions could also be involved. The only proteins known to be specific for the minor spliceosome reside on the U11/U12 di-snRNP particle (Will et al. 2004) and hence are likely to be localized to Complex A. One or more of these proteins could then interact either with the regions of U6atac and U4atac identified here or with proteins bound to the 3' end domain. Experiments to identify such interactions are underway.

METHODS

Construction of U6/U6atac and U4atac expression plasmids

The U6/U6atac and U4atac snRNA expression plasmids were generated by the same methods used previously (Shukla and Padgett 1999; Shukla et al. 2002). A human U6 snRNA gene obtained from J. Manley was mutated by PCR techniques for the U6 snRNA constructs. For the U6atac snRNA constructs, the transcribed portion of the U6 snRNA gene was replaced by human U6atac snRNA sequences or mutants thereof. For U4atac snRNA, the U1 snRNA coding region of a functional U1 gene was replaced by PCR techniques with the coding region of U4atac snRNA amplified from plasmids obtained from J. Steitz. Details of the constructions are available upon request. The sequences of the mutant and wild-type snRNAs 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 and Padgett 1996; Kolosova and Padgett 1997; Inorvaia and Padgett 1998). For these experiments, 0.5 µg of P120 plasmid and 5 µg of each of the snRNA expression plasmids were added to 1×10^6 cells. Where one or more 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 and PCR amplified as described (Kolosova and

Padgett 1997; Inorvaia and Padgett 1998). The products were analyzed by agarose gel electrophoresis followed by staining with ethidium bromide. Independent transfections and analyses gave substantially similar results.

In vitro splicing

Transcription and in vitro splicing of the U12-dependent P120 intron F was carried out as described previously (Dietrich et al. 1997, 2005). Briefly, HeLa cell nuclear extracts were pre-incubated for 15 min at 30°C in the presence of the indicated 2'-O-Me oligonucleotides followed by addition of the ³²P-labeled pre-mRNA and further incubation for 3 h at 30°C. All U12-dependent splicing reactions also contained an anti-U2 2'-O-Me oligo at a concentration of 8 µM (Dietrich et al. 1997, 2005). RNA was extracted and resolved on 8% polyacrylamide/8M urea gels followed by detection of labeled RNA using a Molecular Dynamics Storm imager. For analysis of spliceosome complexes, U12-dependent splicing reactions were incubated for 90 min after addition of pre-mRNA followed by addition of heparin to 0.25 mg/mL and loading onto nondenaturing gels as described (Dietrich et al. 2001).

Antisense 2'-O-methyl oligonucleotides

U2, AUAAGAACAGAUACUACACUUGA;
U12, AUUUUCCUUACUCAUAAG;
U4atac 3', GGGUGUGUUGUUCAGGC;
U6atac 1–20, UCUCUCCUUUCAUACAACAC;
U6atac 3' A, GUAUGCGUGUUGUCAGGCCCGAG;
U6atac 3' B, GUAUGCGUGUUGUCA;
U6atac 3' C, AAUGCCUUAACCGUAUGCGUGU;
U6atac 3' D, UGCCACGAAGUAGGUGGCA AUG; and
U6 27–46 CUCUGUAUCGUCCAAUUUU.

Primers for PCR analysis of in vivo splicing

P120 E6, TTGTGCTGCCCCCTGCTGGGGAGATG; and
P120 E7, TGAGCCCCAAAATCACGCAGAAATTC.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

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