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The intramolecular stem-loop structure of U6 snRNA can functionally replace the U6atac snRNA stem-loop

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ABSTRACT

The U6 spliceosomal snRNA forms an intramolecular stem-loop structure during spliceosome assembly that is required for splicing and is proposed to be at or near the catalytic center of the spliceosome. U6atac snRNA, the analog of U6 snRNA used in the U12-dependent splicing of the minor class of spliceosomal introns, contains a similar stem-loop whose structure but not sequence is conserved between humans and plants. To determine if the U6 and U6atac stem-loops are functionally analogous, the stem-loops from human and budding yeast U6 snRNAs were substituted for the U6atac snRNA structure and tested in an in vivo genetic suppression assay. Both chimeric U6/U6atac snRNA constructs were active for splicing in vivo. In contrast, several mutations of the native U6atac stem-loop that either delete putatively unpaired residues or disrupt the putative stem regions were inactive for splicing. Compensatory mutations that are expected to restore base pairing within the stem regions restored splicing activity. However, other mutants that retained base pairing potential were inactive, suggesting that functional groups within the stem regions may contribute to function. These results show that the U6atac snRNA stem-loop structure is required for in vivo splicing within the U12-dependent spliceosome and that its role is likely to be similar to that of the U6 snRNA intramolecular stem-loop.

Keywords: pre-mRNA splicing; spliceosomes; U12-dependent introns

INTRODUCTION

The recent identification of a minor class of nuclear pre-mRNA introns that are spliced by a distinct alternative spliceosome has provided an unexpected example in which to evaluate the present models of RNA interactions in the spliceosome (reviewed in Tarn & Steitz, 1997; Nilsen, 1998; Burge et al., 1999; Wu & Krainer, 1999). The snRNAs that are involved in splicing this minor (U12-dependent) class of introns in human cells have been shown to be functional analogs of the major (U2-dependent) intron-class spliceosomal snRNAs. 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 in both spliceosomes. The functional similarities of the two sets of snRNAs are given added support by the apparent conservation of RNA–RNA interactions between the pre-mRNA splice sites, U12 snRNA, U6atac snRNA, and U11 snRNA (see Tarn & Steitz, 1997; Nilsen, 1998; Burge et al., 1999; Wu & Krainer, 1999). Several of these interactions are diagramed for the U2-dependent spliceosome (Fig. 1A) and the U12-dependent spliceosome (Fig. 1B).

Among the features that appear to be conserved between the two spliceosomes is an intramolecular stem-loop structure found in both U6 and U6atac snRNAs. This structure is shown for human U6 snRNA in Figure 1A in relation to the conserved helix Ia and Ib elements formed between U6 and U2 snRNAs (Madhani & Guthrie, 1992). As can be seen in Figure 1B, a very similar structure can be drawn for U6atac snRNA that is also juxtaposed to a helix I-like structure formed between U6atac and U12 snRNAs (Tarn & Steitz, 1996b). In addition to the base-paired structures shown in Figure 1, these regions of U6 and U6atac snRNAs also form base pairs with U4 and U4atac snRNAs, respectively, in the U4/U6 and U4atac/U6atac di-snRNPs. The intramolecular stem-loops thus can only be formed following the unpairing of the two snRNAs in the di-snRNP complex (Fortner et al., 1994), an event that

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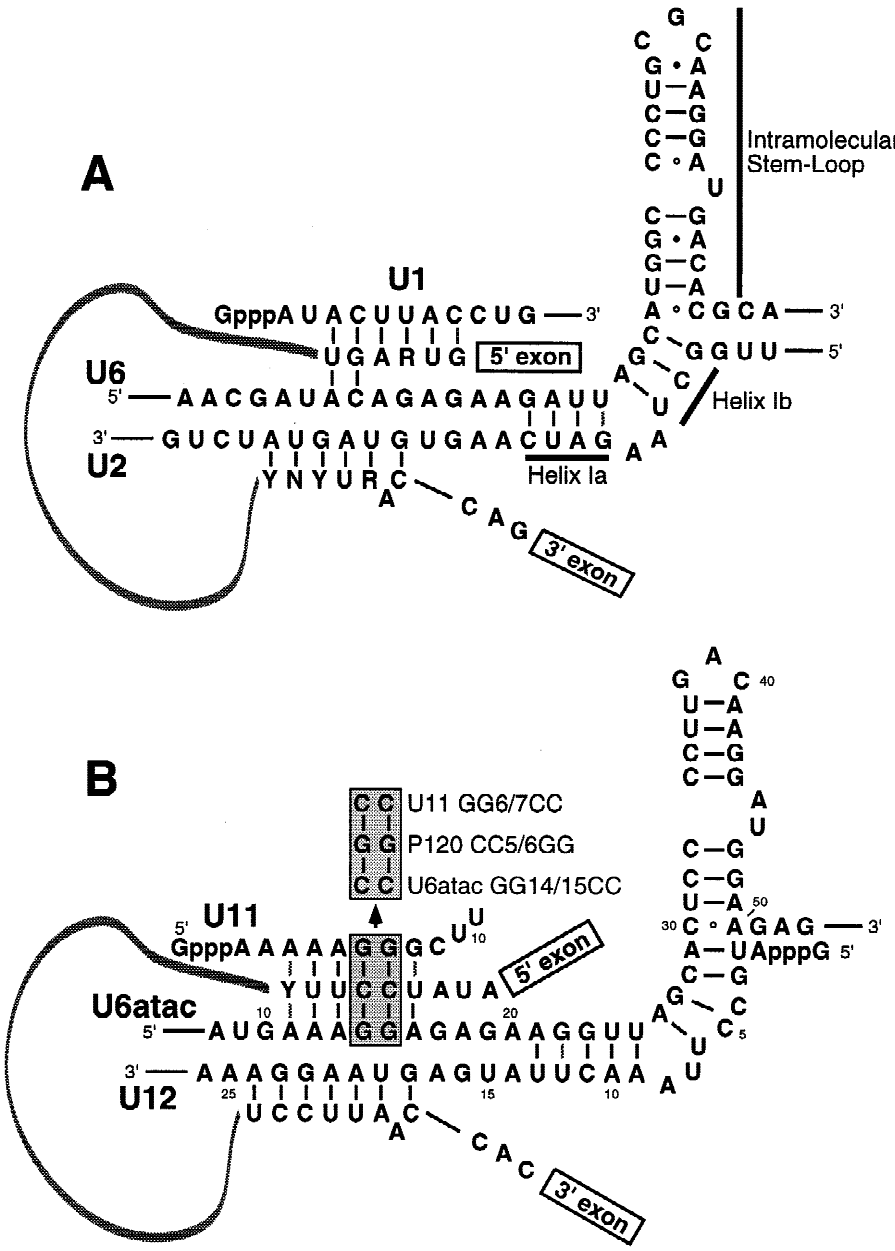

FIGURE 1. Comparison of RNA–RNA interactions in U2- and U12-dependent spliceosomal splicing. **A**: Diagram of the interactions between the pre-mRNA and U1, U2, and U6 snRNAs. Shown are the U2-branch site interaction, the U1 and U6-5′ splice site interactions and the Helix Ia and Ib interactions between U2 and U6 snRNAs. Also shown is the U6 intramolecular stem-loop structure that immediately follows Helix Ib. **B**: Diagram of the analogous interactions between the pre-mRNA and U11, U12, and U6atac snRNAs. Shown are the U12-branch site interaction, the U11 and U6atac-5′ splice site interactions and the U12-U6atac interactions and the U6atac stem-loop structure. Also shown in the shaded boxes are the mutations used in the in vivo mutational suppression assay. The P120 CC5/6GG mutation shown in the middle of the upper box inactivates U12-dependent splicing. The U6atac GG14/15CC mutation restores splicing at the mutant 5′ splice site and the U11 GG6/7CC mutation enhances the level of suppression.

occurs prior to the first step of the splicing reaction (Guthrie, 1991).

The importance of the U6 snRNA stem-loop element for U2-dependent splicing is suggested by the high conservation of this structure throughout the eukaryotic kingdom. Organisms as diverse as humans, plants, and fission yeast have identical U6 snRNA sequences in this region (Brow & Guthrie, 1988). Budding yeast U6 snRNA differs from this sequence in several positions but retains the ability to form a similar structure (see Fig. 2). We have previously shown that the plant and human homologs of U6atac snRNA differ in nearly half the residues in this region, yet appear capable of adopting the same structure (Fig. 2; Shukla & Padgett, 1999). Furthermore, substitution of the human U6atac stem-loop sequence by the plant sequence resulted in a

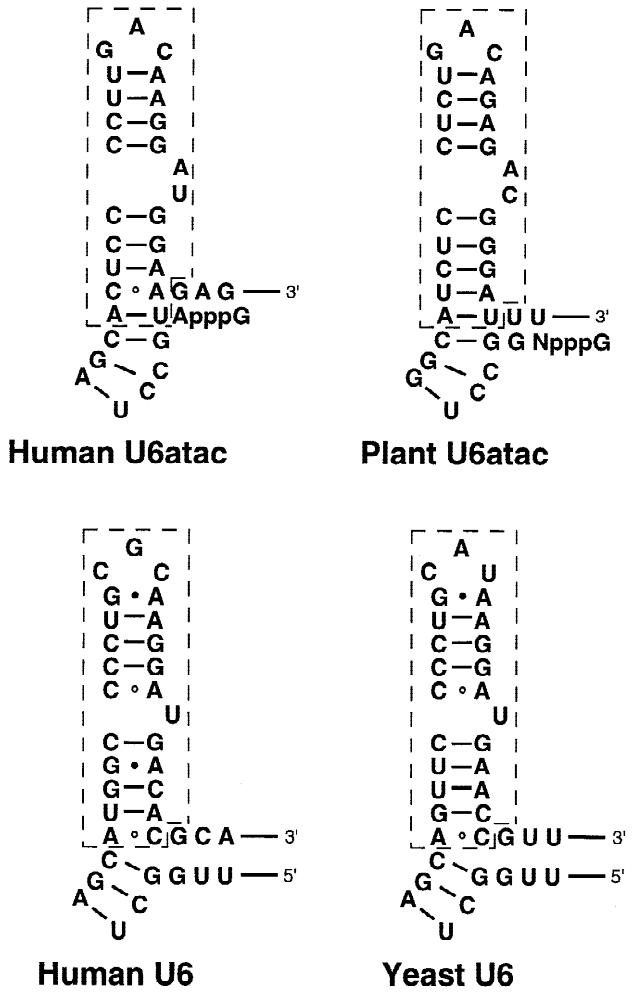

FIGURE 2. Comparison of the intramolecular stem-loop structures of various U6 and U6atac snRNAs. The sequences are from human, *A. thaliana* (plant), and *Saccharomyces cerevisiae* (yeast). In each case the putative helix Ib interactions with the conspecific U2 or U12 snRNAs are shown below the intramolecular structures. The boxed sequences are those that were substituted into the human U6atac snRNA in our previous analysis of the plant U6atac stem-loop (Shukla & Padgett, 1999) or in the work discussed here.

functionally active snRNA when tested in vivo (Shukla & Padgett, 1999).

In addition to the indirect evidence of the importance of the U6 snRNA intramolecular stem-loop provided by phylogenetic conservation, experimental support has also been provided by several studies. Genetic suppression experiments in yeast have shown that formation of the stem-loop structure is required for splicing (Fortner et al., 1994; McPheeters, 1996). Similar experiments in mammalian systems also showed a requirement for this structure (Wolff & Bindereif, 1993; Sun & Manley, 1995, 1997). In an extensive set of experiments, Sun and Manley (1997) used an in vivo approach to show that U6 snRNA function was maintained as long as the base pairing pattern and a critical U residue in the bulge were conserved. Other structural modifications were also compatible with function including pairing of the bulged U residue and extension of the helix by an additional base pair. These results are quite surprising in light of the very high conservation of this region over more than a billion years of evolution.

Results from in vitro modification studies of residues within the U6 intramolecular stem-loop provide additional support for its role in splicing. In both yeast (Fabrizio & Abelson, 1992) and nematode (Yu et al., 1995) in vitro splicing systems, phosphorothioate modification of certain phosphodiester bonds blocks splicing. Such a block could be due to disruption of either RNA–protein interactions or interactions with functional chemical groups such as metal ions required for catalysis or the maintenance of a catalytically active structure (Eckstein, 1985). A recent analysis of one of these positions in yeast U6 snRNA has identified a critical metal-ion binding site in the bulge region (Yean et al., 2000). These and other results have led to speculation that this element of U6 snRNA functions at or near the catalytic center of the spliceosome (reviewed in Nilsen, 1998; Collins & Guthrie, 2000).

Because our earlier experience with substituting the plant U6atac snRNA stem-loop into human U6atac suggested that there was significant sequence flexibility that was still compatible with U6atac function, we next asked if the apparent homology of the U6 and U6atac structures could be extended to the level of in vivo function. Here we show that the substitution of the stem-loop from either human or budding yeast U6 snRNA for the human U6atac snRNA stem-loop results in a fully functional snRNA. We also show that specific bases and the overall structure of the stem loop are required for function.

RESULTS

We have previously developed an in vivo mutational suppressor assay for the function of several of the snRNAs involved in U12-dependent splicing (Hall & Padgett, 1996; Kolossova & Padgett, 1997; Incorvaia & Padgett, 1998; Shukla & 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. For U6atac snRNA, a mutation in the 5′ splice site of a transfected minigene construct, which blocks splicing at the normal sites, is suppressed by cotransfection of expression constructs containing compensatory mutations in U11 and U6atac snRNAs. The elements of this assay are diagramed in Figure 1B. The P120 minigene contains a double mutation in positions 5 and 6 of the 5′ splice site of the U12-dependent intron F (P120 CC5/6GG). This mutation causes a complete loss of splicing activity at the normal 5′ and 3′ splice sites and the concomitant activation of a pair of cryptic splice sites within the intron (Kolossova & Padgett, 1997). In vitro

analysis has shown that this cryptic splicing reaction is catalyzed by the U2-dependent spliceosome (Tarn & Steitz, 1996a). Cotransfection of U6atac and U11 snRNA expression constructs that contain the compensatory mutations shown in Figure 1B restores U12-dependent splicing at the normal 5′ and 3′ splice sites to nearly wild-type levels. This suppression is completely dependent on the U6atac suppressor while the U11 suppressor improves the level of suppression (Incorvaia & Padgett, 1998). This dependence on the addition of an exogenous suppressor U6atac allows us to assay the in vivo effects of mutations at other sites in U6atac snRNA.

We have previously used this assay to show that the intramolecular stem-loop structure of the *Arabidopsis thaliana* U6atac snRNA could function when transplanted into the human U6atac snRNA in spite of sequence differences at 9 of 21 positions (Fig. 2; Shukla & Padgett, 1999). All but one of these differences were located within the regions of the sequence believed to form intramolecular base pairs. Most of the changes within the putatively base paired regions either retained base pairing potential or were accompanied by compensatory changes. The sole difference in the non-base-paired residues was a change from uridine to cytosine in the 2-nt bulge between the two paired segments. The sequence of the top loop was the same in both human and plant U6atac snRNAs.

Unlike the case of the plant U6atac snRNA stem-loop, the U6 snRNA stem-loop appears to be structurally distinct from that of human U6atac snRNA (Fig. 2). In addition to differences in the base pairs of the stems, the sequence of the top loop differs between the two snRNAs. In U6, the loop is either 5 nt or 3 nt closed by a non-Watson–Crick G-A base pair. In U6atac, the loop is 3 nt closed with a U-A base pair. In addition, the bulge region of U6 contains an extra C residue on the 5′ side of the stem when compared to U6atac. This C residue is shown forming a non-Watson–Crick A-C base pair in Figure 2. These divergent features could affect function by altering the overall size or shape of the structure, by changing the angular relationships between the various portions of the structure or by affecting the binding of proteins or RNA elements to the top loop or bulge region. Thus, a functional similarity of these two features, although an attractive hypothesis, does not necessarily follow from a comparison of their structures.

With this in mind, we set out to test the functional relationship between these two elements by inserting the human and budding yeast U6 snRNA stem-loop structures in place of the U6atac snRNA stem-loop and testing the resulting chimeric snRNAs for in vivo splicing activity. Figure 2 shows the sequences and proposed structures of the various snRNA intramolecular stem-loops. The boxes show the regions transplanted into human U6atac snRNA in the chimeric constructs. The precise mutations that were made for the U6/U6atac chimeras are shown in Figure 3A.

In our earlier test of chimeric human/plant U6atac snRNAs, we found that the chimeras were inactive in vivo unless compensating alterations were made to the human U4atac snRNA sequence and this snRNA was coexpressed in vivo with the chimeric U6atac snRNA (Shukla & Padgett, 1999). Thus, for each of the U6/U6atac chimeras, we constructed analogous modified U4atac snRNA expression constructs as shown in Figure 3A. In designing these U4atac constructs, we attempted to maintain the amount of pairing and a similar overall structure to that predicted to form between the native U4atac and U6atac snRNAs.

The chimeric U6/U6atac snRNAs were constructed in the U6atac snRNA GG14/15CC mutant background. As shown in Figure 3A, nine mutations were needed to convert the human U6atac snRNA sequence to the human U6 snRNA sequence and eight mutations were needed to match the yeast U6 snRNA sequence. In both cases, an additional residue, circled in Figure 3A, was inserted to match the U6 snRNA structure.

These expression constructs were cotransfected either singly or together along with the U11 snRNA GG6/7CC suppressor mutant construct and the P120 minigene construct bearing the CC5/6GG 5′ splice site mutation. RNA was extracted from the cells after 48 h and analyzed by reverse transcription using a minigene-specific primer followed by PCR using primers in the exons flanking the U12-dependent P120 intron F.

The results are shown in Figure 3B. As discussed above, the P120 5′ splice site mutation causes the use of an internal pair of cryptic U2-dependent splice sites giving rise to the PCR product labeled “cryptic” (Fig. 3B, lane 4). The level of unspliced RNA is also increased in the mutant. When U11 and U6atac snRNA expression constructs carrying the compensatory mutations were cotransfected with the mutant P120 minigene (Fig. 3B, lane 7), the P120 mutation was suppressed as indicated by the appearance of the correctly spliced product labeled “spliced.”

Figure 3B shows that both the human (lanes 8 and 9) and budding yeast (lanes 11 and 12) U6 snRNA stem-loop structures were functional when substituted for the U6atac stem-loop. In fact, suppressor activity was seen even in the absence of the compensatory U4atac snRNA constructs. These results show that this region of U6 snRNA can functionally substitute for the analogous region of U6atac snRNA.

The apparent tolerance of the U6atac snRNA stem-loop structure for substantial modifications raises the question of the importance of this element in the splicing reaction. Although the conservation of the structural features of this element over the time since the divergence of plants and animals attests to its probable importance, a direct demonstration would also be desirable.

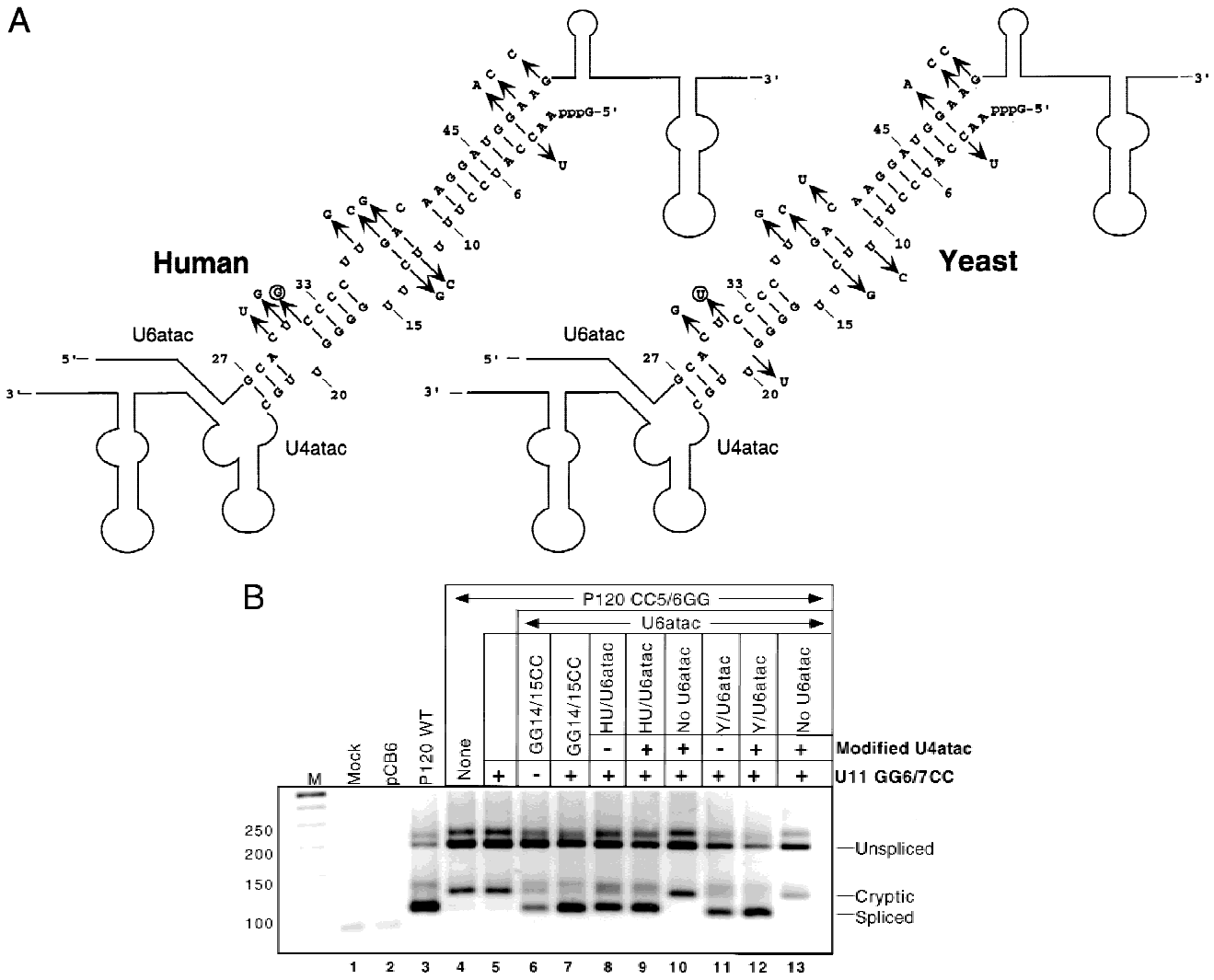

FIGURE 3. In vivo functional analysis of substitution of the human and yeast U6 snRNA stem-loop structures into human U6atac snRNA. **A**: Diagram showing the mutations made in human U6atac snRNA to mimic the human and yeast U6 snRNA stem-loop structures. Nucleotide insertions in U6atac snRNA are circled. Also shown are the compensatory mutations made in U4atac snRNA for each U6atac mutant. Not shown is the U6atac GG14/15CC suppressor mutation that was also included in the constructs. **B**: In vivo splicing assay of the modified snRNAs. The indicated constructs were cotransfected into CHO cells and total RNA was prepared 48 h later. The P120 RNA was reverse transcribed using a transgene-specific primer and the region spanning the U12-dependent intron F was amplified using primers in the adjacent exons. The positions of unspliced and correctly spliced products are shown. The cryptic spliced band is due to splicing between a pair of U2-dependent splice sites located within the P120 F intron. M: molecular size markers; lane 1: RNA from mock transfected CHO cells; lane 2: RNA from CHO cells transfected with the empty pCB6 expression vector; lane 3: RNA from CHO cells transfected with the wild-type P120 minigene. Lanes 4–13 were transfected with the P120 5′ splice site mutant CC5/6GG. Lane 4: the P120 mutant alone; lane 5 was cotransfected with the U11 GG6/7CC suppressor construct; lane 6 was cotransfected with the U6atac GG14/15CC suppressor construct; and lane 7 was cotransfected with both snRNA suppressor constructs. Lanes 8 and 9 were from cells transfected with the human U6/U6atac chimeric snRNA without (lane 8) or with (lane 9) the human U4atac suppressor snRNA construct. Lane 10 was from cells cotransfected with the U11 and human U4atac suppressor snRNA constructs. Lanes 11 and 12 were from cells transfected with the yeast U6/U6atac chimeric snRNA without (lane 11) and with (lane 12) the yeast U4atac suppressor snRNA construct. Lane 13 was from cells cotransfected with the U11 and the yeast U4atac suppressor snRNA constructs.

To address this point, we tested several mutations of this region of human U6atac snRNA in the in vivo suppressor assay. These included a set of three mutants in which a base pair (C35-G43) in the upper part of the stem was disrupted by mutations in each of the two paired residues and then reformed by combining both mutations in a single construct (Fig. 4A). In each case, a compensatory U4atac snRNA mutant was constructed and cotransfected with the U6atac mutant (Fig. 4B). The results in Figure 4C show that the G43C single mutant was inactive for suppression whereas the C35G single mutant showed a substantially reduced level of

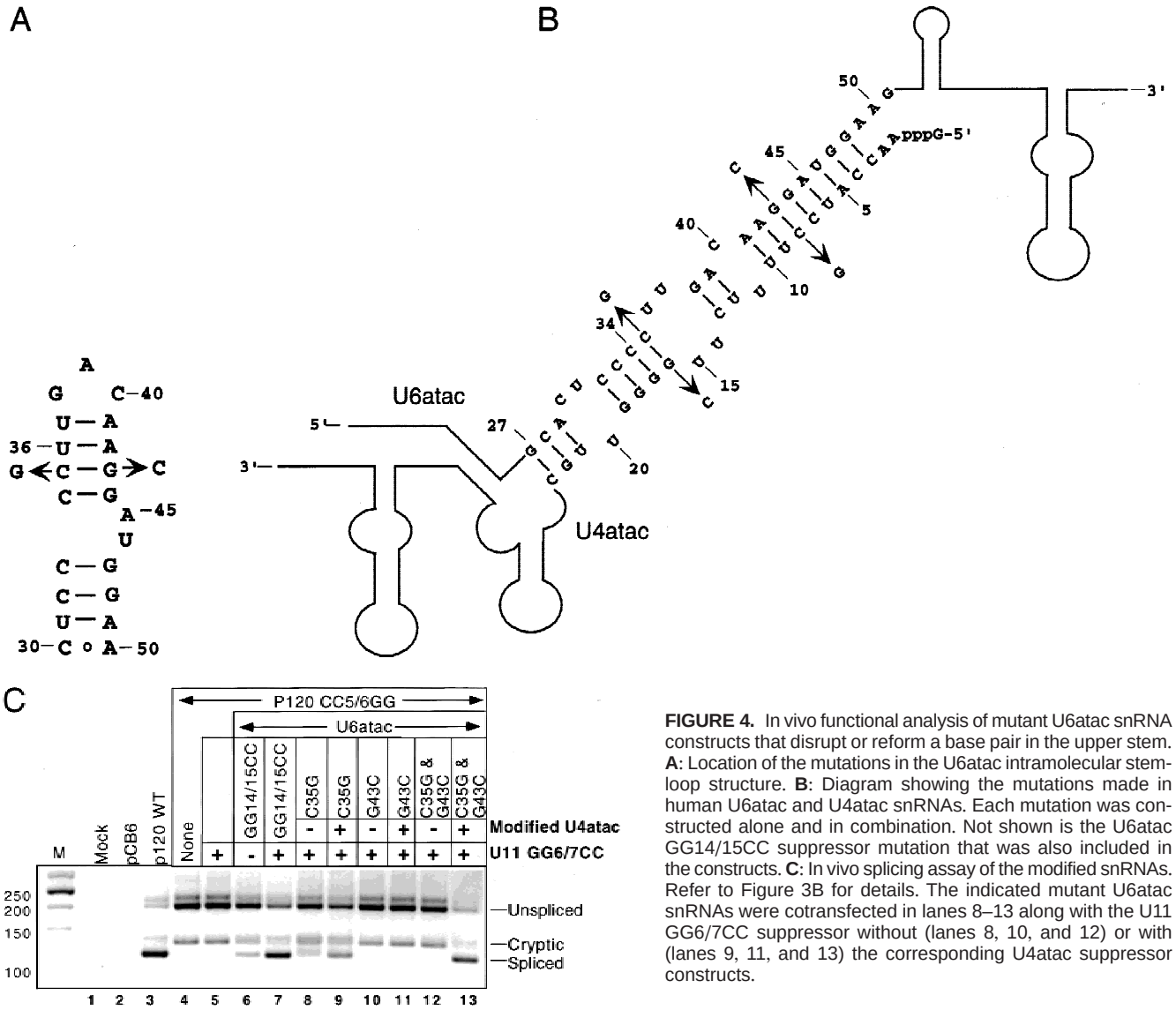

FIGURE 4. In vivo functional analysis of mutant U6atac snRNA constructs that disrupt or reform a base pair in the upper stem. **A**: Location of the mutations in the U6atac intramolecular stem-loop structure. **B**: Diagram showing the mutations made in human U6atac and U4atac snRNAs. Each mutation was constructed alone and in combination. Not shown is the U6atac GG14/15CC suppressor mutation that was also included in the constructs. **C**: In vivo splicing assay of the modified snRNAs. Refer to Figure 3B for details. The indicated mutant U6atac snRNAs were cotransfected in lanes 8–13 along with the U11 GG6/7CC suppressor without (lanes 8, 10, and 12) or with (lanes 9, 11, and 13) the corresponding U4atac suppressor constructs.

suppression. In contrast, the C35G/G43C double mutant suppressed the splicing defect as efficiently as the wild-type stem-loop (Fig. 4C, compare lanes 7 and 13).

We also tested the effects of mutations in the lower stem of the structure. Two base pairs were disrupted by mutations at positions 32 and 33 or positions 47 and 48 (Fig. 5A). In addition, the mutations were combined to restore base pairing potential between these positions. Corresponding mutations in U4atac snRNA were also constructed (Fig. 5B). The in vivo suppression results for these mutants are shown in Figure 5C. The CC32/33AG mutant (Fig. 5C, lane 9) was largely defective for suppression whereas the GG47/48CU mutant (Fig. 5C, lane 11) was fully functional. The combined mutant (Fig. 5C, lane 13) was also fully functional, showing that the defect in the CC32/33AG mutant could be compensated for by restoring base pairing in the lower stem. Note that all three mutants required the coexpression of modified U4atac snRNAs for function.

Both U6 and U6atac snRNAs have a bulge of 1 or 2 nt on one side of the stem-loop structure. In U6atac snRNA, we model this region as a 2-nt bulge consisting of A45 and U46, although alternative structures are possible (Tarn & Steitz, 1996b). To investigate the importance of these nucleotides, we deleted one or both of them in human U6atac snRNA (Fig. 6A) and constructed compensatory U4atac snRNA mutants (Fig. 6B). Deletion of both nucleotides or A45 alone inactivated U6atac (Fig. 6C, lanes 9 and 15). Deletion of U46 alone reduced the suppression activity of U6atac (Fig. 6C, lane 13) whereas mutation of U46 to G was fully functional for suppression of the splicing defect (Fig. 5C, lane 11).

To examine if the U6atac stem-loop serves a strictly structural function or if there might be base-specific interactions involved in its function, we created a U6atac stem-loop sequence in which each residue was converted to its complement (Fig. 7A, mutant I). A com-

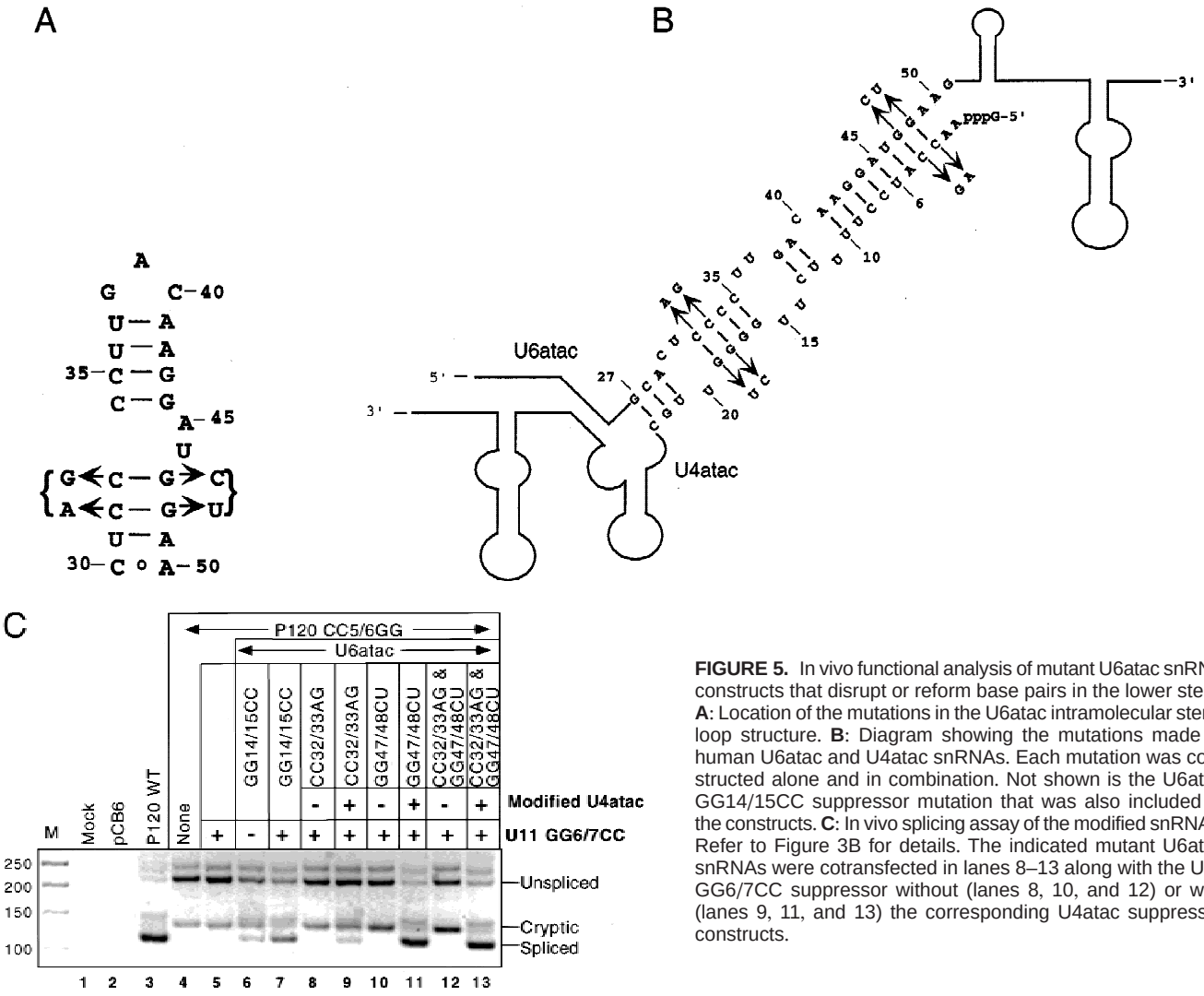

FIGURE 5. In vivo functional analysis of mutant U6atac snRNA constructs that disrupt or reform base pairs in the lower stem. **A**: Location of the mutations in the U6atac intramolecular stem-loop structure. **B**: Diagram showing the mutations made in human U6atac and U4atac snRNAs. Each mutation was constructed alone and in combination. Not shown is the U6atac GG14/15CC suppressor mutation that was also included in the constructs. **C**: In vivo splicing assay of the modified snRNAs. Refer to Figure 3B for details. The indicated mutant U6atac snRNAs were cotransfected in lanes 8–13 along with the U11 GG6/7CC suppressor without (lanes 8, 10, and 12) or with (lanes 9, 11, and 13) the corresponding U4atac suppressor constructs.

pensatory U4atac snRNA construct was also designed. Cotransfection of these snRNA constructs showed no suppression of the splicing defect (Fig. 7B, lane 9). To determine which portion of the stem-loop was responsible for the lack of activity, several combinations of wild-type and complementary sequences were constructed (Fig. 7A, mutants II–VII). These mutants and their corresponding compensatory U4atac snRNA mutants were tested for suppressor activity. As shown in Figure 7B, only mutant VII was positive for suppressor activity. This mutant differed from the other mutants tested in having a wild-type lower stem sequence. All of the mutants in which this region was complementary to the wild type were inactive for suppression.

DISCUSSION

The discovery that two distinct classes of spliceosomal introns coexist within the genomes of most eukaryotes raises a number of intriguing questions. For example, how similar are the mechanisms that serve to splice the two classes and what can the similarities and differences tell us about the mechanism(s) of pre-mRNA splicing? With the identification of the snRNAs that are required to splice the minor class of introns, a partial answer to this question seemed at hand (Tarn & Steitz, 1996b, 1997). The sequences of the minor class snRNAs could be folded into structures that appeared to mimic those of the major class snRNAs. Striking parallels could be drawn between the RNA–RNA interactions that were shown or inferred to be important for spliceosomal splicing.

One example of such a parallel was drawn between the intramolecular stem-loop structures of U6 and U6atac snRNAs that immediately followed regions of interaction with U2 and U12 snRNAs respectively (Tarn & Steitz, 1996b). Although the sequences and some of the structural details were different, the overall architecture consisting of a base-paired stem interrupted by a bulge on one side appeared to be analogous. How-

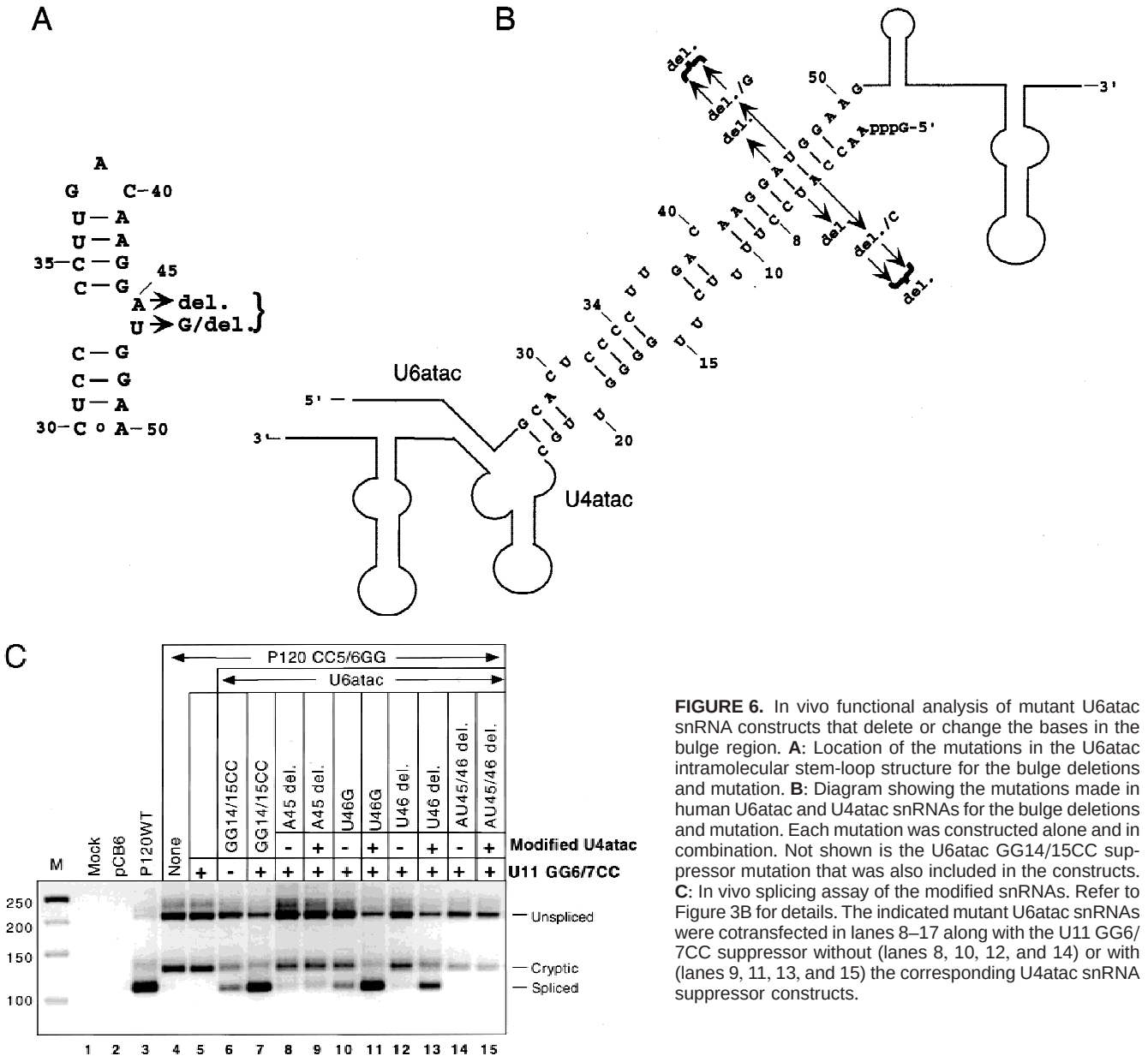

FIGURE 6. In vivo functional analysis of mutant U6atac snRNA constructs that delete or change the bases in the bulge region. **A**: Location of the mutations in the U6atac intramolecular stem-loop structure for the bulge deletions and mutation. **B**: Diagram showing the mutations made in human U6atac and U4atac snRNAs for the bulge deletions and mutation. Each mutation was constructed alone and in combination. Not shown is the U6atac GG14/15CC suppressor mutation that was also included in the constructs. **C**: In vivo splicing assay of the modified snRNAs. Refer to Figure 3B for details. The indicated mutant U6atac snRNAs were cotransfected in lanes 8–17 along with the U11 GG6/7CC suppressor without (lanes 8, 10, 12, and 14) or with (lanes 9, 11, 13, and 15) the corresponding U4atac snRNA suppressor constructs.

ever, there was no direct evidence to show that this region of U6atac snRNA was important for splicing, that it formed such a structure, or that the U6 and U6atac snRNA features were functionally analogous.

The first clues to the functional significance of this region of U6atac came from a comparison of the human and plant U6atac snRNAs (Shukla & Padgett, 1999). This comparison showed that, although the sequences in this region differed by almost 50% between the two organisms, the overall structure was maintained by compensatory mutations in the putative base-paired stem regions whereas the loop and bulge nucleotides were largely conserved. To show that the plant element retained the same function as the human element, a chimeric U6atac snRNA was constructed with the plant stem-loop sequence in the background of the human U6atac snRNA. By adding a suppressor mutation that allowed us to assess the function of this chimeric snRNA in vivo, we showed that the plant stem-loop sequence functioned in the human snRNA (Shukla & Padgett, 1999).

In the work described here, we show that the human U6atac snRNA stem-loop structure is important for in vivo splicing. Several mutations in the suppressor U6atac snRNA abolish in vivo function. These include mutations that are expected to lead to mispairing within the stems and mutations that remove one or both of the bulged nucleotides. The splicing defects of the mispairing mutations could be reversed by restoring the potential for base pairing with compensatory mutations

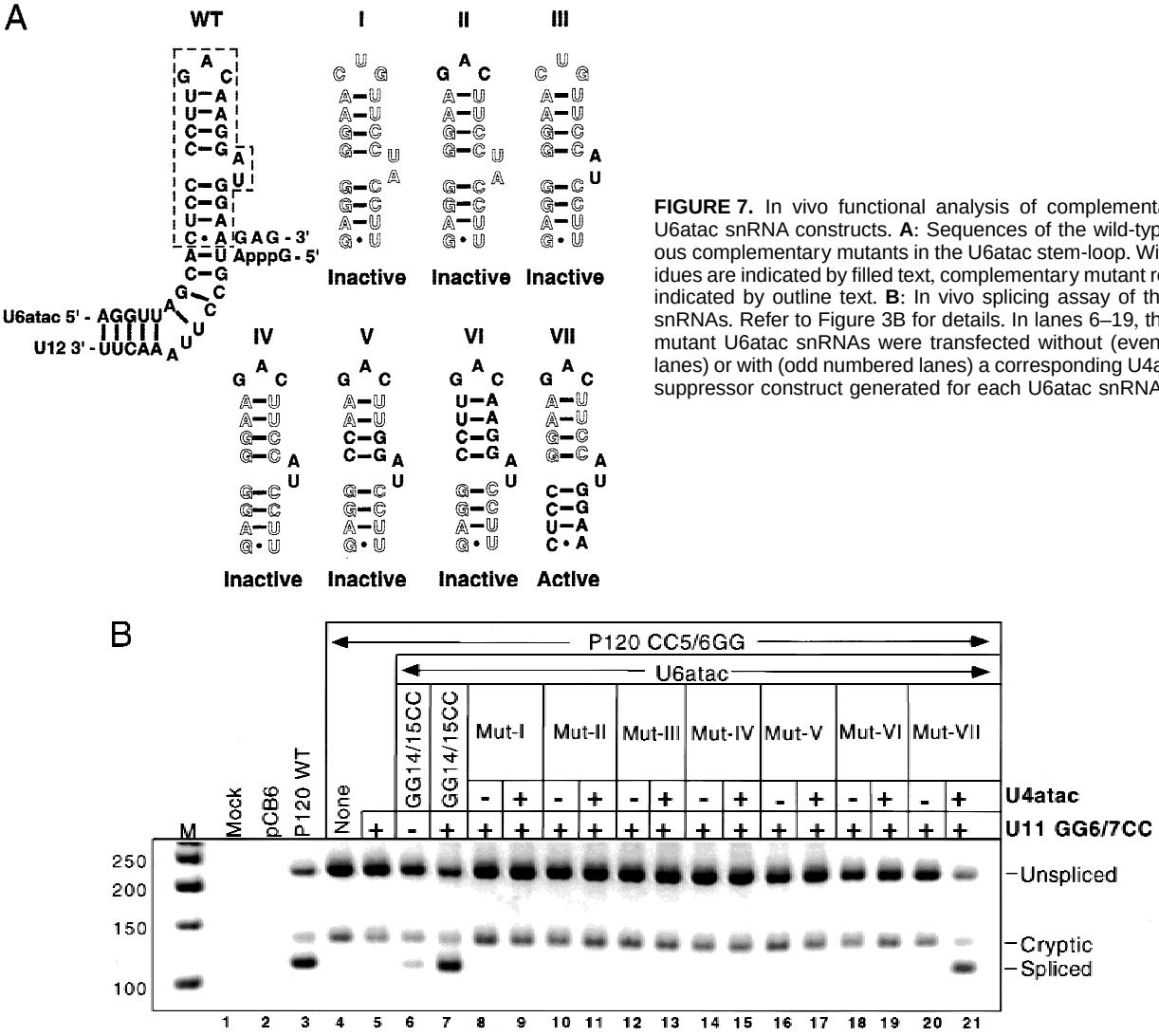

FIGURE 7. In vivo functional analysis of complementary mutant U6atac snRNA constructs. **A**: Sequences of the wild-type and various complementary mutants in the U6atac stem-loop. Wild-type residues are indicated by filled text, complementary mutant residues are indicated by outline text. **B**: In vivo splicing assay of the indicated snRNAs. Refer to Figure 3B for details. In lanes 6–19, the indicated mutant U6atac snRNAs were transfected without (even numbered lanes) or with (odd numbered lanes) a corresponding U4atac snRNA suppressor construct generated for each U6atac snRNA mutant.

on the other side of the stems. These results confirm the prediction of the phylogenetic comparisons between the human and plant sequences.

The finding that in vivo function was preserved as long as the structure of the stem-loop was maintained raised the possibility that its function was strictly structural, that is, there were no base-specific interactions involved in its role in splicing. To test this idea, a completely complementary stem-loop was constructed along with a complementary U4atac snRNA (Fig. 7A, mutant I). This U6atac snRNA had no suppressor function in vivo. The in vivo assay does not allow us to be sure that the changes to the snRNAs only affect activity in the splicing reaction and not the synthesis, processing, distribution, or stability of the snRNAs. Nevertheless, a likely explanation for the inactivity of this mutant is that it disrupts essential base-specific interactions.

To determine which changes were responsible for the inactivity of the complementary stem-loop mutant, several constructs that combined features of the wild-type stem-loop with the complementary mutant were tested (see Fig. 7A, mutants II–VII). The first hypothesis we tested was that the single-stranded top loop and/or bulge sequences needed to be wild type but the stems only needed to pair properly. However, neither correction of the top loop (mutant II) nor the bulge sequences (mutant III) nor correction of both (mutant IV) restored function. An additional mutation of the upper stem to suppress the formation of alternative secondary structures (mutant V) was also unable to restore function as was a mutant with a fully wild-type upper stem (mutant VI). Finally, correction of the lower stem region combined with correction of the top loop and bulge did restore in vivo function (mutant VII).

The stem-loop in mutant VII differs from the wild type in eight positions encompassing all four base pairs of the upper stem. In contrast, all of the constructs with a mutated lower stem were inactive. However, note that the two upper base pairs in the lower stem (C32-G48

and C33-G47) could be altered in the compensatory mutant shown in Figure 5 while retaining function. Likewise the C30-A50 mismatch and the U31-A49 base pair could be simultaneously changed to A30-U50 and C31-G49 base pairs in the plant U6atac stem-loop (Fig. 1; Shukla & Padgett, 1999) without loss of function. It was, therefore, somewhat surprising that the 4-bp mutation was inactive. This suggests that the lower stem may be playing a role in the function of this structural element in addition to base pairing. Exactly which nucleotide or nucleotides are involved in this function is not clear from the mutants tested so far. Nevertheless, the results suggest that this part of the U6atac stem-loop and, by extension, the analogous part of the U6 stem-loop may have a functional as opposed to a strictly structural role in the splicing reaction.

Finally, the proposed functional analogy between the stem-loop structures of U6 and U6atac snRNAs was confirmed by constructing chimeric U6/U6atac snRNAs containing the human or yeast U6 snRNA structures. These chimeric snRNAs were fully functional in vivo in the splice site mutant suppressor assay. Thus, these elements from disparate organisms and from both spliceosomal splicing systems are functionally interchangeable.

In spite of the high conservation of the structure of this element and the apparent conservation of its function as shown by these studies, we do not have a clear idea of what its function might be in the spliceosome. Its location near the sites of splicing chemistry have led to speculation that it plays a role in the active site of the spliceosome (reviewed in Nilsen, 1998; Collins & Guthrie, 2000). Several groups have suggested parallels with features of various ribozymes including the hairpin ribozyme (Tani & Ohshima, 1991; Sun & Manley, 1995) and domain 5 of group II self-splicing introns (see discussions in Sun & Manley, 1997; Costa et al., 1998; Nilsen, 1998). The group II domain 5 comparison is particularly interesting. A recent revision of the proposed structure of the domain 5 stem-loop has emphasized the similarity between it and the U6 (or U6atac) intramolecular stem-loop (Costa et al., 1998). A phosphorothioate modification-interference study of domain 5 in self-splicing identified a phosphate group in the bulge region as important for splicing catalysis (Chanfreau & Jacquier, 1994). This phosphate is located in a very similar position to one identified as important for U6 snRNA function in in vitro pre-mRNA splicing in both yeast (Fabrizio & Abelson, 1992) and nematodes (Yu et al., 1995). Recent investigations of both phosphorothioate diastereomers at this position in yeast U6 snRNA have revealed a metal ion specificity switch for the first step of splicing (Yean et al., 2000). This suggests that U6 snRNA participates in the catalysis of splicing through metal ion coordination and places this stem-loop element at or very near the catalytic center of the spliceosome.

Pyle's group has also suggested that the bulge and lower stem regions of domain 5 serve to position a critical metal ion for group II splicing (Konforti et al., 1998). Such a function would mainly involve the positioning of phosphate groups to coordinate the metal and would be compatible with many but perhaps not all base paired sequences within the stem region. Base-specific interactions would be limited to residues in unpaired regions and functional groups in the major and minor groves of the helical regions. Such a function would fit with the apparent tolerance of the stem regions of U6atac snRNA for many but not all substitutions that maintain base pairing.

More recently, a tertiary interaction has been described between the 5′ splice site region and two C-G or G-C base pairs in the upper helix of domain 5 in a group II intron (Boudvillain et al., 2000). These authors suggest that a similar interaction could take place between the 5′ splice site of spliceosomal introns and conserved C-G base pairs in the upper helix of U6 snRNA. These two C-G base pairs are present in both human and yeast U6 snRNA as well as in human U6atac snRNA (Fig. 2). However, the upper C-G base pair is replaced by a U-A base pair in the plant U6atac snRNA. Furthermore, as shown by mutant VII in Figure 7, mutation of this entire helix in human U6atac snRNA does not abolish in vivo splicing activity. It is possible that both C-G and G-C base pairs could participate in the tertiary interaction proposed by Boudvillain et al. (2000). Additional mutations at these positions of U6atac will be necessary to test this proposed interaction in the U12-dependent spliceosome. Interestingly, mutations in the two C-G base pairs in U6 snRNA were not examined in the extensive study of U6 snRNA by Sun and Manley (1997).

When the results of this work are compared to the similar study of human U6 snRNA by Sun and Manley (1997), some interesting similarities and differences appear. In both studies, single mutations of residues within the base-paired portions of the snRNAs were inactive for splicing in vivo. However, in the case of U6atac, mutation of residues in the 3′ portion of the lower stem showed no defect in splicing, although they could compensate for splicing defective mutations in the other side of the stem. In both studies, splicing defective mutants in the stem regions could be rescued by compensatory mutations restoring the base pairing pattern in the stems.

An interesting difference between these two studies is seen in the effects of mutation or deletion of the U residue in the bulge regions of the two snRNAs. Mutation or deletion of this residue (U74) in U6 abolished function (Sun & Manley, 1997) whereas mutation of U46 in U6atac to G or C (in the plant sequence) as well as deletion had no major effect on function. In the absence of information on the actual conformation of these structures in the spliceosome and on the nature of any

base-specific interactions, it is difficult to judge the significance of this difference.

Another significant difference between the two studies is the apparent lack of the need to compensate for the U6 mutations by altering U4 snRNA. In our U6atac studies, most mutants, including some with single base changes, required the coexpression of compensatory U4atac snRNAs mutants. The mechanistic foundation of this difference is unclear. It might reflect a lower stability of the U4atac/U6atac interaction compared with the U4/U6 interaction. Perhaps the 100-fold greater abundance of U4 and U6 snRNAs compared to U4atac and U6atac snRNAs (Yu et al., 1999) more strongly favors the di-snRNP configuration. Alternatively, the U4atac/U6atac di-snRNP may be a poorer substrate for factors that anneal the two snRNAs or a better substrate for factors that unwind the snRNA duplex. In the yeast system, factors have been identified that appear to catalyze the formation of the U4/U6 di-snRNP and catalyze the unwinding of the duplex (Raghunathan & Guthrie, 1998a, 1998b). One would anticipate the existence of similar activities in mammalian cell nuclei (Laggerbauer et al., 1998).

Two notable exceptions to the requirement for U4atac suppressor constructs are the human and yeast U6 stem-loop transplants into U6atac shown in Figure 3. Although these mutants have many more differences from the wild-type U6atac sequence than most of the other mutants discussed here, they were active in vivo without addition of specific U4atac suppressor constructs. Using the mfold server (www.mfold.wustl.edu; Zuker, 1994), the calculated losses of free energy for the U6 transplants were 10 kcal/mol for the human and 10.5 kcal/mol for the yeast sequences. In contrast, the U46 del mutant, which requires a modified U4atac for activity (Fig. 6), loses only 4.9 kcal/mol of free energy.

A possible explanation for the activity of the U6 transplant mutants is that they are pairing with U4 snRNA rather than U4atac snRNA. The 11-nt region of U6atac snRNA 5′ of the stem-loop differs from U6 snRNA in only one position. As a result, both stems I and II of the U4/U6 pairing could be recapitulated with the HU/U6atac mutant. Similarly, the Y/U6atac mutant could pair to U4 snRNA with a net loss of 2 bp in stem II. As noted above, the data of Sun and Manley (1997) show that the U4/U6 interaction is stable to this number of mutations. Experiments to directly test the idea that U4 snRNA can productively pair with U6atac snRNA are in progress.

The apparent structural similarity between the RNA–RNA interactions in U2- and U12-dependent splicing has been one of the most striking results to come from the comparisons of the two systems. Our demonstration here that an important substructure can be functionally transplanted between the two splicing systems supports the significance of these similarities. The U2- and U12-dependent splicing systems have been separate since before the divergence of plants and animals (Wu et al., 1996). We have suggested that, in fact, they are derived from parallel but distinct splicing systems in ancestral prokaryotic genomes that combined during the genesis of the eukaryotic lineage (Burge et al., 1998). If the U6 and U6atac stem-loop elements function at or near the active sites of both spliceosomes, these results suggest that the active sites are similarly configured. To what extent these similarities are based on common descent, the sharing of spliceosomal factors, or the chemical constraints of the splicing reactions themselves is not clear. Perhaps a detailed comparison to the structure and function of the domain 5 element of group II introns will show how these RNA elements relate to the active sites of the respective systems.

MATERIALS AND METHODS

Construction of U6atac mutants

The U6atac snRNA mutants were made in the 5′ splice site compensatory mutant GG 14/15 CC expression plasmid previously described (Incorvaia & Padgett, 1998) using either the pALTER mutagenesis kit (Promega) or PCR sewing techniques and mutagenic oligonucleotides. All mutations were confirmed by DNA sequencing.

Construction of U4atac expression plasmid

The U4atac expression plasmid was generated by the same method used previously for U11 and U12 snRNAs (Shukla & Padgett, 1999). Briefly, the U1 snRNA coding region of a functional U1 gene was replaced by PCR techniques with the coding region of U4atac snRNA amplified from a U4atac plasmid obtained from J. Steitz. For the mutations studied here, oligonucleotides that included the first 35 nt of U4atac containing the desired mutations were used with a common 3′ oligonucleotide in standard PCR reactions. The PCR products containing either wild-type human U4atac or U4atac containing compensatory mutations to restore base pairing with the various U6atac snRNAs were digested with *Sal*I and *Bgl*II restriction enzymes and ligated into a U1 expression vector from which the U1 coding region had been excised (Bond et al., 1991). 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 & Padgett, 1996; Kolossova & Padgett, 1997; Incorvaia & 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 (Kolossova & Padgett, 1997; Incor-

vaia & Padgett, 1998). The products were analyzed by agarose gel electrophoresis. The DNA bands were visualized using ethidium bromide and photographed using a digital video camera (Kodak). Independent transfections and analyses gave substantially similar results.

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