Randomization–selection analysis of snRNAs in vivo: evidence for a tertiary interaction in the spliceosome

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Putative components of the spliceosomal active site include a bulged helix between U2 and U6 snRNAs (U2–U6 helix I) and the adjacent ACAGAG hexanucleotide in U6. We have developed an in vivo, bimolecular randomization–selection method to functionally dissect these elements. Although a portion of U2–U6 helix I resembles the G-binding site of group I introns, the data are inconsistent with an analogous functional role for this structure in the spliceosome. Instead, analysis of several novel covariants supports the existence of a structure in which the helix I bulge engages in a tertiary interaction with the terminal residue of the U6 hexanucleotide (ACAGAG). Such a higher order structure, together with other known interactions, would juxtapose the two clusters of residues of the U2–U6 complex that are specifically required for the second chemical step of pre-mRNA splicing with the 3' splice site. Indeed, mutations in the residues that participate in the tertiary interaction affect both the efficiency and fidelity of 3' splice site usage.

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The splicing of messenger RNA precursors (pre-mRNAs) occurs in the spliceosome, a large and dynamic ribonucleoprotein complex containing five small nuclear RNAs (U1, U2, U4, U5, and U6 snRNAs) and at least 50 proteins (for review, see Green 1991; Guthrie 1991; Rymond and Rosbash 1992; Moore et al. 1993). Similarities between the two-step chemical pathway of pre-mRNA splicing and that of group II autocatalytic introns have led to the proposal that the former is also fundamentally an RNA-catalyzed process mediated by the snRNA components of the spliceosome (Sharp 1985 1991; Cech 1986; Guthrie 1991). A number of direct RNA–RNA interactions involving the pre-mRNA substrate and the spliceosomal snRNAs have been demonstrated. It is well-established that recognition of the 5' splice site and intron branchpoint involves Watson–Crick base-pairing with U1 and U2 snRNAs, respectively (for review, see Green 1991; Guthrie 1991; Rymond and Rosbash 1992; Moore et al. 1993). More recent studies indicate that U5 also interacts with the pre-mRNA, in this case with the 5' and 3' exons (Newman and Norman 1991, 1992; Wyatt et al. 1992; Sontheimer and Steitz 1993). A non-Watson–Crick interaction between the guanines at the 5' and 3' splice sites has been proposed to function in the second chemical step of splicing (Parker and Siliciano 1993). Several of these interactions have potential counterparts in group II self-splicing introns (for review, see Weiner 1993; Chanfreau and Jacquier 1993).

Our studies have focused on the U6 spliceosomal

snRNA (Madhani et al. 1990; Madhani and Guthrie 1992). This highly conserved molecule is associated with U4 snRNA through an extensive base-pairing interaction, which is disrupted prior to the first chemical step of splicing (for review, see Green 1991; Guthrie 1991; Rymond and Rosbash 1992; Moore et al. 1993). We have previously used a mutational approach in yeast to demonstrate the existence of a conserved base-pairing interaction between U6 and U2 snRNAs that is mutually exclusive with the U4–U6 interaction (Madhani and Guthrie 1992). In this pairing, termed U2–U6 helix I, a conserved sequence in U6 snRNA interacts with sequences in U2 that are just upstream of the branchpoint recognition region of U2. As a result, highly conserved and functionally important residues in U6 can be juxtaposed with the intron branchpoint. Residues that participate in this structure have been shown to be required for cell viability (Madhani et al. 1990), as well as for both chemical steps of splicing in vitro and in vivo (Fabrizio and Abelson 1990; Madhani and Guthrie 1992; McPheeters and Abelson 1992). These properties led us to propose a model for the active site of the spliceosome (Fig. 1) in which U2–U6 helix I might participate directly in chemical steps of splicing (Madhani and Guthrie 1992). Because this helix is mutually exclusive with stem I of the U4–U6 interaction, its formation offers a mechanistic rationale for the destabilization of U4–U6 prior to the chemical steps of splicing (Madhani and Guthrie 1992). Further evidence consistent with a direct

Figure 1. RNA–RNA interactions between the intron and U2 and U6 snRNAs. *Saccharomyces cerevisiae* snRNA sequences are shown. Intron sequences are the *S. cerevisiae* consensus. Depicted are the U2-branchpoint-region helix (Parker et al. 1987; Wu and Manley 1989; Zhuang and Weiner 1989), U2–U6 helix I (Madhani and Guthrie 1992), and the U6–5′-splice-site helix (Sawa and Abelson 1992; Wassarman and Steitz 1992; Kandels-Lewis and Séraphin 1993; Lesser and Guthrie 1993b). Residues in U2 and U6 required at or prior to the first chemical step of splicing are underlined; those involved in step 2 are circled (Fabrizio and Abelson 1990; Madhani and Guthrie 1992; McPheeters and Abelson 1992).

role for this structure in catalysis is suggested by in vitro cross-linking experiments (Sawa and Shimura 1992; Sawa and Abelson 1992; Wassarman and Steitz 1992; Sontheimer and Steitz 1993) and, more recently, genetic suppression experiments (Kandels-Lewis and Séraphin 1993; Lesser and Guthrie 1993b) that demonstrate base-pairing between the highly conserved ACA sequence upstream of U2–U6 helix I and the 3′ portion of the 5′ splice site consensus (Fig. 1A). Notably, the three RNA–RNA interactions depicted in Figure 1 provide a structural mechanism for juxtaposing the branchpoint nucleophile with the 5′ splice site.

U2–U6 helix I exhibits intriguing similarities to structures in self-splicing introns (for review, see Moore et al. 1993; Weiner 1993). We have noted previously a resemblance to domain 5 of group II introns: Both structures are highly conserved, lie just upstream of helices involved in branchpoint recognition, and contain 2-nucleotide bulges that interrupt the 3′ side of their respective helices (Michel et al. 1989b; Madhani and Guthrie 1992). A different analogy was suggested by an in vitro mutational analysis of the AGA sequence in yeast U2 snRNA (McPheeters and Abelson 1992; nucleotides 25–27 in Fig. 1, note that A25 is bulged). On the basis of structural and phenotypic similarities to the AGA sequence in the P7 helix of group I introns, which contains the binding site for the guanosine (G) cofactor (Michel et al. 1989a), it was proposed that the essential role of the AGA sequence in U2 snRNA in the second chemical step of pre-mRNA splicing is to bind to the conserved G found at the 3′ splice site of nuclear introns (McPheeters and Abelson 1992). This putative spliceosomal G-binding motif has been cited as a possible case of convergent evolution (Weiner 1993). Moreover, it has recently been proposed based on stereochemical studies that the spliceosome generates a group I-like catalytic site to execute the second step of splicing (Moore et al. 1993).

Evaluation of the possible roles of snRNAs in catalysis and their relationship to components in self-splicing introns will ultimately require knowledge of the higher order foldings of the structures in which they participate.

In view of the large and dynamic nature of the spliceosome, a potentially powerful strategy for deriving such structural constraints is phylogenetic covariation analysis; this approach has already been applied successfully to diverse RNA molecules (Levitt 1969; Noller and Woese 1981; Woese et al. 1983; Michel et al. 1989b; Michel and Westhof 1990; Romero and Blackburn 1991). In the case of group I introns (Michel and Westhof 1990), sufficient long-range constraints have been obtained in the form of tertiary interactions to allow the determination of a robust three-dimensional structural model, key aspects of which have been confirmed experimentally (for review, see Cech 1993). Comparably detailed models are not yet available for group II introns.

Unfortunately, almost all of the U2 and U6 residues involved in the proposed active site model of the spliceosome (Fig. 1) are phylogenetically invariant, precluding a strictly analogous approach to the identification of tertiary structure (Guthrie and Patterson 1988; C. Guthrie, S. Mian, and H. Roiha, unpubl.). In other systems this limitation has been overcome through the generation of “artificial phylogenies”: novel functional variants selected in vitro from pools of randomized sequences (for review, see Gold et al. 1993; Szostak and Ellington 1993). This approach has been exploited to identify functionally important tertiary RNA interactions in the Rev-responsive element of human immunodeficiency virus type 1 (Bartel et al. 1991) and in the *sunY* group I ribozyme (Green and Szostak 1994). However, there are significant impediments to the application of such methods to the spliceosome. First, the region of interest (Fig. 1) is derived from several different RNA molecules, yet current in vitro methods do not allow for the selection of functional pairs of variants (intermolecular covariants). Second, the efficient reconstitution of splicing in vitro with multiple synthetic RNAs and the iterative selection of functional variants are likely to be difficult and laborious tasks.

To circumvent these limitations, we have employed a method that allows for the single-step selection in vivo of functional covariants of two interacting molecules

from gene pools containing synthetically randomized residues. We have applied this procedure to functionally dissect residues in the U2–U6 helix I region that are involved in the second chemical step of splicing (Fig. 1). The properties of the selected variants do not conform to those predicted by the G-binding site model for the AGA sequence in U2. Instead, analysis of several novel variants supports the existence of a structure in which the conserved A residue of the helix I bulge (AGA; U2–A25) forms a tertiary interaction with the terminal residue of the U6 hexanucleotide (ACAGAG; U6–G52). To our knowledge this represents the first evidence for higher order RNA structure involving spliceosomal snRNAs. We discuss the implications for the active site involved in the second chemical step of splicing.

Results

Randomization–selection strategy

Our strategy is summarized in Figure 2 (for details, see Materials and methods). We first constructed libraries of yeast U6 and U2 genes in which particular residues had been randomized through chemical synthesis. These were used to cotransform a yeast strain, YHM118, in which both chromosomal copies of U2 and U6 are deleted and complemented by a single *URA3*-marked centromere plasmid that contains both wild-type genes. The cotransformants were then replica-plated to media containing 5-fluoro-orotic acid (5-FOA), which selects against the *URA3*-marked wild-type U2–U6 plasmid

(Boeke et al. 1987). Colonies that grow on 5-FOA contain U2 and U6 functional covariants that can then be isolated and analyzed by DNA sequencing. In deciding what residues to randomize in the libraries, we reasoned that residues involved in the same step of splicing would be the most likely to interact with each other. In the U2 and U6 libraries described here, regions that encompass residues required for the second chemical step of splicing were randomized (Fig. 1, nucleotides 51, 52, 58, 59 in U6 and nucleotides 23–26 in U2). In addition, because two of the U2 residues randomized are thought to participate in an alternative intramolecular structure, U2 stem I (Guthrie and Patterson 1988; McPheeters and Abelson 1992), their putative base-pairing partners were also randomized (nucleotides 9 and 10). The total number of residues randomized (in U2 and U6) was limited to 10. If only one combination of these nucleotides is functional, we expect it to be represented once in $\sim 10^6$ variant pairs ($4^{10} = 10^6$), which approaches the upper limit of what is practical to screen in yeast (see Materials and methods).

We selected for functional cotransformants on 5-FOA under two different conditions, which represent arbitrary (but experimentally convenient) thresholds. First, we isolated non-temperature-sensitive variants by demanding growth after 2 days at 30°C and screening replica plates for temperature sensitivity at 37°C. Second, we reduced the stringency of selection by isolating temperature-sensitive variants. This was accomplished by selecting positives after 4 days, followed by screening of replica plates at 37°C. The 60 individual variant pairs (25 non-temperature-sensitive and 35 temperature-sensitive) obtained are listed in Figure 3. Diagrams showing the nucleotides recovered at each randomized position are shown in Figure 4. These sequences were visually inspected for nucleotide covariations.

Analysis of the selected variants

In the conserved hexanucleotide in U6, the last two positions were randomized (ACAGAG; positions 51 and 52). In all variants, the wild-type A residue was observed at position 51 (Fig. 4). In contrast, all three possible nucleotide changes were observed at position 52. The isolation of U6–G52U was not surprising, because this allele is viable at 30°C as a single mutation (Madhani et al. 1990). However, the isolation of the other changes, U6–G52A and U6–G52C, was unexpected as these are unconditionally lethal as single mutations (Madhani et al. 1990). We will return to the basis for this result in the next section.

In helix I, the residues in U2 and U6 flanking and including the 2-nucleotide bulge that separates helix Ia and helix Ib were randomized. We have previously reported evidence for base-pairing between U6–C58 and U2–G26, as well as between U6–A59 and U2–U23 (Fig 1; Madhani and Guthrie 1992). The role of U2–G26 is of particular interest because this residue is the most critical in the proposed G-binding site described in the introductory section (McPheeters and Abelson 1992). In the case of the G-binding site of group I introns, the

Figure 2. Strategy for in vivo selection of functional covariants of two interacting molecules. See Materials and methods for details.

A

U6	51 52	58 59	U2	9 10	23 26
	**	**		**	****
WT	ACAGAGAUGAUCAGCA		WT	UCUUUGCCUUUUGGCCUUAAGC	
030	ACAGAGAUGAUCAGCA		062	UUUUUGCCUUUUGGCC C AGC	
032	ACAGAGAUGAU U AGCA		064	UUUUUGCCUUUUGGCCUUA A AGC	
035	ACAGAGAUGAU U AGCA		067	UUUUUGCCUUUUGGCC C AAAGC	
036	ACAGAGAUGAUCAGCA		068	UUUUUGCCUUUUGGCC A UAGC	
037	ACAGAGAUGAU U AGCA		069	UUUUUGCCUUUUGGCCU U UAGC	
038	ACAGAGAUGAUCAGCA		070	UUUUUGCCUUUUGGCCU A AGC	
039	ACAGAGAUGAU A AGCA		071	UGAUUGCCUUUUGGCCU U UAGC	
040	ACAGAGAUGAU U AGCA		072	UCCUUGCCUUUUGGCC A UAGC	
041	ACAGAGAUGAU U AGCA		073	UUUUUGCCUUUUGGCCU A UAGC	
042	ACAGAGAUGAU A AGCA		074	UGAUUGCCUUUUGGCCU U UAGC	
043	ACAGAGAUGAU U AGCA		075	UUUUUGCCUUUUGGCCU C AGC	
044	ACAGAGAUGAUCAGCA		076	UUUUUGCCUUUUGGCCU G AGC	
045	ACAGAGAUGAU A AGCA		077	UGUUUGCCUUUUGGCC C CAAGC	
047	ACAGAGAUGAU U AGCA		079	UAUUUGCCUUUUGGCC C UAGC	
048	ACAGAGAUGAU A AGCA		080	UAAUUGCCUUUUGGCC C UAGC	
049	ACAGAGAUGAU U AGCA		081	UCUUUGCCUUUUGGCC A AGC	
050	ACAGAGAUGAU U AGCA		082	UAUUUGCCUUUUGGCCU A AGC	
051	ACAGAGAUGAUC G CA		083	UUCUUGCCUUUUGGCC C UAGC	
052	ACAGAGAUGAUCAGCA		084	UCUUUGCCUUUUGGCC G AGC	
053	ACAGAGAUGAUCAGCA		085	UUCUUGCCUUUUGGCCU A AGC	
054	ACAGAGAUGAU U AGCA		086	UCAUUGCCUUUUGGCCU A AGC	
055	ACAGAGAUGAU U AGCA		087	UUUUUGCCUUUUGGCC A UAGC	
056	ACAGAGAUGAU U AGCA		088	UAGUUGCCUUUUGGCCU U UAGC	
057	ACAGAGAUGAU U AGCA		089	UCUUUGCCUUUUGGCCU A UAGC	
058	ACAGAGAUGAU A AGCA		090	UUCUUGCCUUUUGGCC C UAGC	
invariant	AG	R			

B

U6	51 52	58 59	U2	9 10	23 26
	**	**		**	****
WT	ACAGAGAUGAUCAGCA		WT	UCUUUGCCUUUUGGCCUUAAGC	
TS028	ACAGAGAUGAU U CGCA		TS085	UAUUUGCCUUUUGGCCU C UAGC	*
TS029	ACAGACAUGAUCAGCA		TS086	UUUUUGCCUUUUGGCC C CGAGC	*
TS030	ACAGAGAUGAUCAGCA		TS087	UCCUUGCCUUUUGGCCU A CAAGC	*
TS031	ACAGACAUGAUCAGCA		TS088	UCAUUGCCUUUUGGCC C GAGC	*
TS032	ACAGACAUGAUGCGCA		TS089	UCUUUGCCUUUUGGCC G AGC	*
TS033	ACAGACAUGAU A AGCA		TS090	UCAUUGCCUUUUGGCC C GUAGC	*
TS034	ACAGAU A UGAU A GGCA		TS091	UCCUUGCCUUUUGGCC A GUAGC	*
TS035	ACAGACAUGAUCAGCA		TS092	UCAUUGCCUUUUGGCCU G GAGC	*
TS036	ACAGAGAUGAU U CGCA		TS093	UCAUUGCCUUUUGGCC C GAGC	*
TS037	ACAGACAUGAUCAGCA		TS094	UCGUUGCCUUUUGGCC C AGAGC	*
TS038	ACAGAGAUGAU U CGCA		TS095	UCAUUGCCUUUUGGCC C CGAGC	*
TS039	ACAGAGAUGAUC C GCA		TS096	UUUUUGCCUUUUGGCCU C AGAGC	*
TS040	ACAGAU A UGAUC G GCA		TS097	UAUUUGCCUUUUGGCC C GAGC	*
TS041	ACAGAGAUGAU U AGCA		TS098	UACUUGCCUUUUGGCC A CAAGC	*
TS042	ACAGAGAUGAU U AGCA		TS099	UACUUGCCUUUUGGCC A AGAGC	*
TS043	ACAGAAUGAUGAGCA		TS100	UACUUGCCUUUUGGCCU A AGAGC	*
TS044	ACAGAGAUGAUC C GCA		TS101	UUUUUGCCUUUUGGCCU U UAGAGC	*
TS045	ACAGAGAUGAUCAGCA		TS102	UGAUUGCCUUUUGGCCU C AGAGC	*
TS046	ACAGAU A UGAUCAGCA		TS103	UUUUUGCCUUUUGGCCU C UGAGC	*
TS047	ACAGACAUGAUG G CA		TS104	UCAUUGCCUUUUGGCCU C AGAGC	*
TS048	ACAGACAUGAU A AGCA		TS105	UUCUUGCCUUUUGGCCU C UGAGC	*
TS049	ACAGACAUGAU A AGCA		TS106	UUUUUGCCUUUUGGCC C AGAGC	*
TS050	ACAGAGAUGAUC C GCA		TS107	UUCUUGCCUUUUGGCCU U UAGAGC	*
TS051	ACAGAU A UGAUCAGCA		TS108	UGAUUGCCUUUUGGCCU U UAGAGC	*
TS052	ACAGAGAUGAU U AGCA		TS109	A4-13 CCUUUGCC A AGAGAGC	*
TS053	ACAGAGAUGAU U CGCA		TS110	UUUUUGCCUUUUGGCCU G AGAGC	*
TS054	ACAGAU A UGAUCAGCA		TS111	UACUUGCCUUUUGGCCU G UAGAGC	*
TS055	ACAGAGAUGAUCAGCA		TS112	UUGUUGCCUUUUGGCC A AGAGC	*
TS056	ACAGAU A UGAUCAGCA		TS113	UCUUUGCCUUUUGGCCU C UAGAGC	*
TS057	ACAGAGAUGAUC C GCA		TS114	UCAUUGCCUUUUGGCCU A AGAGC	*
TS028'	ACAGAU A UGAU A AGCA		TS060	'UUUUUGCCUUUUGGCCU C GUAGC	*
TS029'	ACAGAGAUGAUCAGCA		TS061	'UGCUUGCCUUUUGGCCU A UAGC	*
TS031'	ACAGAGAUGAU A GGCA		TS063	'UCCUUGCCUUUUGGCCU C UAGC	*
TS033'	ACAGAGAUGAU U AGCA		TS065	'UGUUUGCCUUUUGGCCU C UGAGC	*
TS046'	ACAGAGAUGAUCAGCA		TS078	'UAUUUGCCUUUUGGCC A UAGC	*
invariant	A				

Figure 3. Selected variant pairs. (A) Sequences of non-temperature-sensitive variant pairs selected after 2 days on 5-FOA at 30°C. (B) Sequences of temperature-sensitive variant pairs selected from the U2 and U6 libraries after 4 days on 5-FOA at 30°C. Boldface type indicates changes from wild type. Asterisks (*) indicate variants containing otherwise lethal mutations at U6 nucleotide 52. Prime (') entries are temperature-sensitive variants selected originally after 2 days on 5-FOA at 30°C.

Figure 4. Nucleotides recovered in the variant pairs. (A) Shown in the context of the U2-U6 structure are the nucleotides recovered at each randomized position in the variants shown in Fig. 3A. Large type indicates that only the wild-type nucleotide was recovered at that position. (B) Nucleotides recovered at each randomized position in the variants shown in Fig. 3B.

identity of the G in the AGA sequence is phenotypically more important than base-pairing because of its critical role in substrate binding (Michel et al. 1989a; Yarus et al. 1991b). Therefore, it was of interest to determine whether this was the case in the selected variants. Extensive Watson-Crick covariation was observed between U6 nucleotide 58 and U2 nucleotide 26 (Table 1), strongly supporting the occurrence of base-pairing at this position. To examine in detail the base-pairing requirements at this position, we constructed yeast strains that contained all possible nucleotide combinations at these two positions, using U2 and U6 alleles created by site-directed mutagenesis. These were assayed at 25°C, 30°C,

Table 1. Base appositions seen between U6 nucleotide 58 and U2 nucleotide 26 in functional variants shown in Fig. 3

Variant class	Watson-Crick	G-U wobble	Other ^a
Non-temperature-sensitive variants	25	0	0
Temperature-sensitive variants	26	5	4

Entries refer to the number of covariants of each type.

^aAll are U2-G26A/U6-C58

and 37°C. The phenotypes of these strains are summarized in Table 2 and shown in Figure 5A, for plates incubated at 30°C. As can be seen by examining the diagonals in both Figure 5A and Table 2, all Watson-Crick appositions support cell growth under the conditions tested. Both G-U wobble pairs are functional, though one (U6-C58U/U2-G26) is temperature sensitive at 37°C (Fig. 5A; Table 2). One of the two A-C appositions confers slow, temperature-sensitive growth (Fig. 5A; Table 2); interestingly, rare A-C pairs that replace G-U pairs in rRNA phylogeny are always found at helical junctions (Gutell 1993). No other nucleotide apposition is functional at this position under any of the conditions tested (Fig. 5A; Table 2).

Previously, it was shown that double mutants that affect both G26 and A27 in U2 result in a more severe phenotype *in vitro* than single mutations at either position; a complete inhibition of the second step of splicing was observed (McPheeters and Abelson 1992). This observation was rationalized in terms of a recent "axial" G-binding site proposal for group I introns in which both the G and 3' A of the AGA sequence interact with the G substrate (Yarus et al. 1991a,b; McPheeters and Abelson 1992). However, given the results described above, an alternative possibility is that the severe defect of double mutants at nucleotides 26 and 27 in U2 instead might result from a defect in base-pairing with U6. To test this possibility, we constructed and analyzed a double transversion at these positions (U2-G26C, A27U). As expected, in the presence of wild-type U6, this mutant is lethal at 25°C, 30°C, and 37°C (Fig. 5B shows plates incubated at 30°C). In contrast, in the presence of a U6 allele that restores base-pairing (U6-U57A, C58G), wild-type growth is restored under all conditions tested (Fig. 5B). Moreover, suppression between the U2 and U6 alleles is mutual because the U6 allele is also lethal in the presence of wild-type U2 (Fig. 5B). Taken together with the results shown in Figure 5A and summarized in Table 2, the data indicate that base-pairing at these positions, rather than specific sequence, is crucial for cell viability and, presumably, the second step of splicing.

The other base pair randomized consists of U6 nucle-

Table 2. Phenotypes of base appositions between U6 nucleotide 58 and U2 nucleotide 26

U6 nucleotide 58					
U2 nucleotide		C	U	A	G
26	G	+	ts ^a	—	—
	A	ts ^b	+	—	—
	U	—	—	+	+
	C	—	—	—	+

YHM118 derivatives containing the indicated U2 and U6 allele combinations assayed on 5-FOA after 3 days at 25, 30, and 37°C. (+) Wild-type growth at 25, 30, and 37°C; (—) no growth at 25, 30, and 37°C.

^aWild-type growth at 25 and 30°C and no growth at 37°C.

^bSlow growth at 25 and 30°C and no growth at 37°C. The wild-type nucleotides are indicated in bold.

Figure 5. (A) Analysis of the U6-C59/U2-G26 base pair. Growth on 5-FOA of YHM118 derivatives harboring the indicated U2 and U6 alleles at nucleotides 26 and 58, respectively. Plates were incubated at 30°C for 3 days. (B) Analysis of double mutants in U2-U6 helix Ia. Growth on 5-FOA of YHM118 derivatives harboring the indicated U2 and U6 alleles at nucleotides 26–27 and 57–58, respectively. Plates were incubated at 30°C for 3 days.

otide 59 and U2 nucleotide 23. We demonstrated previously that the deleterious growth phenotypes of U6-A59G, a lethal allele, and U6-A59C, a temperature-sensitive allele, can be specifically suppressed by U2-U23C and U2-U23G, respectively. However, in the presence of wild-type U6, the U2 mutations have no effect on cell growth, although they exhibit a measurable defect in the second step of splicing *in vivo* (Madhani and Guthrie 1992). Thus, base-pairing at this position, although demonstrable, is not essential for cell growth. We expected, therefore, that the selected variants containing the wild-type nucleotide at U6 position 59 would contain all possible nucleotides at position 23 in U2, whereas those that contained U6-A59G would only be found in combination with the compensating U2-U23C mutation.

This is what we observed (Figs. 3 and 4; Table 3). The U6-A59C mutation was not found among the non-temperature-sensitive variants even in combination with the predicted compensatory mutant U2-U23G (Table 3). It does appear in the temperature-sensitive variants but not always in combination with U2-U23G (Table 3). This latter result is expected because in the presence of wild-type U2, U6-A59C exhibits a deleterious effect on growth only above the 30°C temperature used in our selection experiments (Madhani and Guthrie 1992).

In an effort to better understand the structural requirements at this base pair, we used site-directed mutants to construct YHM118 strains containing all possible appositions between U6 nucleotide 59 and U2 nucleotide 23. The growth phenotypes of these strains on 5-FOA are shown in Table 4. Specific suppression of U6-A59C by U2-U23G and of U6-A59G by U2-U23C was observed (Table 4), confirming the occurrence of base-pairing at this position. Because the U6-A59C/U2-U23G combination supports growth at all temperatures tested, the reason for its absence in the non-temperature-sensitive selected variants is unknown but could reflect a defect that becomes apparent only in combination with other changes found in the variants. The U6-A59U site-directed mutation, which was also absent from the selected variants, was found to be inviable, even in combination with the compensatory mutation U2-U23A. The observation that base-pairing is not sufficient to confer growth is consistent with our previous suggestion for an additional role for U6-A59 (Madhani and Guthrie 1992).

Analysis of the variants revealed that the 2-nucleotide bulge (U2 nucleotides 24–25) that separates helix Ia and helix Ib can be replaced by a wide variety of dinucleotides (15/16 of the possible dinucleotides are found; Figs. 3 and 4). To further delineate the minimal features of the bulge required for growth, we constructed single and double nucleotide deletions of the bulge in addition to all possible single nucleotide substitutions. As expected from the selection data, all substitutions in U2 nucleotides 24 and 25 were fully viable (Table 5). Single nucleotide deletions are tolerated, whereas deletion of both nucleotides 24 and 25 is lethal (Table 5). The results at position 25 are consistent with the mild *in vitro* phenotypes of nucleotide substitutions and a point deletion of U2-A25 (McPheeters and Abelson 1992). Finally, we engineered single nucleotide insertions in U6 at the kink (between nucleotides 58 and 59) opposite the bulge; all four insertions are lethal (Table 5). Thus, the bulge appears to be moderately tolerant to mutational insult.

Table 3. Base appositions seen between U6 nucleotide 59 and U2 nucleotide 23 in functional variants shown in Fig. 3

Identity of U6 nucleotide 59	W-C	Other
A (wild type)	21	24
G	6	0
C	1	8
U	0	0

Table 4. Phenotypes of base appositions between U6 nucleotide 59 and U2 nucleotide 23

		U6 nucleotide 59				
U2		C	U	A	G	
nucleotide	G	+	—	+	—	
23	A	—	—	+	—	
	U	ts ^a	—	+	—	
	C	—	—	+	+	

YHM118 derivatives containing the indicated U2 and U6 allele combinations assayed on 5-FOA after 3 days at 25, 30, and 37°C. (+) Wild-type growth at 25, 30, and 37°C; (—) no growth at 25, 30, and 37°C.

^aWild-type growth at 25 and 30°C and no growth at 37°C. The wild-type nucleotides are indicated in bold.

In addition to the regions described above, U2 nucleotides 9 and 10 were randomized in the U2 library to test the importance of base-pairing in the lower portion of U2 stem I. However, because many functional variants are unpaired at either or both randomized positions (Fig. 3), it appears that neither the U2-C9/G26 pair nor the U2-U10/A25 pair is essential for growth. One variant (TS 109) contains a deletion that encompasses this region. These observations are in agreement with previous *in vitro* results in which no requirement for base-pairing in the lower part of the stem was observed (McPheeters and Abelson 1992).

Unexpected variants containing lethal mutations at U6 position 52

As mentioned above, several unexpected temperature-sensitive variants were isolated from the libraries. These contain either an A or a C at nucleotide 52 in U6, which

Table 5. Phenotypes of mutants in the bulge region of U2–U6 helix I

Mutant	Growth
U2-U24C	+
U2-U24A	+
U2-U24G	+
U2-A25G	+
U2-A25C	+
U2-A25U	+
U2-ΔU24	+
U2-ΔA25	+
U2-ΔU24, ΔA25	—
U6-i58G	—
U6-i58A	—
U6-i58U	—
U6-i58C	—

YHM118 derivatives containing the indicated U2 or U6 allele assayed on 5-FOA after 3 days at 25, 30, and 37°C. U2 mutants assayed in combination with wild-type U6; U6 mutants assayed in combination with wild-type U2. (+) Wild-type growth at 25, 30, and 37°C; (—) no growth at 25, 30, and 37°C. i58 represents the insertion of the indicated nucleotide after U6 nucleotide 58.

We performed a secondary selection, starting with U6-G52C. YHM118 was cotransformed with this mutant and the U2 library, and functional variants were selected on 5-FOA at 30°C. On the basis of our initial classification, we expected to obtain representatives of only class IA and class IB, as the other classes contain a flip of the U6-C58/U2-G26 base pair. Twelve functional variants, all of which are temperature sensitive at 37°C, were analyzed (Fig. 7B). As predicted, 11 of the 12 match the consensus from class IA (U23C and A25G); the single exception contains U23C and U24G and, therefore, mimics class IB. Three of the class IA-like suppressors are also labeled class IB because these variants contain a G residue at the first position of the bulge. Further analysis of the class IC variant are described below.

A

Class IA

U6		U2	
	51 52 58 59		9 10 23 26
	** **		** ****
TS029	ACAGACAUGAUCAGCA	TS086	UUUUUGCCUUUUGGCCCGGAUC
TS037	ACAGACAUGAUCAGCA	TS094	UCGUUGCCUUUUGGCCAGGAUC
TS048	ACAGACAUGAUAGCA	TS105	UUCUUGCCUUUUGGCCUGGAUC
TS049	ACAGACAUGAUAGCA	TS106	UUUUUGCCUUUUGGCCAGGAUC
invariant	C		C G

Class IB

U6		U2	
	51 52 58 59		9 10 23 26
	** **		** ****
TS031	ACAGACAUGAUCAGCA	TS088	UCAUUGCCUUUUGGCCGGAUC
TS033	ACAGACAUGAUAGCA	TS090	UCAUUGCCUUUUGGCCGGAUC
invariant	C		A CGU

Class IC

U6		U2	
	51 52 58 59		9 10 23 26
	** **		** ****
TS035	ACAGACAUGAUGAGCA	TS092	UCAUUGCCUUUUGGCCUGGAUC

Class II

U6		U2	
	51 52 58 59		9 10 23 26
	** **		** ****
TS032	ACAGACAUGAUGCGCA	TS089	UCUUUGCCUUUUGGCCAGGAUC
TS047	ACAGACAUGAUGGCA	TS104	UCAUUGCCUUUUGGCCUGGAUC
invariant	C G		CC

B

U2			
	9 10 23 26		
	** ****		
C52SUP06	UCCUUGCCUUUUGGCCAGGAUC	Class	IA
C52SUP07	UUUUUGCCUUUUGGCCCGGAUC		IA
C52SUP08	UCAUUGCCUUUUGGCCAGGAUC		IA
C52SUP09	UCAUUGCCUUUUGGCCAGGAUC		IA
C52SUP10	UCUUUGCCUUUUGGCCAGGAUC		IA
C52SUP11	UCCUUGCCUUUUGGCCCGGAUC		IA/IB
C52SUP12	UCUUUGCCUUUUGGCCAGGAUC		IA
C52SUP13	UACUUGCCUUUUGGCCAGGAUC		IA
C52SUP14	UCUUUGCCUUUUGGCCAGGAUC		IA
C52SUP15	UUUUUGCCUUUUGGCCCGGAUC		IA/IB
C52SUP16	UAAUUGCCUUUUGGCCCGGAUC		IA/IB
C52SUP17	UCUUUGCCUUUUGGCCCGGAUC		IB
invariant	C G		IB
	CG		

Figure 7. Analysis of G52C-containing variants. (A) Four classes of variants isolated from the U2 and U6 libraries that contain the lethal G52C mutation. (B) U2 alleles isolated from the U2 library after cotransformation of YHM118 with U6–G52C and selection at 30°C for 4 days on 5-FOA plates.

To attempt to dissect the class II G52C-containing variants, we constructed a pair of U2 and U6 alleles that contained the nucleotide substitutions common to the two members of this class (U6–G52C, C58G; U2–A25C, G26C). This combination proved to be inviable (data not shown), indicating that additional changes, likely the alterations in the U6–A59/U2–U23 base pair, are necessary for growth.

Specificity of suppression

In principle, the mutations in the bulge region of helix I could function in suppression directly by affecting a physical interaction between this region and nucleotide

52 in U6 or, more indirectly, by forming or disrupting a different interaction. To address this issue, we asked whether or not bulge-region variants that suppress U6–G52C could also suppress U6–G52A and vice versa. Because the G52A-containing variants mimic the structure of the class IC U6–G52C-containing variants, we expected that the former class of bulge-region variants might at least weakly support growth in combination with U6–G52C (and vice versa). In contrast, we expected that the class IA, class IB, and class II U6–G52C-containing variants would not support growth when combined with U6–G52A, because these did not contain the required flip of the U6–G58/U2–G26 base pair found in the G52A-containing variants.

We constructed YHM118 strains in which we combined representative bulge-region variants with all possible nucleotides at U6 position 52 and examined growth of these derivatives on 5-FOA at 25°C, 30°C, 33°C, 35°C, and 37°C. Table 6 summarizes these results, and Figure 8 shows the growth of representative combinations at 30°C. As expected, bulge-region variants originally identified in combination with G52A also support growth in combination with G52C; however, in each case, they suppress G52A more strongly than G52C (Table 6, rows 2–6). For example, the variant shown in Figure 9B suppresses G52A but not G52C at 30°C; but at lower temperatures, weak suppression of G52C is observed (Table 6, row 2). None of these bulge-region variants affect the growth of G52U, a temperature-sensitive mutant (Table 6, rows 2–6; Fig. 8B). The results for the G52C-containing variants are simpler. Each member tested of the class IA, class IB, and class II G52C-containing variants supports growth in the presence of G52C but not in the presence of G52A under any condition tested (Table 6, rows 7–11, 13; Figs. 8C,D,F). Interestingly, the class IA and IB suppressors are lethal in combination with G52U (Table 6, rows 7–11; Figs. 8C,D). The temperature sensitivity of G52U is slightly exacerbated by the class II bulge variant tested (Table 6, row 13; Fig. 8D). As predicted from its structure, the single class IC variant tested suppresses both G52C and G52A, albeit poorly (Table 6, row 7).

In summary, a restricted and specific set of bulge-region variants suppress U6–G52A and U6–G52C. The class I G52C suppressors and all of the G52A suppressors are characterized by the appearance of a G residue in the helix I bulge. In addition to specific substitutions within the bulge, alterations in at least one of the base pairs that flank the bulge are required for growth. Variants isolated initially in combination with G52A preferentially suppress this U6 mutation. However, as expected from their similarity to the class IC G52C-containing variants, weak suppression of G52C was also observed. In contrast, three of the four classes of G52C-containing bulge-region variants are highly allele-specific. That is, no detectable suppression of G52A was observed; moreover, these variants exacerbate the phenotype of G52U. The other class (IC) of G52A-containing variants, which structurally mimics the G52A-containing variants, can support the growth of G52A.

Table 6. Specificity of suppression of G52A and G52C by bulge-region variants

Bulge-region sequence ^a			U6 nucleotide 52			
U6	U2	isolated as a suppressor of	G	A	C	U
WT	WT	N.A.	wt + + + +	—	—	35 +
C58G	TS100	G52A	wt	30++	25 +/—	33 +
C58G	A52SUP1	G52A	wt	33+++	30 +	35 +
C58G	A52SUP17	G52A	wt	30+++	30 +/—	35 +
C58G	A52SUP4	G52A	wt	30++	25 +	35 +
C58G	A52SUP9	G52A	wt	35+/-	30 +	35 +
WT	TS086	G52C (IA)	wt	—	30++	—
WT	C52SUP10	G52C (IA)	wt	—	33+/-	—
WT	C52SUP15	G52C (IA)	wt	—	35+/-	—
WT	C52SUP17	G52C (IB)	wt	—	33+	—
WT	TS088	G52C (IB)	wt	—	30++	—
C58G	TS092	G52C (IC)	wt	30 +	30+	33 + +
C58G	TS104	G52C (II)	wt	—	35+	30 +/—

The highest temperature and degree of growth of the indicated allele combination that was observed is shown (relative to wild type). Boldface entries refer to cognate combinations.

^aThe U6 entries refer to the sequence of the bulge region only (U6 nucleotide 52 varies depending on the column on the right side of the table). The U2 entries refer to the actual variant used in the experiment (see Figs. 6 and 7 for sequences).

Mutation of U2–A25 affects the second step of splicing in vivo

The specificity of suppression demonstrated above is consistent with the notion that U6–G52 and U2–A25 interact directly (see Discussion). Previous work has demonstrated a role for U6–G52 in the second step of splicing: (1) the U6–G52A and G52C (but not G52U) mutations partially inhibit the second step of splicing in vitro (Fabrizio and Abelson 1990), and (2) the U6–G52U mutation can suppress multiple mutations in the AG dinucleotide at the 3' splice site (Lesser and Guthrie 1993b). Considering a direct interaction between U6–G52 and U2–A25, one predicts that U2–A25 might also influence the efficiency and specificity of the second step of splicing. To test these hypotheses, we constructed yeast strains containing either a wild-type U2 gene or one of the three possible nucleotide substitution mutants as the sole copy. These strains were transformed with intron-containing *ACT1–CUP1* fusion constructs containing a wild-type 3' splice site or one of the six possible nucleotide substitutions in the AG dinucleotide at the 3' splice site (Lesser and Guthrie 1993a; Parker and Siliciano 1993). Total RNA was prepared from these strains and analyzed by a primer extension method.

We first examined the splicing of wild-type pre-mRNAs in the U2 mutants. Shown in Figure 9A, lanes 1–4, are the effects of mutations in U2–A25 on the levels of splicing intermediates and products produced by wild-type *ACT1–CUP1*. Quantitation using a PhosphorImager revealed that the efficiency of the second step of splicing in the mutants is decreased to approximately one-half of wild-type as measured by the ratio of the levels of mature mRNA to lariat–intermediate (M/L ratio; data not shown). To confirm this result using an endogenous yeast pre-mRNA, we examined the levels of

lariat–intermediate and mRNA produced from the chromosomal *RP51A* gene (Fig. 9B). In this case, we observed a reduction in the M/L ratio in all three point mutations at U2–A25 to approximately one-third of wild type (Fig. 9B; data not shown). These data demonstrate that alterations of this nucleotide decrease the efficiency of the second step of splicing.

We then examined the effects of the U2 mutations on the splicing of *ACT1–CUP1* fusions that contain mutant 3' splice sites. Strikingly, the U2–A25G mutation partially suppresses the splicing defects produced by all six substitutions in the AG dinucleotide at the 3' splice site (Fig. 9A, lanes 6,10,14,18,22,26). Quantitation revealed a two- to fivefold increase in the M/L ratio in the instances of suppression (data not shown). U2–A25C and A25U have no detectable effect. Thus, like the U6–G52U mutation, U2–A25G relaxes the specificity of the second step of splicing.

Discussion

Creating an artificial phylogeny of spliceosomal snRNAs in yeast

The constraints of higher order folding are essential to the development of robust models for three-dimensional structure. To seek tertiary interactions among putative active site components of the spliceosome (Fig. 1), we used a method that allowed us to select in vivo for functional covariants of two interacting molecules from gene libraries that contain randomized residues (Fig. 2). This method allowed us to create an artificial phylogeny of the U2–U6 helix-I region (Figs. 3 and 4). This method differs from in vitro randomization–selection methods (for review, see Gold et al. 1993; Szostak and Ellington 1993) in several important ways that affect its applica-

Figure 8. Specificity of suppression. Growth on 5-FOA of YHM118 containing all possible nucleotides at U6 position 52 and the indicated bulge variants in U2–U6 helix I. Plates were incubated at 30°C for 4 days.

bility. First, unlike current *in vitro* methods, *in vivo* selection permits the isolation of functional pairs of covariants versus variants of a single molecule. Because each covariant is isolated within a living cell, specific pairs are sequestered from other covariants and are propagated clonally as cells divide. Second, these variants can be isolated in a single selection step; *in vitro* methods are generally iterative because they are limited by the enrichment obtainable in a single cycle of biochemical selection. Moreover, because our selection method is based on a genetic phenotype, its potential usefulness can be extended to molecules other than RNA and to situations where biochemical selections are impractical. Finally, a limitation of our method is that many fewer variants can be introduced into cells (10^6) than can be manipulated *in vitro* (10^{15} ; Bartel and Szostak 1993), thereby limiting the number of residues that can be analyzed in a single experiment. If one desires to analyze all possible variants, selection *in vitro* raises the number of residues that can be randomized from ~ 10 to 25 ($4^{10} = 10^6$; $4^{25} = 10^{15}$). Of course, in both cases, decreasing the level of degen-

eracy would permit the analysis of larger sets of residues (e.g., Green and Szostak 1992).

Does the AGA sequence of U2 function as a group I intron-like G-binding site?

As expected, our analysis of the selected variants revealed Watson–Crick covariation at two positions previously proven to engage in base-pairing (Tables 1 and 3; Madhani and Guthrie 1992). Of particular interest are the data that relate to the U6–C58/ U2–G26 base pair. As discussed in the introductory section, U2–G26 has been proposed to function as part of a group I intron-like G-binding site during the second chemical step of splicing (McPheeters and Abelson 1992). Such a model would be consistent with the recent proposal, based on stereochemical studies, that the spliceosome generates a group I-like catalytic site to execute the second chemical step of splicing (Moore and Sharp 1993). In group I introns, analysis of the putatively homologous base pair (G264–C311 in the *Tetrahymena* rDNA intron) indicates that the identity of the G at position 264 is more important

Figure 9. Mutation of U2-A25 affects the second step of splicing in vivo. (A) Splicing of wild-type and mutant alleles of *ACT1-CUP1*. Autoradiographs of primer extension analysis of RNA isolated from yeast strains containing the indicated allele at U2 nucleotide 25 and either a wild-type *ACT1-CUP1* fusion or one of six nucleotide substitutions in the AG dinucleotide at the 3' splice site (indicated above each set of four lanes in italics). The positions of bands corresponding to mRNA and lariat-intermediate for *ACT1-CUP1* and to U1 snRNA (an internal control) are indicated. The band just above that of mRNA seen in some of the lanes represents cleavage at a downstream UG 3' splice site (Parker and Siliciano 1993). (B) Splicing of *RP51A*. RNAs used in A, lanes 1–4, were analyzed by primer extension using a primer complementary to the second exon of the yeast *RP51A* gene. Bands corresponding to two mRNAs and the lariat-intermediate produced from this gene are indicated. U5 snRNA (not shown) was used as an internal control.

for G binding than is base-pairing of this residue with its partner C311 (Yarus et al. 1991b). This asymmetry is expected because G264 forms hydrogen bonds with the G substrate (Michel et al. 1989a). In contrast, detailed analysis of the U2–G26/ U6–C58 base pair (Fig. 5A; Tables 1 and 2) demonstrates that the identity of U2–G26 can be varied to any other nucleotide so long as base-pairing is maintained. These data are reinforced by our analysis of a double mutant (U2–G26C, A27U), which was shown previously to completely inhibit the second step of splicing in vitro (McPheeters and Abelson 1992). This severe block was proposed to result from the disruption of all hydrogen bonds with G (McPheeters and Abelson 1992) according to a recent axial G-binding site model for group I introns, in which G is bound by hydrogen bonding to A265 as well as G264 (Yarus et al. 1991a,b). However, the growth defect of this U2 allele can be fully suppressed by the U6 double compensatory mutant (Fig. 6B). Thus, our data do not support the hypothesis that the AGA sequence in U2 snRNA functions as a group I-like G-binding site; rather, the critical role of this sequence appears to be a base-pairing interaction with U6 snRNA.

Evidence for a tertiary interaction

In addition to providing further insight into the constraints on established RNA–RNA interactions, our approach permits the identification of nucleotide covariations indicative of previously unknown secondary and/or tertiary interactions. Below we summarize evidence, based on the analysis of several novel variants, for a tertiary interaction between U6–G52 and the helix I bulge.

Novel variants containing lethal substitutions in the ACAGAG hexanucleotide We isolated temperature-sensitive variants from the randomized libraries that contained otherwise lethal mutations, G52A and G52C, in the terminal residue of the ACAGAG hexanucleotide in U6. We demonstrated that specific changes in the bulge region of U2–U6 helix I suppress the lethality of these substitutions. In the case of U6–G52A, an A → G substitution in the second nucleotide of the bulge together with a flip of the adjacent base pair of helix Ia are necessary and sufficient to suppress its lethal phenotype. In the case of U6–G52C, two classes of suppressors could be discerned on the basis of common sequence motifs. Class I contains three subsets (IA, IB, IC), all of which contain a G residue in one of the two residues of the bulge. Class IA and class IB suppressors contain, in addition, a U2–U23C mutation that disrupts the first base pair of helix Ib. Class IC, instead, contains a flip of the U6–C58/U2–G26 base pair and therefore mimics the structure of the G52A-containing variants. The class II bulge variants found in association with G52C are more complex, containing changes in both base pairs that flank the bulge as well as changes in the bulge itself.

Specificity of suppression suggests a direct interaction An important question is whether suppression reflects the formation of a direct tertiary interaction be-

tween U6 nucleotide 52 and the helix I bulge or a more indirect interaction. To address this issue, we examined the specificity of suppression. We found that variants isolated initially in combination with G52A preferentially suppress this U6 mutation. However, as expected from their similarity to the class IC G52C-containing variants, weak suppression of G52C was also observed. In contrast, three of the four classes of G52C-containing bulge-region variants exhibit dramatic specificity: no detectable suppression of G52A was observed; moreover, these variants exacerbate the temperature-sensitive phenotype of G52U. The other class (IC) of G52C-containing variants structurally mimics the G52A-containing variants and, as expected, can support growth of G52A. The substantial specificity of suppression can most simply be explained by proposing a direct interaction between U6 nucleotide 52 and the helix I bulge. In this scenario, the examples of incomplete allele specificity (i.e., the G52A and G52C class IC variants) could be accounted for by the existence of a common element to the physical interaction in the different cases of suppression. Concordant with the latter notion is the observation that several classes of suppressors are characterized by the occurrence of one or two G residues in the helix I bulge; the exception is the more complex G52C-containing class II suppressors.

It should be emphasized that the specific interaction between U6–G52 and the bulge is not absolutely essential for cell growth because when nucleotide 52 is wild type or a U, the sequence requirements in the bulge are flexible (Fig. 3). However, such an asymmetric requirement for nucleotides involved in an RNA–RNA interaction is not unusual. For instance, this pattern is seen in the U6–A59/U2–U23 base pair of U2–U6 helix Ib where a direct Watson–Crick interaction can be demonstrated only when U6–A59 is mutated to a G or a C (Table 4). A second example is seen in U4–U6 stem I, where mutations in U6 have much more dramatic effects on growth than mutations in U4 (Madhani et al. 1990). As with the previous examples, the more stringent requirement for U6–G52 suggests a function for this nucleotide in addition to its interaction with the helix I bulge. This additional function could either be required at the same time as the tertiary interaction or at some other point during the spliceosome cycle.

Model for suppression Any model that accounts for the novel variants described above must rationalize the following observations: (1) the ability of highly specific changes in the bulge region to suppress otherwise lethal mutations in U6 nucleotide 52; (2) a requisite G residue in the 2-nucleotide bulge in the majority of both G52C- and G52A-containing variants; (3) the requirement for alterations in one of the base pairs flanking the bulge; and (4) substantial but partially overlapping specificity of suppression. Although the complexity of the suppressors makes it difficult to derive a single structural model that accounts for all of the data, the following model provides a simple explanation that can account for the four observations summarized above.

We propose that U6–G52 forms a symmetrical heteropurine base pair with U2–A25 (Fig. 10A). Such a base pair is found at the junction of the anticodon and D stems in yeast tRNA^{Phe} (for review, see Saenger 1984). We further propose that the identity of flanking residues influences the stability of this interaction. In this scenario, the G52A suppressors result in an isosteric flip of the U6–G52/U2–A25 heteropurine base pair (Fig. 10B). The required flip of the adjacent Watson–Crick base pair could reflect a necessity for optimal stacking between the proposed U6–G52/U2–A26 base pair and the adjacent base pair, additional hydrogen bonding interactions, or a steric incompatibility between the U6–G52A/U2–A25G apposition with the wild-type base pair in helix Ia. Several studies have documented the influence of flanking residue sequence context on the thermodynamic stability of hydrogen bonding interactions in RNA, including tertiary interactions (for review, see Turner and Bevilacqua 1993). Michel et al. (1990) have observed cooperative interactions between adjacent tertiary interactions (base-triples) in the catalytic core of group I introns.

The class IA-, class IB-, and class IC G52C-containing variants can be rationalized by the creation of a Watson–Crick U6–G52C/U2–A25G base pair that would be a similar but not isomorphous replacement of the original heteropurine apposition (Fig. 10C). Such substitutions have been observed in rRNA phylogeny and are consistent with the similarities in symmetry and backbone geometry between symmetric heteropurine pairs and Watson–Crick pairs (Saenger 1984; Gutell 1993). Presumably, these shared characteristics outweigh in im-

Figure 10. Model for suppression. Comparison of symmetric G-A (A), A-G (B), and C-G (C) base pairs (Saenger 1984). (D) Graphic depiction showing the steric feasibility of the proposed heteropurine pair in the context of U2–U6 helix I.

portance the difference in glycosidic bond distance and account for the accommodation of A–G base pairs in Watson–Crick double helices (Saenger 1984; Gutell 1993). In the case of the class IB suppressors, which contain a G residue at the first position of the bulge, we suggest that U2 nucleotide 24 pairs with U6–G52C instead. Again, how the flanking residues influence suppression is unclear; one possibility is that the unpairing of the first base pair in helix Ib caused by the U2–U23C change found in the class IA and class IB variants augments the flexibility of the sugar–phosphate backbone to allow the closer approach required in the Watson–Crick pair (Fig. 10C). Alternatively, the selection for U2–U23C might reflect an additional stacking or hydrogen bonding interaction. The class II suppressors, however, are not readily rationalized by this model; they may represent a fundamentally different structural solution to the same functional problem. Such novel replacements of tertiary interactions have been observed in group I introns and in tRNA (Hou et al. 1993; Peterson et al. 1993; Green and Szostak 1994).

How does the proposed U6–G52/U2–A25 pairing explain the partially overlapping pattern of suppression specificity (Table 6)? According to the model, the majority of the G52C-containing and all of the G52A-containing variants involve a direct interaction between U6 nucleotide 52 and U2–A25G. The fact that one of the base-pairing partners (U2–A25G) is the same in both cases offers a structural rationale for why the same change in the bulge can, in certain contexts, contribute to suppression of both G52A (by a heteropurine swap) and G52C (by a Watson–Crick replacement). The differential requirement for specific flanking residues could then account for why some suppressors are highly specific whereas others are less so (Table 6).

We have tested the structural feasibility of a direct tertiary interaction between U6–G52 and U2–A25 by constructing an atomic ball-and-stick physical model of the U2–U6 helix I region that includes the upstream ACAGAGA sequence (see Materials and methods). We found that a U6–G52/U2–A25 pair could be accommodated in the context of helix I by requiring that nucleotide 53 in U6 cross the major groove of U2–U6 helix Ia. Short loops capable of crossing the major groove of an A-form helix have been proposed for and observed in H-type RNA pseudoknots (Puglisi et al. 1990; Wyatt et al. 1990; for review, see ten Dam et al. 1992). A computer graphic rendition of this model is shown in Figure 10D. This arrangement was chosen to illustrate that stacking of the G–A pair onto the U6–C58/U2–G26 pair would be sterically permissible (see above). Given the current constraints, other configurations are of course possible. Nonetheless, this exercise illustrates that these two regions can be readily juxtaposed.

Functional implications

The model for a direct interaction between U6–G52 and U2–A25 explains the bulk of the experimental data and is structurally reasonable. Given the complexity of the

suppression data, however, it seems likely that this depiction of the interaction is oversimplified. As with other examples of tertiary interactions proposed on the basis of functional covariation data (Michel and Westhof 1990; Bartel et al. 1991; Green and Szostak 1994), high-resolution structural studies will be necessary to understand its details. Nonetheless, it is interesting to consider a direct interaction between U6–G52 and the helix I bulge in the context of other recent observations. As described in the introductory section, two clusters of residues in U2 and U6 are specifically required for the second chemical step of splicing (circled in Fig. 1A; see introductory section). One cluster involves nucleotides 51 and 52 in U6, the other involves the residues that immediately flank the helix I bulge. Notably, a direct interaction between U6–G52 and U2–A25 would closely juxtapose these two groups of residues. Especially in light of other recent results, it is provocative to consider the possibility that this long-range interaction reflects the formation of an active site involved in the second chemical step. First, cross-linking studies performed in mammalian extracts demonstrate proximity between the second nucleotide of the intron and the equivalent of A51 in U6 prior to and/or during the second step of splicing (Sontheimer and Steitz 1993). Second, a non-Watson–Crick interaction between the conserved Gs at the 5' and 3' ends of the intron has been shown to be important for the second step of splicing (Parker and Siliciano 1993). If these interactions occur simultaneously such that the 5' and 3' splice sites are juxtaposed and placed in proximity to A51 in U6 (Fig. 11), then an interaction between U6–G52 and U2–A25 would facilitate the recruitment of the second group of residues critical for the second step of splicing to this region. Thus, we suggest that the tertiary interaction proposed here might function in a network of

Figure 11. Network of RNA–RNA interactions involving the 3' splice site. The intron is shown in lariat–intermediate form. In addition to the interactions shown in Fig. 1, the non-Watson–Crick between the guanines at the 5' and 3' splice site is depicted here (Parker and Siliciano 1993), as well as a site-specific cross-link between the second residue of the 5' splice site and U6–A51 (Sontheimer and Steitz 1993), U5–exon interactions (Newman and Norman 1992; Wyatt et al. 1992; Sontheimer and Steitz 1993), and the proposed tertiary interaction between U6–G52 and the bulge of U2–U6 helix I (this paper).

RNA–RNA interactions that together form an active site involved in 3' splice site cleavage and exon ligation.

Intriguingly, mutation of the residues that participate in this tertiary structure affects not only the efficiency of the second step of splicing but its fidelity as well (Fabrizio and Abelson 1990; Lesser and Guthrie 1993b; Fig. 9). Specifically, both the U6–G52U and U2–A25G mutations can partially reverse the splicing defect produced by several different mutations in the AG dinucleotide at the 3' splice site. In the context of the model presented in Figure 11, this apparent relaxation of specificity might be the result of alterations in the proposed tertiary interaction in a manner that can in some way loosen or change the fit of the 3' splice site into the spliceosomal active site such that noncanonical 3' splice sites can now be accommodated. The precise structural mechanism by which this might occur is unclear; one possibility is that the U2 and U6 mutations cause increased structural flexibility in the active site. A precedent for this notion exists in proteins: crystallographic analyses of mutations that relax the specificity of a bacterial serine protease suggest that increased active site flexibility underlies effects of mutations that decrease specificity (Bone et al. 1991). In any case, the present data demonstrate that both of the residues that participate in the proposed tertiary interaction can influence events at the 3' splice site and are consistent with the notion that this higher order interaction functions intimately in the second step of splicing.

Materials and methods

Strain construction

Yeast procedures used in this study have been described previously (Guthrie and Fink 1991). YHM118 [*a leu2 lys2 trp1 his3 ura3 ade2 snr6::LEU2 snr20::LYS2 pU2U6U[SNR6 (SNR20 URA3 CEN)]*] was constructed as follows: Two parental strains were employed. One contains a disruption of the chromosomal U6 gene, YHM2 [*a leu2 lys2 trp1 his3 ura3 ade2 snr6::LEU2 pSX6U[SNR6 URA3 CEN]*] and the other, YHM111 [*a lys2 trp1 his3 ura3 ade2 snr20::LYS2 pU2U[SNR20 URA3 CEN]*], contains a disruption of the chromosomal U2 gene. Each contains a complementing URA3-marked plasmid containing the wild-type snRNA gene. YHM2 and YHM111 were mated, and diploids were selected on 5-FOA which also cures them of both wild-type plasmids. These were transformed with pU2U6U, which contains the wild-type U2 and U6 genes on a single URA3-marked plasmid. Following sporulation, haploid progeny were identified that contained both U2 and U6 gene disruptions and the complementing plasmid. Growth of this strain on 5-FOA, which selects against the wild-type U2 and U6 genes, was shown to be dependent on the introduction of a functional copy of U2 and a functional copy of U6.

Construction of U2 and U6 gene libraries containing randomized residues

U2 and U6 DNA fragments containing randomized residues were created by polymerase chain reaction of U2 and U6 DNAs using synthetic oligonucleotide primers that were fully degenerate at specified positions. These fragments were used to replace the respective wild-type sequences in snRNA gene-containing plasmids that contain restriction sites either flanking

the coding sequence of U6 (pSX6; Madhani et al. 1990) or the conserved region of U2 (pES143ΔB; Madhani and Guthrie 1992).

Oligonucleotide synthesis was performed on a 0.05 μmole scale on a Cyclone DNA synthesizer (Milligen) using reagents and conditions recommended by the manufacturer. Because of the differential reactivities of the nucleoside phosphoramidites, a biased mixture of 30% A, 30% C, 20% T, and 20% G phosphoramidites was used at the randomized positions; this ratio has been observed to result in an equal incorporation (25% each) of the four monomers (D. Bartel, pers. comm., see below).

To construct the U2 fragment, a 5' primer was used that spans positions +1 to +36 of the U2 gene in pES143ΔB (with respect to the transcription start site) in the sense strand and contains randomized residues at positions 9, 10, 23, 24, 25, and 26. The 3' primer used spans residues +266 to +227 on the antisense strand (Madhani and Guthrie 1992). Amplification was performed exactly as before (Madhani and Guthrie 1992) except that 1 ng of a template that corresponds to an inviable U2 allele (U2–G26U) was used and 35 cycles of amplification were performed. A mutant template was used so as to avoid any possible contamination with wild-type U2 sequences. To generate more DNA, a second amplification was performed using the same primers. This consisted of two 100-μl reactions in which 20 μl of the first reaction was amplified for 20 cycles.

To construct the U6 fragment, we used a mutagenic primer that spans positions +21 to +62 on the sense strand of the U6 gene in pSX6 and contains randomized residues at positions 51, 52, 58, and 59. The 3' primer spans residues +129 to +96 on the antisense strand. These two primers were used in a first round of PCR in which 1 ng of a mutant template (U6–C60G) was amplified for 35 cycles, as above. Forty microliters of this reaction was further amplified in two 100-μl reactions for 20 cycles using the same 3' primer and a 5' primer that spans positions –12 to +33 (Madhani and Guthrie 1992).

The U2 and U6 fragments were extracted with phenol/chloroform to remove the polymerase and spin-chromatographed twice on 1-ml columns G-25 Sephadex-equilibrated in TE buffer (10 mM Tris-HCl at pH 8.0, 0.1 mM EDTA) to remove unincorporated nucleotides. The U2 fragments were treated with 200 units each of *Bam*HI and *Eco*RI for 5 hr at 37°C. The U6 fragment was treated with 40 units of *Sph*I and 200 units of *Xho*I for 5 hr at 37°C. These were then heated to 70°C for 20 min to inactivate the endonucleases and spin-chromatographed twice on G-50 Sephadex equilibrated in TE to remove the short end oligonucleotides produced by digestion. Samples were precipitated with ethanol and resuspended in 50 μl of TE.

To avoid contamination with wild-type sequences, the vectors pSX6 and pES143ΔB were modified so that the wild-type snRNA sequences between the introduced restriction sites were removed and replaced with unrelated stuffer DNA. The resulting derivatives, pΔSX and pΔES, contain 1.6- and 1.3-kb stuffer fragments, respectively, that are derived from the yeast gene *PRP16*. pΔSX was digested with *Sph*I and *Xho*I. Similarly, pΔES was digested with *Eco*RI and *Bam*HI. The vector fragments were separated from the stuffer fragments by agarose gel electrophoresis. DNA was recovered from gels using GeneClean (Bio 101).

Libraries were constructed by ligation of 200 ng of gel-purified vector DNA with 50 ng of the appropriate insert fragment in a volume of 20 μl containing 50 mM Tris (pH 7.6), 10 mM MgCl₂, 5 mM DTT, 50 μg/ml of BSA, 0.1 U/μl of T4 DNA ligase, and 1 mM ATP. Following incubation for 16 hr at 16°C, the reactions were heated to 70°C for 5 min and cooled on ice. Reaction products were introduced into *Escherichia coli* strain DH5α via electroporation (Dower et al. 1988). The resulting U6 library contains 2.8×10^5 independent clones (each variant represented 1×10^3 times); the U2 library contains 4.1×10^5 independent

clones (each variant represented 1×10^2 times). Control ligations lacking insert indicated negligible background. Unlike pΔSX, pΔES cannot replicate in yeast. Therefore, the U2 library was subcloned en masse into the yeast vector pSE362 (*HIS3*, *CEN*) using *Sall* and *SacI* sites that flank the U2 gene. Little, if any, complexity was lost during this subcloning step because $>10^6$ transformants were recovered. DNA sequencing of the pool DNA revealed that the appropriate residues had been randomized. Moreover, at these positions, we observed an even distribution of nucleotides except for G, which was underrepresented approximately twofold.

Selection experiments

For transformation, competent YHM118 was prepared from 1 liter of an exponentially growing culture using a modification of the lithium acetate method (Schiestl and Gietz 1989). Cells were pelleted by centrifugation, washed once with 20 ml of 0.1 M lithium acetate in TE, and, finally, resuspended in 1 ml of 0.1 M lithium acetate in TE to form a concentrated paste. Prior to use, 0.5 ml of 50% glycerol was added and the cells were frozen by an overnight incubation at -70°C . The use of highly concentrated, frozen cells was found to increase cotransformation efficiency ~ 10 -fold. In the initial selection experiments, 1 ml of thawed competent cells was transformed with 5 μg of each library. Transformants (10^5) were selected on 10 SD-HIS-TRP plates at 30°C for 3 days. These were replica-plated to prewarmed 5-FOA plates that were then incubated for either 2 or 4 days at 30°C . Approximately 0.5% and 2% of the transformants, respectively, grew on 5-FOA. These were replica-plated to prewarmed YEPD plates that were then incubated at 37°C for 2 days to test for temperature sensitivity. Non-temperature-sensitive variants that grew initially after 2 days on 5-FOA and temperature-sensitive variants that grew after 4 days on 5-FOA were selected for further analysis. Following colony purification, the U2 and U6 plasmids were recovered from the variants using standard methods (Guthrie and Fink 1991) and were used to retransform YHM118 to verify the growth phenotype. The DNA sequences of the randomized regions of a total of 60 U2 functional variants and their 60 U6 partners were determined.

Secondary selections in which specific lethal U6 mutants were used in combination with the U2 library were accomplished by cotransformation of YHM118 followed by selection of the resultant transformants on 5-FOA for 4 days at 30°C . In these cases, $\sim 3\%$ of the transformants grew on 5-FOA. These were analyzed as above.

RNA analysis

The *ACT1-CUP1* fusions used in this work have been described elsewhere (Lesser and Guthrie 1993a; Parker and Siliciano 1993). Primer extension analysis was performed as described previously (Madhani and Guthrie 1992).

Model construction

Parts for the RNA physical model were purchased from Maruzen, Inc. (Tokyo). Helices were constrained into A-form using spacers and supports supplied by the manufacturer. A model was built that corresponds to the sequences shown in Figure 1. To examine the tertiary interaction suggested by the results, we assumed the symmetric G-A pair between U6 nucleotide 52 and U2 nucleotide 25 discussed above. This was found to be sterically feasible in many different conformations, including one in which the base pair is stacked on the end of helix Ia. Computer modeling was done using the interactive modeling program SYBYL (Tripos Associates). In this case, only helix I and the upstream AGA sequence were modeled.

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