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Invariant U2 RNA Sequences Bordering the Branchpoint Recognition Region Are Essential for Interaction with Yeast SF3a and SF3b Subunits

DONG YAN AND MANUEL ARES, JR.*

Biology Department, Sinsheimer Laboratories, University of California, Santa Cruz, Santa Cruz, California 95064

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U2 small nuclear RNA (snRNA) contains a sequence (GUAGUA) that pairs with the intron branchpoint during splicing. This sequence is contained within a longer invariant sequence of unknown secondary structure and function that extends between U2 stem I and stem IIa. A part of this region has been proposed to pair with U6 in a structure called helix III. We made mutations to test the function of these nucleotides in yeast U2 snRNA. Most single base changes cause no obvious growth defects; however, several single and double mutations are lethal or conditional lethal and cause a block before the first step of splicing. We used U6 compensatory mutations to assess the contribution of helix III and found that if it forms, helix III is dispensable for splicing in *Saccharomyces cerevisiae*. On the other hand, mutations in known protein components of the splicing apparatus suppress or enhance the phenotypes of mutations within the invariant sequence that connect the branchpoint recognition sequence to stem IIa. Lethal mutations in the region are suppressed by Cus1-54p, a mutant yeast splicing factor homologous to a mammalian SF3b subunit. Synthetic lethal interactions show that this region collaborates with the DEAD-box protein Prp5p and the yeast SF3a subunits Prp9p, Prp11p, and Prp21p. Together, the data show that the highly conserved RNA element downstream of the branchpoint recognition sequence of U2 snRNA in yeast cells functions primarily with the proteins that make up SF3 rather than with U6 snRNA.

Nuclear pre-mRNA splicing is a dynamic process marked by an intricate program of RNA-RNA, RNA-protein, and protein-protein interactions (for reviews, see references 6, 43, and 47–49). Five small nuclear RNAs (snRNAs) (U1, U2, U4, U5, and U6), packaged with proteins into small nuclear ribonucleoprotein particles (snRNPs), are essential components of spliceosome. The snRNAs, especially U2 and U6, are intimately associated with the reactive regions of the intron during the chemical reactions of splicing (6, 43, 47–49), supporting the hypothesis that the splicing reactions are RNA catalyzed (14, 55). Construction of the catalytically active spliceosome is heavily dependent on the activity of numerous protein factors, including snRNP proteins (29, 47, 48). Thus, snRNA sequences contain information necessary for interaction with the substrate, each other, and the proteins that guide them to their places in the activated spliceosome.

A web of RNA-RNA interactions holds U2, U6, the 5′ splice site, and the branchpoint together for the first step of splicing (for reviews, see references 6, 43, 48, and 49). In both *Saccharomyces cerevisiae* and mammals, the GUAGUA sequence of U2 snRNA base pairs with the intron branchpoint (51, 66, 71). Two other stretches of U2 RNA are involved in forming helices with U6 snRNA. The 5′ end of U2 snRNA base pairs with the 3′ end of U6 snRNA to form U2-U6 helix II (Fig. 1) (28), a structure that is important for splicing in mammalian cells (18, 67) but can be changed without greatly compromising splicing or cell growth in yeast cells (21, 22, 40, 44, 46). The 3′ half of U2 snRNA stem-loop I base pairs with the U6 snRNA nucleotides just downstream of the invariant ACAGAG sequence to form helix I (Fig. 1) (41), a structure important for splicing in both systems (19, 21, 40, 41, 59). Helix I, together with the U6 ACAGAG sequence-5′ splice site base pairing (35, 38, 53, 62), juxtaposes the functionally important U2 and U6 residues with the intron branchpoint and 5′ splice site (6, 43, 48, 49) (Fig. 1). Recently, support for a third interaction (helix III) proposed to involve U6 sequences upstream of the ACAGAG and U2 sequences downstream of the branchpoint interaction region (50, 60) has been obtained by using mammalian cells (59).

Numerous proteins play roles in the elaborate assembly pathway that leads to the formation of the catalytically active spliceosome. In mammals, U2 snRNA is associated with a large set of proteins in the functional 17S form of the U2 snRNP (8, 58). This set of proteins includes the multisubunit splicing factors SF3a and SF3b, which are necessary for spliceosome assembly (7, 8, 11, 12) and which require the 5′ half of U2 snRNA for their binding to the snRNP (8). Yeast splicing factors Prp9p (1, 37), Prp11p (15, 39), and Prp21p (3, 16) correspond to the SF3a subunits (7, 9, 11; for a review, see reference 29) and are dependent on U2 RNA for their association with the assembling spliceosome (1, 2, 52, 63). Cus1p, identified as a suppressor of a yeast U2 cold-sensitive mutation, is also required for spliceosome assembly (64) and is a yeast homolog of the mammalian SF3b subunit identical to SAP 145 (26, 64). Stem-loop IIa is a U2 RNA structure required for spliceosome assembly (70), and genetic results suggest that its role is mediated through yeast SF3a and SF3b (52, 63, 64). Thus, in both systems, a conserved set of U2 proteins requires U2 RNA sequence for correct spliceosome assembly.

Two stretches of invariant U2 nucleotides connect the branchpoint interaction region with stem-loop I and stem-loop IIa (4, 27). To study the roles of this highly conserved sequence, we tested the ability of mutant U2 snRNA to support

* Corresponding author. Mailing address: Sinsheimer Laboratories, University of California, Santa Cruz, Santa Cruz, CA 95064. Phone: (408) 459-4628. Fax: (408) 459-3737. Electronic mail address: ares@darwin.ucsc.edu.

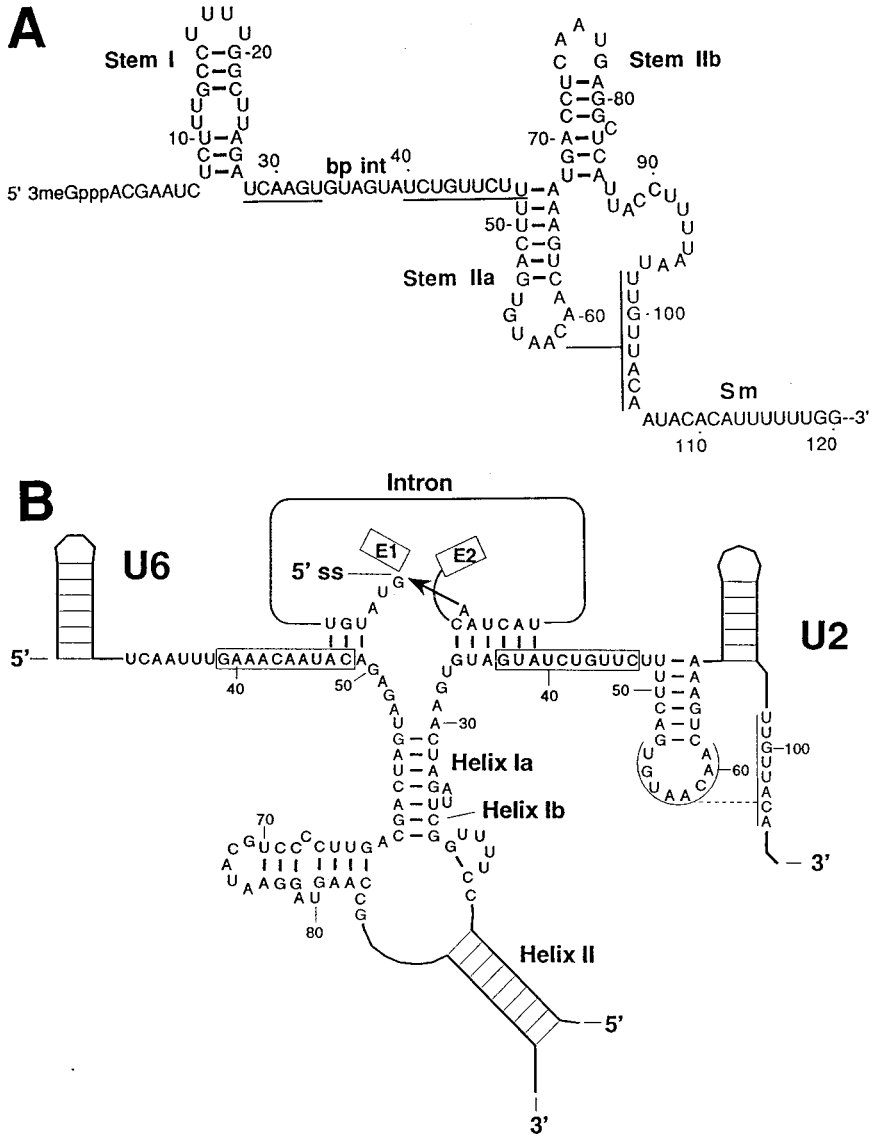

FIG. 1. U2 RNA structure during splicing. (A) Secondary structure of the 5′ half of the U2 snRNA in *S. cerevisiae* (5). Underlined are the nucleotides mutated in this study. The branchpoint interaction sequence labeled bp int. The Sm site labeled Sm. (B) Helix I and helix II interactions between U2 and U6 snRNAs in the spliceosome (6). Exon 1 and exon 2 are abbreviated as E1 and E2 in boxes. U2 and U6 sequences boxed are the nucleotides potentially involved in U2-U6 helix III.

growth and splicing in yeasts. We found that while some single and multiple mutations are lethal and block the first step of splicing, most single mutations are viable and, unlike their mammalian counterparts (68), do not significantly affect splicing (see also reference 44). In an exhaustive test of the proposed U2 and U6 helix III (59, 60), we combined multiple lethal mutations in U2 with compensatory mutations in U6. This analysis and measurement of splicing efficiency in cells carrying various combinations of nonlethal U2 and U6 mutations revealed no contribution to splicing of helix III in yeast cells. In contrast, several lethal U2 mutations in residues 40 to 45 of this region are suppressed by *CUS1-54*, a mutation in a yeast splicing factor homologous to a mammalian SF3b subunit (64). In addition, many aphenotypic mutations in this region are synthetic lethal with *prp5*, *prp9*, *prp11*, and *prp21*, showing that these nucleotides are required to function with other factors including SF3a and a putative RNA helicase (29). We propose that the extremely conserved nucleotides downstream of the branchpoint interaction sequence contribute to splicing primarily through their interactions with proteins rather than through an interaction with U6 snRNA to form helix III.

MATERIALS AND METHODS

Yeast strains and growth conditions. Yeast (*S. cerevisiae*) cells were grown by standard procedures (56). Strain HI70 (*MAT***a** *leu2-3,112 ura3-52 his4-619 lys2 snr20*::*URA3* carrying the pseudo-wild-type U2 gene on YCpLYS2-U2C121U) grows as wild type (5). HI70 derivatives used to test for the growth phenotypes were created by transformation of a set of *LEU2* centromere plasmids carrying different U2 alleles by the lithium acetate method (32). The transformants were streaked on α-aminoadipate (α-AA) (17) at 30°C to select for loss of the *LYS2* plasmid carrying the pseudo-wild-type U2 gene. These were further tested on yeast extract-peptone-dextrose (YEPD) at 18, 23, 30, and 37°C for 2 to 5 days to assay for growth phenotypes. The transformants that failed to shuffle on α-AA at 30°C were also streaked on α-AA at 18, 23, and 37°C for 5 to 7 days. The U44A mutant did not grow well on α-AA; therefore, the growth phenotype of U44A in the absence of wild-type U2 was assayed with strain BJ81 (see below).

Strain BJ81 is *MAT***a** *leu2-3,112 ura3-52 trp1 pep4-3 prb1 prc1* and contains a glucose-repressible wild-type U2 allele integrated into the chromosome; thus, it

is galactose dependent for growth (46). After introduction of a *LEU2* plasmid bearing an appropriate mutant U2 allele, the cells can be grown on glucose-containing medium to repress the expression of the wild-type U2 gene and reveal any phenotypes caused by the mutant U2 snRNA. To deplete wild-type U2 snRNA from cells, cultures of BJ81 carrying different U2 alleles on *LEU2*-marked plasmids were grown in synthetic complete medium containing 2% galactose (SCGAL) to stationary phase. Cells were pelleted and diluted into glucose-containing rich medium (YEPD) for 15 h at various temperatures. Cultures were harvested at an optical density at 600 nm of 0.5 to 1.8 for isolation of total RNA as described previously (5). Suppression of U2 mutations by *CUS1-54* was assayed in strain BJ81. The strain was transformed with different mutant U2 alleles on a *LEU2* centromere plasmid and with *CUS1-54* or *CUS1*$^+$ on a *TRP1* centromere plasmid, and suppression of U2 mutations was tested by growing the cells on glucose medium at various temperatures.

To test for synthetic lethal interactions between U2 mutations and *prp* mutations, we used strains carrying the temperature-sensitive *prp* mutations and containing the glucose-repressible chromosomal U2 gene as described previously (63). Plasmids carrying U2 mutations were introduced into these strains, and transformants were selected on SCGAL plates lacking the appropriate amino acid at 26°C. Transformants were streaked on YEPD plates and grown at 26°C to score synthetic lethality. If a strain cannot grow on glucose-containing medium, the combination of the U2 mutation and the *prp* mutation it carries is scored as synthetic lethal.

To test for helix III, U2 and U6 alleles of various combinations were transformed into strain DK10. Strain DK10 is *MAT***a** *his3*Δ *lys2*Δ *leu2-3,112 ura3-52 trp1 pep4-3 prb1 prc1 snr20*::*LYS2 snr6*::*HIS3* and carries pU2U6U (the *URA3* plasmid carrying the wild-type U2 and U6 minigenes [42]). The strain was constructed as follows. Parental strain HI227 (*MAT***a** *his3*Δ *lys2*Δ *leu2-3,112 ura3-52 trp1 pep4-3 prb1 prc1*) was first constructed from IH1097 by sequential application of the two-step integration and excision replacement technique with cloned copies of a *his3* deletion and a *lys2* deletion. HI227 was sequentially transformed with pU2U6U and a *HIS3*-disrupted U6 gene fragment, which was created by inserting the 1.8-kb *HIS3* *Bam*HI piece into the *Bcl*I site of the marked U6 gene in pSX6 Bluescript (40). The transformants that did not subsequently grow on 5-fluoroorotic acid (5-FOA) (10) were transformed with a U6 gene on a *TRP1* plasmid and retested on 5-FOA. Those that grew on 5-FOA when provided with a wild-type U6 gene were retained. These were transformed with a *LYS2*-disrupted U2 gene fragment (57), and the transformants that could grow on 5-FOA only after the introduction of functional U2 and U6 genes were retained. Southern analysis confirmed that the disruption of the chromosomal U2 and U6 genes had occurred, and this strain is designated DK10.

To create yeast strains with different combinations of U2 and U6 genes, DK10 was transformed with the desired U2 and U6 plasmids and then streaked on 5-FOA at 30°C to select for cells that lost the *URA3* plasmid which carries the wild-type U2 and U6 genes. Colonies were streaked from 5-FOA plates onto YEPD plates and incubated at 18, 23, 30, and 36°C for 3 to 8 days to test for growth phenotypes. Transformants that could not shuffle on 5-FOA plates at 30°C were also shuffled at 18, 23, and 36°C for 5 to 9 days. To assay for the splicing defects of the U2 and U6 mutations in strain DK10, cells were grown at 30°C in liquid YEPD after being shuffled on 5-FOA plates. Total RNA was isolated from cultures at an optical density at 600 nm of 0.5 to 1.8.

Construction of a library of U2 mutations. For cassette mutagenesis, a *LEU2*-marked yeast-*Escherichia coli* shuttle plasmid carrying the 3′ half of the U2 gene (31) was modified. In the first step, the unique *Bgl*II site in the autonomously replicating sequence (ARS) was disrupted by digestion with *Bgl*II, end filling, and ligation. Next, a U2 gene fragment spanning the 5′ half of the gene was mutated with oligonucleotide U2BP by oligonucleotide-directed mutagenesis (36), and the mutant U2 gene fragment was introduced into the *LEU2* plasmid, regenerating the altered U2 gene with *Bgl*II and *Pvu*II restriction sites at positions +24 and +59 of the U2 gene and lacking the nucleotides between these two sites. This plasmid is called pU2-BP. The U2BP oligonucleotide sequence is 5′ ATTGAG GTCATTTCAGCTGCAGATCTAAGCCAAAAG 3′.

A pool of U2 gene fragments containing randomized residues was made by in vitro DNA synthesis with a degenerate synthetic oligonucleotide template and a 5′ primer. A template spanning positions +60 to +20 of the antisense strand of the U2 gene was synthesized with 3.1% degeneracy at each position. A 5′ primer complementary to positions +15 to +29 of the U2 gene was used for DNA synthesis as follows. Primer (400 pmol) and 200 pmol of degenerate oligonucleotide template were mixed in 12 μl of water and heated to 70°C for 5 min. Then 2 μl of 10× Klenow buffer (500 mM Tris-Cl [pH 8.0], 100 mM $MgCl_2$, 10 mM dithiothreitol, 500 mg of bovine serum albumin per ml) was added, and the reaction mixture was slowly cooled to room temperature (23°C) and incubated at this temperature for 1 h. After addition of 4 μl of 5 mM each deoxynucleoside triphosphate [dNTP], 2 μl of Klenow fragment of DNA polymerase I (5 U/μl; Life Science), and 10 μCi of [α-^{32}P]dATP (Amersham), the reaction mixture was incubated at 23°C for 1 h. Then 5 U of Klenow fragment of DNA polymerase I, together with 1 μl of 10× Klenow buffer and 6 μl of water, was added, and the reaction mixture was incubated for another 3 h. The reaction was stopped by adding 3 μl of 0.5 M EDTA. The reaction mixture was phenol-chloroform extracted and ethanol precipitated in the presence of 0.3 M sodium acetate (pH 7.0). The DNA was resuspended in 40 μl of reaction mixture containing 1× Klenow buffer, 1 mM each dNTP, and 10 U of Klenow fragment of DNA polymerase I and incubated at 30°C for 15 min to ensure that the ends were blunt. The reaction was stopped by addition of EDTA. After phenol-chloroform extraction, the duplex DNA was precipitated. Following *Sau*3AI digestion, the duplex DNA fragment was purified on a 15% native polyacrylamide gel. The band corresponding to 36 bp of double-stranded DNA was eluted in 0.5 M ammonium acetate–1 mM EDTA–0.1% sodium dodecyl sulfate at 37°C overnight and ethanol precipitated.

Library construction was done by two sequential ligations. First, 2.5 μg of pU2-BP was digested with *Bgl*II and ligated in a 20-μl final volume to a threefold excess of the gel-purified randomized DNA duplex fragment with T4 DNA ligase. The mixture was phenol-chloroform extracted and ethanol precipitated. The DNA was then digested with *Pvu*II and ligated at low DNA concentration to favor plasmid circularization. After ligation, the ligation reaction mixtures were heated to 65°C for 10 min to inactivate the ligase and were digested again with *Bgl*II. One-tenth of the reaction mixture was used to transform *E. coli* via electroporation (20). The resulting library had more than 20,000 independent colonies, about two-thirds of which have inserts as assayed by restriction enzyme digestions on 110 individual plasmids. The results of sequencing 72 colonies containing insert suggest that about 92% have a correctly inserted fragment and that the library contains 25% of wild-type and 46% of single, 17% of double, 3% of triple, and 1% of quadruple mutations. The other 8% of the insert-containing plasmid lacks the 5′ nucleotide of the template oligonucleotide, resulting in deletion of A-61.

To obtain other mutations in the U2 and U6 sequence, we employed standard oligonucleotide-directed mutagenesis methods (36). The 0.95-kb *Hin*dIII fragment containing the 5′ end of the U2 gene was subcloned into the *Hin*dIII site of the pBluescript KS− vector (Stratagene). Mutagenesis was performed, and the U2 fragments were subcloned into YCpLEU U2-3′ (31). To generate U6 mutations, pBluescript KS- vector containing the U6 gene (40) was used to introduce double and triple mutations. The mutated U6 fragments were subcloned into vector pSX6 (40). All the U6 single-mutation plasmids were kindly provided by C. Guthrie (38).

In vivo chemical probing. In vivo chemical modification of cellular RNA was carried out as described previously (5, 70). Strain BJ81 carrying U2 mutations were grown to saturation in SCGAL dropout liquid medium at 30°C and were then shifted to YEPD medium at different temperatures for 15 h before being used in modification.

RNA extraction and primer extension. RNA extraction and primer extension by reverse transcriptase were carried out as described previously (5). ^{32}P-end-labeled oligonucleotide YSTR was used in the primer extension reactions of the chemically modified U2 RNA. Primer extension will stop or pause at the modified nucleotides. An oligonucleotide complementary to the second exon of both of the pre-U3A and pre-U3B transcripts was end labeled and used to measure splicing efficiency by primer extension. The inefficiently spliced *MAT***a***1* (45) pre-mRNA was assayed with end-labeled oligonucleotide MATa1-29 primer complementary to the second exon (70). The *MAT***a***1* pre-mRNA has no G residues between the 3′ end of the primer and the AG at the 3′ splice site of the first intron. Primer extensions in which dideoxycytidine is used to replace deoxycytidine resolves spliced and unspliced transcripts as follows: on unspliced *MAT***a***1* pre-mRNA, primer extension gives a shorter, 38-nucleotide product, whereas on the mature *MAT***a***1* transcripts lacking the first intron extension produce a longer, 70-nucleotide cDNA terminating at the last G in exon 1. cDNA products were quantitated with a Molecular Dynamics PhosphorImager. All of the gel images were generated with Adobe Photoshop. Oligonucleotide primers and their annealing temperatures are as follows: MATa1-29, 5′ GTTGCTCT ACTTTAGTCAAATTACTTTCC 3′ (57°C); SNR17A and SNR17B U3, 5′ CC AAGTTGGATTCAGTGGCTC 3′ (58°C); SNR7 U5 primer: 5′ AAGTTCCAA AAAATATGGCAAGC 3′ (37°C); and YSTR primer: 5′ ATTATTTTGGGTG CCAA 3′ (37°C).

RESULTS

Effects of mutations in the highly conserved region of U2 containing the branchpoint interaction sequence. To obtain single and multiple mutations in U2, we constructed a library in a *LEU2*-marked centromere plasmid with the U2 gene sequence randomized at positions 30 through 61. Since the library contains a high percentage (46%) of single-residue substitutions, U2 mutants with single base changes in regions of interest were identified by sequencing individual plasmids from the library. Additional mutations were made by oligonucleotide-directed mutagenesis. Strains carrying the U2 mutations were constructed by plasmid shuffling of a U2 knockout strain with a wild-type U2 gene on a counterselectable *LYS2* plasmid (5). Viable colonies were streaked on YEPD plates at different temperatures to score growth defects caused by the U2 mutations, and the results are summarized in Table 1. Strikingly, most single mutations and a number of double mutations in

TABLE 1. Growth phenotypes of U2 mutations[a]

U2 allele	Growth phenotype at[b]:			
	18°C	23°C	30°C	37°C
WT[c]	++++	++++	++++	++++
U28Δ	−	−	−	−
C29Δ	−	−	−	−
C29U, A30C	++++	++++	++++	++++
G32A	++++	++++	++++	++++
U33A	++++	++++	++++	++++
U33Δ	+++	+++	++	−
U40A or G	++++	++++	++++	++++
C41G	++++	++++	++++	+++
U42A, C, G, or Δ	++++	++++	++++	++++
G43A	++++	++++	++++	++++
U42G, G43A	++++	++++	++++	++
U40A, G43A	−	−	−	−
U40G, G43C	−	−	−	−
U40A, G43C	−	−	−	−
U40G, G43A	−	−	−	−
U44A[d]	−	+	++++	++++
U44C	++++	++++	++++	++++
U44G	−	±	±	+
U45A	+++	++++	++++	++++
U45C	++++	++++	++++	++++
U45G	++	++++	++++	++++
C41A, U45C	+	++	++	−
G43A, U44C, U45C	−	−	±	−
C46A or U	++++	++++	++++	++++
U47A	++++	++++	++++	++++
U48G	++++	++++	++++	++++
C41A, U48A	+++	++++	++++	++
U47G, U48A	++++	++++	++++	++++
A61Δ	+	++	++	−

[a] Strains were constructed in HI70 as described in Materials and Methods, and colonies were streaked on YEPD plates at various temperatures.

[b] ++++, wild-type growth; −, no growth. Intermediate growth is indicated by fewer plus signs.

[c] WT, wild type.

[d] The U44A mutation was tested in strain BJ81 at 37°C instead of 37°C (see Materials and Methods).

this region produce no detectable growth defects, despite the absolute lack of phylogenetic variation observed at many positions in the sequence (27, 31). Several single base changes cause growth defects. Deletion of either U-28 or C-29 is lethal and gives rise to a subtle dominant slow-growth phenotype. In contrast, the double mutation C29U A30C is not noticeably defective. In vitro, the U-28 deletion causes a block in splicing at a step after spliceosome assembly but before the first step of splicing (44). Deletion of U-33 results in temperature-sensitive growth (Table 1).

The strongest effect of a single base change on the 3′ side of the U2 branchpoint interaction sequence is the dominant cold-sensitive phenotype caused by U44A. Further analysis of this mutation (see below) indicates that the phenotype is due to a novel RNA misfolding. A second distinct misfolding is caused by changing U-44 to G, explaining the more severe recessive cold-sensitive phenotype of this mutation. Subtle phenotypes are observed with U45A and U45G (slightly cold sensitive) and with C41G (slightly heat sensitive). Multiple mutations give a variety of phenotypes. Double mutations such as U42G G43A and U47G U48A cause relatively robust growth phenotypes, whereas C41A U45C and C41A U48A give rise to temperature-sensitive phenotypes (Table 1). Four different double nucleotide substitutions made at U-40 and G-43 are lethal, as is the triple substitution at positions 43 to 45, although the component single base substitutions we tested have no growth defect (Table 1). We conclude that the nucleotides downstream of the branchpoint interaction sequence have an essential function in splicing. Primer extension analysis of splicing indicates that the mutations block the first step of splicing in vivo (data not shown).

U6 mutations restoring the proposed helix III fail to suppress U2 mutations downstream of the branchpoint interaction region. The highly conserved nucleotides 40 to 46 in U2 snRNA have been proposed to form U2-U6 helix III (59, 60). In transfected mammalian cells, disruption of this pairing blocks splicing and restoration of potential for pairing rescues splicing of a reporter intron dependent on a cotransfected suppressor U2 (59). To test for a role for helix III in *S. cerevisiae*, we attempted to determine whether the essential function lost in the lethal multiple mutations in this region of U2 could be rescued by compensatory U6 mutations. Because the sequence of the U6 strand in the proposed helix III is not conserved between *S. cerevisiae* and humans, ambiguity arises when assigning potential pairing partners in the proposed helix. We diagrammed four more possible pairing interactions between U2 and U6 in the helix III region (Fig. 2) (60). In all five models, U2 U-40 always pairs with U6 A-45 and U2 G-43 always pairs with U6 C-43 (Figs. 2A and B).

To test these models, yeast strains carrying different combinations of U2 and U6 mutations were constructed and their growth was assayed (Table 2). Whereas all of the multiple U2 mutations (U40G G43A, U40A G43C, and G43A U44C U45C) were lethal in combination with the wild-type U6 allele over a range of temperatures, all of the multiple U6 mutations (C43U A45C, C43G A45U, and A41G A42G C43U) were viable at all of the temperatures tested (Table 2). When the U2 multiple mutations were combined with the U6 mutations designed to restore helix III, no suppression was observed. We conclude that the loss of essential function of the multiple U2 mutations cannot be rescued by restoration of the potential to form helix III. We also tested U44A and U45G to determine whether their cold-sensitive growth defects could be suppressed by compensatory U6 mutations (Table 2). Again, no suppression was observed, indicating that the sublethal loss of U2 function observed in the U44A and U45G mutations could not be suppressed by restoration of the potential to form helix III.

To determine whether helix III contributes to splicing efficiency, we measured splicing of the *MAT***a***1* first intron, an inefficiently spliced intron, in cells carrying different combinations of U2 and U6 single mutations (Fig. 2C; Table 3). We also measured the efficiency of splicing of pre-U3 RNA (Table 3). Single mutations in U2 and U6 alone have either subtle or undetectable splicing defects, as shown by the ratio of spliced to unspliced *MAT***a***1* RNA and by the ratio of pre-U3A relative to U5 (Table 3). Combination of U2 and U6 mutations failed to reveal a pattern of effects on splicing efficiency consistent with a contributory role of helix III in splicing. We conclude that disruption of the potential to form helix III results in little or no detectable effect on splicing in *S. cerevisiae*.

Among organisms for which the sequences of U2 and U6 are known, *S. cerevisiae* has one of the weakest helix III analogs (Fig. 2A). A U6 double mutation (A40G A44G) would add two extra G · C base pairs in the *S. cerevisiae* helix III and make it identical to the human version of the helix (Fig. 2A). This U6 double mutation has no effect on cell growth (Table 2), indicating that hyperstabilization of the potential helix III in *S. cerevisiae* does not significantly interfere with splicing.

Lethal mutations in positions 40 to 45 of U2 can be suppressed by *CUS1-54*. We recently characterized a dominant suppressor of cold-sensitive U2 mutations which encodes

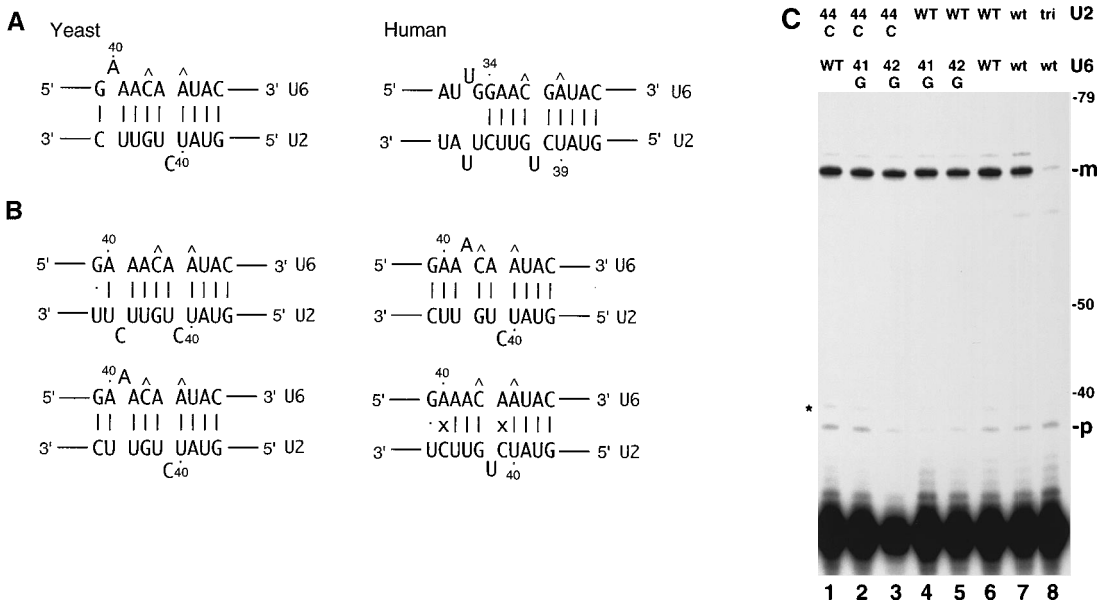

FIG. 2. The U2-U6 base-pairing schemes to form helix III. (A) The proposed helix III base-pairing models in *S. cerevisiae* and humans (60). Numbering is according to *S. cerevisiae* and human sequences, respectively. (B) Other possible base-pairing interactions between U2 and U6 snRNAs in the proposed helix III region in *S. cerevisiae*. ^ indicates the U2-U6 base pairs that are present in all the models. (C) Analysis of in vivo splicing defects by using the *MAT***a***1* first intron. Total RNA was from strain DK10 carrying the indicated combinations of U2 and U6 alleles. Bands that correspond to unspliced (p) and spliced (m) *MAT***a***1* transcripts are indicated. Note that unspliced *MAT***a***1* pre-mmRNA gives rise to a shorter, 38-nucleotide product, whereas the mature *MAT***a***1* transcripts lacking the first intron extension produce a longer 70-nucleotide product (see Materials and Methods). Asterisk indicates a band generated by the use of an upstream 5′ splice site (3a). For comparison, RNA isolated from strain BJ81 expressing only the wild-type (wt) U2 (lane 7) or the lethal U2 G43A U44C U45C triple mutations (tri) (lane 8) are shown. See Table 3 for quantitation.

Cus1-54p, a protein homologous to the mammalian U2 snRNP protein SAP 145 (26, 64). The Cus1-54p suppressor protein is a mutant version of an essential splicing factor, Cus1p, that rescues a discrete subset of mutations in U2 stem IIa (64). To determine whether *CUS1-54* can rescue the lethal mutations in positions 40 to 45, we introduced either a control *CUS1*$^+$ (nonsuppressing wild-type gene) or the *CUS1-54* suppressor allele on a *TRP1* centromere plasmid into a yeast strain carrying the glucose-repressible wild-type U2 gene in the chromosome and a plasmid bearing the mutant U2 gene and tested for growth on glucose (Table 4). All four lethal double mutations at positions 40 and 43, as well as the triple mutation at residues 43 to 45, are rescued by *CUS1-54*, whereas neither of the cold-sensitive U44G and U44A mutations are suppressed. These data argue that the nucleotides immediately downstream of the GUAGUA sequence interact with Cus1p in the execution of U2 snRNP function. In addition, the two mutations at U-44 block U2 function by mechanisms distinct from that of the double and triple mutations (see below), because they are not rescued by *CUS1-54*, despite their less severe phenotype (Table 4).

Synthetic lethal interactions between *prp* gene products and silent U2 mutations. In addition to suppression, functional interactions between gene products can be detected by the phenomenon of synthetic lethality, whereby double-mutant cells have a phenotype far more severe than that associated with either of the component single mutations (30). Such interactions have been used to identify gene products of the splicing apparatus that cooperate with each other (2, 24, 52, 63). Allele-specific synthetic lethality has been used to demonstrate cooperative function of U2 snRNA structures with the DEAD-box protein Prp5p and the yeast SF3a subunits (63). To determine whether sequences adjacent to the branchpoint interaction sequence are also required for the cooperative functions of Prp5p or yeast SF3a with U2, we tested the growth phenotypes of strains containing U2 mutations and temperature-sensitive *prp* alleles at the permissive temperature, 26°C. Strains carrying *prp4*, *prp5*, *prp8*, *prp9*, *prp11*, or *prp21* in combination with U2 mutations adjacent to the branchpoint interaction sequence were tested, and the results are shown in Table 5. Whereas the U2 mutations cause no growth defect in the wild-type *PRP* strain at 26°C or in either of the *prp4* or *prp8* mutant strains, lethality is observed when U2 mutations in residues 3′ to the branchpoint interaction region are combined with *prp5*, *prp9*, *prp11*, or *prp21*. Two mutations upstream of the branchpoint interaction sequence show no synthetic lethality with any of the *prp* mutations tested. These results indicate that the functions of both the DEAD-box protein Prp5p and the yeast homologs of the splicing protein complex SF3a (Prp9p, Prp11p, and Prp21p) are dependent on the sequences immediately downstream of the branchpoint interaction region of U2 snRNA (Table 5), as well as on stem-loops IIa and IIb (52, 63) (Fig. 3).

Intragenic suppressors of the dominant cold-sensitive mutation U44A reveal a novel alternative folding of U2. The U44A mutants display a dominant cold-sensitive phenotype (Fig. 4). Although U44G is also cold sensitive, it is recessive, whereas U44C is aphenotypic, suggesting that it is the substitution of A rather than the loss of U at this position that contributes most to the special phenotype (Table 1; Fig. 4B). In addition, neither mutation is suppressed by compensatory mutations in U6 or the *CUS1-54* suppressor (Tables 2 and 4). Previous analysis of yeast U2 (5, 69, 70) and U6 (13, 23, 54) snRNAs have identified cold-sensitive RNA mutations and suppressors that affect the stability of competing alternative intramolecular RNA structures relative to the structure most

TABLE 2. Testing for helix III by genetic suppression experiments[a]

U2 allele	U6 allele	Growth phenotype at[b]:			
		18°C	23°C	30°C	36°C
WT[c]	WT	++++	++++	++++	++++
U40A, G43C	WT	−	−	−	−
U40G, G43A	WT	−	−	−	−
G43A, U44C, U45C	WT	−	−	±	−
Null	WT	−	−	−	−
WT	C43G, A45U	++++	++++	++++	++++
WT	C43U, A45C	++++	++++	++++	++++
WT	A41G, A42G, C43U	++++	++++	++++	+++
WT	Null	−	−	−	−
U40A, G43C	C43G, A45U	−	−	−	−
U40G, G43A	C43U, A45C	−	−	−	−
G43A, U44C, U45C	A41G, A42G, C43U	−	−	−	−
U44A	WT	+	++	++++	+++
U44A	A41U	+	++	++++	+++
U44A	A42U	+	++	++++	+++
WT	A41U	++++	++++	++++	++++
WT	A42U	++++	++++	++++	++++
WT	A40G, A44G	++++	++++	++++	++++
WT	WT	++++[d]	++++	++++	++++
C45G	WT	+[d]	ND[e]	++++	++++
C45G	A41C	+[d]	ND	++++	++++
WT	A41C	++++[d]	ND	++++	++++

[a] Strain DK10 was transformed with combinations of U2 and U6 alleles on plasmids and shuffled on 5-FOA. Colonies were then streaked on YEPD plates, and growth phenotypes are indicated.
[b] ++++, wild-type growth; −, no growth; intermediate growth is indicated by fewer plus signs.
[c] WT, wild type.
[d] Growth at 14°C.
[e] ND, not determined.

important for function. The effects of such mutations are thought to be attributed to the hyperstabilization of a competing structure, and we searched U2 for the potential of U44A to hyperstabilize an internal U2 structure. Indeed, U2 nucleotides 25 to 30 and nucleotides 42 to 47, except U28 to U44, are complementary to each other (Fig. 4A). The U44A mutation could hyperstabilize this alternative U2 intramolecular structure by changing this U-U pair to a U-A pair. To test this idea, mutations that change U-28 to A or C were introduced into the U44A allele. Consistent with our hypothesis, strains carrying U44A and either the U28A or the U28C second mutation grew like the wild type (Fig. 4B). As shown by primer extension, the levels of unspliced U3A and U3B RNAs in the strains carrying the U2 U28A U44A or U28C U44A double mutations were restored to wild-type values as well (Fig. 4C). To test the hyperstabilization model further, we made a U28C U44G double mutation, which should stabilize the alternative folding even more (Fig. 4A). Indeed, this mutant had a more severe dominant cold-sensitive phenotype than U44A (Fig. 4B). Taken together, the results suggest that hyperstabilization of an interaction between U2 nucleotides 25 to 30 and nucleotides 42 to 47 causes dominant cold-sensitive growth defects.

To obtain biochemical evidence for misfolding, we probed the structure of the U-44 mutants in vivo (5, 70) by using strains conditionally expressing only the mutant RNA. The U44G mutant is cold sensitive, and the mutant U2 RNA is misfolded (Fig. 5), similar to what is observed with other cold-sensitive mutations (70). The perturbation detected in U44G does not involve the pairing of nucleotides 42 to 47 with nucleotides 25 to 30 detected genetically in U44A; rather, it involves

TABLE 3. Splicing efficiency in strains carrying U2 and U6 alleles predicted to disrupt or restore helix III[a]

U2 allele	U6 allele	Spliced/unspliced *MAT***a***1* ratio[b]	Pre-U3A/U5 ratio[c]
WT	WT	1	1
U40A	WT	0.76	0.9
U40A	A45U	0.78	1.1
WT	A45U	0.86	1.8
U40G	WT	0.69	1.2
U40G	C43U, A45C	0.57	1.5
WT	C43U, A45C	0.57	2.3
C41G	WT	0.54	2.2
C41G	A44C	0.52	2.5
WT	A44C	0.65	1.8
G43A	WT	0.85	2.8
G43A	C43U, A45C	0.84	3.0
WT	C43U, A45C	0.69	2.1
U44C	WT	0.38	1.5
U44C	A41G	0.40	1.8
U44C	A42G	0.41	1.5
WT	A41G	0.65	1.1
WT	A42G	0.68	2.4

[a] Radioactivity in the bands corresponding to the unspliced (p) and spliced (m) *MAT***a***1* transcripts in Fig. 2C was measured with a Molecular Dynamics Phosphor Imager.
[b] The ratio of spliced to unspliced *MAT***a***1* transcripts was normalized to the ratio observed in strain DK10 carrying the wild-type (WT) U2 and U6 genes. Strain BJ81 carrying only the wild-type gene or the lethal triple mutation (U2-G43A, U44C, U45C) was included in the assay. When the ratios of wild-type spliced to unspliced *MAT***a***1* RNA are normalized to 1, the ratio in the triple mutation is 0.08, indicating that the assay has a dynamic range of about 12.5-fold.
[c] The ratio of pre-U3A to U5 (gel not shown) was also normalized to the ratio observed in strain DK10 carrying wild-type U2 and U6 genes.

TABLE 4. U2 mutations in positions 40 to 45 are suppressed by *CUS1-54*[a]

U2 allele	*CUS1* allele	Growth phenotype at[b]:			
		18°C	26°C	30°C	34°C
WT[c]	*CUS1*$^+$	++++	++++	++++	++++
	CUS1-54	++++	++++	++++	+++
U40A, G43A	*CUS1*$^+$	+	+	+	−
	CUS1-54	++	++++	++++	+++
U40A, G43C	*CUS1*$^+$	+	+	+	+
	CUS1-54	++++	++++	++++	+++
U40G, G43A	*CUS1*$^+$	−	+	++	+
	CUS1-54	++	++++	++++	+++
U40G, G43C	*CUS1*$^+$	++	+	−	−
	CUS1-54	++++	++	++	+
G43A, U44C, U45C	*CUS1*$^+$	+	+	+	+
	CUS1-54	++	+++	++	+
U44A	*CUS1*$^+$	−	+	+	++
	CUS1-54	−	+	+	+
U44G	*CUS1*$^+$	−	−	−	+
	CUS1-54	−	−	−	+

[a] Strain BJ81 carrying mutant U2 allele and either wild-type *CUS1* or the *CUS1-54* allele was grown on YEPD plates for 3 days at 26, 30, and 34°C and for 5 days at 18°C.

[b] ++++, wild-type growth; −, no growth. Intermediate growth is indicated by fewer plus signs.

[c] WT, wild type.

extension of stem IIa and disruption of stem IIb by pairing of nucleotides 42 to 47 to the 5′ strand of stem IIb (Fig. 5C). The situation is different in the U44A mutant, which displays only subtle differences from the wild-type folding pattern (Fig. 5A and B) despite its more severe growth phenotype. The reactivity of C-9 increases slightly (Fig. 5A). This change would be expected if the 42 to 47 region pairs with the 25 to 30 region: C-9 becomes more reactive because it is no longer efficiently protected by base pairing with G-26 (Fig. 4A). The expected reduced reactivity of C-29 and A-30, as well as C-46, is not observed in the bulk of the U2 population in this mutant (Fig. 5A). This suggests that the misfolded population is a small fraction of the total U2 snRNP sampled by the chemical probe and that a large fraction of the mutant U2 RNA is folded similarly to the wild type. In the suppressors (U28A U44A and U28C U44A), the increased reactivity at C-9 is reversed, restoring the pattern observed in wild-type U2 (Fig. 5A).

The severe dominant cold-sensitive U44A mutant seems paradoxical. It is completely suppressed by mutations that block the alternative folding, yet direct probing of the structure in vivo (even at the restrictive temperature) reveals that only a small fraction of the mutant RNA is misfolded (Fig. 5A; data not shown). This suggests that if the defect is caused by the novel misfolding, the misfolded form is likely to be occurring in a smaller, dynamic subset of U2 snRNPs in the cell and hence is not well represented in the total mutant U2 population sampled in vivo. We conclude that the different cold-sensitive phenotypes of U44A and U44G are caused by different RNA-misfolding events. The folding defect in U44G is obvious; however, the misfolding of U44A may occur only at a critical step during the function of U2 in the assembled spliceosome.

A potential helix formed from conserved U2 residues is dispensable for splicing in *S. cerevisiae*. The complementarity between positions 25 to 30 and 42 to 47 that is hyperstabilized in the U44A mutant includes dynamic, highly conserved residues that form part of the U2-U6 helix I (41). It is possible that the defects shown by the lethal double mutations in the region downstream of the branchpoint interaction sequence (Table 1) are due to loss of this interaction. To test whether the complementarity between positions 25 to 30 and positions 42 to 47 (Fig. 4A) contributes to U2 function, we combined two compensatory dinucleotide substitutions in U2 (C29U A30C and U42G G43A), which disrupt 2 bp in the alternative folding (Table 1). The U42G G43A double mutant grows slowly at 37°C, and this defect is not suppressed in the compensatory C29U A30C U42G G43A quadruple mutant (data not shown). We conclude that a potential interaction between residues 25 to 30 and 42 to 47 of U2 snRNA generates a dominant growth defect when it is hyperstabilized but that is not essential for U2 function in *S. cerevisiae*.

DISCUSSION

We have analyzed the highly conserved regions of U2 adjacent to the branchpoint recognition sequence. Most of the residues adjacent to the branchpoint recognition sequence are invariant in the known phylogenetic collection of U2 gene sequences. Despite this, many of the single mutations we tested did not significantly affect yeast cell growth (Table 1). We measured the effects of the single U2 mutations on efficiently and inefficiently spliced introns and found no significant inhibition of splicing (Table 3). In contrast, equivalent single nucleotide changes in mammalian U2 gene eliminate small t splicing (68). Combined with an in vitro study (44), our in vivo results indicate that the yeast splicing apparatus is sufficiently robust that only the unusual single mutation in this region of U2 is incompatible with function.

We identified six single mutations that cause severe growth defects. Three (U28Δ, C29Δ, and U33Δ) are in the sequences upstream of the branchpoint recognition sequence (Table 1) (44), one (A61Δ) is in loop IIa (Fig. 1A) (5), and two others are at position 44 and give rise to cold-sensitive growth and splicing. Double and triple U2 mutations cause more severe effects on growth. Double substitutions involving positions 29 and 30, 41 and 48, 42 and 43, or 47 and 48 are not greatly inhibited; however, all double-mutant combinations we tested at 40 and

TABLE 5. Results of synthetic lethal growth assay[a]

U2 allele	*prp* mutation[b]						
	PRP	*prp4*	*prp8*	*prp5*[c]	*prp9*[c]	*prp11*	*prp21*
WT	++++	++++	++++	++++	++++	++++	++++
G32A	++++	++++	++++	++++	++++	++++	++++
U33A	++++	++++	++++	++++	++++	++++	++++
U40A	++++	++++	++++	−	−	−	−
U40G	++++	++++	++++	−	−	−	++
C41G	++++	++++	+++	−	−	−	−
U42A	++++	++++	++++	+++	++++	+++	++++
U42C	++++	++++	++++	++++	++++	++++	++++
U42G	++++	++++	++++	++	−	+++	++++
U45C	++++	++++	++++	+	−	+	++++
C46A	++++	++++	++++	−	−	−	++++
C46U	++++	++++	++++	+	−	−	++
U47A	++++	++++	++++	−	−	−	−

[a] Strains were streaked on YEPD plates at 26°C for 3 days. The growth of strains carrying mutant U2 alleles was scored by comparing their growth with the appriate (corresponding) strain transformed with the wild-type (WT) U2 gene.

[b] ++++, growth of the indicated *prp* strain carrying the wild-type U2 plasmid; −, no growth. Intermediate growth is indicated by fewer plus signs.

[c] The *prp5* strain carries the *prp5-3* allele, and the *prp9* strain carries the *prp9-2* allele.

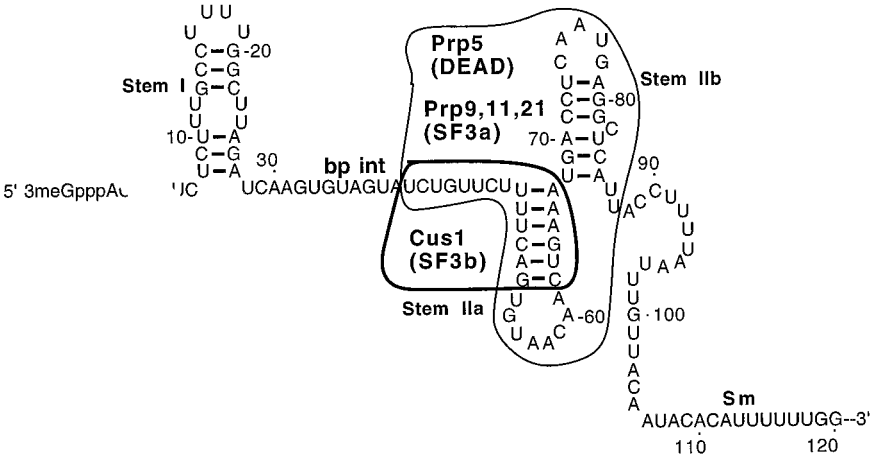

FIG. 3. Summary of genetic interactions between U2 snRNA and proteins. Shown is the 5′ end of U2 RNA (5). U2 nucleotides and proteins are boxed together to indicate the regions on U2 snRNA where U2 mutations that interact with the indicated proteins are located. Thick line, Cus1-54 suppression. Thin line, *prp5*, *prp9*, *prp11*, and *prp21* synthetic lethality (29, 52, 63, 64).

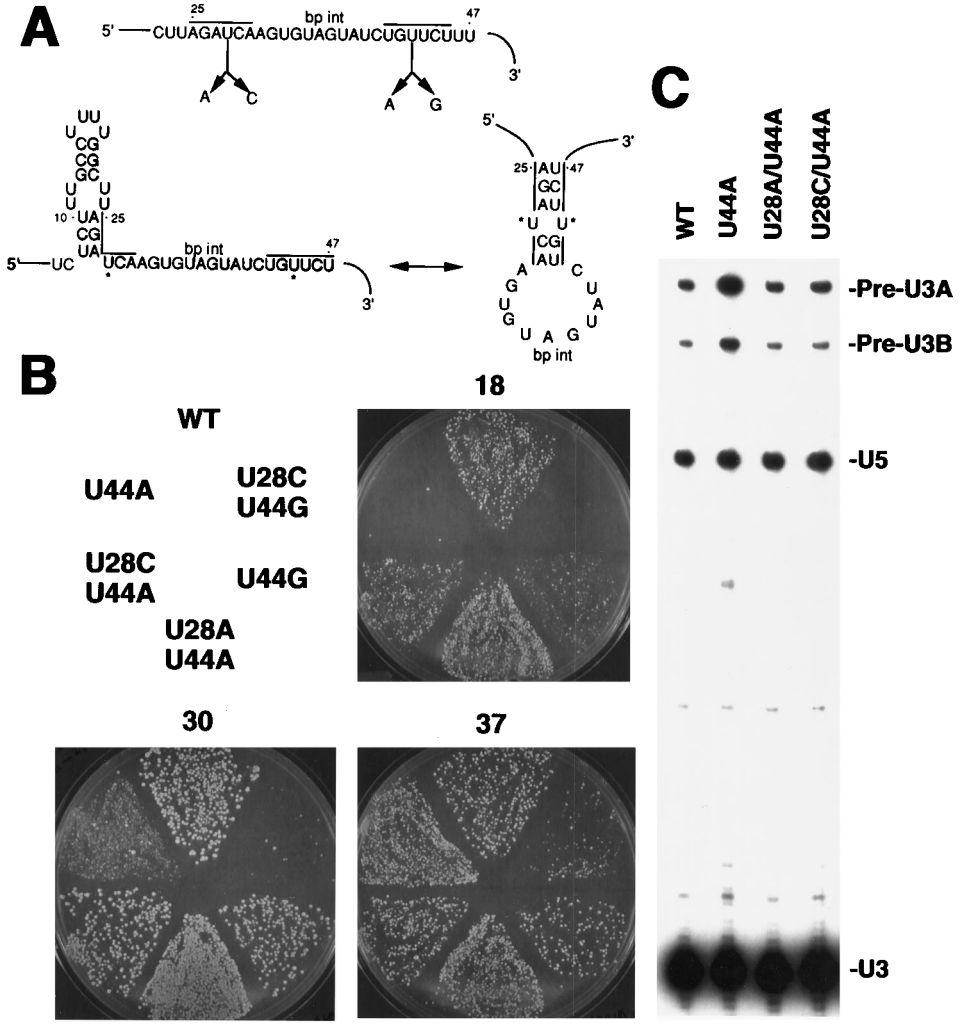

FIG. 4. Intragenic suppressors of a dominant cold-sensitive mutation (A) Diagram of a novel alternative folding in U2 snRNA. Mutations that affect complementarity between residues 25 to 30 and 42 to 47 are indicated. (B) Growth of cells carrying both a wild-type (WT) U2 gene and dominant mutant U2 gene on a plasmid. Strain HI70 was transformed with the indicated mutant U2 on a *LEU2* plasmid, split into aliquots, and plated directly on synthetic complete plates minus leucine for 2 to 5 days at the temperatures indicated. Mutant plasmids that give rise to transformants at 37°C but not at lower temperatures carry dominant cold-sensitive mutations. (C) Analysis of splicing of pre-U3 RNA in vivo. Total RNA was isolated from strain BJ81 expressing only the indicated U2 alleles. Bands that correspond to pre-U3A, pre-U3B, mature U3, and U5 are indicated. The U5 RNA level was measured as an internal control.

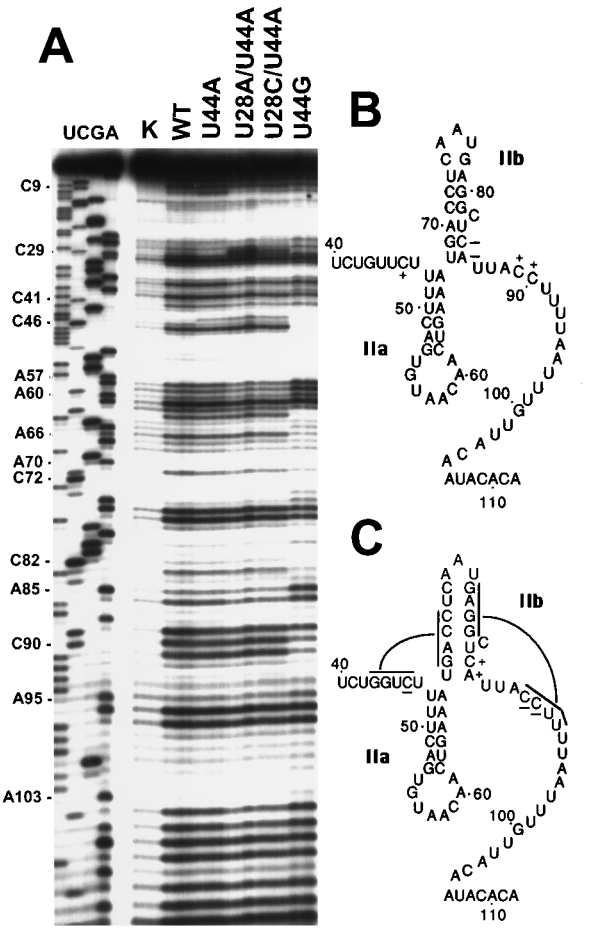

FIG. 5. (A) In vivo chemical probing of mutant U2 snRNA with dimethyl sulfoxide (DMS). Sites of modification were mapped by primer extension relative to an RNA sequence ladder. An end-labeled primer complementary to U2 downstream of the Sm site was annealed and extended on total unmodified wild-type (WT) RNA in the presence of ddATP (lane U), ddGTP (lane C), ddCTP (lane G), ddTTP (lane A), on RNA from cells treated with DMS modification stop buffer prior to addition of DMS as a control (lane K), or on RNA from strains expressing only the indicated mutant U2 RNA modified with DMS. Duplicate samples were loaded in adjoining lanes. At left is the position of migration of the dideoxynucleotide-generated stop at the indicated position in U2 RNA. The reverse transcriptase stop produced by the RNA template modification is 1 nucleotide shorter than that produced by incorporation of a dideoxynucleotide. (B) Secondary-structure model of the wild-type U2 snRNA (5). (C) Secondary structure of the U2 U44G mutation. Lines indicate the nucleotides involved in the alternative base pairing. Changes in DMS reactivity are indicated by plus and minus signs.

43 are lethal, as is a triple substitution at 43 to 45 (Table 1). Taken together, the data show that the invariant sequences adjacent to the branchpoint recognition region of U2 are mutable but contain information essential for splicing.

It has been proposed that the 40 to 46 region of U2 snRNA forms a third base-pairing interaction (helix III) with U6 nucleotides just upstream of the ACAGAG box (50, 60). This model has recently been supported by a test in the mammalian system (59). Single base changes in the nucleotides that make up the proposed helix III region of a suppressor mammalian U2 snRNA inhibit splicing of a reporter simian virus 40 small t intron in transfected mammalian cells (68), and this inhibition can be suppressed by compensatory U6 mutations (59). Curiously, although mutations in this region of yeast U2 snRNA reduce splicing in vitro (44), nucleotide changes in U6 residues thought to participate in helix III cause neither growth defects in yeast cells (38) nor significant splicing defects in yeast extracts (21), *Xenopus* oocytes (61), or mammalian cell extracts (65) reconstituted with mutant U6 snRNA. In addition, several results suggest that both the U6 and U2 strands of the proposed helix III structure in *S. cerevisiae* are involved primarily in interactions with proteins. First, the U6 contribution to the proposed helix III is probably a critical binding site for the product of the *PRP24* gene (25, 34). Second, we show (Tables 4 and 5) that the U2 strand of the proposed helix III participates in functional interactions with three distinct splicing factors, yeast SF3a, Prp5p, and Cus1p. Our tests of helix III in *S. cerevisiae* did not reveal a contribution to splicing (Tables 2 and 3). Thus, although a contributory role of the U2-U6 helix III in mammalian splicing can be demonstrated by using a modified simian virus 40 small t intron and mutant U2 and U6 snRNAs (59), the generality of helix III function in splicing is not yet established.

The differences observed between the yeast and mammalian systems may reflect differing sensitivities of the assays used, as discussed for related situations (19, 59, 67, 68). Alternatively, helix III may exist in *S. cerevisiae* but may function adequately with very few base pairs (59). If this is the case, the loss of growth we observe is due to some other function of those residues, because compensatory U6 mutations fail to improve it (Table 2). As discussed previously (59), helix III may have a contributory role in splicing which may be revealed only upon analysis of inefficiently spliced introns (see also reference 33); however, careful measurement of splicing of an inefficiently spliced yeast intron failed to reveal such a contribution (Table 3). A possibility not previously considered is that the suppression data convincingly observed in the human system (59) may represent rescue of defective U2 and U6 RNA by de novo generation of a stabilizing structure that improves the very poor splicing of the reporter intron in the suppressor U2-U6 system. Such a structure would not have to carry out a sophisticated function but could simply act to occupy RNA strands that otherwise fold to occlude important residues.

Instead of interactions with U6, residues 40 to 48 of yeast U2 interact with known splicing factor mutations (Fig. 3). A mutation in *CUS1* (64), the gene encoding the yeast homolog of the human U2 snRNP protein SAP 145 (26), efficiently suppresses the U2 double mutations at positions 40 and 43 and positions 41 and 45, as well as the triple mutation at positions 43 to 45 (Table 4). SAP 145 is very probably a component of SF3b (12), indicating that nucleotides 40 to 45 function to activate the U2 snRNP for spliceosome assembly. Functions of Prp5p, as well as the yeast homolog of SF3a, composed of the products of the *PRP9*, *PRP11*, and *PRP21* genes, are dependent on the identity of residues 40 to 45, as shown by synthetic lethal interactions between silent U2 mutations at these positions and the temperature-sensitive alleles of *prp5*, *prp9*, *prp11*, and *prp21* at permissive temperature (Table 5). The sum of available data from *S. cerevisiae* suggests that the invariant nucleotides that connect the branchpoint interaction region with stem-loop IIa have a critical role in interactions with known splicing factors. Because stem-loop IIa interacts similarly with mutations in *CUS1*, *PRP5*, *PRP9*, *PRP11*, and *PRP21*, we suggest that the interaction between SF3 and U2 is dependent on both stem-loop IIa and the conserved sequence just 5′ to it at positions 40 to 48. These interactions are summarized in Fig. 3.

Two single mutations in the 40 to 45 region of U2 cause significant growth defects that are not suppressed by *CUS1-54* or compensatory U6 mutations. One of these mutations (U44A) is suppressed by intragenic second mutations pre-

dicted to remove the potential to form stable alternative structures. Most of the mutant U2 population displays no gross structural alteration in the structure-probing experiment (Fig. 5A), but the U44A defects are completely suppressed by the disruption of complementarity between residues 28 and 44 of U2 (Fig. 4). This indicates that the hyperstabilization of the extended pairing between U2 residues 25 to 30 and 42 to 47 blocks splicing. The dominant nature of the block (Fig. 4B) and the small proportion of misfolded RNA detectable in the whole-cell structure-probing experiment (Fig. 5A) suggest that it occurs in a blocked splicing complex that sequesters a limiting splicing component, as postulated for other dominant U2 mutations (46, 51). Because the U2 RNA structure stabilized by U44A is incompatible with the formation of the essential U2-U6 helix I (41), we propose that U44A executes its dominant negative effect by blocking U2-U6 helix I formation during the activation of the spliceosome.

The sequences carrying the branchpoint interaction region of U2 appear to have multiple functions, as might be expected of residues placed near the attacking group in the first chemical step of splicing. Upstream of the branchpoint interaction sequence, only single base deletions, but not substitutions, lead to lethal growth defects in vivo (Table 1) and a strong block to splicing in vitro at a later step of spliceosome assembly (44). Downstream of the branchpoint interaction region, U2 nucleotides are required for optimal interaction with splicing factors SF3a and SF3b (Tables 4 and 5; Fig. 3) and may interact with U6 to rescue splicing of certain introns (59). The importance of conservation of these residues is not limited to their positive roles, however, as certain nucleotide substitutions cause RNA misfolding to interfere with function (Fig. 4 and 5). Further analysis of the roles of these invariant residues is necessary to determine the details of their contribution to the structure and function of the spliceosome.

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REFERENCES

1. **Abovich, N., P. Legrain, and M. Rosbash.** 1990. The yeast PRP6 gene encodes a U4/U6 small nuclear ribonucleoprotein particle (snRNP) protein, and the PRP9 gene encodes a protein required for U2 snRNP binding. Mol. Cell. Biol. **10:**6417–6425.
2. **Abovich, N., X. C. Liao, and M. Rosbash.** 1994. The yeast MUD2 protein: an interaction with PRP11 defines a bridge between commitment complexes and U2 snRNP addition. Genes Dev. **8:**843–854.
3. **Arenas, J. E., and J. N. Abelson.** 1993. The *Saccharomyces cerevisiae PRP21* gene product is an integral component of the prespliceosome. Proc. Natl. Acad. Sci. USA **90:**6771–6775.

3a. **Ares, M., Jr.** Unpublished data.

4. **Ares, M., Jr., and A. H. Igel.** 1989. Phylogenetic comparison of U2 small nuclear RNA sequences suggests a pseudoknotted structure. UCLA Symp. Mol. Cell. Biol. **94:**13–23.
5. **Ares, M., Jr., and A. H. Igel.** 1990. Lethal and temperature-sensitive mutations and their suppressors identify an essential structure element in U2 small nuclear RNA. Genes Dev. **4:**2132–2145.
6. **Ares, M., Jr., and B. Weiser.** 1995. Rearrangement of snRNA structure during assembly and function of the spliceosome. Prog. Nucleic Acid Res. Mol. Biol. **50:**131–159.
7. **Behrens, S.-E., F. Galisson, P. Legrain, and R. Luhrmann.** 1993. Evidence that the 60-kDa protein of 17S U2 small nuclear ribonucleoprotein is immunologically and functionally related to the yeast PRP9 splicing factor and is required for the efficient formation of prespliceosomes. Proc. Natl. Acad. Sci. USA **90:**8229–8233.
8. **Behrens, S.-E., K. Tyc, B. Kastner, J. Reichelt, and R. Luhrmann.** 1993. Small nuclear ribonucleoprotein (RNP) U2 contains numerous additional proteins and has a bipartite RNP structure under splicing conditions. Mol. Cell. Biol. **13:**307–319.
9. **Bennett, M., and R. Reed.** 1993. Correspondence between a mammalian spliceosome component and an essential yeast splicing factor. Science **262:** 105–108.
10. **Boeke, J. D., J. Trueheart, G. Natsoulis, and G. R. Fink.** 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. **154:** 164–175.
11. **Brosi, R., K. Groning, S.-E. Behrens, R. Luhrmann, and A. Kramer.** 1993. Interaction of mammalian splicing factor SF3a with U2 snRNP and relation of its 60-kD subunit to yeast PRP9. Science **262:**102–105.
12. **Brosi, R., H.-P. Hauri, and A. Kramer.** 1993. Separation of splicing factor SF3 into two components and purification of SF3a activity. J. Biol. Chem. **268:**17640–17646.
13. **Brow, D. A., and R. M. Vidaver.** 1995. An element in human U6 RNA destabilizes the U4/U6 spliceosomal RNA complex. RNA **1:**122–131.
14. **Cech, T. R.** 1985. RNA splicing: three themes with variations. Cell **43:** 713–716.
15. **Chang, T.-H., M. W. Clark, A. J. Lustig, M. E. Cusick, and J. Abelson.** 1988. RNA 11 protein is associated with the yeast spliceosome and is localized in the periphery of the cell nucleus. Mol. Cell. Biol. **8:**2379–2393.
16. **Chapon, C., and P. Legrain.** 1992. A novel gene, spp91-1, suppresses the splicing defect and the pre-mRNA nuclear export in the prp9-1 mutant. EMBO J. **11:**3279–3288.
17. **Chattoo, B., F. Sherman, T. Fejellstedt, D. Menhnert, and M. Ogur.** 1979. Selection of *lys2* mutants of the yeast *Saccharomyces cerevisiae* by the utilization of alpha-aminoadipate. Genetics **93:**51–65.
18. **Datta, B., and A. M. Weiner.** 1991. Genetic evidence for base pairing between U2 and U6 snRNA in mammalian mRNA splicing. Nature (London) **352:**821–824.
19. **Datta, B., and A. M. Weiner.** 1993. The phylogenetically invariant ACAG AGA and AGC sequence of U6 small nuclear RNA are more tolerant of mutation in human cells than in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:** 5377–5382.
20. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:** 6127–6145.
21. **Fabrizio, P., and J. Abelson.** 1990. Two domains of yeast U6 small nuclear RNA required for both steps of nuclear precursor messenger RNA splicing. Science **250:**404–409.
22. **Fabrizio, P., D. S. McPheeters, and J. Abelson.** 1989. *In vitro* assembly of yeast U6 snRNP: a functional assay. Genes Dev. **3:**2137–2150.
23. **Fortner, D. M., R. G. Troy, and D. A. Brow.** 1994. A stem/loop in U6 RNA defines a conformational switch required for pre-mRNA splicing. Genes Dev. **8:**221–233.
24. **Frank, D., B. Patterson, and C. Guthrie.** 1992. Synthetic lethal mutations suggest interactions between U5 small nuclear RNA and four proteins required for the second step of splicing. Mol. Cell. Biol. **12:**5197–5205.
25. **Ghetti, A., M. Company, and J. Abelson.** 1995. Specificity of Prp24 binding to RNA: a role for Prp24 in the dynamic interaction of U4 and U6 snRNAs. RNA **1:**132–145.
26. **Gozani, O., J. G. Patton, and R. Reed.** 1994. A novel set of spliceosome-associated proteins (SAPs) and the essential splicing factor PSF bind stably to pre-mRNA prior to catalytic step II of the splicing reaction. EMBO J. **13:** 3356–3367.
27. **Guthrie, C., and B. Patterson.** 1988. Spliceosomal snRNAs. Annu. Rev. Genet. **22:**387–419.
28. **Hausner, T. P., L. M. Giglio, and A. M. Weiner.** 1990. Evidence for base-pairing between mammalian U2 and U6 small nuclear ribonucleoprotein particles. Genes Dev. **4:**2146–2156.
29. **Hodges, P. E., and J. D. Beggs.** 1994. U2 fulfills a commitment. Curr. Biol. **4:**264–267.
30. **Huffaker, T. C., M. A. Hoyt, and D. Botstein.** 1987. Genetic analysis of the yeast cytoskeleton. Annu. Rev. Genet. **21:**259–284.
31. **Igel, A. H., and M. Ares, Jr.** 1988. Internal sequences that distinguish yeast from metazoan U2 are unnecessary for pre-mRNA splicing. Nature (London) **334:**450–453.
32. **Ito, H., Y. Fakuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
33. **Jacquier, A., J. R. Rodriguez, and M. Rosbash.** 1985. A quantitative analysis of the effects of 5′ junction and TACTAAC box mutants and mutant combinations on yeast mRNA splicing. Cell **43:**423–430.

34. **Jandrositz, A., and C. Guthrie.** 1995. Evidence for a Prp24 binding site in U6 snRNA and in a putative intermediate in the annealing of U6 and U4 snRNAs. EMBO J. **14:**820–832.
35. **Kandels-Lewis, S., and B. Seraphin.** 1993. Role of U6 snRNA in 5′ splice site selection. Science **262:**2035–2039.
36. **Kunkel, T.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
37. **Legrain, P., C. Chapon, and F. Galisson.** 1993. Interactions between PRP9 and SPP91 splicing factors identify a protein complex required in prespliceosome assembly. Genes Dev. **7:**1390–1399.
38. **Lesser, C. F., and C. Guthrie.** 1993. Mutations in U6 snRNA that alter splice site specificity: implications for the active site. Science **262:**1982–1988.
39. **Lin, R.-J., A. J. Lustig, and J. Abelson.** 1987. Splicing of yeast nuclear pre-mRNA *in vitro* requires a functional 40S spliceosome and several extrinsic factors. Genes Dev. **1:**7–18.
40. **Madhani, H. D., R. Bordonne, and C. Guthrie.** 1990. Multiple roles for U6 snRNA in the splicing pathway. Genes Dev. **4:**2264–2277.
41. **Madhani, H. D., and C. Guthrie.** 1992. A novel base-pairing interaction between U2 and U6 snRNAs suggests a mechanism for the catalytic activation of the spliceosome. Cell **17:**803–817.
42. **Madhani, H. D., and C. Guthrie.** 1994. Randomization-selection analysis of snRNAs in vivo: evidence for a tertiary interaction in the spliceosome. Genes Dev. **8:**1071–1086.
43. **Madhani, H. D., and C. Guthrie.** 1994. Dynamic RNA-RNA interactions in the spliceosome. Annu. Rev. Genet. **28:**1–26.
44. **McPheeters, D. S., and J. Abelson.** 1992. Mutational analysis of the yeast U2 snRNA suggests a structural similarity to the catalytic core of group I introns. Cell **71:**819–831.
45. **Miller, A.** 1984. The yeast MATa1 gene contains two introns. EMBO J. **3:** 1061–1065.
46. **Miraglia, L., S. Steiwert, A. H. Igel, and M. Ares, Jr.** 1991. Limited functional equivalence of phylogenetic variation in small nuclear RNA: yeast U2 RNA with altered branchpoint complementarity inhibits splicing and produces a dominant lethal phenotype. Proc. Natl. Acad. Sci. USA **88:**7061–7065.
47. **Moore, M. J., C. C. Query, and P. A. Sharp.** 1993. Splicing of precursors to mRNA by the spliceosome, p. 303–357. *In* R. Gesteland and J. Atkins (ed.), The RNA world. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **Newman, A.** 1994. Small nuclear RNAs and pre-mRNA splicing. Curr. Opin. Cell Biol. **6:**360–367.
49. **Nilsen, T. W.** 1994. RNA-RNA interactions in the spliceosome: unraveling the ties that bind. Cell **78:**1–4.
50. **Orum, H., H. Nielsen, and J. Engberg.** 1991. Spliceosomal small nuclear RNAs of *Tetrahymena thermophila* and some possible snRNA-snRNA base-pairing interactions. J. Mol. Biol. **222:**219–232.
51. **Parker, R. A., P. G. Siliciano, and C. Guthrie.** 1987. Recognition of the TACTAAC box during mRNA splicing in yeast involves base pairing to the U2-like snRNA. Cell **49:**229–239.
52. **Ruby, S. W., T.-H. Chang, and J. Abelson.** 1993. Four yeast spliceosomal proteins (PRP5, PRP9, PRP11, and PRP21) interact to promote U2 snRNP binding to pre-mRNA. Genes Dev. **7:**1909–1925.
53. **Sawa, H., and J. Abelson.** 1992. Evidence for a base-pairing interaction between U6 small nuclear RNA and 5′ splice site during the splicing reaction in yeast. Proc. Natl. Acad. Sci. USA **89:**11269–11273.
54. **Shannon, K. W., and C. Guthrie.** 1991. Suppressors of a U4 snRNA mutation define a novel U6 snRNP protein with RNA-binding motif. Genes Dev. **5:**773–785.
55. **Sharp, P. A.** 1985. On the origin of RNA splicing and introns. Cell **42:** 397–400.
56. **Sherman, F., G. Fink, and J. Hicks.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
57. **Shuster, E. O., and C. Guthrie.** 1988. Two conserved domains of yeast U2 snRNA are separated by 945 nonessential nucleotides. Cell **55:**41–48.
58. **Staknis, D., and R. Reed.** 1994. Direct interactions between pre-mRNA and six U2 small nuclear ribonucleoproteins during spliceosome assembly. Mol. Cell. Biol. **14:**2994–3005.
59. **Sun, J., and J. L. Manley.** 1994. A novel U2-U6 snRNA structure is necessary for mammalian mRNA splicing. Genes Dev. **9:**843–854.
60. **Takahashi, Y., S. Urushiyama, T. Tani, and Y. Ohshima.** 1993. An mRNA-type intron is present in the *Rhodotorula hasegawae* U2 small nuclear RNA gene. Mol. Cell. Biol. **13:**5613–5619.
61. **Vankan, P., C. McGuigan, and I. W. Mattaj.** 1990. Domains of U4 and U6 snRNAs required for snRNP assembly and splicing complementation in *Xenopus* oocytes. EMBO J. **9:**3397–3404.
62. **Wassarman, D. A., and J. A. Steitz.** 1992. Interactions of small nuclear RNAs with precursor messenger RNA during *in vitro* splicing. Science **257:**1918–1925.
63. **Wells, S. E., and Ares, M., Jr.** 1994. Interactions between highly conserved U2 small nuclear RNA structures and Prp5p, Prp9p, Prp11p, and Prp21p proteins are required to ensure integrity of the U2 small nuclear ribonucleoprotein in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **14:**6337–6349.
64. **Wells, S. E., M. Neville, M. Haynes, J. Wang, H. Igel, and M. Ares, Jr.** *CUS1*, a suppressor of cold sensitive U2 snRNA mutations, is a novel yeast splicing factor homologous to human SAP145. Genes Dev., in press.
65. **Wolff, T., R. Menssen, J. Hammel, and A. Bindereif.** 1994. Splicing function of mammalian U6 small nuclear RNA: conserved positions in central domain and helix I are essential during the first and second step of pre-mRNA splicing. Proc. Natl. Acad. Sci. USA **91:**903–907.
66. **Wu, J., and J. L. Manley.** 1989. Mammalian pre-mRNA branch site selection by U2 snRNP involves base pairing. Genes Dev. **3:**1553–1561.
67. **Wu, J., and J. L. Manley.** 1991. Base pairing between U2 and U6 snRNs is necessary for splicing of a mammalian pre-mRNA. Nature (London) **352:** 818–821.
68. **Wu, J., and J. L. Manley.** 1992. Multiple functional domains of human U2 small nuclear RNA: strengthening conserved stem I can block splicing. Mol. Cell. Biol. **12:**5464–5473.
69. **Zavanelli, M., J. Britton, H. Igel, and M. Ares, Jr.** 1994. Mutations in an essential U2 small nuclear RNA structure cause cold sensitive U2 small nuclear ribonucleoprotein function by favoring competing U2 RNA structures. Mol. Cell. Biol. **14:**1689–1697.
70. **Zavanelli, M. I., and M. Ares, Jr.** 1991. Efficient association of U2 snRNPs with pre-mRNA requires an essential U2 RNA structural element. Genes Dev. **5:**2521–2533.
71. **Zhuang, Y., and A. M. Weiner.** 1989. A compensatory base change in human U2 snRNA can suppress a branch site mutation. Genes Dev. **3:**1545–1552.