Mutations in U6 snRNA That Alter Splice Site Specificity: Implications for the Active Site

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What determines the precise sites of cleavage in the two transesterification reactions of messenger RNA (mRNA) splicing is a major unsolved question. Mutation of the invariant G (guanosine) at position 5 of 5′ splice sites in *Saccharomyces cerevisiae* introns activates cleavage at nearby aberrant sites. A genetic approach was used to test the hypothesis that a base-pairing interaction between the 5′ splice site and the invariant ACAGAG sequence of U6 is a determinant of 5′ splice site choice. Mutations in U6 or the intron (or both) that were predicted to stabilize the interaction suppressed aberrant cleavage and increased normal cleavage. In addition, a mutation in the ACAGAG sequence suppressed mutations of the 3′ splice site dinucleotide. These data can fit a model for the spliceosomal active site comprised of a set of RNA-RNA interactions between the intron, U2 and U6.

Nuclear precursor-mRNA (pre-mRNA) splicing is the process by which introns are removed from mRNA precursors to form functional mature mRNAs. This process is mediated by the spliceosome, a complex ribonucleoprotein machine composed of five small nuclear RNAs (U1, U2, U4, U5, and U6) and numerous proteins. Introns are removed by means of two consecutive transesterification reactions. In step 1, the 2′-OH of the intron branch point adenosine attacks the phosphodiester bond at the 5′ splice site, resulting in the formation of free exon 1 and the lariat intermediate. In step 2, the 3′-OH of exon 1 attacks the 3′ splice site, resulting in ligation of exon 1 to exon 2 and the release of the excised intron lariat (*1*, *2*). Introns are defined only by three short consensus sequences: the 5′ splice site, the branch point region, and the 3′ splice site. It is not understood how the spliceosome correctly identifies boundaries between exons and introns and selects the precise location of the sites of covalent modification.

There is now considerable information on the role of RNA-RNA interactions in this identification process. The 5′ splice site and branch point sequences of the intron are recognized by base-pairing interactions with U1 (Fig. 1A) (*3*, *4*) and U2 (*5*), respectively. U1 binds at an early step in spliceosome assembly and commits the pre-mRNA to the splicing pathway (*6*). Genetic and biochemical data place U5 in close proximity to the 5′ and 3′ exon sequences (*7–10*). In special cases, in which the authentic 5′ splice site has been mutated, Watson-Crick base-pairing interactions between U5 and the 5′ region of the precursor mRNA play a role in activating aberrant cleavage sites (*8*).

Mutation studies have shown that the highly conserved 5′ splice site of *Saccharomyces cerevisiae* has several roles in the splicing pathway (*11*). Mutation of the invariant G at intron position 5 (/GUAUGU) (*12*), like other 5′ splice site mutations, decreases the efficiency of the first catalytic step. However, changes at this position are the only intron mutations that activate aberrant cleavage sites. The location and frequency of aberrant cleavage depend on the particular intron mutated as well as the specific intron mutation (*13*). Restoration of complementarity between the 5′ splice site and U1, by the introduction of compensatory changes in U1, did not prevent aberrant cleavage; rather, it increased cleavage at both the normal and aberrant sites (Fig. 1B) (*4*). Thus, while the base-pairing interaction between the invariant C at position 4 of U1 and the invariant G at position 5 of the 5′ splice site is important for the efficiency of the first catalytic step of splicing, intron position 5 must also be recognized by some other factor in order to specify the location of the cleavage reaction (*4*).

An excellent candidate for such a second factor is U6. Crosslinking experiments in mammalian (*10*) and yeast (*14*) extracts indicated that intron position 5 is close to a conserved domain of U6. Two distinct U6-5′ splice site base-pairing interactions have been proposed on the basis of these experiments (*15*). In both models, ACA sequences in U6 base pair with the UGU conserved at positions 4 to 6 of yeast introns. In model I (Fig. 1C), which is based on a psoralen crosslink between U6 and pre-mRNA (*10*), the ACA residues (nucleotides 47 to 49) are in the invariant ACAGAG sequence. In model II (Fig. 1D), which is based on ultraviolet (UV) crosslinks between U6 and lariat intermediates (*14*), the ACA residues (nucleotides 42 to 44) are located just upstream of the ACAGAG sequence and are relatively nonconserved (*16*, *17*). The regions of U6 modeled to base pair with the 5′ splice site are immediately upstream of a helix (helix I) formed by base-pairing interactions between U6 and U2 (*18*). This helix can juxtapose the essential ACAGAG sequence of U6 (*19*, *20*) with the branch point interaction domain of U2 (*18*). Thus, the proposed U6-5′ splice site interactions might serve to juxtapose the branch point, which is the nucleophile that attacks the 5′ splice site (*21*), with the 5′ cleavage site.

We used a genetic approach to test the role of U6 in the specificity of 5′ splice site cleavage. Mutations predicted to stabilize the interactions in model I suppressed aberrant cleavage and increased normal cleavage. Conversely, enhancement of the interaction in model II led to an increase in cleavage at the aberrant site activated by a mutation at intron position 5. We thus propose that base pairs between the intron (positions 4 to 6) and U6 (nt 47 to 49) serve to align correctly the 5′ splice site with the intron branch point during the first catalytic step. We also found that mutation of the last nucleotide of the ACAGAG sequence can suppress the second step block imposed by mutations of the 3′ splice site dinucleotide (AG/). These data, together with other recent results (*18*, *22*, *23*), suggest a model for the catalytic center of the spliceosome.

Genetic test of the models of interactions between U6 and the intron. In order to identify potential base-pairing interactions between U6 and the 5′ splice site, we generated strains homozygous for U6 point mutations (*24*) and transformed them with ACT1-CUP1 reporters (Fig. 2A) (*25*) that contained either a wild-type intron (Fig. 2B) or intron position 5 mutation (G5A or G5C) (*26*). We monitored the amount of cleavage at either the normal (NL) or aberrant (AB) cleavage sites by using a pair of reporters—one that produced an in-frame mRNA when cleavage was at the normal site (Fig. 2C) and the other that produced an in-frame mRNA when cleavage was at the aberrant site (Fig. 2D) (*27*). The ACT1-CUP1 fusion protein allowed cells to grow in the presence of copper in a dosage-dependent

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manner. Thus, in a cup1Δ background, it is possible to monitor mRNA by monitoring copper resistance of strains harboring the reporter constructs (*28*). For example, we could detect that the G5A mutation activated more aberrant cleavage than the G5C mutation, because wild-type strains transformed with the G5A reporter for aberrant cleavage grew at 0.25 mM copper, but wild-type strains transformed with the G5C reporter for aberrant cleavage grew at 0.1 mM copper.

If activation of the aberrant cleavage site results from disruption of a normal base-pairing interaction between U6 and position 5 of the 5′ splice site, restoration of the proposed base-pairing interaction should inhibit cleavage at the aberrant site. The most direct test of this prediction would be, in the case of model I (Fig. 1C), to determine whether mutations at U6 position 48, which should restore complementarity to intron position 5, could prevent cleavage at the aberrant site. However, strains are inviable when the sole copy of U6 is mutated at position 48 (*19*). Moreover, when expressed on low-copy plasmids in the presence of the chromosomal copy of U6, mutations at U6 position 48 did not influence the splicing phenotypes conferred by intron position 5 mutations, as determined by primer extension and copper resistance assays (*29*). This is not surprising in that these U6 alleles are poorly expressed when assayed in the presence of the chromosomal copy of U6 (*19*).

An additional prediction of model I is that cleavage at the aberrant site might be suppressed if the proposed base-pairing interaction between U6 and the 5′ splice site were strengthened by extending the complementarity between U6 positions 47 to 49 (ACA) and intron positions 4 to 6 (UGU) to include U6 positions 45 and 46 and intron positions 7 and 8 (Fig. 3A). Thus, U6 positions 45 and 46 were mutated to A45G,U46A to create complementarity to actin intron positions U7 and C8 (Fig. 3A). If these nucleotides are in close proximity to the 5′ splice site, they should be able to base pair with the intron, thereby stabilizing the normal U6-intron base-pairing interaction (model I) and inhibiting cleavage at the aberrant site (*30*).

As predicted, when either the G5C or G5A reporter was transformed into the U6-A45G,U46A strain, cleavage at the normal site was increased but cleavage at the aberrant site was decreased, as assayed by copper resistance (Fig. 3B). In fact, the copper resistance of these mutant U6 strains, transformed with reporters for normal cleavage, was as high as that of a wild-type strain transformed with a wild-type 5′ splice site reporter (Fig. 3B). Primer extension analyses (Fig. 3C) confirmed that the changes in copper resistance resulted from alterations in the amount of mature mRNAs generated by cleavage at each of the two sites and indicated that the amount of cleavage at the normal site relative to the aberrant site was at least ten times greater in the U6-A45G,U46A strain than in the U6–wild-type strain (*31*).

A second test of the U6-intron interaction was conducted by mutating the G5A intron positions 7 and 8 (U7C,C8G) (Fig. 3A). Unexpectedly, the introduction of these mutations into a nonconserved region of the intron (*32*) inhibited splicing in either the wild-type or the U6-A45G,U46A strains. Nonetheless, the U6-A45C,U46G mutation, which was predicted to restore complementarity between U6 and the altered intron (Fig. 3A) (*30*), restored splicing efficiency, increased cleavage at the normal site, and decreased cleavage at the aberrant site as assayed by copper resistance (Fig. 3B). Primer extension analyses indicated that the amount of cleavage at the normal site relative to the aberrant site was at least 20 times greater in the U6-A45C,U46G strain than in the U6–wild-type strain (*31*) (Fig. 3C). Although we cannot demonstrate a direct base-pairing interaction between intron position 5 and U6, the specificity of inhibition of

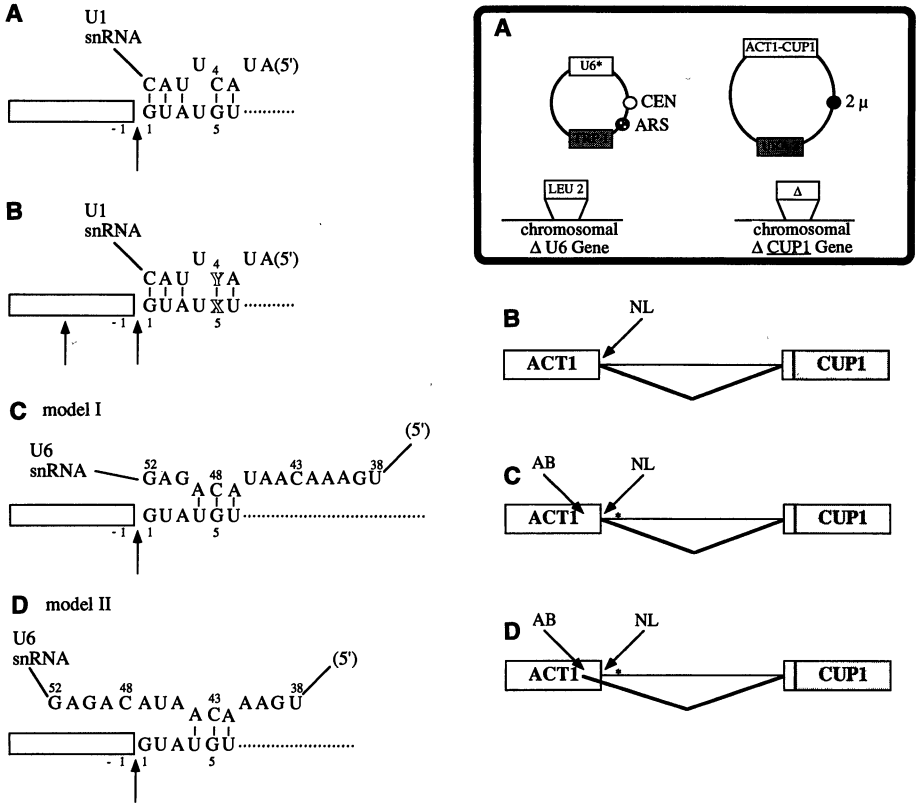

Fig. 1 (left). Base-pairing interactions between the 5′ splice site and U1 and U6 snRNAs. Shown is the complementarity between the *S. cerevisiae* 5′ splice site consensus sequence and either U1 (A,B) or U6 (C,D). The open rectangle represents exon 1; the arrow between positions 1 and −1 indicates the normal cleavage site. (**A**) The 5′ end of U1 is complementary to and base pairs with the 5′ splice site. (**B**) Disruption of this interaction by intron position 5 mutations, depicted by the open-faced X, results in cleavage at an aberrant splice site upstream of the 5′ splice site as represented by the upstream arrow. Restoration of complementarity by the introduction of point mutations in U1 at position 4, depicted by the open-faced Y, does not prevent aberrant cleavage. (**C**) Model I (*10*) proposes that the invariant ACA sequence of U6 (nt 47 to 49, in yeast) base pairs with positions 4 to 6 of the 5′ splice site. (**D**) Model II (*14*) proposes that an upstream ACA sequence of U6 (nt 42 to 44, in yeast) base-pairs with positions 4 to 6 of the 5′ splice site. **Fig. 2 (right).** General strategy for assaying effects of U6 point mutations on intron mutations. (**A**) Shown is the yeast strain YCL47 which contains a deletion of both the chromosomal copies of U6 and CUP1. Low-copy (CEN) plasmids carrying U6 point mutations (*) (*24*) were introduced into this strain by plasmid shuffle methods (*18*) and then ACT1-CUP1 reporters on high copy (2 μm) plasmids (*25*) were introduced into the strain by standard yeast transformation protocols; copper-resistance was then determined (*28*). (**B**) The wild-type reporter plasmid measures cleavage at the normal (NL) cleavage site in the absence of any 5′ splice site mutations. The white boxes represent ACT1 sequences that flank the intron; the filled-in boxes represent CUP1 sequence (*28*). Depicted is a pair of reporter constructs, containing intron position 5 mutations (*), whose copper-resistance reflects cleavage at the normal (NL) (**C**) or aberrant (AB) (**D**) cleavage sites (*27*).

aberrant cleavage by extension of the region of complementarity between U6 and the 5′ splice site supports the hypothesis that base-pairing is required for accurate cleavage.

The above results suggest that mutations at intron position 5 activate aberrant cleavage by weakening the normal interaction with U6 at the 5′ splice site. Several observations suggested that potential, fortuitous base-pairing interactions between nearby sequences in U6 and the mutated intron might re-phase the interaction and thus account for the location of the aberrant site. As we pointed out earlier, the location and efficiency of cleavage at aberrant sites depend on the intron into which the position 5 mutation is introduced (*13*). The base-pairing interaction proposed in model II was based on U6 crosslinking to the yeast actin intron (*14, 15*), which allows a more extended region of complementarity between U6 and the intron (Fig. 4A). Model II aligns the UGU of the 5′ splice site with an ACA sequence (nt 42 to 44) five nucleotides upstream of the ACA (nt 47 to 49) aligned in model I (Fig. 1C), which is the distance between the normal and aberrant cleavage sites in actin. In fact, the position of the aberrant cleavage in the actin intron can be rationalized by the base-pairing interaction proposed in model II since, as in model I, the conserved C at position 48 in U6 is complementary to the G residue five nucleotides downstream of the aberrant site (Fig. 4B).

Since aberrant cleavage can be inhibited by strengthening the base-pairing interaction proposed in model I, strengthening the base-pairing interaction in model II should increase aberrant cleavage. Therefore, as a test, we transformed U6 strains homozygous for each of the position 43 mutations with the intron G5A and G5C reporters (Fig. 2) (*33*). Only the U6-C43G strain exhibited increased copper resistance, relative to the wild-type strain, when transformed with the G5C reporter for cleavage at the aberrant site (Table 1). This mutant strain also exhibited decreased copper resistance relative to the wild-type strain when transformed with the G5C reporter for cleavage at the normal site (Table 1). Primer extension analyses confirmed that the U6-C43G mutation resulted in increased cleavage at the aberrant site activated by the intron G5C mutation (Fig. 5). In contrast, in the case of the G5A mutant intron, the U6-C43G mutation decreased cleavage at both the normal and aberrant sites (Table 1 and Fig. 5). The observed allele specificity for increasing aberrant cleavage with the G5C intron is consistent with model II since U6-C43G, unlike U6-C43A and U6-C43U, should increase the stability of the alternative base-pairing interaction (Fig. 4C) (*34*).

To complete our analysis of this region of U6, all possible point mutations were introduced at positions 38 to 46 (*33*), except for A38U and A45G, because the crosslinking experiments (*10, 14*) indicat-

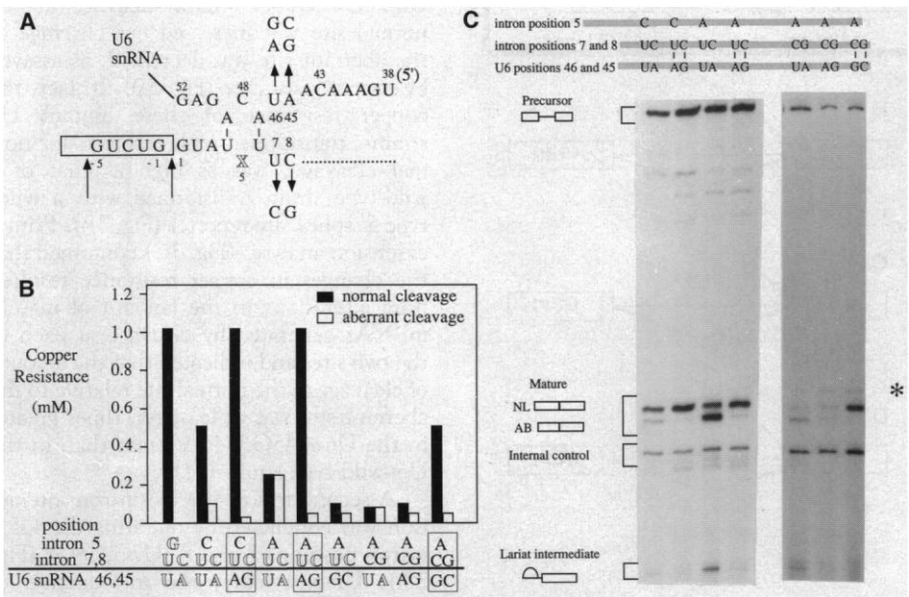

Fig. 3. A test of the base-pairing interaction proposed in model I (*10*). (**A**) Disruption of the potential U6-5′ splice site base-pairing interaction proposed in model I by the introduction of intron position 5 mutations. Mutations at position 5, depicted by the open-faced X, result in activation of aberrant cleavage at position −5 as shown by the upstream arrow. Mutations were introduced at U6 positions 45 and 46 and actin intron positions 7 and 8 to test the prediction that increasing the base-pairing region between these two RNAs should decrease activation of aberrant cleavage. (**B**) The copper resistance of U6-wild-type and U6-position 45,46 mutant strains transformed with ACT1-CUP1 reporters. The first two bars of the histogram reflect copper resistance of a U6-wild-type strain transformed with a wild-type intron ACT1-CUP1 reporter. At the bottom of the graph is indicated the specific sequence of the intron reporter and U6; the boxed combinations indicate when complementarity between U6 and the 5′ splice site should be restored. The open-faced nucleotides represent wild-type sequence. (**C**) Primer extension analysis of RNA isolated from U6-wild-type or U6-45,46 mutant strains carrying intron position 5,7,8 mutant ACT1-CUP1 reporters. Each lane is labeled with the specific genotype of the strain (U6 allele and intron reporter construct) from which the RNA was isolated. The products of the primer extension assays (*49*) are indicated. RNA was isolated from strains carrying a reporter plasmid which generates an in-frame mRNA when cleavage is at the aberrant site. Since out-of-frame mRNAs are significantly less stable than in-frame mRNAs, the ratio of the levels of aberrant mRNA relative to normal mRNA is skewed in favor of the aberrant mRNA (*28*). The * marks a new band that appears when the intron U7C,C8G mutations are introduced (*50*).

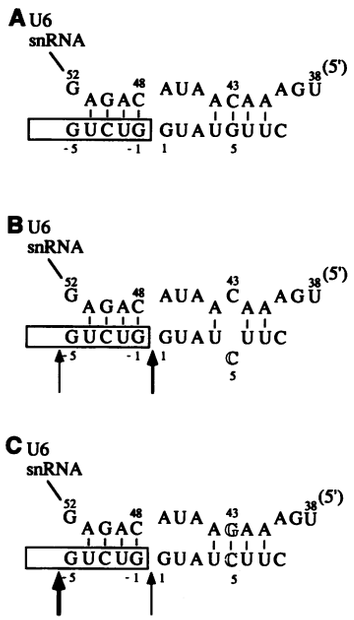

Fig. 4. A test of the base-pairing interaction proposed in model II (*14*). (**A**) The extended base-pairing interaction that can be drawn between the actin intron and U6 (model II). (**B**) The pairing in (A) may be favored when mutations are introduced at intron position 5 (G5C), disrupting the normal base-pairing interaction proposed in model I (Fig. 1C). This alternative base-pairing interaction could activate aberrant cleavage as indicated by the arrow at −5. (**C**) Increasing the complementarity between the G5C intron and U6 by the introduction of the C43G mutation, increased cleavage at the aberrant site and decreased cleavage at the normal site (Fig. 5) as depicted by the thickness of the arrows demarcating the two alternative cleavage sites.

ed that this region of U6 was close to the pre-mRNA. Although many mutations in this region had slight effects on splicing when combined with mutations at intron position 5 (*35*), no change other than C43G increased the absolute amount of cleavage at the aberrant site. All three strains homozygous for point mutations at U6 position 39 exhibited increased copper resistance relative to the wild-type strain when transformed with the intron position G5A reporter for normal cleavage (Table 1). Only the G39C mutation increased cleavage at the normal site with the G5C reporter and decreased cleavage at the aberrant site with both the G5A and G5C reporters (Table 1) (*36*). We do not understand how these mutations at position 39 influence cleavage site choice although the lack of allele specificity suggests that the mechanism differs from that of U6-C43G. It may be relevant that in *Ascaris* this location in U6 is activated for aberrant attack by the branch point when mutations are introduced into U6 upstream of the ACAGAG sequence (*37*).

The base-pairing interaction proposed in model I (Fig. 1C) closely juxtaposes the invariant ACAGAG sequence of U6 with the 5′ splice site. We analyzed the phenotypes conferred by the four U6-ACAGAG mutant strains that are viable at 30°C (A47G, A47U, G50C, and G52U) (*19*) on the position 5 mutant intron reporters. None of these strains conferred a phenotype when splicing the wild-type actin intron; however, all exacerbated the splicing defect of G5A and G5C introns. The position 47 mutations decreased cleavage at both the normal and aberrant cleavage sites, A47G>A47U (Fig. 6A), a result consistent with the severity of their blocks to the first catalytic step in vitro (*20*). The U6-G50C mutation is the only mutation that almost abolishes cleavage at the normal site with both the G5C and G5A reporters, but only decreases cleavage at the aberrant site (G5A>G5C) (Fig. 6A). In addition, the G52U mutation decreased cleavage of pre-mRNA with a wild-type 5′ splice site only slightly, if at all, when assayed either in vivo (*29*) or in vitro (*20*), but when combined with the intron position 5 mutant reporters the U6-G52U mutation almost abolished cleavage at both the normal and aberrant sites (Fig. 6A). Thus, there was a synergistic effect observed when the intron position 5 and the U6-G52U mutations were combined. Taken together with the severe phenotype conferred by the G50C mutation, these results are consistent with model I, in which residues 50 to 52 of U6 are most closely juxtaposed to the cleavage site, and thus likely to function in the first catalytic step.

Suppression of 3′ splice site mutations. Intron position 1, like intron position 5, has at least two roles in the splicing pathway. Position 1 mutations decrease the efficiency of the first catalytic step and result in a complete block to the second catalytic step (*38, 39*). Mutations of the 3′ splice site also inhibit the second step (*8, 38*). Parker and Siliciano (*23*) have shown that specific changes at the first (G1) and last (G303) intron positions of the actin intron can suppress the block to the second catalytic step conferred by each mutation, suggesting a direct interaction between G1 and G303. Model I juxtaposes U6 position 52 close to intron position 1 (Fig. 1C), which is presumably in proximity to G303. Moreover, certain mutations in U6-A51 and U6-G52 inhibit the second step of splicing in vitro (*20*). To test whether this region of U6 is involved in the recognition of the 3′ splice site AG/, we considered the possibility that viable U6 mutations in the invariant ACAGAG sequence (*16, 17, 19*) could suppress the second step phenotypes conferred by 5′ or 3′ splice site mutations (or both). For reporters, we used actin-LacZ constructs carrying mutations at intron position G1

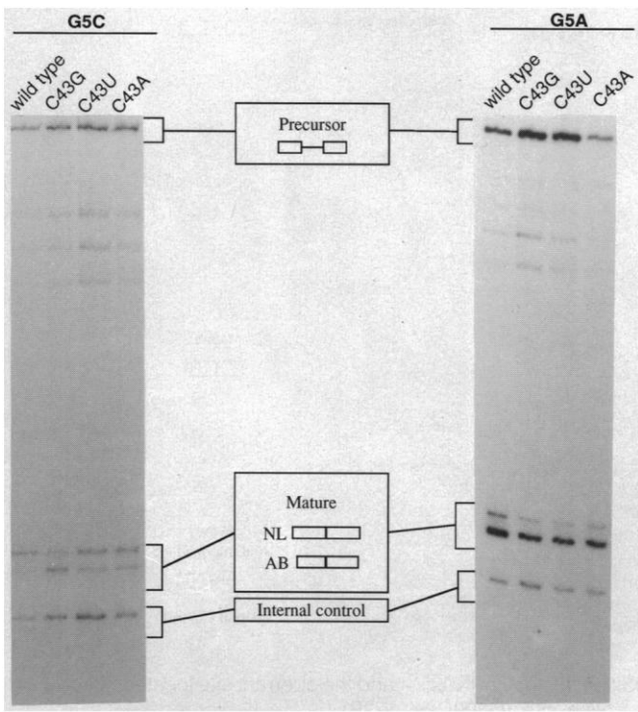

Fig. 5. Primer extension analysis of RNA isolated from U6 position 43 mutant strains transformed with G5A and G5C reporters. Total RNA was isolated from either a wild-type or position 43 mutant U6 strain transformed with the G5A or G5C reporter which generates an in-frame mRNA when cleavage is at the aberrant site. Each lane is labeled with the specific U6 strain and the intron reporter from which the RNA was isolated and the products of the primer extension assays are indicated on the side of the figure (*49*).

Table 1. Summary of copper resistance (mM) of U6 strains transformed with either normal or aberrant reporters.

U6 snRNA	Copper resistance of reporters*	
	G5C	G5A
Wild type	.50/.10	.25/.25
C43G	.10/.13	.10/.08
C43U	.25/.08	.18/.10
C43A	.50/.10	.25/.18
G39C	1.0/.08	.50/.08
G39A	.50/.10	.50/.25
G39U	.50/.10	.50/.25

*The numerator of the ratio represents the level of copper-resistance of a strain transformed with a reporter construct whose growth reflects cleavage at the normal site, while the denominator of the ratio represents the level of copper resistance of the same strain transformed with a reporter construct whose growth reflects cleavage at the aberrant site (*28*).

Table 2. Effects of U6-ACAGAG mutations on 5′ and 3′ splice site mutations in the actin intron.

1 302 303 305*
G/GUAUGU----------UACUAAC---------UAG/AG

Intron mutation‡	β-Galactosidase of wild-type and U6 snRNA mutants†			
	Wild type	A47G	G50C	G52U
G1A	–	–	–	–
G303C	+	–	–	++
G303U	++	++	+	+++
G303A	+/++	+	–	+++
A302C	++	++	+	++
A302U	++	++	–	+++
G1A/G303C	+++	++	–	+
G1A/G303U	+/–	–	–	–
G1A/G303A	+	–	–	–

*All 3′ splice site mutations were analyzed in a G305C background to avoid activation of an aberrant cleavage site at actin exon position 305. †β-galactosidase was determined by overlay assays (*48*) performed on three independent transformants. ‡The U6 snRNA mutants do not affect splicing of a wild-type intron actin-LacZ fusion.

or in the 3′ splice site (A302,G303) or both (*23*, *40*). The U6-G52U mutation was able to weakly suppress the block to the second catalytic step conferred by all three point mutations at G303 (Table 2); in addition, we observed suppression of A302U, but not A302C. In contrast to this suppression with G52U, the U6-G50C and U6-A47G mutations exacerbated the phenotype conferred by the 3′ splice site mutations as seen by a decrease in β-galactosidase (G50C>>A47G) (Table 2). Primer extension analyses of RNA isolated from these U6 mutants, transformed with the G303C ACT1-CUP1 reporter (*23*, *41*), confirm that suppression conferred by the U6-G52U mutation was due to increased cleavage at the mutated 3′ splice site (Fig. 6B) (*42*).

Both the U6-G52U and the intron G1A mutations suppress the block to the second step conferred by the intron G303C mutation. Thus, it is possible that all three nucleotides interact during the second catalytic step of splicing. However, the U6-G52U mutation is not able to suppress the 3′ splice site mutations when combined with an intron position 1 mutation (Table 1). Primer extension analyses of RNA isolated from strains transformed with the G1A-G303C ACT1-CUP1 reporter (*41*) revealed that the loss of suppression was due to a decrease in the efficiency of the first catalytic step, as demonstrated by a decrease in the accumulation of lariat intermediate (Fig. 6C). Thus, it is not possible to evaluate, on a genetic basis, whether U6 position 52 and actin intron positions 1 and 303 interact simultaneously during the second catalytic step. Nonetheless, mutations at position 52 decrease the splicing efficiency of introns mutated at position 1 (Fig. 6C) (*43*) or position 5 (Fig. 6A), yet increase splicing of introns mutated at the 3′ splice site (Fig. 6B). These results imply that U6 position 52 has different roles during each catalytic step of splicing.

Function of 5′ splice site–U6 base-pairing interaction. As shown above, the specificity of 5′ splice site cleavage can be enhanced or reduced by mutations predicted to alter two distinct base-pairing interactions between U6 and the 5′ splice site in the actin intron in *S. cerevisiae*. When invariant position G5 of the intron is altered, mutations predicted to stabilize the interaction shown in model I suppress aberrant cleavage and increase cleavage at the normal site. Conversely, enhancement of the interaction drawn in model II leads to an increase in cleavage at the aberrant site. We propose that the pairing between the conserved UGU at intron positions 4 to 6 (in *S. cerevisiae*) and the invariant ACA at positions 47 to 49 of U6 is a determinant of the proper position of cleavage at the 5′ splice site. This conclusion is consistent with the observation that aberrant cleavage activated by position 5 mutants in the yeast *rp51A* intron was partially suppressed by compensatory changes in U6 position 48 (*44*).

In yeast, only mutations in the invariant G at position 5 activate aberrant cleavage (*11*). Thus the G·C base pair between intron position 5 and C48 of U6 is likely to play a particularly important role in maintaining the fidelity of cleavage site choice. Although 5′ splice site sequences in mammals are considerably more degenerate, position 5 is a G in 85 percent of mammalian introns (*1*). In addition, the base-pairing interaction in model I (Fig. 1C) provides an explanation for the conservation of a U at position 4 of yeast introns (*11*) even though this would prevent base-pairing with the invariant U in U1 (Fig. 1A); thus yeast can form three consecutive base pairs between U6 and the 5′ splice site. Conversely, in mammals this potential interaction would be shortened to two base pairs because the consensus mammalian intron contains an A at position 4 and thereby would be increased in complementarity to U1.

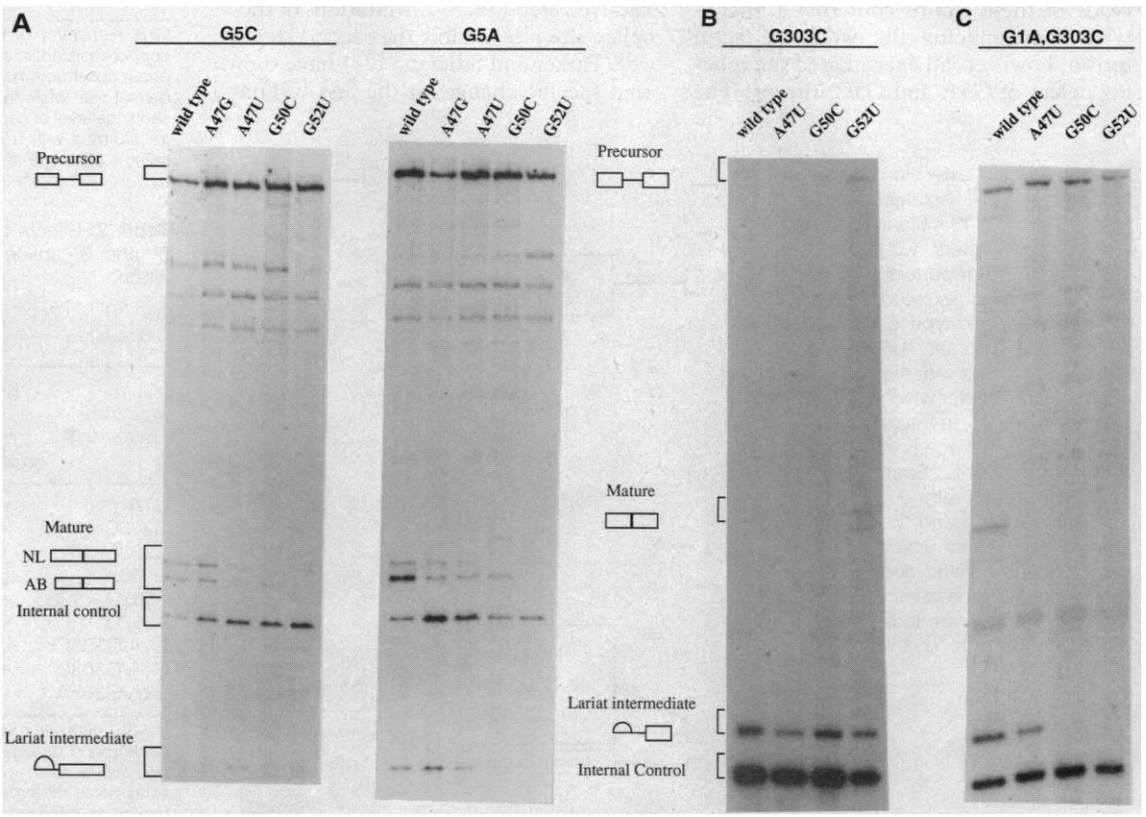

Fig. 6. Primer extension analyses of the RNA isolated from U6-ACAGAG mutant strains transformed with 5′ and 3′ splice site mutant reporters. (**A**) Splicing of the intron G5C and G5A reporters for aberrant cleavage by strains mutated in the U6 ACAGAG sequence. (**B**) Splicing of the intron G303C reporter (*23*, *40*, *41*) by strains mutated in the U6 ACAGAG sequence. (**C**) Splicing of the intron G1A-G303C reporter (*23*, *40*, *41*). Total RNA was isolated from strains containing either U6-wild-type or a U6-ACAGAG mutant transformed with the ACT1-CUP1 reporter designated at the top of each panel. Each lane is labeled with the genotype with respect to the U6 of the specific strain from which the RNA was isolated (*49*). The products of the primer extension assays are indicated at the left side. On longer exposures it is possible to detect both normal and aberrant mRNAs in the G52U lane in (A) in addition to the mRNAs resulting from cleavage at both the mutated 3′ splice site (G303C) and the aberrant site located five nucleotides upstream in the intron (*42*) in (B).

Our experiments also provide an explanation for the location of the aberrant cleavage site activated by mutations of actin at position 5 (*45*). Presumably, the 5′ splice site region of actin can fortuitously form an extended base-pairing interaction with nonconserved sequences in U6 upstream of the invariant ACA. This alternative base-pairing mode would be favored by disruption of the normal base-pairing interaction in position 5 mutants and would be further stabilized by the creation of a G·C base pair between intron position G5C and U6-C43G. This alternative pairing would juxtapose U6-48C with G1 instead of G5 (Fig. 4). Thus, in both normal and aberrant cases, cleavage would occur five nucleotides upstream of the base pair formed with C48 of U6. Although the alternative base-pairing interaction between G5C and the U6-C43G mutant is similar to that proposed for an interaction between U6 and wild-type introns (model II), the crosslinks on which this proposal was based were identified only in the lariat intermediate (*14*); thus model II may reflect a valid U6-5′ splice site interaction that occurs at a different step in the splicing pathway.

Other factors are also likely to contribute to the fidelity of 5′ cleavage. For example, an alternative base-pairing scheme similar to that in model II cannot account for the location of the aberrant cleavage site in position 5 mutants of the *rp51A* intron. Mutations in the invariant loop of U5 activate aberrant cleavage when the intron is mutated at position 1 by base pairing with exon sequences adjacent to the newly activated aberrant 5′ splice site (*8*). Also, the sequences just upstream of the aberrant cleavage sites activated by intron position 5 mutations in *act1* and *rp51A* are complementary to these invariant U5 nucleotides, suggesting that the abnormal cleavage events may be favored by interactions with both U6 and U5.

In summary, three of the five spliceosomal small nuclear RNAs (U1, U5, and U6) can participate in the identification and use of the 5′ splice site. On the basis of kinetic analysis (*9, 10*) and the mutually exclusive base-pairing interactions (Fig. 1A compared to C and D), it seems likely that the U1 and U6 interactions with the 5′ splice site are sequential (*10, 14*), whereas the U5 and U6 interactions may occur simultaneously.

RNA-RNA interactions with the branch point sequence and the 5′ and 3′ splice sites. A model for the spliceosomal active site has been proposed as a result of genetic suppression studies in which a previously undescribed pairing interaction between U6 and U2 (helix I) was identified (*18*). Because formation of this helix is mutually exclusive with the pairing of U6 to U4, which is thought to be the negatively regulated form of U6 (*16*), this rearrangement could correspond to the catalytic activation of the spliceosome (*18*). The further significance of helix I derived from its location immediately upstream of the branch point recognition sequence in U2 and immediately downstream of the highly conserved (*16, 17*) and essential (*19, 20*) nucleotides of the ACAGAG sequence in U6. However, this model left unspecified how the 5′ splice site could be delivered to the presumptive catalytic core. The newly demonstrated base-pairing interaction between the invariant ACAGAG sequence and the 5′ splice site (Fig. 7) can serve the critical function of aligning the 5′ splice site of the substrate with the branch point nucleotide, which is the attacking nucleophile in the first chemical step (*21*).

The configuration shown in Fig. 7 can also accommodate RNA-RNA interactions that occur in the second catalytic step of splicing. Extension of the short 5′ splice site-U6 helix would result in the apposition of the last three residues of the ACAGAG sequence (G50 to G52) with the first three nucleotides of the intron (/GUA). Consistent with this alignment, mammalian crosslinking experiments (*22*) have provided evidence that the last A of the ACAGAG sequence (A51 in yeast) is in close proximity to intron position 2 (/GU). This A-U crosslink is induced in the lariat intermediate but not in the pre-mRNA. Furthermore, as described above, genetic suppression studies have demonstrated interactions between G1 and G303, the first and last nucleotides of the actin intron, during the second catalytic step (*23*). These studies imply that the first and last positions of the intron and the last two nucleotides of the ACAGAG sequence are in proximity during the second step of splicing. This is consistent with the previous demonstration that mutations of residues 51 and 52 of ACAGAG inhibit the second step of splicing of a wild-type actin intron in vitro (*20*).

We have now demonstrated suppression of mutations in the last two positions of the actin intron (AG/) by mutation of G52 in the ACAGAG sequence. The U6-G52U mutation does not exhibit allele specificity; that is, it can suppress the second step block in all G303 mutations as well as one A302 mutation. The more restricted allele specificity of the G1-G303 suppression spectrum probably indicates a direct, but non-Watson-Crick, interaction between these two nucleotides (*23*). Whether the suppression of 3′ splice site mutations by U6-G52U also reflects a direct, albeit complex, set of RNA-RNA interactions bridging G52 to both A302 and G303 cannot be determined by our

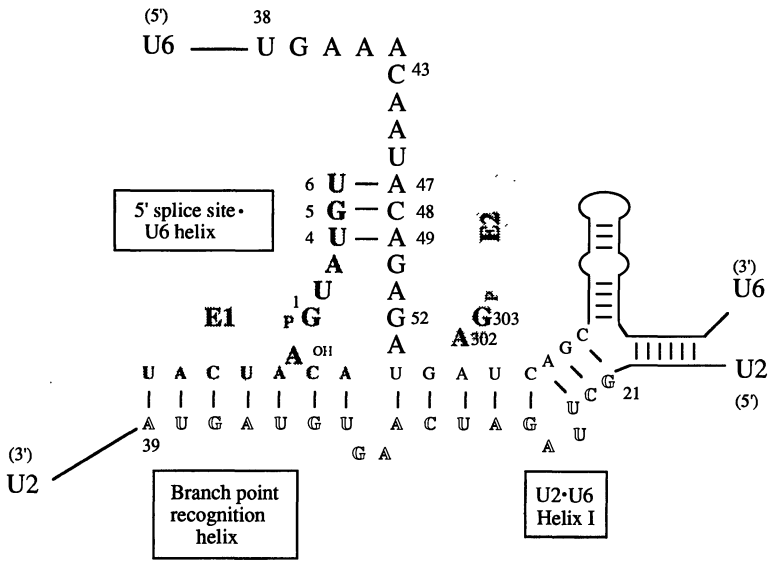

Fig. 7. U6 base-pairing interactions juxtapose the branch point A with the 5′ and 3′ splice sites. The pre-mRNA is shaded and the 5′ and 3′ splice site nucleotides as well as the branch point A are drawn in a larger font and are numbered according to their positions in the yeast actin gene. E1 and E2 represent exon 1 and exon 2, respectively, and the U2 sequence is shown in open-faced print. The proposed base-pairing interaction between U6 positions 47-49 and intron positions 4-6, described in this manuscript (model I), is shown along with four other previously identified helices, indicated in smaller font or line drawings. The interactions between U6 and the intron can act to juxtapose the splice sites in close proximity to the putative catalytic residues of the spliceosome. The four previously identified RNA·RNA helices in the spliceosome are (i) the branch point recognition helix (*5*), (ii) the U2·U6 helix I (*18*), (iii) the U2·U6 helix II (*51*), and (iv) the intramolecular U6 helix (*16, 17, 52*).

experiments. Nonetheless, this interpretation is at least consistent with the genetic and biochemical data linking actin intron G1 to G303 (*23*) and U6-A51 to actin intron U2 (*22*), respectively.

In summary, the 5′ and 3′ splice sites can be modeled as juxtaposed with one another and with the ACAGAG region of U6 by a set of RNA-RNA interactions (Fig. 7) that appear to comprise Watson-Crick as well as noncanonical pairings (*23*, *46*). Since the interactions described above encompass both the first and second chemical steps, this model suggests that the catalytic sites for the two steps might be substantially overlapping (*18*), although not identical (*21*, *47*).

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24. U6 point mutations were generated by polymerase chain reaction (PCR) amplification with primers that contained the desired nucleotide changes (*18*). The mutant U6 genes were cloned into the pSX6 low copy centromere containing vector (*18*).
25. Intron point mutations were generated by PCR amplification with primers that contained the desired nucleotide change (*28*). The ACT1-CUP1 intron fusions under the control of the glucose-6-phosphate-dehydrogenase (GPD) promoter (*28*) were cloned into the high copy (2 μm) RS423 or RS426 vectors [R. S. Sikorski and P. Hieter, *Genetics* **122**, 19 (1989)].
26. Specific intron or small nuclear RNA (snRNA) mutations are represented by a number flanked by letters. The number represents the position of the mutation in the RNA. The letter preceeding the number represents the wild-type sequence and the letter following defines the mutation.
27. Cleavage at the aberrant site normally results in the accumulation of lariat intermediate, because a G is required at intron position 1 in the second catalytic step 2 [B. Seraphin and M. Rosbash, *Cell* **63**, 619 (1990)]. We have changed the U at position −5 in actin (the aberrant cleavage site) to G. This allows the aberrant lariat intermediate to proceed through the second catalytic step, and a mRNA five nucleotides shorter than the normal mRNA is produced.
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30. Although the mutations introduced at U6 positions 45 and 46 should create two new contiguous base pairs in model I, they only negligibly strengthen the base-pairing interaction proposed in model II, in that they would introduce only a single noncontiguous A·U or G·U base pair.
31. Primer extension products were quantified by means of a phosphor-imaging system (Molecular Dynamics), and the data were analyzed with ImageQuant software. The numerical results are based on analysis of three independent transformants. The degree of suppression was determined by comparing the change in the ratio of mRNA generated by cleavage at the normal site relative to the aberrant site in one strain relative to another.
32. A comparison of the sequence of 35 introns of *S. cerevisiae* reveals no sequence conservation at intron positions 7 and 8 (D. N. Frank and C. Guthrie, unpublished data).
33. None of these U6 point mutants conferred a detectable growth phenotype when assayed at 18°, 25°, 30°, or 37°C, nor did they influence splicing of a wild-type intron reporter as assayed by copper resistance.
34. That the U6-C43U mutant did not increase aberrant cleavage activated by the intron G5A mutation may be due to the greater stability provided by a G·C base pair in wild-type and U6-C43G intron-G5C interactions.
35. Mutations in this region decreased the splicing efficiency of introns mutated at position 5 but did not greatly alter the relative amount of cleavage at the aberrant compared to the normal site.
36. Primer extension analyses confirmed that cleavage was increased at the normal site and decreased at the aberrant site.
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40. All actin 3′ splice site mutations were also mutated at position 305 of the pre-mRNA to prevent activation of an aberrant cleavage site (*23*).
41. The intron position 1 and 303 (or both) mutant ACT-CUP1 reporter constructs were provided by P. Siliciano. The construction of these plasmids has been described (*23*). These ACT1-CUP1 fusions contain about 50 additional nucleotides of endogenous actin exon 2 as compared to the ACT1-CUP1 reporters used earlier in our work.
42. Primer extension analyses have shown that when a wild-type strain is transformed with an ACT1-CUP1 reporter that has been mutated at position 303 (G303C, G305C), cleavage occurs very weakly at the mutant 3′ splice site and at an aberrant 3′ splice site (UG/) located upstream from the normal site (*23*). U6-G52U increases cleavage at both the mutated 3′ splice site (G303C) and also at the aberrant 3′ cleavage site (UG/) located five nucleotides upstream from the normal site. In contrast, the intron G1A mutation only increases cleavage at the normal site. The ability of U6-52U to increase cleavage at both the mutated and aberrant sites is consistent with the observation that this mutation does not exhibit allele-specific suppression.
43. Primer extension analyses conducted on RNA isolated from U6-ACAGAG mutant strains transformed with the G1A ACT1-CUP1 reporter confirmed that the G50C and G52U strains decrease the efficiency of the first catalytic step when the intron is mutated at position 1 of the 5′ splice site.
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45. Sawa and Abelson (*14*) have pointed to the possibility that mutation of intron position 5 could lead to incorrect positioning of the catalytic center as a result of disruption of a U6-intron interaction.
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49. Total RNA was analyzed by primer extension with a ^{32}P-end-labeled oligonucleotide complementary to CUP1, which is able to detect pre-mRNA and lariat intermediate and mature mRNA (*28*). A labeled oligonucleotide complementary to U1 was also included in the reaction as an internal control for the amount of RNA in each reaction (*28*). The products were analyzed by electrophoresis through a 6 percent denaturing acrylamide gel with subsequent autoradiography.
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