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A Novel Base-Pairing Interaction between U2 and U6 snRNAs Suggests a Mechanism for the Catalytic Activation of the Spliceosome

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Summary

Prior to the chemical steps of mRNA splicing, the extensive base-pairing interaction between the U4 and U6 spliceosomal snRNAs is disrupted. Here, we use a mutational analysis in yeast to demonstrate a conserved base-pairing interaction between the U6 and U2 snRNAs that is mutually exclusive with the U4–U6 interaction. In this novel pairing, conserved sequences in U6 interact with a sequence in U2 that is immediately upstream of the branch point recognition region. Remarkably, the residues in U6 that can be consequently juxtaposed with the intron substrate include those that have been proposed previously to be catalytic. Both the first and second steps of splicing are inhibited when this base-paired structure is mutated. These observations, together with the high conservation of the U2–U6 structure, lead us to propose that it might be a component of the spliceosomal active site.

Introduction

The chemical pathway that accomplishes the removal of introns from nuclear messenger RNA (mRNA) precursors involves two sequential transesterifications (for reviews see Green, 1991; Guthrie, 1991). Cleavage at the 5′ splice site is accompanied by the formation of a 5′–2′ phosphodiester linkage between the 5′ end of the intron and an adenosine in the intron. In the second step of the reaction, the 3′ splice site is cleaved with the concomitant formation of the ligated exons and the excision of the intron as a lariat stucture. Since this chemistry is shared by group II self-splicing introns, it has been suggested that nuclear mRNA splicing is fundamentally an RNA-catalyzed process (Sharp, 1985, 1991; Cech, 1986).

Much attention has been devoted to the five evolutionarily conserved small nuclear RNAs (U1, U2, U4, U5, and U6 snRNAs) that are required for nuclear mRNA splicing (for reviews see Green, 1991; Guthrie, 1991), partly because of their potential role as direct mediators of the reaction. Packaged by proteins into small nuclear ribonucleoprotein particles (snRNPs), the RNAs assemble onto an intron-containing substrate in an ordered pathway to form the spliceosome, in which the chemical steps of the reaction take place. In yeast and mammals, recognition of the 5′ splice site and intron branch point are mediated in part through Watson–Crick base pairing with U1 and U2 snRNAs, respectively (for reviews see Green, 1991; Guthrie, 1991). Recent studies suggest that, like U1 and U2, U5 also interacts with the pre-mRNA, in this case with the 5′ and 3′ exons (Newman and Norman, 1991, 1992).

In contrast with U1, U2, and U5, little is known about the specific functions of the U4–U6 snRNP. These RNAs are found base paired to each other, forming a single particle (Bringmann et al., 1984; Hashimoto and Steitz, 1984; Siliciano et al., 1987; Brow and Guthrie, 1988). U6 is unusual among the spliceosomal snRNAs in its high degree of phylogenetic conservation, the yeast molecule being 80% identical to its human homolog over half its length (Brow and Guthrie, 1988). On the basis of phylogenetic comparisons, it has been proposed that the U4–U6 base-pairing interaction consists of two intermolecular helices termed stem I and stem II (Figure 1; Brow and Guthrie, 1988); the stem I interaction had been previously observed in a psoralen cross-linking study (Rinke et al., 1985). Studies in yeast and metazoan systems have provided strong evidence for this model and demonstrated that both stems are required for the formation of the U4–U6 snRNP (Hamm and Mattaj, 1989; Bindereif et al., 1990; Vankan et al., 1990; Shannon and Guthrie, 1991). Despite its stability, the U4–U6 base-pairing interaction is dynamic: after the assembly of the spliceosome, the interaction is disrupted, and upon native gel electrophoresis U4 is released from the spliceosome (Pikielny et al., 1986; Cheng and Abelson, 1987; Lamond et al., 1988). Complexes that lack U4 are the first in which splicing intermediates and products are found (Pikielny et al., 1986; Konarska and Sharp, 1987; Cheng and Abelson, 1987; Lamond et al., 1988). Importantly, U4 snRNA apparently does not participate in the subsequent chemical steps of splicing, since a spliceosomal intermediate that lacks U4 has been shown to be functional (Yean and Lin, 1991).

The temporal correlation between the release of U4 and the appearance of reaction intermediates, the dispensability of U4 prior to the chemical steps of splicing, and the remarkable size and sequence conservation of U6 are consistent with the hypothesis that U6 participates directly in catalysis and that a primary function of U4 is to sequester U6 in an inert conformation (Guthrie and Patterson, 1988). The disruption of the U4–U6 interaction would then liberate specific residues in U6 to function directly in splicing. By postulating dual constraints on residues in U6 (base pairing with U4 and participation in catalysis), this model explains why nucleotides in U6 that base pair with U4 are more conserved than their partners in U4 (Guthrie and Patterson, 1988; Figure 1). It has also been suggested that the mRNA-type introns that interrupt a handful of fungal U6 snRNA genes arose through reverse splicing accidents in which introns integrated into a proximal component of the catalytic machinery, namely U6 snRNA (Brow and Guthrie, 1989; Tani and Ohshima, 1991; reviewed in Guthrie, 1991; Figure 1).

We have previously conducted a genetic analysis of the U6 molecule in the yeast Saccharomyces cerevisiae (Madhani et al., 1990). By combining mutagenesis of the U6 gene with a screen for mutants that are deleterious for cell growth, we identified two stretches of nucleotides that are particularly sensitive to point mutations: the ACAGAG hex-

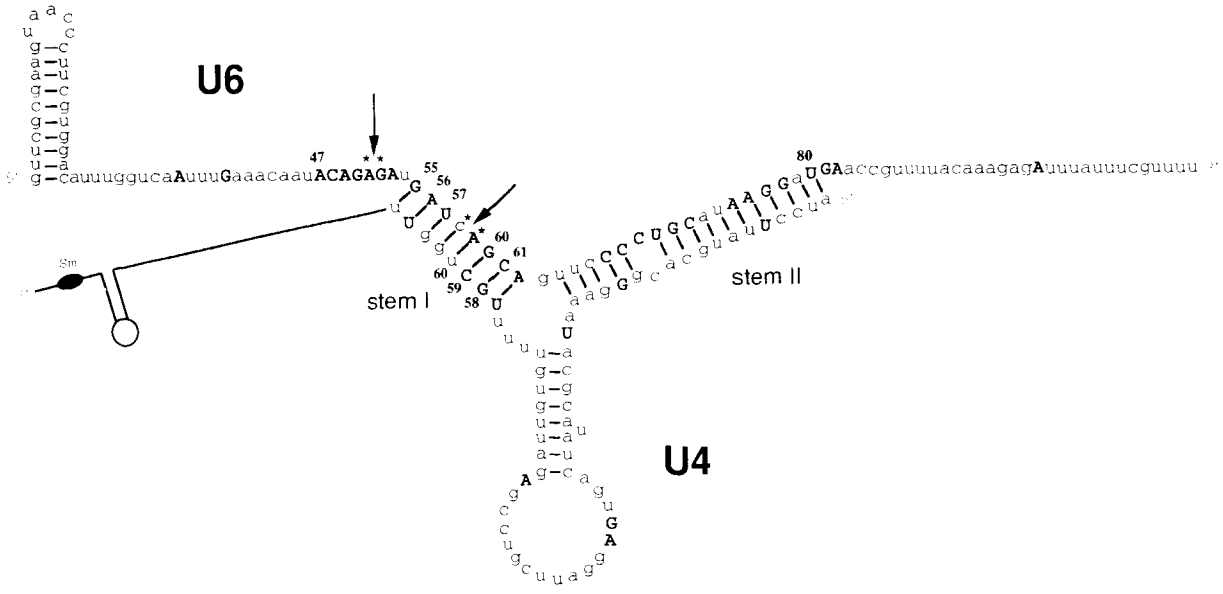

Figure 1. S. cerevisiae U4 and U6 snRNAs

Phylogenetically invariant residues are shown in uppercase and bold (based on alignments of Guthrie and Patterson, 1988; C. G., S. Mian, and H. Roiha, unpublished data). Asterisks mark residues specifically required for the second step of splicing in vitro (Fabrizio and Abelson, 1990). Arrows mark the locations of mRNA-type introns in the U6 genes of Schizosaccharomyces pombe (Tani and Ohshima, 1989) and Rhodosporidium dacryoidum (Tani and Ohshima, 1991). The essential ACAGAG and AGC sequences mentioned in the text span positions 47–52 and 59–61, respectively.

anucleotide in the central domain (nucleotides 47–52) and the AGC sequence (nucleotides 59–61) in stem I of the U4–U6 interaction domain (Madhani et al., 1990; Figure 1). The modest disruption of the U4–U6 base-pairing interaction caused by point mutants in the stem I region was shown not to be responsible for the lethal phenotypes of these mutants since analogous mutants in the stem I region of U4 have no effect on cell growth (nucleotides 58–60; Figure 1); moreover, compensatory mutants in U4 designed to restore base pairing fail to suppress the lethality of the U6 mutants (Madhani et al., 1990). We thus concluded that nucleotides in the stem I region of U6 have a role(s) in addition to base pairing with U4. A mutational analysis of the yeast U6 snRNA has also been described by Fabrizio and Abelson (1990). Using a cell-free system to assay the splicing activities of synthetic U6 mutant RNAs, Fabrizio and Abelson (1990) identified a virtually identical set of nucleotides as being important for U6 function in vitro. In addition, these studies revealed that while most functionally important nucleotides are required at or prior to the first chemical step of splicing, mutants at four positions lead to varying degrees of inhibition of the second chemical step of splicing (Fabrizio and Abelson, 1990; asterisks in Figure 1).

These studies of the yeast U6 snRNA and similar analyses of metazoan U6 snRNAs (Vankan et al., 1990, 1992; Bindereif and Green, 1990; Wolff and Bindereif, 1992) have identified regions of the molecule required at multiple steps of the splicing pathway, including spliceosome assembly and the two chemical steps of the reaction; however, the specific molecular interactions that underlie these requirements have yet to be elucidated. In particular, two key mechanistic questions remain: first, what is the function of the dynamically unstable U4–U6 base-pairing interaction, and, second, what are the specific roles of the mutationally sensitive nucleotides in U6 snRNA? Here we report experiments that provide important insight into these issues. Based on genetic suppression analyses, we propose a structural model for the active site of the spliceosome, in which previously proposed catalytic residues of U6 (ACAGAG and AGC) are directly juxtaposed with the branch point recognition region of U2. The predominant feature of this structure is an intermolecular helix, whose formation requires the displacement of U4 snRNA from U4–U6 stem I and its replacement by a highly conserved sequence in U2. Biochemical experiments described herein and elsewhere (Fabrizio and Abelson, 1990; D. S. McPheeters and J. Abelson, personal communication) indicate that residues that form this U2–U6 helix are important for both chemical steps of splicing in vivo and in vitro.

Results

U2–U6 Base-Pairing Model

Starting with the premises that the essential nucleotides in U6 are components of the spliceosomal active site and that the release of U6 from U4 activates U6 for participation in catalysis, we searched for base-pairing interactions between U6 and other snRNAs that would juxtapose these residues of U6 with the intron. We noticed that nucleotides in the stem I region of U6 (nucleotides 54–61 in yeast) are complementary to a highly conserved region of U2 snRNA (nucleotides 21–30 in yeast) that is itself immediately upstream of the sequence in U2 that is known to base pair with the intron branchsite region (Figure 2; Parker et al.,

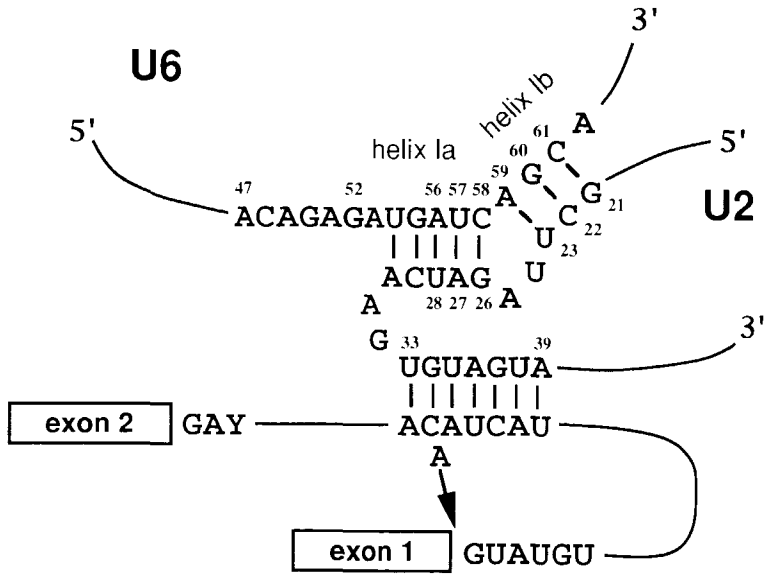

Figure 2. U2–U6 Base-Pairing Model

Shown is the Watson–Crick complementarity between nucleotides 54–61 of yeast U6 snRNA and nucleotides 21–30 of yeast U2 snRNA. Also depicted is the established interaction between nucleotides 33–39 of yeast U2 and the yeast intron branch point region consensus UACUAACA (Parker et al., 1987). The branch point adenosine is shown attacking the 5′ splice site during the first chemical step of splicing. In yeast, the 5′ splice site consensus sequence is GUAUGU. The 3′ splice site (YAG) is usually 30–50 nt downstream of the branch point.

1987). The resulting structure comprises two intermolecular helices (labeled helix Ia and helix Ib in Figure 2) connected by a 2 nt bulge. This structure is distinct from a previously identified base-pairing interaction between a more 5′ region of U2 and the 3′ end of U6 (which we refer to below as U2–U6 helix II; Hausner et al., 1990; Wu and Manley, 1991; Datta and Weiner, 1991). U2–U6 helix I can closely juxtapose essential residues in U6 with the intron branch point (Figure 2). Its functional requirement would also explain our previous genetic results that suggest a dual role for nucleotides in U6 that participate in U4–U6 stem I (Madhani et al., 1990). If the deleterious phenotypes of mutants in this region of U6 are due to the disruption of base pairing with U2, then their effects on growth would be predicted to be suppressed by supplying cells with compensatory mutants in U2 that restore Watson–Crick complementarity.

Phenotypes of U6 snRNA Mutants Can Be Suppressed by Restoring Complementarity with U2 snRNA

To test the U2–U6 helix I model, we employed a haploid S. cerevisiae strain (YHM1) that contains a wild-type U6 gene on a centromere-bearing plasmid marked with the *URA3* gene and a deletion of the chromosomal U6 coding sequence (Figure 3; Madhani et al., 1990). This strain

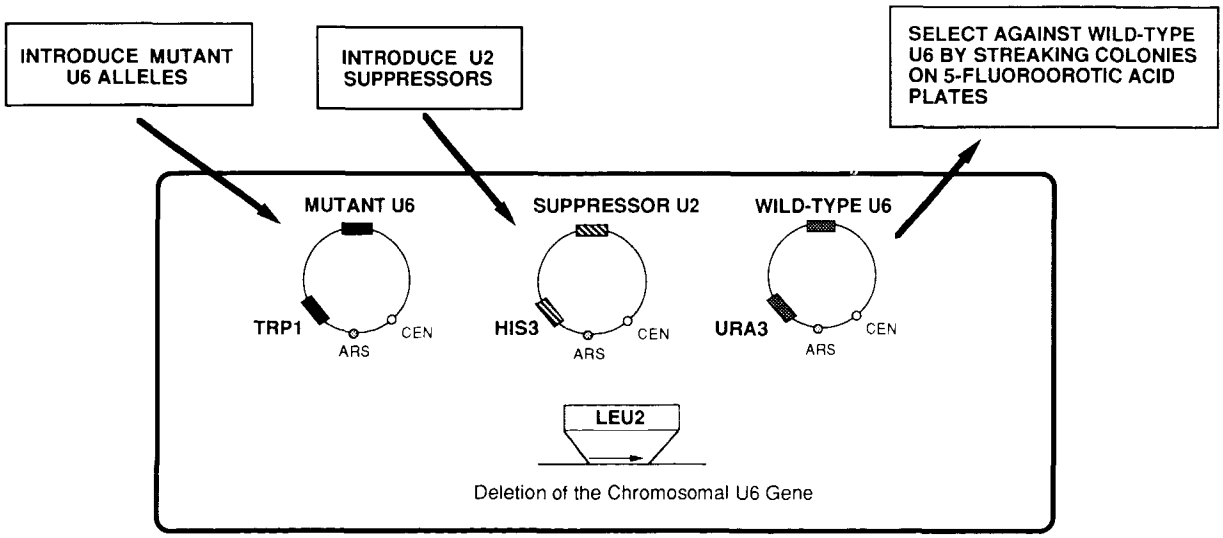

Figure 3. Plasmid Shuffle Assay for Mutants

The yeast strain YHM1 is depicted (Madhani et al., 1990). This strain contains a deletion of the chromosomal U6 coding sequence that is complemented by a wild-type U6 gene carried on a centromeric plasmid marked with the yeast *URA3* gene. U6 mutants, either alone or together with U2 compensatory mutants or noncompensatory mutants, can be introduced by transformation. The phenotype of the introduced U6 mutant can then be assessed by streaking transformants to plates containing 5-FOA, which selects for loss of the *URA3*-marked plasmid (Boeke et al., 1987).

Table 1. Summary of Genetic Suppression Experiments

U6 Allele	U2 Allele[a]	U2 Vector[b]	Temperature[c]	Growth[d]
A56U,U57A	Null	CEN	30	−
	WT	CEN	30	−
	A27U,U28A	CEN	30	+
	A27C,U28G	CEN	30	−
	A27G,U28G	CEN	30	−
	U23C	CEN	30	−
	U23G	CEN	30	−
A56C,U57C	Null	CEN	33	−
	WT	CEN	33	−
	A27G,U28G	CEN	33	+
	A27U,U28A	CEN	33	−
	A27C,U28G	CEN	33	−
	U23C	CEN	33	−
	U23G	CEN	33	−
A56C,U57G	Null	CEN	33	−
	WT	CEN	33	−
	A27C,U28G	CEN	33	+
	A27U,U28A	CEN	33	−
	A27G,U28G	CEN	33	−
	U23C	CEN	33	−
	U23G	CEN	33	−
C58U	Null	2μm	37	−
	WT	2μm	37	−
	G26A	2μm	37	+
	U23C	2μm	37	−
	U23G	2μm	37	−
C58A	Null	CEN	31.5	−
	WT	CEN	31.5	−
	G26U	CEN	31.5	+
	U23C	CEN	31.5	−
	U23G	CEN	31.5	−
A59C	Null	CEN	35	−
	WT	CEN	35	−
	U23G	CEN	35	+
	U23C	CEN	35	−
	A27U,U28A	CEN	35	−
	A27C,U28G	CEN	35	−
A59G	Null	CEN	30	−
	WT	CEN	30	−
	U23C	CEN	30	+
	U23G	CEN	30	−
	A27U,U28A	CEN	30	−
	A27C,U28G	CEN	30	−
G60U	Null	CEN	18–37	−
	WT	CEN	18–37	−
	C22A	CEN	18–37	−
	C22A	2μm	18–37	−
G60C	Null	CEN	18–37	−
	WT	CEN	18–37	−
	C22G	CEN	18–37	−
C61G	Null	CEN	37	−
	WT	CEN	37	−
	G21C	CEN	37	−
	G21C	2μm	37	−

Mutants in the S. cerevisiae U6 and U2 snRNAs are indicated by the wild-type (WT) nucleotide and its position followed by the identity of the mutant nucleotide.

[a] Additional copy of U2 snRNA introduced into YHM1. Null refers to the introduction of a vector plasmid containing no U2 gene.

[b] CEN indicates a centromeric (low copy) yeast vector; 2μm indicates a high copy yeast vector.

[c] Except for U6-A56U, U57A, and U6-A59G, suppression was generally much poorer or not observed at temperatures (shown in degrees centrigrade) higher than that indicated.

[d] Growth of the YHM1 derivatives on 5-FOA plates after 3 days.

can be transformed with plasmids encoding various alleles of U6 and U2, and the growth phenotypes of transformants can be assayed by streaking colonies onto 5-fluoroorotic acid (5-FOA)-containing plates, which select for cells that have lost the wild-type *URA3*-marked plasmid (Figure 3; Boeke et al., 1987). This strain also contains a wild-type chromosomal copy of U2; since compensatory mutations are expected to be gain-of-function alleles, they should be dominant over the endogenous wild-type gene. For each U6 mutant described below, we first examined the growth phenotype over a range of temperatures (25°C–37°C), and then tested the effects of compensatory U2 mutants under conditions where a given U6 mutant is lethal. To determine whether or not suppression reflected the restoration of base pairing per se, we also examined the effects of noncompensatory U2 mutants. If base pairing is occurring, one predicts only compensatory mutants to act as suppressors. As described below in detail (and summarized in Table 1), in each of seven cases, we were successful in suppressing the phenotype of a given U6 mutant with the predicted compensatory mutant in U2. Conversely, in none of 29 cases tested did a noncompensatory mutant or wild-type U2 have effects at all temperatures tested. Some "noncognate" U2 mutants can actually act as weak suppressors by partially restoring base pairing (e.g., through the restoration of 1 of 2 disrupted base pairs); however, at higher temperatures, suppression is only seen with the true cognate U2 allele. For purposes of clarity, only data from the most stringent conditions (i.e., the highest temperature at which suppresion was observed) are presented below.

Positions 56 and 57

Since previous mutagenesis data indicated that single point mutations at nucleotides 56 and 57 in U6 had relatively mild effects on splicing in vitro (Fabrizio and Abelson, 1990), we decided to construct double point mutations at these positions. Changing nucleotides 56 and 57 from AU to UA (U6-A56U,U57A) results in lethality at 30°C (Figure 4A). This phenotype can be suppressed by a U2 mutant that restores base pairing (U2-A27U,U28A) as predicted by the model (Figure 4B); the observed growth is equivalent to that seen with the parental wild-type strain (data not shown). On the other hand, suppression is not observed with four noncompensatory U2 mutants (U2-A27C,U28G; U2-A27G,U28G; U2-U23C; U2-U23G; Figures 4C–4F). Importantly, the failure of the noncognate U2 alleles to suppress is not due to their inability to function per se since each of them can suppress their cognate mutation in U6 (see below). As expected, the introduction of an additional wild-type U2 gene has no effect on the growth of U6-A56U,U57A or any other U6 mutant (Table 1).

A different mutant combination at the same dinucleotide (U6-A56C,U57C) is lethal at 33°C (Figure 4G). Introduction of the U2 compensatory mutant (U2-A27G,U28G) suppresses the growth defect (Figure 4H). However, U2-A27U,U28A, which could suppress U6-A56U,U57A, fails to suppress this mutant (Figure 4I). Likewise, three other noncompensatory mutants (U2-A27C,U28G; U2-U23C; U2-U23G) also fail to suppress (Figures 4J–4L).

Finally, changing positions 56 and 57 in U6 from AU to

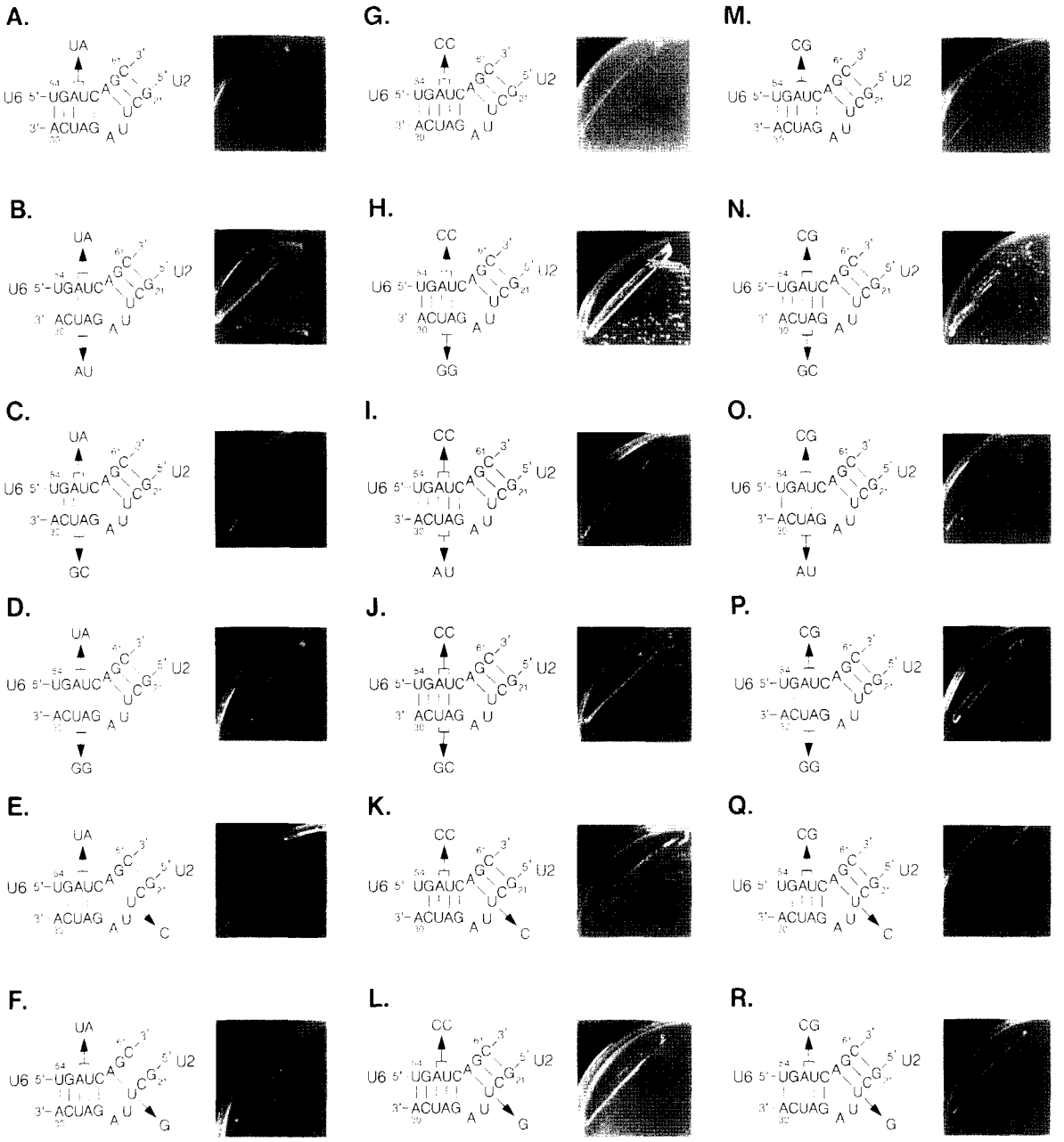

Figure 4. Specific Suppression of U6 Mutants at Positions 56–57 by Compensatory U2 Mutants

Shown is the growth on 5-FOA of YHM1 derivatives containing the indicated U6 mutant either on its own ([A], [G], and [M]), in the presence of the predicted compensatory mutant in U2 that restores base pairing according to Figure 2 ([B], [H], and [N]), or in the presence of noncompensatory U2 alleles ([C]–[F], [I]–[L], and [O]–[R]). (A)–(F) (U6-A56U,U57A) were incubated at 30°C for 3 days. (G)–(R) (U6-A56C,U57C; U6-A56C,U57G) were incubated at 33°C for 3 days.

CG (U6-A56C,U57G) is also lethal at 33°C (Figure 4M). Again, the appropriate compensatory U2 mutant (U2-A27C,U28G) suppresses the growth defect of the U6 mutant (Figure 4N). However, four noncognate U2 alleles (U2-A27U,U28A; U2-A27G,U28G; U2-U23C; U2-U23G) do not suppress (Figures 4O–4R).

Position 58

Mutation of C58 to a U results in lethality at 37°C (Figure 5A). As before, we attempted to suppress this defect using the compensatory mutant in U2 (U2-G26A); however, no suppression was observed (data not shown). Considering the possibility that the suppressor was inadequately expressed or assembled, we placed U2-G26A on a high copy plasmid. As shown in Figure 5B, U2-G26A on a high copy plasmid suppresses the growth defect of U6-C58U. This effect is also allele-specific since wild-type U2 as well as two noncognate suppressors, U2-U23C and U2-U23G (on high copy plasmids), do not suppress U6-C58A (Figures

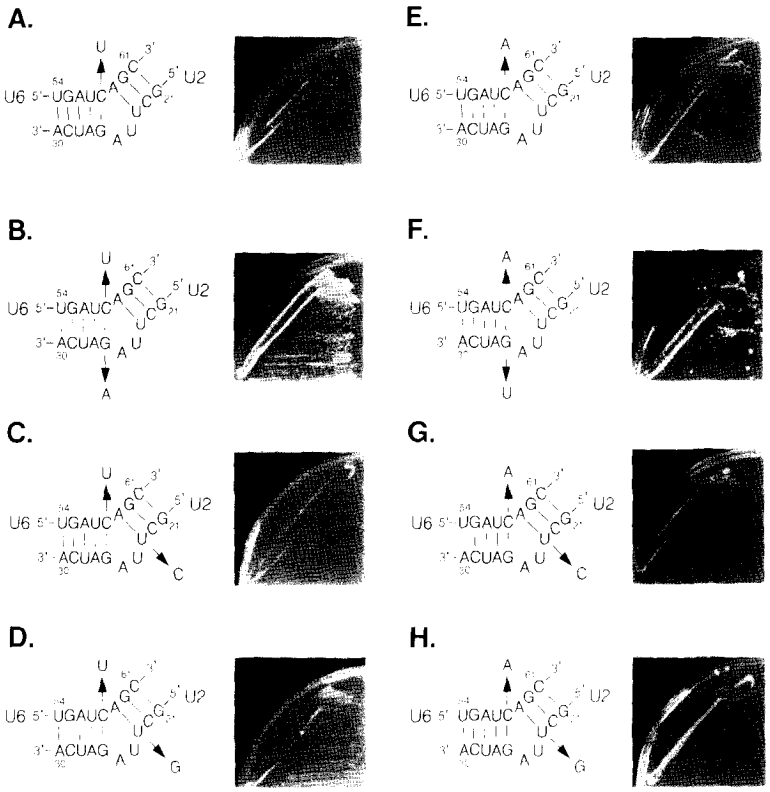

Figure 5. Specific Suppression of U6 Mutants at Position 58 by Compensatory U2 Mutants

The growth of the indicated mutants at position 58 in U6 was assayed as in Figure 4. Mutants were assayed on their own ([A] and [E]), in the presence of compensatory mutants in U2 that are predicted to restore base pairing ([B] and [F]), or in the presence of noncompensatory U2 alleles ([C], [D], [G], and [H]). (A)–(D) (U6-C58U) were incubated at 37°C for 3 days; (E)–(H) (U6-C58A) were incubated at 31.5°C for 3 days. U2 mutants used in (B)–(D) were carried on high copy plasmids (see Table 1).

5C and 5D). The latter mutants (U2-U23C and U2-U23G) do, however, suppress their cognate U6 mutants (data not shown).

We also constructed a different mutant at position 58, C58A. This mutant is lethal under all conditions tested (Figure 5E; data not shown). Introduction of the compensatory U2 mutant (on a low copy plasmid), G26U, allows growth at 31.5°C (Figure 5F). However, two noncompensatory mutants in U2 fail to suppress (U2-U23C; U2-U23G), again demonstrating the specificity of the interaction (Figures 5G and 5H).

Position 59

Mutation of A59 to C is lethal at 35°C (Figure 6A). It can be suppressed by the compensatory mutant U2-U23G (Figure 6B). In contrast, three noncompensatory U2 mutants (U2-U23C; U2-A27U,U28A; U2-A27C,U28G) do not have any effect (Figures 6C–6E). Similarly, mutation of A59 to a G is lethal under all conditions tested (Figure 6F shows a plate incubated at 30°C; data not shown). We found that the U2 suppressor, U2-U23C, suppresses this growth defect at all temperatures (Figure 6G shows suppression at 30°C). On the other hand, three noncompensatory U2 mutants (U2-U23G; U2-A27U,U28A; U2-A27C, U28G) fail to show any effect at any temperature tested (Figures 6H–6J show plates incubated at 30°C).

Positions 60 and 61

Mutation of G60 to a U or a C is lethal under all conditions (Madhani et al., 1990). Introduction of the cognate U2 suppressors (U2-C22A; U2-C22G) fails to suppress the lethality of these mutants (Table 1). In one case tested (U2-C22A), suppression is still not observed when the U2 mutant is placed on a high copy plasmid (Table 1). Similarly, C61G, which is lethal at 37°C (Madhani et al., 1990), is also not suppressed by U2-G21C either on a low copy plasmid or on a high copy plasmid (Table 1).

Comparison of the Growth Phenotypes of U2 and U6 Mutants

There are several potential explanations for why suppression was not observed at positions 60 and 61, including poor expression of the U2 suppressors or a lack of a base-pairing requirement at these positions. Another possibility is that the U6 residues have essential roles in addition to base pairing with U2 (or vice versa). To begin to address this issue, we examined the growth phenotypes of strains that contained comparable U2 and U6 mutants in helix Ia and Ib as their sole copy of the respective gene. We reasoned that if the only roles of two nucleotides were to base pair with each other, then mutation of either should have an equally deleterious effect on cell growth. However, if a particular nucleotide has an additional role, then its alteration should have a more severe effect than an analogous change in its base-pairing partner.

Two strains were used. The U6 mutants were assayed as above in YHM1. The U2 mutants were assayed in an analogous strain (YHM111) that contains a deletion of the chromosomal U2 gene and a wild-type U2 gene on a *URA3*-marked centromere plasmid. As before, the phenotypes of mutants were assessed by first transforming these strains with U6 and U2 mutants that disrupt helix Ia or Ib (see

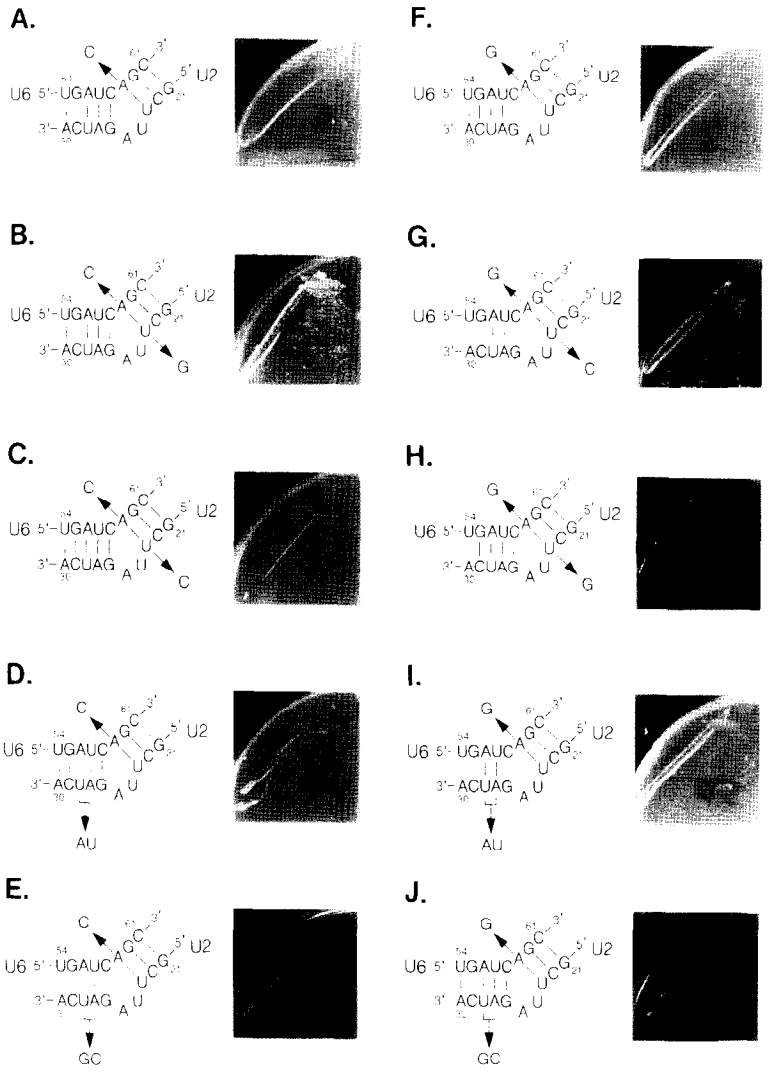

Figure 6. Specific Suppression of U6 Mutants at Position 59 by Compensatory U2 Mutants

The growth of the indicated mutants at position 59 in U6 was assayed as in Figure 4. Mutants were assayed on their own ([A] and [F]), in the presence of compensatory mutants in U2 that are predicted to restore base pairing ([B] and [G]), or in the presence of noncompensatory U2 alleles ([C]–[E] and [H]–[J]). (A)–(E) (U6-A59C) were incubated at 35°C for 3 days; (F)–(J) (U6-A59G) were incubated at 30°C for 3 days.

Figure 2) and then observing the growth of transformants streaked to 5-FOA plates and incubated at 25°C, 30°C, or 37°C.

As shown in Table 2, mutants that disrupt helix Ia have similar phenotypes regardless of whether the alteration is in U6 or in U2. For instance, U6-A56U,U57A and U6-A57C,U58G are lethal at each temperature tested, and the analogous mutants in U2 (U2-A27U,U28A and U2-A27C,U28G) exhibit the same phenotype. Likewise, U6-A56C, U57C grows poorly at 25°C, well at 30°C, but not at 37°C, and the comparable mutant in U2 (U2-A27C,U28C) shows the identical pattern (Table 2). Finally, mutants in U6-C58 and in its predicted pairing partner have similar effects on growth. Note that the C58U mutant, which results in a U–G "wobble" base pair, exhibits a relatively mild (temperature-sensitive) phenotype (Table 2).

We observed a different pattern in helix Ib. As described above, mutants at position 59 in U6 are lethal or temperature sensitive (Table 2) and can be suppressed by the predicted compensatory changes in U2 at position 23. In striking contrast, we observed that the mutants at position 23 in U2 (U2-U23C; U2-U23G) have no effect on growth on their own (Table 2). It is also notable that the U6-A59G mutant, which should allow the formation of a G–U base pair with U2-U23, exhibits a more deleterious phenotype than A59C, which completely disrupts base pairing; this suggests that something in addition to the stability of base pairing with U2 at this position is important for cell growth.

At position 60 in U6, mutants are lethal (Table 2) but cannot be suppressed by compensatory mutants at position 22 in U2 (see above); the U2 mutants at this position exhibit no growth defects on their own (Table 2). Finally, the most complex situation is observed at position 61 in U6 and its predicted pairing partner, position 21 in U2. We observed that while mutation of either is deleterious, changing U6 (U6-C61G) results in temperature-sensitive growth, while the U2 mutant (U2-G21C) is cold sensitive (Table 2).

In summary, while mutants in U2–U6 helix Ia exhibit similar phenotypes regardless of whether the alteration is made in U6 or in U2, we observe a marked asymmetry in phenotypes of helix Ib. In particular, changes to nucleo-

Table 2. Comparison of Phenotypes of U2 and U6 Mutants That Disrupt Helix I

Alleles	Growth 25°C	30°C	37°C
U6 allele			
Null	−	−	−
Wild-type	+	+	+
A56U,U57A	−	−	−
A56C,U57C	±	+	−
A56C,U57G	−	−	−
C58A	−	−	−
C58U	+	+	−
A59C	+	+	−
A59G	−	−	−
G60C	−	−	−
G60U	−	−	−
C61G	+	+	−
U2 allele			
Null	−	−	−
Wild-type	+	+	+
A27U,U28A	−	−	−
A27C,U28C	±	+	−
A27C,U28G	−	−	−
G26A	−	±	−
G26U	−	−	−
U23G	+	+	+
U23C	+	+	+
C22G	+	+	+
C22A	+	+	+
G21C	−	−	+

Mutants are designated as in Table 1. Plus indicates wild-type growth, plus/minus indicates poor growth, and minus indicates no growth. Plates were scored after 3 days.

Table 3. Splicing Defects Exhibited by U2 Mutants: Quantitation of Pre-mRNA and Lariat Intermediate

U2 Allele	Relative Levels Pre-U3A	Pre-U3B	Pre-RP51A	RP51A LI
G21C	1.2	1.5	4.7	3.0
U23C	1.0	0.9	2.0	5.6
U23G	1.0	0.7	2.9	7.6
G26U	1.8	2.7	1.6	3.6
G26A	0.6	0.6	0.9	2.4
A27U,U28A	3.1	3.4	1.3	1.0
A27C,U28G	3.7	6.9	1.7	0.5
Wild-type	1.0	1.0	1.0	1.0

Radioactivity in the gels that correspond to the autoradiograms displayed in Figures 7B and 7C was quantitated using a Molecular Dynamics phosphor screen and a phosphorimager. The relative amounts of pre-mRNA, lariat intermediate, and the U5 snRNA (internal control) were determined. The values for pre-mRNA and lariat intermediate (LI) were then normalized for the amount of RNA analyzed in each reaction, as reflected by the levels of the U5 internal control. In the table, the levels for wild type are arbitrarily set at 1.0; the levels shown for the mutants therefore reflect fold increases over wild type.

tides 59 and 60 in U6 are much more deleterious than changes in their predicted base-pairing partners, nucleotides 22 and 23 in U2.

Mutants in U2 Inhibit Both Steps of Splicing In Vivo

In a previous in vitro study of mutants in the yeast U6 molecule, it was found that while most deleterious mutants in U4–U6 stem I affect the first step of splicing, mutation of two nucleotides (nucleotides 58 and 59) inhibit the second chemical step of the reaction (Fabrizio and Abelson, 1990). In light of our model, it seemed likely that mutants in residues in U2 that engage in base pairing with these nucleotides in U6 would also affect both steps of splicing in vivo. Indeed, independent experiments by D. S. McPheeters and J. Abelson (personal communication) indicated that this was the case in vitro.

To test this in vivo, we constructed a yeast strain that contains a wild-type U2 gene that is under the control of the *GAL1–GAL10* upstream activating sequence (UAS), which allows one to regulate the transcription of U2 by growing cells in different carbon sources (Figure 7A). This strategy has been used previously to regulate snRNA expression (Patterson and Guthrie, 1987; Séraphin and Rosbash, 1989; Miraglia et al., 1991). Into this strain, we introduced either wild-type U2 or U2 mutants at positions 21, 23, 26, and 27–28. Cultures of these transformants were shifted from galactose-containing media to glucose-containing media for 15 hr, which causes transcriptional repression of the *GAL*-regulated U2 gene and, as a consequence, depletion of the regulated wild-type U2 snRNP from the cell (see Experimental Procedures). We then harvested total RNA from these strains and analyzed the splicing of endogenous transcripts by a primer extension method.

We first examined the splicing of the intron-containing *SNR17A* and *SNR17B* genes, which each encode the nucleolar U3 snRNA (Myslinski et al., 1990). By using a single ^{32}P-end-labeled primer that is complementary to the second exons of both genes, we were able to measure the levels of unspliced U3A and U3B RNAs. The reactions also included a primer complementary to U5 snRNA as an internal control for the amounts of RNA analyzed in each reaction. The results are shown in Figure 7B and are quantitated in Table 3.

In the control sample, we examined RNA from depleted cells that contained an additional copy of a wild-type U2 gene. This allowed us to determine the background levels of unspliced pre-U3A and pre-U3B RNAs (Figure 7B, lane 8). The U2 mutants are shown in Figure 7B (lanes 1–7). Three mutants (U2-G26U; U2-A27U,U28A; U2-A27C, U28G) show a 2- to 7-fold accumulation of pre-U3A and U3B (Figure 7B; Table 3). A slight increase in pre-mRNA levels is also seen in U2-G21C (Figure 7B; Table 3). However, no discernable accumulation is seen in U2-U23C, U23G or G26A (Figure 7B).

Because of the architecture of the *SNR17* genes, the band produced by primer extension of the U3 lariat intermediates (the product of the first step of splicing) comigrates with mature U3 snRNA (Myslinski et al., 1990). Therefore, to determine whether the second step of splicing was affected by the U2 mutants, we examined the splicing of a different gene, *RP51A*, which encodes a ribosomal protein (Teem and Rosbash, 1983). The RNA samples used in the experiment described above were ana-

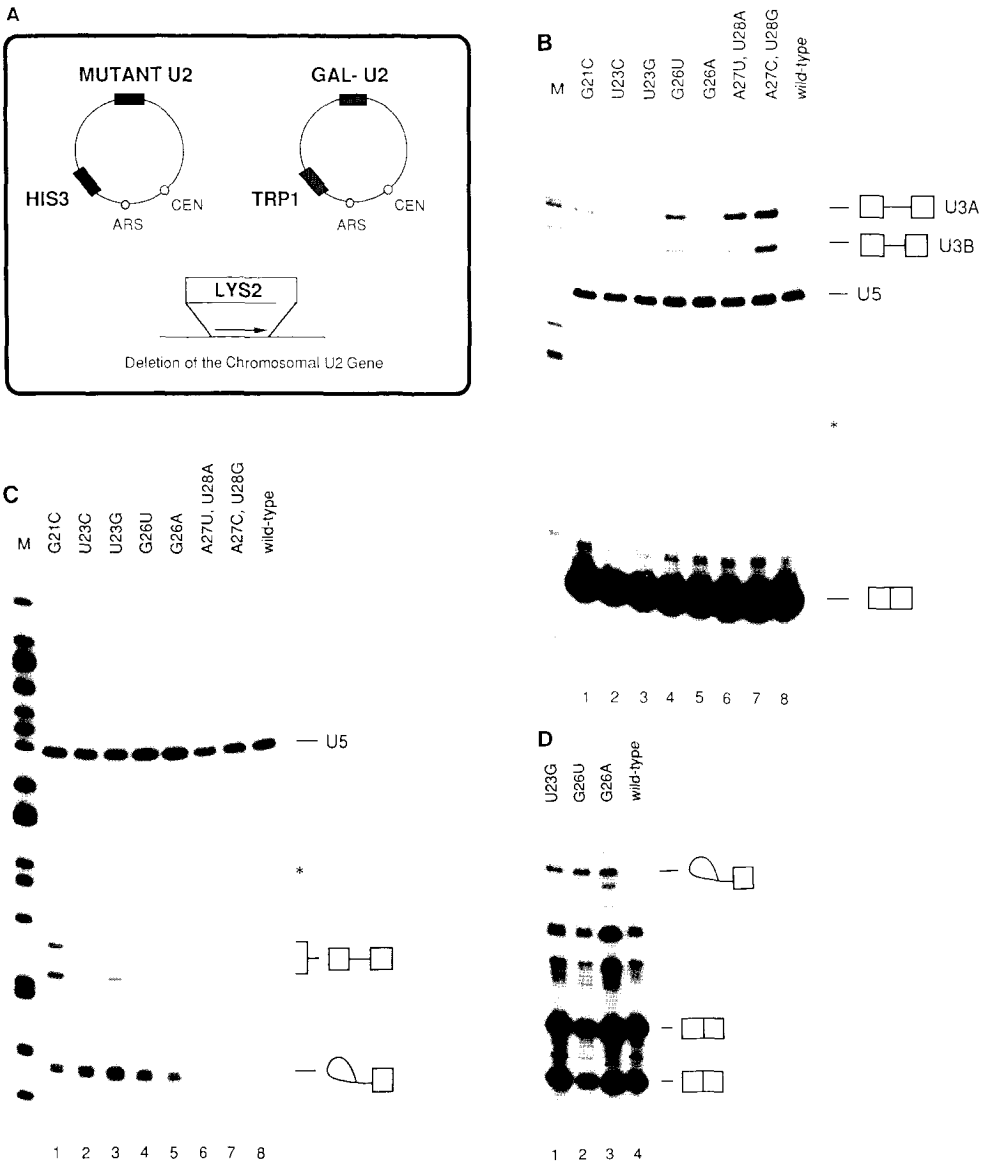

Figure 7. Mutants in U2 Inhibit Both Steps of Splicing In Vivo

(A) A strain (YHM113) containing a deletion of the chromosomal U2 gene that is complemented by a *GAL* UAS-regulated U2 gene is depicted. Plasmids that encode mutant U2 alleles or wild-type U2 were individually introduced into this strain by transformation. Cultures of the resulting strains were shifted from galactose media to glucose media for 15 hr to deplete cells of wild-type U2 snRNP.

(B) Analysis of in vivo splicing defects: *SNR17A* and *SNR17B*. Total RNA from the strains described in (A) was analyzed by a primer extension method using a ^{32}P-end-labeled oligonucleotide complementary to the second exons of the yeast *SNR17A* and *SNR17B* genes (Myslinski et al., 1990). A labeled oligonucleotide complementary to U5 snRNA was also included in the reaction as an internal control for the amounts of RNA in each reaction. The products were analyzed by electrophoresis through a 6% denaturing acrylamide gel followed by autoradiography. Bands that correspond to unspliced pre-U3A, pre-U3B, mature U3, and mature U5 snRNAs are indicated. U3 lariat intermediate comigrates with mature U3 snRNA. Boxes indicate exon sequences, and lines depict intron sequences. The band (marked by an asterisk) below that which corresponds to full-length U5 is the result of a reverse transcriptase strong stop due to secondary structure in U5 snRNA. M indicates markers (pBR322/HpaII). The U2 alleles analyzed are indicated above each lane.

(C and D) Analysis of in vivo splicing defects: *RP51A*. In (C), the RNA samples used in (B) were analyzed using an oligonucleotide complementary to the intron of the yeast *RP51A* gene (Teem and Rosbash, 1983). Bands that correspond to unspliced pre-mRNA (note there are two transcription start sites for the *RP51A* gene), lariat intermediate, and the U5 internal control are indicated. Since the intron primer can, in principle, also hybridize to the excised intron lariat, we examined several of the same RNA samples using a primer complementary to the second exon of *RP51A* (D) and confirmed that the observed signal is due to the accumulation of lariat intermediate (bands not designated in [D] are probable reverse transcriptase strong stops commonly seen with this primer).

lyzed using primers complementary to the intron and 3′ exon of this gene (Figures 7C and 7D). The levels of pre-mRNA and lariat intermediate for the wild-type control are shown in Figure 7C (lane 8). Strikingly, mutants at positions 21, 23, and 26 cause the accumulation of lariat intermediate (Figures 7C and 7D; Table 3), indicating inhibition of the second step of splicing. In addition, several mutants exhibit increases in the level of unspliced *RP51A* pre-mRNA (Figure 7C; Table 3). Curiously, the mutants that show the most pronounced effect on the levels of this pre-mRNA (G21C, U23C, U23G) exhibit little or no accumulation of pre-U3 (Table 3). Differences in the response of different pre-mRNAs to perturbations of the spliceosome have been observed previously (e.g., Patterson and Guthrie, 1987); however, their bases have yet to be elucidated.

In summary, these data indicate that residues U2-G21, U2-U23, and U2-G26 are particularly important for the second step of splicing in vivo. In addition, mutations at positions 21, 23, 26, and 27–28 can also inhibit the first step of splicing, as manifested by the accumulation of unspliced pre-mRNA.

Discussion

A Novel U2-U6 Base-Pairing Interaction

We have identified a novel base-pairing interaction between the stem I region of yeast U6 snRNA and the sequence just upstream of the branch point interaction region in yeast U2 snRNA (U2–U6 helix I; Figure 2). In seven instances, we have been able to suppress the growth phenotypes of U6 mutants that disrupt the predicted base-pairing interaction with compensatory U2 mutants that restore base pairing (Figures 4–6). Conversely, in each of the 29 cases tested, noncompensatory U2 mutants (as well as wild-type U2) do not suppress the phenotypes of the U6 mutants. These data provide strong evidence for the structural model presented in Figure 2. Biochemical data (Figure 7) show that the integrity of this helix is required for efficient splicing in vivo.

Although many of the nucleotides in U2–U6 helix I can be altered as long as base pairing is maintained, several observations suggest that particular nucleotides have additional functions. First, suppression is almost never complete. As described in Results, specific suppression by compensatory U2 mutants can be achieved for many U6 mutants (positions 56–59) but only at certain temperatures, above which growth cannot be observed. For other mutants, such as those at positions 60 and 61 in U6, we do not observe suppression under any conditions. Although we cannot rule out the possibility that there is no base-pairing requirement at these positions, the potential for base pairing between U6 nucleotides 60–61 and U2 nucleotides 21–22 is universally conserved. This fact and the evidence for base pairing at the adjacent position (U6-A59 and U2-U23) lead us to favor the alternative interpretation, namely that nucleotides 60 and 61 in U6 engage in base pairing but also have an additional essential role.

Second, in contrast with U2–U6 helix Ia, mutants in U2–U6 helix Ib exhibit a marked asymmetry in phenotypes. For example, while our data indicate base pairing between U6-A59 and U2-U23, mutants in U6-A59 are either lethal or temperature sensitive, while altering U2-U23 has no effect on growth (Table 2). This observation can be rationalized by proposing that U6-A59 has a function in addition to base pairing with U2-U23.

Since the residues in U6 that participate in U2–U6 helix I also base pair with U4 (Figure 1), this may explain some of the observations described above. However, point mutants in U4 that disrupt the U4–U6 stem I interaction do not affect cell growth (Madhani et al., 1990); thus, it is likely that residues in U6 that are involved in the U4–U6 stem I and U2–U6 helix I interactions also engage in other functionally important interactions that have yet to be identified.

U2–U6 Helix I May Be a Component of the Spliceosomal Active Site

The requirement of U2–U6 helix I for cell growth and splicing raises the question of whether the structure participates directly in catalysis or plays an indirect role, promoting the formation of a spatially or temporally distant active site. Below we describe characteristics of the U2–U6 structure that lead us to favor the former possibility.

Potential Juxtaposition of Candidate Catalytic Residues in U6 with Intron

The potential position in space of U2–U6 helix I relative to the intron is consistent with it being a component of the active site. That is, the U2–U6 structure can directly juxtapose two key regions of U6 (the ACAGAG hexanucleotide in the central domain and the AGC sequence in U4–U6 stem I) with the intron branch point (Figure 2). Indeed, we and others have previously proposed that these two regions of U6 contain particularly attractive candidates for catalytic residues based on their mutational sensitivity, the biochemical properties of mutants in these sequences, and their coincidence with intron insertion sites (Madhani et al., 1990; Fabrizio and Abelson, 1990; reviewed in Guthrie, 1991).

Is the U2–U6 helix formed at a time consistent with a role in catalysis? As discussed in detail below, the U2–U6 interaction likely forms after assembly of the spliceosome but prior to catalysis, consistent with a direct role. Presumably, for U2–U6 helix I to be juxtaposed with the intron branch point, it must occur simultaneously with the U2 branch point region interaction. The timing of this U2 intron interaction has not been precisely established. Recently, however, cross-links between U2 and the branch point region have been observed in fully assembled spliceosomes; these were induced by either ultraviolet light (Sawa and Shimura, 1992) or a psoralen reagent (Wassarman and Steitz, 1992). Cross-links between U6 and the intron were also identified in these experiments. These have been localized to a region just upstream of the ACAGAG hexanucleotide in U6 and to a sequence downstream of the 5′ splice site (Sawa and Shimura, 1992; H. Sawa and J. Abelson, personal communication; Wassarman and Steitz, 1992). The potential simultaneous formation of U2–U6 helix I and the U2 branch point region helix would offer a structural basis for the observed proximity between U6 and the 5′ splice site.

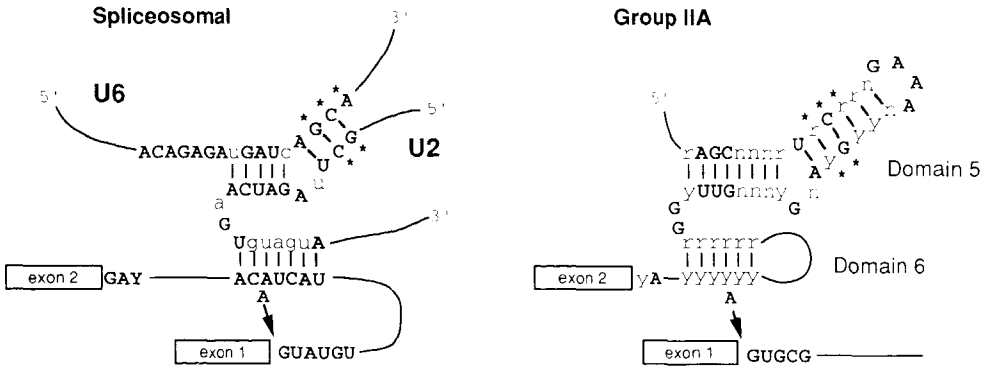

Figure 8. Structural Similarity between the Spliceosome and Group II Introns

On the left, U2 and U6 snRNAs are shown together with a yeast consensus intron as in Figure 2 (phylogenetically invariant residues are indicated in uppercase bold). The consensus sequence for domains 5 and 6 of group IIA introns is shown on the right (Michel et al., 1989). Highly conserved nucleotides in group IIA introns are shown in uppercase bold; conserved purines and pyrimidines are denoted by r and y, respectively, and variable sequences are denoted by n. Asterisks next to nucleotides in both structures indicate a limited identity in primary sequence.

Phylogenetic Conservation

U2–U6 helix I exhibits extraordinary phylogenetic conservation, consistent with the view that it is an active site element. Virtually all of the nucleotides in the structure are identical in all organisms for which sequences are available, including the highly divergent kinetoplastid protozoa (Guthrie and Patterson, 1988; C. G., S. Mian, and H. Roiha, unpublished data). Two nucleotides in helix Ia, U6-U54 and U6-C58, are not universally conserved (Figure 2); however, these nucleotides covary to just one other pattern (to A54 and U58) among all species examined (Guthrie and Patterson, 1988; Roiha et al., 1989; C. G., S. Mian, and H. Roiha, unpublished data). In fact, the U6 molecules of all metazoans for which sequences are available display the A54,U58 pattern, which results in a U2–U6 helix Ia that is 1 bp shorter than in Saccharomyces (and replaces the U6-C58–U2-G26 base pair with a U6-U58–U2-G26 pair). The only other nonconserved nucleotide, U2-U24, is found in the 2 nt bulge between U2–U6 helix Ia and helix Ib (Figure 2). It varies only to an A in all characterized metazoans (Guthrie and Patterson, 1988; C. G., S. Mian, and H. Roiha, unpublished data).

Residues in U2-U6 Helix I Are Important for Both Steps of Splicing

Nucleotides in U6 that we have shown here to be in U2–U6 helix I have been demonstrated previously to be important for both steps of splicing in vitro (Fabrizio and Abelson, 1990). Based on our model, one might expect that mutants in residues in U2 that participate in the structure would also inhibit both chemical steps of splicing. This was borne out in our in vivo analysis (Figure 7). Notably, we observed that mutation of positions 21, 23, and 26 in U2 results in the accumulation of lariat intermediate in vivo, while changes at positions 27–28 result only in the accumulation of unspliced pre-mRNA. Some mutants that inhibit the second step of splicing also exhibit an accumulation of unspliced pre-mRNA. Our results with U2 are in general agreement with recent experiments that demonstrate that alteration of position 26 and, to a lesser extent, position 27 in yeast U2 inhibit the second step of splicing in vitro (D. S. McPheeters and J. Abelson, personal communication). Although we do not observe any accumulation of lariat intermediate in mutants that change position 27, this apparent discrepancy may reflect differences in the assays employed (in vitro versus in vivo). Mutants at position 23 in U2 have yet to be tested for activity in vitro (D. S. McPheeters and J. Abelson, personal communication).

Given the potential proximity to the intron and strong phylogenetic conservation of U2–U6 helix I, the requirement of these base-paired residues for both steps of splicing could reflect the direct involvement of this helix in the catalysis of both reactions. If true, this raises the question why some residues are particularly important for the first chemical step of splicing, while other, adjacent nucleotides are more important for the second step. A parsimonious explanation would be that the catalytic sites for the two steps are largely overlapping but also contain distinct components specific to each step. Such a model is consistent with the fact that the nucleophiles that participate in the transesterification reactions (a 2′ hydroxyl in the first step and a 3′ hydroxyl in the second step) are similar but not identical. This hypothesis may also account for the observed differences in the importance of the 2′ hydroxyl groups that lie adjacent to the cleaved phosphodiester bonds at the 5′ versus 3′ splice sites (Moore and Sharp, 1992).

Structural Similarity with Domains 5 and 6 of Group II Self-Splicing Introns

In addition to similarities in the chemistry of splicing between group II autocatalytic introns and the spliceosome, two specific structural analogies have been suggested to support an evolutionary relationship between these systems. The first involves recognition of the intron branch point. In group II introns, the branch point adenosine is specified in part by being bulged out of an intramolecular helix termed domain 6 (Figure 8; Schmelzer and Schweyen, 1986). Similarly, in the spliceosome, the branch point adenosine is also found bulged out of a helix, in this case an intermolecular helix involving U2 snRNA (Figure 8;

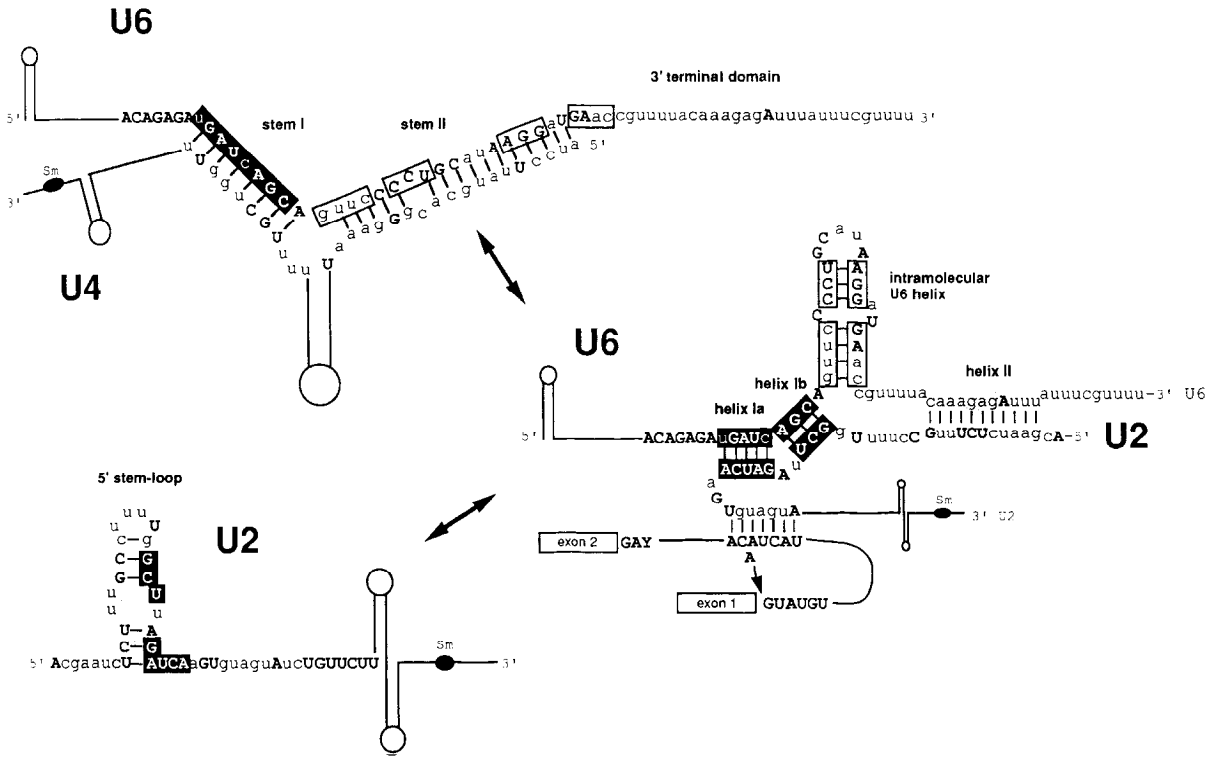

Figure 9. Conformational Isomers and Phylogenetic Conservation of U2, U4, and U6 snRNAs

Base-pairing interactions between U4 and U6 and U2 and U6, as well as intramolecular structures, are shown. Uppercase bold nucleotides represent those that are phylogenetically invariant (Guthrie and Patterson, 1988; C. G., S. Mian, and H. Roiha, unpublished data). Nucleotides in the U4–U6 structure and in the U2 intramolecular structure that engage in base pairing in the U2–U6 structure are shaded accordingly. Structural domains of the molecules discussed in the text are indicated.

Parker et al., 1987; Wu and Manley, 1989; Zhuang and Weiner, 1989). The second analogy arises from studies demonstrating that the conserved loop of U5 snRNA can base pair with exon sequences immediately adjacent to the 5′ and 3′ splice sites (Newman and Norman, 1992). These authors suggest that the U5 loop is analogous to the D3 loop in group II introns, which is also known to base pair with the 5′ and 3′ exons (Newman and Norman, 1992; Jacquier and Michel, 1987; Jacquier and Jacquesson-Breuleux, 1991). While these analogies suggest similarities in the mechanism of splice site selection between the two systems, neither of these examples involves highly conserved sequences in group II introns such as those that one might expect to be involved in catalysis.

In light of the results described in this paper, we considered whether there might be additional structural similarities between the two systems involving highly conserved sequences. A good candidate for a catalytic element in group II introns is domain 5, which is the most conserved domain of these introns. It immediately precedes domain 6 (the branch point region helix), and its integrity is essential for self-splicing (Jarrell et al., 1988; Koch et al., 1992). Domain 5 consists of two helices connected by a 2 nt bulge; it is also highly conserved in primary sequence (Figure 8; Michel et al., 1989). In our model of the spliceosomal snRNAs, the branchsite helix is also immediately preceded by a helix–2 nt bulge–helix structure that involves residues that are virtually phylogenetically invariant (Figure 8). There is also some limited sequence identity between helix Ib and the potentially analogous region in domain 5 (indicated by asterisks in Figure 8). These structural similarities suggest the possibility that the U2–U6 structure and the U2 branch point interaction represent homologs of group II intron domains 5 and 6, respectively. Further evaluation of the similarities between the two systems will likely come from an understanding of the specific tertiary contacts that position the branch point adenosine as well as the 5′ and 3′ splice sites within the respective conserved core structures.

RNA Structural Rearrangements: A General Paradigm for Spliceosome Assembly

Although the dynamic nature of the extensive U4–U6 base-pairing interaction has been known for some time (Pikielny et al., 1986; Cheng and Abelson, 1987; Konarska and Sharp, 1987; Lamond et al., 1988), the purpose of its cyclical disruption and reformation has been enigmatic. In that the novel U2–U6 structure described here and stem I of the U4–U6 base-pairing interaction are mutually exclusive (Figure 9), we propose that an important function of the unwinding of U4–U6 is to allow the formation of U2–U6 helix I. Since the U4–U6 interaction is known to be disrupted after the assembly of the spliceosome but prior to the chemical steps of the reaction (Pikielny et al., 1986;

Cheng and Abelson, 1987; Lamond et al., 1988), it is reasonable to propose that the U2–U6 interaction forms during the window of time after U4–U6 destabilization but prior to (or concomitant with) the chemical steps of the reaction.

The U2–U6 helix I structure involves sequences in U6 that are (initially) part of stem I of the U4–U6 interaction. In principle, the U4–U6 stem II interaction could be maintained after formation of the U2–U6 structure. However, since U4 can be released from functional spliceosomes prior to catalysis (Yean and Lin, 1991), stem II is presumably also disrupted prior to the first step of splicing. A rearrangement involving stem II is supported by phylogenetic covariation analyses, which indicate base pairing between the first few nucleotides of the stem II region and the beginning of the 3′ terminal domain (C. G., S. Mian, and H. Roiha, unpublished data). An intramolecular U6 helix that incorporates this observation is depicted in Figure 9; this pairing is mutually exclusive with stem II of the U4–U6 interaction. This stem loop has also been proposed by Brow and colleagues as part of a larger secondary structure model for free (nonspliceosomal) U6 snRNP based on chemical modification studies (R. Troy and D. A. Brow, personal communication). Interestingly, we have previously described a mutant whose biochemical properties may be explained by this structure. The lethal mutant U6-U80G is apparently blocked in the transition from the free U6 snRNP to the U4–U6 snRNP (Madhani et al., 1990; Figure 1). This mutation would affect the U that bulges out of the intramolecular U6 helix in Figure 9. Replacing U80 with a G would permit base pairing with the C on the other side of the bulge, increasing the stability of the putative hairpin structure (Figure 9). Since the unwinding of this structure would be necessary to reform the U4–U6 stem II interaction, the hyperstabilization of the intramolecular helix caused by the U6-U80G mutation potentially explains the failure of this mutant U6 to associate with U4. Further experiments will be necessary to test this model; specifically, mutations that disrupt the intramolecular U6 helix are predicted to suppress the phenotypes of U6-U80G. Indeed, it has been recently shown that the phenotype of a different hyperstabilizing mutant can be suppressed by a destabilizing mutant elsewhere in the helix (D. Fortner and D. A. Brow, personal communication).

According to this view, the unwinding of the U4–U6 snRNAs could promote two RNA rearrangements: a U4–U6 stem I to U2–U6 helix I isomerization and a U4–U6 stem II to U6 intramolecular stem isomerization. As proposed above, the former transition could serve to juxtapose candidate catalytic residues in U6 with the intron. In addition, the base pairs in U2–U6 helix I and in the intramolecular U6 structures may also have another role. Once the base pairs that hold together U4 and U6 are disrupted, some alternative interaction is presumably necessary to prevent the immediate reassociation of the unwound strands. The formation of alternative base pairs in U2–U6 helix I and the U6 intramolecular helix may be significant in this regard.

The region of U2 snRNA in U2–U6 helix I is thought to participate in an intramolecular stem (Figure 9; Keller and Noon, 1985). In addition to the conserved complementarity of bases in the two halves of the stem, the existence of this hairpin is suggested by Watson–Crick covariation of the sequence of 1 bp in the top part of the structure (Guthrie and Patterson, 1988) and the inaccessibility of this region of RNA to oligonucleotide probes (Lamond et al., 1989). Evidence that this structure may be dynamic comes from observations in mammals that demonstrate base pairing involving the 5′ half of the U2 stem loop and the 3′ terminal domain of U6 (U2–U6 helix II in Figure 9). This interaction, which was inferred from a psoralen cross-linking study (Hausner et al., 1990), requires the unwinding of the U2 5′ stem loop. Its functional importance was subsequently demonstrated in transfected mammalian cells (Wu and Manley, 1991; Datta and Weiner, 1991). However, since this pairing can be disrupted without detectable effect in yeast (Fabrizio et al., 1989; Madhani et al., 1990; Bordonné and Guthrie, 1992), its role in this organism may be a more subtle one, perhaps serving to assist in the opening up of the U2 5′ stem to facilitate interaction between its 3′ half and the helix I region of U6. This may be reflected in the relatively low primary sequence conservation of U2–U6 helix II (Figure 9). Interestingly, in mammalian nuclear extracts, the accessibility of the U2 5′ stem loop to oligonucleotide hybridization is increased when a 2′-O-methyl RNA oligonucleotide is annealed to the branch point region interaction domain of U2 (Lamond et al., 1989). A provocative interpretation of this observation is that the U2–branch point region interaction promotes the formation of the U2–U6 interaction during spliceosome assembly by activating the unwinding of the U2 5′ stem loop.

In summary, the current evidence suggests a minimum of three RNA conformational rearrangements that occur during spliceosome assembly. Presumably, these events must be reversed for the snRNPs to be recycled and used in the next round of splicing. How are these events accomplished and regulated? Two families of known or suspected RNA-dependent ATPases that also share homology to ATP-dependent RNA helicases are required for splicing in yeast (for reviews see Guthrie, 1991; Schmid and Linder, 1992); no physiological RNA substrate for any of these proteins has been identified. Similarly, an RNA binding (RNP consensus) protein family member, PRP24, has been implicated in regulation of the U4–U6 base pairing cycle (Shannon and Guthrie, 1991); its binding sites have yet to be elucidated. The dynamic RNA–RNA interactions described above constitute excellent candidate substrates for these proteins. The requirements for ATP hydrolysis at many steps of spliceosome assembly could in part reflect a need for energy to facilitate such RNA rearrangement reactions. In turn, the ordered nature of spliceosome assembly argues that these reactions are tightly regulated with respect to timing and directionality.

Experimental Procedures

Yeast Methods and Strains

All yeast procedures including plasmid shuffle assays were performed using standard methods (Guthrie and Fink, 1991). YHM1 (*MATa ura3 his3 lys2 trp1 leu2 snr6::LEU2 YCp50–SNR6*) has been described previously (Madhani et al., 1990). YHM111 (*MATa trp1 ura3-52 ade2-101 his3 lys2 snr20::LYS2* pSE360–*SNR20*) was constructed by trans-

forming the diploid strain ES218 (Shuster and Guthrie, 1988) with a plasmid encoding the wild-type U2 gene, followed by sporulation. Both YHM1 and YHM111 are descendants of S288C. The *GAL*–U2 strain (YHM113) was created by transformation of YHM111 with pGAL–U2 (see below) followed by removal of the wild-type U2 plasmid by the streaking of transformants to 5-FOA plates.

U6 and U2 Plasmids

The low copy shuttle vectors used in this study, pSE358 (*TRP1*, CEN) and pSE362 (*HIS3*, CEN), were obtained from S. Elledge (Baylor University). pSE358 is a precursor to pUN10, and pSE362 is identical to pUN90 (Elledge and Davis, 1988). For high copy experiments, U2 mutants were subcloned into the 2μm-based *HIS3* vector, pRS423 (a gift from J. Li).

We have previously introduced SphI and XhoI restriction sites just upstream and downstream of the U6 coding sequence to facilitate mutagenesis (Madhani et al., 1990). The plasmid pSX6 contains this U6 gene derivative cloned into pSE358 (Madhani et al., 1990). For mutagenesis of U2, we used a U2 derivative that contains a single nucleotide insertion at its 5′ end that creates an EcoRI site and a BamHI linker in place of a segment of the large nonessential domain of yeast U2 (see Figure 1 in Shuster and Guthrie, 1990). pES143 contains the SalI–SmaI fragment containing this derivative in pBluescript (Strategene Cloning Systems, La Jolla, California). In this study we employed a derivative, pES143ΔB, which contains a deletion of a BamHI site in the pBluescript polylinker. Construction of *GAL* UAS-regulated U2 gene was accomplished by replacment of the SalI–DraIII fragment of pES143, which contains all U2 sequences upstream of the putative TATA box, with the SauIIIA–DdeI fragment of the *GAL1–GAL10* intergenic region (Schnieder and Guarente, 1991). The SalI–SacI fragment was cloned into pSE358 to yield pGAL–U2.

Mutagenesis Strategy

Mutants in U6 and U2 derivates described above were created by polymerase chain reaction amplification of the respective DNAs using primers that contained the desired nucleotide changes. For the U2 mutants, this was accomplished in one step using primers that overlapped the EcoRI site at the 5′ end of the modified U2 gene in pES143 (5′ primer spans positions +1 to +36 relative to the transcription start site of the sense strand) and the BamHI site that is downstream of the U2 Sm site (3′ primer spans +266 to +227 on the antisense strand). For the U6 mutants, a two-step protocol was used. In the first amplification reaction, we used a mutagenic primer that extends from positions +21 to +62 on the sense strand of the relative to the transcription start site and a 3′ primer that extends from positions +129 to +96 on the antisense strand. One-tenth of this reaction was used in a second amplification using a 5′ primer spanning positions −12 to +33 and the same 3′ primer as used before. Reaction conditons were as follows: 1 μM primers, 200 μM dNTPs, 0.06 U/μl Amersham Hot Tub Polymerase, 2 ng/μl template DNA (pSX6 or pES143ΔB) in 1 × buffer supplied with Hot Tub polymerase. Total volume was 50 μl. After an initial denaturation at 94°C for 3 min and an annealing step at 35°C for 2 min, amplifications were performed for 20 cycles using a two temperature-step protocol: 1 min at 50°C and 45 s at 94°C.

The resulting reaction products were preciptated with ethanol and resuspended in 40 μl of water. For the U6 mutants, the DNA was treated with SphI and XhoI and was cloned into the SphI and XhoI sites of pSX6. For the U2 mutants, the DNA was treated with EcoRI and BamHI and used to replace the same fragment in pES143ΔB. A fragment containing the U2 gene was then cloned into the polylinker of pSE362 using SacI and SalI. In all cases, the amplified region was sequenced to confirm the identity of each mutant.

Depletion of U2 snRNA Using *GAL*-U2

The *GAL*–U2 strain (and derivatives) was grown to midlog phase in minimal media containing 2% galactose and 2% sucrose. Cells were pelleted by centrifugation and resuspended in minimal media containing 2% glucose. Cultures were maintained in midlog phase (OD_{600} = 0.1–1.0) by dilution with glucose-containing media. After 15 hr, cells were pelleted and frozen prior to subsequent RNA isolation.

RNA Isolation and Analysis

Total RNA was isolated from yeast using the hot phenol method (Kohrer and Domdey, 1991). RNA was analyzed using the primer extension protocol described by Patterson and Guthrie (1991). The following ^{32}P-end-labeled primers were used: *SNR17A* and *SNR17B* (U3 snRNA; Myslinski et al., 1990): 5′-CCAAGTTGGATTCAGT-3′ (complementary to exon 2 of both genes); *RP51A* (Teem and Rosbash, 1983): 5′-GTATGACTTTATTGCGCATGTCGACTC-3′ (intron primer) and 5′-CGCTTGACGGTCTTGGTTC-3′ (exon 2 primer); *SNR7* (U5 snRNA; Patterson and Guthrie, 1987): 5′-AAGTTCCAAAAAATATGGCAAGC-3′.

The sizes of the extension products shown in Figures 7B, 7C, and 7D that are generated by primer extension of the endogenous RNAs are as follows: *SNR17* exon 2 primer: pre-U3A = 237 nt, pre-U3B = 210 nt, and lariat intermediate and U3 snRNA = 81 nt; *RP51A* intron primer: pre-mRNA = 93 nt and 103 nt and lariat intermediate and excised lariat = 73 nt; *RP51A* exon 2 primer: lariat intermediate = 87 nt and mRNA = 49 nt and 59 nt; *SNR7* primer: U5 = 180 nt.

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