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Genetic Interactions Between the Yeast RNA Helicase Homolog Prp16 and Spliceosomal snRNAs Identify Candidate Ligands for the Prp16 RNA-Dependent ATPase

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ABSTRACT

Pre-mRNA splicing occurs in a large and dynamic ribonucleoprotein complex, the spliceosome. Several protein factors involved in splicing are homologous to a family of RNA-dependent ATPases, the so-called DEAD/DEAH proteins. A subset of these factors exhibit RNA helicase activity *in vitro*. The DEAD/DEAH proteins involved in splicing are thought to mediate RNA conformational rearrangements during spliceosome assembly. However, the RNA ligands for these factors are currently unknown. Here, we present genetic evidence in *Saccharomyces cerevisiae* for a functional interaction between the DEAH protein Prp16, and the U6 and U2 spliceosomal snRNAs. Using a library of mutagenized U6 snRNA genes, we have identified 14 strong suppressors of the cold-sensitive (cs) allele, *prp16-302*. Remarkably, each suppressor contains a single nucleotide deletion of 1 of the 6 residues that lie immediately upstream of a sequence in U6 that interacts with the 5′ splice site. Analysis of site-directed mutations revealed that nucleotide substitutions in the adjacent U2-U6 helix I structure also suppress *prp16-302*, albeit more weakly. The U6 suppressors tested also partially reverse the phenotype of two other cs alleles, *prp16-1* and *prp16-301*, but not the four temperature-sensitive alleles tested. Finally, overexpression of each cs allele exacerbates its recessive growth phenotype and confers a dominant negative cs phenotype. We propose that the snRNA suppressors function by destabilizing an interaction between the U2-U6 complex and a hypothetical factor (X), which is trapped by cs mutants of *PRP16*. The phenotypes of overexpressed *prp16* alleles are consistent with the model that this trapped interaction inhibits the dissociation of Prp16 from the spliceosome. We discuss the intriguing possibility that factor X is Prp16 itself.

INTRONS are removed from messenger RNA precursors within a large and dynamic ribonucleoprotein complex, the spliceosome, in which the two chemical steps of the splicing reaction (5′ splice site cleavage/lariat formation and 3′ splice site cleavage/exon ligation) take place (reviewed in MOORE *et al.* 1993; RYMOND and ROSBASH 1992; GREEN 1991). The spliceosome is formed by the ordered assembly of four small nuclear ribonucleoprotein particles (U1, U2, U5, U4-U6 snRNPs), together with numerous extrinsic protein factors, onto the intron-containing substrate. Similarities in chemistry between nuclear pre-mRNA splicing and group II autocatalytic splicing have led to the proposal that the former is fundamentally an RNA-catalyzed process performed by the snRNA components of the spliceosome (SHARP 1985; CECH 1986). Indeed, numerous RNA-RNA interactions involving the pre-mRNA substrate and the spliceosomal snRNAs have been identified (reviewed in MOORE *et al.* 1993; WEINER 1993; RYMOND and ROSBASH 1992; GREEN 1991).

Our studies have focused on the role of the highly conserved U6 snRNA (MADHANI *et al.* 1990; MADHANI and GUTHRIE 1992). We have previously employed a mutational approach in *Saccharomyces cerevisiae* to demonstrate a base-pairing interaction between U2 and U6 snRNAs that is mutually exclusive with the extensive U4-U6 base-pairing interaction (MADHANI *et al.* 1990; MADHANI and GUTHRIE 1992). In this novel pairing, termed U2-U6 helix I, a conserved sequence in U6 snRNA interacts with sequences in U2 that are immediately upstream of the branchpoint recognition region of U2. As a result, functionally important residues in U6 can be juxtaposed with the intron branchpoint. Residues that form this structure have been shown to be required for cell viability (MADHANI *et al.* 1990; MADHANI and GUTHRIE 1992) and 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 (Figure 1) in which U2-U6 helix I might participate directly in chemical steps of splicing (MADHANI and GUTHRIE 1992). Because it is mutually exclusive with the U4-U6 interaction, the existence of U2-U6 helix I offers a mechanistic rationale for the destabilization of the U4-U6 interaction that occurs prior to the chemical steps of splicing (reviewed in MOORE *et al.* 1993; RYMOND and ROSBASH 1992; GREEN 1991). Other biochemical and genetic studies, which indicate a direct interaction between the ACA sequence in U6 snRNA (nucleotides (nt) 47–49) and a portion of the

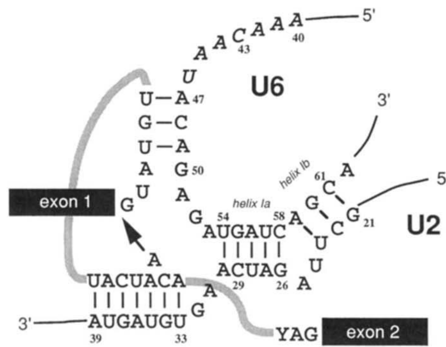

FIGURE 1.—RNA-RNA interactions between U2 snRNA, U6 snRNA and the pre-mRNA. Shown are the U2-branchpoint interaction (PARKER *et al.* 1987), U2-U6 helix I (MADHANI and GUTHRIE 1992) and the U6-5′ splice site interaction (WASSARMAN and STEITZ 1992; SAWA and SHIMURA 1992; SAWA and ABELSON 1992; LESSER and GUTHRIE 1993; KANDELS-LEWIS and SÉRAPHIN 1993). The sequences for the snRNAs are from *S. cerevisiae*, and the intron sequences reflect the *S. cerevisiae* consensus. "Y" indicates a pyrimidine. Residues 40–46 in U6 (underlined in Figure 3) are italicized.

5′ splice site consensus sequence (Figure 1), are also consistent with the view that U2-U6 helix I is an active site component of the spliceosome (SAWA and SHIMURA 1992; WASSARMAN and STEITZ 1992; SAWA and ABELSON 1992; LESSER and GUTHRIE 1993; KANDELS-LEWIS and SÉRAPHIN 1993). These studies also demonstrate another dynamic conformational rearrangement involving the swapping of mutually exclusive base-pairing partners, in this case the exchange of the 5′ splice site between U1 and U6 snRNAs.

The prominent role of dynamic RNA-RNA interactions in splicing suggests that one important class of functions for the estimated >50 proteins required for splicing is likely to be the catalysis and regulation of these RNA structural rearrangements. Of particular interest are a family of RNA-dependent ATPases which mediate many of the ATP-dependent steps of splicing (reviewed in SCHMID and LINDER 1992). These factors (Prp2, Prp5, Prp16, Prp22, Prp28), which were identified through the use of genetics in *S. cerevisiae*, have been classified into two subfamilies on the basis of conserved sequence motifs (DEAD and DEAH box families). Members of these families are involved in diverse biological processes; several have been shown to exhibit ATP-dependent RNA helicase activity *in vitro* (reviewed in SCHMID and LINDER 1992; see also LEE and HURWITZ 1993). However, of the DEAD/DEAH splicing factors tested (Prp2, Prp16 and Prp28), none exhibit RNA helicase activity (SCHWER and GUTHRIE 1991; B. SCHWER and C. GUTHRIE, unpublished; KIM *et al.* 1991; E. STRAUSS and C. GUTHRIE, unpublished). A reasonable explanation is that the DEAD/DEAH splicing factors are capable of unwinding activity only when bound to specific RNA ligands in the spliceosome. A major step toward testing this hypothesis would be the idenfication of such ligands.

Prp16, the prototypical member of the DEAH box family (BURGESS *et al.* 1990), is required at or prior to the second chemical step of splicing and is known to interact transiently with the spliceosome (SCHWER and GUTHRIE 1991). The original allele of *PRP16*, *prp16-1*, was isolated as a dominant suppressor of an intron branchpoint mutation (COUTO *et al.* 1987). More recent studies have demonstrated that the rate of ATP hydrolysis by Prp16 influences the accuracy of branchpoint recognition by regulating the use of a discard pathway for aberrant lariat intermediates (BURGESS and GUTHRIE 1993). Other experiments demonstrate that ATP hydrolysis by Prp16 directly or indirectly causes a conformational change in the spliceosome that leads to the protection of the 3′ splice site from oligonucleotide-directed RNase H cleavage (SCHWER and GUTHRIE 1992a). Finally, the original branchpoint suppressor allele, *prp16-1*, has been shown to exhibit a dominant negative phenotype when overexpressed; this phenotype is exacerbated at low temperatures (SCHWER and GUTHRIE 1992b). The *prp16-1* allele also exhibits a recessive cold-sensitive (cs) defect when expressed on a low copy vector (SCHWER and GUTHRIE 1992b). The molecular basis for the dominant negative phenotype was revealed by *in vitro* studies that demonstrated that the purified Prp16-1 protein is capable of binding to the spliceosome, but is deficient in ATP hydrolysis and release from the spliceosome (SCHWER and GUTHRIE 1992b). Indeed, Prp16-1 functions as a dose-dependent dominant inhibitor of splicing *in vitro* (SCHWER and GUTHRIE 1992b). These studies have allowed us to uncouple three functions of Prp16: (1) binding to the spliceosome, (2) nucleotide hydrolysis required for a conformational change and (3) release from the spliceosome. While the outlines of the Prp16 cycle have been formulated, the function of Prp16 is not understood, in part because the RNA ligand of this protein in the spliceosome remains unknown.

The requirement for specific residues of U2 and U6 snRNAs in the second chemical step of splicing (see above) suggests these RNAs as possible candidates for RNA ligands of Prp16. Here we describe studies aimed at detecting a functional interaction between the U2-U6 complex and Prp16. We reasoned that mutants in an RNA ligand for Prp16 might suppress the defect of a *prp16* mutant by decreasing the stability of a target RNA-RNA duplex and thus easing the requirement for helicase activity. Alternatively, given that at least some helicases undergo an ATP-driven cycle of nucleic acid binding and release (reviewed in LOHMAN 1993), there might exist a class of mutants of Prp16 that are defective in an RNA release step. In this case, it should be possible to counteract this defect by mutations in an RNA ligand of Prp16 that weaken the interaction with the protein.

We report the isolation and characterization of mutants in the U2 and U6 snRNAs that suppress cs but not temperature-sensitive (ts) mutations of *PRP16*. The

TABLE 1

S. cerevisiae strains used in this study

Strain	Genotype	Derivation	Source
YS78	**a** *ade2 his3 leu2 lys2 prp16::LYS2 trp1 ura3* pSB2 (*PRP16 URA3 CEN*)		BURGESS (1993)
YSN131	α *ade2 his3 leu2 lys2 prp16-302 ura3*		S. NOBLE (unpublished)
YSN132	α *ade2 his3 leu2 prp16-302 ura3*		S. NOBLE (unpublished)
YHM1	**a** *ade2 his3 leu2 lys2 trp1 ura3 snr6::LEU2* pSX6U (*SNR6 URA3 CEN*)		MADHANI *et al.* (1990)
YHM111	**a** *ade2 his3 lys2 ura3 snr20::LYS2* pU2U (*SNR20 URA3 CEN*)		MADHANI and GUTHRIE (1992)
YHM118	**a** *ade2 his3 leu2 lys2 snr20::LYS2 snr6::LEU2 ura3* pU2U6U (*SNR6 SNR20 URA3 CEN*)		MADHANI and GUTHRIE (submitted)
YHM145	**a** *ade2 his3 leu2 lys2 prp16-302 trp1 ura3 snr6::LEU2* pSX6U (*SNR6 URA3 CEN*)	YSN132 × YHM1	This study
YHM151	α *ade2 his3 leu2 lys2 prp16-302 ura3 snr20::LYS2* pU2U (*SNR20 URA3 CEN*)	YSN131 × YHM111	This study
YHM187	α *ade2 his3 leu2 lys2 prp16-302 snr20::LYS2 snr6::LEU2 ura3* pU2U6U (*SNR6 SNR20 URA3 CEN*)	YHM145 × YHM151	This study

data can be most simply accommodated by a model in which the snRNA suppressors function by destabilizing an interaction between the U2-U6 complex and a hypothetical factor (X), which is trapped in cold-sensitive mutants of *PRP16*. The effects of overexpressed *prp16* alleles are consistent with the hypothesis that this trapped interaction inhibits the dissociation of Prp16 from the spliceosome. In the simplest case, factor X is Prp16 itself, and the U2 and U6 alleles function by weakening the interaction with Prp16, as in the second model described above.

MATERIALS AND METHODS

Yeast methods: All yeast genetic manipulations including media preparation, crosses, plasmid shuffle assays, plasmid recovery and transformations were performed according to published methods (GUTHRIE and FINK 1991). Strain genotypes, derivations and sources are summarized in Table 1. The pluses shown in Tables 2–6 refer to relative colony size, with "–" indicating no observable growth. Scoring was based on visual comparisons of photographs of plates. These were done blindly, *i.e.*, without reference to the identity of the strains involved.

Plasmid construction: Conditional *PRP16* alleles on centromere-containing plasmids have been described previously, with the exception of *prp16-302*. This allele was transferred to a plasmid vector by gap repair of a wild-type *Prp16* gene in a *prp16-302* strain. YSN131, a gift from S. NOBLE, was transformed with a fragment of pSB62 (*PRP16 HIS3 CEN*) that was missing the 5′ two-thirds of the *PRP16* coding sequence. Of 13 HIS^+ transformants assayed, all were Cs^-, suggesting that the *prp16-302* mutation is located in the N-terminal two thirds of the protein. A plasmid recovered from one of these strains was shown to confer cold sensitivity when used to replace the wild-type *PRP16* allele in YS78 using the plasmid shuffle method. This allele was also subcloned into pSE358 (*TRP1 CEN*) using the *Eco*RI and *Sph*I site that flank the *PRP16* fragment. The resulting plasmid was used in the experiments described in the text. Overexpression constructs encoding conditional *PRP16* alleles were made by swapping the *Sac*I-*Sac*I fragment of pG16 (pG1-*PRP16*) with the same fragment from the centromere plasmid-borne alleles.

U6 mutant library: The U6 mutant library used has been described previously (MADHANI *et al.* 1990). This library was constructed through degenerate chemical synthesis of the U6 coding sequence. It is highly representative, containing mutants in all regions of U6 (MADHANI *et al.* 1990; H. D. MADHANI and C. GUTHRIE, unpublished; P. RAGHUNATHAN and C. GUTHRIE, unpublished). In addition to containing nucleotide substitutions, the library also contains single nucleotide deletions at a lower frequency (as determined by DNA sequencing of randomly selected isolates).

Site-directed mutagenesis: U2 and U6 site-directed mutants were either obtained from our published collection (MADHANI *et al.* 1990; MADHANI and GUTHRIE 1992) or created using synthetic oligonucleotides and a polymerase chain reaction-based strategy described previously (MADHANI and GUTHRIE 1992).

RESULTS

Since Prp16 interacts transiently with the spliceosome (SCHWER and GUTHRIE 1991), we reasoned that mutant versions of Prp16 that retain the ability to bind the spliceosome might be the most useful for genetic suppression studies involving snRNAs. As described above, the dominant negative phenotype of overexpressed *prp16-1* can be rationalized by its ability to bind the spliceosome but not function in a subsequent step. To identify similar alleles, we tested our existing collection of plasmid-borne conditional lethal *prp16* alleles for this phenotype. Three cs [*prp16-1* (COUTO *et al.* 1987; SCHWER and GUTHRIE 1992b); *prp16-301* (BURGESS and GUTHRIE 1993); *prp16-302* (S. NOBLE and C. GUTHRIE, unpublished)] and four ts alleles of *PRP16* [*prp16-2* (VIJAYRAGHAVAN *et al.* 1989; SCHWER and GUTHRIE 1991; BURGESS and GUTHRIE 1993); *prp16-201, -202, -205* (BURGESS 1993)] were cloned into pG1 (*pGPD, TRP1,* 2μ), the vector used previously to overexpress *prp16-1* (SCHWER and GUTHRIE 1992b; SCHENA *et al.* 1991). These plasmids were introduced into a yeast strain, YS78, in which the chromosomal *PRP16* gene is disrupted and complemented by the wild-type *PRP16* gene on a *URA3*-marked centromere plasmid (BURGESS 1993). As controls, the recessive phenotype of each allele was assayed in parallel.

Table 2 summarizes the growth phenotypes of each allele at various temperatures when (1) expressed on a centromere plasmid as the sole copy, (2) overexpressed

TABLE 2

Growth of YS78 derivatives containing alleles of *PRP16*

Allele	*prp16* (*CEN-ARS*) *prp16::LYS2*							P_{GPD}-*prp16* (2μ) *prp16::LYS2*							P_{GPD}-*prp16* (2μ) *prp16::LYS2* PRP16 (*CEN-ARS*)						
	16°	18°	25°	30°	33°	35°	37°	16°	18°	25°	30°	33°	35°	37°	16°	18°	25°	30°	33°	35°	37°
PRP16	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	++
prp16-2	NT	+++	+++	+++	++	++	–	NT	NT	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	++
prp16-201	NT	+++	+++	+++	++	++	–	NT	NT	+++	+++	+++	+++	–	+++	+++	+++	+++	+++	+++	++
prp16-202	NT	+++	+++	+++	++	++	–	NT	NT	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	++
prp16-205	NT	+++	+++	+++	++	++	–	NT	NT	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	++
prp16-1	–/+	++	+++	+++	+++	+++	++	–/–/+	+	+++	+++	+++	+++	++	–/+	–/+	+++	+++	+++	+++	++
prp16-301	–	+	++	+++	+++	+++	+	–	–/+	+++	+++	+++	+++	++	++	++	+++	+++	+++	+++	++
prp16-302	–	–/+	+++	+++	+++	+++	++	–	–	+++	+++	+++	+++	++	–/+	+	+++	+++	+++	+++	++

The first set of columns describes the growth at various temperatures of strains containing conditional *prp16* alleles on pSE358 (*TRP1*, *CEN*) as the sole allele. The second set of columns describes the growth of strains containing pG1-borne alleles (*TRP1*, 2μ) as the sole copy. The third set of columns, in which dominant negative effects are shown, describes the growth of strains in which the indicated pG1-borne allele is in a strain containing wild-type *PRP16* on a centromere vector. Growth in the first two sets of columns was assayed on 5-FOA at 25, 30, 33, 35 and 37°. For the assessment of growth in the cold, transformants were cured of the wild-type plasmid on 5-FOA at 30°, and then streaked to YEPD plates which were incubated for the 5 days at 18° or 12 days at 16°. This resulted in more sensitive and reproducible results. To test for dominant negative phenotypes, transformants were assayed on SD-TRP-URA to select for both the pG1-borne allele and the wild-type allele, which is on pSE360 (*URA3*, *CEN*). NT, not tested.

in the absence of wild-type *PRP16* or (3) overexpressed in the presence of wild-type *PRP16* (to assay for dominant negative phenotypes). Two patterns of phenotypes are apparent. First, all of the cs, but none of the ts alleles of *PRP16* exhibit a dominant negative effect when overexpressed in the presence of wild-type *PRP16* (Table 2). Second, the growth defects of cs alleles expressed in the absence of wild-type *PRP16* are consistently exacerbated by overexpression; in contrast, each of the four ts alleles tested is at least partially self-suppressed by overexpression (Table 2). We note that, in the cases of the cs alleles, the severity of the dominant negative effect of a particular allele does not correlate precisely with the severity of its cold-sensitivity in the absence of wild-type *PRP16*. For example *prp16-301* and *prp16-302* result in a more pronounced cs defects than *prp16-1*, but less dramatic dominant negative phenotypes (Table 2).

Thus, based on the criterion of dominant negativity, the products of the cs alleles of *PRP16* appeared to be the most likely to retain some ability to interact with the spliceosome at the restrictive temperature. For our initial experiments, we chose the *prp16-302* allele because it exhibited the tightest cs defect. As a source of snRNA mutants, we employed a library of U6 alleles created previously through degenerate chemical synthesis of the entire coding sequence (MADHANI *et al.* 1990). This highly representative bank exists on a *TRP1*-marked centromere plasmid. Since snRNA mutant alleles have often been observed to be poorly expressed when required to compete with the wild-type allele (E. SHUSTER and C. GUTHRIE, unpublished; MADHANI *et al.* 1990), our experiments were designed to permit the recovery of recessive suppressors in U6 of *prp16-302*. We constructed a haploid yeast strain, YHM145, which contains *prp16-302*, a disruption of the chromosomal U6 gene, and a wild-type copy of the U6 gene on a *URA3*-marked centromere plasmid. Using this strain, we could replace the wild-type U6 allele with mutant versions using the plasmid shuffle technique (BOEKE *et al.* 1987). In this method, plasmids encoding mutants are introduced by transformation, and the wild-type *URA3*-containing plasmid is selected against using 5-fluoroorotic acid (5-FOA).

Into YHM145, we introduced the U6 mutant library by transformation. Approximately 10^4 transformants were selected at 30° on SD-TRP plates. These were then replica-plated to 5-FOA-containing plates and placed at 30° for 2 days in order to select for cells that lacked the wild-type U6 plasmid. Finally, colonies that grew on 5-FOA were replica-plated to YEPD plates which were placed at a non-permissive temperature for *prp16-302*, 16°, in order to select for suppressors. Colonies that grew after 7 days of incubation were purified by streaking onto YEPD plates followed by incubation at 18°. The U6 plasmids were recovered from these candidates for further analysis. Fourteen plasmids were identified that

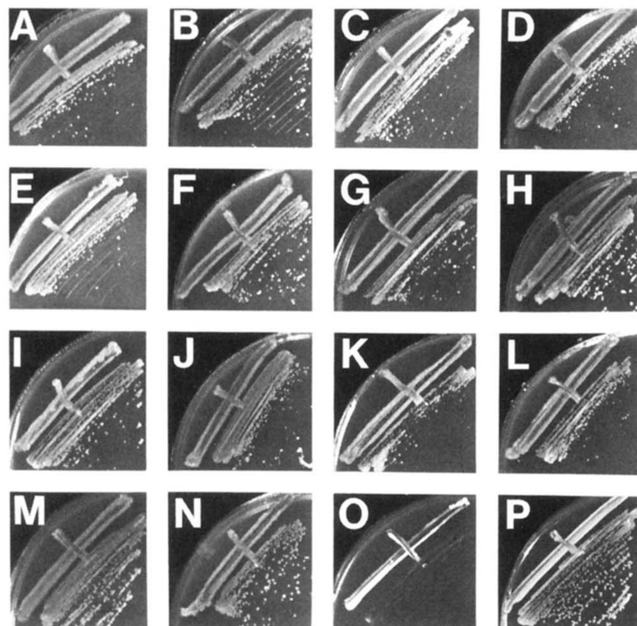

FIGURE 2.—Suppressors of *prp16-302*. Growth of YHM145 derivatives containing (as their sole copy of U6) the 14 suppressors isolated from the U6 mutant library (panels A–N). Panel O shows the growth of YHM145 transformed with a vector control. Panel P shows the growth of a wild-type sister spore (YHM146). YEPD plates were incubated at 18° for 5 days.

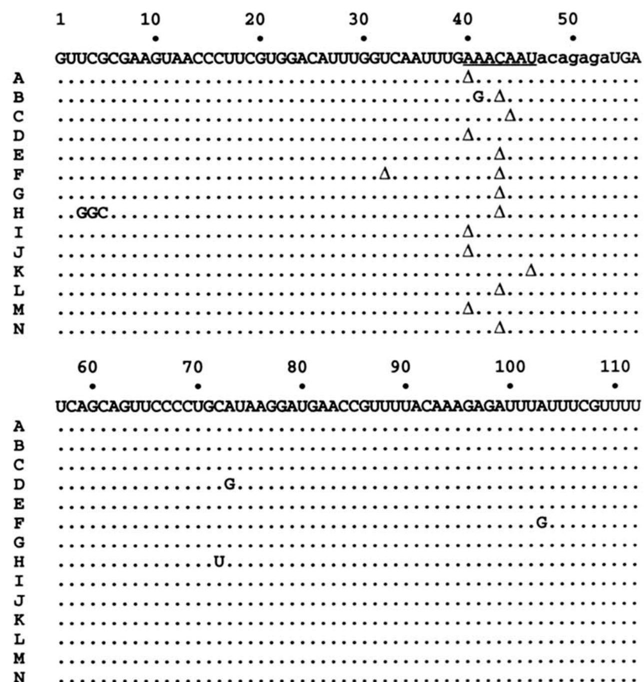

FIGURE 3.—Sequence of the U6 coding sequence for the suppressor alleles shown in Figure 2. Sequences A–N correspond to suppressors shown in Figure 2, panels A–N, respectively. Note that each of the 14 suppressors contains a point deletion in nt 40–46 (underlined), which lie immediately 5′ to the conserved ACAGAGA sequence (shown in lowercase). By convention, deletions in nucleotide runs are referred to by the most 5′ residue.

suppressed the cs defect of *prp16-302* upon retransformation of YHM145. The relative growth of these alleles is shown in Figure 2. As can be seen, the suppressors substantially alleviate the cs defect of *prp16-302*, restoring growth close to wild-type levels (Figure 2). The sequence of the U6 coding region was determined for each mutant (Figure 3). Remarkably, all 14 suppressors contain a single nucleotide deletion of one of the 7 nucleotides that lie upstream of the 5′ splice site binding sequence in U6 snRNA (Figure 3; see also Figure 1). This region exhibits high phylogenetic conservation, but contains few invariant residues (GUTHRIE and PATTERSON 1988; C. GUTHRIE, S. MIAN and H. ROIHA, unpublished). Four of the mutants contain additional changes. It is unlikely that these account for the suppressor phenotype, however, since in each case we recovered other suppressors that contain the same single residue deletion in nt 41–46 of U6 in the absence of the additional alterations (Figure 3). Given that the U6 library contains both nucleotide substitutions and deletions (MADHANI *et al.* 1990), the observed enrichment of point deletions, *vs.* substitutions, is likely to be significant. To confirm this notion, we examined the effects of five nucleotide substitutions in this region (A41G; A42C; A44U; A45U; U46G; Table 3). As expected, all suppress *prp16-302* much more poorly than the point deletions (Table 3). The more potent suppressor activity of the deletion mutants might reflect the fact that they not only alter a sequence but also change the relative spacing of the adjacent sequences.

Since the suppressor screen required that the U6 alleles be fully functional (we demanded growth on 5-FOA after two days at 30° prior to selecting for suppressors), we considered the possibility that slow growing and/or conditional-lethal U6 alleles in the region might also function as suppressors but would have been selected against in the screen. We took advantage of our collection of site-directed mutants (MADHANI *et al.* 1990; MADHANI and GUTHRIE 1992; H. D. MADHANI, unpublished) to determine whether or not mutations in the adjacent ACAGAGA sequence or in the U2-U6 helix I region of U6 and U2 snRNAs (Figure 1) could also suppress *prp16-302*. We constructed a haploid *prp16-302* strain, YHM187, that contains disruptions of both the chromosomal U2 and U6 genes complemented by a single *URA3*-marked centromere plasmid that carries both wild-type genes. We used this strain to replace the wild-type U2 gene or the wild-type U6 gene with mutant versions using the plasmid shuffle technique. In the case of the mutant U6 alleles, YHM187 was simultaneously transformed with a U6 mutant and a wild-type U2 gene prior to streaking on 5-FOA. Similarly, to assay U2 mutants, these were introduced together with wild-type U6 prior to streaking on 5-FOA. The resulting strains were assayed for growth in the cold (Table 3). Several alterations in the conserved ACAGAGA sequence in U6 (in nt 47, 50, 52 and 53) result in very low levels of sup-

TABLE 3

Suppressor and recessive growth phenotypes of U6 and U2 alleles

U6 allele	U2 allele	Suppression of *prp16-302*	Growth in the presence of wild-type *PRP16* 18°	25°	30°	37°
WT	WT	–	++++	++++	++++	+++
ΔA40	WT	+++	++++	++++	++++	+++
ΔC43	WT	+++	++++	++++	++++	+++
ΔA44	WT	+++	++++	++++	++++	+++
ΔU46	WT	+++	++++	++++	++++	+++
A41G[a]	WT	+	++++	++++	++++	+++
A42C[a]	WT	+	++++	++++	++++	+++
A44U[a]	WT	+	++++	++++	++++	+++
A45U[a]	WT	–/+	++++	++++	++++	+++
U46G[a]	WT	+	++++	++++	++++	+++
ΔA47[b]	WT	–/+	NT	++++	++++	+
A47U[b]	WT	–	NT	++++	++++	+
G50C[b]	WT	–/+	NT	++++	++++	+++
G52U[b]	WT	–/+	NT	++++	++++	+
A53C[b]	WT	–/+	NT	++++	++++	+++
A53G[b]	WT	–/+	NT	++++	++++	+++
U54A	WT	–	++++	++++	++++	+++
G55C	WT	–/+	++++	++++	++++	+++
G55C, A56U	WT	++	++++	++++	++++	+++
A56G, U57G	WT	++	++++	++++	++	–
C58U	WT	–	++++	++++	++++	–
A59C	WT	–	+	++	++	–
C61U	WT	–	++++	++++	++++	+
WT	G21U	–	++++	++++	++++	+++
WT	C22A	–	++++	++++	++++	+++
WT	U23C	–	++++	++++	++++	+++
WT	U23G	–	++++	++++	++++	+++
WT	A27C, U28C	–/+	+++	+++	+++	–
WT	U28A, C29G	+	+++	+++	+++	–
WT	C29G	–	++++	++++	++++	+++
WT	A30U	–	++++	++++	++++	+++
Control	Control	++++	NA	NA	NA	NA

The left two columns list the U6 and U2 alleles tested. The third column shows the growth of YHM187 derivatives containing site-directed mutants in U6 or U2 snRNAs as the sole copy of the respective gene. YEPD plates were incubated for 9 days at 18 °. Control: YHM187 containing wild-type U6, wild-type U2, and pSB2 (*PRP16 URA CEN*). The four columns on the right indicate the recessive growth phenotypes of the U2 and U6 alleles. Other alleles were assayed using the strain YHM118. FOA plates were scored after 3 days at the indicated temperature, except for the 18 °C plates, which were scored after 5 days. WT, wild-type. NT, not tested; NA, not applicable.

[a] Data of Lesser and Guthrie (1993).

[b] Data of Madhani *et al.* (1990).

pression (Table 3). Interestingly, double substitutions in U2-U6 helix Ia confer moderate levels of suppression, both in the case of alterations to U6 (G55C, A56U; A56G, U57G) and U2 (A27C, U28C; U28A, C29G; Table 3). The U2 alleles, however, are weaker suppressors than the U6 alleles. Also shown in Table 3 are the phenotypes of the U2 and U6 alleles in the presence of wild-type *PRP16*. This was done using the strain YHM118, which is identical to YHM187 except that it contains a wild-type chromosomal allele of *PRP16*. There appears to be no correlation between the ability of a given U2 or U6 allele to support growth of otherwise wild-type cells and their ability to suppress *prp16-302* (Table 3).

As described in the Introduction, one mechanism by which suppression of mutant *PRP16* alleles might occur is through the weakening of a target helix. It was therefore of interest to determine whether the reduction in stability of helix Ia was responsible for suppression in these cases. Consequently, we assayed the effects of combinations of U2 and U6 alleles that disrupt base-pairing in helix Ia, restore wild-type stability, or increase the predicted stability of helix Ia. Nine different combinations were tested, including wild-type. Figure 4 shows the predicted structure of helix Ia in each of these allele combinations. Next to each is shown the growth of the corresponding YHM187 derivative on YEPD plates at 18° after 9 days. In contrast to our expectations, each of the variants suppresses *prp16-302*, irrespective of the predicted stability of the helix (Figure 4, B–I). Although variants that preserve the integrity of helix Ia suppress more strongly (compare Figure 4, B–E with F–I), this correlation might simply reflect the fact that the disruption of helix Ia is deleterious to cell growth (Madhani and Guthrie 1992), independent of its effects on *prp16-302*. In any case, since suppression can occur in both mutants that destabilize helix Ia and in those that have the opposite effect, there is no consistent correlation between suppressor activity and change in helix stability. It is therefore likely that some other effect of nucleotide changes in this region is responsible for suppression. We

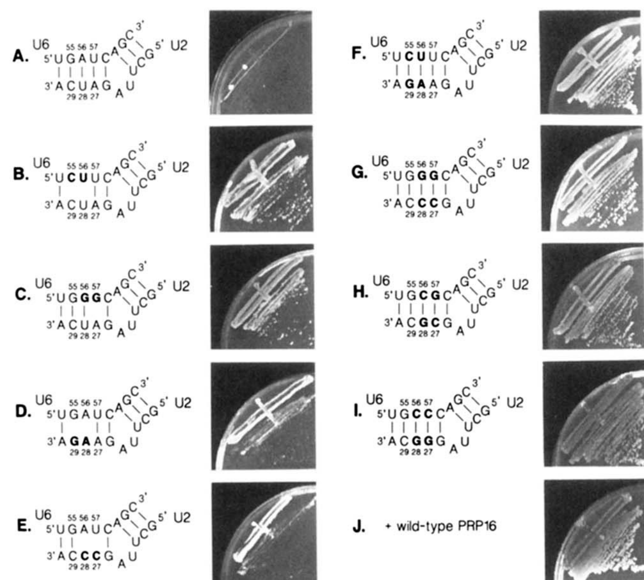

FIGURE 4.—Growth of YHM187 derivatives containing the indicated alterations in U2-U6 helix Ia. Growth on YEPD was assessed after 9 days at 18°. A longer incubation time was used with this strain compared to YHM145 because the *prp16-302* phenotype is slightly more severe in this background. The strain shown in J contains wild-type copies of U2, U6 and *PRP16*.

note that our analysis of U2 was limited to site-directed mutants in the U2-U6 helix I region; it is therefore possible that suppressors in other regions of U2 exist.

Our suppressor screens were based on the assumption that snRNA mutants capable of suppressing *PRP16* mutants would function better in the absence of competition from the wild-type snRNA allele; however, for technical reasons (see below), it was desirable to have alleles that functioned dominantly. To this end, we tested representative U6 alleles from the recessive screen for their ability to suppress *prp16-302* in the presence of a wild-type U6 allele. We employed a YS78 derivative in which the wild-type copy of *PRP16* was replaced by *prp16-302*. This strain, which contains a wild-type chromosomal copy of the U6 gene, was transformed with five strong U6 suppressor alleles. As shown in Table 4, each allele is capable of suppressing *prp16-302* in the presence of wild-type U6. In two of five cases (U6-ΔC43 and U6-ΔA44), suppression is as effective as in the absence of wild-type U6, and produces close to wild-type growth. However, the other three U6 suppressors function more weakly is in the presence of wild-type U6 than in its absence (Table 4).

This observation facilitated tests of a different question: is suppression specific for the *prp16-302* allele or are other alleles also suppressed? The availability of dominantly acting U6 suppressors allowed us to test their effects on isogenic *prp16* strains created using the plasmid shuffle method. The analysis above demonstrated that cs versions of Prp16 produce dominant negative effects when overexpressed, consistent with

TABLE 4

Comparison of suppression of *prp16-302* by U6 suppressor alleles in the absence and presence of wild-type U6

U6 allele	U2 allele	Suppression in absence of wild-type U6	Suppression in presence of wild-type U6
WT	WT	−	−
ΔA40	WT	+++	+
ΔC43	WT	+++	+++
ΔA44	WT	+++	+++
ΔU46	WT	+++	++
A56G, U57G	WT	++	+
Control	Control	++++	++++

YHM187 derivatives were cotransformed with the indicated alleles (shown in the first two columns), and growth was assessed after 9 days at 18 °. The third column refers to the growth of YHM187 derivatives containing indicated U6 and U2 alleles and have been cured of the wild-type U2-U6 plasmid. The fourth column refers to the growth of YHM187 derivatives containing indicated U6 and U2 alleles and also contain the wild-type U2-U6 plasmid. SD −URA and SD −TRP plates were incubated for 9 days at 18 °. Control refers to YHM187 containing WT U6, WT U2, and pSB2 (*PRP16 URA CEN*). WT, wild type.

some ability to interact with the spliceosome, whereas overexpression of ts alleles does not have this effect. This disparity suggests that the two types of conditional alleles are blocked at different steps. Therefore, we expected that snRNA suppressors of *prp16-302* would suppress other cs *PRP16* alleles but not ts alleles. To test this hypothesis, we constructed YS78 derivatives containing each of the ts and cs *PRP16* alleles on pSE358 (*TRP1, CEN*) as the sole copy in the presence or absence of one of two strong suppressors, U6-ΔC43 and U6-ΔA44. As shown in Table 5, U6-ΔC44 partially suppresses *prp16-1* and *prp16-301* in addition to *prp16-302*. U6-ΔC43 suppresses *prp16-301* and *prp-302* but not *prp16-1*. The inability of U6-ΔC43 to suppress *prp16-1* likely reflects its generally weaker suppressor activity compared to U6-ΔA44 (Table 5). In contrast, no suppression by either U6 mutant of ts alleles of *PRP16* alleles was observed (Table 5).

Finally, we asked whether or not overexpressed cs alleles of *PRP16* can be suppressed by U6-ΔC43 or U6-ΔA44. YS78 derivatives containing pG1-borne *prp16-1*, *prp16-301* and *prp16-302* as the sole copy of *PRP16* were transformed with either a vector control, or one of the two U6 alleles. In the case of *prp16-1* and *prp16-301*, no suppression was observed (Table 6). Slight suppression of the cs defect of *prp16-302* was seen at 18° but not at 16° (Table 6). Thus overexpression of cs alleles of *PRP16* in some way antagonizes suppression.

DISCUSSION

This study was motivated by the recent realization that the assembly and function of the spliceosome is accompanied by a series of RNA rearrangements. These findings (reviewed in WEINER 1993; MOORE *et al.* 1993) suggest that a key role for at least one class of protein factors involved in splicing is in the mediation and control of these RNA conformational transitions. Members of the

TABLE 5

Allele specificity of suppression

Allele	pSE358 (vector) *prp16* (*CEN-ARS*) *prp16-LYS2*							U6ΔC43 (*CEN-ARS*) *prp16* (*CEN-ARS*) *prp16-LYS2*							U6ΔA44 (*CEN-ARS*) *prp16* (*CEN-ARS*) *prp16-LYS2*						
	16°	18°	25°	30°	33°	35°	37°	16°	18°	25°	30°	33°	35°	37°	16°	18°	25°	30°	33°	35°	37°
PRP16	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	++
prp16-2	NT	+++	+++	+++	++	++	–	NT	+++	+++	+++	++	++	–	NT	+++	+++	+++	++	++	–
prp16-201	NT	+++	+++	+++	++	++	–	NT	+++	+++	+++	++	++	–	NT	+++	+++	+++	++	++	–
prp16-202	NT	+++	+++	+++	++	++	–	NT	+++	+++	+++	++	++	–	NT	+++	+++	+++	++	++	–
prp16-205	NT	+++	+++	+++	++	++	–	NT	+++	+++	+++	++	++	–	NT	+++	+++	+++	++	++	–
prp16-1	–/+	++	+++	+++	+++	+++	++	–/+	++	+++	+++	+++	+++	++	**+**	++	+++	+++	+++	+++	++
prp16-301	–	+	+++	+++	+++	++	+	–	**++**	+++	+++	+++	++	+	–	**++**	+++	+++	+++	++	+
prp16-302	–	–/+	+++	+++	+++	+++	++	–	**+**	+++	+++	+++	+++	++	**–/+**	**++**	+++	+++	+++	+++	++

Shown are the effects on growth at various temperatures of U6 suppressor alleles on YS78 derivatives containing mutant alleles of *PRP16* on pSE358 (*TRP1, CEN*) as the sole allele. Assays were performed as in Figure 1. Bold indicates differences with the data in the first set of columns. The somewhat weaker suppression of *prp16-302* by the U6 suppressors observed in YS78 may reflect differences in strain background. NT, not tested.

DEAD/DEAH family of helicase-like proteins are good candidates for such factors. However, despite an increasing understanding of their temporal action in the splicing pathway, nothing is known regarding their specific biological ligands.

Prp16, the prototype of the DEAH family, has been particularly well characterized (COUTO *et al.* 1987; BURGESS *et al.* 1990; SCHWER and GUTHRIE 1991, 1992a,b; BURGESS and GUTHRIE 1993). Prp16 binds to the spliceosome prior to the second step of splicing, subsequently performs a function that requires ATP hydrolysis, and is then apparently released from splicing complexes prior to or concomitant with the second chemical step of splicing. Given that the purified protein is an RNA-dependent ATPase (SCHWER and GUTHRIE 1991), Prp16 presumably interacts with one or more RNA molecules in the spliceosome. We hypothesized that U6 and U2 snRNAs might interact with Prp16 because specific residues on both RNAs are critical for the second chemical step of splicing. Consequently, we set out to hunt for functional interactions between these RNAs and Prp16.

We took advantage of an existing collection of conditional lethal alleles of *PRP16* for these studies. One mutant protein, Prp16-1, is known to bind spliceosomes *in vitro* but not function in a subsequent step (SCHWER and GUTHRIE 1992b). As expected from this *in vitro* behavior, this allele also causes a dominant negative phenotype when overexpressed *in vivo* (SCHWER and GUTHRIE 1992b). Reasoning that alleles capable of interacting with the spliceosome would be the most useful for detecting functional interactions with snRNAs, we identified two additional cs alleles of *PRP16* that also exhibit a dominant negative phenotype when overexpressed (*prp16-301* and *prp16-302*; Table 2). By analogy to *prp16-1*, we expect that these mutant proteins also retain some ability to bind the spliceosome but are deficient in a subsequent activity; confirmation of this notion will require the purification and *in vitro* analysis of these mutant molecules. Assuming this to be the case, the most severe cs allele, *prp16-302*, was chosen for a mutant hunt to identify suppressors in U6 snRNA.

Using a synthetically mutagenized library of U6 genes, we identified 14 cs^+ suppressors of *prp16-302*. Remarkably, each contains a single nucleotide deletion in one of the seven residues upstream of the 5′ splice site-binding sequence in U6 snRNA (Figures 1–3; nt 40–46). Further analysis identified somewhat weaker suppressors of *prp16-302* in U2-U6 helix Ia (Figure 1, Table 3). We had initially considered the notion that cold sensitivity might be due to the inability of a mutant Prp16 to unwind a target helix. However, suppression cannot be due to the disruption of helix Ia, because both mutants that destabilize helix Ia and mutants that hyperstabilize helix Ia were found to result in suppression (Figure 4). We found that all three of the cs *PRP16* alleles could be

TABLE 6

Overexpression of cs *PRP16* alleles antagonizes suppression by U6 mutants

		Plasmid-borne U6 gene					
		None		U6-ΔC43		U6-ΔA44	
Allele	Vector type	16°	18°	16°	18°	16°	18°
prp16-1	2μ	−/−/+	+	−/−/+	+	−/−/+	+
prp16-1	*CEN*	−/+	++	−/+	++	**+**	++
prp16-301	2μ	−	−/+	−	−/+	−	−/+
prp16-301	*CEN*	−	+	−	**++**	−	**++**
prp16-302	2μ	−	−	−	**−/+**	−	**−/+**
prp16-302	*CEN*	−	−/+	−	**+**	**−/+**	**++**

Compared is the growth of YS78 derivatives containing the indicated *PRP16* allele on pG1 (P_{GPD}-2μ) *vs.* a low copy vector (*CEN-ARS*) as the sole copy. Compared is the effects of vector, U6-ΔC43 and U6-ΔA44. Bold indicates differences from the "vector" columns.

suppressed by one or both of the two strong U6 suppressor alleles tested, suggesting that these cs alleles share a common defect. In contrast, ts alleles of *PRP16* were not suppressed. Finally, we observed that overexpression of cs *PRP16* alleles exacerbates their recessive growth defect (Table 2) and antagonizes suppression by the U6 suppressors (Table 6).

The observation that mutations in many different nucleotides in U6 and U2 snRNAs in the U2-U6 helix I region (Figure 1) confer suppressor activity suggests that disruption of an interaction involving these residues is responsible for suppression. Shown in Figure 5A is a model in which a factor, X, is proposed to interact with the U2-U6 complex, induce a change in its conformation, and then be released. We suggest that the release step becomes rate-limiting for cell growth in the cold in the presence of a cs mutant of Prp16. Disruption of this interaction would overcome the block to the release of X and allow splicing to proceed. In this model, X could be a protein, an RNA, or Prp16 itself.

In the context of the first two cases (X = another protein or RNA), one rationalization for the data would be that Prp16 competes with the U2-U6 complex for X. Cs mutants of Prp16 would be defective in binding X (*e.g.*, due to a defect in ATPase activity) and would result in the accumulation of a U2-U6-X complex. The suppressors would function by weakening the interaction between U2-U6 and X which, as a result, would partially restore the equilibrium with Prp16. Given that the cs versions of Prp16 are likely to bind the spliceosome effectively (see above), this hypothetical competition between Prp16 and the U2-U6 complex would have to occur within or on the surface of the spliceosome. This model is similar to one proposed to explain genetic and biochemical interactions among U4, U6 and the U6 snRNP protein Prp24 (SHANNON and GUTHRIE 1991). In that study it was observed that mutations that destabilize the U4-U6 base-pairing interaction result both in a cs growth defect and the accumulation of a U4-U6-Prp24 complex, a likely intermediate in assembly of the U4-U6 snRNP. The cold sensitivity produced by this bottleneck can be partially suppressed *in vivo* by mutations in the

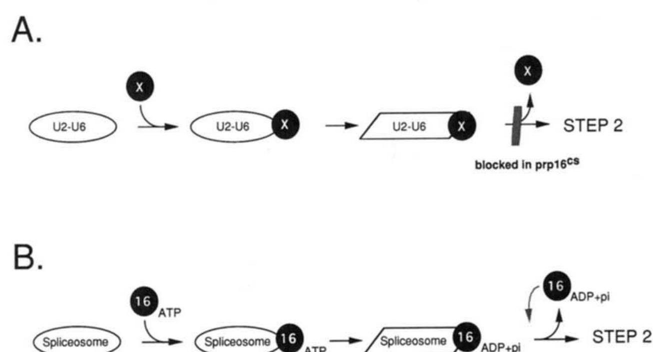

FIGURE 5.—Model for suppression. (A) Hypothetical interaction between U2-U6 and factor X. A factor, X, is proposed to interact with the U2-U6 complex and mediate a conformational change (depicted as a change from an ellipse to a parallelogram). In cs *PRP16* alleles, it is proposed that the release of X from the U2-U6 complex becomes rate-limiting in the cold. Mutants in U2 and U6 suppress this cs defect by weakening the interaction with X, promoting its release. X could be Prp16, another protein, or an RNA. (B) Prp16 cycle. Depicted is the binding of Prp16 to the spliceosome (SCHWER and GUTHRIE 1991), a conformational change induced by ATP hydrolysis (SCHWER and GUTHRIE 1992a)–represented a change in shape of the spliceosome (ellipse to parallelogram)–and dissociation of Prp16 from the spliceosome prior to or concomitant with the second chemical step of splicing (SCHWER and GUTHRIE 1991).

U6 gene or in an RNA-binding motif in *PRP24* that each demonstrably disrupt the U6-Prp24 interaction. Based on these results, it was suggested that the destabilization of the U6-Prp24 interaction partially restores an equilibrium with the weakened U4-U6 complex, allowing the formation of sufficient U4-U6 snRNP to promote cell growth (SHANNON and GUTHRIE 1991). Interestingly, nt 40–46 of U6, the site of the strongest *prp16* suppressors, have also been implicated as a binding site for Prp24 in free (non-spliceosomal) U6 snRNP (SHANNON and GUTHRIE 1991; A. JANDROSITZ and C. GUTHRIE, unpublished). Whether Prp24 could function as factor X is unclear since it is not known whether Prp24 is present in the spliceosome. However, mutations in an RNA binding motif in *PRP24* that weaken the Prp24-U6 interaction (SHANNON and GUTHRIE 1991) fail to suppress

prp16-302 (H. D. Manhani, and C. Guthrie, unpublished). Moreover, no suppressors of *prp16-302* were isolated with changes in a second region implicated in Prp24 binding in the 3′ terminal domain of U6 (Figure 3; Shannon and Guthrie 1991). Finally, models of this type (*i.e.*, those that invoke competition between the U2-U6 complex and Prp16) do not readily explain why overexpression of cs versions of Prp16 exacerbates cold sensitivity.

A second possibility is that X is Prp16 itself. As shown in Figure 5B, Prp16 is known to bind to the spliceosome prior to the second chemical step of splicing and, upon ATP hydrolysis, induce a conformational change in the spliceosome that leads to protection of the 3′ splice site (Schwer and Guthrie 1991, 1992a). At some point, the protein is released from the spliceosome so that it can function in the next splicing cycle (Schwer and Guthrie 1991). In this model, the cs alleles of Prp16 would be defective in release of the protein from the U2-U6 complex. The U2 and U6 mutations would destabilize this direct interaction, promoting the dissociation of Prp16. This model can also explain why overexpression of cs *prp16* alleles exacerbates their phenotype. Following the initial release of a mutant Prp16 from the spliceosome, high levels of the protein would promote its rapid rebinding, thereby preventing subsequent productive steps. This would have the effect of worsening the primary defect of these alleles, slow release from the spliceosome.

In addition to rationalizing several important aspects of the genetic data, this second model makes several testable predictions. First, release of Prp16 from spliceosomes should be defective in the cs alleles. For Prp16-1, it is known that the protein binds the spliceosome but is blocked in a subsequent step prior to its release (Schwer and Guthrie 1992b). However, whether the release step *per se* or a prior step is defective has not been determined. Second, the observed *in vitro* RNA-dependent ATPase defect of the purified Prp16-1 protein (Schwer and Guthrie 1992b) is predicted to be due, at least in part, to a defect in RNA release as opposed to defects in ATP binding or the chemical step of nucleotide hydrolysis. A defect in RNA release would manifest itself as a decrease in the apparent steady-state rate of ATP hydrolysis if dissociation of the protein from RNA is required in order to initiate a second ATPase cycle. More sophisticated kinetic studies will be required to determine whether this is the case. Finally, if the snRNA suppressors act by destabilizing an interaction with Prp16, one predicts that the same ends could be achieved by a different means: amino acid changes in Prp16 that destabilize the interaction with RNA. Indeed, such intragenic suppressors of the cs defect of *prp16-1* can easily be isolated; of four analyzed, all contain different additional amino acid changes in the helicase-related domain of Prp16 (S. Burgess and C. Guthrie, unpublished).

Although several aspects of the data can most simply be explained by proposing that Prp16 interacts directly with the U2-U6 complex, additional experiments will be required to critically test this model. Nonetheless, it is interesting to consider what the role of such an interaction might be. Of particular importance is the function of ATP hydrolysis by Prp16. Burgess and Guthrie (1993) have demonstrated that the rate of ATP hydrolysis by Prp16 influences the accuracy of intron branchpoint recognition by regulating the use of a discard pathway for aberrant lariat intermediates. In their kinetic model, ATP hydrolysis plays an additional, but unspecified, role in the productive branch of the pathway (Burgess and Guthrie 1993). One possibility is that the energy of ATP hydrolysis is converted into RNA binding energy so as to activate Prp16 for binding to the U2-U6 complex. Consistent with this notion, recent experiments demonstrate that ATP hydrolysis by the DEAD box family member eIF4A leads to a form of the protein with greatly increased affinity for RNA (Pause *et al.* 1993). If, as we have proposed, the U2-U6 complex functions as part of a spliceosomal active site (Madhani and Guthrie 1992), such an activated form of Prp16 could serve to promote an RNA conformational rearrangement required prior to or during the second chemical step. This possibility would be consistent with the observed Prp16-dependent protection of the 3′ splice observed during *in vitro* splicing (Schwer and Guthrie 1991). Understanding the precise function of the RNA-dependent ATPase activity of Prp16 will require a description of the changes in active site structure that are likely to occur between the two chemical steps of splicing (Moore and Sharp 1993), and the relationship between these conformational shifts and the nucleotide-regulated states of Prp16.

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