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Genetic interactions between the 5′ and 3′ splice site consensus sequences and U6 snRNA during the second catalytic step of pre-mRNA splicing

CATHERINE A. COLLINS[1,2] and CHRISTINE GUTHRIE[1]

[1]Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143, USA

ABSTRACT

The YAG/ consensus sequence at the 3′ end of introns (the slash indicates the location of the 3′ splice site) is essential for catalysis of the second step of pre-mRNA splicing. Little is known about the interactions formed by these three nucleotides in the spliceosome. Although previous observations have suggested that the G of the YAG/ interacts with the first nucleotide of the /GUA consensus sequence at the 5′ end of the intron, additional interactions have not been identified. Here we report several striking genetic interactions between A+3 of the 5′ /GUA with Y–3 of the 3′ YAG/ and G50 of the highly conserved ACAGAG motif in U6 snRNA. Two mutations in U6 G50 of the ACAGAG can weakly suppress two mutations in A+3 of the 5′ /GUA. This suppression is significantly enhanced upon the inclusion of a specific mutation Y–3 in the 3′ YAG/. RNA analysis confirmed that the severe splicing defect observed in A+3 and Y–3 double mutants can be rescued to near wild-type levels by the mutations in U6 G50. The contributions of each mutation to the genetic interaction and the strong position specificity of suppression, combined with previous findings, support a model in which the 5′ /GUA and the GAG of U6 function in binding the 3′ YAG/ during the second catalytic step.

Keywords: 3′ splice site recognition; active site; catalysis; model; *Saccharomyces cerevisiae*; spliceosome; tertiary interaction

INTRODUCTION

The removal of introns from pre-messenger RNA (pre-mRNA) is catalyzed by the spliceosome, a large ribonucleoprotein machine comprised of small nuclear RNAs (snRNAs) and at least 60 proteins. The pre-mRNA splicing reaction consists of two chemical steps. In the first step, the 5′ phosphate of the intron (the 5′ splice site) is cleaved by nucleophilic attack from a 2′ hydroxyl group within the intron. In the second step, the 3′ hydroxyl group of the 5′ exon attacks the 3′ phosphate of the intron (the 3′ splice site), resulting in ligation of the two exons and excision of the intron. There is great interest in understanding how the spliceosome catalyzes these two phosphoryl transfer reactions, and in how the sites of chemistry are accurately specified, particularly because spliceosomal RNA appears to play a large role in carrying out these tasks (Nilsen, 1994; Collins & Guthrie, 2000).

An understanding of the mechanism of spliceosome catalysis remains elusive, in part because the active form of the spliceosome, a transient species that forms upon each intron after many conformational rearrangements of its RNA components, has been difficult to isolate for detailed structural and biochemical analysis. In contrast, there is no technical barrier to characterizing the functional effect of mutations in spliceosomal components. Much of our understanding of the structure of the catalytic core comes from analysis of genetic interactions between spliceosomal mutations. In particular, many RNA–RNA interactions within the spliceosome have been inferred from observations of genetic suppression (Madhani & Guthrie, 1994a; Newman, 1994; Nilsen, 1994).

Some of these RNA–RNA interactions have provided substantial information about how the site of cleavage for the first chemical step, the 5′ splice site, is accurately specified. The 5′ intron consensus sequence /GUAUGU (the slash indicates the location of the 5′ splice site) forms base-pairing interactions with a se-

Reprint requests to: Christine Guthrie, Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143, USA; e-mail: guthrie@cgl.ucsf.edu.

[2]Present address: Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110, USA.

quence in U1 snRNA (reviewed in Rosbash & Séraphin, 1991). These interactions help direct the assembly of a spliceosome upon the intron. Later, U1 interactions with the 5′ /GUAUGU are replaced by interactions with U6 snRNA (reviewed in Staley & Guthrie, 1998; Murray & Jarrell, 1999). Watson–Crick base-pairing interactions between some of the 5′ intron consensus residues (/GUAUGU) and some of the residues in U6 snRNA ACAGAG motif play a role in determining the site of cleavage (Kandels & Séraphin, 1993; Lesser & Guthrie, 1993).

In contrast to the 5′ splice site, we know very little about how the 3′ splice site is recognized and specified. Although the 3′ YAG/ sequence is essential for the second step (Y denotes a pyrimidine nucleotide; the slash indicates the 3′ splice site), no base-pairing interactions with snRNAs have been implicated in recognizing this sequence at the second step. An intriguing genetic observation suggests that 3′ YAG/ sequence is recognized, at least in part, by nucleotides in the 5′ consensus sequence. Specifically, intron mutations G+1**a** and G−1**c** (5′ /GUA to /**a**UA and 3′ YAG/ to YA**c**/) can reciprocally suppress each other (Parker & Siliciano, 1993; Chanfreau et al., 1994; Deirdre et al., 1995). Also, a number of introns have now been discovered in higher eukaryotes that contain naturally covariant 5′ /**A**UA and 3′ YA**C**/ sequences (Sharp & Burge, 1997; Burge et al., 1998). The simplest explanation for this striking observation is that the terminal intron Gs form a specific interaction that can be functionally substituted by an interaction between A and C. Although formal proof requires structural analysis, the genetic data strongly imply the existence of a direct physical interaction.

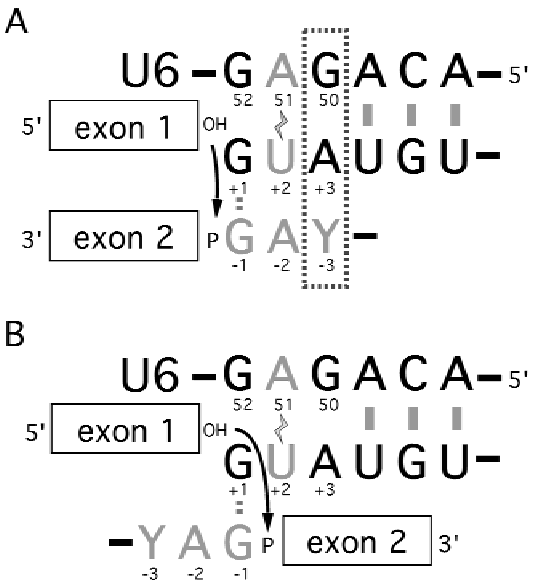

FIGURE 1. Possible alignments of 5′ and 3′ consensus intron nucleotides and the U6 ACAGAG motif (yeast nt 47–52), during the second catalytic step of splicing. Model **A** and Model **B** illustrate two of many possible orientations of the 5′ and 3′ intron nucleotides. Both models accommodate previously observed crosslinking interactions of U+2 to U6 A51 (Sontheimer & Steitz, 1993; Kim & Abelson, 1996), indicated by the lightning bolt, and a genetic suppression interaction between intron nucleotides G+1 and G−1 (Parker & Siliciano, 1993), indicated by the short dotted line. This study describes genetic interactions between A+3, U−3, and U6 G50, which can be juxtaposed (in the rectangle) in Model **A**. In gray are nucleotides that, when mutated, can be suppressed by alleles of *prp8* (Umen & Guthrie, 1996; Collins & Guthrie, 1999; Siatecka et al., 1999). For simplicity, the branchpoint adenosine, which is physically attached to intron nucleotide G+1 during the second step, is not shown.

There is a particularly strong sequence conservation and requirement at the second step for the first and last *two* nucleotides of the intron, 5′ /**GU**A and 3′ Y**AG**/ (Newman et al., 1985; Aebi et al., 1986, 1987; Fouser & Friesen, 1986; Vijayraghavan et al., 1986; Fouser & Friesen, 1987). A previous study found that most combinations of mutations in U+2 and A−2 strongly exacerbate each other (Ruis et al., 1994). Likewise, experiments with 3′ splice site competition reporters suggest that some mutations in A+3 worsen the efficiency of splicing to sites with mutations in Y−3 (Deirdre et al., 1995). Although these results are consistent with the existence of additional interactions between the 5′ and 3′ ends of the intron and an orientation of strands as modeled in Figure 1A, observations of allele-specific genetic suppression, which can more strongly suggest the existence of a direct interaction, were not observed. Current data are consistent with many possible alignments of the 5′ and 3′ ends of the intron; an example is in Figure 1B.

If an interaction between the 5′ and 3′ splice site consensus sequences does occur, several observations suggest that it is likely to involve residues in the ACAGAG motif of U6 snRNA. First, U6 A51 (yeast nomenclature) of the ACAGAG crosslinks to 5′SS +2U (lightning bolt in Fig. 1) before both the first and second steps of splicing (Sontheimer & Steitz, 1993; Kim & Abelson, 1996). Second, mutational analysis has revealed a second step requirement for U6 G50, A51, and G52 of the ACAGAG (Fabrizio & Abelson, 1990; Madhani et al., 1990). It is thus possible that these nucleotides in U6 function concomitantly with the 5′ /GUA and 3′ UAG/. Indeed, pairwise genetic interactions have been observed between U6 G52 and intron nucleotides G+1 and G−1 (Lesser & Guthrie, 1993; Luukkonen & Séraphin, 1998). Last, mutations in a highly conserved protein, Prp8, can simultaneously suppress mutations (in gray type in Fig. 1) in the 3′ YAG/, U+2 of the 5′ /GUA sequence, and U6 A51 (Umen & Guthrie, 1996; Collins & Guthrie, 1999; Siatecka et al., 1999). We have previously suggested that Prp8 influences a tertiary interaction between the affected nucleotides (Collins & Guthrie, 1999).

A possible (although not mandatory) prediction for a tertiary interaction is that some combinations of mutations in the interacting residues might suppress each other. We have conducted a search for such genetic

suppressor interactions between the 5′ and 3′ ends of the intron, together with nucleotides in the U6 ACA GAG motif. We have taken advantage of the *ACT1-CUP1* splicing reporter system to measure the effects of intron mutations in vivo without affecting the viability of the cell (Lesser & Guthrie, 1993). Accurate splicing of the *ACT1* intron allows for expression of the *CUP1* gene product, metallothionein, which chelates toxic copper ions in a dose-dependent manner (Fogel et al., 1988; Jeyaprakash et al., 1991). The splicing efficiency of a mutated reporter intron can be measured indirectly in a strain containing wild-type or mutant U6 snRNA by determining the concentration of copper that the strain can tolerate. The splicing efficiency can also be determined by directly measuring the relative levels of splicing precursor, product, and intermediate species of *ACT1-CUP1* RNA in the strain.

Through this analysis, we have found a number of genetic interactions between residues in the U6 ACA GAG sequence with residues in the 5′ and 3′ splice site consensus sequences. Very strong suppression arises from two related combinations of specific mutations in A+3 of the 5′ /GUA, U−3 of the 3′ YAG/, and U6 G50 of the ACAGAG motif (boxed in Fig. 1A). This functional evidence supports the existence of a tertiary interaction, and strongly supports an antiparallel alignment of 5′/GUA and 3′ YAG/ splice site consensus sequences with the U6 ACAGAG motif as depicted in Figure 1A.

RESULTS

Some 5′ and 3′ intron mutations act synergistically

The effects of double mutations in U+2 and A−2 (/G**x**A—U**x**G/) have been previously reported: all double mutant combinations are less efficient for splicing than single mutants (Ruis et al., 1994). We generated all possible double mutations A+3 and U−3 (/GU**x**—**x**AG/) in *ACT1-CUP1* reporters (Materials and Methods). The effects measured by copper resistance are summarized in Table 1. Most of the double mutations are not significantly worse than the most severe single mutation. However, two double mutations, /GU**c**—**a**AG/ and /GU**c**—**g**AG/, were far more severe than either of the single mutations (shaded in Table 1).

Suppression of 5′ and 5′–3′ mutations by U6 mutations

The copper resistance (Fig. 2) conferred by the *ACT1-CUP1* reporters were compared in strains containing wild-type or viable mutations in U6 (G50**c**, G50**u**, and G52**u**; Madhani et al., 1990) as the sole copy of U6. For some mutant reporters, the copper growth phenotype is suppressed by U6 G50**c** and G50**u**. Highlighted in Figure 2 with black outline, some mutant reporters confer better growth on copper when U6 is mutated to G50**c** and/or G50**u** than when U6 is wild type. G50**c** and G50**u** increase the copper resistance of /GU**c**—UAG/ 2–2.5-fold (Fig. 2; Table 2), and /GU**g**—UAG/ less than 2-fold (Fig. 2; data not shown). However, these U6 mutations worsen the copper resistance of /GU**u**—UAG/ (Fig. 2).

TABLE 1. Copper resistance of *ACT1-CUP1* reporters containing mutations in 5′ A+3 and 3′ U−3 (mM $CuSO_4$).

		3′ splice site			
		UAG/	**c**AG/	**a**AG/	**g**AG/
5′ splice site	/GUA	2.0	1.5	1.75	0.1
	/GU**c**	0.3	0.3	0.025	0.025
	/GU**g**	0.2	0.1	0.2	0.1
	/GU**u**	0.5	0.2	0.2	0.05

The highest copper concentration tolerated by strains containing each /GU**x**—**x**AG/ mutant reporter was determined in at least three independent replica-plating assays. Shaded are double mutants that are substantially worse than either of the respective single mutants.

A particularly striking result was that the copper resistance of one double mutant reporter /GU**c**—**a**AG/ was very strongly increased (20-fold by U6 G50**c** and 30-fold by G50**u** (Table 2; asterisk in Fig. 2). This is a significant enhancement over the 2–2.5-fold effects of G50**c** and G50**u** on the single mutant /GU**c** reporter. This suggests that Y−3 of the 3′ YAG/ strongly influences the interaction between the 5′ /GUA and U6.

The strong suppression is specific to the identity of the 3′ splice site mutation, because the similarly severe defect of /GU**c**—**g**AG/ (Table 1) is not suppressed by the U6 mutations (Fig. 2). We also asked whether additional reporters containing 5′ /GU**c** combined with other 3′ splice site mutations could be suppressed. Of the mutations tested, /GU**c**—UA**a**/, /GU**c**—UA**c**/, and /GU**c**—UA**u**/, none were suppressed by U6 G50 mutations (data not shown). Thus the strong suppression is specific to the /GU**c**—**a**AG/ combination of intron mutations.

Strong *position* specificity in suppression by U6 mutations

The /GU**x**—**x**AG/ reporters are only suppressed by U6 G50**c** and/or G50**u**. The nearby U6 G52**u** mutation exacerbates the copper resistance of many /GU**x**—**x**AG/ mutant reporters, but does not suppress any (Fig. 2). In contrast, U6 G52**u** can suppress mutations in G−1 of the 3′ YAG/ (Lesser & Guthrie, 1993; data not shown). The U6 G50**u**, G50**c**, and G52**u** mutations were also assayed with all of the /G**x**A—U**x**G/ *ACT1-CUP1* reporters. No suppression was observed (data not shown). Thus suppression is very specific to position, consistent with an alignment of the 3′ YAG/ with the 5′ /GUA and U6, as drawn in Figure 1A.

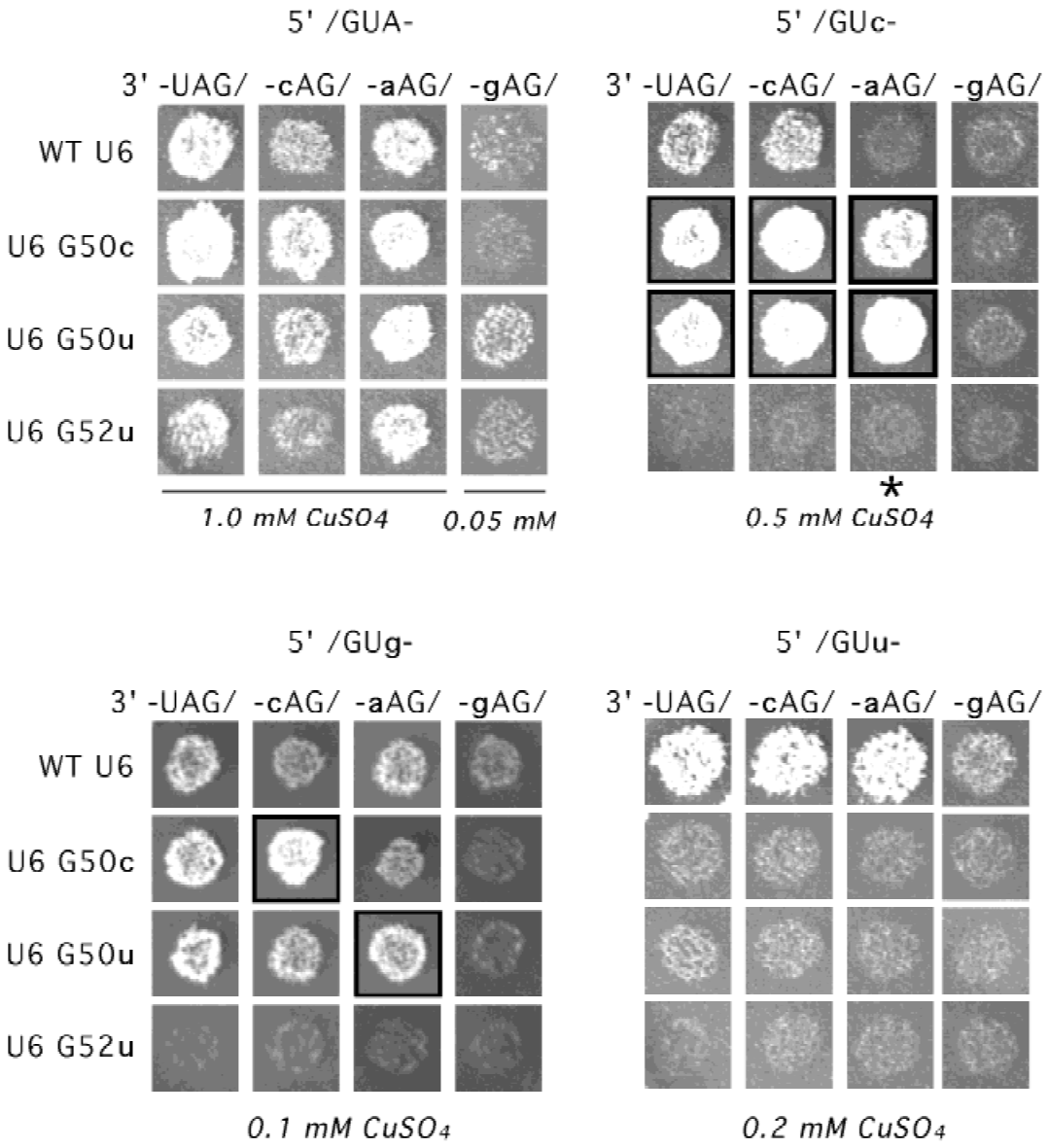

FIGURE 2. Copper growth of combinations of mutations in intron nucleotides A+3 and U−3 with viable mutations in U6. For each *ACT1-CUP1* reporter, the copper growth phenotype is shown at a concentration of copper that is limiting for growth when the strain contains wild-type U6 (top row in each quadrant). Each column shows yeast patches containing wild-type or mutant U6 that were replica-plated onto the same copper plates. Black outlined boxes highlight the observations in which U6 G50**c** and/or G50**u** mutations confer a higher copper resistance to (suppress) specific reporters, and which were observed reproducibly in at least three independent assays. The asterisk highlights the particularly strong suppression conferred by both U6 G50**c** and G50**u** to the /GU**c**—**a**AG/ reporter.

U6 50c and 50u increase the splicing efficiency of the /GUc—aAG/ intron

We confirmed that the strong increase in copper resistance conferred by the /GU**c**—**a**AG/ reporter with U6 50**c** and 50**u** was indeed due to an increased splicing efficiency, and that this splicing takes place at the correct junctions. RNA was isolated (in triplicate) from each strain in Figure 2. The splicing efficiency of the *ACT1-CUP1* reporter RNA was assayed by primer extension analysis, using a ^{32}P-labeled oligo that hybridizes to the second exon of the reporter. Figure 3 and Table 2 show measurements (in the presence of wild-type or mutant alleles of U6) for the 5′ and 3′ single mutant reporters, /GU**c**—UAG/ and /GUA—**a**AG/, and the /GU**c**—**a**AG/ double mutant reporter.

In the presence of wild-type U6, the /GU**c**—UAG/ and the /GU**c**—**a**AG/ reporters are very defective for splicing, showing reduced levels of spliced *ACT1-CUP1* mRNA, consistent with the low levels of copper resistance, and increased levels of lariat intermediate and precursor species. The U6 G50**c** and G50**u** mutations confer a modest increase in the splicing efficiency of the /GU**c**—UAG/ reporter (1.7- and 2.5-fold; Table 2),

TABLE 2. Quantitation of suppression of splice site mutations by mutations in U6.

		(a) Copper resistance		(b) Total splicing efficiency		(c) Second-step efficiency	
Reporter intron	U6 allele	[CuSO4] tolerated	Fold increase	[Mature]/[Precursor]	Fold increase	[Mature]/[Lariat Int.]	Fold increase
/GUA—UAG/	WT U6	2.0	—	13 ± 1.7	—	122 ± 46	—
	G50c	1.75	0.9×	7.3 ± 0.8	0.6×	43 ± 5.0	—
	G50u	1.5	0.75×	5.6 ± 0.5	0.4×	67 ± 0.5	—
/GU**c**—UAG/	WT U6	0.3	—	1.3 ± 0.2	—	4.7 ± 1.0	—
	G50c	0.5	**1.7×**	1.8 ± 0.3	**1.4×**	37 ± 12	**8×**
	G50u	0.75	**2.5×**	1.7 ± 0.3	**1.3×**	55 ± 11	**12×**
/GUA—**a**AG/	UT U6	1.75	—	10.7 ± 0.8	—	31.6 ± 6.7	—
	G50c	1.5	0.9×	6.3 ± 0.05	0.6×	9.3 ± 0.8	0.3×
	G50u	1.5	0.9×	5.1 ± 0.3	0.5×	39.0 ± 4.0	1.2×
/GU**c**—**a**AG/	UT U6	0.025	—	0.2 ± 0.02	—	0.06 ± 0.005	—
	G50c	0.5	**20×**	2.2 ± 0.1	**11×**	4.5 ± 0.3	**75×**
	G50u	0.75	**30×**	3.9 ± 0.4	**20×**	12.8 ± 0.8	**213×**

In column (a) the level of copper resistance (mM copper tolerated for growth) for each strain was measured (three independent times). In column (b) the total splicing efficiency is estimated by the ratio of steady state levels of mature to precursor species (as described in Pikielny & Rosbash, 1985), measured by phosphorimager analysis. These numbers are the average of two to six independent primer extension experiments. For (c), the efficiency of the second catalytic step of splicing as estimated by the ratio of mature to lariat intermediate levels (Fouser & Friesen, 1986). Estimates for cases in which U6 G50**c** and G50**u** confer an increase in the splicing efficiency (compared to WT U6) are indicated in bold.

and a very striking increase in the efficiency of the /GU**c**—**a**AG/ reporter (20- and 30-fold; Table 2). The strong increase in levels of mature, accompanied by a significant decrease in the accumulated lariat intermediate species (Fig. 3, last two lanes) suggests that U6 mutations greatly enhance the efficiency of the second step for the /GU**c**—**a**AG/ reporter. Upon quantitation, we measured a >200-fold increase in the efficiency of the second step (Table 2, column c).

We confirmed that the mRNA species that accumulates when /GU**c**—**a**AG/ is suppressed by the U6 mutations, which is of the correct size, is indeed created by splicing to the correct junctions. We gel purified the primer extension product and amplified it by PCR for sequencing analysis (see Materials and Methods). The sequence corresponds to wild-type mRNA, generated by splicing to the correct 5′ and 3′ junctions.

A smaller primer extension product (asterisk in Fig. 3) is abundant for the /GU**c**—**a**AG/ reporter (and, not shown, for a few other mutant reporters) in the presence of wild-type U6, but disappears when the splicing defect is suppressed by the U6 mutations. It does not appear to be a splicing product from cryptic 5′ and/or 3′ splice sites (see Materials and Methods). Because we see this primer extension product for a few other reporter mutants that exhibit strong splicing defects (data not shown), we suspect it reflects a degraded form of an intron-containing species.

DISCUSSION

We have explored the possibility that nucleotides in the 5′ and 3′ intron consensus sequences functionally interact with each other and/or nucleotides in the ACA GAG motif of U6 snRNA. A possible (although not mandatory) prediction for this hypothesis is that a specific combination of mutations in the interacting residues may be able to functionally rescue (suppress) each other. To ask whether suppression can occur, we have systematically evaluated the effect of combining mutations in the 5′ and 3′ splice site residues with viable mutations in U6.

We have described several instances of genetic suppression. One set of observations provides support for an interaction between the 5′ intron nucleotide A+3 with G50 of U6. Two mutations in U6 G50, G50**c** and G50**u**, weakly suppress two mutations in position +3 of the 5′ splice site, 5′ /GU**c** (2–2.5-fold) and 5′ /GU**g** (less than 2-fold). These results complement previous observations of interactions between adjacent nucleotides: Watson–Crick base pairing between U+4 and U6 A49 (Kandels & Séraphin, 1993; Lesser & Guthrie, 1993); photocrosslinking between U+2 and U6 A51 (Sontheimer & Steitz, 1993; Kim & Abelson, 1996); and strong exacerbation of splicing defects upon combining mutations in G+1 and U6 G52 (Lesser & Guthrie, 1993; Luukkonen & Séraphin, 1998). However, this is the first

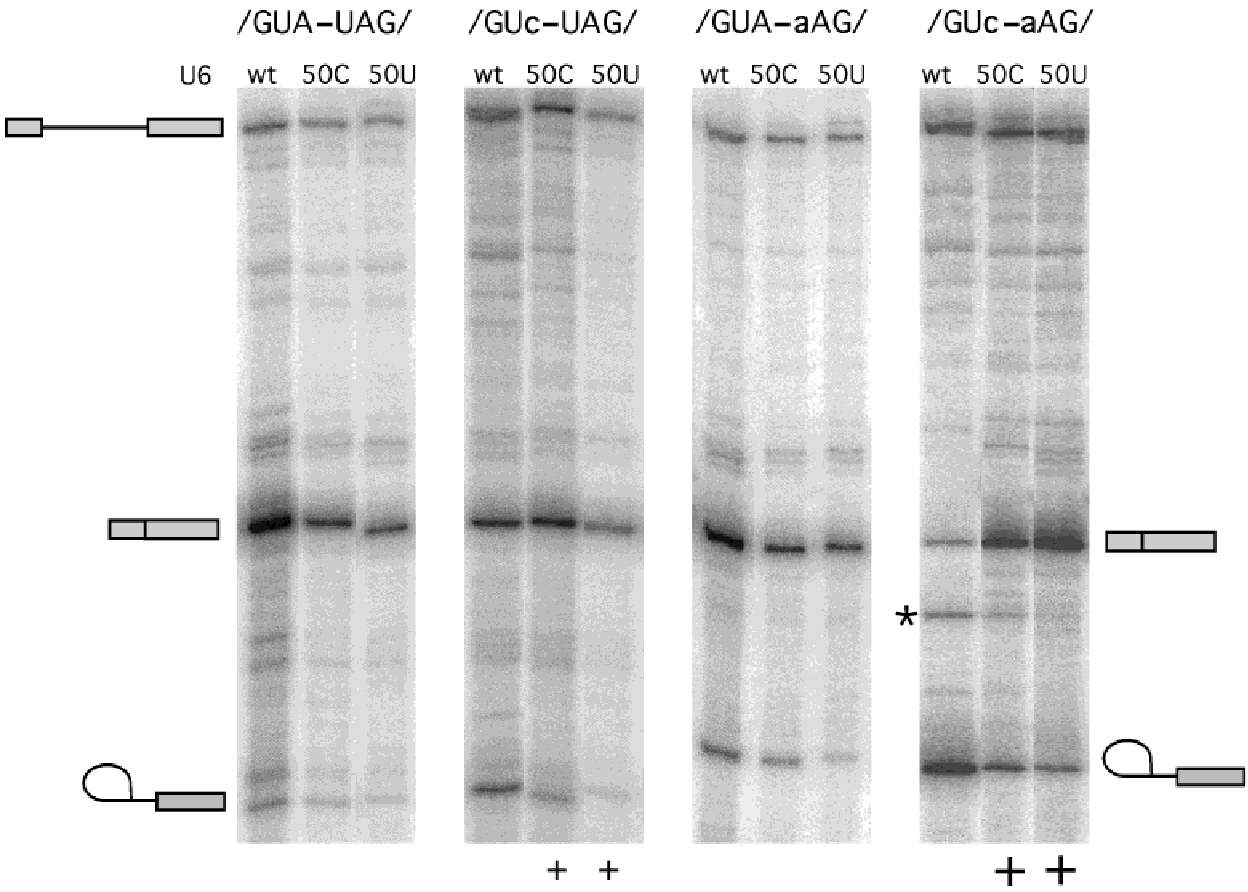

FIGURE 3. Primer extension analysis of suppression. The migration of primer extension products, using an oligo that hybridizes to exon 2 (CUP1-CC4), is indicated for mature, lariat intermediate, and precursor species in cartoon on the side of the gel. Cases in which U6 mutations increase the splicing of the mutant *ACT1-CUP1* reporter (see Table 2) are indicated by + symbols. Although some of the lanes have been moved with respect to one another for clarity, all of the reactions were conducted within the same experiment, and exposed together on one phosphorimager screen. The asterisk denotes an unknown primer extension product seen for the /GU**c**—**a**AG/ reporter, discussed in the text.

report of genetic suppression between A+3 and U6 G50. A previous study using an *RP51* intron reporter reported that the U6 G50 mutations worsen the splicing defect of the 5′SS /GU**g** mutation (Luukkonen & Séraphin, 1998). It is possible that this small difference from our data reflects differences in the sensitivities of the *ACT1* and *RP51* intron reporters. Taken together, these results suggest the existence of an extended interaction between the 5′ /GUAUGU intron consensus sequence with the ACAGAG motif of U6. Although only part of the interaction consists of Watson–Crick base pairing, the remaining nucleotides appear to interact in a pairwise fashion (A+3 with U6 G50; U+2 with U6 A51; and G+1 with U6 G52).

We were interested in determining whether the interaction between the 5′ /GUA and U6 functions to recognize nucleotides in the 3′ YAG/ consensus sequence. A particularly striking set of observations of suppression supports this hypothesis: the severe splicing defect of a specific combination of 5′ and 3′ intron mutations (/GU**c**—**a**AG/) was suppressed to near wild-type levels of splicing by U6 G50**c** (a 20-fold increase in copper resistance) and U6 G50**u** (a 30-fold increase). The combination of 5′ and 3′ mutations in the /GU**c**—**a**AG/ reporter causes a particularly strong splicing defect in the presence of wild-type U6 (Table 1; Fig. 2). Thus, although the degree of copper resistance conferred by U6 G50**c** and G50**u** upon the /GU**c**—**a**AG/ reporter is similar to that of the /GU**c**—YAG/ reporter (Fig. 2; Table 2), the fold suppression is far greater for the double mutant /GU**c**—**a**AG/ reporter. Although the 3′ mutation alone in the /GUA—**a**AG/ reporter is not severe, the U6 G50 mutations worsen its splicing (Fig. 3; Table 2). In summary, the suppression we observe upon combining three mutations is much stronger than the effects of any two mutations. These results suggest that 5′ A+3, 3′ Y−3, and U6 G50 directly influence each other.

Does the allele specificity of suppression reflect a direct interaction?

The strong suppression is specific to one combination of 5′ and 3′ mutations (/GU**c**—**a**AG/). However, both viable mutations U6 G50**u** and G50**c** confer high levels of suppression to this double-mutant reporter. To compare effects of the G50**a** mutation (which is inviable as the sole copy of U6 snRNA in the cell) with the G50**c** and G50**u** mutations, we assayed the effects of each mutation on all of the mutant *ACT1-CUP1* reporters in the presence of an additional wild-type copy of U6 (data not shown). Whereas the U6 G50**c** and G50**u** mutations can dominantly suppress /GU**c**—**a**AG/, the U6 G50**a** mutation did not suppress any of the mutant *ACT1-CUP1* reporters. Previous in vitro analysis suggests that the failure of U6 G50**a** to suppress is not due to defects in spliceosome assembly, because the mutant snRNA can assemble into spliceosomes and exhibits a block to the splicing of wild-type introns at the second step (Fabrizio & Abelson, 1990; Madhani et al., 1990).

Two explanations are currently possible for the observation that *both* G50**c** and G50**u** suppress /GU**c**—**a**AG/. One is that suppression of /GU**c**—**a**AG/ by the U6 mutations occurs through the formation of a specific interaction between the three mutated residues, and that both pyrimidine mutations C and U are able to form this interaction, whereas the A mutation is not. An alternative explanation is that the U6 G50**c** and G50**u** mutations create mild disruptions in the fidelity of splicing in a way that is particularly advantageous for the /GU**c**—**a**AG/ double mutant intron. In contrast, the G50**a** mutation may not allow for suppression because it severely disrupts splicing. Although the first scenario for suppression requires a direct interaction between the three residues, the second scenario for suppression could take place either via a direct interaction, or via additional interactions and possibly additional factors.

To further explore the possibility of a direct interaction, we asked whether the genetic suppression results could reflect the formation of a standard base triple. Specifically, we asked whether the A+3, Y−3, and U6 G50 residues could be modeled into a base triple (containing at least two hydrogen bonds between each base) that could be structurally mimicked by a base triple between the A+3**c**, Y−3**a**, and U6 G50**u** or G50**c** mutations (see Materials and Methods). Although we found no satisfactory triple models, many other forms of direct RNA–RNA interaction remain possible, but cannot be evaluated with the current genetic data.

Because U6 G50**c** and G50**u** confer weak suppression to several /GU**x** and /GU**x**—**x**AG/ mutations, we favor the second explanation for suppression: Rather than allowing specific interactions to form, the G50**c** and G50**u** mutations loosen the stringency of requirements for certain nucleotides at 5′ position +3 and 3′ position −3. If specificity is loosened via mild perturbations in the space surrounding these nucleotides, it is unlikely to be entirely lost. Some particularly severe mutations, such as 3′ **g**AG/, may remain unrescuable, and other mutations, such as 5′ /GU**u**, might suffer negative rather than permissive effects. To understand the basis for strong suppression of /GU**c**—**a**AG/, one needs to determine why this combination of 5′ and 3′ intron mutations is so severe. This combination may allow or cause the formation of an aberrant interaction, which the U6 G50 mutations can relieve. Ultimately, structural analysis is required to resolve these issues.

Position specificity of genetic interactions suggests an orientation of intron sequences

The suppression we observe is highly specific to position. Only mutations in U6 G50 can suppress +3 and +3/−3 intron mutations. The mutation G52**u**, which has been previously observed to suppress −1 mutations and exacerbate +1 mutations (Lesser & Guthrie, 1993; Luukkonen & Séraphin, 1998), either did not affect or exacerbated the splicing of all the +3/−3 and +2/−2 mutations. [We did not observe the previously reported weak suppression of −2 mutations (Lesser & Guthrie, 1993), perhaps due to slight differences between the β-galactosidase and *CUP1* constructs and assays.] We also detected no effect for mutations in U6 A51 (which are dominant negative) upon any +3 and/or −3 mutations (data not shown). The mutations in U6 G50 had no effect upon any of the +2/−2 mutations, nor upon +3/−1 mutations (data not shown). Thus suppression by U6 G50c and G50u appears specific to mutations in intron positions +3 and −3.

When combined with previously observed evidence for an interaction between the G+1 and G−1 intron nucleotides (Parker & Siliciano, 1993; Chanfreau et al., 1994; Deirdre et al., 1995), even an indirect interaction between A+3, Y−3, and U6 G50 suggests an alignment of strands as drawn in Figure 1A. The model in Figure 1A is also supported by previously characterized genetic interactions of Prp8, a highly conserved protein that can be crosslinked to nucleotides within and near the 5′ /GUA, 3′ YAG/, and to U6 near the ACAGAG motif (Teigelkamp et al., 1995; Umen & Guthrie, 1995a; Chiara et al., 1996, 1997; Reyes et al., 1996; Vidal et al., 1999). A specific class of *prp8* alleles can simultaneously suppress the second-step defect of mutations in the 3′ YAG/ sequence, U+2 of the 5′ GUA sequence, and in U6 A51 of the ACAGAG motif (Collins & Guthrie, 1999; Siatecka et al., 1999). The simplest explanation for these results is that Prp8 influences a tertiary interaction between the affected nucleotides. These nucleotides, in gray, are juxtaposed in Figure 1A. The observations reported here of strong genetic sup-

pression through mutations in RNA nucleotides (in the presence of wild-type Prp8), supports the idea that Prp8 acts upon a tertiary RNA structure (rather than influencing different nucleotides independently of one another).

The model in Figure 1A is also attractive because the orientation of the 5′ /GUA and 3′ YAG/ sequences could allow for a similar orientation for the reactive sites of the two steps of splicing, the 5′ splice site and the 3′ splice site. Because Figure 1 depicts the second chemical step of splicing, the 5′ splice site (immediately preceding the 5′ GUA) has already been cleaved, and the scissile phosphate of the 3′ splice site (immediately downstream of the 3′ YAG) is about to be cleaved. The possible orientation of the two sites invites some interesting speculation about the mechanism by which the spliceosome catalyzes the two steps of splicing. Although the two steps have different chemical requirements (Moore & Sharp, 1993; Sontheimer et al., 1997; Gordon et al., 2000), a favored hypothesis is that both steps are catalyzed by one spliceosomal structure, which could undergo modest conformational changes between the two steps (Steitz & Steitz, 1993; Umen & Guthrie, 1995b; Staley & Guthrie, 1998). If the two splice sites are similarly oriented, the same catalytic components of the spliceosome (e.g., the same metal ions) might be able to act upon each site via modest conformational changes between the two steps. In the contrasting orientation in Figure 1B, the two sites of chemistry are separated by the G+1 and G−1 interacting nucleotides, and when modeled in three dimensions, would lie much farther apart in space.

A mechanism for recognizing the 3′SS YAG/

The model in Figure 1A suggests a mechanism for recognizing the 3′ YAG/ sequence and orienting the 3′ splice site for the second catalytic step of splicing, while simultaneously providing an explanation for the defects in the second step conferred by mutations in the 5′ /GUA and in the interacting U6 GAG nucleotides (Newman et al., 1985; Aebi et al., 1986, 1987; Fouser & Friesen, 1986; Vijayraghavan et al., 1986; Fouser & Friesen, 1987; Fabrizio & Abelson, 1990; Madhani et al., 1990; Luukkonen & Séraphin, 1998). Understanding the exact nature of the structure formed by the 5′ /GUA, 3′ YAG/, and the U6 ACAGAG, and the contributions to this structure made by Prp8 awaits higher-resolution structural analysis of the catalytic core. Based on previously reported genetic and crosslinking interactions with the 3′SS YAG/, other RNA nucleotides from U2/U6 helix I are likely to participate in this structure (Madhani & Guthrie, 1994b; Newman et al., 1995; Chang & McPheeters, 2000).

Although we suggest here that the 3′ YAG/ is recognized by RNA interaction (in conjunction with Prp8), we note that previous studies suggest that the 3′ YAG is recognized at multiple stages of splicing. In metazoans, the protein U2AF35 functions in recognizing the 3′ YAG/ early during spliceosome assembly (Merendino et al., 1999; Wu et al., 1999; Zorio & Blumenthal, 1999). This early protein-based recognition of the 3′ YAG sequence is not absolutely required for spliceosome assembly or the first chemical step of splicing (Frendewey & Keller, 1985; Reed & Maniatis, 1985; Rymond & Rosbash, 1985; Rymond et al., 1987). Rather than functioning in catalysis, this early recognition may ensure that the spliceosome assembles upon an intron that is competent to undergo splicing subsequently. The 3′ YAG might be recognized again in the process of selecting the correct 3′ YAG sequence for splicing (Umen & Guthrie, 1995b). The distance of a 3′ YAG sequence from the branchpoint, the presence of an upstream polypyrimidine tract, and the protein Slu7 function in the process of selecting a 3′ YAG among competing YAG sequences (Patterson & Guthrie, 1991; Frank & Guthrie, 1992; Brys & Schwer, 1996; Chua & Reed, 1999). Whether recognition of the 3′ YAG during selection is mediated by the 5′ /GUA and U6 GAG (and Prp8) or by different or additional interactions is not known. [A previous study of the proposed interaction between the G+1 and G−1 detected only mild effects in the *selection* of a 3′ splice site over competing sites (Luukkonen & Séraphin, 1997).] Because the spliceosome is thought to have evolved from an RNA enzyme (Sharp, 1985), Figure 1A provides an attractive model for interactions within an RNA-based catalytic core, that could function during catalysis of the second step.

MATERIALS AND METHODS

Strains and reagents

All +3, −3 single and double mutant *ACT1-CUP1* reporters (pCC168 through pCC183, and pCC188 through pCC191) were constructed in the pGAC14 (*TRP*) vector by QuickChange mutagenesis (Stratagene). For all of these reporters, position 305 has also been mutated to eliminate a cryptic AG 3′ splice site: (UAG/AG to UAG/A**u**). Each construct was sequenced to confirm identity of the introduced mutations. The +2, −2 double mutant *ACT1-CUP1* reporters, described originally in Ruis et al. (1994), were provided by Paul Siliciano. U6 mutants (pCC134–137, 145, 146, and 108) were subcloned pSE362 (CEN, HIS) from constructs described in Madhani et al. (1990). *prp8* alleles were carried on pRS424 (2 μm, TRP) plasmids (Collins & Guthrie, 1999), or pRS313 (CEN, HIS) (pCC197, pCC198, and pCC125).

The *cup1* deletion strains are derived from the strains described in Lesser and Guthrie (1993). U6 mutants were assayed in the double deletion strain yCC30: *cup1*Δ*::ura3, U6*Δ*::LEU2; his3, ura3, leu2, trp1; WT U6-URA*, constructed by Cammie Lesser. U6 WT or mutant plasmids (in pSE362) were cotransformed into this strain with each *ACT1-CUP1* reporter. The WT U6-URA plasmid was then shuffled out by growth on 5-FOA at room temperature.

Identification of cDNA products

Primer extension products mRNA (using radiolabeled CUP1-CC4 as the primer) for /GU**c**—**a**AG/ *ACT1-CUP1* mRNA in the presence of wild-type and mutant U6 were gel purified. We used an oligo to the 5′ end of exon 1 (OAK10) together with the exon 2 CUP1-CC4 oligo to PCR amplify the product with *Taq* polymerase. The PCR products were cloned into pCR2.1 TOPO using the Topo TA cloning strategy (Stratagene), and three independent isolates were sequenced. The same procedure was applied to the smaller primer extension product (asterisk in Fig. 3) generated for /GU**c**—**a**AG/ in the presence of wild-type U6. PCR with the exon primers yielded a product of the same size and sequence of the wild-type mRNA (possibly reflecting small levels in the gel slice), but did not generate a smaller product, which would be expected if an upstream 5′ splice site and/or downstream 3′ splice site were utilized.

Oligos

The following oligos were used in this work:

CUP1-CC4: 5′-GCAGCTACCACATTGGCATTG-3′;
OAK-10: 5′-GGATCCCCGGCAGCT-3′.

Copper growth, primer extension

Copper resistance and primer extension assays, to measure the splicing efficiency of the *ACT1-CUP1* reporters, were conducted as previously described in Collins and Guthrie (1999).

Modeling of potential base interactions

We were assisted by Bernhard Walberer and Alan Frankel at the University of California, San Francisco, who constructed a structural database of all possible RNA base pair and base triple interactions (involving at least two hydrogen bonds for each base; Walberer, 2000). We screened this database for possible G(U650)—A(+3)—U(−3) triples that had structural similarity (in the location of glycosidic bonds) to potential C—C—A or U—C—A triples for the mutated residues. We also evaluated the ability of the 5′ /GUA, 3′ YAG/, and U6 ACAGAG sequences to form a standard triple helix, in which each triple displayed structural overlap with the adjacent positions. We did not find any compelling base triples that were consistent with genetic observations.

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