Identification of a U2/U6 helix Ia mutant that influences 3' splice site selection during nuclear pre-mRNA splicing

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ABSTRACT

Base substitutions in U2/U6 helix I, a conserved base-pairing interaction between the U6 and U2 snRNAs, have previously been found to specifically block the second catalytic step of nuclear pre-mRNA splicing. To further assess the role of U2/U6 helix I in the second catalytic step, we have screened mutations in U2/U6 helix I to identify those that influence 3' splice site selection using a derivative of the yeast actin pre-mRNA. In these derivatives, the spacing between the branch site adenosine and 3' splice site has been reduced from 43 to 12 nt and this results in enhanced splicing of mutants in the conserved 3' terminal intron residue. In this context, mutation of the conserved 3' intron terminal G to a C also results in the partial activation of a nearby cryptic 3' splice site with U as the 3' terminal intron nucleotide. Using this highly sensitive mutant substrate, we have identified a mutation in the U6 snRNA (U57A) that significantly increases the selection of the cryptic 3' splice site over the normal 3' splice site and augments its utilization relative to that observed with the wild-type U2 or U6 snRNAs. In a previous study, we found that the same U6 mutation suppressed the effects of an A-to-G branch site mutation in an allele-specific fashion. The ability of U6–U57 mutants to influence the fidelity of both branch site and 3' splice site recognition suggests that this nucleotide may participate in the formation of the active site(s) of the spliceosome.

Keywords: RNA splicing; splicing fidelity; U2 RNA; U6 RNA

INTRODUCTION

The similarity in the chemical steps involved in nuclear pre-mRNA splicing to those of self-splicing group II introns has led to the hypothesis that nuclear pre-mRNA splicing may be a fundamentally RNA-catalyzed reaction (Cech, 1983; Sharp, 1985). According to this hypothesis, interactions between conserved pre-mRNA intron sequences and the highly conserved spliceosomal snRNAs form the active sites for catalysis. Consistent with this hypothesis, a large number of biochemical and genetic experiments have now demonstrated that the spliceosomal snRNAs play important roles in recognition of the 5' splice site and branch site sequences (Madhani & Guthrie, 1994b; Jacquier, 1997; Nilsen, 1998) and participate directly in the first catalytic step of splicing through the coordination of a catalytic Mg^{+2} ion (R.-J. Lin, pers. comm.).

The possible role(s) of the spliceosomal snRNAs in the recognition of the conserved YAG/ sequence at the 3' splice site and in the catalysis of the second step of nuclear pre-mRNA splicing remain uncertain, however. In yeast, the first two bases of the 3' exon adjacent to the 3' splice site are able to base pair with nucleotides in loop I of the U5 RNA, suggesting an intimate involvement of this snRNA in the transesterification reaction at the 3' splice site (Newman & Norman, 1992). However, because exon sequences adjacent to the 3' splice site are not conserved (Burge et al., 1999; Spingola et al., 1999), and substantial changes in the sequence of loop I of U5 do not significantly affect the second step of splicing (O'Keefe et al., 1996; Segault et al., 1999), the general importance and nature of these interactions in 3' splice site recognition are unresolved. Biochemical and genetic data have also suggested that a portion of the highly conserved A₄₇CAGAGA₅₃ sequence (yeast numbering) of the U6 snRNA is likely involved in the second catalytic step (Fabrizio & Abelson, 1990; Madhani et al., 1990). Consistent with a possible role for these nucleotides in recognition of the 3' splice site, the G52U mutant nonspecifically suppresses the ef-

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fects of 3' splice site mutations (Lesser & Guthrie, 1993b). The most significant RNA–RNA interaction involved in 3' splice site selection thus far discovered does not involve any of the spliceosomal snRNAs, but instead involves the formation of a non-Watson–Crick base pairing interaction between the first and last intron residues (Parker & Siliciano, 1993; Deirdre et al., 1995).

Activation of the spliceosome for catalysis is thought to be triggered by formation of a base-pairing interaction between U2 and U6 snRNAs (U2/U6 helix I; Fig. 1), which juxtaposes residues in U6 that interact with the 5' splice site with sequences in U2 that interact with the branch site (Madhani & Guthrie, 1992; Lesser & Guthrie, 1993b). A central role of U2/U6 helix I in formation of the active site for the second catalytic step was first suggested by the finding that several mutations in the "core" region of this bipartite helix specifically inhibit the second catalytic step (Fig. 1; Fabrizio & Abelson, 1990; Madhani & Guthrie, 1992; McPheeters & Abelson, 1992). The actual close proximity of U2 residue U23 in U2/U6 helix I to the scissile phosphodiester bond at the 3' splice site prior to the second step has been shown by crosslinking analysis (Newman et al., 1995). Genetic evidence has suggested the formation of a tertiary interaction between U2 nucleotide A25 in the bulge of U2/U6 helix I and G52 in the conserved U6 A₄₇CAGAGA₅₃ sequence (Madhani & Guthrie, 1994a). One attractive feature of this proposed tertiary interaction between U6–G52 and U2–A25 is that it would juxtapose the two clusters of residues in the U2 and U6 snRNAs that are specifically required for the second chemical step. Interestingly, the U2–A25U mutant, like the U6–G52U mutant, nonspecifically suppresses the effects of 3' splice site mutations.

FIGURE 1. Model for U2/U6 helix I in *S. cerevisiae*. Nucleotides in U2/U6 helix I implicated as having important roles in the second catalytic step are shown in bold and form the "core" of U2/U6 helix I. Other U2/U6 helix I nucleotides that have not been experimentally implicated in the second catalytic step are shown in gray. Base substitutions at nucleotides shown in upper case, as well as phosphorothioate substitution of the phosphate between U6 positions 58 and 59, have been demonstrated to substantially inhibit the second catalytic step of splicing in vitro. Nucleotide U23 of the U2 snRNA (underlined) has been shown to crosslink to the first base of the 3' exon prior to the second catalytic step. Nucleotide U57 of the U6 snRNA, shown in this report to influence selection of the 3' terminal intron nucleotide, is circled. Solid lines indicate experimentally demonstrated individual Watson–Crick base pairs, and dotted lines indicate proposed base pairs.

An important role for the bulge in U2/U6 helix I is also suggested by the finding that, although deletions removing either base in the bulge are not lethal, a mutant that deletes both nucleotides is inviable (Madhani & Guthrie, 1994a). In vitro reconstitution experiments have also suggested that the bulge region of U2/U6 helix I may potentially participate in coordination of a catalytic metal ion specifically required for the second step (Fabrizio & Abelson, 1992). Although the viability of compensatory mutants in U2/U6 helix I has suggested that this helix is unlikely to specifically interact with the 3' splice site (Madhani & Guthrie, 1994a), functionally redundant interactions between the U2 and U6 RNAs (Field & Friesen, 1996; Luukkonen & Séraphin, 1998), as well as other spliceosomal components (Frank et al., 1992; Xu et al., 1996, 1998), could obscure the detection of such specific interactions.

Genetic and biochemical experiments have shown that several factors specifically required for the second step, including Slu7p, Prp18p, and Prp22p, are dispensable for splicing of pre-mRNAs in which the 3' splice site is in close proximity to the branch site (Frank & Guthrie, 1992; Brys & Schwer, 1996; Zhang & Schwer, 1997; Schwer & Gross, 1998). To reduce the possible requirements for these factors in 3' splice site selection in vivo, we have used derivatives of the yeast actin intron in which the spacing between the branch site and 3' splice site has been reduced from its natural distance of 43 nt to 12 nt. In contrast to the full-length actin intron, mutations in the 3' terminal intron nucleotide of these shortened derivatives splice very efficiently, and in some cases cause the partial activation of a nearby cryptic 3' splice site. We have used one of these mutant substrates to screen for U2/U6 helix I mutants that influence 3' splice site selection. This screen has identified a single base substitution in U2/U6 helix I (U6–U57A) that is able to both alter the ratio of normal-to-cryptic 3' splice site use and increase the overall splicing of substrates containing uridine as the 3' terminal intron nucleotide. Interestingly, this same U6 mutant was previously identified as an allele-specific suppressor of A → G branch site mutations (McPheeters, 1996). These results are consistent with a previously proposed model for RNA-based catalysis by the spliceosome in which a single site is responsible for binding of the branch site nucleotide in the first step and the 3' terminal intron residue in the second step (Steitz & Steitz, 1993), and suggest that U6–U57 contributes to the formation of this binding site.

RESULTS

Efficient utilization of mutant 3' splice sites in a derivative of the yeast actin intron

To facilitate the identification of mutations in U2/U6 helix I that affect recognition of the 3' splice site, de-

derivatives of the *ACT1-CUP1* reporter gene fusions developed by Lesser and Guthrie (1993a) in which the spacing between the branch site and 3' splice site was either 43 or 12 nt were used. The relevant features of the *ACT1-CUP1* constructs used are compared in Figure 2A. The sequence between the branch site and 3' splice site of the full-length ACT_{43} construct is identical to that found in the wild-type yeast actin intron (Ng & Abelson, 1980). The ACT_{43} construct is also identical to the *ACT1-CUP1* fusions used in previous studies (Lesser & Guthrie, 1993a, 1993b; Madhani & Guthrie, 1994a; McPheeters, 1996), except that the 3' exon sequence has been altered so that the next (Y)AG is 34 nt downstream from the normal 3' splice site (see Materials and methods). The ARS_{12} (actin reduced spacing) constructs are identical to the ACT_{43} constructs in all respects, except that the interval between the branch site adenosine and 3' splice site has been reduced from its natural distance of 43 nt to 12 nt (Fig. 2A). To assay the effects of decreasing the interval between the branch site and 3' splice site, primer-extension assays were performed using an end-labeled 3' exon primer and RNAs harvested from cells harboring wild-type versions of either the ACT_{43} or ARS_{12} constructs (Fig. 2B, lanes 1 and 5). Both wild-type constructs were found to produce mRNA species of identical length, indicating that each used the expected 3' splice site at position 303 (see Fig. 2A). Interestingly, the amount of spliced mRNA produced by the wild-type ARS_{12} substrate was consistently found to be approximately 2.6-fold higher than the amount produced by the wild-type ACT_{43} substrate. This increase in spliced mRNA appears to be caused, at least in part, by an approximately sevenfold increase in the efficiency of the second step as determined by the ratio of mature to lariat intermediate RNA (M/L; Fouser & Friesen, 1986; data not shown). No significant difference in the apparent efficiency of the first step, as measured by the ratio of mature plus lariat intermediate to precursor RNAs ($[M + L]/P$), was observed between the wild-type ACT_{43} and ARS_{12} substrates (data not shown).

To assay the effects of base substitutions in the 3' terminal intron nucleotide (position 303), mutant derivatives of the ACT_{43} and ARS_{12} substrates were made. Consistent with previous observations using similar *ACT-CUP1* fusions (Lesser & Guthrie, 1993a, 1993b; Madhani & Guthrie, 1994a; Parker & Siliciano, 1993), only very low amounts of mRNA are produced by any of the 3' terminal intron nucleotide mutants in the ACT_{43} constructs (Fig. 2B, lanes 2–4). Strikingly, significantly higher levels of total spliced mRNA (25–33% the level of the wild-type ARS_{12} substrate) were produced by each of the 3' terminal intron nucleotide mutants in the ARS_{12} constructs (Fig. 2B, lanes 6–8). Surprisingly, ~60% and ~25% of the total spliced mRNA produced with the ARS_{12} -C₃₀₃ and ARS_{12} -U₃₀₃ substrates, respectively, is two bases shorter than that observed with the

FIGURE 2. Primer extension analysis of the effects of altering the branch site to 3' splice site spacing on the splicing of substrates containing each of the four possible 3' terminal intron nucleotides. **A:** Sequences of the 3' splice site region of the wild-type ACT_{43} and ARS_{12} *ACT1-CUP1* fusions. The conserved yeast branch site sequences and 3' splice site sequence are shown in upper case. The branch site adenosine is marked with an asterisk and the positions of 3' splice site cleavages with arrows. The sequence of the 34 nt not shown in the ACT_{43} construct is identical to those found in the wild-type yeast actin intron; they are 5'-attgcttcattctttgttgctatattatgt-3'. The sequence of the 3' exon adjacent to the 3' splice site in both constructs is identical, and has been altered to eliminate any nearby, potentially competing AG dinucleotides (see Materials and methods). **B:** Primer extension assays of the *in vivo* splicing of the wild-type (G₃₀₃) and mutant (N₃₀₃) ACT_{43} and ARS_{12} *ACT1-CUP1* fusions were performed using the primer GAC-SEQ as previously described (McPheeters, 1996) and a U1 snRNA specific primer as an internal loading control. RNA species are labeled as P: pre-mRNA; M: mRNA; U1: U1 internal loading control; and L: lariat-3' exon intermediate. The predicted size of primer extension product resulting from cleavage at the closest (Y)AG sequence 34 nt downstream from the normal 3' splice site for both the ACT_{43} and ARS_{12} substrates is 167 nt. The predicted sizes of the primer-extension products for the ACT_{43} / ARS_{12} substrates are as follows: pre-mRNA: 501/470 nt; mRNA: 201/201 nt; lariat intermediate: 135/104 nt; debranched lariat intermediate: 392/361 nt. The size of the U1E primer-extension product is 153 nt. Small amounts of an ~213 nt primer extension product that may result from 3' cleavage at position +30 from the branch site nucleotide in the ACT_{43} constructs are visible in lanes 2–4, but the origin of these bands has not been investigated further.

wild-type ARS₁₂ substrate (Fig. 2B, lane 5). This shorter mRNA was found to result from the activation of a cryptic 3' splice site at position U₃₀₅ (Fig. 2B, lanes 7,8; see Fig. 2A), as primer-extension analysis using an intron specific oligonucleotide showed the position of 5' splice site cleavage to be identical to wild type (data not shown). In addition, mutation of position 305 in the ARS₁₂-C₃₀₃ substrate was found to abolish cryptic 3' splice site use (see Fig. 5). No significant use of this cryptic 3' splice site was observed with either the wild-type ARS₁₂ substrate (Fig. 2B, lane 5), the ARS₁₂-A₃₀₃ substrate (Fig. 2B, lane 6), or any of the ACT₄₃ substrates (Fig. 2B, lanes 1–4). Aside from use of the cryptic 3' splice site at position 305 in the ARS₁₂ constructs, no significant cleavage at the next (Y)AG sequence 34 nt downstream, or any other sequences, was observed (Fig. 2B; data not shown).

Screening base substitutions in U2/U6 helix I for effects on 3' splice site selection

To explore the possible involvement of nucleotides in U2/U6 helix I in recognition of the 3' terminal intron nucleotide, we screened 44 out of the 48 total possible base substitutions in U2/U6 helix I for their effects on the splicing of the ARS₁₂-C₃₀₃ substrate. This substrate was chosen because it was expected that any U2/U6 helix I mutants specifically affecting recognition of the 3' terminal intron residue might influence 3' splice site cleavage at C₃₀₃ and/or U₃₀₅, and thus alter the ratio of normal to cryptic 3' splice site usage. The yeast strain constructed for the screening of U2/U6 helix I mutants, Δ U2 Δ U6 Δ CUP (see Materials and methods), contains disruptions of the chromosomal copies of the U2 (*SNR20*) and U6 (*SNR6*) genes, and the wild-type U2 and U6 genes are both supplied on a single-copy *URA3* plasmid, permitting counterselection using 5-fluoroorotic acid (Boeke et al., 1987). The effects of all 23 possible viable or conditionally lethal U2 mutants in U2/U6 helix I on splicing of the ARS₁₂-C₃₀₃ substrate were examined by cotransformation of Δ U2 Δ U6 Δ CUP with the U2 mutant and wild-type U6 plasmids and subsequent loss of the wild-type U2/U6 plasmid. Similarly, the effects of two of the four possible inviable U2 mutants were examined by cotransformation of the mutant U2 plasmid with a U6 plasmid expressing the predicted compensatory mutant (Madhani & Guthrie, 1992, 1994a). RNAs prepared from these strains were then assayed by primer extension and the results quantitated by phosphorimager analysis (Fig. 3A; data not shown). Although many of the U2 mutants were found to cause a significant decrease in the total amount of spliced mRNA, none were found to significantly alter the ratio of normal to cryptic 3' splice site use. In agreement with previous results (Madhani & Guthrie,

1994a), the U2–A25G mutant was found to partially suppress the second-step splicing defect of the ARS₁₂-C₃₀₃ substrate, and increased utilization of both the normal and cryptic 3' splice sites (Fig. 3A, lane 14). Interestingly, several of the single base U2 mutants in the core region of U2/U6 helix I which exhibit significant second step defects in vitro (G26C and A27G; McPheeters & Abelson, 1992) did not exhibit severe second-step defects in the presence of the predicted compensatory U6 mutant in this assay (Fig. 3A, lanes 17, 21).

The effects of 13 out of the 14 possible viable or conditionally lethal U6 mutants in U2/U6 helix I were examined by cotransformation of Δ U2 Δ U6 Δ CUP with wild-type U2 and mutant U6 plasmids and subsequent loss of the wild-type U2/U6 plasmid. For the six possible inviable U6 mutants in U2/U6 helix I, mutant U6 genes were expressed from a multi-copy (2 μ) plasmid in the presence of the wild-type U6 RNA expressed from a single-copy (CEN ARS) plasmid (Kandels-Lewis & Séraphin, 1993). The in vivo growth phenotype of the U6-A59U mutant, which strongly inhibits the second step in vitro (Fabrizio & Abelson, 1990) has not been reported, and this mutant was not included in our screen. Our screen of the effects of U6 mutants on splicing of the ARS₁₂-C₃₀₃ substrate is shown in Figure 3B; quantitative data was collected by phosphorimager analysis. Like many of the U2 mutants (Fig. 3A), a significant number of the U6 mutants caused substantial decreases in the total amount of spliced mRNA (Fig. 3B). Strikingly, one U6 mutant (U57A; Fig. 3B, lane 9) was found both to cause the almost exclusive use (>90% compared to ~60% for wild-type U6) of the cryptic 3' splice site at U₃₀₅ relative to the normal 3' splice site at C₃₀₃, and to increase total mature mRNA production approximately 1.3-fold over that observed with wild-type U6. Measurement of the ratio of mature to lariat intermediate RNA (M/L) showed that the U6–U57A mutation increases the efficiency of the second step of splicing of the ARS₁₂-C₃₀₃ substrate approximately threefold over wild-type U6 (data not shown). Only one other U6 mutant, U57G (Fig. 3B, lane 11), significantly increased use (~80%) of the cryptic 3' splice site compared to wild-type U6. However, in contrast to the U6–U57A mutant, the U6–U57G mutant produced only 20% as much total spliced mRNA as wild-type U6, and decreased the efficiency of the second step approximately threefold compared to wild-type U6. Interestingly, although the proposed pairing partner of U6–U57 in U2/U6 helix I is U2–A27 (Madhani & Guthrie, 1992), neither of the base substitutions tested at this position (U2–A27C and U2–A27G; Fig. 3A, lanes 21 and 22) increased mRNA production or significantly altered the ratio of normal to cryptic 3' splice site use. These results suggest that the U6–U57A mutation may either increase the utilization of introns con-

FIGURE 3. Screening of U2/U6 helix I base substitution mutants for their effects on the splicing of the ARS₁₂-C₃₀₃ substrate (see text for details). RNA species are labeled in Figure 2, but only a portion of the gel is shown. **A:** Screen of U2 mutants. **B:** Screen of U6 mutants.

taining U as the 3' terminal nucleotide, or increase the preferred branch site to 3' splice site spacing.

Characterization of altered 3' splice site utilization by the U6–U57A mutant

To further characterize the effects of U6–U57 mutants on 3' splice site recognition, primer-extension assays of the *in vivo* splicing of all possible U6–U57 mutants with ARS₁₂ substrates containing all four possible 3' terminal intron nucleotides were performed (Fig. 4). None of the U6–U57 mutants were found to activate cleavage at the cryptic 3' splice site or to significantly affect the amount of spliced mRNA produced with the wild-type ARS₁₂-G₃₀₃ intron (Fig. 4, lanes 1–4). In contrast, with each of the three mutant ARS₁₂ substrates, the U6–U57A mutant consistently activated cleavage at the cryptic 3' splice site at position U₃₀₅ (Fig. 4, lanes 6, 10, and 14). With both the ARS₁₂-A₃₀₃ and ARS₁₂-U₃₀₃ sub-

strates, the U57A mutant did not significantly alter the level of cleavage at the normal site (position 303), but instead enhanced cleavage at the cryptic site (position 305). This result is particularly striking with the ARS₁₂-U₃₀₃ substrate (Fig. 4, lane 14), and suggests that, with the U57A mutation, the branch site to 3' splice site spacing required for cleavage at the more proximal site (U₃₀₃) may be unfavorable relative to that of the more distal (U₃₀₅) 3' splice site. The U6–U57G mutant was also observed to weakly activate cleavage at the cryptic 3' splice site, but in contrast to the U57A mutant, dramatically decreased the overall amount of spliced mRNA compared to wild-type U6 (Fig. 4, lanes 8, 12, and 16). The U6–U57C mutant consistently reduced mRNA levels and did not cause activation of cryptic 3' splice site cleavage (Fig. 4, lanes 7, 11, and 15).

As noted above, the increased use of the cryptic 3' splice site in the ARS₁₂-A₃₀₃, ARS₁₂-C₃₀₃, and ARS₁₂-U₃₀₃ substrates by the U6–U57A mutant suggests that

FIGURE 4. The U6–U57A mutant activates use of the cryptic 3' splice site at U305 in introns containing mutations of the 3' terminal intron nucleotide. Effects of U6–U57 mutants on splicing of wild-type and ARS₁₂ substrates containing all possible 3' terminal intron nucleotides (N₃₀₃) were assayed by primer-extension analysis. RNA species are labeled in Figure 2, but only a portion of the gel is shown.

this U6 mutation may either increase use of uridine as the 3' terminal intron nucleotide, or increase the preferred branch site to 3' splice site spacing. The branch site to 3' splice site spacing for both position 303 and 305 (12 and 14 nt, respectively) is less than the optimal branch site to 3' splice site spacing of 18–22 nt observed *in vivo* (Luukkonen & Séraphin, 1997) or the mean spacing of 39 nt determined by genomic analysis (Spingola et al., 1999). To test if the preferred branch site to 3' splice site spacing alone was altered by the U6–U57A mutant, position 305 was changed to C, and the effect of the U57A mutant on the ratio of cryptic splice site usage was assayed in the ARS₁₂-(C₃₀₃ + C₃₀₅) double mutant substrate (Fig. 5). Although cryptic 3' splice site use was observed with the original ARS₁₂-C₃₀₃ substrate (Fig. 5, C₃₀₃ + U₃₀₅, lane 1), and cleavage at this site was greatly enhanced by the U57A mutant (Fig. 5, lane 2), no cryptic 3' splice site cleavage in the C₃₀₃ + C₃₀₅ mutant substrate is observed with either wild-type U6 or the U57A mutant (Fig. 5, lanes 3 and 4). With the C₃₀₃ + C₃₀₅ substrate, the U6–U57A mutant decreases 3' splice site cleavage at C₃₀₃ relative to wild-type U6 (Fig. 5, lanes 3 and 4), suggesting that the increase in cryptic 3' splice site usage by the U57A mutant with the ARS₁₂-C₃₀₃ substrate (Fig. 5, lanes 1 and 2) results from both decreased use of C and increased use of U as the 3' terminal intron nucleotide.

The ARS₁₂-C₃₀₃ mutant was chosen for our screen of U2/U6 mutants influencing 3' splice site usage because of its ability to efficiently use either C or U as the 3' terminal intron nucleotide (Fig. 2). In a previous study,

FIGURE 5. The U6–U57A mutation does not activate cleavage at the cryptic 3' splice site in a C₃₀₃ + C₃₀₅ double mutant ARS₁₂ substrate. RNA species are labeled in Figure 2, but only a portion of the gel is shown.

we identified the U6–U57A mutant as a weak, but specific suppressor of an A-to-G branch site mutation (McPheeters, 1996). Using copper-resistance assays, the U6–U57A mutant was found not to affect the splicing of the C₃₀₃ mutant in the context of a full-length actin intron. To determine if the apparent increase in utilization of 3' splice sites with a terminal U could be observed in the context of a full-length actin intron, we used a more sensitive primer-extension assay to examine the effects of the U6–U57A mutation on the splicing of the ACT₄₃-U₃₀₃ construct. Figure 6 shows this analysis with RNAs isolated from cells harboring either wild-type or U57A mutant U6 plasmids and ACT₄₃ plasmids containing each of the four possible 3' terminal intron nucleotides. Compared to wild-type U6, the U6–U57A mutant did not significantly increase the splicing of any of the 3' terminal intron nucleotide mutants. A slight increase (~1.4-fold) in the splicing of the ACT₄₃-U₃₀₃ substrate was visible in darker exposures (Fig. 6, lower panel; data not shown). This overall increase is similar to the ~1.3-fold increase in overall splicing observed with the ARS₁₂-C₃₀₃ and ARS₁₂-U₃₀₃ substrates (Fig. 4), and is consistent with the U6–U57A mutation increasing the utilization of uridine as the 3' terminal intron nucleotide. As previously observed with the wild-type full-length actin intron (McPheeters, 1996), the U6–U57A mutant (Fig. 6, lane 5) caused a moderate decrease (~30%) in the level of mature mRNA relative to wild-type U6 with the ACT₄₃-G₃₀₃ substrate (Fig. 6, lane 1).

FIGURE 6. U57A enhances the weak splicing of the full-length ACT43-U₃₀₃ substrate. The figure shows a comparison between RNAs expressing either wild-type U6 or the U6-U57A mutant on splicing of full-length ACT₄₃ substrates containing all possible 3' terminal intron nucleotides. In the top panel, RNA species are labeled as in Figure 2. The lower panel shows only the mRNA band from a darker exposure of the gel.

DISCUSSION

Accurate recognition of both the 5' splice site and the branch site sequences in nuclear pre-mRNA introns appears to rely upon multiple, sequential recognition steps (Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993b; Berglund et al., 1997; Staley & Guthrie, 1998). In contrast to the factors and steps involved in 5' splice site and branch site recognition, the identity and roles of the factors, as well as the number of individual steps involved in 3' splice site recognition is much less well understood (Umen & Guthrie, 1995b). Recent experiments have clearly established that in metazoan introns, the 3' splice site AG is recognized in a sequence-specific manner during prespliceosome formation by U2AF³⁵ (Merendino et al., 1999; Wu et al., 1999; Zorio & Blumenthal, 1999). Although no homolog of U2AF³⁵ exists in *Saccharomyces cerevisiae* (Abovich & Rosbash, 1997), we have recently found an ~110-kDa yeast protein that crosslinks to the 3' splice site following prespliceosome formation and thus may be a candidate for an early 3' splice site recognition factor (McPheeters et al., 2000). Although a 3' splice site is not absolutely required for the first step in yeast (Ry-

mond & Rosbash, 1985), recognition of the 3' splice site prior to the first catalytic step could facilitate its subsequent utilization in the second catalytic step.

Prior to the second step of nuclear pre-mRNA splicing, the 3' splice site in the intron lariat intermediate must be selected and correctly positioned relative to the 3'-OH group of the excised 5' exon. At least six yeast proteins have thus far been identified that may play roles in 3' splice site selection during the second step (Frank & Guthrie, 1992; Schwer & Guthrie, 1992; Ansari & Schwer, 1995; Jones et al., 1995; Umen & Guthrie, 1995a, 1995b, 1995c; Schwer & Gross, 1998; McPheeters et al., 2000). Prp16p, Prp17p, Slu7p, Prp18p, and Prp22p appear to function in two steps to facilitate a conformational rearrangement in the spliceosome that is thought to reflect the identification of the 3' splice site and its positioning within the active site for the second catalytic step. Although several of these factors, including Prp8, Prp16, Prp22, and Slu7, can be crosslinked to the 3' splice site (Umen & Guthrie, 1995c; McPheeters et al., 2000), it is unknown if any of these proteins interacts with the 3' splice site in a sequence-specific manner.

We have demonstrated that, in derivatives of the yeast actin intron in which the interval between the branch site adenosine and 3' splice site has been substantially reduced, 3' terminal intron nucleotide mutants are spliced efficiently, and utilize either the expected 3' splice site or a nearby cryptic 3' splice site (Fig. 2). Both the ARS₁₂-C₃₀₃ and ARS₁₂-U₃₀₃ mutants maintain a YA dinucleotide preceding the 3' terminal intron nucleotide at both the normal as well as the cryptic 3' splice site. In contrast, neither the wild-type ARS₁₂-G₃₀₃, nor the ARS₁₂-A₃₀₃ mutant contains a YA sequence preceding the cryptic 3' splice site, and this deviation from the 3' splice site consensus sequence (Burge et al., 1999; Spingola et al., 1999) likely forms the basis for the lack of cryptic 3' splice site usage in these constructs. These results highlight an apparent redundancy of interactions within the spliceosome, and suggest that interactions involving only a YA sequence located close to the branch site are sufficient to dictate 3' splice site choice. Although the precise nature of the interactions involving the YA of the 3' splice site is uncertain, both Prp8p and U6-A51 may be involved (Collins & Guthrie, 1999; Siatecka et al., 1999). Additional contributions to the efficient recognition of these mutant 3' splice sites may come from the interaction of the U5 RNA with 3' exon sequences (Newman & Norman, 1992). It has also been suggested that the non-Watson-Crick interaction between terminal intron residues (Parker & Siliciano, 1993) may extend to include interactions between the penultimate intron adenosine (YAG/) and the second intron nucleotide (/GU), but tests of this proposal have thus far been negative (Ruis et al., 1994).

The efficient splicing of 3' terminal intron nucleotide mutants in the ARS₁₂ substrates, where the distance

between the branch site and 3' splice site is very short, contrasts with their inefficient splicing in the parental ACT₄₃ substrate (Fig. 2). We propose that this effect may reflect the *in vivo* bypass of a recognition step(s) involving Slu7p, Prp18p, and/or Prp22p, as the requirement for these proteins *in vitro* can be alleviated by the use of pre-mRNA substrates having a short branch site to 3' splice site spacing (Brys & Schwer, 1996; Zhang & Schwer, 1997; Schwer & Gross, 1998; see also Frank & Guthrie, 1992). This hypothesis is consistent with a previous proposal that sequences upstream from the yeast actin 3' splice site may normally serve as the binding site for a negatively acting splicing factor (Kivens & Siliciano, 1996). Bypass of an early step(s) in a multiple-step recognition pathway could permit more direct and less accurate 3' splice site recognition by components in the active site of the spliceosome.

Our screen of base substitution mutants in U2/U6 helix I affecting 3' splice site utilization with the ARS₁₂-C₃₀₃ substrate (Fig. 2) has demonstrated that most mutants decrease the overall production of mature mRNA and the efficiency of the second step (Fig. 3; data not shown). This decrease in splicing efficiency is consistent with these mutants directly affecting 3' splice site recognition by destabilizing the formation of Watson-Crick base pairs in U2/U6 helix I. Because several of the bases in U2/U6 helix I are involved in multiple and mutually exclusive base-pairing interactions (Madhani & Guthrie, 1992), it is also possible that decreased splicing efficiency observed may be related to defects in other stages of splicing, rather than being directly attributable to perturbations of interactions involving the 3' splice site. Additionally, some of the U2/U6 helix I mutants may indirectly affect 3' splice site recognition by perturbing the interaction of this helix with other

spliceosomal components involved in the second catalytic step.

Our screen has identified a single U2/U6 helix I mutant, U6-U57A, that affects recognition of the 3' splice terminal intron nucleotide. In a previous study, we found that U57 mutants were capable of suppressing the first- and/or second-step splicing defects caused by nonadenosine branch sites in a unique, allele-specific manner (McPheeters, 1996). In our present experiments, we have shown that the U6-U57A mutant increases the splicing of substrates containing uridine as the 3' terminal intron nucleotide (Figs. 3, 4, and 6) and decreases the splicing of substrates containing cytidine as the 3' terminal intron nucleotide (Fig. 5) compared to wild-type U6.

The ability of U57 mutants to influence both branch site and 3' splice site selection is consistent with the proposal that U2/U6 helix I forms at least a portion of the active site for the second catalytic step of splicing (Madhani et al., 1990; Madhani & Guthrie, 1992, 1994a; McPheeters & Abelson, 1992; Newman et al., 1995; McPheeters, 1996) and with the assignment of U57 as an active site component in the general two-metal-ion mechanism for catalytic RNA proposed by Steitz & Steitz (1993). The application of this model to the splicing of nuclear pre-mRNA introns by the spliceosome is shown in Figure 7.

This model proposes the existence of three specific binding sites for the reactive groups of the substrate. In the first catalytic step, the 2' hydroxyl group of the branch site adenosine bound in site 2 attacks the phosphodiester bond at the 5' splice site, which is bound by sites 1 and 3. Between the first and second catalytic steps, a rearrangement of the active site (Schwer & Guthrie, 1992; Moore & Sharp, 1993) occurs that re-

FIGURE 7. General two-metal-ion mechanism for catalytic RNA applied to the splicing of nuclear pre-mRNA introns by the spliceosome (redrawn from Steitz & Steitz, 1993) showing likely contributions of the spliceosomal snRNAs to the formation of the proposed binding sites.

sults in a remodeling of site 2 so that it now interacts with the conserved guanosine at the 3' terminus of the intron during the second step of the reaction (Fig. 7). Our data showing that U57 mutants can affect the recognition of the branch site residue in the first catalytic step and the 3' terminal intron nucleotide in the second catalytic step are consistent with U6 nucleotide U57 contributing to the formation of binding site 2 in this model.

The specific role of U57 in recognition of the branch site residue and 3' splice site remains uncertain, however. The allele-specific suppression of both branch site and 3' splice site mutants by U57 mutants suggests that this base may directly function in the binding of substrates to site 2 (Fig. 7). Although it is possible that U57 interacts with the branch site and/or 3' terminal intron nucleotide through specific hydrogen-bonding interactions, additional data will be required to determine if such specific contacts exist. Alternatively, U57 mutants may act indirectly by perturbing the structure of the active site or the interactions of other factors involved in 3' splice site recognition. The effects of U57 mutants on the fidelity of pre-mRNA splicing may therefore be similar to ribosomal RNA mutants that reduce translational accuracy by altering the affinity of the A-site for cognate and noncognate aminoacyl-tRNAs, or by altering the rate of NTP-hydrolysis and factor release (see Green & Noller, 1997).

MATERIALS AND METHODS

Plasmids

pACT₄₃ (*ADE2 CEN ARS*) was constructed by first subcloning the *ACT1-CUP1* *EcoRI-PvuII* fragment from p423-*ACT1-CUP1* (McPheeters, 1996) into *EcoRI-PvuII*-cut pASZ12 (Stotz & Linder, 1990), followed by PCR-mediated alteration of the 3' exon sequence to eliminate any closed spaced AG dinucleotides downstream of the normal 3' splice site. In pACT₄₃, the 3' exon sequence between the 3' splice site and *KpnI* sites (underlined) was changed from that found in pGAC14 (Lesser & Guthrie, 1993a) to 5'-tagataacataaaaaa cactcatggtacc-3'. pARS₁₂ (*ADE2 CEN ARS*) was constructed by using a deletion bearing 3' splice site region oligonucleotide for PCR and recloning of this PCR product into *KpnI*-cut pACT₄₃. The length of the intron in the ARS₁₂ constructs is 272 nt compared to 303 nt in the parental ACT₄₃ constructs (see Fig. 2). 3' terminal intron nucleotide mutants were constructed using PCR and oligonucleotides bearing the appropriate mutations using standard procedures. U6 mutants in pSX6T (*SNR6 TRP1 CEN ARS*; Madhani et al., 1990) were described previously (McPheeters, 1996). For multicopy expression of inviable U6 mutants, *BamHI* fragments from the appropriate U6 mutants in pSX6T were subcloned into *BamHI* cut pRS424 (*TRP1*, 2 μ). Construction of U2 mutants was performed by subcloning *EcoRI-EcoNI*-cut PCR fragments from the appropriate pT7U2 mutant plasmids (McPheeters & Abelson, 1992) into pCAU2. pCAU2 (*SNR20 HIS3 CEN ARS*) is a derivative of pRS313 con-

taining the wild-type yeast U2 (*SNR20*) gene (this laboratory) with a single base insertion at the 5' end to create a unique *EcoRI* site (see Shuster & Guthrie, 1990).

Yeast strains

The strain Δ U2 Δ U6 Δ CUP (DM1998: *MATa trp1 his3 ade2 leu2 ura3 lys2 CUP1::ura3 SNR6::LEU2 SNR20::LYS2 pU2U6U [CEN ARS SNR6 SNR20 URA3]*) was constructed by mating the strain Δ U6 Δ CUP (*MATa trp1 his3 ade2 leu2 ura3 lys2 CUP1::ura3 SNR6::LEU2 p[URA3 CEN ARS SNR6]*, kindly supplied by Hiten Madhani) to strain Δ U2 Δ CUP (DM1979: *MATa trp1 his3 ade2 leu2 ura3 lys2 CUP1::ura3 SNR20::LYS2 pU2URA[URA3 CEN ARS SNR20]*, this laboratory). Transformations were performed as described by McPheeters (1996) and cotransformations of U2 and U6 plasmids were performed as described in Madhani & Guthrie (1994a).

Yeast RNA preparation and primer extension

Total yeast RNA preparation and primer extension analysis were performed as described in McPheeters (1996), except that the oligonucleotide U1E (sequence: 5'-gcgccgtatgtgtgtg tgacc-3'), complementary to the yeast U1 snRNA, was used for an internal loading control. To map the position of 5' splice site cleavage, the 5' end-labeled intron-specific oligonucleotide IN (McPheeters & Abelson, 1992) was used. Primer-extension reactions using different RNA preparations were repeated at least twice to verify the reproducibility of the results and data were analyzed using a Molecular Dynamics PhosphorImager. The amounts of each RNA product were normalized to the band resulting from the internal control done with a U1 snRNA specific primer, U1E.

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REFERENCES

- Abovich N, Rosbash M. 1997. Cross-intron bridging interactions in the yeast commitment complex are conserved in mammals. *Cell* 89:403-412.

- Ansari A, Schwer B. 1995. SLU7 and a novel activity, SSF1, act during the PRP16-dependent step of yeast pre-mRNA splicing. *EMBO J* 14:4001–4009.
- Berglund JA, Chua K, Abovich N, Reed R, Rosbash M. 1997. The splicing factor BBP interacts specifically with the pre-mRNA branch-point sequence UACUAAAC. *Cell* 89:781–787.
- Boeke JD, Trueheart J, Natsoulis G, Fink GR. 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol* 154:164–175.
- Brys A, Schwer B. 1996. Requirement for SLU7 in yeast pre-mRNA splicing is dictated by the distance between the branchpoint and the 3' splice site. *RNA* 2:707–717.
- Burge CB, Tuschl T, Sharp PA. 1999. Splicing of precursors to mRNAs by the spliceosomes. In: Gesteland RF, Cech TR, Atkins JF, eds. *The RNA world*, 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 525–560.
- Cech TR. 1983. RNA splicing: Three themes with variations. *Cell* 34:713–716.
- Collins CA, Guthrie C. 1999. Allele-specific genetic interactions between Prp8 and RNA active site residues suggest a function for Prp8 at the catalytic core of the spliceosome. *Genes & Dev* 13:1970–1982.
- Deirdre A, Scadden J, Smith CW. 1995. Interactions between the terminal bases of mammalian introns are retained in inosine-containing pre-mRNAs. *EMBO J* 14:3235–3246.
- Fabrizio P, Abelson J. 1990. Two domains of yeast U6 small nuclear RNA required for both steps of nuclear precursor messenger RNA splicing. *Science* 250:404–409.
- Fabrizio P, Abelson J. 1992. Thiophosphates in yeast U6 snRNA specifically affect pre-mRNA splicing in vitro. *Nucleic Acids Res* 20:3659–3664.
- Field DJ, Friesen JD. 1996. Functionally redundant interactions between U2 and U6 spliceosomal snRNAs. *Genes & Dev* 10:489–501.
- Fouser LA, Friesen JD. 1986. Mutations in a yeast intron demonstrate the importance of specific conserved nucleotides for the two stages of nuclear mRNA splicing. *Cell* 45:81–93.
- Frank D, Guthrie C. 1992. An essential splicing factor, SLU7, mediates 3' splice site choice in yeast. *Genes & Dev* 6:2112–2124.
- Frank D, Patterson B, Guthrie C. 1992. Synthetic lethal mutations suggest interactions between U5 small nuclear RNA and four proteins required for the second step of splicing. *Mol Cell Biol* 12:5197–5205.
- Green R, Noller HF. 1997. Ribosomes and translation. *Annu Rev Biochem* 66:679–716.
- Jacquier. 1997. RNA-catalysed RNA processing. In: Krainer AR, ed. *Eukaryotic mRNA processing*. New York: IRL Press. pp 1–36.
- Jones MH, Frank DN, Guthrie C. 1995. Characterization and functional ordering of Slu7p and Prp17p during the second step of pre-mRNA splicing in yeast. *Proc Natl Acad Sci USA* 92:9687–9691.
- Kandels-Lewis S, Séraphin B. 1993. Role of U6 snRNA in 5' splice site selection. *Science* 262:2035–2039.
- Kivens W, Siliciano PG. 1996. RNA sequences upstream of the 3' splice site repress splicing of mutant yeast ACT1 introns. *RNA* 2:492–505.
- Lesser CF, Guthrie C. 1993a. Mutational analysis of pre-mRNA splicing in *Saccharomyces cerevisiae* using a sensitive new reporter gene, CUP1. *Genetics* 133:851–863.
- Lesser CF, Guthrie C. 1993b. Mutations in U6 snRNA that alter splice site specificity: Implications for the active site. *Science* 262:1982–1988.
- Luukkonen BG, Séraphin B. 1997. The role of branchpoint-3' splice site spacing and interaction between intron terminal nucleotides in 3' splice site selection in *Saccharomyces cerevisiae*. *EMBO J* 16:779–792.
- Luukkonen BG, Séraphin B. 1998. A role for U2/U6 helix Ib in 5' splice site selection. *RNA* 4:915–927.
- Madhani HD, Bordonne R, Guthrie C. 1990. Multiple roles for U6 snRNA in the splicing pathway. *Genes & Dev* 4:2264–2277.
- Madhani HD, Guthrie C. 1992. A novel base-pairing interaction between U2 and U6 snRNAs suggests a mechanism for the catalytic activation of the spliceosome. *Cell* 71:803–817.
- Madhani H, Guthrie C. 1994a. Randomization-selection analysis of snRNAs in vivo: Evidence for a tertiary interaction in the spliceosome. *Genes & Dev* 8:1071–1086.
- Madhani HD, Guthrie C. 1994b. Dynamic RNA–RNA interactions in the spliceosome. *Annu Rev Genet* 28:1–26.
- McPheeters DS. 1996. Interactions of the yeast U6 RNA with the pre-mRNA branch site. *RNA* 2:1110–1123.
- McPheeters DS, Abelson J. 1992. Mutational analysis of the yeast U2 snRNA suggests a structural similarity to the catalytic core of group I introns. *Cell* 71:819–831.
- McPheeters DS, Schwer B, Muhlenkamp P. 2000. Interaction of the yeast DEXH-box RNA helicase, Prp22p, with the 3' splice site during the second step of nuclear pre-mRNA splicing. *Nucleic Acids Res* 28:1313–1321.
- Merendino L, Guth S, Bilbao D, Martinez C, Valcarcel J. 1999. Inhibition of msl-2 splicing by Sex-lethal reveals interaction between U2AF35 and the 3' splice site AG. *Nature* 402:838–841.
- Moore MJ, Sharp PA. 1993. Evidence for two active sites in the spliceosome provided by stereochemistry of pre-mRNA splicing. *Nature* 365:364–368.
- Newman AJ, Norman C. 1992. U5 snRNA interacts with exon sequences at 5' and 3' splice sites. *Cell* 68:743–754.
- Newman AJ, Teigelkamp S, Beggs JD. 1995. snRNA interactions at 5' and 3' splice sites monitored by photoactivated crosslinking in yeast spliceosomes. *RNA* 1:968–980.
- Ng R, Abelson J. 1980. Isolation and sequence of the gene for actin in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 77:3912–3916.
- Nilsen TW. 1998. RNR–RNA interactions in nuclear pre-mRNA splicing. In: Simons RW, Grunberg-Manago M., eds. *RNA structure and function*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 279–307.
- O'Keefe RT, Norman C, Newman AJ. 1996. The invariant U5 snRNA loop 1 sequence is dispensable for the first catalytic step of pre-mRNA splicing in yeast. *Cell* 86:679–689.
- Parker R, Siliciano PG. 1993. Evidence for an essential non-Watson–Crick interaction between the first and last nucleotides of a nuclear pre-mRNA intron. *Nature* 361:660–662.
- Ruis BL, Kivens WJ, Siliciano PG. 1994. The interaction between the first and last intron nucleotides in the second step of pre-mRNA splicing is independent of other conserved intron nucleotides. *Nucleic Acids Res* 22:5190–5195.
- Rymond BC, Rosbash M. 1985. Cleavage of 5' splice site and lariat formation are independent of 3' splice site in yeast mRNA splicing. *Nature* 317:735–737.
- Schwer B, Gross CH. 1998. Prp22, a DEXH-box RNA helicase, plays two distinct roles in yeast pre-mRNA splicing. *EMBO J* 17:2085–2094.
- Schwer B, Guthrie C. 1992. A conformational rearrangement in the spliceosome is dependent on PRP16 and ATP hydrolysis. *EMBO J* 11:5033–5039.
- Segault V, Will CL, Polycarpou-Schwarz M, Mattaj JW, Branlant C, Lührmann R. 1999. Conserved loop I of U5 small nuclear RNA is dispensable for both catalytic steps of pre-mRNA splicing in HeLa nuclear extracts. *Mol Cell Biol* 19:2782–2790.
- Sharp PA. 1985. On the origin of RNA splicing and introns. *Cell* 42:397–400.
- Shuster EO, Guthrie C. 1990. Human U2 snRNA can function in pre-mRNA splicing in yeast. *Nature* 345:270–273.
- Siatecka M, Reyes JL, Konarska MM. 1999. Functional interactions of Prp8 with both splice sites at the spliceosomal catalytic center. *Genes & Dev* 13:1983–1993.
- Spingola M, Grate L, Haussler D, Ares M Jr. 1999. Genome-wide bioinformatic and molecular analysis of introns in *Saccharomyces cerevisiae*. *RNA* 5:221–234.
- Staley JP, Guthrie C. 1998. Mechanical devices of the spliceosome: Motors, clocks, springs, and things. *Cell* 92:315–326.
- Steitz TA, Steitz JA. 1993. A general two-metal-ion mechanism for catalytic RNA. *Proc Natl Acad Sci USA* 90:6498–6502.
- Stotz A, Linder P. 1990. The ADE2 gene from *Saccharomyces cerevisiae*: Sequence and new vectors. *Gene* 95:91–98.
- Umen JG, Guthrie C. 1995a. A novel role for a U5 snRNP protein in 3' splice site selection. *Genes & Dev* 9:855–868.
- Umen JG, Guthrie C. 1995b. The second catalytic step of pre-mRNA splicing. *RNA* 1:869–885.
- Umen JG, Guthrie C. 1995c. Prp16p, Slu7p, and Prp8p interact with

- the 3' splice site in two distinct stages during the second catalytic step of pre-mRNA splicing. *RNA* 1:584–597.
- Wu S, Romfo CM, Nilsen TW, Green MR. 1999. Functional recognition of the 3' splice site AG by the splicing factor U2AF35. *Nature* 402:832–835.
- Xu D, Field DJ, Tang SJ, Moris A, Bobechko BP, Friesen JD. 1998. Synthetic lethality of yeast slt mutations with U2 small nuclear RNA mutations suggests functional interactions between U2 and U5 snRNPs that are important for both steps of pre-mRNA splicing. *Mol Cell Biol* 18:2055–2066.
- Xu D, Nouraini S, Field D, Tang SJ, Friesen JD. 1996. An RNA-dependent ATPase associated with U2/U6 snRNAs in pre-mRNA splicing. *Nature* 381:709–713.
- Zhang X, Schwer B. 1997. Functional and physical interaction between the yeast splicing factors Slu7 and Prp18. *Nucleic Acids Res* 25:2145–2152.
- Zorio DA, Blumenthal T. 1999. Both subunits of U2AF recognize the 3' splice site in *Caenorhabditis elegans*. *Nature* 402:835–838.