RNA (2002), *8*:997–1010. Cambridge University Press. Printed in the USA.

DOI: 10.1017.S1355838202025013

The conserved central domain of yeast U6 snRNA: Importance of U2-U6 helix I_a in spliceosome assembly

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ABSTRACT

In the pre-mRNA processing machinery of eukaryotic cells, U6 snRNA is located at or near the active site for pre-mRNA splicing catalysis, and U6 is involved in catalyzing the first chemical step of splicing. We have further defined the roles of key features of yeast U6 snRNA in the splicing process. By assaying spliceosome assembly and splicing in yeast extracts, we found that mutations of yeast U6 nt 56 and 57 are similar to previously reported deletions of U2 nt 27 or 28, all within yeast U2-U6 helix I_a. These mutations lead to the accumulation of yeast A1 spliceosomes, which form just prior to the Prp2 ATPase step and the first chemical step of splicing. These results strongly suggest that, at a late stage of spliceosome assembly, the presence of U2-U6 helix I_a is important for promoting the first chemical step of splicing, presumably by bringing together the 5′ splice site region of pre-mRNA, which is base paired to U6 snRNA, and the branchsite region of the intron, which is base paired to U2 snRNA, for activation of the first chemical step of splicing, as previously proposed by Madhani and Guthrie [*Cell*, 1992, *71*: 803–817]. In the 3′ intramolecular stem-loop of U6, mutation G81C causes an allele-specific accumulation of U6 snRNP. Base pairing of the U6 3′ stem-loop in yeast spliceosomes does not extend as far as to include the U6 sequence of U2-U6 helix I_b, in contrast to the human U6 3′ stem-loop structure.

Keywords: divalent metal; phosphorothioates; pre-mRNA; Prp2; snRNPs; splicing

INTRODUCTION

During normal gene expression in eukaryotic cells, gene-interrupting sequences, or introns, are removed from the pre-messenger RNA transcripts of an intron-containing gene via pre-mRNA splicing (reviewed in Nilsen, 1998; Burge et al., 1999; Hastings & Krainer, 2001). The presence of an intron in pre-mRNA triggers its association with a mostly preformed complex of five small nuclear ribonucleoprotein particles (snRNPs) to form a spliceosome (Stevens et al., 2002). The RNA–RNA and RNA–protein interactions within the spliceosome are dynamic and involve all five spliceosomal snRNPs, named U1, U2, U4, U5, and U6 snRNPs (reviewed in Will & Lührmann, 2001), and additional spliceosomal proteins. This dynamic complex catalyzes the excision of the intron in two chemical steps: (1) nucleophilic cleavage of the 5′ splice site with concurrent formation of a lariat intron intermediate, and (2) ligation of the mRNA exons with simultaneous cleavage of the 3′ splice site to release the lariat intron. In a fully assembled spliceosome, U6 snRNA interacts with the 5′ splice site via base pairing, and U2 snRNA base pairs with the nucleophilic branchsite region, to help mediate the first chemical step of splicing (Fig. 1C).

Previous studies in various eukaryotic systems have identified several functional elements in U6 snRNA that are important for pre-mRNA splicing, and these are briefly described here (refer to Fig. 1). It was recently determined that the 3′ terminal 12 nt of human U6 snRNA are necessary and sufficient for the binding of Lsm proteins in human U6 snRNP (Achsel et al., 1999). Similarly, the 3′ terminus of yeast U6 snRNA was shown to be necessary for the binding of yeast Lsm proteins (Mayes et al., 1999; Vidal et al., 1999). Prp24 protein is a component of yeast U6 snRNP (Shannon & Guthrie, 1991; Stevens et al., 2001). Although its binding site on U6 snRNA in not clear, the footprint of recombinant Prp24 protein on U6 RNA transcript has been reported (Ghetti et al., 1995). Prior to joining the spliceosome, free U6 snRNP undergoes rearrangement of its secondary RNA structure as it forms a duplex with U4 snRNA. In this process, the U6 3′ stem-loop is unwound, and its loop sequence is important for U4-U6

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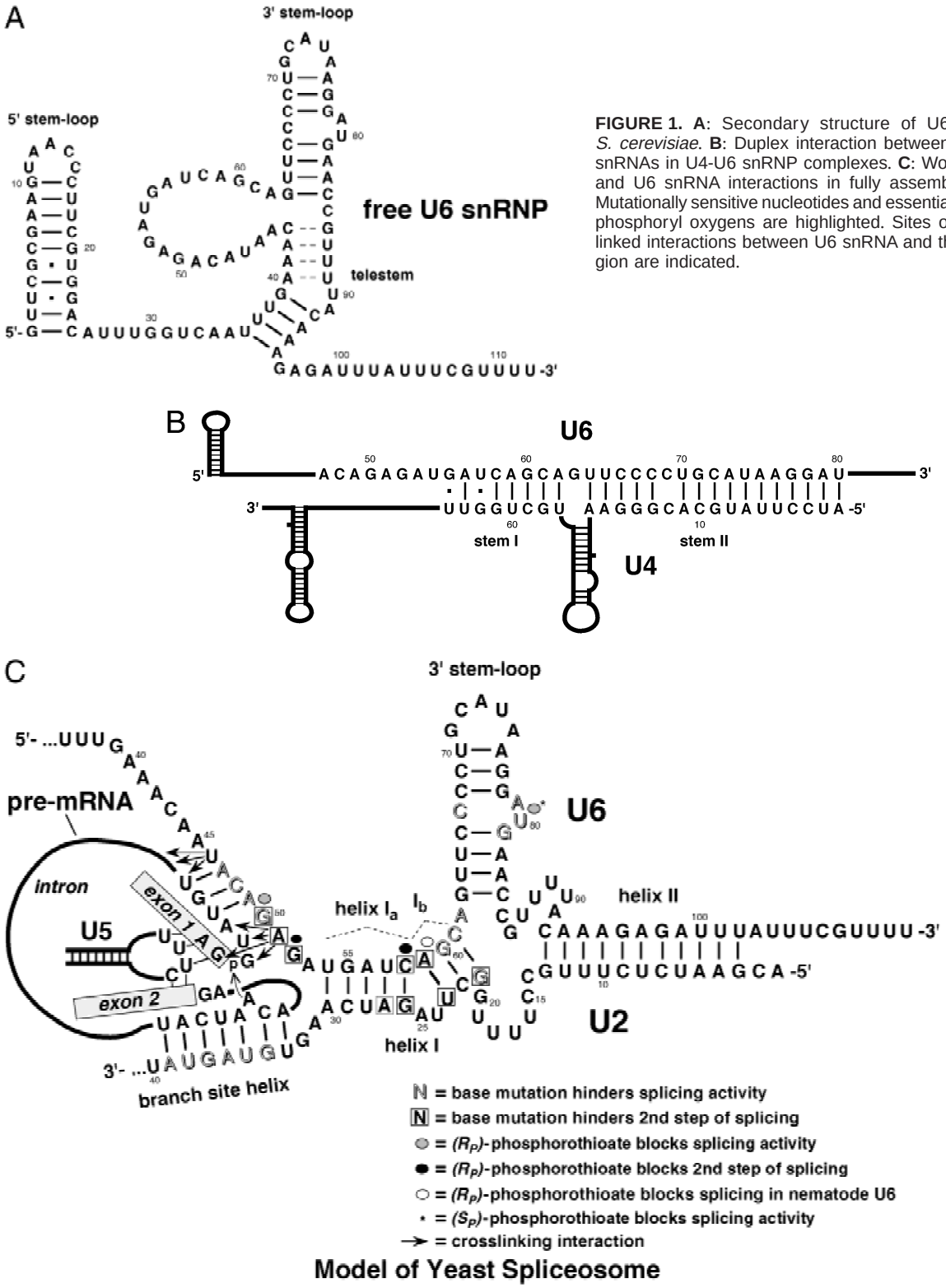

FIGURE 1. A: Secondary structure of U6 snRNA in yeast *S. cerevisiae*. **B**: Duplex interaction between yeast U4 and U6 snRNAs in U4-U6 snRNP complexes. **C**: Working model for U2 and U6 snRNA interactions in fully assembled spliceosomes. Mutationally sensitive nucleotides and essential pro-R_P and pro-S_P phosphoryl oxygens are highlighted. Sites of previously cross-linked interactions between U6 snRNA and the 5′ splice site region are indicated.

di-snRNP assembly (Wolff & Bindereif, 1993). U4-U6 di-snRNP joins U5 snRNP to assemble the "tri-snRNP," which then joins U2 snRNP and U1 snRNP to form the tetra- and penta-snRNPs, respectively, which can bind pre-mRNA to form a spliceosome (Stevens et al., 2002).

In an early stage of spliceosome maturation, a base-paired interaction between U1 snRNA and the 5′ splice site is dissociated as a subset of these 5′ splice site nucleotides becomes base paired with the conserved ACAGAG trinucleotide of U6 snRNA (nt 47–49 in yeast;

Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993; Staley & Guthrie, 1999). U6 snRNA is dissociated from U4 snRNA, and the liberated central domain of U6 refolds to regenerate the U6 3′ stem-loop, which is required for the first chemical step of splicing (Wolff & Bindereif, 1993). Upstream and downstream of this U6 stem-loop, U6 sequences base pair with sequences of U2 snRNA to form U2-U6 helices I and II. The ability to form either U2-U6 helix II or helix I_b is essential in yeast (Field & Friesen, 1996); therefore helices II and I_b may serve a redundant function, presumably to stabilize the U6 3′ intramolecular stem-loop required in active spliceosomes (Wolff & Bindereif, 1993; Fortner et al., 1994; Sun & Manley, 1997). In mammalian spliceosomes, U2-U6 helix III forms downstream (with respect to the U2 strand) of the U2/pre-mRNA branchsite helix (Sun & Manley, 1995); however, U2-U6 helix III may not form in yeast (Yan & Ares, 1996). The existence and functional importance of U2-U6 helices I, II, III and the U6 3′ stem-loop were proven in human cells (Datta & Weiner, 1991; Wu & Manley, 1991; Sun & Manley, 1995), and at least portions of these helices, except helix III, were shown to exist in yeast spliceosomes (Madhani & Guthrie, 1992; Madhani, 1993; Field & Friesen, 1996). However, these and other studies have also shown that the U6 helices are tolerant of mutational disruptions (see also Hamm et al., 1989; Fabrizio & Abelson, 1990, Vankan et al., 1990, 1992; McPheeters & Abelson, 1992; Wolff & Bindereif, 1993, 1995; Wolff et al., 1994; McPheeters, 1996; Sun & Manley, 1997).

In fully mature spliceosomes, a backbone phosphate group in the 3′ stem-loop of U6 RNA (at the A79-U80 linkage in yeast U6) is required for splicing activity (Fabrizio & Abelson, 1992; Yu et al., 1995) and was recently shown to bind a divalent metal ion, presumably Mg^{2+}, which is essential for catalyzing the first chemical step of splicing (Yean et al., 2000). Four other backbone phosphate groups in U6 were found to be important for the first and/or second chemical step of splicing in yeast and nematodes, and these phosphates are located at mutationally sensitive nucleotides (Fabrizio & Abelson, 1992; Yu et al., 1995). For the second chemical step of splicing, the ACAGAG sequence of U6 is important, as are nucleotides around the bulge of U2-U6 helix I in both yeast and HeLa extracts (Fabrizio & Abelson, 1990; Madhani & Guthrie, 1992; McPheeters & Abelson, 1992; Wolff et al., 1994). Indeed, it is clearly suggested from the second-step defects for point mutations around the helix I bulge and from the pattern of compensatory mutations that suppressed the corresponding growth defects that base pairing directly adjoining the bulge is especially important for the second chemical step of splicing. Maintenance of this base pairing between the bulge-adjoining nucleotides, that is, between U6 nt 58 and U2 nt 26 and between U6 nt 59 and U2 nt 23, was found to be sufficient for growth. A wide variety of base-pairing combinations was viable, whereas most base-pair disruptions were lethal (Madhani & Guthrie, 1994).

Several questions remain about the roles of the U6 helices in splicing. For example, at what steps of splicing are the yeast U6 helices necessary? How many of the U6 helices occur simultaneously? Are the helices reconfigured between the first and second chemical steps? We analyzed the effects of mutational disruptions in U2-U6 helix I_a and in the U6 intramolecular 3′ stem-loop. Our data strongly suggest that the presence of U2-U6 helix I_a is important at a late stage of spliceosome assembly, confirming the hypothesis that formation of this helix promotes spliceosome activation (Madhani & Guthrie, 1992). We also show that base pairing within the U6 3′ stem-loop of fully assembled yeast spliceosomes is not extended to include the U6 sequence of U2-U6 helix I_b, in contrast to the human U6 3′ stem-loop structure (Sun & Manley, 1995). In free U6 snRNPs, mutations in the bulged region of the 3′ stem-loop of U6 strongly inhibit U4-U6 snRNP assembly.

RESULTS

Mutational disruption of U2-U6 helix I_a blocks spliceosome maturation at a late stage

Previous studies in yeast showed that growth defects for base pair disruptions in U2-U6 helix I were rescued by making compensatory mutations that restore base pairing in the helix (Madhani & Guthrie, 1992). Analysis of these growth defects showed some rather weak defects in the first chemical step of splicing for U3A/U3B pre-mRNA in vivo, defects that we note correlated with the 30 °C growth defects. However, the RP51A pre-mRNA in the same strains did not show a similar pattern of first-step defects, but rather showed some obvious second-step defects that did not correlate with the growth defects at 30 °C. These second-step blocks appeared to be stronger than the accompanying first-step defects, suggesting that a strong second-step block in vivo might also cause a modest accumulation of unspliced pre-mRNA, perhaps by blocking turnover of the stalled spliceosomes. Such a scenario might explain the weak first-step defects observed for U3A/U3B splicing. Unfortunately, the second-step products for U3A/U3B pre-mRNA splicing could not be visualized or quantified in the assay. Therefore, it was unclear whether the U2-U6 helix I disruptions blocked the first step or the second step of splicing or both.

Using our in vitro splicing assay, we examined the same U6 double mutations of helix I_a that had been rescued by compensatory U2 mutations in yeast by Madhani and Guthrie (1992), and we assayed additional helix I_a U6 mutations as well. In this standard assay, endogenous U6 snRNA in yeast extract is depleted as described Materials and Methods, and ali-

quots of the U6-depleted extract are reconstituted by adding synthetic, mutant U6 RNA. The reconstituted extracts are then assayed for their ability to catalyze splicing of ^{32}P-labeled actin pre-mRNA. The U6 double mutations that previously showed compensatory effects are A56U,U57A, A56C,U57G, and A56C,U57C. The new mutations in the U6 strand of helix I_a are U54A,G55C and G55C,A56C,U57C. As controls, we included the U6 point mutations C58G and A59U, which are both strong second-step blocks (Fabrizio & Abelson, 1990). To block single-strand specific, exonucleolytic degradation of synthetic U6 RNAs in extract, we incorporated four 5′-3′ phosphorothioate linkages between the last 5 nt at the 3′ end of each synthetic U6 RNA (thus each phosphorothioate is racemic). These substituents were found to have no detectable effect on splicing activity (Ryan et al., 2002). The splicing assays are shown in Figure 2 and summarized in Table 1. Our in vitro results correlate well with the previously reported growth defects for the U6 double mutations (Madhani & Guthrie, 1992); that is, the A56C,U57C mutant was viable at 30 °C and reconstituted 47% of wild-type U6 splicing activity (Fig. 2, lane 7), whereas the A56U,U57A and A56C,U57G mutants were not viable at 30 °C and reconstituted only 10% and 28% of wild-type U6 splicing activity, respectively (Fig. 2, lanes 5 and 6; Table 1). The adjacent double mutation U54A,G55C showed no splicing defect (Fig. 2, lane 4). Extending the A56C,U57C disruption mutation by one more nucleotide in U6 G55C,A56C,U57C caused a loss of splicing activity (Fig. 2, lane 8). Native gel analysis revealed that this triple mutation interfered with U4-U6 snRNP assembly well before the stage of A complex assembly, as suspected (see Fig. 6A, lane 3).

TABLE 1. Splicing yields for mutations in the U2-U6 helix I sequence of U6 RNA in yeast extract.

U6 RNA mutations[a]	Relative splicing yields[b]
U54A,G55C	98%
A56U,U57A	10%
A56C,U57G	28%
A56C,U57C	47%
G55C,A56C,U57C	4%
C58G	42%[c]
A59U	99%[c]
Wild type	100%

[a]The last four 5′-3′ linkages at the 3′ terminus are racemic phosphorothioates.
[b]Splicing yields for reconstituted, synthetic U6 RNAs are normalized relative to the yield for reconstituted, synthetic wild-type U6 RNA as a control.
[c]Strongly blocked at second chemical step of splicing.

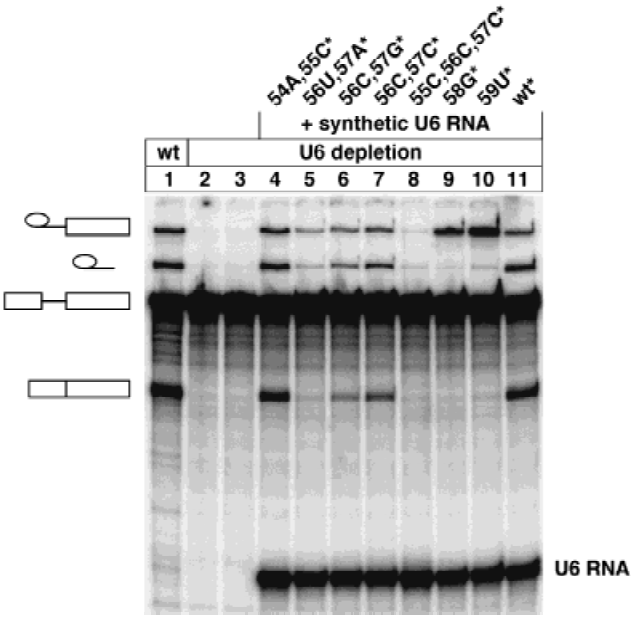

FIGURE 2. Splicing of ^{32}P-labeled actin pre-mRNA in U6-depleted yeast extract reconstituted with mutant and wild-type U6 RNAs. U6 mutations were introduced in the yeast U6 sequence of U2-U6 helix I (nt 54–61). Yeast splicing extract was treated (lanes 2–11) or not treated (lane 1) with a DNA oligonucleotide (d1) complementary to U6 snRNA. After incubation at 34 °C to allow native RNase H digestion of endogenous U6 snRNA, aliquots of the U6-depleted extract were reconstituted by adding synthetic, ^{32}P-labeled U6 RNA as indicated. In synthetic U6 RNAs marked with an ∗, the last four 5′-3′ linkages at the 3′ ends were racemic phosphorothioates. Splicing of ^{32}P-labeled actin pre-mRNA substrate was assayed at 25 °C for 30 min. This substrate has one intron and gives rise to exon1 and lariat-exon2 intermediates after the first chemical step of splicing and to excised lariat intron and spliced mRNA products after the second chemical step (as indicated pictorially). Total nucleic acid for each sample was separated on a denaturing polyacrylamide gel. The U6-depleted extract was assayed twice in lanes 2 and 3 for residual splicing activity, and the averaged background signal was used to correct the levels of splicing products assayed in lanes 4–11.

The lethal U6 double mutations in U2-U6 helix I_a showed first-step splicing defects in our in vitro assay. To determine more specifically which step of spliceosome assembly or splicing was affected in vitro, we analyzed the deleterious double and triple U6 mutations of helix I_a using a standard native gel assay for spliceosome assembly (Cheng & Abelson, 1987). In these experiments, the reconstituted U6 RNAs have a very low specific activity to allow unambiguous detection of spliceosomal complexes containing the high-specific-activity, ^{32}P-labeled actin pre-mRNA substrate in the standard assay. When the standard pre-mRNA substrate is added to wild-type yeast extract, several spliceosome intermediates are formed around pre-mRNA, and these have been characterized biochemically. The pre-mRNA substrate initially associates with U1•U2•U4-U6•U5 penta-snRNP or with U1 snRNP and U2•U4-U6•U5 tetra-snRNP (Stevens et al., 2002) to form the "commitment complex" in which the U1 snRNP has specifically bound to the pre-mRNA and has committed it to the splicing pathway (Séraphin & Rosbash, 1989; Du & Rosbash, 2001). Following the formation of the commitment complex, U2 snRNA binds to the branch sequence of the pre-mRNA substrate to form a complex that partially dissociates on nondenaturing gels to

become the B complex (using terminology adopted for yeast complexes; Cheng & Abelson, 1987). RNA and protein factors of the U4-U6•U5 tri-snRNP component specifically bind to the substrate, thereby producing the A2-1 complex, and this immature spliceosome undergoes further rearrangement, including dissociation of the U4-U6 base pairing, to form a complex of slower mobility, the A1 complex (Yean & Lin, 1991). A DEAH-box ATPase, Prp2, triggers another rearrangement to an intermediate of faster mobility, SS3, which is the fully assembled, or mature, spliceosome (Kim & Lin, 1996). The fully assembled spliceosome readily undergoes the first chemical step of splicing to form the A2-2 complex. This is followed by the A2-3 complex, which has undergone the second chemical step. The SS3 intermediate and the A2 complexes, including A2-1, A2-2, and A2-3, comigrate on the standard native gel. Spliceosomal complexes are identified by inspection of the native gel with reference to a wild-type control lane in which the fully assembled A1 complex appears at the top of the lane (Fig. 3). Because the A complexes are U6 dependent, they are readily identified via comparison with a U6-depleted control lane in which A complexes are absent or nearly so, and the B complex appears at the top of the lane (Fig. 3, lanes 2 and 3).

The spliceosome assembly assay for the splicing-defective helix I_a mutants is shown in Figure 3. The B complex is readily apparent in the U6-depleted extract (Fig. 3, lanes 2 and 3), and the A1 and A2 complexes are clearly resolved when wild-type extract is treated with EDTA to accumulate A2-1 and A1 complexes (Fig. 3, lane 9; Cheng & Abelson, 1987). Without EDTA treatment, the wild-type extract shows mostly A2 complexes (Fig. 3, lane 1), as the abundance of the A1 complex is often very low and difficult to discern. The three double mutations in U2-U6 helix I_a, A56C,U57C, A56C,U57G, and A56U,U57A, all show a striking accumulation of A1 complexes (Fig. 3, lanes 6–8) relative to reconstitution with wild-type U6 RNA (Fig. 3, lane 4). The triple mutant 55–57polyC did not assemble any appreciable amounts of A complexes (Fig. 3, lane 5) as expected, as we had found this triple mutant to be severely defective in U4-U6 snRNP assembly (see below). For all of the helix I_a mutations in Figure 3, the severity of the spliceosome assembly defects (i.e., accumulations of A1 and B complexes relative to the wild-type U6 control) correlates with the severity of the splicing defects observed (cf. Fig. 2, lanes 5–8, vs. Fig. 3, lanes 8–5, respectively). To confirm that the A1 complexes are appropriately identified and do not contain extraneous snRNPs, namely the U1 and U4 snRNPs (cf. Gottschalk et al., 2001), we repeated the experiment in Figure 3 with nonradiolabeled actin pre-mRNA and electroblotted the native gel to a membrane for sequential northern probing with U1, U4, U6, and actin pre-mRNA probes. The A1 complexes contained actin pre-mRNA and U6 snRNA but not U1 or U4 snRNA

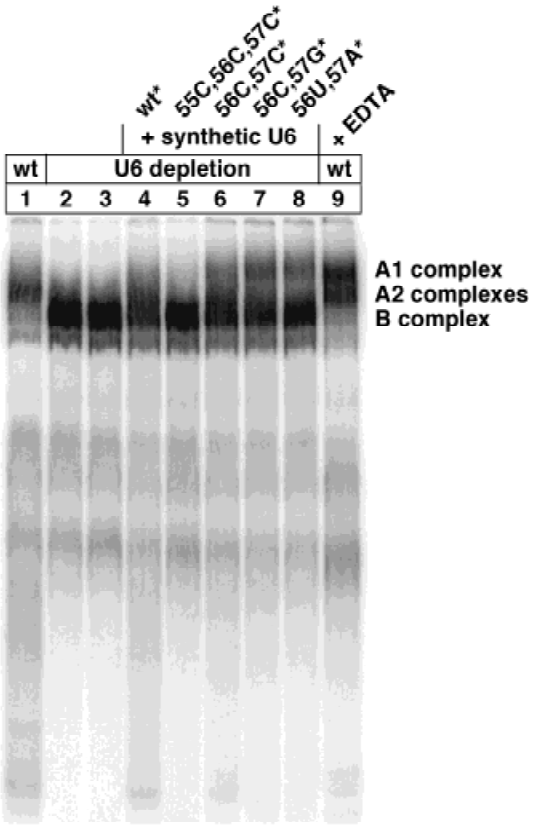

FIGURE 3. Spliceosome assembly of ^{32}P-labeled actin pre-mRNA substrate in U6-depleted yeast extract reconstituted with mutant and wild-type U6 RNAs. Mutations were introduced in the yeast U6 sequence of U2-U6 helix I_a (nt 54–58). Samples were prepared as in Figure 2 except that native complexes were separated on a non-denaturing 3% polyacrylamide (29:1) gel and synthetic U6 RNAs had a very low specific activity that did not interfere with observation of the high-specific-activity, ^{32}P-labeled actin pre-mRNA substrate. As a control, EDTA was added to a sample of yeast extract (2 mM EDTA final concentration) to block the splicing activity of A1 complexes and thus provide a visual marker for A1 and A2 complexes on the gel (Cheng & Abelson, 1987). In synthetic U6 RNAs marked with an ∗, the last four 5′-3′ linkages at the 3′ ends were racemic phosphorothioates.

(data not shown) as expected for normal A1 complexes as characterized previously (Cheng & Abelson, 1987).

The unusual accumulation of A1 complexes for the three U6 double mutations within the helix I_a sequence suggests that the presence of U2-U6 helix I_a is required for the progression of A1 spliceosomes to SS3 spliceosomes prior to the first chemical step of splicing. Growth defects for the same U6 double mutations had been rescued by compensatory mutations in U2 RNA in yeast, thus demonstrating that helix I_a exists, and various splicing defects were observed for these corresponding U2 double mutations in vivo (Madhani & Guthrie, 1992). However, to establish that helix I_a is required in A1 complexes and not at some other step that was rescued by compensatory mutations in vivo requires that we demonstrate a similar requirement for the U2 sequences of helix I_a in A1 complexes. Results that suggest that the U2 strand of helix I_a is required specifically

in A1 complexes were previously obtained in studies of the effects of U2 mutations on splicing (McPheeters & Abelson, 1992). Point deletions A27Δ and U28Δ in yeast U2 RNA hindered splicing by accumulating A1 complexes. These and the U6 double mutations mentioned above are the only snRNA mutations known to hinder splicing at this particular stage. U2 nt 27 and 28 are, in fact, base paired with U6 nt 56 and 57 in helix I_a. Therefore, the idea that U2-U6 helix I_a is required for the progression of A1 complexes to SS3 complexes, just prior to the first chemical step of splicing, is strongly supported by (1) the unusual requirement for U2 and U6 helix I_a nucleotides specifically in A1 complexes, (2) the importance of base pairing between these nucleotides as shown by compensatory mutations in vivo, and (3) the correlation between first-step splicing defects and growth defects in Madhani and Guthrie's (1992) compensatory mutational study of helix I_a. Thus, we obtained results that strongly suggest that U2-U6 helix I_a is required just before, and conceivably at, the first chemical step of splicing.

Extensive mutation of the 3′ stem-loop of yeast U6 snRNA

In previous studies of the role of the U6 3′ stem-loop in splicing, specific mutations in the upper portion of the human U6 stem-loop did not impede assembly of spliceosomes but hindered splicing, and this hindrance was relieved by making compensatory mutations to restore base pairing (Wolff & Bindereif, 1993). This result demonstrated that the upper half of the stem is important in active spliceosomes. Most point mutations that disrupt a base pair in the U6 3′ stem-loop showed little or no splicing effect (Fabrizio & Abelson, 1992; Wolff & Bindereif, 1993, 1995; McPheeters, 1996), except in the case of a sensitive splicing assay of the human 3′ stem-loop in which nearly every point disruption blocked splicing (Sun & Manley, 1997). In contrast, point mutations that hyperstabilize the 3′ stem-loop have a deleterious effect on splicing by interfering with the assembly of U6 snRNP into U4-U6 snRNP complexes (Madhani et al., 1990; Wolff & Bindereif, 1993, 1995; Fortner et al., 1994). If the 3′ stem-loop is not mutated to a deleterious hyperstabilization or disruption, it is tolerant of changes at nearly every position, especially those that maintain base pairing (Fabrizio & Abelson, 1992; Wolff & Bindereif, 1995; McPheeters, 1996; Sun & Manley, 1997). Similarly, the 3′ stem-loop of human U6atac snRNA of the U12-dependent spliceosomes can be functionally replaced in vivo by mutating it to either the human or yeast U6 3′ stem-loop sequence, thus introducing nine or eight mutations, respectively, and two or one fewer Watson–Crick base pairs, respectively, while adding one additional nucleotide to the bulged region (Shukla & Padgett, 2001).

As a follow-up to the previously reported point mutational studies for yeast U6, we tested whether the U6 3′ stem-loop is sensitive to multimutational disruptions using our in vitro splicing assay, and if so, whether a particular step of splicing is affected. The results are presented in Figure 4 and Table 2, part A. We found that in yeast extract, the two base paired regions of the 3′ stem-loop of U6 are fairly tolerant of extensive mutational disruption, but complete disruption of either region had a more severe effect (4–14% splicing yields for 68G,69G,70A and for 81C,82U,83U,84G mutations; Fig. 4, lanes 7 and 10, respectively). Interestingly, the most deleterious mutations were in the bulged region of the 3′ stem, especially the U80G and G81C mutations, which severely blocked splicing (Fig. 4, lanes 8 and 9). Also, the bulge-adjoining C68G,C69G disruption was nearly twofold more severe than the disruption that was 1 bp further away from the bulge, C69G,U70A (Fig. 4, cf. lanes 5 and 6). We conclude that mutations at the central bulge have a more deleterious effect than do multiple disruption mutations at other positions in the stem-loop.

To further investigate the deleterious effects of the G81C point mutation, we tested the splicing activity of all mutations of this nucleotide and found that the G81C mutation was the most deleterious of this group (Fig. 5, lanes 6–8; Table 2, part B). Within the C66-G81 base pair, mutations C66G and C66U had weaker effects on splicing (Fig. 5, lanes 4 and 5) than the opposing G81C, G81U, and G81A point mutations (Fig. 5, lanes 6–8).

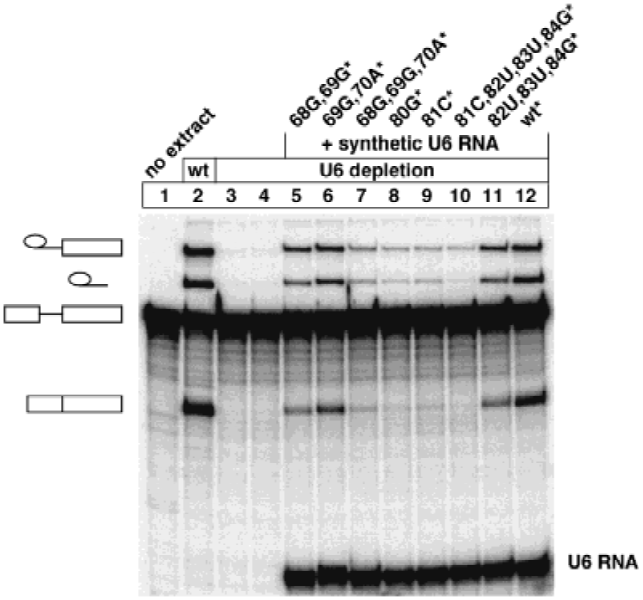

FIGURE 4. Splicing of ^{32}P-labeled actin pre-mRNA in U6-depleted yeast extract reconstituted with mutant and wild-type U6 RNAs. U6 mutations were introduced in the 3′ stem-loop (nt 62–85) in yeast U6 RNA. Samples were prepared as in Figure 2. In synthetic U6 RNAs marked with an ∗, the last four 5′-3′ linkages at the 3′ ends were racemic phosphorothioates.

TABLE 2. Splicing yields for mutations in the U6 3′ stem-loop and U2-U6 helix I_b sequence of U6 RNA in yeast extract.

U6 RNA mutations[a]	Relative splicing yield[b]
Part A	
C68G,C69G	34%
C69G,U70A	60%
C68G,C69G,U70A	14%
U80G	5%
G81C	8%
G81C,A82U,A83U,C84G	4%
A82U,A83U,C84G	42%
Wild type	100%
Part B	
C66G	73%
C66U	66%
G81C	5%
G81U	38%
G81A	57%
C66G,G81C	6%
C66G,G81U	23%
C66U,G81A	29%
Wild type	100%
Part C	
G60C	2%
G60U	2%
C61G	10%
G86C	127%
U87G	122%
G60C,U87G	0.1%
G60U,U87G	1%
C61G,G86C	3%
Wild type	100%

[a]The last four 5′-3′ linkages at the 3′ terminus are racemic phosphorothioates.

[b]Splicing yields for reconstituted, synthetic U6 RNAs are normalized relative to the yield for reconstituted, synthetic wild-type U6 RNA as a control.

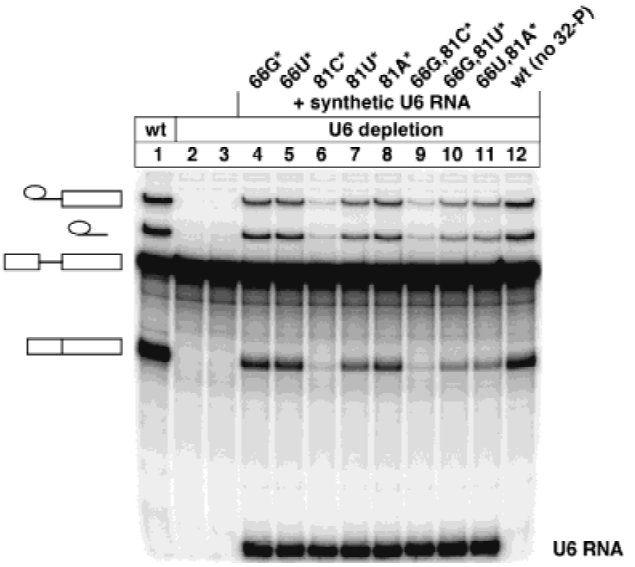

FIGURE 5. Splicing of ^{32}P-labeled actin pre-mRNA in U6-depleted yeast extract reconstituted with mutant and wild-type U6 RNAs. U6 mutations were introduced at a base pair in the bulged region of this stem-loop (nt 66 and 81) in yeast U6 RNA. Samples were prepared as in Figure 2. In synthetic U6 RNAs marked with an ∗, the last four 5′-3′ linkages at the 3′ ends were racemic phosphorothioates. The wild-type U6 RNA was not ^{32}P-labeled in this experiment.

Surprisingly, the C66U-G81 wobble pairing (Fig. 5, lane 5) was reproducibly more deleterious than the C66G-G81 mismatch (Fig. 5, lane 4), demonstrating that disruption of the C66-G81 base pair is not the primary cause of the G81C splicing defect. Furthermore, restoration of base pairing for the G81C mutation did not suppress its splicing defect (C66G,G81C; Fig. 5, lane 9; Table 2, part B), and restoration of base pairing for the G81U and G81A mutations exacerbated their splicing defects (C66G,G81U and C66U,G81A; Fig. 5, lanes 10 and 11) to an extent consistent with the additive effects of the individual point mutations (Fig. 5, lanes 4, 5, 7, and 8; Table 2, part B). These results suggest that the G81C mutation activates a deleterious interaction with the 3′ stem-loop in an allele-specific manner that does not correlate with mutational base-pair disruption in the stem-loop.

We analyzed the U4-U6•U5 tri-snRNP assembly profiles for the U6 G81C and U80G point mutations as well as their abilities to bind to Prp24 and Lsm proteins. These results are presented in Figure 6A and Table 3.

It was known from previous work that U80G elicits a splicing defect by introducing an extraneous G–C pair in the bulge of the U6 3′ stem-loop, thereby hyperstabilizing the stem-loop and interfering with its unwinding, which is necessary for U4-U6 base pairing during spliceosome assembly. As a result of this effect, an accumulation of free U6 snRNP was observed on glycerol gradients for the U80G mutation (Madhani et al., 1990). Accordingly, we observed aberrantly high levels of free U6 snRNP for this mutation on a native gel (Fig. 6A, lane 5; Fig. 6B, lane 4). Similarly, the U6 G81C mutation caused a striking accumulation of free U6 snRNP on the native gels (Fig. 6A, lane 6; Fig. 6B, lane 5), but with a somewhat faster mobility than for the free U6.U80G snRNP. (The reason for this mobility difference is not known.) Northern blot analysis of the native gel, which was repeated with nonradiolabeled U80G and G81C U6 RNA and probed sequentially with radiolabeled U4, U5, and U6 probes, positively identified both accumulated bands as mutant free U6 snRNPs (data not shown). Consistent with these accumulations of free U6 snRNPs, we found that the U80G and G81C mutations both showed aberrantly high levels of the mutant U6 RNA bound to Prp24 and Lsm proteins (Table 3).

A reported study of compensatory mutations in human U2 and U6 snRNPs provided evidence for a U6 3′ stem-loop extension that is mutually exclusive with most of U2-U6 helix I_b (Sun & Manley, 1995). Furthermore, mutational analysis failed to provide support for the existence of helix I_b in human cells. However, for splice-

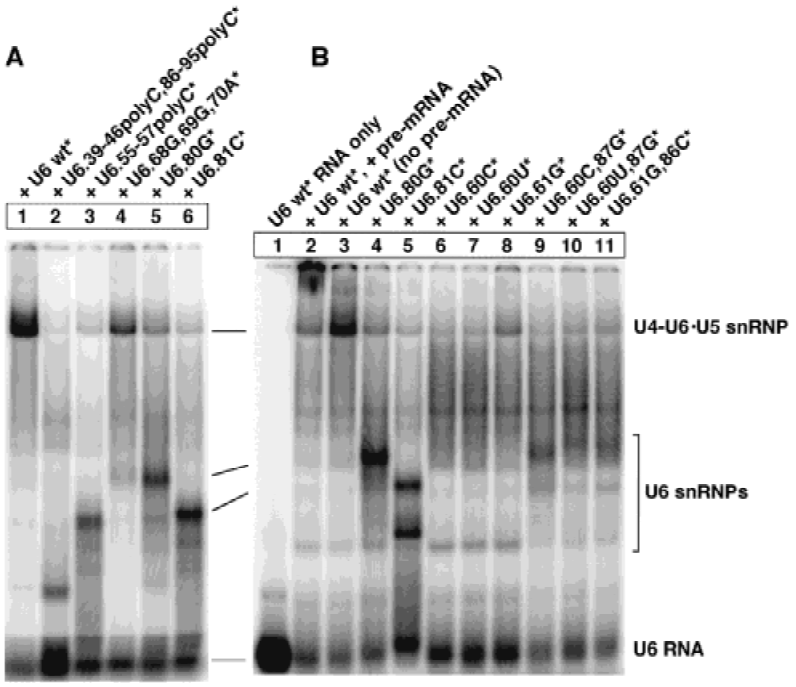

FIGURE 6. A: U4-U6•U5 snRNP assembly of U6-depleted yeast extract reconstituted with mutant and wild-type ^{32}P-labeled U6 RNAs. U6 mutations were introduced in the telestem sequences (nt 36–43 and 86–95) and the 3′ stem-loop (nt 62–85) of yeast U6 RNA and in the U6 sequence of U2-U6 helix I_a (nt 54–58). Yeast splicing extract was treated with d1 oligonucleotide to digest endogenous U6 snRNA as described in Figure 2. Aliquots of the U6-depleted extract were reconstituted by addition of synthetic, ^{32}P-labeled U6 RNA as indicated. Samples were incubated at 23 °C for 20 min and then loaded onto a nondenaturing 4% polyacrylamide (79:1) gel for separation of the U6 snRNP-containing complexes as well as free U6 RNAs. **B:** U4-U6•U5 snRNP assembly of U6-depleted yeast extract reconstituted with mutant and wild-type ^{32}P-labeled U6 RNAs. U6 mutations were introduced in the bulged region of the 3′ stem-loop (nt 80 and 81) of yeast U6 RNA and in the U6 sequence of U2-U6 helix I_b (nt 59–61). Samples were prepared as in **A**. In synthetic U6 RNAs marked with an ∗, the last four 5′-3′ linkages at the 3′ ends were racemic phosphorothioates.

osomes in yeast and for U12-dependent spliceosomes in HeLa extract, the existence of U2-U6 helix I_b or its equivalent is strongly supported by compensatory mutational studies in yeast and by psoralen crosslinking in HeLa extract, respectively (Madhani & Guthrie, 1992; Madhani, 1993; Tarn & Steitz, 1996). We were interested in knowing whether the splicing defects for point mutations G60C and C61G discovered in yeast U6 (Fabrizio & Abelson, 1990; Madhani et al., 1990) might be suppressed by introducing compensatory mutations at U6 nt G86 and U87, thereby extending base pairing of the 3′ stem-loop of yeast U6 (Fig. 1C), which could be analogous to the 3′ stem-loop extension found in human U6 RNA. Perhaps both U2-U6 helix I_b and the U6 3′ stem-loop extension, which are mutually exclusive interactions, are important at different times in the splicing cycle. This hypothesis was also suggested by the severity of the U6 point mutations G60C and C60U, which have a much more deleterious effect on splicing than point mutations of their corresponding U2 base pairing partner, C22, in helix I_b.

We observed the same splicing defects for yeast U6 RNA mutations G60C, G60U, or C61G (Table 2, part C) as previously reported (Fabrizio & Abelson, 1990), and

TABLE 3. Data summary for mutations in the 3′ stem-loop and 3′ terminal domain of yeast U6 RNA.

U6 RNA mutations	Splicing yields[a]	U4-U6•U5 yield[b]	Polyoma-Lsm 4 I.P.[c]	Prp 24(HA)$_3$ I.P.[d]
U80G	5%	23%, 23%[e,f]	280%	940%, 900%[f]
G81C	8%, 5%[f]	9%, 6%[e,f]	270%	690%, 630%[f]
87–89polyC[g]	102%, 114%[f]	105%[g]	42%	140%[g]
Wild type[h]	100%	100%	100%	100%

[a] Splicing yields for reconstituted, synthetic U6 RNAs are normalized relative to the yield for reconstituted, synthetic wild-type U6 RNA as a control.
[b] Yield of U4-U6•U5 tri-snRNP assembled from reconstituted U6 RNA, separated by native gel electrophoresis and normalized relative to the U6 wild-type control.
[c] Relative amount of reconstituted U6 RNA coimmunoprecipitated on anti-polyoma protein G-Sepharose beads, normalized relative to the U6 wild-type control.
[d] Relative amount of reconstituted U6 RNA coimmunoprecipitated with 12CA5 antibodies on protein A-Sepharose beads, normalized relative to the U6 wild-type control.
[e] U4/U6-U5 tri-snRNP assembly gel showed aberrantly high levels of free U6 snRNP relative to the U6 wild-type control.
[f] Yield in a duplicate experiment.
[g] Average of three or more trials.
[h] The last four 5′-3′ linkages at the 3′ terminus are racemic phosphorothioates.

we further found that these point mutations caused accumulations of the naked mutant U6 RNAs on native polyacrylamide gels (Fig. 6B, lanes 6–8) as well as accumulations of poorly resolved snRNPs in the region of U4-U6 snRNP mobility on these gels (Raghunathan & Guthrie, 1998). Mutation of nt G86 and U87 individually to G86C or U87G did not hinder splicing, in sharp contrast to the opposing G60 and C61 mutations; rather, their splicing activities were unusually enhanced relative to wild-type U6 RNA (Table 2, part C, G86C and U87G). Similarly, we found that mutation of an overlapping sequence, 87–89polyC, also caused a modest but unusual enhancement of in vitro splicing activity (Table 3; see also Fig. 4A, lane 7, of Ryan et al., 2002). When the point mutation of G60 or C61 was combined with the potentially base pairing mutation of U87 or G86, respectively, rather than finding suppression of the splicing defects of G60C, G60U, or C61G, we found a small but detectable exacerbation of these splicing defects (Table 2, part C). We also note that rather than observing accumulations of the naked U6 RNAs on the native gel as was found for the G60 and C61 point mutations, we observed modest accumulations of free U6 snRNPs that migrated close to hyperstabilized U6.U80G snRNP (Fig. 6B, lanes 9–11). This suggests that the potentially base-pairing mutations at the base of the U6 3′ stem-loop extended and hyperstabilized the stem-loop and thus hyperstabilized the mutant free U6 snRNPs. These putatively hyperstabilized U6 snRNPs were less active in splicing than expected from the individual point mutations (Table 2, part C), further suggesting that the mutant 3′ stem-loops are likely extended and hyperstabilized. If so, these stem-loop extensions are expected to be deleterious to the splicing pathway, as are other mutations that hyperstabilize the 3′ stem-loop of yeast U6 RNA, for example, point mutations A62G, C67A, A79G, and U80G (Fabrizio & Abelson, 1990; Madhani et al., 1990; Fortner et al., 1994; McPheeters, 1996).

DISCUSSION

U2-U6 helix I_a is required in fully assembled yeast spliceosomes

The formation of U2-U6 helix I was proposed to be important for bringing together the U6-5′ splice site and U2-branchpoint regions during spliceosome assembly for catalytic activation of the first chemical step of splicing (Madhani & Guthrie, 1992). U2-U6 helix I_a forms in both yeast and mammalian spliceosomes (Madhani & Guthrie, 1992; Sun & Manley, 1995) and is conserved in U12-dependent spliceosomes (Tarn & Steitz, 1996). Mutations in U2 and U6 nucleotides around the bulge in helix I are lethal in yeast (Madhani & Guthrie 1992, 1994; McPheeters, 1996), and several point mutations specifically blocked the second chemical step of splicing in yeast extracts (Fabrizio & Abelson, 1990; McPheeters & Abelson, 1992). Similar mutational effects were observed in HeLa nuclear extract (Wolff et al., 1994), although some of the same mutations showed no splicing defects in HeLa cells or in *Xenopus* oocytes (Vankan et al., 1992; Datta & Weiner, 1993). The growth defects in yeast were suppressed by incorporating compensatory mutations to restore base pairing around the bulge in helix I (Madhani & Guthrie, 1992, 1994). Whereas U6 nt 58 and 59 at the bulge are required for the second chemical step, the adjacent U6 nt 60 and 61 in helix I_b were shown to be important for spliceosome assembly (Fabrizio & Abelson, 1990). Curiously, the U2 nucleotides that are base paired with U6 nt 59 and 60 in helix I_b have markedly different mutational effects on splicing than their U6 partners. The mutational effects of U2 nt 22 and 23 are generally weaker than those of their U6 counterparts, and mutations of U2 nt 23 can produce first-step effects or not, depending on the particular pre-mRNA substrate (Madhani & Guthrie, 1992, 1994). This asymmetry of mutational effects implies that the chemical features of U6 nucleotides A59 and G60 of helix I_b are more important for splicing than is base pairing within helix I_b.

In the minor class of U12-dependent spliceosomes, U6atac is predicted to base pair with U12 to form a helix analogous to U2-U6 helix I, based on sequence similarities and on psoralen crosslinking studies (Tarn & Steitz, 1996; Frilander & Steitz, 2001). However, the unusually far-reaching pattern of crosslinking sites in and around U12-U6atac helix I_a in the psoralen studies raises questions about whether these crosslinks were formed within a normal helical structure or in a differently arranged binding pocket for psoralen. Clearly the two strands of U12-U6atac helix I_a were near each other at the moment of crosslinking, but these crosslinks may have formed in a precursor or successor of helix I_a in which the U12 and U6atac strands were held in proximity, perhaps by binding to a common protein or protein cluster, without being base paired. Our findings about the importance of U2-U6 helix I_a in spliceosome assembly most likely apply to the U12-dependent spliceosomes as well, but additional studies are necessary to confirm this.

Our results demonstrate that mutational disruptions of helix I_a in yeast spliceosomes block spliceosome assembly within A1 complexes, at or near the Prp2 step, which occurs at a late stage of assembly and just prior to the first chemical step of splicing. As mentioned, these helix I_a disruptions (at U6 nt 56 and 57) were found by Madhani and Guthrie (1992) to cause growth defects that were suppressed by restoring helix I_a base pairing. We observed that these growth defects correlate best with their reported first-step splicing defects for splicing of U3A/U3B pre-mRNA in vivo. Our in vitro splicing results for the same U6 helix I_a mutations correlate with these reported growth defects: Two

of the mutants (A56U,U57A and A56C,U57G) showed more severe growth defects than the third (A56C,U57C), and we observed a parallel correlation for the severity of their splicing defects in vitro. The only other snRNA mutations known to block spliceosome assembly at the stage of A1 complexes are point deletions of either nt 27 or 28 in U2 snRNA (McPheeters & Abelson, 1992), and these nucleotides are in fact base paired with U6 nt 56 and 57 in helix I_a. Therefore, mutations and point deletions within the central U2-U6 base pairs of helix I_a inhibit spliceosome assembly by inhibiting A1 complexes from progressing to SS3 complexes. It is now rather clear that helix I_a is required for the progression of A1 to SS3 complexes, just prior to the first chemical step of splicing. To prove this point, however, it is necessary to show that the first-step splicing defects of helix I_a U6 mutations (our data) or U2 mutations (Madhani & Guthrie, 1992), rather than their growth defects, are specifically suppressed by introducing compensatory mutations that restore helix I_a base pairing. Nonetheless, our results strongly suggest that the presence of U2-U6 helix I_a is important at a late stage of spliceosome assembly, presumably to help bring together the U6-5′ splice site and U2-branchpoint regions that carry the reactive nucleotides for the first chemical step of splicing.

Although our data show that helix I_a is not required until the progression of A1 to SS3 spliceosomes, it is entirely possible that the helix actually forms before this stage. The requirement for helix I_a in the A1 to SS3 progression suggests that the formation of helix I_a may be catalyzed by Prp2 protein, a member of the DEAH-box family of ssRNA-stimulated ATPases (Kim et al., 1992). Prp2 protein makes direct contact with the pre-mRNA substrate in A1 complexes (Teigelkamp et al., 1994), and it remodels A1 complexes (also called SS2 complexes) to faster mobility SS3 intermediates, which are poised for the first chemical step of splicing (Kim & Lin, 1996). In an alternative suggestion, the requirement for helix I_a at or near the Prp2 step could imply that helix I_a must be present to allow Prp2 activity, perhaps even to be a substrate for Prp2 protein. More experiments are needed to sort out whether there is a direct connection between helix I_a and Prp2 or not. This question is a salient one because it has been known for some time that Prp2 mediates a dramatic rearrangement of the spliceosome to initiate the first chemical step (Cheng & Abelson, 1987; Lin et al., 1987; Prp2 was formerly called Rna2), yet the structural nature of this rearrangement and its relationship to the catalytic activation of splicing are still unknown.

Mutational analysis of the 3′ intramolecular stem-loop of yeast U6 RNA

One of the difficulties encountered when studying mutational effects in the 3′ stem-loop of U6 RNA (nt 63–84 in yeast) relates to the fact that the 3′ stem-loop is present in free U6 snRNPs, is unwound and base-paired with U4 snRNA in U4-U6 snRNPs, and is formed again during spliceosome assembly. Several studies have shown that mutations in the 3′ stem-loop can have a deleterious effect on splicing. In both yeast and mammalian assays of 3′ stem-loop mutations at 18–30 °C, mutations that hyperstabilize the 3′ stem-loop were found to inhibit U4-U6 snRNP assembly (and thus splicing) whereas point disruptions were generally well tolerated (Madhani et al., 1990; Fabrizio & Abelson, 1992; Wolff & Bindereif, 1993, 1995; Fortner et al., 1994; McPheeters, 1996). Conversely, the opposite was found to be the case in mammalian cells at 37 °C, that is, hyperstabilizing mutations were well tolerated for splicing whereas point disruptions were generally deleterious (Sun & Manley, 1997). However, it is not clear whether these point disruptions interfered with U4-U6 snRNP assembly or a later stage of spliceosome assembly or splicing. It is clear, however, that the U6 3′ stem-loop requires the proper balance of stability in order to function properly in splicing. Hyperstabilizing the 3′ stem-loop can be inhibitory at lower temperatures (e.g., in yeast at 18 °C) whereas destabilization can be deleterious at higher temperatures (e.g., in mammalian cells at 37 °C). The U6 3′ stem-loop was also found to be quite tolerant of mutational changes, including transverted base pairs, at several positions tested. Aside from the importance of disrupting the 3′ stem-loop for U4-U6 snRNP assembly, the 3′ stem-loop is functional in fully assembled spliceosomes, according to two mutational studies. In one of these studies, mutations in the upper portion of the U6 3′ stem-loop did not inhibit spliceosome assembly in HeLa extract but did inhibit splicing (Wolff & Bindereif, 1993). This inhibition was alleviated by incorporating compensatory mutations to restore base pairing in the 3′ stem-loop, thus demonstrating the existence and functional importance of at least the upper portion of the U6 3′ stem-loop in active spliceosomes. In another study, a genetic screen for yeast U6 mutations that enhanced the use of nonadenosine branchsites in splicing found several suppressor mutations among the lower base pairs of the U6 3′ stem-loop (McPheeters, 1996). These results corroborate the importance of the 3′ stem-loop in active spliceosomes.

In our experiments, we have made more extensive disruption mutations in the 3′ stem-loop than in previous studies. Mutations in the upper portion of the stem-loop are expected to affect U4-U6 snRNP assembly as this region base pairs with U4 RNA during assembly; however, mutations downstream of U80 in the 3′ strand of the lower U6 stem-loop are outside of the U4-U6 pairing region. We were surprised to find that nearly complete mutational disruptions of either the upper or lower parts of the U6 3′ stem-loop were fairly well tolerated in splicing assays (34–60% relative splicing

yields). However, the point mutation G81C strongly blocked splicing in vitro, consistent with previous observations for this mutation in the context of other 3′ terminal domain mutations (Fabrizio et al., 1989). We note that the bulge-adjacent guanosine equivalent to G81 is conserved in the U6atac 3′ stem-loop (Tarn & Steitz, 1996). The severity of the G81C splicing defect in our experiments was allele specific and was not due to disruption of the C66-G81 base pair in general. By an unknown mechanism, the G81C mutation causes a substantial accumulation of free U6 snRNP that is deficient in assembling further into U4-U6 snRNPs.

At the base of the yeast U6 3′ stem-loop is a trinucleotide sequence, nt 59–61, that is usually represented as base paired with U2 RNA in U2-U6 helix I_b. The helix I bulge-adjoining base pair of helix I_b (U6.A59-U2.U23) was shown to exist by compensatory mutational analysis (Madhani & Guthrie, 1992), and similar evidence for the U6.C61-U2.G21 base pair of helix I_b came subsequently (Madhani, 1993). However, it has been known that double mutational disruption of the two helix I_b base pairs involving U2 nt 21 and 22 has very little effect on splicing in vitro (McPheeters & Abelson, 1992). Mutations of the U6 nucleotides of these two base pairs were far more deleterious for splicing than were mutations of the complementary U2 nucleotides. This asymmetry of mutational effects suggested that alternative base pairings might occur for these U6 nucleotides at different stages of splicing, perhaps including a base pairing analogous to that found for the human U6 3′ stem-loop (Sun & Manley, 1995). In human cells, compensatory base pair analysis showed no evidence for the two bulge-distal base pairs of helix I_b, rather both U6 nucleotides of this region were found to be base paired to U6 nucleotides across the bottom of the U6 3′ stem-loop, thereby extending the human U6 3′ stem-loop at its base. In yeast U6 RNA, we found no analogous compensatory suppression of the splicing defects of G60U or C61G by introducing U87G or G86C, respectively, to extend base pairing at the bottom of the U6 3′ stem-loop (Table 2, part C). Rather, these extensions of the 3′ stem-loop produced an unusual accumulation of the mutant, free U6 snRNP that comigrated on native gels near U6.U80G snRNP, which is defective in splicing due to hyperstabilization of its 3′ stem-loop. This observation suggests that extension of the yeast U6 3′ stem-loop, as found in human U6, is inhibitory for splicing in yeast due to 3′ stem-loop hyperstabilization in free U6 snRNPs. As the U6 3′ stem-loop requires the proper balance of stability to function properly in splicing, the difference in stability between the yeast and human 3′ stem-loops may correlate with the different optimal growth temperatures for yeast and human cells. Surprisingly, the U87G or G86C mutation alone caused a small but reproducible enhancement of splicing (Table 2, part C), as we had observed for the comparable 87–89polyC mutation (Table 3). Such enhancement of in vitro splicing is unusual and suggests that these nucleotides can be involved in inhibitory interactions in vitro when the nucleotides are wild type. These inhibitory interactions could include extensions of the U6 3′ stem-loop in yeast. We conclude that mutations of nt 86 and 87 can alleviate mildly inhibitory interactions of these nucleotides in fully wild-type yeast spliceosomes in vitro.

Hypothesis for the function of yeast U6 snRNA in spliceosomes

All of our current data, including that presented in our companion report (Ryan et al., 2002), are consistent with the idea that U6 snRNA primarily catalyzes splicing by helping to fold the pre-mRNA into a reactive conformation while presenting a few essential U6 phosphate groups in a productive three-dimensional arrangement to effect catalysis via metal ions (Steitz & Steitz, 1993; Sontheimer et al., 1997). Recently, incorporation of a nonbridging (S_P)-phosphorothioate at the A79-U80 linkage in U6 RNA was found to block splicing at precisely the stage of the first chemical step (Yean et al., 2000). This phosphorothioate block was substantially alleviated for the first chemical step by adding thiophilic divalent metal ions, Mn^{2+} or Cd^{2+}. Therefore, in yeast U6 RNA, the pro-S_P phosphoryl oxygen of U80 binds a divalent metal ion that is important for catalysis of the first chemical step of splicing. A tantalizing possibility is that U6 RNA may bind this divalent ion in the active site of the spliceosome to directly catalyze the splicing reaction. Related to this, it has been known that two or three pro-R_P phosphoryl oxygens of U6 are required for the first chemical step of splicing, and two other such pro-R_P oxygens of U6 are required for the second chemical step (Fabrizio & Abelson, 1992; Yu et al., 1995). It is possible that some of these U6 phosphate groups, and perhaps others as yet unidentified in U6 and/or U2 snRNA, present metal ions in the active site to help mediate the chemical events of splicing.

We recently found no individual 2′-hydroxyl group in the conserved central domain of U6 RNA to be required for splicing under normal conditions in vitro (Kim et al., 1999). Only one nucleotide in yeast U6 is intolerant of every mutation, A51, and this second-step-specific adenosine is attached to a phosphate group required for the second chemical step. In human U6, the adenosine equivalent to A51 is mildly affected by an A-to-G mutation specifically, suggesting that this position requires a purine (Wolff et al., 1994). Dominant-negative growth defects of A51 mutations were recently found to be suppressed by mutations in Prp8, a highly conserved and important spliceosomal protein that interacts with both 5′ and 3′ splice sites (Collins & Guthrie, 1999; Siatecka et al., 1999). Most of the mutationally sensitive yeast U6 nucleotides are clustered near the essential U6 phosphate groups. Second-step-specific

nucleotides are clustered in two areas near the essential second-step phosphate groups. We found no evidence of any additional second-step mutational effects in any of the yeast U6 helices. Perhaps most of the known mutational sensitivities of U6 nucleotides are related to their roles in maintaining the active conformations of the essential phosphate groups for presentation of catalytic metal ions to the pre-mRNA substrate.

A recent finding that specific mutations of U57 of U6 increase the usage of aberrant 3′ splice sites as well as aberrant branchsites (Chang & McPheeters, 2000) could result from the fact that U57 is adjacent to the essential, pro-R_P phosphoryl oxygens located between nt 58, 59, and 60 of yeast U6 RNA. It is possible that specific mutations of U57 perturb the usual conformations of these essential phosphate groups and that U57 itself is not an essential component of the active site, especially considering that point mutations of U57 have very little effect on splicing activity (Fabrizio & Abelson, 1990; McPheeters, 1996). Although previous attempts failed to demonstrate that the essential pro-R_P phosphoryl oxygens of U6 interact with catalytic metal ions (Fabrizio & Abelson, 1992; Yean et al., 2000; our unpubl. data using Cd^{2+}), it was recently demonstrated that a successful metal rescue experiment requires a kinetic system in which the rate-limiting step depends on metal ion catalysis rather than on a conformational change (Sontheimer et al., 1999). Further knowledge about spliceosomal structures, their functional elements and rearrangements, and the kinetics of the multistep process will help to elucidate the dynamic role of U6 snRNA in pre-mRNA splicing.

MATERIALS AND METHODS

Synthesis of U6 RNAs for in vitro experiments

U6 RNA oligonucleotides were chemically synthesized on an Applied Biosystems synthesizer model 380A or were commercially obtained from Dharmacon Research, Inc. For in-house syntheses, nucleotide phosphoramidites were commercially obtained from Glen Research. Full-length U6 RNA was synthesized in four pieces: nt 1–38, 39–59, 60–79, and 80–112. U6 DNA oligonucleotides were synthesized to be complementary to U6 RNA nt 22–112 for annealing of U6 RNA pieces. All synthetic RNA and DNA oligonucleotides were purified via denaturing polyacrylamide gel electrophoresis. Synthetic U6 RNA oligonucleotides require a 5′ terminal monophosphate group for RNA ligation. The oligonucleotide pieces of each U6 RNA, except for the 5′ piece, were 5′-phosphorylated as a group (5–10 pmol of each piece) using 5 U of T4 polynucleotide kinase (NEB), 50 μM ATP, and $\sim$50 μCi of [γ-^{32}P]ATP (Amersham or ICN) in the supplied enzyme buffer (NEB) at 39 °C for 1 h. The phosphorylated RNA mixture was quenched with 2.5 mole-equivalent of EDTA per mole of MgCl and phenol extracted. To the aqueous phase was added the 5′ piece of U6 RNA and the complementary U6 DNA oligonucleotide. Total nucleic acid was precipitated by adding mussel glycogen carrier (Boehringer Mannheim), 0.25 vol. of 8 M ammonium acetate and 3 vol. of ethanol. The precipitated nucleic acid was dissolved in 20 mM Tris-HCl, pH 8, 1 mM EDTA solution, and then the U6 RNA pieces were annealed to the complementary U6 DNA oligonucleotide in situ, and the annealed U6 RNA pieces were ligated by T4 DNA ligase (Amersham, 5U/μL stock), all as described by Moore and Sharp (1992), except that the ligation mixture was $\sim$0.5 μM for each RNA and DNA oligonucleotide (0.95 mole-equiv. of DNA).

Reconstitution of U6 snRNA in yeast extracts and splicing activity assay

Yeast whole-cell extract was prepared according to Lin et al. (1985) for "wild type" extract, strain EJ101. Epitope-tagged Prp24$(HA)_3$ and polyoma-Lsm4 yeast extracts were prepared according to Ansari and Schwer (1995) using a mortar and pestle in a liquid N_2 bath and adding protease inhibitors to the liquid N_2-filled mortar prior to pulverizing together with the frozen yeast cell–buffer suspension (1 mM PMSF, 10 μg/mL leupeptin, and 1 μg/mL pepstatin final concentration). We also used KOAc, pH 7.9, instead of KCl in the extract buffer. Extracts were depleted of endogenous U6 snRNA according to Fabrizio et al. (1989) by incubating the extract with an optimized amount of d1 oligonucleotide (typically 0.08–0.3 μM) at 33–34 °C for 25 min, then put on ice. To reconstitute the extract with synthetic U6 RNA, the U6-depleted extract was divided into 4-μL samples, and 1 μL of ligated U6 RNA (2–20 nM, in TE, pH 7.5) was added to each. These levels of added U6 RNA are well above saturating levels for achieving maximal splicing (Fabrizio et al., 1989; our unpubl. results), and the presence of excess, naked U6 RNA is routinely visible in such saturated samples, as observed on nondenaturing gels, regardless of the presence of any U6 mutations. To assay the splicing activity in the U6-reconstituted extracts, [5′-^{32}P]uridine-labeled actin pre-mRNA (1 μL of 2 nM actin in 2$\times$ splicing buffer) was added to each sample and incubated at 23 °C for 30 min. The reactions were terminated by digestion and extraction of proteins as described by Lin et al. (1985). The radiolabeled pre-mRNA splicing products were separated on a 7% denaturing polyacrylamide (29:1) gel (7 M urea, 1$\times$ TBE) to assay the formation of splicing intermediates and products. The sum of the levels of splicing intermediates and products (determined by PhosphorImager analysis with proper background correction) constitute the yield that is normalized relative to the yield for synthetic wild-type U6 RNA control.

Spliceosome complex formation

To assay the assembly of spliceosomes in vitro, nondenaturing polyacrylamide gel electrophoresis was performed as described by Cheng and Abelson (1987). After the 30-min splicing reaction (as above), 1 μL of heparin (8 μg/μL) and 1 μL of nondenaturing gel loading buffer (25% glycerol, 0.25% xylene cyanole) were added per 2 μL of yeast extract stock in each splicing sample, and these were loaded directly onto the nondenaturing 3% polyacrylamide (30:1) gel (18.5 $\times$ 24 $\times$ 0.8 cm). Electrophoresis was conducted at 200 V for 6.5 h at $\sim$4 °C with recirculating buffer.

U4-U6•U5 tri-snRNP assembly assay

To assay the assembly of U4-U6•U5 tri-snRNPs in vitro, nondenaturing polyacrylamide gel electrophoresis was performed as described by Raghunathan and Guthrie (1998). We prepared high-specific-activity, synthetic U6 RNAs as above, except that one internal oligonucleotide piece of U6 was 5′-phosphorylated with 4 mole-equivalent of [γ-^{32}P]ATP (>7,000 Ci/mmol; ICN) and no cold ATP, whereas the other internal ligation sites were 5′-phosphorylated with 1 mM ATP before combining the oligonucleotide pieces of U6 RNA during phenol extraction. Wild-type yeast extract was reconstituted with the high-specific-activity, ^{32}P-labeled U6 RNA as above and incubated at 23 °C for 20 min, then put on ice.

ACKNOWLEDGMENTS

We thank present and past members of the Abelson and Guthrie laboratories for helpful discussions and Scott Stevens, Tracy Johnson, Stephen Rader, Jon Staley, and R.J. Lin for comments on the manuscript. This work was supported by a National Institutes of Health grant GM32637 to J.A. and by a Caltech Division of Biology Fellowship to D.E.R.

Received April 22, 2002; returned for revision May 17, 2002; revised manuscript received June 10, 2002

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