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A role for U2/U6 helix Ib in 5′ splice site selection

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ABSTRACT

Selection of pre-mRNA splice sites is a highly accurate process involving many trans-acting factors. Recently, we described a role for U6 snRNA position G52 in selection of the first intron nucleotide (+1G). Because some U2 alleles suppress U6-G52 mutations, we investigated whether the corresponding U2 snRNA region also influenced 5′ splice site selection. Our results demonstrate that U2 snRNAs mutated at position U23, but not adjacent nucleotides, specifically affect 5′ splice site cleavage. Furthermore, all U2 position U23 mutations are synthetic lethal with the thermosensitive U6-G52U allele. Interestingly, the U2-U23C substitution has an unprecedented hyperaccurate splicing phenotype in which cleavage of introns with a +1G substitution is reduced, whereas the strain grows with wild-type kinetics. U2 position U23 forms the first base pair with U6 position A59 in U2/U6 helix Ib. Restoration of the helical structure suppresses 5′ splice site cleavage defects, showing an important role for the helix Ib structure in 5′ splice site selection. U2/U6 helix Ib and helix II have recently been described as being functionally redundant. This report demonstrates a unique role for helix Ib in 5′ splice site selection that is not shared with helix II.

Keywords: pre-mRNA processing; RNA catalysis; splice site selection; yeast

INTRODUCTION

Splicing of nuclear pre-mRNAs occurs through two sequential transesterfication reactions. Initially, a 2′ OH group on a conserved intronic adenosine residue (the branch point adenosine) is activated and acts as a nucleophile on the phosphodiester bond between the 5′ exon (exon 1) and the intron. This results in a free exon 1 intermediate carrying a 3′ OH group, and an intron–exon 2 lariat intermediate where the 5′ splice site is linked to the branch point adenosine through a 5′-2′ phosphodiester bond. The second step is initiated through a nucleophilic attack by the activated 3′ OH group on exon 1, on the phosphodiester bond between the intron and exon 2. This reaction leads to excision of the intron lariat and exon ligation (for review see Green, 1991; Guthrie, 1991; Moore et al., 1993; Will & Luhrmann, 1997). The chemistry of the splicing pathway is identical to that of group II self-splicing introns, where splicing also occurs through a lariat intermediate. This and other observations indicate that group II self-splicing and nuclear pre-mRNA splicing may be evolutionarily related (Sharp, 1985; Cech, 1986; Weiner, 1993; Michel & Ferat, 1995).

Nuclear pre-mRNA splicing takes place in a dynamic ribonucleoprotein particle termed the spliceosome. The core of the spliceosome consists of five small nuclear ribonucleoprotein particles (snRNP), each consisting of one small nuclear RNA (snRNA, U1, U2, U4, U5, and U6) and several protein components. In addition, several non-snRNP protein factors are required for splicing. The spliceosome is sequentially assembled on short, conserved intron sequences located at the cleavage sites and the branch point. Initially, the 5′ splice site is recognized by base pairing with the 5′ end of U1 snRNA (Zhuang & Weiner, 1986; Séraphin et al., 1988; Siliciano & Guthrie, 1988). The branch point sequence is identified first by a protein factor SF1/BBP (Arning et al., 1996; Abovich & Rosbash, 1997; Berglund et al., 1997), later by base pairing with U2 snRNA (Parker et al., 1987; Wu & Manley, 1989; Zhuang & Weiner, 1989; Query et al., 1994; Pascolo & Séraphin, 1997). Upon addition of U4/U6.U5 tri-snRNP, some RNA–RNA rearrangements have to take place before the mature spliceosome is formed (reviewed in Madhani & Guthrie, 1994a). The U1 snRNA base pairing is probably displaced by the U5 and U6 snRNAs at the 5′ splice site (Kandels-Lewis & Séraphin, 1993; Konforti et al., 1993; Lesser & Guthrie, 1993). In the nucleo-

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plasm, U4 and U6 snRNPs assemble in a di-snRNP through extensive Watson–Crick base pairing between their snRNA components. This pairing is disrupted upon or after U4/U6.U5 addition to the spliceosome, and the U6 snRNA forms two helices with U2 snRNA—helix I and helix II—separated by an intramolecular U6 stem loop (Datta & Weiner, 1991; Wu & Manley, 1991; Madhani & Guthrie, 1992; Sun & Manley, 1995; Field & Friesen, 1996) (Fig. 1A). U2/U6 helix I is interrupted by a two-nucleotide bulge in U2 snRNA, dividing it into helix Ia and Ib (Madhani & Guthrie, 1992) (Fig. 1A,B). Although base pairing between the U2 and U6 snRNA components is essential to form helix Ia, the helix Ib structure is not essential (Madhani & Guthrie, 1992; Field & Friesen, 1996). The U2 snRNA residues forming helix Ib, although conserved in sequence, are not sensitive to mutations (Madhani & Guthrie, 1992; Field & Friesen, 1996). In contrast, the corresponding U6 snRNA residues are invariant, and nucleotide substitutions lead to a block in splicing and cell death (Madhani & Guthrie, 1992). Helix II is less conserved than helix I and is entirely dispensable in yeast (Field & Friesen, 1996). In *Saccharomyces cerevisiae*, it was genetically demonstrated that U2/U6 helix Ib was essential when the U2/U6 helix II was disrupted, indicating a functional redundancy between them (Field & Friesen, 1996). More recently, a genetic interaction between U2/U6 helix Ib and the invariant loop I of U5 snRNA has also been described (Xu et al., 1998). Due to its strong conservation, U2/U6 helix I has been proposed to be a component of the active site of the spliceosome (Brow & Guthrie, 1989; Fabrizio & Abelson, 1990; Madhani & Guthrie, 1992). The existence of a third helix connecting U2 and U6 snRNA has been proposed (Sun & Manley, 1995). However, its contribution to the splicing reaction in the yeast system is unclear (Li & Brow, 1996; Yan & Ares, 1996).

Accurate 5′ splice site cleavage is determined at least in part by RNA–RNA interactions surrounding the cleavage site. U5 snRNA has been demonstrated to interact with exon 1 sequences adjacent to the phosphodiester bond to be transesterified (nt −1 to −3 from the cleavage site) (Newman & Norman, 1991, 1992; Wyatt et al., 1992; Cortes et al., 1993; Sontheimer & Steitz, 1993). However, this interaction is not essential because deletion of the U5 snRNA loop does not prevent 5′ splice site cleavage in vitro (O'Keefe et al., 1996). U6 snRNA base pairs with intron sequences at positions +4 to +6 downstream of the 5′ cleavage site (Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993). In a weakened intron context, we have demonstrated that the U5 and U6 snRNAs act cooperatively in determining which phosphodiester bond to cleave (S. Kandels-Lewis & B. Séraphin, in prep.). Recently, we also demonstrated a genetic interaction between U6 snRNA positions 50 and 52, and the 5′ splice site positions +3 and +1, respectively (Luukkonen & Séraphin, 1998). Significantly, we identified a U6 snRNA mutation (U6-G52U) that could distinguish between different 5′ cleavage sites based on the identity of the first intron nucleotide. This showed that U6 positions 50–52 may be involved in recognition of the first three nucleotides of the intron for the first splicing step, data further corroborated by crosslinking studies both in yeast and mammalian extracts (Sontheimer & Steitz, 1993; Kim & Abelson, 1996).

Based on genetic suppression data, Madhani and Guthrie (1994b) suggested a genetic interaction between U6 snRNA position G52 and U2 snRNA residue A25 that is situated in the bulge between U2/U6 helices Ia and Ib (Fig. 1A). Mutating U2-A25 led to a weak second step block, and the U2-A25G allele could suppress a number of 3′ splice site mutations. Because we had evidence for a role of U6-G52 in selection of the first intron nucleotide, we wanted to test if the U2/U6 helix I bulge region was also involved in 5′ splice site cleavage. We found no evidence for an interaction between the intron position +1 and U2-A25. Surprisingly, mutations in U2-U23 specifically affected 5′ splice site cleavage of introns with a base substitution in position +1. U2-U23 transversions could discriminate between cleavage sites based on the identity of the first intron nucleotide. To our knowledge, this is the first report implicating U2 snRNA in 5′ splice site selection. Further analyses revealed that the helix Ib structure affects 5′ splice site selection. This function is not redundant with a function of U2/U6 helix II.

RESULTS

Involvement of U2-U23 in 5′ splice site cleavage

We have previously demonstrated that U6 position 52 affects selection of the 5′ splice site position +1 (Luukkonen & Séraphin, 1998). Because U6-G52 mutations are suppressed by substitution at and around U2-A25 (Madhani & Guthrie, 1994b), we wanted to investigate the effect of these mutations on 5′ splice site cleavage. Among the suppressors of U6-G52 mutations selected by Madhani and Guthrie (1994b), mutations in U2 snRNA positions 23–25 from UUA to CAG and related sequences were recurrent. Therefore, we constructed yeast strains harboring a unique copy of U2 snRNA with the 23–25 sequence UUA (wt), CAG, or the related UAG or CAA (Fig. 1B). These mutations did not lead to any growth phenotype (data not shown). The strains were transformed with reporter plasmids harboring the RP51A intron fused to a β-galactosidase reporter gene (Teem & Rosbash, 1983). To investigate the role of the U2 snRNA alleles in 5′ splice site cleavage, we employed an assay based on aberrant 5′ splice site cleavage (Séraphin & Rosbash, 1990; Kandels-Lewis & Séraphin, 1993; Luukkonen & Séraphin, 1998). Briefly, a mutation in the RP51A intron position +5G to

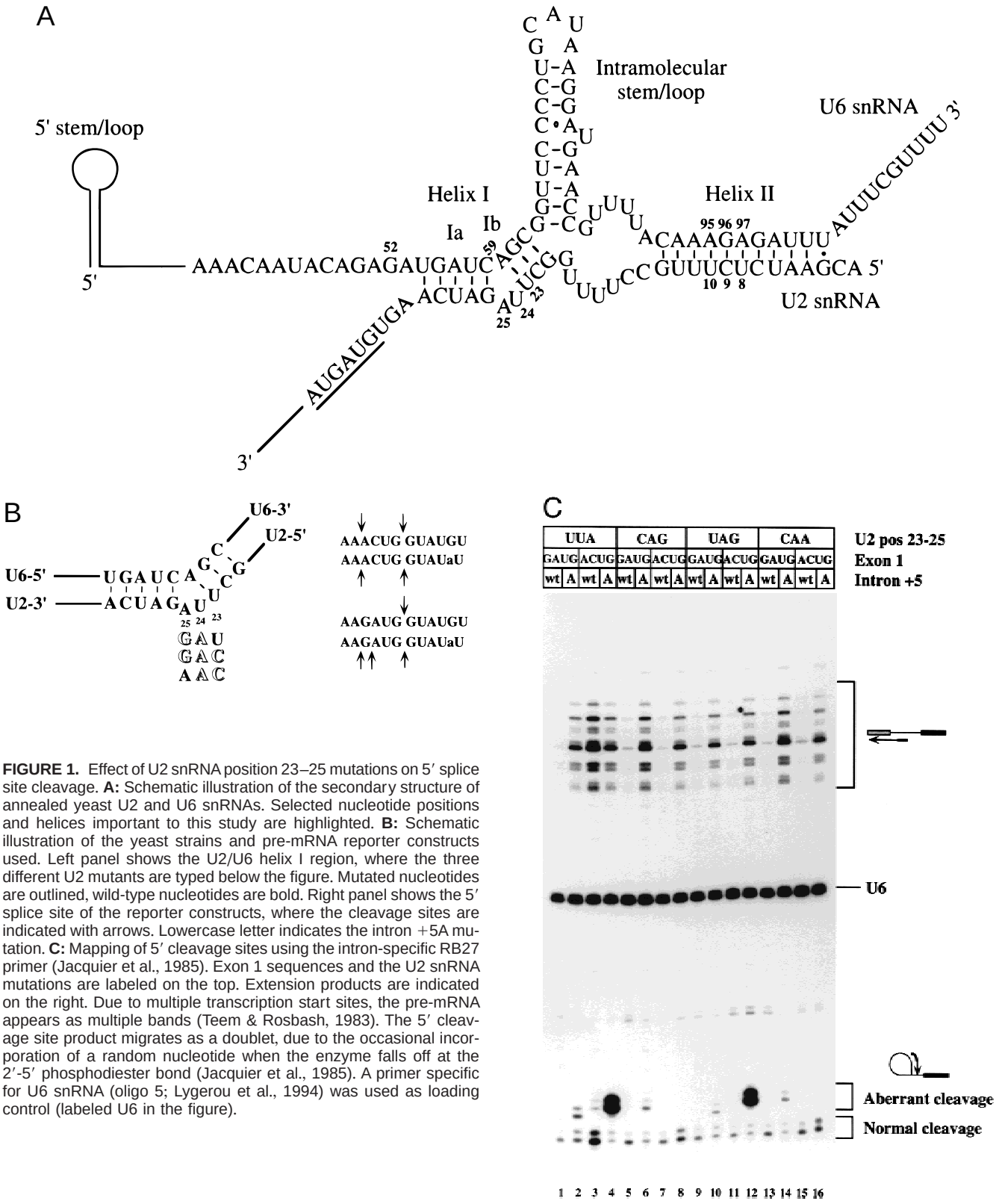


FIGURE 1. Effect of U2 snRNA position 23–25 mutations on 5′ splice site cleavage. **A:** Schematic illustration of the secondary structure of annealed yeast U2 and U6 snRNAs. Selected nucleotide positions and helices important to this study are highlighted. **B:** Schematic illustration of the yeast strains and pre-mRNA reporter constructs used. Left panel shows the U2/U6 helix I region, where the three different U2 mutants are typed below the figure. Mutated nucleotides are outlined, wild-type nucleotides are bold. Right panel shows the 5′ splice site of the reporter constructs, where the cleavage sites are indicated with arrows. Lowercase letter indicates the intron +5A mutation. **C:** Mapping of 5′ cleavage sites using the intron-specific RB27 primer (Jacquier et al., 1985). Exon 1 sequences and the U2 snRNA mutations are labeled on the top. Extension products are indicated on the right. Due to multiple transcription start sites, the pre-mRNA appears as multiple bands (Teem & Rosbash, 1983). The 5′ cleavage site product migrates as a doublet, due to the occasional incorporation of a random nucleotide when the enzyme falls off at the 2′-5′ phosphodiester bond (Jacquier et al., 1985). A primer specific for U6 snRNA (oligo 5; Lygerou et al., 1994) was used as loading control (labeled U6 in the figure).

A disrupts the U6 snRNA–pre-mRNA base pairing interaction (Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993), and allows for activation of cryptic 5′ cleavage sites three nucleotides into exon 1 (Jacquier et al., 1985). Different aberrant cleavage sites can be created by appropriate mutations in exon 1 (Séraphin & Rosbash, 1990; S. Kandels-Lewis & B. Séraphin, in prep.). Analyses of these aberrant cleavage substrates

have revealed that they are substrates of the normal splicing machinery (Séraphin & Rosbash, 1990), that cleavage is dependent on sequences surrounding the site (Luukkonen & Séraphin, 1998; S. Kandels-Lewis & B. Séraphin, in prep.), and that cleavage site selection is dependent on the U5 and U6 snRNAs (Kandels-Lewis & Séraphin, 1993; S. Kandels-Lewis & B. Séraphin, in prep.).

To investigate the effects of U2 position 23–25 on 5′ splice site cleavage, we transformed the U2 snRNA mutant strains with reporter plasmids harboring different exon 1 sequences (GAUG or ACUG), in combination with a wild-type or +5A mutated intron sequence (Fig. 1B). The GAUG exon 1 sequence displays aberrant cleavage both at position −3A and −4G (Séraphin & Rosbash, 1990) (Fig. 1B), whereas the ACUG exon 1 sequence is very efficiently cleaved at the −4A position (Fig. 1B). These reporter constructs allowed us to determine position and sequence requirements for 5′ splice site cleavage in the U2 snRNA mutant strains. Total RNA was prepared and splicing analyzed by primer extension using an exon 2-specific primer. Pre-mRNAs harboring wild-type intron sequences appeared to be unaffected by the U2 mutations (Séraphin & Rosbash, 1990; data not shown). The +5A mutation led to accumulation of pre-mRNA, and a reduction in mature mRNAs (Séraphin & Rosbash, 1990; data not shown). For the ACUG construct, this mRNA reduction appeared stronger in the U2-CAG and CAA strains (data not shown).

To investigate how 5′ splice site cleavage was affected, the RNAs were probed with the intron-specific RB27 primer (Jacquier et al., 1985; Fig. 1C). This primer detects pre-mRNA and intron lariats. The GAL10–CYC1 promoter fused to the reporter gene has multiple transcription initiation sites, and extension of pre-mRNAs consequently results in multiple bands (Teem & Rosbash, 1983). Extension products originating from primers bound to lariats appear as doublets, because the reverse transcriptase occasionally incorporates a random nucleotide before falling off at the 5′-2′ bond (Jacquier et al., 1985). Consistent with previous findings, constructs containing a wild-type intron sequence generated no (GAUG) or only a low level (ACUG) of aberrantly cleaved pre-mRNA (Fig. 1C, odd lanes). When position +5G of the intron was changed to an A, the GAUG pre-mRNA was cleaved at position −3A and weakly at −4G, whereas for the ACUG pre-mRNA, large amounts of lariat intermediate cleaved at position −4A accumulated in both the wild-type and U2-UAG strains (Fig. 1C, lanes 2, 4, 10, and 12). Strikingly, in the U2-CAG and CAA strains, aberrant cleavage of the GAUG reporter occurred only at position −4G, and aberrant cleavage of the ACUG construct was virtually abolished (Fig. 1C, lanes 6, 8, 14, and 16). This phenotype is identical to that observed for the U6-G52U transversion (Luukkonen & Séraphin, 1998). The two U2 alleles that inhibited aberrant cleavage have a CA at positions 23–24. However, U24A is present also in the UAG allele, suggesting that only the U23C transition may be responsible for the phenotype. The absence of a phenotype with the U2-UAG allele also demonstrates that inhibition of aberrant cleavage is not a general phenotype associated with all mutations in this conserved region of U2 snRNA, but rather a specific effect of substitutions in some positions (also see below).

To confirm that the phenotype resulted from the change at U2 position U23, we constructed all substitutions of U2 snRNA at this position. Consistent with previous observations (Madhani & Guthrie, 1992), these three U2 snRNA mutants functionally complemented a U2 gene disruption (data not shown). The growth rates of the mutant strains were determined in liquid culture. U2-U23C conferred a wild-type growth rate, whereas the U2-U23 transversions led to a slightly reduced growth rate (data not shown). Analysis of 5′ splice site cleavage in these strains using the GAUG reporter revealed that U2-U23C strongly inhibited both the −3A and −4G aberrant cleavage events, whereas U2-U23G and U2-U23A blocked cleavage at −3A, but not at −4G (data not shown, also see Fig. 4C). This confirms that U2 snRNA position U23 is specifically involved in 5′ splice site selection.

U2 position 23 affects cleavage at the normal position

The results presented above indicate that substitutions in U2 snRNA position U23 affect selection of aberrant cleavage sites. We decided to analyze whether U2-U23 could, like U6-G52U (Luukkonen & Séraphin, 1998), also affect cleavage at the normal site. For this purpose, yeast strains harboring the various substitutions at U2 position U23 were transformed with reporter constructs having all possible intron position +1G mutations, in a wild-type exon 1 context. Analysis of splicing efficiency by primer extension using an exon 2-specific primer revealed that the U2-U23 base substitutions did not significantly affect the level of mRNA resulting from cleavage of wild-type introns (data not shown). As reported previously (Aebi et al., 1987; Parker & Siliciano, 1993; Chanfreau et al., 1994; Luukkonen & Séraphin, 1997), all +1G substitutions led to a dramatic decrease in mRNA production (data not shown). The effect on 5′ splice site cleavage was analyzed using the intron-specific primer (Fig. 2). Cleavage of a wild-type 5′ splice site or a +1U was not affected by the U2 mutations (Fig. 2, odd lanes). The U2-U23C mutation led to a decrease in cleavage at +1A (Fig. 2, lane 6), and all substitutions severely reduced cleavage at a +1C (Fig. 2, lanes 4, 8, 12, and 16). These data show that U2-U23 mutants, like U6-G52U, affect cleavage of introns with a substitution in position +1 (Luukkonen & Séraphin, 1998). Significantly, the U2 snRNA substitu-

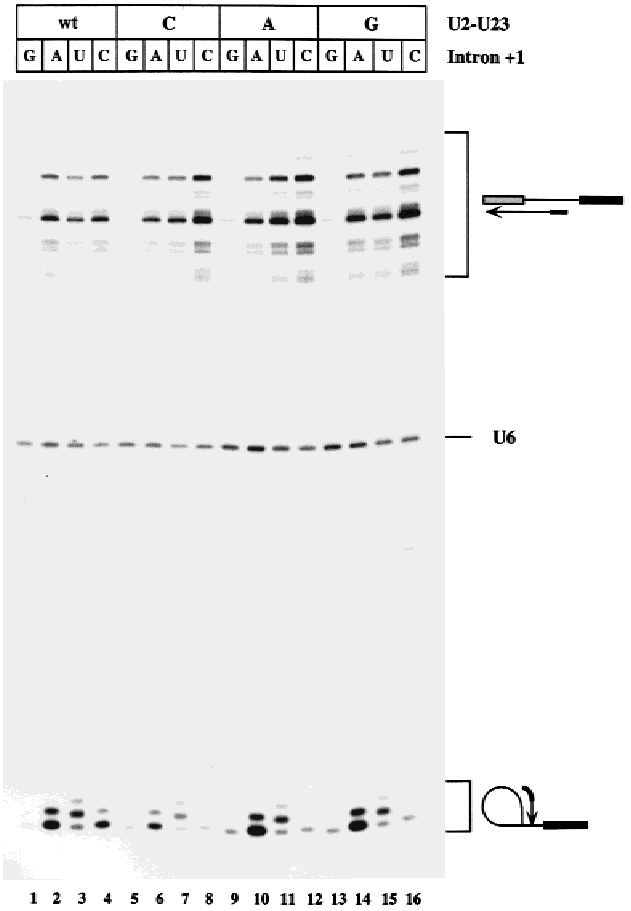


FIGURE 2. U2-U23 substitutions affect cleavage of introns harboring a single point mutation at position +1. Primer extension analysis of the reporter constructs are indicated on the top of the figure using the intron-specific RB27 primer. Intron and U2 snRNA sequences are shown on the top of the figure. The different extension products are labeled on the right.

tions do not selectively inhibit cleavage at aberrant 5′ splice sites, but rather affect cleavage events at mutated 5′ splice sites regardless of their position. As a control, we transformed the U2 snRNA mutant strains with reporter constructs harboring all single point mutations in the branch point adenosine. No effect on mRNA production from these constructs was detected (data not shown), indicating that the phenotype observed is 5′ splice site specific.

U6 position A59 affects both steps of splicing

U2-U23 pairs with U6-A59 in U2/U6 helix Ib (Madhani & Guthrie, 1992). To test if the splicing phenotype resulted from the U2 substitution or from disruption of helix Ib, a yeast strain containing the only viable substitution at U6 position A59 (A59C; Madhani & Guthrie, 1992) was generated. The A59C strain is thermosensitive (Madhani & Guthrie, 1992, 1994b; data not shown) and cells were grown at 23 °C. This strain and the isogenic wild-type strain were transformed with the ACUG, GAUG, and GGUG exon 1 reporter constructs having the wild-type or +5A intron sequence. The GGUG exon 1 sequence is aberranty cleaved both at −3G and −4G and, in addition, one or both of these aberrant cleavage events is associated with selection of an aberrant branch point (Séraphin & Rosbash, 1990). Analysis of splicing using an exon 2-specific primer revealed a slight decrease in mRNA production from pre-mRNAs harboring a wild-type or +5A intron sequence (data not shown). However, mRNAs resulting from aberrant −4G cleavage in the GAUG strain and both the aberrant −3G- and −4G-cleaved mRNAs from the GGUG construct were not detectable (data not shown). For all constructs, U6-A59C partially blocked the second splicing step as evidenced by an increased level of lariat intermediate (data not shown), as has been observed previously in vitro (Fabrizio & Abelson, 1990).

To analyze how these reporter constructs were cleaved, the RNAs were analyzed with the intron-specific primer (Fig. 3). U6-A59C significantly reduced, but did not abolish, −4A cleavage of the ACUG construct (Fig. 3, compare lane 2 with lane 8). However, all aberrant cleavage events for the GAUG and GGUG constructs were eliminated (Fig. 3, compare lanes 4 and 6 with lanes 10 and 12). Thus, U6-A59C exhibited a negative effect on all aberrant cleavage sites irrespective of the nature of the base following that site. It differs in this respect from the transversion mutants at the complementary U2 snRNA position U23 (Figs. 2, 4; and data not shown). In addition, U6-A59C increased the level of product arising from cleavage at the normal site (Fig. 3, compare lanes 1–6 with 7–12). This results from the partial block to the second splicing step associated with the U6-A59C mutation (see above, and Fabrizio & Abelson, 1990).

U2/U6 helix Ib structure is essential for accurate 5′ splice site selection

To investigate the relevance of the helix Ib structure for the cleavage phenotype that we have observed, we constructed yeast strains harboring all possible U2-U23/U6-A59 double mutations. As previously shown, all substitutions in U2-U23 were viable, and complementing the U6-A59C mutation with U2-U23G led to suppression of the thermosensitive phenotype (Madhani & Guthrie, 1992, 1994b; and data not shown). Also the U2-U23C/U6-A59G double mutants grew without any apparent phenotype, whereas the complementary U2-U23A/U6-A59U double substitutions, and all other combinations, were not viable (data not shown, and Madhani & Guthrie, 1992, 1994b). These experiments confirm a base paring interaction between U2-U23 and U6-A59 (Madhani & Guthrie, 1992).

To analyze how restoration of helix Ib affected splicing, the yeast strains were transformed with the GAUG

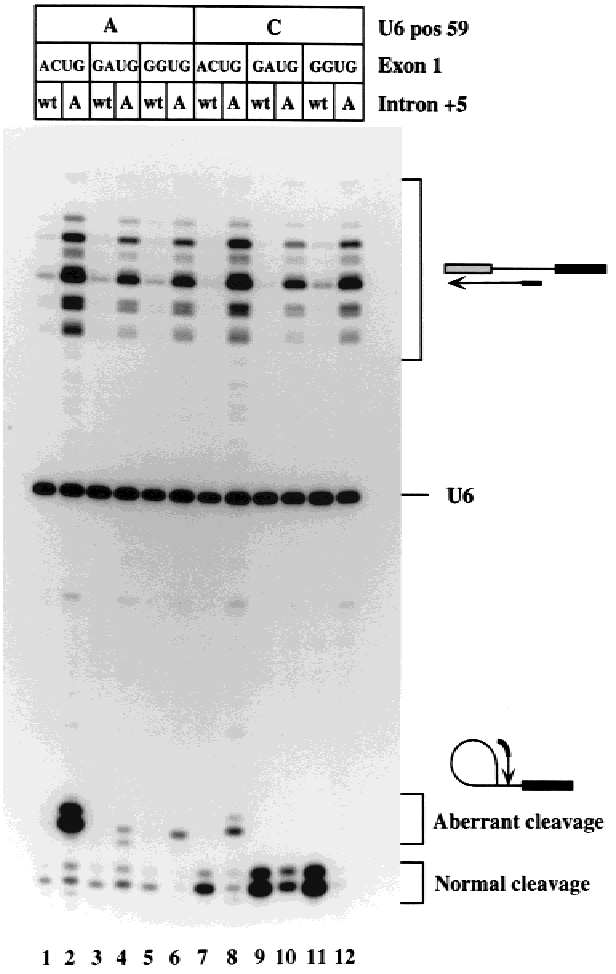


FIGURE 3. U6-A59C affect both steps of splicing in vivo. Primer extension analysis of the reporter constructs indicated on top of the figure, using the intron-specific RB27 primer. Extension products are shown on the right.

wt and +5A constructs (Fig. 4A). This construct was used because some U2-U23 substitutions can distinguish between the −3A and −4G cleavage sites (see Fig. 1C; and data not shown), which is not the case for the U6 substitution tested (Fig. 3). Total RNA was prepared and splicing analyzed by the exon 2-specific EM38 primer (Luukkonen & Séraphin, 1997) (Fig. 4B). Splicing from the normal 5′ splice site was not significantly affected in any of the strains (Fig. 4B). In the U6-A59C mutant strain, some exon 2–lariat intermediate accumulated, both with the wild-type and +5A intron (Fig. 4B, lanes 11–12; see also above). The mRNA resulting from aberrant cleavage at position −4G was not apparent in most strains, except for the wild-type and U2-U23C/U6-A59G strains (Fig. 4B, lanes 2 and 8, bands indicated with arrows) (Séraphin & Rosbash, 1990; Luukkonen & Séraphin, 1998).

5′ Splice site cleavage was analyzed by the intron-specific primer (Fig. 4C). As reported above, aberrant cleavage was strongly reduced in the U2-U23C/U6-wt strain, but very low levels of −3A cleavage were still detected (Fig. 4C, lane 4; and data not shown). Interestingly, in the U2-U23C/U6-A59G strain, aberrant cleavage was restored to wild-type efficiency (Fig. 4C, lane 8). This experiment demonstrates that restoration of helix Ib in the U2-U23C/U6-A59G double mutant is sufficient to suppress alterations in 5′ splice site cleavage caused by U2-U23C. In the U2-U23G/U6-wt strain, aberrant cleavage occurred solely at position −4G (Fig. 4C, lane 6, see also above), whereas, in the U2-U23G/U6-A59C and U2-wt/U6-A59C strains, no aberrant cleavage was detected (Fig. 4C, lanes 10 and 12). Therefore, even if restoration of the helical structure suppressed the growth phenotype associated with U6-A59C, this was insufficient to rescue the splicing phenotype. This indicates that this nucleotide position is required for another function in addition to forming U2/U6 helix Ib.

U2/U6 helix II does not affect 5′ splice site cleavage

It has recently been proposed that U2/U6 helix Ib is functionally redundant with U2/U6 helix II, because helix Ib is only essential when helix II is disrupted (Field & Friesen, 1996). Therefore, we investigated the effects of U2/U6 helix II mutations in the 5′ splice site cleavage assay. Strains U2-wt/U6-wt, U2-2d/U6-wt, and U2-wt/U6-6d, harboring mutations in U2 snRNA position 8–10 or U6 snRNA position 95–97 that disrupt U2/U6 helix II (Field & Friesen, 1996; kind gift of D. Field and J. Friesen), were transformed with the ACUG and GAUG cleavage substrates (Fig. 5A). Total RNA was prepared and analyzed by primer extension using the exon 2-specific primer (Fig. 5B). ACUG mRNAs resulting from normal cleavage of both wild-type introns and +5A-mutated introns appeared slightly reduced in the U6-6d and U2-2d mutant strains (Fig. 5B, compare lanes 1 and 2 with lanes 3–6). Splicing of the GAUG wt intron construct was not affected by the U6-6d mutation (Fig. 5B, compare lanes 7 and 9), however, mRNA levels decreased significantly in the U2-2d mutant strain (Fig. 5B, compare lanes 7 and 11). In combination with the +5A mutation, the reduction in mRNA levels in the U2-2d strain was less pronounced (Fig. 5B, lanes 8, 10, and 12). Significantly, for both the wild-type intron ACUG and GAUG constructs, the levels of pre-mRNA decreased dramatically in the U2-2d strain (Fig. 5B, lanes 5 and 11). This is suggestive of an early block in spliceosome assembly leading to degradation of the pre-mRNA.

To test how 5′ splice site cleavage was affected by the U2/U6 helix II mutations, the RNAs were probed with the intron-specific primer. Aberrant cleavage of the ACUG construct was largely unaffected in the U6-6d strain, whereas cleavage in the U2-2d strain was somewhat reduced (Fig. 5C, lanes 1–6). Cleavage of the

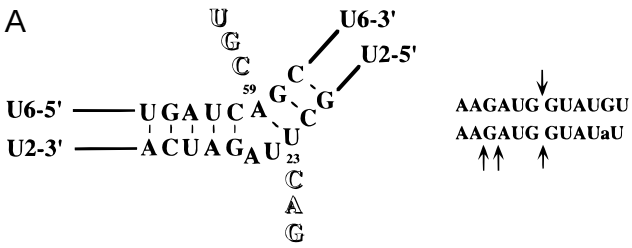
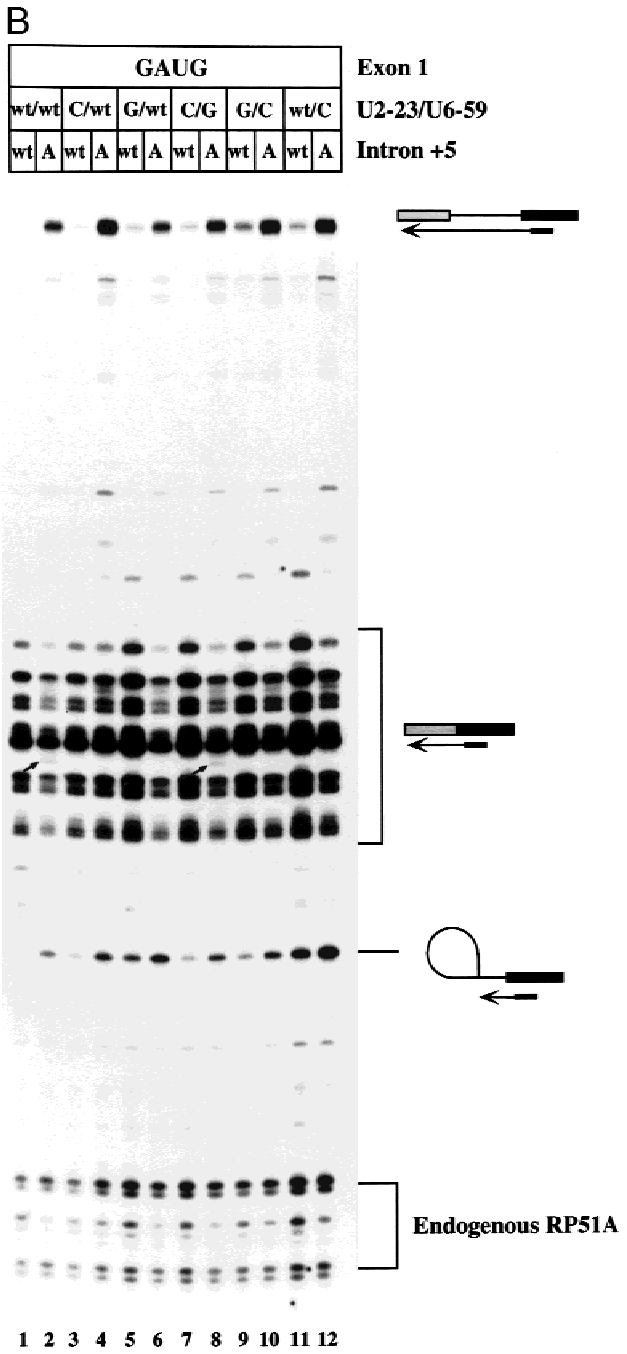
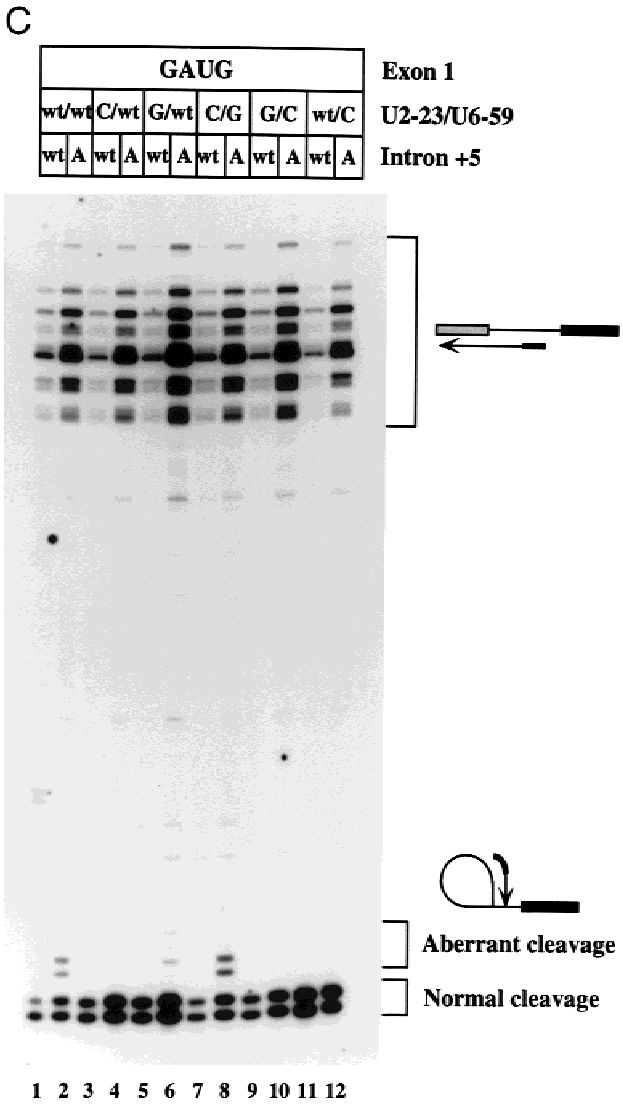


FIGURE 4. U2/U6 helix Ib is required for accurate 5′ splice site cleavage. **A:** Schematic illustration of the yeast strains and constructs used. Labeled as in Figure 1A. **B:** Splicing efficiency of the GAUG reporter construct in the U2-U23–U6 A59C mutated strains, determined by primer extension analysis using the exon 2-specific EM38 primer. The different extension products are labeled on the right. **C:** Effect of U2-U23–U6-A59 double mutants on aberrant cleavage determined by primer extension analysis using the intron-specific RB27 primer. Panel labeled as in B.





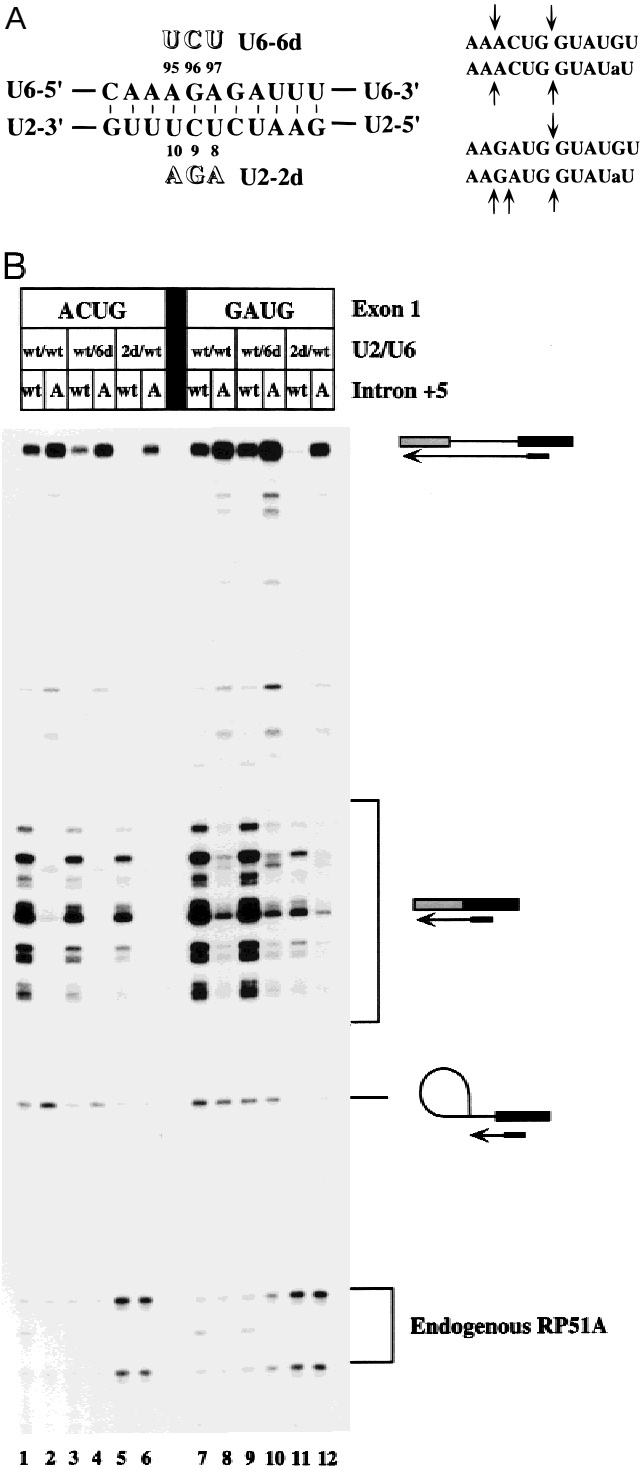


FIGURE 5. U2/U6 helix II mutations do not alter 5′ splice site specificity. **A:** Schematic illustration of the yeast strains and constructs used. Labeled as in Figure 1A. **B:** Analysis of splicing efficiency by primer extension, using the exon 2-specific EM38 primer. Extension products are indicated on the right. **C:** Analysis of 5′ cleavage sites using the intron-specific RB27 oligo. Panel labeled as in B.

GAUG construct was also unaffected by the U6-6d mutations, whereas the U2-2d mutations appeared to exert a stronger effect on this substrate (Fig. 5C, lanes 7–12). However, both the normal and aberrant cleavage sites were similarly affected in the U2-2d strain, demonstrating that the inhibition is neither position nor sequence specific (Fig. 5C, lanes 11 and 12). The experiment demonstrates that disruption of the U2/U6 helix II structure does not affect the specificity of 5′ splice site selection. Cleavage of both the normal and aberrant site is affected equally in the U2-2d strain, showing that there is no sequence-specific inhibition. Given the decrease in pre-mRNA levels (Fig. 5B,C, lanes 5 and 11), the effect of the U2-2d mutation is more likely to be a consequence of an early block in spliceosome assembly, rather than a late block at the

time of splicing catalysis. Because high levels of +5A-mutated pre-mRNA still accumulated in the U2-2d strain (Fig. 5B,C, lanes 6 and 12), this suggests that the block is subsequent to U1 snRNP or U6 snRNP addition to the 5′ splice site, but most likely involves U2 snRNP. Preliminary data obtained by nondenaturing gel electrophoresis analysis suggests that the spliceosome assembly pathway is blocked prior to, or concomitantly with, pre-spliceosome formation in a U2-2d, but not U6-6d or wild-type, extract (data not shown).

U2 snRNA position 23 substitutions are synthetic lethal with U6-G52U

We have previously shown an involvement of U6-G52 in selection of the first intron nucleotide (Luukkonen & Séraphin, 1998). The phenotype observed with some U2 position U23 mutations is strongly reminiscent of that obtained with U6-G52U, implicating a role also for U2-U23 in 5′ splice site selection. One explanation for this redundancy is that U6-G52 forms a G-binding pocket with U2/U6 helix Ia/Ib residues, where many individual groups contribute to substrate binding and maintenance of the pocket's structure (see Discussion). This is further supported by the tertiary interaction between U6-G52 and U2-A25, which is not essential for 5′ splice site selection, but may constitute an important structural determinant. We reasoned that if both U6-G52 and U2-U23 are involved in binding the first intron nucleotide, U2-U23 substitutions should be synthetically lethal with the thermosensitive U6-G52U allele. To test this, we transformed U2 and U6 snRNA-encoding plasmids harboring all possible substitutions in positions U2-U23 and U6-G52 into yeast strain BSY597 containing a single copy of the U2 and U6 snRNA wild-type alleles on URA3 marked plasmid. Two independent transformants were subcloned once to SD plates and then on 5-FOA plates and incubated at 23 °C. Growth was scored after 3, 6, and 9 days incubation (Table 1A). In a wild-type U6 snRNA background, all substitutions in U2-U23 were viable (Table 1A and data not shown). In a wild-type U2 snRNA background, U6-G52U is the only viable substitution (Table 1A; Madhani et al., 1990; Luukkonen & Séraphin, 1998). However, with any substitution in U2-U23, the U6-G52U allele is no longer viable (Table 1A; Madhani & Guthrie, 1994b). This supports a shared role for U6-G52 and U2-U23 in 5′ splice site selection. However, it is not possible to exclude the synthetic lethal effect being on the second, rather than the first, step of splicing.

To control for the specificity of the effect, we tested whether other U2 position 23–25 substitutions could suppress or exacerbate the phenotype of U6 position G52 mutations. For this purpose, we co-transformed the U2 position 23–25 constructs (Fig. 1A) with plasmids harboring all possible substitutions in U6-G52. Growth was scored as above (Table 1B). U6-G52U was perfectly viable with the U2 23–25 allele UAG, but not CAA, strengthening the specific synthetic lethal phenotype between U2-U23C and U6-G52U (Table 1B). However, U6-G52U was suppressed weakly by the U2 23–25 allele CAG (Table 1B). U6-G52C was also suppressed by U2 23–25 allele CAG and CAA, but not with U2 23–25 UAG (Table 1B). In contrast, U6-G52A was suppressed weakly by the U2 23–25 UAG allele (Table 1B). This shows that U2-U23C in combination with U2-U24A can suppress transversions in U6-G52, whereas the U2-U24A and U2-A25G combination specifically suppresses the U6-G52A transition. These data do not support the model of a single tertiary interaction between U6-G52 and U2-A25 proposed earlier (Madhani & Guthrie, 1994b). Our previous data on the involvement of U6-G52 in 5′ splice site cleavage (Luukkonen & Séraphin, 1998) and the data presented here strongly point toward a shared role of U6-G52 and the U2-23–25 sequence in 5′ splice site selection.

TABLE 1. Synthetic lethality between U6 position G52 and U2 position U23 mutations and U2 position 23–25 mutations.[a]

A. U6 position G52 and U2 position U23 mutations

		U2 Position 23			
		G	A	**U**	C
U6 Position 52	**G**	+	+	+	+
	A	−	−	−	−
	U	−	−	+	−
	C	−	−	−	−

B. U6 position G52 and U2 position 23–25 mutations

		U2 Position 23–25			
		UUA	CAG	**U**AG	CA**A**
U6 Position 52	**G**	+	+	+	+
	A	−	−	+**	−
	U	+	+*	+	−
	C	−	+	−	+**

[a] Yeast strains were subcloned to SD plates containing 5-FOA at 23 °C, and growth scored after 3, 6, and 9 days. − = no growth after 9 days incubation. * = growth only after 6 days incubation, ** = growth only after 9 days incubation. Wild-type nucleotides are shown in bold face.

DISCUSSION

The U2 part of helix Ib is insensititve to mutations, and the helical structure is essential only when U2/U6 helix II is disrupted (Field & Friesen, 1996). Here we have uncovered a role for U2 snRNA and helix Ib in 5′ splice site cleavage.

Intriguingly, our results indicate that U2-U23C is more accurate than wild type in the selection of the 5′ splice site, inhibiting cleavage of introns with base substitutions at the position following the cleavage site (+1C, +1A, aberrant sites), while exerting no negative effects on growth. This hyperaccurate splicing phenotype is

reminiscent of the hyperaccurate translation mutants described for *Escherichia coli* (Gorini, 1971). However, in the latter case, the mutants grew at a slower rate than wild type, explaining the counter-selection for this mutation. In the case of the U2-U23C mutant, it is unclear why it should not be selected if it prevents aberrant cleavage events. It is possible that it confers a too subtle growth phenotype to be detected, or that it is disadvantageous under conditions we have not tested. The U2-U23G mutant has stronger growth and splicing phenotypes than U2-U23C. Strikingly, it can discriminate between two closely spaced 5′ splice sites depending on the identity of the first intron nucleotide, in a manner similar to the U6-G52U allele (Luukkonen & Séraphin, 1998). This indicates that components of helix Ib are intimately involved in selection of the first intron nucleotide, or in the catalysis of the first step. U2-U23C does not display this specificity without the U2-U24A substitution, indicating that the helix Ia/Ib bulge may be another component involved in this process (see below).

The three U6 snRNA residues forming helix Ib are phylogenetically invariant, and have been proposed to play a role in splicing catalysis. It is possible that this occurs independently of their base pairing interaction with U2 snRNA in helix Ib. A dual role for these U6 snRNA nucleotides would be consistent with our observation that restoring the U6-A59C base pair with U2-U23G restores the growth phenotype, but not the splicing phenotype, of U6-A59C. The only A59 substitution that is not viable when base paired with U2 position 23 is A59U. One possibility is that the U6-A59U mutant snRNA fail to make optimal contact with U4 snRNA, leading to a block in spliceosome assembly. However, in the yeast in vitro depletion-reconstitution assay developed by Fabrizio et al. (1989) and Fabrizio and Abelson (1990), the A59U mutant U6 snRNAs were efficiently incorporated into spliceosomes (Fabrizio & Abelson, 1990). Alternatively, when U2/U6 helix I is formed, U6-A59U may form a Watson–Crick base pair with U2-A25, distorting the helical structure. In the in vitro depletion-reconstitution assay, all substitutions in position A59 led to a second step block. Although both U6-A59C and A59G permitted low levels of splicing, the U6-A59U transversion was completely blocked after the first step (Fabrizio & Abelson, 1990). The nonbridging Rp-oxygen of the phosphodiester bonds between G58 and A59, and A59 and G60 are essential for splicing activity in yeast and nematode in vitro systems (Fabrizio & Abelson, 1992; Yu et al., 1995). It is possible that these oxygens are involved in coordinating one or more magnesium ions for both steps of splicing. In that case, the invariant U6 nt 59–61 may contribute additional chemical groups to the metal ion binding site(s), or may be bound to an RNA or protein factor, thereby positioning the site relative to the substrate. Because we have evidence for one of the reactants for the first step being in close proximity to these residues, it is tempting to speculate that the putative magnesium-binding site is directly involved in the first transesterification reaction.

The U2-A25 nucleotide that interacts with the highly conserved U6-G52 is not essential for splicing, and mutating positions U2-U24 and A25 does not have any effect on cleavage of sensitized substrates. This indicates that U6 position G52 has an essential role in splicing independently of the formation of a putative tertiary contact with U2-A25 (Madhani & Guthrie, 1994b). Further support of this comes from the observation that U6-G52U is viable with only a weak splicing phenotype, whereas U6-G52U would not be able to form the proposed symmetrical heteropurine base pair with U2-A25 (Madhani & Guthrie, 1994b). Furthermore, yeast cells harboring the U6-G52U/U2-A25G double mutation are also perfectly viable (Table 1B). Indeed, the tertiary interaction may only be required when the essential function of U6-G52 is impaired. We have previously presented evidence for a non-Watson–Crick interaction between U6-G52 and the first nucleotide of the intron involved in 5′ splice site selection (Luukkonen & Séraphin, 1998). Here we present additional evidence for the involvement of a conserved but non-essential nucleotide (U2-U23) in selection of the first intron nucleotide. How are these observations reconciled? The U6-G52/U2-A25 interaction brings U2/U6 helix Ib close to the intron position +1. Assuming that the essential role of U6-G52 is in binding the intron +1G for the first step, the role of the U2/U6 suppressors of U6-G52 mutations may be to enable or facilitate an interaction between +1G and a mutated U6-G52. Alternatively, the helix Ia/Ib bulge region may provide an alternative binding site for the +1G, or may contribute single hydrogen bonds to compensate for those lost by the U6-G52 mutation. In a variation of the latter case, the +1G will be bound in a pocket formed by both U6-G52 and components of the U2/U6 helix Ia/Ib bulge region. All components of this region, including the first base pair of helix Ib, are not important for 5′ splice site cleavage of wild-type introns or introns with a G as first nucleotide, but are required for aberrant cleavage of +1A introns, especially in the weaker exon 1 −3 or −4 positions (Figs. 1C, 4C). We and others have demonstrated previously that there is some redundancy in the RNA–RNA interactions surrounding the 5′ splice site at the time of cleavage, so that all components of a 5′ splice site do not have to be recognized for accurate cleavage to occur (Newman & Norman, 1991, 1992; Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993; Luukkonen & Séraphin, 1998; S. Kandels-Lewis & B. Séraphin, in prep.).

Many residues surrounding the U2/U6 helix Ia/Ib bulge give rise to a second step block when mutated (Fabrizio & Abelson, 1990; Madhani & Guthrie, 1992). Furthermore, the first nucleotide of exon 2 of the CYH2

lariat intermediate could be crosslinked to U2-U23 in a yeast extract, indicating that the 3′ splice site is in close proximity to U2/U6 helix Ib for the second step (Newman et al., 1995). It has been proposed that the intron terminal nucleotides interact before the second splicing step, inferring that they are within hydrogen bonding distance (Parker & Siliciano, 1993; Chanfreau et al., 1994; Scadden & Smith, 1995). If this is the case, the +1G may still be bound close to, or in, the U2/U6 helix I bulge at the time of exon ligation. Consequently, the last intron nucleotide is likely to be bound by the same residues, or residues adjacent to those holding the +1G in place. This would explain why mutating these residues may affect either the first or second splicing step, and why the exon 2 nucleotide is crosslinked to a nucleotide involved in 5′ splice site selection. Indeed, in the catalytically active domain V of group II self-splicing introns, certain residues are required for both catalytic steps (Chanfreau & Jacquier, 1994). This indicated two distinct but overlapping catalytic sites for the two splicing steps, a theory that has also been brought forward for nuclear pre-mRNA splicing (Lesser & Guthrie, 1993; Moore & Sharp, 1993; Sontheimer & Steitz, 1993; Steitz & Steitz, 1993; Sontheimer et al., 1997). A base substitution of U6-G52 is associated with a second step block in some splicing systems (Fabrizio & Abelson, 1990). It is not unlikely that this nucleotide is involved in 3′ splice site selection. Alternatively, or in addition, the 5′ splice site/U6 snRNA helix is likely to be proofread prior to the second transesterfication reaction (Luukkonen & Séraphin, 1998).

U2/U6 helix I has been proposed to be homologous to domain V of group II self-splicing introns (Madhani & Guthrie, 1992; Chanfreau & Jacquier, 1994; Boulanger et al., 1995). Based on mutagenesis data and chemical modification analyses, the bottom part of domain V was suggested to be the counterpart of U2/U6 helix Ib (Chanfreau & Jacquier, 1994; Boulanger et al., 1995). We have tested if mutations in the base pair homologous to U2-U23/U6-A59 (A816-U847 of the aI5 intron) affect 5′ splice site cleavage using a sensitized 5′ cleavage substrate (Jacquier & Jacquesson Breuleux, 1991). We did not detect any effect on cleavage with mutations in either or both nucleotides (data not shown). However, because the reaction only took place in a high-salt buffer, and 5′ splice site cleavage occurred by hydrolysis rather than transesterification, we cannot rule out that, under more physiological conditions, these nucleotides have a role in 5′ splice site cleavage.

MacPheeters (1996) recently described U6 snRNA suppressors of branch point mutations located in U2/U6 helix Ia. These data suggested that the nucleophile for the first splicing step may also be close to U2/U6 helix I. Our results bring together the U6-G52/U2-A25 tertiary interaction with our previous observation on the involvement of U6-G52 in recognition of the 5′ splice site +1G. The interaction between U2/U6 helix Ib and the 5′ splice site, and the indication that U2/U6 helix Ia may be close to the branch point adenosine strongly supports a catalytic role for U2/U6 helix I in splicing. Interestingly, in the fission yeast *Schizosaccharomyces pombe*, the U6 snRNA gene contains an intron inserted between nucleotides U52 and A53 (C58 and A59, in *S. cerevisiae*) (Tani & Ohshima, 1989). It was proposed that the intron insertion resulted from an aberrant splicing event, followed by reverse transcription and recombination. This observation suggested that the U6 snRNA region including C58 and A59 was a part of the catalytic center of the spliceosome. Here we demonstrate that this region in the U2/U6 helix I context is interacting with the 5′ splice site +1G for the first step of splicing, strengthening the aberrant splicing hypothesis significantly.

MATERIALS AND METHODS

Plasmids and strains

DNA manipulations were performed using standard protocols (Sambrook et al., 1989). Enzymes were obtained from New England Biolabs unless otherwise stated. Site-directed mutagenesis was performed on single-stranded DNA using the dut$^-$ung$^-$ procedure (Kunkel, 1985). All mutations were confirmed by DNA sequencing using T7 DNA polymerase (Pharmacia) or Sequenase (USB). A completely sequenced fragment containing each mutation was subcloned to the relevant vector to minimize the risk of unwanted mutations. Mutagenesis of the U6 snRNA sequence (Kandels-Lewis & Séraphin, 1993) and U2 snRNA sequence (Pascolo & Séraphin, 1997) was performed as described previously. All plasmids were propagated in *E. coli* strains MC1066 or MH1.

Analysis of the U2-23–25 and U2-U23 mutant strains were performed in yeast strain BSY325 (MATa, ade2, arg4, leu2, trp1, ura3, snr20::TRP1, pBS499 (pCEN-URA3–SNR20)). Experiments with U6-A59C were done in strain DAB017 (MATα, can1, his4, leu2, trp1, ura3, SNR6::LEU2, YCpEP6 (URA3)) (Brow & Guthrie, 1988), and the U2-U23/U6-A59 experiments in strain BSY597 (MATα, trp1–289, ura3–52, arg4, leu2-3,112, ade2, snr20::ADE2, snr6::ARG4, pBS1367 (CEN-URA-U2/U6)). The U2/U6 helix II experiments were done in strain YDF426 (U2/U6 wt) [MATα, ade2, his3, snr20::HIS3, leu2, snr6::ADE2, trp1, ura3, can1, pU2 (CEN-TRP-U2), pU6 (CEN-LEU-U6)]. The U2-2d and U6-6d strains only differ by the presence of pU2-2d and pU6-6d instead of the wild-type plasmid. Plasmid shuffling was performed according to standard protocols. All yeast transformations were made according to the protocol of (Ito et al., 1983).

RNA extraction and analysis

Total RNA was prepared from cells grown in liquid minimal medium with 2% lactate/glycerol to an OD_{600} of 0.5–1 and subsequently induced with 2% galactose for 3 h. RNA extractions were performed essentially according to Pikielny and Rosbash (1985). Primer extension analysis was performed as described (Pikielny & Rosbash, 1985), using the

RP51A exon 2 primer EM38 (Luukkonen & Séraphin, 1997) and intron primer RB27 (Jacquier et al., 1985).

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