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D. S. McPheeters

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DAVID S. McPHEETERS

Department of Biochemistry, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106-4935, USA

ABSTRACT

The small nuclear RNA (snRNA) components of the spliceosome have been proposed to catalyze the excision of introns from nuclear pre-mRNAs. If this hypothesis is correct, then the snRNA components of the spliceosome may interact directly with the reactive groups of pre-mRNA substrates. To explore this possibility, a genetic screen has been used to identify potential interactions between the U6 RNA and the pre-mRNA branch site. Notably, the selection yielded mutants in two regions of the yeast U6 RNA implicated previously in the catalytic events of splicing. These mutants significantly increase the splicing of pre-mRNA substrates containing non-adenosine branch sites. U6 mutants in U2/U6 helix Ia show strong allele-specific interactions with the branch site nucleotide and interact with *PRP16*, a factor implicated previously in branch site utilization. The other mutants cluster in the intramolecular helix of U6 and suppress the effects of branch site mutations in a nonallele-specific fashion. The locations of these mutants may define positions important for binding of the U6 intramolecular helix to the catalytic core of the spliceosome.

Keywords: messenger RNA; RNA; RNA catalysis; RNA splicing; U2 RNA

INTRODUCTION

The removal of introns from nuclear pre-mRNAs occurs via two sequential transesterification reactions that are catalyzed by a large complex termed the spliceosome. Assembly of spliceosomes on intron-containing pre-mRNA substrates occurs through a highly ordered, multistep pathway requiring a large number of factors, including the U1, U2, U4/U6, and U5 small nuclear ribonucleoprotein particles (snRNPs). Mechanistic similarities in the self-splicing of group II introns and the splicing of nuclear pre-mRNA introns has led to the proposal that these processes may be related evolutionarily, and that the RNA components of the U1, U2, U4/U6, and U5 snRNPs may be responsible for the catalytic reactions of nuclear pre-mRNA splicing (Sharp, 1985; Cech, 1986). If nuclear pre-mRNA splicing is an RNA-catalyzed process, then the spliceosomal snRNAs should both bind metal ions involved in catalysis and interact directly with the reactive groups of the pre-mRNA substrate (see Steitz & Steitz, 1993).

Extensive biochemical and genetic studies of the U1, U2, U4, U5, and U6 snRNAs have demonstrated that, during both spliceosome assembly and catalysis, a complicated series of conformational rearrangements occurs (see reviews by Moore et al., 1993; Nilsen, 1994; Umen & Guthrie, 1995). In the first step of spliceosome assembly, the U1 snRNP binds to the intron via base pairing of the U1 snRNA to the 5′ splice site. Subsequently, the U2 snRNA binds by base pairing with the pre-mRNA branch site sequence. In yeast, the branch site sequence, UACUAAC, is very highly conserved and branch formation occurs at the last adenosine residue of this sequence during the first catalytic step. Following U2 binding, a U4/U5/U6 tri-snRNP complex joins the pre-spliceosome. In this tri-snRNP complex, the U4 and U6 RNAs are base paired extensively with each other. Following binding of the U4/U5/U6 tri-snRNP to the pre-spliceosome, the U5 snRNP interacts with exon sequences adjacent to the 5′ splice site. Prior to, or concomitant with, the first catalytic step, the U1-5′ splice site interaction is replaced by a U6-5′ splice site interaction and the extensive base pairing between the U4 and U6 snRNAs is disrupted, destabilizing the association of U4 with the spliceosome. The disruption of the extensive base pairing between U4 and U6 permits the formation of interactions between the U2 and U6 snRNAs that are mutually exclusive with the U4/U6 base pairing interaction. This complicated network of snRNA–pre-mRNA and

Reprint requests to: David S. McPheeters, Department of Biochemistry, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106-4935, USA; e-mail: mcpheete@biocserver.cwru.edu.

snRNA–snRNA interactions involving the U2, U5, and U6 RNAs is thought to juxtapose the 5′ splice site with the pre-mRNA branch site sequence for the first catalytic step of splicing (Madhani & Guthrie, 1994c; Ares & Weiser, 1995; Umen & Guthrie, 1995).

Following the first step, a poorly defined conformational rearrangement of the RNAs in the active site of the spliceosome occurs. Evidence for this rearrangement comes from studies involving chemical modification of the reactive groups at the 5′ and 3′ splice sites (Moore & Sharp, 1992, 1993), a change in the accessibility of the 3′ splice site to oligonucleotide-mediated RNase H cleavage (Schwer & Guthrie, 1992), and the existence of mutations in the U2 and U6 RNAs that specifically block the second catalytic step (Fabrizio & Abelson, 1990; Madhani et al., 1990; Madhani & Guthrie, 1992; McPheeters & Abelson, 1992). Although the 5′ splice site and branch site are not involved obviously in the chemistry of the second catalytic reaction, mutations affecting these sequences can specifically block this step (Parker & Guthrie, 1985; Hornig et al., 1986; Query et al., 1995). A direct interaction between the terminal bases of nuclear pre-mRNA introns involved in the second catalytic step has been suggested from experiments showing reciprocal suppression of 5′ and 3′ splice site mutants (Parker & Siliciano, 1993). These observations suggest that the 2′-5′ branch of the lariat intermediate formed in the first catalytic step may be involved in the recognition of the 3′-terminal base of the intron.

Despite the identification of many of the structural elements involved in the formation of the spliceosomal active site(s), specific RNA–RNA interactions involved in the recognition of the reactive groups at the 5′ and 3′ splice sites, as well as the branch site, are not well understood. Recent studies have shown, however, that the unpaired (or bulged) state of the branch site residue in the U2/pre-mRNA branch site helix is an important determinant for its selection as the nucleophile in the first step of splicing (Query et al., 1994). Systematic variation of the chemical groups on the branch site adenosine has also demonstrated that this residue is recognized at least three times during a splicing reaction: in spliceosome assembly, and in both the first and second transesterification reactions (see Hornig et al., 1986; Ruby & Abelson, 1988; Query et al., 1995). The factors involved in the recognition of the branch site nucleotide have not yet been discerned, although several candidates have been identified (MacMillan et al., 1994).

The U6 RNA is the most highly conserved of the spliceosomal snRNAs and has been proposed to function in the catalysis of nuclear pre-mRNA splicing (Brow & Guthrie, 1989). This proposal has been supported by mutational analyses of U6 (Fabrizio & Abelson, 1990; Madhani et al., 1990), crosslinking studies (Sontheimer & Steitz, 1993), and the dynamic interactions of U6 with U2 in the active spliceosome (Madhani & Guthrie, 1992). In particular, the involvement of U6 in selection of the 5′ splice site and its possible role in positioning this sequence near the branch site adenosine for the first catalytic step (Kandels-Lewis & Seraphin, 1993; Lesser & Guthrie, 1993b) raises the possibility that U6 may be involved in recognition of the branch site adenosine. Crosslinking of the last residue in the highly conserved U6 ACAGA sequence to nearly invariant uridine at the second position of the 5′ splice site (GU) during the second step (Sontheimer & Steitz, 1993) provides further evidence for a close association of U6 with the branch site. To test the hypothesis that U6 may interact with the branch site adenosine, I have used a genetic screen to identify mutations in the U6 RNA that influence the utilization of non-adenosine branch sites during the catalytic steps of splicing. Previously, U2/U6 helix Ia and the U6 intramolecular helix had been implicated in catalytic function. In the present study, mutations in both these regions of U6 have also been found to influence utilization of non-adenosine branch sites.

RESULTS

Selection of U6 mutants that increase the utilization of non-adenosine branch sites

To facilitate the identification of U6 mutants that may affect the recognition of the branch site nucleotide, the sensitive *ACT1-CUP1* reporter gene system developed by Lesser and Guthrie (1993a) was used. In this system, the intron-containing fragment of the yeast *ACT1* gene is fused upstream of the *CUP1* gene, which encodes yeast copper metallothionein. The *CUP1* protein binds copper in a stoichiometric fashion and its expression results in increased resistance to copper. In strains harboring a deletion of the chromosomal *CUP1* gene, expression of the *ACT1-CUP1* fusion, and hence the level of copper resistance, is dependent upon splicing of the actin intron. With this system, growth on copper-containing media accurately reflects the amount of spliced *ACT1-CUP1* mRNA produced (Lesser & Guthrie, 1993a). In addition to providing a sensitive assay to monitor splicing in vivo, this system allows for the genetic selection of mutations that improve splicing of mutant introns (Burgess & Guthrie, 1993; Lesser & Guthrie, 1993a).

A series of *ACT1-CUP1* plasmids containing all three possible base substitutions at the *ACT1* branch site (position 259) were constructed and transformed into a yeast strain (ΔCUPΔU6) containing deletions of both the chromosomal *CUP1* and U6 genes. Transformants were then tested using a plate assay to determine the level of copper resistance conferred by each of the *ACT1-CUP1* fusions. In this assay, it was found that the copper resistance of each of the strains carrying an

ACT1-CUP1 plasmid with a mutant branch site was significantly lower than that of the strain containing a wild-type *ACT1-CUP1* plasmid (Table 1). The levels of the various *ACT1-CUP1* RNA species in each of these strains were also quantitated using a primer extension assay with a 3′ exon-specific primer (Fig. 1). When the levels of mature *ACT1-CUP1* RNA (M) produced are normalized to the amount of an internal control RNA (either U2 or U6), the amounts are consistent with the levels of copper resistance determined by the plating assay (Table 1). Neglecting possible differences in the rate of lariat intermediate degradation of the different branch site mutants, the apparent efficiency for use of the four branch nucleotides in the first step is A > G ≫ U > C (see legend to Table 1). The relatively efficient use of a G branch site in the first step in yeast contrasts sharply with results obtained in mammalian extracts, in which G branch sites are used very inefficiently (Hornig et al., 1986; Query et al., 1995) The apparent efficiency for use of the four branch nucleotides in the second step is A > C ≫ U > G, similar to that observed in mammals. In mammals, the branch site sequence, YURAC, is much less highly conserved, and branch formation at the purine immediately upstream of the 3′-most adenosine in this sequence has been observed (see Query et al., 1994). In the yeast *ACT1-CUP1* fusions, branch formation occurs exclusively at the penultimate position of the branch site sequence, UACUANC, with all four branch nucleotides (Fig. 1).

In order to obtain U6 mutants that affect utilization of mutant branch sites in either the first or second steps of splicing, a pool of U6 mutants was screened for

TABLE 1. Characteristics of *ACT1-CUP1* fusions containing the four different branch site mutations.[a]

Plasmid	Copper resistance (mM)	M/U2	M/U6	Apparent efficiency 1st step	Apparent efficiency 2nd step
pRS423 (vector)	$0.013^{+/-}$	–	–	–	–
p423-A259WT	>1.0	15.2	1.4	1.00	1.00
p423-A259C	0.18	0.9	0.07	0.28	0.75
p423-A259G	0.05	0.2	0.02	0.78	0.03
p423-A259U	0.05	0.1	0.01	0.39	0.09

[a] Copper resistance of the strains was determined by plate assays as described in the legend to Table 2. Levels of *ACT1-CUP1* pre-mRNA (P), lariat intermediate (L), and mature mRNA (M) in Figure 1 were quantitated using a phosphorimager. The close correlation of the ratio of mature mRNA to both the U2 and U6 RNAs (M/U2 and M/U6) shows the validity of using these RNAs as internal standards. The apparent efficiency with which each branch nucleotide is used in the first step is defined as the total amount of *ACT1-CUP1* RNA that has undergone the first step divided by the total amount of all *ACT1-CUP1* RNA species normalized to the value determined for the wild-type branch site.

$$\text{Apparent efficiency (step 1)} = \frac{(M + L)/(M + L + P)_{\text{mutant}}}{(M + L)/(M + L + P)_{\text{wild-type}}}.$$

The apparent efficiency with which each branch nucleotide is used in the second step is defined as the total amount of *ACT1-CUP1* RNA that has undergone the second step divided by the total amount of all *ACT1-CUP1* RNA species that have undergone the first step normalized to the value determined for the wild-type branch site.

$$\text{Apparent efficiency (step 2)} = \frac{(M)/(M + L)_{\text{mutant}}}{(M)/(M + L)_{\text{wild-type}}}.$$

These parameters may not accurately reflect the true efficiency of each step, because potential degradation of the intermediate species is not included in the calculations (see Fouser & Friesen, 1986).

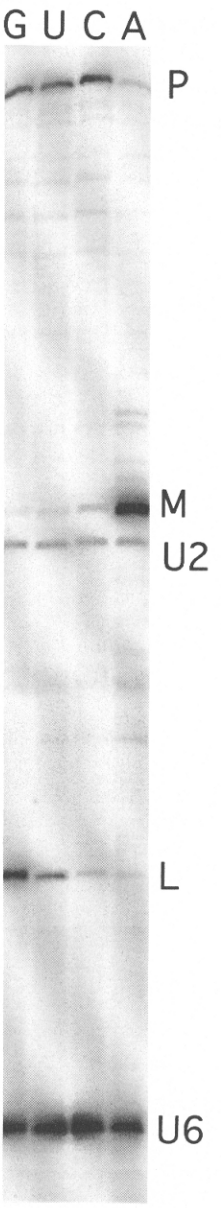

FIGURE 1. Primer extension analysis of RNAs prepared from ΔCUPΔU6 cells harboring *ACT1-CUP1* plasmids containing G, U, C, or A branch site residues (plasmids p423-A259G, p423-A259U, p423-A259C, and p423-A259WT, respectively). For this analysis, the 3′ *ACT1-CUP1* exon-specific primer GACSEQ was used. *ACT1-CUP1* products are noted as follows: P, precursor; M, mature mRNA; L, lariat intermediate. The U2-specific oligo U2SEQ and the U6-specific oligo U6NU were also included as internal controls in the reactions products denoted U2 and U6, respectively. See Table 1 for quantification of these data.

potential suppressors of either C or G branch site mutations. The strategy for the genetic selection of U6 mutants that increase the utilization of G branch sites is shown in Figure 2. The U6 mutant pool is depicted in Figure 3, and was created by combining individual plasmids corresponding to more than 160 different lethal, conditionally lethal, and viable U6 mutants. The U6 mutant pool was transformed into a ΔCUPΔU6 strain containing the *ACT1-CUP1* fusion plasmid with a G branch site mutation (p423-A259G/ΔCUPΔU6). This strain normally grows on plates containing copper concentrations up to 0.05 mM (Table 1). Following transformation with the U6 mutant pool, individual colonies were tested by streaking at a copper concentration (0.075 mM) that is restrictive for growth of the parent strain. Because disruption of any potential interactions between U6 and the branch site residue could severely affect normal splicing and result in lethality, the wild-type U6 plasmid in the ΔCUPΔU6 strain was maintained during the initial screen. This

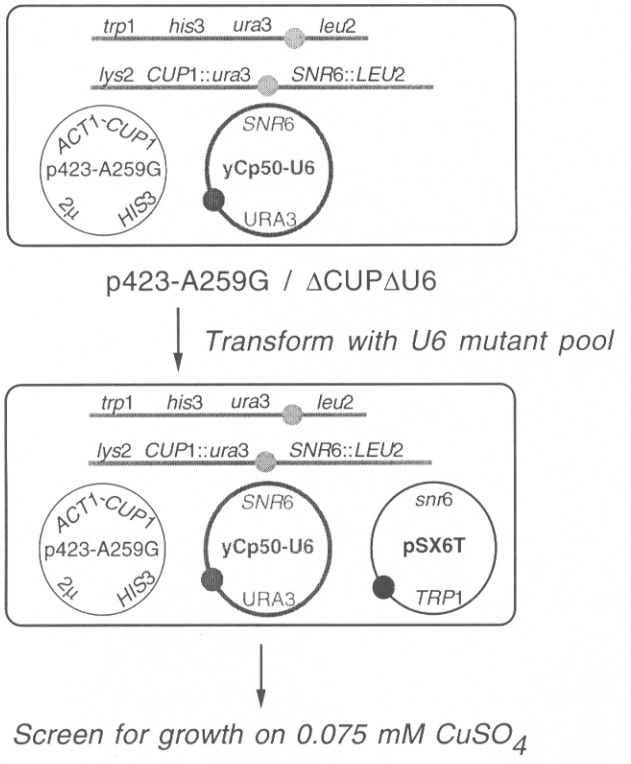

FIGURE 2. Selection scheme for the isolation of U6 mutants capable of increasing production of mature *ACT1-CUP1* mRNA from an *ACT1-CUP1* pre-mRNA containing a G branch site mutation. The strain p423-A259G/ΔCUPΔU6 contains an *ACT1-CUP1* fusion with the A259G branch site mutation in the *ACT1* intron. Normally, this strain does not form colonies on plates containing more than 0.05 mM $CuSO_4$. Following transformation of this strain with a pool of approximately 160 different U6 mutants (see Fig. 3) carried on the plasmid pSX6T (*TRP1 CEN*), cells were plated on media lacking histidine and tryptophan to select for cells containing both the ACT1-CUP1 fusion plasmid and the mutant U6 plasmid. Transformants were then picked and tested for growth on plates containing a concentration of copper (0.075 mM) restrictive for growth of the parent strain.

imposed the requirement that any U6 mutants capable of suppressing the effects of a G branch site mutation must also be dominant over wild-type U6.

From an initial screen of 1,200 transformants, 21 colonies were found to be capable of growth at the restrictive copper concentration. The U6 plasmids were recovered from these strains and sequenced to identify the mutation present. At this stage, 14 different U6 mutations were identified. To confirm that these U6 mutations were responsible for the increase in copper resistance, the individual mutant U6 plasmids were retransformed into the parent strain (p423-A259G/ΔCUPΔU6). Upon retesting, 13 of the 14 U6 mutants were found to confer increased copper resistance. Growth assays of the dominant G branch site suppressors identified in this screen on copper-containing plates are shown in Figure 4. Interestingly, none of selected U6 G branch site suppressor mutants produced either a lethal or conditionally lethal phenotype when tested by the plasmid shuffle assay (see Fig. 3). To test whether the copper resistance of these strains could be improved by expressing solely the mutant U6 RNA, cells that had lost the wild-type U6 plasmid were selected by plating the strains on media containing 5-fluoro-orotic acid (5-FOA, Sikorski & Boeke, 1991). Copper plate assays of several of the strains expressing only the mutant U6 RNA are shown in Figure 5 and the results are summarized in Table 2. None of the U6 mutants selected for the ability to improve splicing of the A to G branch site mutation showed a significant increase in copper resistance following loss of the wild-type U6 plasmid (data not shown). The 1.5–2-fold increase in copper resistance conferred by the U6 G branch site suppressors is similar to the levels of suppression seen with several mutant alleles of *PRP16* (Burgess & Guthrie, 1993; see below).

An identical screen for suppressors of the A259C branch site mutation was also performed. In this case, because the p423-A259C/ΔCUPΔU6 parent strain normally grows on plates containing up to 0.18 mM copper sulfate (Table 1), the suppressors were selected on plates containing 0.25 mM copper sulfate. Twelve different U6 mutants that permitted growth of this strain at a restrictive copper concentration (0.25 mM) were identified. Strikingly, many of the U6 suppressors of the C branch site mutation were found to be identical to the suppressors of the G branch site mutation isolated previously (see Table 2). Only one of the selected U6 C branch site suppressor mutants, U57C, confers a conditionally lethal phenotype (see Fig. 3).

The observation that many of the same U6 mutants were obtained as suppressors of either G or C branch site mutations (Table 2) suggested that these positions in U6 may act indirectly to affect branch site utilization. Direct interactions of U6 with the branch site residue would be expected to result in allele-specific suppression of branch site mutations. To rigorously test the al-

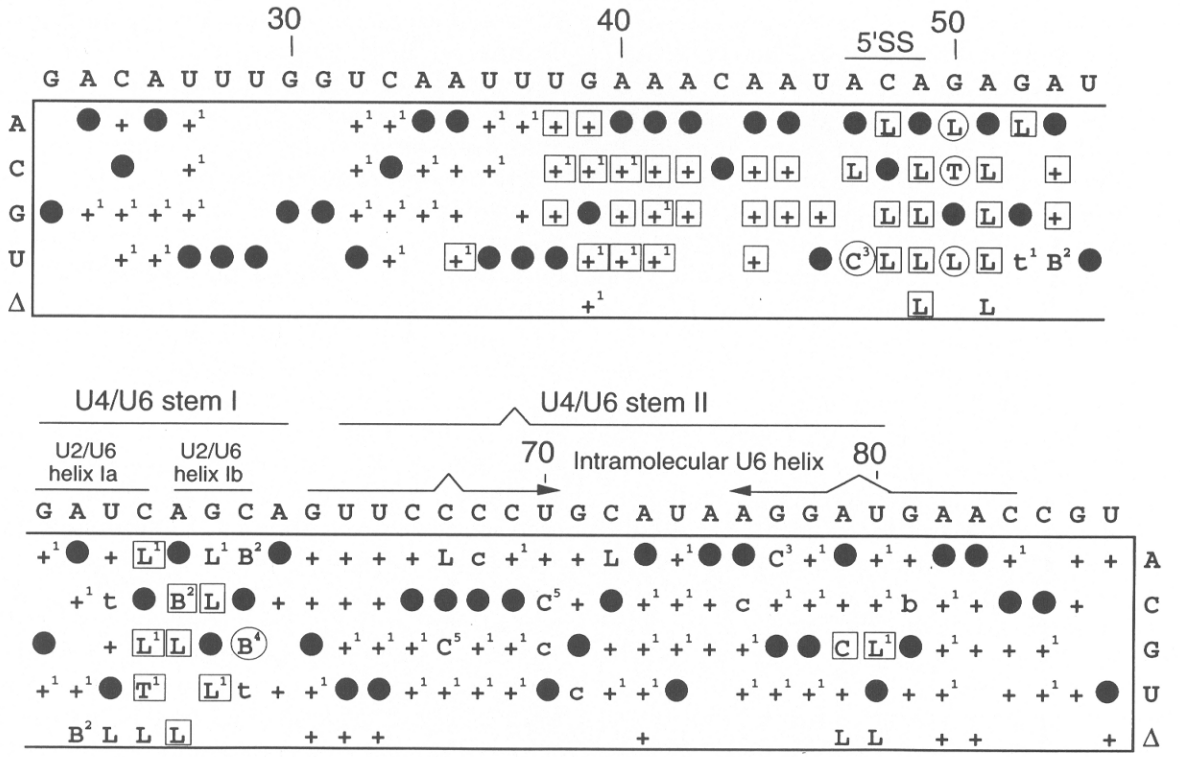

FIGURE 3. Composition and in vivo characterization of the U6 mutant pool. Individual U6 mutants in the plasmid pSX6T were transformed into the strain YHM1 (Madhani et al., 1990). At least two independent transformants were then tested for growth on plates containing 5-FOA at 18°, 25°, 30°, and 37 °C. The wild-type U6 sequence is indicated across the top and a matrix showing the phenotypes of the mutants tested is shown below. **Δ** indicates deletion of a nucleotide and ● indicates that the wild-type base is present at that position. Growth on 5-FOA is scored as follows: **+**, cells grow well at all temperatures tested; **L**, lethal at all temperatures; **T**, temperature sensitive (*ts*) growth (cells do not grow at 37°); **t**, weak *ts* (weak growth at 37°); **C**, cold sensitive (*cs*) growth (cells do not grow at 18°); **c**, weak cs (weak growth at 18°); **B**, cells both *cs* and *ts* (cells grow only at 25° and 30°); **b**, weak *cs* and *ts* (weak growth at 18 °C and/or 37 °C, see footnotes); [1], not tested at 18°; [2], weak growth at 18°; [3], dead at 18° and 25°, sick at both 30° and 37°; [4], grows well only at 30° (weak growth at 25°); [5], grows well only at 30° and 37°. Boxes indicate that, for a particular mutant, the present analysis agrees with those described previously by either Lesser and Guthrie (1993b), Madhani and Guthrie (1994b), Madhani et al. (1990), Madhani and Guthrie (1992), or Fortner et al. (1994). Open circles indicate that, for a particular mutant, slight differences (i.e., weak growth versus no growth) from results reported previously by others were observed.

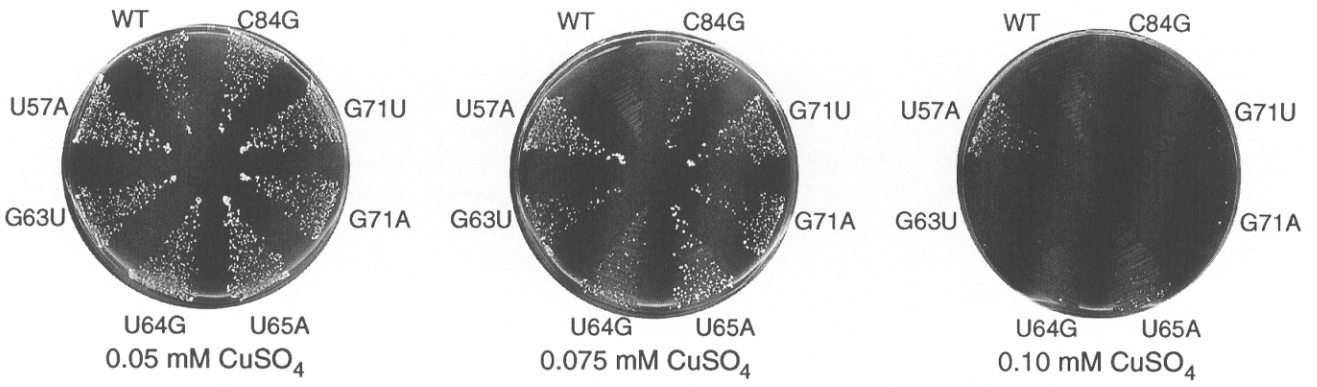

FIGURE 4. Copper plate assays of selected U6 G branch site suppressor mutants. The strain p423-A259G/ΔCUPΔU6 was transformed with U6 plasmids containing the indicated U6 allele. Transformants were picked and resuspended in a small volume of water before streaking on plates containing the indicated concentrations of copper sulfate. U6 G branch site suppressor mutants isolated in the original selection, but not shown in this figure, are: G63A, U65G, G71C, A82U, A83C, and A83G.

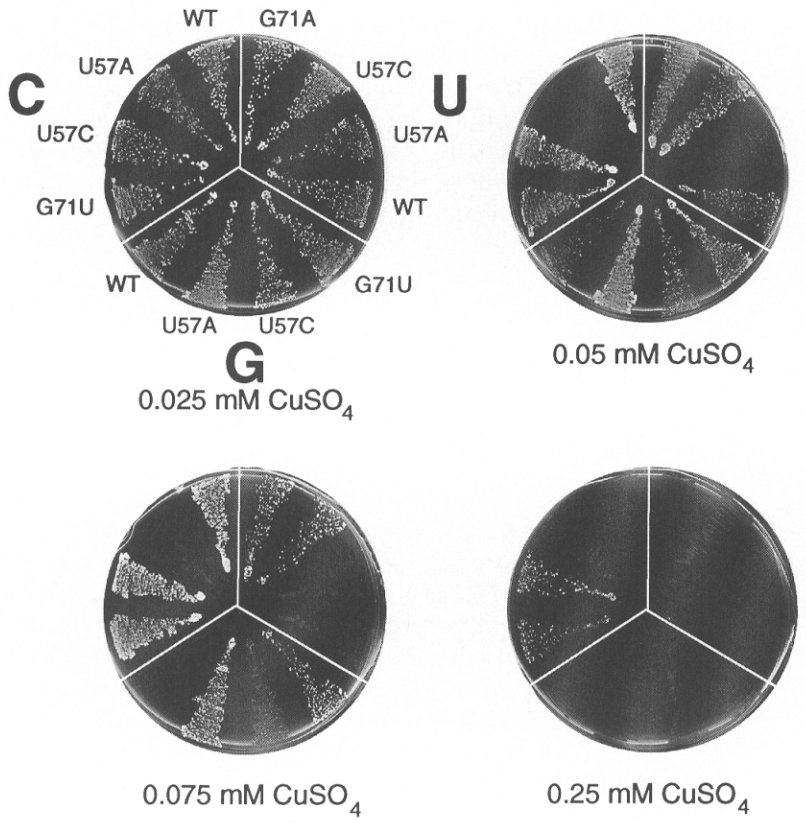

FIGURE 5. Copper plate assays of U6 branch site suppressor mutants in strains containing the either a C, G, or U branch site mutation in the *ACT1-CUP1* fusion and expressing only the indicated U6 RNA. The strains p423-A259G/ΔCUPΔU6, p423-A259C/ΔCUPΔU6, or p423-A259U/ΔCUPΔU6 were transformed with U6 plasmids containing the indicated U6 allele. Transformants were then plated on 5-FOA to select for cells that had lost the wild-type U6 plasmid. Resultant colonies were then picked and resuspended in a small volume of water before streaking on plates containing the indicated concentrations of copper sulfate.

lele specificity of U6 G and C branch site suppressor mutants, each was transformed into ΔCUPΔU6 strains harboring *ACT1-CUP1* fusions with either G, C, or U branch site mutations. Following loss of the wild-type U6 plasmid from these transformants, the level of copper resistance of each was determined (Fig. 5; Table 2). As expected based on the initial screens, most of the U6 C and G branch site suppressor mutants were found to be nonallele specific and caused an approximately twofold increase in copper resistance of strains harboring *ACT1-CUP1* fusions with either G, C, or U branch site mutations (Table 2). Notably, the mutations that confer nonallele specific suppression of branch site mutations are all located in the intramolecular helix of U6 (Fig. 7; see the Discussion). In contrast, mutations at position U57 of U6 exhibit allele-specific suppression of branch site mutations.

The U57A mutant was identified initially in the screen for suppressors of the G branch site mutation, whereas the U57C mutant was selected as a suppressor of a C branch site mutation. When the U57A mutant was assayed with *ACT1-CUP1* fusions containing C and U branch site mutations in the absence of the wild-type U6 plasmid, the copper resistance of these strains was significantly lower than with wild-type U6 (Fig. 5; Table 2). Because the U57A mutant was viable, and did not noticeably decrease the copper resistance of strains containing a wild-type *ACT1-CUP1* fusion (Table 2), the U57A mutant must be compatible with either A or G branch sites, but not with C or U branch sites. In contrast, the U57C mutant increased the copper resistance of strains containing either a C or U branch site, but had no effect on a G branch site (Fig. 5; Table 2). The U57G mutant did not suppress the effects of either G or U branch site mutations, and lowered the copper resistance of a strain containing a C branch site mutation (Table 2). Primer extension analysis revealed that the striking allele specificity of the U57 mutants is due to differential effects of these mutants on the first and second steps of splicing (see below).

U6 suppressors do not affect splicing of introns with 5′ or 3′ splice site mutations

To determine whether any of the U6 branch site suppressor mutants could suppress the effects of other intron mutations, copper plate assays were performed with several different mutant *ACT1-CUP1* reporter gene fusions (Table 3). Importantly, none of the U6 branch site suppressor mutants increased the copper resistance of strains containing *ACT1-CUP1* fusions with mutations at either the 5′ (G1A) or 3′ (G303C) splice sites. On the other hand, U6-U57 mutants had significant effects on the splicing of mutants at positions C256 and U257 in the branch site sequence ($UAC_{256}U_{257}A\underline{A}C$), whereas the U6-G63U, -U65A and

TABLE 2. Maximum copper concentrations (mM) at which strains containing the A, C, G, or U branch site residues in the intron of the *ACT1-CUP1* reporter gene construct in the ΔCUPΔU6 strain grew when expressing only the indicated U6 allele.[a]

	Branch site nucleotide			
Plasmid	A	G	C	U
U6-WT	>1.0	0.05	0.18	0.05
U6-WT + prp16-1	>1.0	0.05	**0.40**	0.05
U6-U57A	>1.0	**0.10**	(0.025)	(0.025)
U6-U57C	>1.0	(0.05)	0.30	**(0.10)**
U6-U57G	NT	(0.05)	(0.05)	(0.05)
U6-G63A	>1.0	**0.10**	**0.40**	**(0.10)**
U6-G63U	>1.0	0.075	**0.40**	(0.075)
U6-U64G	NT	**0.10**	**0.40**	**(0.10)**
U6-U65A	>1.0	**0.10**	**0.40**	**(0.10)**
U6-U65G	>1.0	**0.10**	**0.40**	**(0.10)**
U6-C66G	>1.0	(0.05)	(0.18)	(0.05)
U6-G71A	>1.0	0.075	**0.40**	**(0.10)**
U6-G71C	>1.0	**0.10**	**0.40**	**(0.10)**
U6-G71U	NT	**0.10**	**0.40**	**(0.10)**
U6-A82G	>1.0	(0.075)	**0.40**	(0.075)
U6-A82U	>1.0	**0.10**	(0.25)	**(0.10)**
U6-A83C	NT	**0.10**	(0.18)	**(0.10)**
U6-A83G	NT	**0.10**	(0.25)	**(0.10)**
U6-C84G	NT	**0.10**	**0.40**	**(0.10)**
U6-C84U	NT	(0.075)	**0.40**	**(0.10)**

[a] For comparison, the copper resistance of ΔCUPΔU6 strains containing both a wild-type U6 plasmid and a plasmid harboring the dominant *prp16-1* allele is included. Strains were tested by streaking on plates containing 0, 0.013, 0.025, 0.05, 0.075, 0.1, 0.15, 0.18, 0.25, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 mM copper sulfate and were incubated at 30 °C for 3 days before scoring. Tests of U6 mutants not obtained in the original suppressor isolation screens are indicated in parentheses and NT designates the plasmid was not tested. The maximum copper concentrations for each branch site mutation at which growth is supported by the U6 suppressors are indicated in boldface type.

TABLE 3. Allele specificity of the U6 branch site suppressor mutants.[a]

	Intron mutation					
U6 allele	G1A	C256A	C256G	U257A	U257C	G303C
WT	0.05	0.18	0.075	0.05	0.30	0.05
U57A	0.05	**0.05**	**0.025**	0.05	0.18	0.05
U57C	0.05	**0.40**	**0.25**	**0.10**	0.40	0.05
G63U	0.05	0.18	0.075	0.075	0.30	0.05
U65A	0.05	0.15	0.05	0.05	0.30	0.05
G71A	0.05	0.30	0.10	0.075	0.30	0.05

[a] Maximum copper concentrations (mM) at which strains containing the indicated intron mutations in the *ACT1-CUP1* reporter gene grew when expressing only the indicated U6 allele (see legend to Table 2). Results indicating at least a twofold increase or decrease in the copper resistance conferred by U6 mutations upon the mutant intron are shown in boldface type.

-G71A mutants had little or no effect. The U57A mutant lowered the copper resistance of three of the four C256 and U257 mutants tested. In contrast, the U57C mutant improved the splicing of these mutants significantly (Table 3). This general suppression of branch site recognition sequence mutants by the U57C mutant is similar to that observed previously with several mutant alleles of *PRP16* (see Burgess & Guthrie, 1993), a factor implicated previously in branch site utilization, suggesting a potential interplay between U57 and *PRP16*.

Interactions of the U6 branch site suppressor mutants with *PRP16*

PRP16 was identified originally in a genetic screen for dominant suppressors of an A to C branch site mutation (Couto et al., 1987). Subsequent studies have shown that *PRP16* is a member of the DEAH family of putative RNA helicases required for the second catalytic step of splicing, and it has been proposed that *PRP16* is involved in a discard pathway for aberrant lariat intermediates (see review by Umen & Guthrie, 1995). Because the original *PRP16* mutant, *prp16-1*, is dominant, a direct comparison of the level of suppression shown by *prp16-1* with the U6 branch site suppressors in the ΔCUPΔU6 strain was possible (see Table 2). Although no suppression of either the G or U branch sites by the dominant *prp16-1* mutation was observed, *prp16-1* did increase the copper resistance of strain containing a C branch site mutation by approximately twofold (Table 2). Notably, the increase in copper resistance conferred by many of the U6 C branch site suppressor mutants is identical to that conferred by the *prp16-1* mutation.

Recently, Madhani and Guthrie (1994a) provided evidence for a possible genetic interaction between *PRP16* and the U6 RNA by showing that single-base deletions in the region immediately upstream of the conserved ACAGA sequence could suppress the cold-sensitive phenotype of the *prp16-302* mutation. Several mutations in U2/U6 helix Ia were also found to suppress the cold-sensitive phenotype of *prp16-302*, albeit more weakly. To examine possible interactions of *PRP16* with the U6 branch site suppressor mutants, all of the U6 mutants listed in Table 2 were transformed into a yeast strain (YHM145) containing both the *prp16-302* mutation and a deletion of the chromosomal U6 gene. Transformants lacking the wild-type U6 plasmid were then selected by plating on 5-FOA. Whereas the *prp16-302* mutation is cold sensitive (Madhani & Guthrie, 1994a) and the U6-U57C mutant is weakly temperature sensitive (Fig. 3), the *prp16-302*/U6-U57C double mutant was inviable at all temperatures (data not shown). The synergistic lethality of the U6-U57C and *prp16-302* mutations suggests a functional interaction between U6 and *PRP16*. Further evidence for an interaction be-

tween U57 and *PRP16* is provided by the observation that the U57A mutation weakly suppressed the cold-sensitive phenotype of *prp16-302*. The U57G mutation was neither synergistically lethal with, nor suppressed the cold-sensitive phenotype of, *prp16-302* (data not shown). The U6 branch site suppressor mutants in the stem and loop of the intramolecular helix of U6 (Table 2) also failed to exhibit synergistic lethality with *prp16-302* or suppress its cold-sensitive phenotype (data not shown). Taken together, these results show a direct interaction of *PRP16* with nt U57 of U6, and suggest that the intramolecular helix of U6 does not interact with *PRP16*.

To investigate further the relationship between the U6 branch site suppressor mutants in the U2/U6 helix Ia region and the U6 intramolecular helix, several combinations of double mutants in these regions were constructed. It was predicted that, if the U6 mutants increase the splicing of substrates of non-adenosine branch sites by affecting different aspects of branch site utilization, then pairwise combinations should show additive effects. On the other hand, if the mutants affect the same aspect of branch site utilization, then the pairwise combinations should not have additive effects. As summarized in Table 4, strains harboring combinations of mutants at position U57 and position G71 in the loop of the intramolecular helix exhibit consistent and significant increases in their copper resistance over the single mutants. With *ACT1-CUP1* fusions containing C and U branch site mutations, the copper resistance of strains expressing only the U6-U57A RNA is significantly lower than with wild-type U6 (Fig. 5; Table 2). Curiously, this negative effect of the U57A mutation is not rescued by combinations involving either the G71A, G71C, or G71U mutations (Table 4). This observation suggests the effects of mutations in U2/U6 helix I on non-adenosine branch site utilization may be dominant to the effects of mutations in the U6 intramolecular helix. Overall, these results suggest strongly that these mutants affect different aspects of branch site utilization, and that the effects of the mutations in the loop of the intramolecular helix of U6 do not reflect interactions with *PRP16*. Only one combination of mutations in the lower base paired portion and loop of the intramolecular helix (U65A+G71A) led to increased levels of copper resistance (Table 4). The U65G+G71A and U65G+G71C double mutants in the C branch site-containing strain exhibited lower copper resistance than the corresponding single mutants and may reflect impaired function of these double mutants. These results suggest that, in most cases, mutations in the stem and loop of the U6 intramolecular helix affect similar aspects of branch site utilization.

Many of the mutations in U6 selected as suppressors of C and G branch site mutations (Table 2) are located in the lower base paired region of the U6 intramolecular helix (see Fig. 7) and are predicted to disrupt this pairing. To address whether the suppressor phenotype is due to disruption of structure or to the alteration of the sequence in this region, two triple mutants were constructed. The first mutant (CAA) changes the sequence $G_{63}U_{64}U_{65}$ to CAA, whereas the second mutant (UUG) changes the sequence $A_{82}A_{83}C_{84}$ to UUG. Each triple mutant is predicted to completely abolish pairing of the lower portion of the U6 intramolecular helix. In addition, these mutants are expected to destabilize the interaction of U6 with the U4 RNA. Both of the triple mutants exhibited temperature-sensitive phenotypes, and the CAA mutant was cold-sensitive as well. Consistent with the proposed base pairing of this region in the U6 intramolecular helix, a sextuple mutant (CAA+UUG) restored growth at high temperatures (data not shown). Using the copper plate assay, neither triple mutant was able to suppress the effects of C, G, or U branch site mutations. In contrast, the sextuple mutant (CAA+UUG) was able to suppress the effects of C, G, and U branch site mutations (data not shown). These results demonstrate that the alteration of the sequence in the lower portion of the U6 intramolecular helix, and not the disruption of structure, is responsible for the branch site suppressor phenotype.

TABLE 4. Interactions between U6 branch site suppressors.[a]

	Branch site nucleotide		
U6 allele	G	C	U
WT	0.05	0.18	0.05
U57A	0.10	0.025	0.025
U57C	0.05	0.30	0.10
U64G	0.10	0.40	0.10
U65A	0.10	0.40	0.10
U65G	0.10	0.40	0.10
G71A	0.075	0.40	0.10
G71C	0.10	0.40	0.10
G71U	0.10	0.40	0.10
U64G+G71U	0.10	0.40	0.10
U65A+G71A	**0.15**	**0.50**	**0.15**
U65G+G71A	0.10	0.30	0.10
U65G+G71C	0.10	0.30	0.10
U57A+G71A	**0.15**	0.05	0.05
U57A+G71C	**0.18**	0.05	0.05
U57A+G71U	**0.18**	0.05	0.05
U57C+G71A	0.10	**0.50**	**0.15**
U57C+G71C	0.10	**0.50**	**0.15**
U57C+G71U	0.10	**0.60**	**0.15**

[a] Maximum copper concentrations (mM) at which strains containing the indicated intron mutations in the *ACT1-CUP1* reporter gene grew when expressing only the indicated U6 allele (see legend to Table 2). Results indicating significant increases in the copper resistance of double mutants over that exhibited by single mutants are shown in boldface type.

In vivo RNA analysis shows that the U6 mutants affect both steps of splicing

Primer extension analysis was used to quantitate the levels of *ACT1-CUP* pre-mRNA (P), lariat intermediate (L), and mature mRNA (M) in strains expressing only the mutant U6 and containing *ACT1-CUP1* fusions with the different branch site mutations. An example of this assay is shown in Figure 6 and a summary of the quantitative results is presented in Table 5. Significantly, none of the U6 branch site suppressors caused branch formation at the adenosine residue upstream of the normal branch site residue (Fig. 6; data not shown). Consistent with the modest increase in copper resistance conferred by the suppressor U6 mutants, a 1.5–2.7-fold increase in mature *ACT1-CUP1* mRNA levels (ΔM) was observed in these strains compared to strains expressing the wild-type U6 RNA. Each of the U6 branch site suppressor mutants had significant effects on the first and/or second steps of splicing of fusion RNAs containing branch site mutations, but had little effect on the splicing of the fusions containing an adenosine branch site. The first and second step effects were measured by comparing changes in the ratios of mature mRNA to pre-mRNA [Δ(M/P)] and mature mRNA to lariat intermediate [Δ(M/L)], respectively, in the U6 mutants relative to wild-type U6 (Table 5). A change in the ratio of lariat to pre-mRNA [Δ(L/P)] also reflects first-step effects, but only if there is no significant effect on the second step (Fouser & Friesen, 1986). The U6 suppressor mutants at position G71 consistently increased the relative efficiencies of both steps compared to wild-type U6. The effects of the branch site suppressor mutations at position U57 were much more complex. The observed allele specificity of the U57A mutation (Fig. 5; Table 2) resulted from a dramatic inhibition of the first step of splicing in RNAs containing either C or U branch sites. In contrast, the U57A mutation

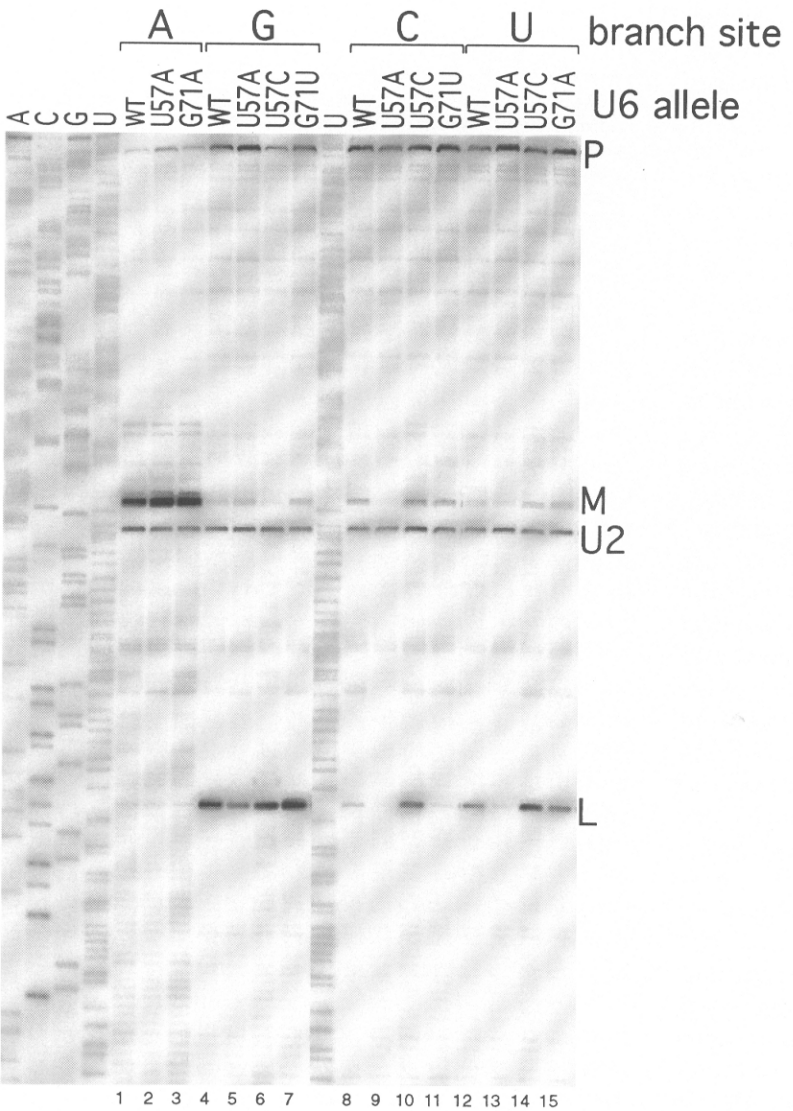

FIGURE 6. Primer extension assays of RNAs isolated from cells harboring a C, G, or U branch site mutation in the *ACT1-CUP1* fusion and expressing only the indicated U6 RNA. The 3′ *ACT1-CUP1* exon-specific primer GACSEQ was used. The U2-specific primer U2SEQ was also included in the reactions as an internal control. *ACT1-CUP1* products are noted as follows: P, precursor; M, mature mRNA; L, lariat intermediate.

TABLE 5. Quantitative analysis of the effects of the U6 mutants on splicing of the wild-type and mutant branch site ACT1-CUP1 fusions.[a]

Branch site nucleotide	U6 allele	ΔM	Δ(T)	Δ(M/P)	Δ(L/P)	Δ(M/L)
A	WT	1.0	1.0	1.0	1.0	1.0
	U57A	0.9 ± 0.3	1.1 ± 0.4	0.7 ± 0.2	1.2 ± 0.5	0.7 ± 0.4
	G71A	1.0 ± 0.2	1.1 ± 0.2	0.7 ± 0.2	1.6 ± 1	0.7 ± 0.3
G	WT	1.0	1.0	1.0	1.0	1.0
	U57A	1.8 ± 0.3	0.4 ± 0.1	1.0 ± 0.6	0.3 ± 0.04	4.4 ± 1.1
	U57C	0.7 ± 0.4	0.6 ± 0.4	1.8 ± 0.7	2.1 ± 0.4	0.7 ± 0.3
	G71U	1.8 ± 0.4	0.9 ± 0.2	2.4 ± 1.5	1.4 ± 0.7	1.7 ± 0.3
C	WT	1.0	1.0	1.0	1.0	1.0
	U57A	0.07 ± 0.03	0.6 ± 0.1	0.05 ± 0.02	0.1 ± 0.03	0.5 ± 0.3
	U57C	1.5 ± 0.2	1.1 ± 0.2	2.5 ± 0.7	21.4 ± 9.6	0.1 ± 0.02
	G71U	1.9 ± 0.2	0.8 ± 0.1	1.8 ± 0.4	0.7 ± 0.3	2.9 ± 0.9
U	WT	1.0	1.0	1.0	1.0	1.0
	U57A	0.5 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	0.1 ± 0.01	4.8 ± 0.2
	U57C	1.9 ± 0.7	1.2 ± 0.1	2.5 ± 0.1	2.3 ± 0.1	1.1 ± 0.1
	G71A	2.7 ± 0.1	1.3 ± 0.2	2.4 ± 0.2	1.2 ± 0.2	2.1 ± 0.2

[a] RNAs were prepared from three independent transformants of each of the indicated U6 alleles into the four different *ACT1-CUP1* fusion strains following selection of strains lacking the wild-type U6 plasmid on 5-FOA. Levels of *ACT1-CUP1* pre-mRNA (P), lariat intermediate (L), and mature mRNA (M) were determined by primer extension as shown in Figure 6 and quantitated using a phosphorimager. The background value immediately above each band was subtracted from the value obtained for each band. In each case, the numbers shown indicate the average change ± 1 standard deviation for each of the U6 mutants compared to RNA prepared from the same strain expressing only the wild-type U6 RNA. For determination of changes in the amounts of total (ΔT, where T = P + M + L) and mature ACT1-CUP1 RNA (ΔM), the amounts of RNA were first normalized to the amount of U2 RNA in each sample. As discussed in detail in Fouser and Friesen (1986), a change in the ratio of mature to lariat intermediate RNA, defined as $\Delta(M/L) = (M/L)_{mutant\ U6}/(M/L)_{wild\text{-}type\ U6}$, is directly proportional to the efficiency of the second step of splicing. Normally, the ratio of mature to pre-mRNA, M/P, is proportional to the efficiency of the first step of splicing only if the rate of the second step is much greater than the rate of intron degradation (Fouser & Friesen, 1986). In the analysis above, the additional assumption was also made that the rate of intron degradation is independent of the U6 allele. If this is true, then a comparison of this ratio in the U6 mutant relative to wild-type U6, Δ(M/P), is directly proportional to the change in efficiency of the first step. Analysis of the U6-U57C mutant in the C branch site-containing strain suggests that this assumption may not be valid in this case.

had little or no effect on the first step of splicing of the G branch site fusion RNA. The suppression of the G branch site mutation by U57A resulted from a large (~4-fold) increase in the efficiency of the second step of splicing (Table 5). A similar increase in the efficiency of the second step of splicing of the U branch site containing RNA by the U57A mutation was also observed. The U57C mutation, which was found to increase the copper resistance of strains containing U and C, but not G, branch sites (Table 2), appeared primarily to increase the efficiency of the first step (Table 5). Interestingly, this mutant caused the accumulation of a large amount of lariat intermediate in the C branch site containing RNA (Fig. 6). This dramatic accumulation appeared to result partially from a large (~10-fold) decrease in the efficiency of the second step of splicing [Δ(M/L), Table 5], but could also reflect a decrease in the rate of degradation of the mutant lariat intermediate. If degradation of the C branch site containing lariat intermediate is decreased by the U57C mutation, then an increase in the relative amount of total *ACT1-CUP1* RNA would be expected. However, no significant increase in the total amount of *ACT1-CUP1* RNA species (ΔT) was observed (Table 5).

DISCUSSION

This report describes a hitherto unknown genetic interaction of the pre-mRNA branch site residue with the U6 RNA. By exploiting yeast genetics, mutations in the U6 RNA that significantly increase the utilization of substrates containing non-adenosine branch sites have been identified. Although initial recognition of the pre-mRNA branch site residue occurs at a very early stage in the assembly of the spliceosome, prior to the association of U6, the identity of the branch site residue is important for both the first and second catalytic steps of splicing (Hornig et al., 1986; Ruby & Abelson, 1988; Query et al., 1995). The U6 mutants described in the current study affect both the first and second catalytic steps of splicing with substrates containing non-adenosine branch sites. In these mutants, the overall production of spliced RNA from substrates containing non-adenosine branch sites increased 1.5–2.7-fold (Table 5). Strikingly, the U6 mutants that affect branch site utilization are located in two regions of the U6 RNA implicated previously in catalytic function.

As illustrated in Figure 7, the branch site suppressor mutants are located in the region of U6 involved in

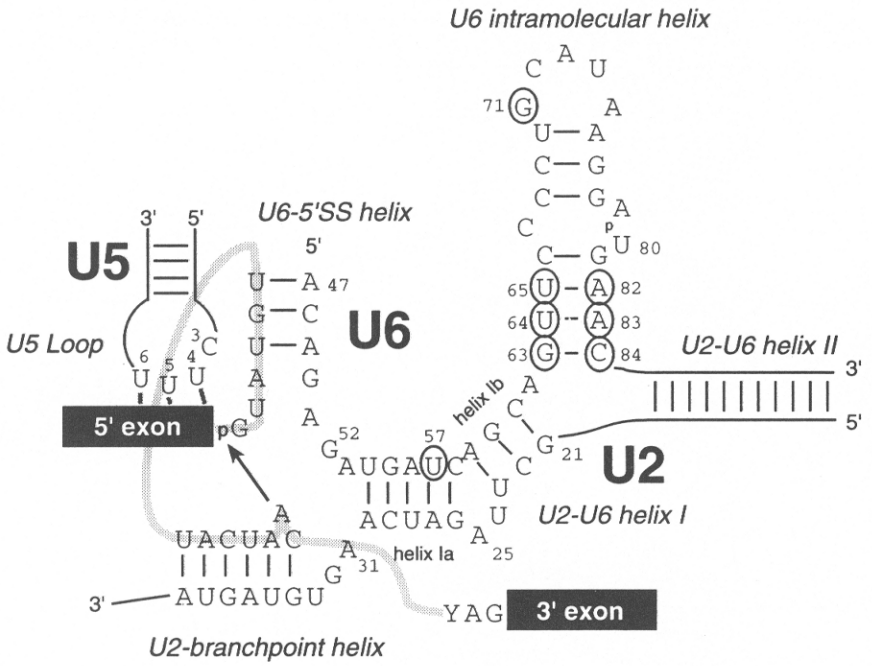

FIGURE 7. Locations of branch site suppressor mutations in the yeast U6 RNA. A model depicting the RNA–RNA interactions in the spliceosome prior to the first catalytic step is shown (redrawn from Umen & Guthrie, 1995). Nucleotides altered in the U6 branch site suppressors are circled (see Table 2 for exact identities of the alterations).

formation of U2/U6 helix Ia and the U6 intramolecular helix. Previous studies have established that both the identities and pairing of several bases in U2/U6 helix Ia are important for the catalytic events of nuclear pre-mRNA splicing, particularly the second step (Fabrizio & Abelson, 1990; Madhani et al., 1990; Madhani & Guthrie, 1992; McPheeters & Abelson, 1992). Previous studies of U57 mutants using an in vitro U6 reconstitution system have failed to reveal significant effects on either step of splicing with a substrate containing an adenosine branch site (Fabrizio & Abelson, 1990). Using the sensitive *ACT1-CUP1* reporter gene system, mutants at position U57 exhibited an unusual pattern of allele specificity in suppression of branch site mutations. Although the U57A mutant suppressed the effects of a G branch site mutation, it not only failed to increase, but actually decreased the splicing of substrates containing either a C or U branch site. In contrast, the U57C mutant suppressed C and U branch site mutations, and had no significant effect on G branch site mutations (Fig. 5; Tables 2, 5). Although the basis for this allele specificity is not entirely clear, primer extension analysis showed that, whereas the increased utilization of G branch sites in the U57A mutant was due to an increase in the efficiency of the second step, the inhibition of C and U branch sites was due to a decrease in the efficiency of the first step (Fig. 6; Table 5). The effects of the U57C mutation also were complex. This mutation both increased the efficiency of the first step and decreased the efficiency of the second step. These opposing effects were most pronounced with a substrate containing the C branch site mutation. Despite the allele-specific effects of U57 mutants on the first and second steps of splicing, the lack of significant in vivo (Table 2) or in vitro phenotypes of U57 mutants (Fabrizio & Abelson, 1990) as well as the interactions of U57 mutants with *PRP16*, suggests that the interaction(s) of U57 with the branch site are indirect.

PRP16 was isolated originally as a dominant suppressor of an A to C branch site mutation (Couto et al., 1987). Subsequent studies have shown that *PRP16* is a member of the DEAH family of putative RNA-dependent helicases (Burgess et al., 1990), and that this protein is required specifically for the second catalytic step of splicing in vitro (Schwer & Guthrie, 1991). Following the first catalytic step, ATP hydrolysis by *PRP16* promotes a conformational change that results in protection of the 3′ splice site from oligonucleotide-mediated RNase H cleavage (Schwer & Guthrie, 1992); this conformational change may involve interaction of the 3′ splice site with the branch of the lariat intermediate (Umen & Guthrie, 1995). It has been proposed that, for lariat intermediates containing non-adenosine branch sites, the conformational change (LI → LI*) that results in protection of the 3′ splice site becomes rate-limiting, and is in kinetic competition with an ATP-dependent discard pathway controlled by *PRP16* (Burgess et al., 1990; Burgess & Guthrie, 1993). The synergistic lethality of the U6-U57C mutant with the *prp16-302* mutant, and the suppression of the cold-sensitive growth phenotype of *prp16-302* by the U57A mutant, may reflect a physical interaction between these two components. The interaction of U57 and *PRP16* suggests that some of the effects of mutations at U57 on non-adenosine branch site utilization are mediated at least par-

tially through effects on *PRP16*. Suppression of the second step defect in the G branch site mutant may result from a decrease in the rate of ATP hydrolysis by *PRP16* caused by the U6-U57A mutant. As proposed previously by Madhani and Guthrie (1994a), following ATP hydrolysis by *PRP16*, the U57A mutation may weaken binding of *PRP16* to the spliceosome, thereby suppressing the cold-sensitive phenotype of the *prp16-302* mutation. Because *PRP16* is required only for the second step of pre-mRNA splicing in vitro (Schwer & Guthrie, 1991), it is unlikely that the first-step effects of the U6-U57 mutants are mediated through *PRP16*. The basis of the profound first-step effects of U6-U57 mutants are not understood. However, because another putative RNA helicase (*PRP2*) that is closely related to *PRP16* is required for the first catalytic step in vitro (Kim & Lin, 1993), it is possible that the first-step effects of the U6-U57 mutants may involve this factor.

In current models for RNA–RNA interactions within the active site of the spliceosome (see Fig. 7), U6-U57 is thought to interact with nt A27 of the U2 snRNA through formation of a Watson–Crick base pair. In vivo, the mutants U2-A27G and U2-A27U are lethal (D.S. McPheeters, unpubl. data), and in vitro, they moderately inhibit the second catalytic step of splicing (McPheeters & Abelson, 1992). When assayed for their ability to suppress the effects of branch site mutations in a dominant fashion using a copper plate assay similar to that shown in Figure 3, none of the single-base substitutions of U2-A27 suppresses the effects of branch site mutations (D.S. McPheeters, unpubl. data). The complexity of the interactions involving U6-U57 and U2-A27 is also evidenced by the fact that the lethal phenotypes of U2-A27G and U2-A27U mutants can be suppressed by U6-U57A, -U57C, -U57G, and U6-U57A, -U57C mutants, respectively (D.S. McPheeters, unpubl. data). This pattern of suppression is clearly not consistent with formation of a standard Watson–Crick base pair between U6-U57 and U2-A27.

The majority of the U6 mutations selected for the ability to suppress the effects of non-adenosine branch site mutations are located in the intramolecular helix (Fig. 7). Previous studies have demonstrated that this helix is involved in conformational changes of the U6 RNA, the assembly of the U4/U6 snRNP, and the first step of splicing (Wolff & Bindereif, 1993, 1995; Fortner et al., 1994). In addition, it has been shown that phosphorothioate substitution of the phosphate of nt U80 blocks the first step of splicing completely (Fabrizio & Abelson, 1992; see also Yu et al., 1995). On the basis of limited sequence homology and the phosphorothioate substitution experiments, the intramolecular helix of U6 has been proposed to be homologous to the bulged domain 5 helix of autocatalytic group II introns (Chanfreau & Jacquier, 1994; Yu et al., 1995). In group II introns, domain 5 plays an essential role in both catalytic steps of splicing, and portions of both the helical and loop regions are involved in binding of this helix to the core of the intron (Chanfreau & Jacquier, 1994; Abramovitz et al., 1996). In addition, it has been shown recently that several 2′ hydroxyls in the helical region of domain 5 are involved in catalysis (Abramovitz et al., 1996).

Most of the selected U6 branch site suppressors lie in the lower portion of the U6 intramolecular helix (Fig. 7). In fact, the results reported here show that almost any sequence change in this region of U6 results in acquisition of the branch site suppressor phenotype (Table 2). Furthermore, it was shown that it is the alteration of the sequence in this region, and not the disruption of base pairing, that increases the ability to use non-adenosine branch sites. These results suggest that the U6 branch site suppressor mutants in the lower portion of the intramolecular helix do not contact the branch site residue directly, but rather may affect the binding of this helix to a component of the catalytic core of the spliceosome (see below). Although the primary sequence of the lower portion of the U6 intramolecular helix is not absolutely conserved, the sequence is restricted into three families. In yeast, the sequence of nt 63–65 is predominantly GUU, whereas in all other organisms, it is either UGG or CUC (see Guthrie & Patterson, 1988; Roiha et al., 1989). Curiously, this variation in sequence is many times coordinated with a change at position 58 (see Roiha et al., 1989), immediately adjacent to the other position selected as a branch site suppressor mutant, U57.

The other branch site suppressor mutants are at position G71 in the loop of the U6 intramolecular helix (Fig. 7). All substitutions at position G71 show a branch site suppressor phenotype (Table 2). Little is known about the function, if any, of this loop sequence during the catalytic steps of splicing. Modification interference experiments have shown this loop to play a role in the formation of the U4/U6 snRNP (Wolff & Bindereif, 1993). Mutational analysis indicates that most single-base mutations in this loop sequence do not produce noticeable growth defects (Fig. 3), suggesting that, if the loop sequence in the yeast U6 intramolecular helix plays a role in either U4/U6 assembly or catalysis, it is either nonessential or functionally redundant. In group II splicing, the loop sequence of domain 5 is involved in binding this helix to the catalytic core of the intron (Costa & Michel, 1995).

The complete lack of noticeable growth defects (Fig. 3) and lack of allele specificity in the suppression of non-adenosine branch site mutations (Table 2) suggests that the branch site suppressor mutations in the U6 intramolecular helix act indirectly to influence branch site utilization. The increased suppression by double mutants in the U6 intramolecular helix and U2/U6 helix Ia (Table 4), and the lack of genetic interactions between mutants in the intramolecular helix with *prp16-302*, suggests that the U6 intramolecular helix acts indepen-

dently of *PRP16* to influence branch site utilization. I suggest that, in a manner similar to that shown for domain 5 of group II self-splicing introns (see above), the U6 branch site suppressors in the intramolecular helix of U6 act by affecting the binding of this helix to the catalytic core of the spliceosome. The weakened binding of the intramolecular helix to the catalytic core in the branch site suppressor mutants may affect the positioning of groups in the helix involved in catalysis. Phosphorothioate interference experiments have shown that the phosphate of residue U80, located in the bulge of the intramolecular helix, is absolutely essential for the first step of splicing (Fabrizio & Abelson, 1992; see also Yu et al., 1995); no data concerning the requirement for this phosphate in the second step are available. The U6 branch site suppressor mutants in the intramolecular helix may affect the positioning of the essential phosphate of U80 and facilitate the accommodation of non-adenosine branch residues in the active site(s). In the second step, the ability of the U6 mutants to accommodate non-adenosine branches into the active site may increase the rate of the LI → LI* transition, and, therefore, decrease the utilization of the proposed *PRP16*-mediated discard pathway.

In summary, mutations at eight positions of the yeast U6 RNA have been identified that allow increased utilization of non-adenosine pre-mRNA branch sites during the catalytic steps of splicing. These mutants appear to act indirectly through at least two different mechanisms to influence the fidelity of branch site utilization.

MATERIALS AND METHODS

Strains

The following yeast strains were used in this study and were obtained from H. Madhani. YHM1, MATa ura3 his3 lys2 trp1 leu2 snr6::LEU2 YCp50-SNR6 (Madhani et al., 1990); YHM145, MATa ade2 his3 leu2 lys2 prp16-302 trp1 ura3 snr6::LEU2 pSX6U (SNR6 URA3 CEN) (Madhani & Guthrie, 1994a); ΔCUPΔU6, MATα trp1 his3 ade2 leu2 ura3 lys2 CUP1::ura3 SNR6::LEU2 p(URA3 CEN ARS SNR6).

Oligonucleotides

A large number of synthetic oligonucleotides containing randomized positions were used for the construction of the U6 mutant pool (see Fabrizio et al., 1989). Other oligonucleotides used in this study were: U6S2, 5′-tttttgcatgcatgtgttcgcgaagtaaccct-3′; U6X2, 5′-tttttctcgaggataaaaaaaaaacgaaataaatctc-3′; U2SEQ, 5′-cattagcggaaaacaacttgc-3′; U6NU, 5′-gtaaaacggtucatccttatgcaggggaacugctgatcauctctg-3′; GACSEQ, 5′-gcagctaccacattggc-3′; GACS2, 5′-tttctgcagcatacgatttaggtgacactatagg ttgtaattctgtaaatcta-3′; GACS3, 5′-tttgaattcttggtttcttcagact-3′.

Plasmids

Wild-type and mutant p423-*ACT1-CUP1* fusion plasmids were constructed by subcloning of the *Eco*R 1–*Nae* I fragments of the pGAC-X plasmids described by Burgess and Guthrie (1993) into the *Eco*R 1–*Sma* I sites of pRS423 (Sikorski & Hieter, 1989). U6 mutants were either obtained as pT7U6 constructs from P. Fabrizio (Fabrizio & Abelson, 1990) and subcloned into pSX6T (see below) or were constructed in a two-step procedure involving, first, cloning of the mutants under control of a T7 promoter into pUC18 (pT7U6; see Fabrizio et al., 1989). In the second step, the U6 coding region of the pT7U6 mutant plasmids was PCR amplified using the oligonucleotides U6S2 and U6X2 and subcloned into the *Xho* I–*Sph* I sites of pSX6T (Madhani et al., 1990). The coding region in all clones was sequenced to confirm the presence of the desired mutation. To create the U6 mutant pool, equal volumes of individual plasmid preparations from each mutant were combined.

Yeast transformation

Yeast transformations were performed as described in Schiestl and Gietz (1989).

Copper assays and selection methods

Plates containing synthetic complete media with various levels of copper sulfate were prepared as described in Lesser and Guthrie (1993a). For copper plate assays, transformants were picked from selective plates and resuspended in 100–400 μL of sterile water. This suspension was then used for streaking plates. In the initial selection of U6 mutants, plasmids from candidate suppressors were recovered from cells using standard methods (Guthrie & Fink, 1991), sequenced, and the original stock of each mutant plasmid retransformed into the appropriate *ACT1-CUP1*/ΔCUPΔU6 strain to verify the suppressor phenotype.

RNA analysis

Cells were grown in the appropriate selective media at 30 °C to approximately 3×10^6 cells/mL and RNAs were prepared as described in Lesser and Guthrie (1993a). Primer extension reactions were performed as described in McPheeters and Abelson (1992) using 9 μg of each yeast RNA. RNA for *ACT1-CUP1* fusion sequencing reactions was prepared by in vitro transcription of a template generated by PCR amplification of p423-A259WT using the oligonucleotides GACS2 and GACS3. Primer extension on ACT1-CUP1 RNA was done using the primer GACSEQ, on the U2 RNA using the primer U2SEQ, and on the U6 RNA using the primer U6NU. Data from these experiments were collected on a Molecular Dynamics PhosphorImager.

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