Multiple Functions of *Saccharomyces cerevisiae* Splicing Protein Prp24 in U6 RNA Structural Rearrangements

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ABSTRACT

U6 spliceosomal RNA has a complex secondary structure that includes a highly conserved stemloop near the 3′ end. The 3′ stem is unwound when U6 RNA base-pairs with U4 RNA during spliceosome assembly, but likely reforms when U4 RNA leaves the spliceosome prior to the catalysis of splicing. A mutation in yeast U6 RNA that hyperstabilizes the 3′ stem confers cold sensitivity and inhibits U4/U6 assembly as well as a later step in splicing. Here we show that extragenic suppressors of the 3′ stem mutation map to the gene coding for splicing factor Prp24. The suppressor mutations are located in the second and third of three RNA-recognition motifs (RRMs) in Prp24 and are predicted to disrupt RNA binding. Mutations in U6 RNA predicted to destabilize a novel helix adjacent to the 3′ stem also suppress the 3′ stem mutation and enhance the growth defect of a suppressor mutation in RRM2 of Prp24. Both phenotypes are reverted by a compensatory mutation that restores pairing in the novel helix. These results are best explained by a model in which RRMs 2 and 3 of Prp24 stabilize an extended intramolecular structure in U6 RNA that competes with the U4/U6 RNA interaction, and thus influence both association and dissociation of U4 and U6 RNAs during the splicing cycle.

NUCLEAR pre-mRNA splicing is performed by a ribonucleoprotein complex called the spliceosome, which is assembled on each intron. Five small nuclear RNAs (snRNAs) are essential constituents of the spliceosome: U1, U2, U4, U5, and U6. Each snRNA associates with specific proteins to form a small nuclear ribonucleoprotein complex (snRNP). Early in spliceosome assembly, U6 RNA base-pairs with U4 RNA to form the U4/U6 snRNP complex (Hashimoto and Steitz 1984; Rinke *et al.* 1985; Brow and Guthrie 1988). A tri-snRNP complex, comprising the U4/U6 snRNP and the U5 snRNP, then forms and associates with the pre-spliceosome, which consists of the pre-mRNA and U1 and U2 snRNPs (reviewed in Will and Lührmann 1997; Burge *et al.* 1999). For the spliceosome to become catalytically active, U4 RNA must dissociate from U6 RNA and may leave the spliceosome (Yean and Lin 1991). Concomitant with or soon after U4 RNA release, U6 RNA base-pairs with U2 RNA (reviewed in Madhani and Guthrie 1994; Ares and Weiser 1995; Nilsen 1998) and the first catalytic step of splicing occurs. After splicing, the U6/U2 RNA interaction is disrupted, and free U6 RNA is recycled to the U4/U6 snRNP.

U6 RNA undergoes several conformational changes during spliceosome assembly and activation. In the free U6 snRNP, *Saccharomyces cerevisiae* (hereafter "yeast") U6 RNA contains at least three intramolecular helices: the 5′, central, and 3′ stem (Fortner *et al.* 1994; Figure 1). The central and 3′ stems must unwind for U6 RNA to base-pair with U4 RNA, and the 3′ stem likely reforms upon U6 RNA base-pairing with U2 RNA. To study the conformational changes of U6 RNA, we created a mutation, A62G, in yeast U6 RNA. The U6-A62G mutation, which creates a G-C base pair, results in (1) hyperstabilization of the 3′ stem, (2) a defect in U4/U6 complex assembly, and (3) cold-sensitive growth (Fortner *et al.* 1994). Overexpression of U4 RNA in the U6-A62G strain restores U4/U6 complex to wild-type levels but does not fully suppress the cold sensitivity. Thus, the U6-A62G mutation affects not only U4/U6 complex assembly, but at least one additional process as well.

To further characterize the defects caused by the U6-A62G mutation, spontaneous suppressors of the cold-sensitive growth were selected. Of 109 suppressor strains obtained, 31 contain a mutation in U6 RNA (Fortner *et al.* 1994). Some of these *cis*-suppressors fully revert the U4/U6 complex assembly defect caused by the A62G mutation, likely by destabilizing the 3′ stem of U6 RNA. Others, however, have an intermediate or negligible effect on U4/U6-A62G complex assembly. Most of these latter mutations fall within two regions of U6 RNA: positions 38–43 and 86–95. We previously noted that these regions have the potential to base-pair and form a stem in U6 RNA (Brow and Vidaver 1995), here named the telestem to reflect the fact that the interaction occurs across a distance. If present simultaneously with the central stem loop of free U6, the telestem would form a pseudoknot structure (Figure 1). Our previous

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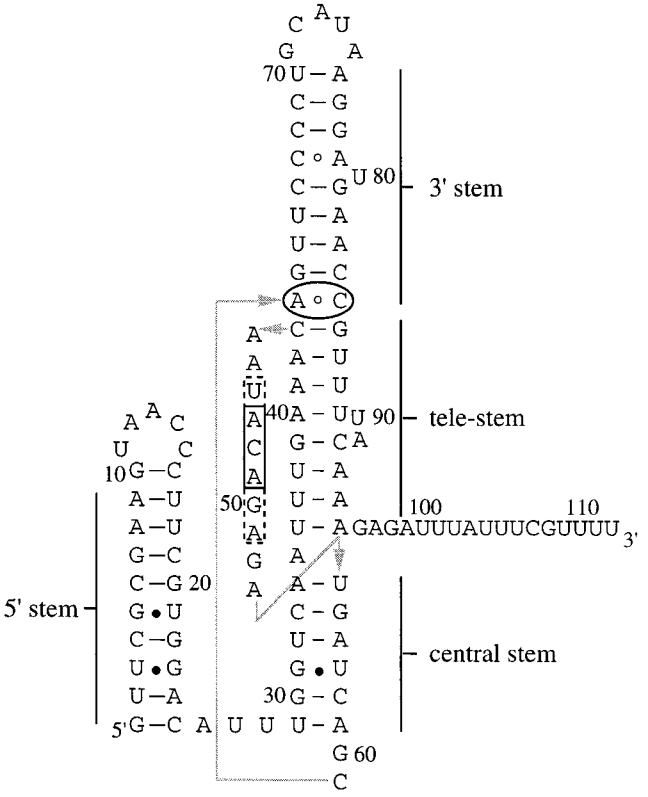


Figure 1.—Proposed secondary structure of yeast U6 RNA in the U6 snRNP. The telestem includes residues 36–43 and 86–95. The site of the 62/85 base pair, hyperstabilization of which leads to a cold-sensitive phenotype, is indicated with an oval. Arrows represent phosphodiester bonds. The boxed residues interact with the pre-mRNA 5′ splice site; solid lines indicate proven base pairs (reviewed in Nilsen 1998), dashed lines indicate proposed interactions (Li and Brow 1996; Spingola *et al.* 1999).

in vitro studies on the human U4/U6 complex showed that a competing intramolecular structure in human U6 RNA, the extended 3′ stem, destabilizes the human U4/U6 complex (Brow and Vidaver 1995). We suggested that the telestem in yeast U6 RNA performs an analogous destabilizing function.

Evidence from chemical modification studies (Jandrositz and Guthrie 1995) supports the notion that the U6 RNA telestem exists in purified U6 snRNPs. U6 RNA bases 40–43, which reside in the 5′-most strand of the telestem, are protected in the U6 snRNP (but not in deproteinized U6 RNA) from reagents that modify unpaired bases. It was proposed that the essential U6 snRNP protein, Prp24 (Shannon and Guthrie 1991), binds directly to bases 40–43 because mutations in the third RNA-recognition motif (RRM) of Prp24 also abolish protection. We suggest instead that the protection of U6 RNA bases 40–43 is due to base-pairing in the telestem, and that the stem requires Prp24 for stabilization. The fact that a mutation in the 3′ strand of the telestem (U6-A91G) eliminates the ability of U6 RNA to be coimmunoprecipitated by anti-Prp24 antibodies (Shannon and Guthrie 1991) and abolishes protection of bases 40–43 (Jandrositz and Guthrie 1995) supports our suggestion that the telestem is stabilized by Prp24. However, direct evidence for the telestem base-pairing interaction has not been reported, nor has any genetic interaction between Prp24 and U6 RNA. Furthermore, our proposal that the telestem/Prp24 complex promotes U4/U6 dissociation appears to contradict other studies that implicate Prp24 in U4/U6 association (Ghetti *et al.* 1995; Raghunathan and Guthrie 1998b).

Here we report strong evidence for the involvement of Prp24 in both U4/U6 complex association and dissociation. Extragenic suppressors of the U6-A62G mutations map to RRMs 2 and 3 of Prp24. Although the suppressor mutations inhibit wild-type U4/U6 complex assembly, they partially relieve the U4/U6 complex assembly defect caused by the U6-A62G mutation. This finding supports the proposal of Shannon and Guthrie (1991) that wild-type Prp24 brings U4 and U6 RNAs into close proximity but inhibits their base-pairing, because the suppressor mutations in Prp24 seem to disrupt both functions. However, relief of the U4/U6-A62G complex assembly defect is not the mechanism of suppression, because the cold sensitivity of a U6 RNA 3′ stem mutant that does not affect U4/U6 complex assembly is nevertheless suppressed by the Prp24 mutations. Rather, the following results suggest that suppression is due to inhibition of U4/U6 complex dissociation via decreased stabilization of the telestem. First, the suppressor mutations in Prp24 map to the predicted RNA contact surface of RRMs 2 and 3 and include residues known to directly bond with RNA in model RRMs. Second, mutations in U6 RNA that disrupt the telestem also suppress U6-A62G cold sensitivity, and at least one compensatory mutation reverts suppression. Third, a suppressor mutation in RRM2 of Prp24 confers a synthetic lethal phenotype when combined with mutations in the U6 RNA telestem, but viability is restored upon restoration of telestem base-pairing potential. Our results support a model in which the telestem/Prp24 interaction antagonizes U4/U6 base-pairing and plays an important role in U4/U6 dissociation during spliceosome activation.

MATERIALS AND METHODS

Plasmid constructions and site-directed mutagenesis: Mutant alleles of *SNR6* (except as described below) were generated by oligonucleotide-directed mutagenesis of plasmids p-539H6 (Brow and Guthrie 1990), p-539H6-A62G (Fortner *et al.* 1994), or p-539H6-G39A,A62G (constructed from a spontaneous *cis*-suppressor allele; Fortner *et al.* 1994). Single-stranded DNA was obtained and mutagenesis performed essentially as described (Kunkel *et al.* 1987; Vieira and Messing 1987). Resulting clones were confirmed by sequencing with oligonucleotide U6G. Mutant alleles of *SNR6* were cloned into the yeast shuttle vector pSE358 (*CEN4*, *TRP1*; derivative of pUN10; Elledge and Davis 1988) by isolating the *Eco*RI-

*Sph*I fragment from each allele and ligating it into *Eco*RI-*Sph*I-digested pSE358.

The U89C mutation in *SNR6* was created by PCR amplification of p-539H6 using the U6-U89C oligonucleotide as the downstream primer and U6PCR5′ as the upstream primer. The resulting PCR product was cut with *Fok*I and ligated to an isolated fragment containing the downstream portion of *SNR6* from *Fok*I-digested pSE358-*SNR6* (Fortner *et al.* 1994). This ligated fragment was then subjected to PCR amplification using the U6PCR5′ and U6PCR3′ oligonucleotides as primers. The resulting PCR product was cut with *Eco*NI and *Bst*BI and ligated into *Eco*NI-*Bst*BI-digested pSE358-*snr6-A62G* (Fortner *et al.* 1994) to create pSE358-*snr6-A62G,U89C*. The resulting DNA was then transformed into *Escherichia coli* and clones were sequenced as above. pSE358-*snr6-A40G,A62G,U89C* was generated by isolating the *Eco*RI-*Bcl*I fragment from pSE358-*snr6-A40G,A62G* (Fortner *et al.* 1994) and subsequent ligation into *Eco*RI-*Bcl*I-digested pSE358-*snr6-A62G,U89C*. The resulting DNA was then transformed into *E. coli* and clones were sequenced as above. pRS317-*snr6-A62U/C85A* was previously described (Fortner *et al.* 1994). pRS426-*snr6-A62G* was constructed by isolating the *Eco*RI-*Sac*I fragment from pRS317-*snr6-A62G* (Fortner *et al.* 1994) and ligating it into *Eco*RI-*Sac*I-digested pRS426 (Sikorski and Hieter 1989).

pRS314-*PRP24* was constructed by isolating the *Spe*I-*Sna*BI fragment from pUN50-*PRP24* (*CEN4*, *URA3*; a kind gift from K. Shannon and C. Guthrie; Shannon and Guthrie 1991) and subsequent ligation into *Spe*I-*Hinc*II-digested pRS314 (*CEN6*, *TRP1*; Sikorski and Hieter 1989). pRS313-*PRP24* was constructed by isolating the *Sac*I-*Xho*I fragment from pRS314-*PRP24* and subsequent ligation into *Sac*I-*Xho*I-cut pRS313 (*CEN6*, *HIS3*; Sikorski and Hieter 1989). pRS314-*PRP24* and pRS313-*PRP24* were amplified and mutagenized by oligonucleotide-directed mutagenesis as above. pRS314-*PRP24* was mutagenized using oligonucleotides PRP24-RRM1sub, PRP24-RRM2sub, and PRP24-RRM3sub. pRS313-*PRP24* was mutagenized with PRP24-A474C and PRP24-T769A. The resulting DNA was then transformed into *E. coli* and clones were sequenced using primers PRP24-920-904 and PRP24-Seq1. The pRS314-*PRP24-RRM3sub* clones that resulted from this mutagenesis contained a deletion of a base pair at position 741, so site-directed mutagenesis was carried out using the PRP24-RRM3sub+A primer to add back the deleted base pair. All mutant *PRP24* alleles were subcloned into a wild-type *PRP24* plasmid with the same vector backbone to ensure that only the desired mutation was present.

The *ADE2* gene was obtained from pRS316-*ADE2* (a kind gift from Phil James, UW-Madison) by digesting with *Ecl136*II and *Cla*I. This fragment was ligated into pUN50-*PRP24* that had been digested with *Cla*I and *Sna*BI to remove part of the Prp24 coding sequence, to yield plasmid pUN50-*prp24-Δ1::ADE2*. pRS306-*prp24-T769A* was created by isolating the *Sac*I-*Xho*I fragment from pRS313-*prp24-T769A* and subsequent ligation into *Sac*I-*Xho*I-cut pRS306 (*URA3* integrating plasmid; Sikorski and Hieter 1989).

Yeast strains and genetic methods: Mutant alleles of *SNR6* cloned into pSE358 were tested for their ability to function as the sole U6 RNA gene in the cell by transformation (Gietz *et al.* 1995) into a yeast strain containing an insertion in the chromosomal copy of *SNR6* (DAB016; *MAT***a** *leu2 trp1 his4 ura3 snr6::LEU2* [YCpEP6]; Brow and Guthrie 1988). Trp^+ transformants of DAB016 were streaked onto synthetic dropout medium containing 0.75 mg/ml 5-fluoroorotic acid (5-FOA) to select for loss of the *URA3*-marked wild-type *SNR6* plasmid. Resulting colonies were streaked to YEPD plates at the temperatures indicated in the figure legends. All strains generated as spontaneous suppressors of U6-A62G cold sensitivity are derivatives of DAB016 containing pSE358-*snr6-A62G* (Fortner *et al.* 1994).

Construction of a *PRP24* chromosomal disruption strain was as follows: pUN50-*PRP24* was transformed into PJ51-3A (*MAT***a** *his3 leu2 trp1 ura3 met2 can1 ade2 lys2*; a kind gift from Phil James, UW-Madison) and a Ura^+ transformant was selected to create LL100. To introduce the *ADE2* gene into the chromosomal locus of *PRP24*, a linear *Bam*HI-*Sph*I fragment from pUN50-*prp24-Δ1::ADE2* was transformed into LL100, and Ade^+ transformants were selected. Disruption of the *PRP24* locus was verified by Southern blotting. The resulting Prp24 chromosomal disruption strain was named LL101 (*MAT***a** *his3 leu2 trp1 ura3 met2 can1 ade2 lys2 prp24-Δ1::ADE2* [pUN50-*PRP24*]). Mutant alleles of *PRP24* cloned into pRS314 or pRS313 were tested for their ability to function as the sole Prp24 gene by transformation into LL101, selection on −Trp or −His medium, respectively, and subsequent plating to synthetic dropout medium containing 5-FOA. A strain containing chromosomal disruptions of the U6 and Prp24 genes was generated by mating LL101 with a strain containing a replacement of the U6 gene (MWK023; *MAT*α *his3 leu2 trp1 ura3 met2 can1 ade2 lys2 snr6Δ::LEU2* [YCp50-39D6]; Eschenlauer *et al.* 1993). Ade^+Leu^+ diploids were selected and then sporulated by growth in liquid YEPA medium. Resulting spores were screened for Ade^+Leu^+ and 5-FOA-sensitive phenotypes. An Ade^+Leu^+ 5-FOA-sensitive spore was propagated and named LL200 (*MAT***a** *his3 leu2 trp1 ura3 met2 can1 ade2 lys2 prp24Δ1::ADE2 snr6Δ::LEU2* [pUN50-*PRP24*], [YCp50-39D6]). Mutant alleles of *PRP24* cloned into pRS313 were tested for their ability to function in the presence of *SNR6* alleles carried on pSE358 by cotransformation into LL200, selection on −Trp-His medium, and subsequent plating to synthetic dropout medium containing 5-FOA.

The *prp24-F257I* allele was introduced into the *PRP24* chromosomal locus via homologous recombination (Scherer and Davis 1979). MWK033 (*MAT***a** *his3 leu2 trp1 ura3 met2 can1 ade2 lys2 snr6Δ::LEU2* [pRS317-539H6 (*CEN6*, *LYS2*)], a derivative of MWK023; Eschenlauer *et al.* 1993) was transformed with *Rsr*II-linearized pRS306-*prp24-T769A*, and Ura^+ colonies were selected. Integration of this plasmid by homologous recombination at the *PRP24* locus results in a strain with a tandem duplication of the *PRP24* gene, separated by the *URA3* gene. To screen for such strains, the following assay was performed: Ura^+ colonies were grown overnight in liquid −Ura medium, and a quick DNA preparation for PCR was performed (Ling *et al.* 1995). PCR amplification was carried out using the oligonucleotides PRP24-END and URA3-1 as primers; a product is expected only if the integration event occurred at the *PRP24* locus. Southern blotting was performed on PCR-positive clones to verify integration at the *PRP24* locus. The RRM2-RRM3 region of *PRP24* from resulting cultures was amplified using the oligonucleotides PRP24-RRM2-5′ and PRP24-920-904, and PCR products were digested with *Tsp509*I, which cuts the mutant *PRP24* allele but not the wild-type allele. Those colonies that appeared to contain the *prp24-F257I* allele in addition to wild-type *PRP24* were mated to MWK038, an isogenic strain carrying wild-type *SNR6* on a *TRP1*-marked plasmid (pRS314), and Lys^+Trp^+ diploids were selected. Diploids were plated to synthetic dropout medium containing 5-FOA to select for cells that had undergone homologous intrachromosomal recombination such that one of the tandem *PRP24* alleles "popped out." Resulting colonies were screened by the amplification/restriction assay to determine if the mutation was maintained, and those with the mutation were then transformed with pRS316-*snr6-A62G* (*URA3*-marked centromeric plasmid) and plated to medium lacking uracil. The resulting colonies were plated to synthetic dropout medium containing 2 mg/ml α-aminoadipate to select for loss of the *LYS2*-marked wild-type *SNR6* plasmid. The resulting yeast were sporulated and tetrads dissected. Spores were rep-

lica plated to −Ura and −Trp media to determine which spores contained only wild-type *SNR6* (*TRP1* marked) or *snr6-A62G* (*URA3* marked). Spores that appeared to have only the *snr6-A62G* allele were assayed for the presence or absence of the Prp24-F257I mutation by the amplification/restriction screen described above. A spore that appeared to have the mutant *prp24* allele, based on the screen, was subjected to PCR to amplify the *PRP24* locus and the PCR product was sequenced directly to confirm the identity of the *PRP24* allele. The resulting strain was named RMV022 (*MATα his3 leu2 trp1 ura3 met2 can1 ade2 lys2 snr6Δ::LEU2 prp24-F257I* [pRS316-*snr6-A62G*]). Mutant alleles of *SNR6* carried on pSE358 were tested for their ability to function as the sole U6 RNA gene in the *prp24-F257I* integrant cells by transformation, selection on −Trp media, and subsequent plating to synthetic dropout medium containing 5-FOA.

Oligonucleotides:

URA3-1: 5′-dGGATAATGCGTTTAGCGG;
U6G: 5′-dCGAGACAATTTTCTATTCGAG;
U6-U36A,U37A: 5′-dCTGTATTGTTTCATTTTGACCAAAT GTCC;
U6-A41U,A42U: 5′-dCATCTCTGTATTGAATCAAATTGACC;
U6-U87A,U88A: 5′-dCTCTTTGTAATTCGGTTCATCC;
U6-U89C: 5′-dCTCTTTGTAGAACGGTTCATCC;
U6-C92U: 5′- dCGAAATAAATCTCTTTATAAAACGGTTC;
U6-A94U,A95U: 5′-dCGAAATAAATCTCAATGTAAAACGG TTC;
PRP24-RRM2-5′: 5′-dCGCCATAGTACAGAATGCACATTA TGG;
PRP24-577-596: 5′-dCCAATCCGCTGGAAAAGTCG;
PRP24-920-904: 5′-dGCTCTTACGCGATGCGAG;
PRP24-Seq1: 5′-dGGAGTATGGACATCACGC;
PRP24-Seq4: 5′-dCGCCCCTCTAGTGTGGC;
PRP24-END: 5′-dCTAGGTGAGTAGACTAACCG;
PRP24-A474C: 5′-dCGATTCAATACAAGCAGCAGGTTCGC TTAC;
PRP24-T769A: 5′-dCTGTTGTGCAATTATGGTTTTTG;
PRP24-RRM1sub: 5′-dCGCTGAAGAAGAACGCTCGTGCTG CAGCTATTGAATTTGCC;
PRP24-RRM2sub: 5′-dCGATTCAATACAAGCGCTAGGGCT GCTGCTATCGATGTTAC;
PRP24-RRM3sub: 5′-dGAGCACAGTTTCAATGCTTGTGCT GCAGCTATGGTTTTTG;
PRP24-RRM3sub+A: 5′-dGAAAGAGCACAGTTTCAAT GCTTG;
U1-SH, U6-SH (Kuhn *et al.* 1999);
U4B, U6D (Li and Brow 1993);
U4PCR5′, U4PCR3′, U6PCR5′, U6PCR3′ (Li and Brow 1996).

RNA analysis: Total cellular RNA was obtained as previously described (Treco 1989). All strains were grown at the indicated temperatures in YEPD broth. Solution hybridization was done essentially as described (Li and Brow 1993) using 1.5–2 μg of RNA and 0.1 pmol ^{32}P-labeled oligonucleotide U1-SH, U6-SH, or U6D, and/or U4B in each reaction. Oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (United States Biochemical, Cleveland) and separated from unincorporated nucleotides using a TE MIDI Select-D, G-25 microcentrifuge spin column (5 prime → 3 prime) according to manufacturer instructions. Oligonucleotide/RNA hybrids were separated by electrophoresis at 4°, 300 V on a 10-cm-tall, 1-mm-thick, 6 or 9% polyacrylamide nondenaturing gel [30:1 acrylamide/bisacrylamide (BDH Electran) in 50 mm Tris-borate, pH 8.3 and 1 mm EDTA] for ~1–1.5 hr. Gels were exposed to Biomax MR film (Eastman Kodak, Rochester, NY) with or without a Cronex Lightning-Plus screen (DuPont, Wilmington, DE). Gels were quantitated using a Molecular Dynamics (Sunnyvale, CA) Phosphorimager and ImageQuant software.

Isolation and characterization of *SNR14* and *PRP24* alleles: Complementation of the temperature-sensitive phenotype of suppressor *snr6-A62G*, *snr14-A16G* (DMF110) was performed using plasmid YCp50-*SNR14* (*CEN4*, *URA3*; Shannon and Guthrie 1991). Dideoxy sequencing of U4 RNA from total cellular RNA of this strain was performed as described (Brow and Guthrie 1990) using the U4B oligonucleotide. The U4 coding sequence from DMF110 was cloned into the *Eco*RI site of pUC118 by amplifying genomic DNA (Hoffman and Winston 1987) with primers U4PCR5′ and U4PCR3′. The M13 universal primers (New England Biolabs, Beverly, MA) were used to sequence the U4 RNA coding sequence. Complementation of the temperature-sensitive phenotypes of suppressor strains *snr6-A62G, prp24-R158S* (DMF111) and *snr6-A62G, prp24-F257I* (DMF112) was performed using plasmid pUN50-*PRP24*. The region of *PRP24* containing RRM3 was sequenced from gel-purified fragments generated by amplification of genomic DNA with primers PRP24-577-596 and PRP24-920-904. The region of *PRP24* containing RRMs 1 and 2 was sequenced from amplified DNA generated with primers PRP24-Seq1 and PRP24-Seq4. Nonconditional U6-A62G suppressor strains were screened for mutations in *PRP24* by PCR amplification and sequencing of RRMs 1–3 using the primers PRP24-Seq1 and PRP24-920-904.

RESULTS

Identification of extragenic suppressors of U6-A62G: Only 31 of 109 spontaneous suppressors of the U6-A62G cold sensitivity are *cis*-acting (Fortner *et al.* 1994); thus the other 78 are in genes other than *SNR6*. Four of the extragenic suppressor strains, DMF110–113, also displayed a temperature-sensitive phenotype (Figure 2). In one strain, DMF113, the weak temperature sensitivity and suppression are not linked, so this strain was not studied further. In another strain, DMF110, complementation of the temperature sensitivity was observed upon transformation with a plasmid containing the wild-type U4 RNA gene (data not shown). Sequencing of U4 RNA and its gene from DMF110 revealed a single mutation, A16G (Figure 3). Interestingly, U4-A16G is the only identified *cis*-acting suppressor of the cold-sensitive phenotype of the U4-G14C mutation, which destabilizes stem II of the U4/U6 complex (K. Shannon and C. Guthrie, personal communication). While the mechanism of suppression by U4-A16G is unknown, the ability of a U4 RNA mutation to suppress the cold sensitivity of U6-A62G strongly suggests that the cold-sensitive block occurs when U4 is present, *i.e.*, between formation and dissociation of the U4/U6 complex.

The two remaining temperature-sensitive suppressor strains, DMF111 and DMF112, were tested for complementation by *PRP24*. After transformation with a plasmid bearing the wild-type *PRP24* gene, the two suppressor strains were able to grow at wild-type rates at 37° (data not shown), suggesting that the *PRP24* locus contains the suppressor mutations in these strains. Sequencing of fragments amplified from genomic DNA with primers flanking the three RRMs of *PRP24* (Figure 4A)

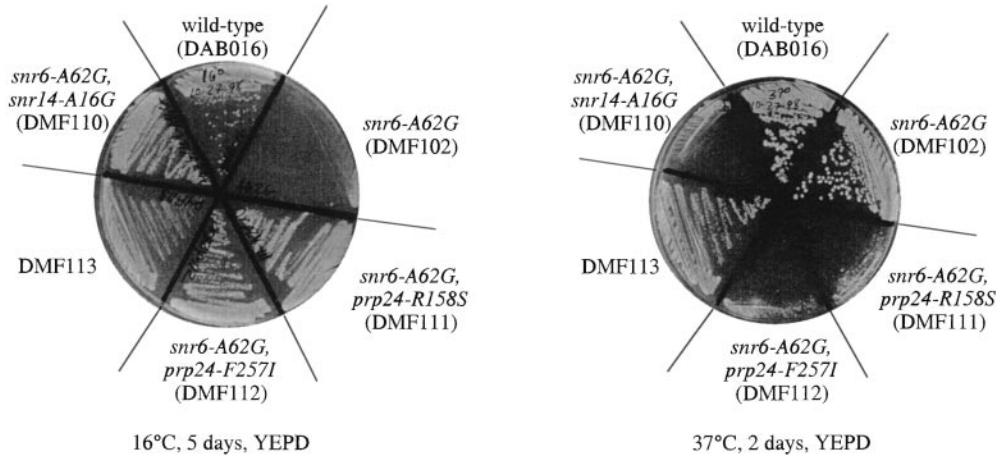


Figure 2.—Four extragenic suppressors of the cold-sensitive growth defect of *snr6-A62G* have temperature-sensitive phenotypes. Shown are the parental, wild-type strain; the original *snr6-A62G* strain; and the spontaneous suppressor strains DMF110–113, incubated on YEPD medium at 16° for 5 days and at 37° for 2 days. Identified mutations are indicated (see text).

revealed a single base change in RRM2 of one strain and in RRM3 of the other. The RRM2 mutation results in a change of an arginine codon at amino acid position 158 to a serine codon, while the RRM3 mutation results in a change of a phenylalanine codon at amino acid position 257 to an isoleucine codon (Figure 4B, solid boxes).

To determine if some of the nonconditional suppressor strains also have mutations in Prp24, we sequenced ~900 bp (containing all three RRMs) of the *PRP24* gene from 12 randomly selected *trans*-suppressor strains. In 5 of these 12 strains, a mutation in either RRM2 or RRM3 of *PRP24* was found (Figure 4B, open boxes). This result implies that approximately half of the 78 extragenic suppressor strains contain mutations in Prp24. These mutations represent the first genetic interaction identified between U6 RNA and Prp24.

The availability of the crystal structure of the amino-terminal RRM of the U1A protein (Oubridge *et al.* 1994) allowed us to model the positions of the Prp24 suppressor mutations onto the RRM structure (Figure 4C). Strikingly, the mutations localize to the RNA-binding surface of the motif (reviewed in Varani and Nagai 1998). For example, arginine 158 of Prp24 is predicted to occupy a position analogous to arginine 52 of the U1A protein, which hydrogen-bonds directly to two bases in cocrystals with its target RNA (Oubridge *et al.* 1994). In the U2B″ protein, the equivalent arginine side chain also interacts directly with the RNA target (Price *et al.* 1998). The R52Q mutation in U1A has previously been shown to destroy specific RNA binding (Nagai *et al.* 1990); thus we expect the R158S mutation to reduce binding of Prp24 to its target RNA(s). Phenylalanine 257 of Prp24 is predicted to be equivalent to phenylalanine 56 of U1A, which base-stacks with the target RNA (as does the analogous phenylalanine residue in U2B″),

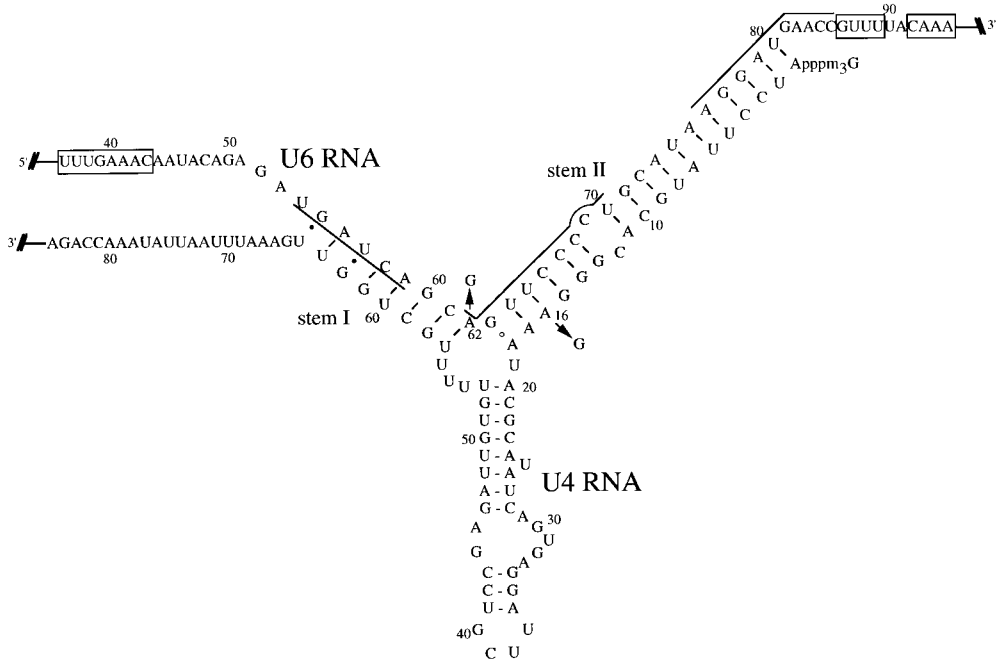
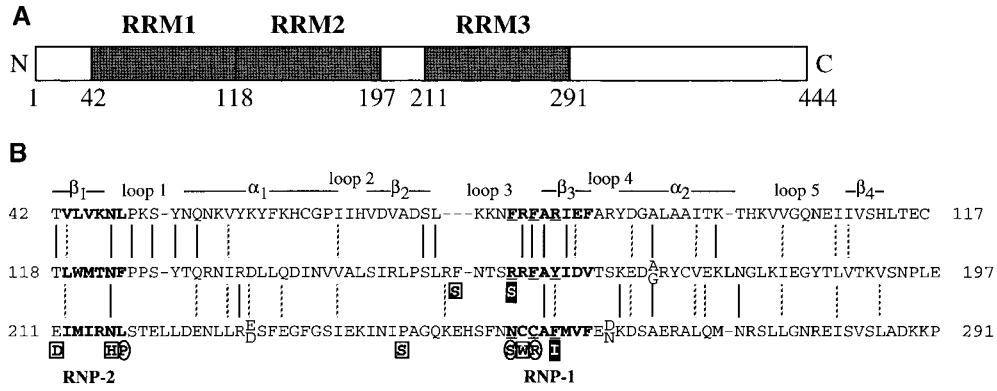


Figure 3.—Secondary structure model of the yeast U4/U6 RNA interaction. Positions of the U6-A62G cold-sensitive mutation and U4-A16G suppressor mutation are indicated by arrows. The boxed regions of U6 RNA form the telestem, the overlined regions form the 3′ stem, and the underlined region forms the 3′ strand of the central stem (see Figure 1).



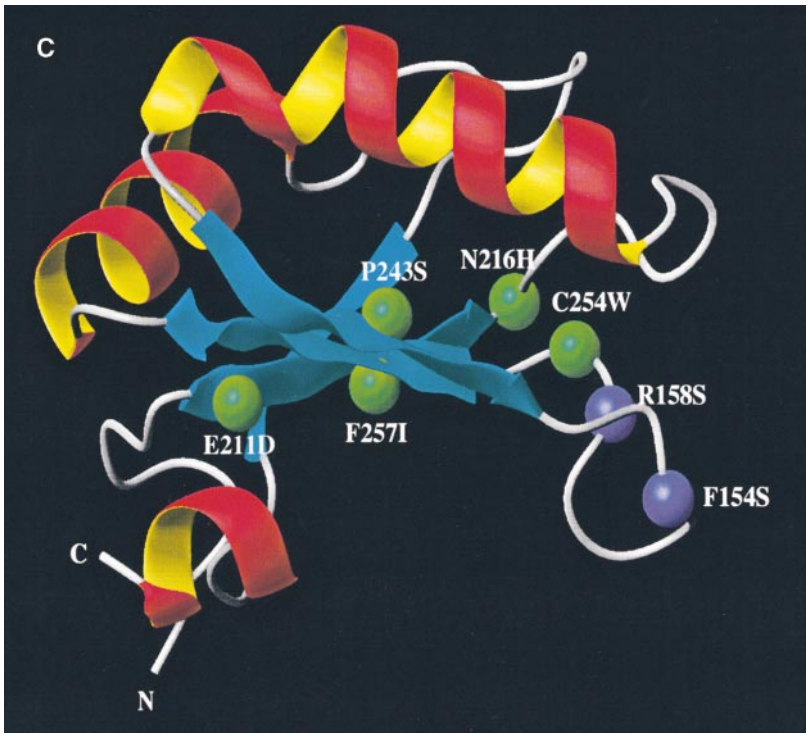


Figure 4.—Sites of suppressor mutations in Prp24. (A) Schematic diagram of the primary structure of Prp24. Numbers refer to amino acid residues. The RNA-recognition motifs (RRMs) are shown as shaded boxes. RRM1 and RRM2 overlap by one amino acid, residue 118. (B) Amino acid sequence of Prp24 RRMs 1–3, with the mutations discussed in the text indicated. Where the deduced amino acid sequence from the parental DAB016 strain differs from that in the yeast genome database, the DAB016 sequence is on the bottom. All spontaneous suppressor mutations are single-site substitutions. U6-A62G-suppressor mutations are boxed, with a black background indicating an additional, temperature-sensitive phenotype. Suppressors of U4-G14C (Shannon and Guthrie 1991) are outlined in ovals. The sites of triple-alanine substitutions made in each RNP-1 domain are underlined. Solid vertical lines indicate identical amino acids conserved between the RRMs. Dashed vertical lines indicate similar amino acids (I = V = L; F = Y = W; Q = N; Q = E; D = E; N = D; R = K; S = T). Likely regions of β-strands, loops, and α-helices are indicated (based on the alignment from Kenan *et al.* 1991). (C) Locations of U6-A62G suppressor mutations in Prp24 RRMs 2 and 3 were modeled on the crystal structure of the N-terminal RRM of U1A (Oubridge *et al.* 1994) using MolMol. The α-carbons of mutant residues in RRMs 2 and 3 are indicated as purple and green spheres, respectively. A side view of the RNA-contact surface is shown, with the RNA-binding site at the bottom.

and the F56L mutation in the related U1-70K protein greatly decreases RNA binding (Kenan *et al.* 1991). Because the F257I mutation would destroy Prp24's ability to base-stack this residue with its target RNA, it is likely that this mutation weakens RNA binding. The other suppressor mutations are also predicted to lie on the RNA-binding face, so nonconservative changes in these residues are also likely to result in decreased binding of Prp24 to its target RNA(s).

prp24-R158S and -F257I are each sufficient for suppression of U6-A62G cold sensitivity and confer temperature sensitivity: To confirm that the *PRP24* mutations identified in the temperature-sensitive strains are responsible for suppression of the U6-A62G cold-sensitive phenotype, we introduced each of these mutations into plasmid-borne *PRP24* by site-directed mutagenesis. The plasmid bearing *prp24-R158S* or *prp24-F257I* was introduced, along with a plasmid containing *snr6-A62G*, into a strain (LL200) with chromosomal Prp24 and U6 gene disruptions complemented by wild-type *PRP24* and *SNR6* alleles on separate *URA3*-marked plasmids. After plating to medium containing 5-FOA to select for loss of the wild-type *PRP24* and *SNR6* plasmids (plasmid shuffle), both the *prp24-R158S* and *prp24-F257I* alleles were found to be capable of suppressing the U6-A62G growth defect at 16° (Figure 5A), showing that each mutation is responsible for the suppressor phenotype of the original strains.

The 37° growth phenotypes of *prp24-R158S* and *prp24-F257I* were tested in a strain (LL101) containing a chromosomal *PRP24* disruption, complemented by wild-type *PRP24* on a *URA3*-marked plasmid, with the wild-type

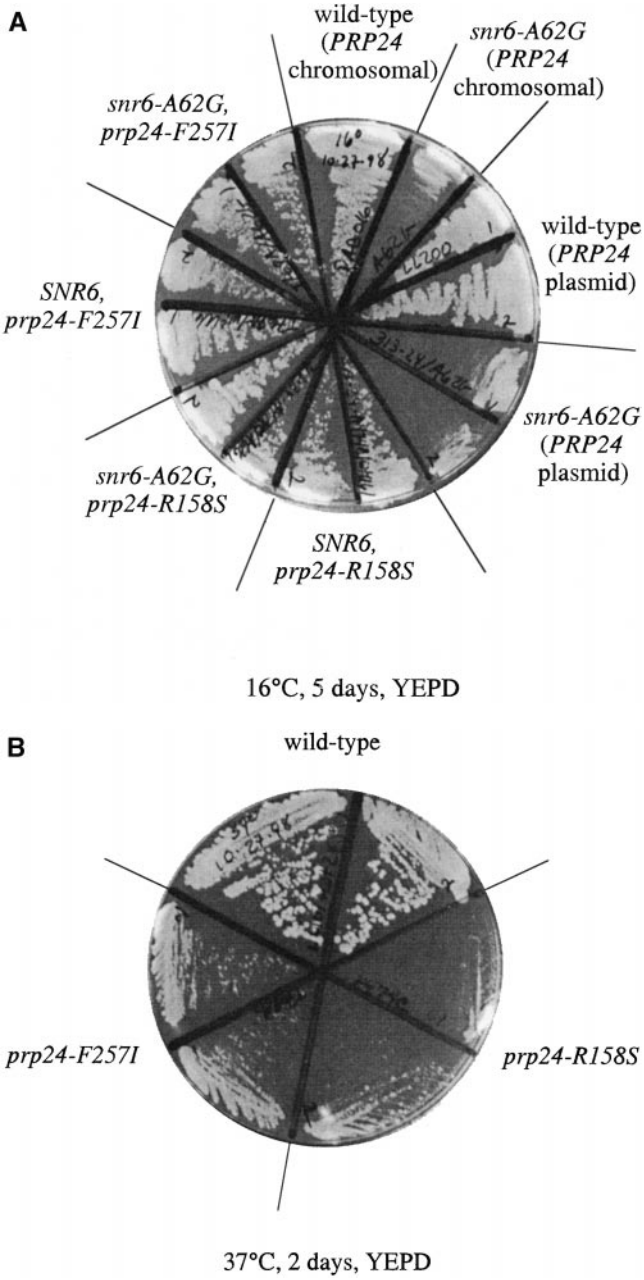


Figure 5.—Phenotypes of single-site mutants *prp24-R158S* and *prp24-F257I*. (A) *prp24-R158S* and *prp24-F257I* each suppress the U6-A62G cold sensitivity. Each allele of *PRP24* was introduced, along with *SNR6* or *snr6-A62G*, into a strain with chromosomal disruptions of the Prp24 and U6 genes (LL200) and plated to 5-FOA to select against the wild-type Prp24 and U6 genes carried on *URA3*-marked plasmids. Shown are the resulting strains plated to YEPD at 16° for 5 days. Two colonies were plated from each transformation. A wild-type *PRP24* strain (DAB016) with *SNR6* or *snr6-A62G* is included as a control. (B) *prp24-R158S* and *prp24-F257I* each confer a temperature-sensitive phenotype. Each allele of *PRP24* was introduced into a strain with a chromosomal disruption of the Prp24 gene (LL101) and subsequently plated to 5-FOA to select against the wild-type Prp24 gene carried on a *URA3*-marked plasmid. Shown are two clones of each resulting strain plated to YEPD at 37° for 2 days.

U6 gene at the chromosomal locus. After plasmid shuffle, both *prp24-R158S* and *prp24-F257I* conferred a temperature-sensitive growth phenotype, although the phenotype caused by the *prp24-F257I* allele was less severe (Figure 5B), showing that each of these individual mutations is responsible for the associated temperature sensitivity of the original suppressor strains. Because the temperature-sensitive phenotypes appear in the presence of wild-type U6 RNA, the temperature sensitivity is dependent on the mutant Prp24 protein itself rather than on a specific interaction of Prp24 with U6-A62G RNA.

To test if the temperature sensitivity of the *prp24-F257I* allele is more severe when restricted to single copy, the *prp24-F257I* allele was integrated into the chromosome. The integrated allele exhibits both suppression of the U6-A62G cold sensitivity and the temperature-sensitive phenotype (data not shown). Colony sizes of these cells appeared slightly smaller at 37° than those from the strain containing the plasmid-borne *prp24-F257I* allele, indicating a modest effect of copy number on the temperature-sensitive growth phenotype.

Substitution of three highly conserved residues in RRMs 2 and 3, but not RRM1, of Prp24 severely affects growth: The identification of U6-A62G suppressor mutations in Prp24-RRM2 provides the first evidence for the requirement of this RRM in Prp24 function. The suppressor mutations in RRMs 2 and 3 presumably decrease the RNA binding activity of Prp24. However, because the selection demanded that the suppressor strains retain Prp24's essential function, the suppressor mutations may be hypomorphic, *i.e.*, the mutated RRMs may retain some RNA-binding activity. To systematically test if each RRM is essential, we created mutant RRMs in which three of the RNP-1 consensus residues are changed to alanine (see Figure 4B). These residues correspond to positions 52, 54, and 56 of the human U1A protein, which interact directly with the RNA target (see above). Thus, we expect that the triple-alanine mutations eliminate RNA-binding activity of each RRM of Prp24.

Each mutant *prp24* allele was transformed into a strain (LL101) that contains a chromosomal Prp24 gene disruption complemented by wild-type *PRP24* on a *URA3*-marked plasmid. After selection against the wild-type *PRP24*, the triple-alanine substitution in RRM1 (*prp24-RRM1sub*) has no appreciable effect on cell growth at any temperature tested (Figure 6). Therefore, the mutations in this RRM, predicted to greatly destabilize RNA binding, do not significantly affect cell viability. The triple-alanine mutation in RRM2 (*prp24-RRM2sub*) causes a lethal phenotype at all temperatures tested (Figure 6 and data not shown). This growth defect is likely due to disruption of RNA-binding activity of the RRM; however, we cannot rule out the possibility that the mutation causes destabilization of the protein. The analogous substitution in RRM3 (*prp24-RRM3sub*) leads to a severe temperature-sensitive growth phenotype, allowing growth at 18°, with only minimal growth at 30°

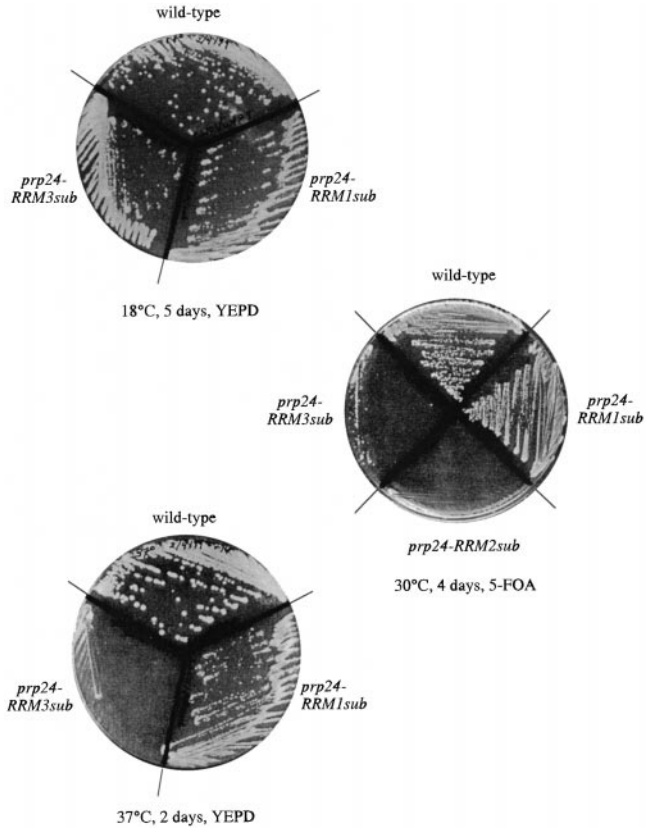


Figure 6.—Recessive growth phenotypes of *prp24-RRMsub* alleles. The *prp24-RRM1sub* mutant grows normally at all temperatures tested. The *prp24-RRM2sub* mutant does not survive at any temperature tested. The *prp24-RRM3sub* mutant is viable at 18° and only weakly so at 30°. Shown are YEPD plates incubated for 5 days at 18° or 2 days at 37°, and a 5-FOA-containing plate incubated at 30° for 4 days.

(Figure 6). The severe temperature sensitivity observed is consistent with a function for RRM3 in stabilizing an RNA structure by binding; at low temperature such stabilization may not be required. Interestingly, *prp24-RRM3sub*, like the single-site mutant *prp24-F257I*, suppresses U6-A62G cold sensitivity (data not shown), further suggesting that a decrease in binding of Prp24 to its target RNA(s) results in suppression of U6-A62G. The phenotypes of the RRM2- and RRM3-mutant strains show that these RRMs have functions in the cell that influence viability, and because these RRMs also interact genetically with U6 RNA, it seems likely that interaction with U6 RNA is at least one of the functions required for viability.

To test if the functions of RRMs 2 and 3 of Prp24 are physically separable, we assessed whether *prp24-RRM2-sub* and *prp24-RRM3sub* can rescue one another *in trans.* The two alleles, on differently marked plasmids, were transformed into the strain (LL101) described above. After selection against the wild-type *PRP24*, the strain shows a phenotype identical to the *prp24-RRM3sub* strain, showing that the two alleles do not complement one another *in trans* and *prp24-RRM3sub* is dominant to *prp24-RRM2sub.* This result implies that the essential functions of RRMs 2 and 3 are required in the same molecule.

Mutations in Prp24 affect U4/U6 complex assembly *in vivo*, but suppression of mutations in the U6 3′ stem occurs independently of this effect: As a first step toward understanding the mechanism of suppression of U6-A62G cold sensitivity by mutant Prp24 proteins, we used nondenaturing gel analysis (see Figure 7A) to examine the levels of U4/U6 complex, free U4, and free U6 RNAs in *prp24* mutant cells grown overnight at 37° (Table 1). We examined RNA levels in the presence of either a chromosomal (LL101) or plasmid-borne (LL200) U6 gene. Both *prp24-R158S* and *prp24-F257I* confer a moderate U4/U6 complex assembly defect when the U6 gene is chromosomal as judged by the increased ratio of free to total U4 RNA (Table 1). This result corroborates *in vivo* (Lygerou *et al.* 1999) and *in vitro* (Ghetti *et al.* 1995; Raghunathan and Guthrie 1998b) evidence that Prp24 is involved in the assembly of the U4/U6 complex. However, in *prp24* mutant strains with the U6 gene provided on a plasmid, a three- to fourfold increase in U6 RNA is seen, and the ratio of free U4 to U4/U6 complex is normal or even subnormal (Table 1). Presumably, increased expression of U6 RNA drives U4/U6 complex assembly by mass action. We have not examined the mechanism of U6 RNA overexpression in the *prp24* strains, but it is likely due to amplification of the low-copy-number centromeric plasmid (Bitoun and Zamir 1986).

We next tested if the mutant *prp24* alleles influence U4/U6 complex assembly in the U6-A62G strain when grown at 18°. These experiments were carried out only in the presence of a plasmid-borne U6 gene. Both of the mutant alleles of *prp24* partially correct the U4/U6-A62G complex assembly defect (Table 2). As was seen in *prp24* mutant strains grown at 37°, U6 RNA is overexpressed from plasmid-borne *SNR6* alleles in *prp24* mutant strains grown at 18° (Table 2). However, suppression of the U4/U6-A62G complex assembly defect is not due to overexpression of U6 RNA, because merely providing the U6-A62G gene on a high-copy plasmid suppresses neither the cold-sensitive phenotype nor the U4/U6-A62G complex assembly defect in a wild-type *PRP24* strain despite sixfold overexpression of U6-A62G (data not shown).

From the results described above, it is not clear if partial correction of the U4/U6-A62G assembly defect is the mechanism of suppression of cold-sensitive growth by the Prp24-RRM2 and -RRM3 mutants or simply occurs coincident with suppression. To address this issue, we examined a different U6 RNA 3′ stem hyperstabilizing mutant, U6-A62U/C85A. The U6-A62U/C85A strain has a more severe cold-sensitive phenotype than U6-A62G (Fortner *et al.* 1994) yet does not display an appreciable U4/U6 complex assembly defect (Figure

7A; compare lane 2 with lanes 3 and 4). We asked whether *prp24-R158S* or *prp24-F257I* can suppress U6-A62U/C85A cold sensitivity; both alleles do so (Figure 7B). This result clearly shows that the *prp24-R158S* and *prp24-F257I* mutations suppress hyperstabilization of the 62/85 base pair in U6 RNA, and that this suppression occurs at a stage other than U4/U6 complex assembly.

***Cis*-acting suppressors of U6-A62G define a novel U6 RNA structure:** The results described above are consistent with a model of Prp24 function first proposed by Shannon and Guthrie (1991), in which Prp24 brings U4 and U6 RNAs into proximity but antagonizes their base-pairing interaction. Our results suggest that Prp24 antagonizes U4/U6 pairing by stabilizing a competing intramolecular structure in U6 RNA. The 3′ stem of yeast U6 RNA is mutually exclusive with U4/U6 pairing (see Figures 1 and 3). Analogy to the dynamics of the human U4/U6 complex (Brow and Vidaver 1995) suggests that the telestem also contributes to a competing intramolecular structure. The isolation of many *cis*-acting suppressors of U6-A62G that are expected to disrupt the telestem (Fortner *et al.* 1994) supports this hypothesis.

We tested whether the proposed U6 RNA telestem forms *in vivo* via compensatory mutational analysis. If suppression of U6-A62G is due to disruption of the telestem, then additional mutations that restore base-pairing should revert suppression. Two individual base pairs (39/92 and 40/89) and two sets of adjacent base pairs (36/95,37/94 and 41/88,42/87) were tested (Figure 8A). Each of the mutations in one or the other strand of the duplex suppressed the U6-A62G cold sensitivity (Fortner *et al.* 1994; Figure 8B; data not shown) as expected if destabilization of the telestem is responsible for the suppression phenotype. One pair of compensatory mutations reverted the suppression phenotype: that which restored the 36/95,37/94 pairing (Figure 8B). Compensatory mutations in each of the other base pairs tested did not revert suppression (data not shown). These results indicate that at least the lower portion of the telestem in yeast U6 RNA forms *in vivo*, and that disruption of this structure is one mechanism of suppression of the U6-A62G cold sensitivity. The inability of compensatory mutations at the other locations to revert suppression does not disprove the existence of the upper telestem, because these positions may be sites where sequence-specific binding of a protein(s) is required for telestem formation and/or stabilization (Brow and Vidaver 1995; see below).

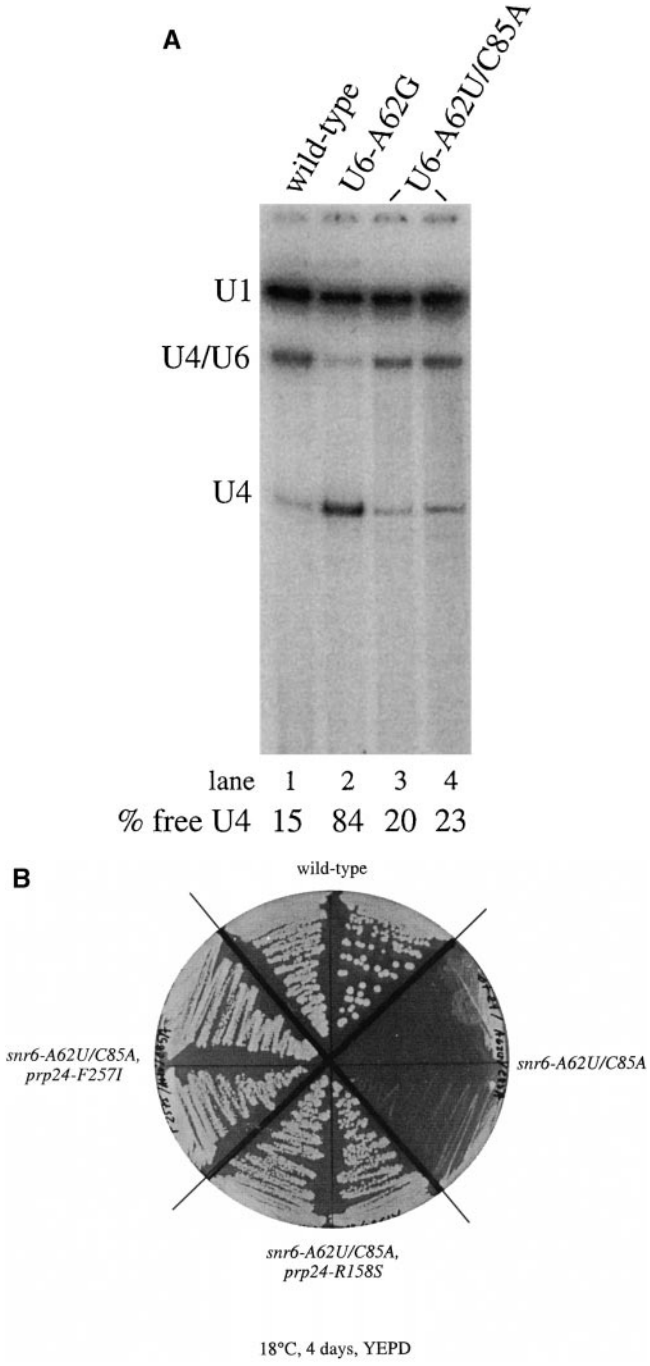


Figure 7.—The U6-A62U/C85A cold sensitivity is suppressed by *prp24-R158S* and *prp24-F257I*. (A) U4 RNA is not significantly impaired in its assembly with U6-A62U/C85A RNA. Total cellular RNA from strains grown at 30° expressing the indicated U6 RNAs was hybridized in solution to a ^{32}P-labeled oligonucleotide complementary to U4 RNA (U4B) and U1 RNA (U1-SH) and electrophoresed on a nondenaturing gel. The conditions used preserve the U4/U6 complex (Li and Brow 1993). The positions of U4/U6 and free U4 hybrids with the U4 oligonucleotide, and U1 with the U1 oligonucleotide (as a loading control) are indicated and the percentage of free U4 RNA is displayed below each lane. Two separate RNA preparations from the same strain were run in lanes 3 and 4. (B) *prp24-R158S* and *prp24-F257I* each suppress the U6-A62U/C85A cold sensitivity. Each allele of *PRP24* was introduced, along with *snr6-A62U/C85A*, into a strain with chromosomal disruptions of the Prp24 and U6 genes (LL200), and plated to 5-FOA to select against the wild-type Prp24 and U6 genes carried on *URA3*-marked plasmids. Shown are two clones of each resulting strain plated to YEPD at 18° for 4 days.

TABLE 1

prp24-R158S and prp24-F257I each confer a U4/U6 complex assembly defect at 37°

PRP24 allele	*SNR6* allele	*SNR6* location	Relative amount of U4	Relative amount of U6	Free U4/ total U4
Wild type	Wild type	Chromosome	1.0	1.0	0.092 ± 0.01
R158S	Wild type	Chromosome	0.75 ± 0.11	0.78 ± 0.05	0.28 ± 0.08
F257I	Wild type	Chromosome	0.90 ± 0.18	0.77 ± 0.16	0.34 ± 0.13
Wild type	Wild type	Plasmid	0.86 ± 0.33	0.63 ± 0.09	0.12 ± 0.07
R158S	Wild type	Plasmid	0.83 ± 0.17	2.9 ± 1.6	0.018 ± 0.016
F257I	Wild type	Plasmid	0.87 ± 0.26	4.4 ± 0.42	0.06 ± 0.07

Averages and standard deviations from three or more experiments using at least two RNA preparations are shown.

If destabilization of the telestem suppresses U6-A62G cold sensitivity solely by allowing U6 RNA to base-pair with U4 RNA more efficiently, we would expect substantial reversion of the U4/U6-A62G complex assembly defect in the presence of telestem mutations. However, in accordance with the behavior of spontaneous telestem mutants (Fortner *et al.* 1994), U4/U6 complex assembly was only marginally rescued by site-directed mutations that disrupt the telestem as evidenced by an intermediate fraction of free U4 RNA (40–50% free U4; data not shown). Because the telestem mutations only partially revert the U4/U6-A62G assembly defect, yet fully suppress the U6-A62G cold sensitivity, the telestem must influence a step other than U4/U6 complex assembly.

Synthetic interactions between mutant *PRP24* and *SNR6* alleles: Modification/protection studies suggest that the region comprising the 5′ strand of the telestem is a binding site for Prp24 (Ghetti *et al.* 1995; Jandrositz and Guthrie 1995), while immunoprecipitation studies implicate residue 91, which lies in the 3′ strand of the telestem helix, in Prp24 binding (Shannon and Guthrie 1991). If the telestem of U6 RNA is stabilized by Prp24 binding, then mutations in Prp24 that disrupt binding to the telestem are predicted to be suppressors of U6-A62G cold sensitivity.

To test if U6-A62G suppressor mutations in RRMs 2 or 3 disrupt Prp24 binding to the telestem, we asked if either the *prp24-R158S* or *prp24-F257I* temperature sensitivity is enhanced by mutations that disrupt the base-pairing potential of the U6 RNA telestem. The *prp24-R158S* plasmid-borne allele was combined with plasmids bearing various mutant alleles of *SNR6* in a strain with chromosomal *PRP24* and *SNR6* disruptions (LL200). When combined with the *prp24-R158S* mutant, mutations that disrupt the base-pairing potential of the telestem of U6 RNA result in synthetic lethality or severely impaired viability (Figure 9). The presence of the U6-A62G mutation does not alter the synthetic interaction (data not shown). Furthermore, when the base-pairing potential of the telestem is restored by compensatory mutation, enhancement is abolished, underscoring the importance of base-pairing rather than sequence. Because these results are independent of the U6-A62G mutation, the telestem appears to form in wild-type U6 RNA as well as in U6-A62G RNA. We carried out a similar experiment with the strain containing the integrated allele of *prp24-F257I*. When mutations in the telestem of U6 RNA were introduced into this strain, no phenotypic changes were observed (Figure 9). The observation that synthetic enhancement of the *prp24-R158S* phenotype but not the *prp24-F257I* phenotype

TABLE 2

prp24-R158S and prp24-F257I each partially correct the U4/U6-A62G complex assembly defect at 18°

PRP24 allele	*SNR6* allele	*SNR6* location	Relative amount of U4	Relative amount of U6	Free U4/ total U4
Wild type	Wild type	Plasmid	1.0	1.0	0.15 ± 0.04
R158S	Wild type	Plasmid	1.2 ± 0.33	3.0 ± 1.3	0.064 ± 0.02
F257I	Wild type	Plasmid	1.2 ± 0.39	2.4 ± 1.5	0.10 ± 0.04
Wild type	A62G	Plasmid	0.61 ± 0.14	1.6 ± 0.93	0.64 ± 0.05
R158S	A62G	Plasmid	0.86 ± 0.06	3.6 ± 1.8	0.28 ± 0.05
F257I	A62G	Plasmid	1.3 ± 0.57	4.3 ± 1.9	0.28 ± 0.03

Averages and standard deviations from three or more experiments using at least two RNA preparations are shown.

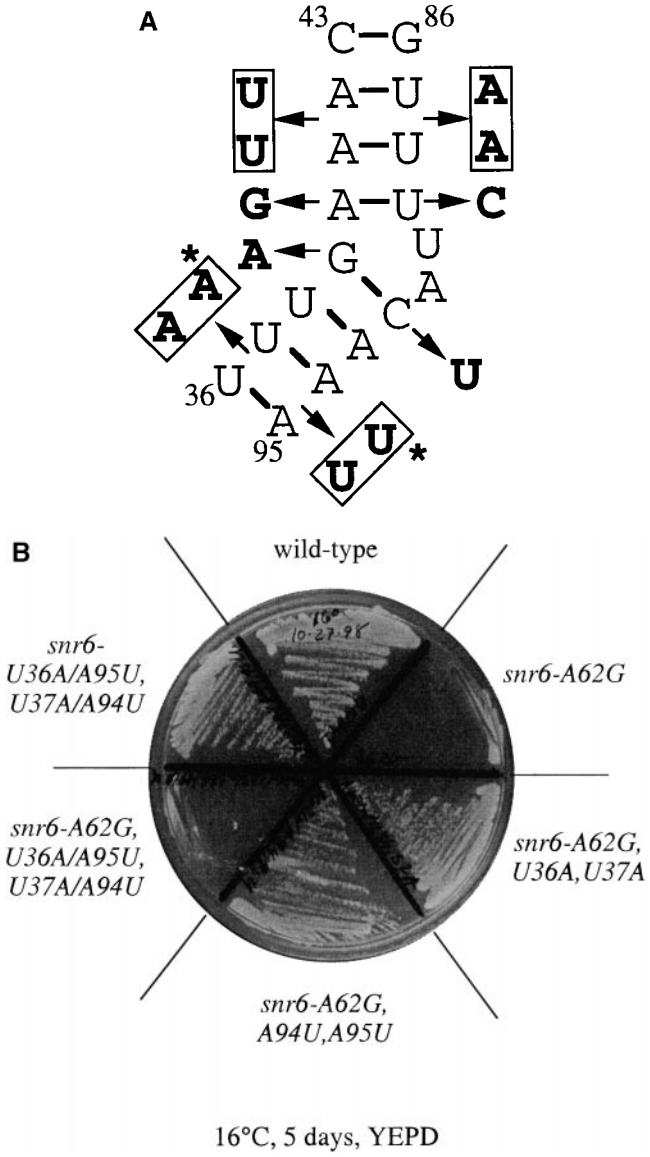


Figure 8.—Compensatory mutational analysis of the yeast U6 RNA telestem. (A) Sites of tested compensatory mutations in the yeast U6 RNA telestem. Shown is the secondary structure of the proposed telestem. Arrows indicate tested base substitutions (double mutations are boxed). In all cases, mutations on a single side of the telestem resulted in suppression of U6-A62G cold sensitivity. The combination of compensatory mutations marked with asterisks reverted suppression. (B) Compensatory mutational analysis supports formation of part of the telestem *in vivo.* The strains shown are isogenic (DAB016) except for their *SNR6* allele, which is indicated. The strains were incubated on YEPD medium at 16° for 5 days.

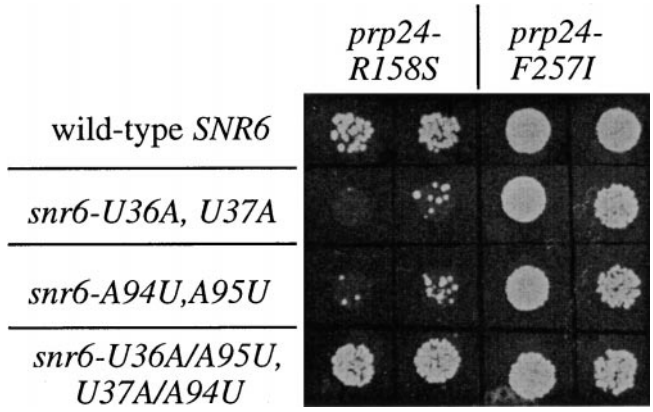


Figure 9.—The *prp24-R158S* mutation exhibits an enhanced phenotype when combined with mutations in the telestem of U6 RNA, while the *prp24-F257I* mutation does not. The *prp24-R158S* allele was introduced, along with various *SNR6* alleles, into a strain with chromosomal disruptions of the Prp24 and U6 genes (LL200), and plated to 5-FOA to select against the wild-type Prp24 and U6 genes carried on *URA3*-marked plasmids. The *prp24-F257I* integrant strain was transformed with various *SNR6* alleles and plated to 5-FOA to select against the U6 gene carried on a *URA3*-marked plasmid. Two clones of each resulting strain are shown. The plate was incubated at 27° for 3 days.

occurs when telestem base-pairing is disrupted suggests that RRM2 has a greater influence on the stabilization and/or formation of the telestem than does RRM3.

DISCUSSION

Mutations in Prp24 give insight into essential functions of the protein: We have identified missense mutations in RRMs 2 and 3 of Prp24 that suppress the cold sensitivity resulting from the A62G mutation in U6 RNA. Furthermore, we find that triple alanine substitutions in RRMs 2 and 3 cause severe growth defects. RRM 3 of Prp24 was previously shown to be important for U6 binding (Shannon and Guthrie 1991; Jandrositz and Guthrie 1995), but no function had been ascribed to RRM2. We conclude that RRMs 2 and 3 perform important, nonredundant functions that likely include binding to U6 RNA. Although the triple alanine substitution in RRM1 of Prp24 has no effect on cell growth, we cannot exclude the possibility that RRM1 has an essential function. For example, RRM1 may participate in protein-protein interactions that are not perturbed by the alanine substitutions in the RNP-1 consensus.

One role of RRMs 2 and 3 of Prp24 is to promote U4/U6 complex assembly. Both *prp24-R158S* and *prp24-F257I* mutants confer a U4/U6 complex assembly defect as judged by an increased fraction of free U4 RNA when the U6 gene is chromosomal. These results are in agreement with earlier *in vitro* studies (Ghetti *et al.* 1995; Raghunathan and Guthrie 1998b) and with the finding of Lygerou *et al.* (1999) that the *prp24-1* mutation, which has not been mapped, inhibits U4/U6 complex assembly *in vivo*. Interestingly, in *prp24-R158S* and *prp24-F257I* mutant strains harboring a plasmid-borne U6 gene, free U4 RNA levels are low and U6 RNA levels are greatly elevated. This observation indicates that wild-type U6 RNA overexpression can drive U4/U6 complex assembly in mutant *PRP24* strains. From this we infer that Prp24's primary role in U4/U6 complex assembly

is to increase the probability of interaction of the RNAs, likely by binding to each, either directly or through another protein.

Although the RRM2 and RRM3 mutations in Prp24 inhibit wild-type U4/U6 complex assembly, they nevertheless partially correct the U4/U6 complex assembly defect caused by U6-A62G. The observation that overexpression of U6-A62G RNA suppresses neither the cold-sensitive phenotype nor corrects the U4/U6-A62G complex assembly defect leads us to propose that the rate-limiting step in U4/U6-A62G complex assembly is base-pairing rather than the apposition of the two RNAs. Weakened binding of Prp24 to U6-A62G RNA presumably destabilizes the telestem, thus allowing U6-A62G RNA to adopt a conformation favorable for pairing with U4 RNA.

It is clear, however, that Prp24 also functions later in the splicing cycle. Prp24-mediated suppression of the cold sensitivity caused by hyperstabilization of the U6 RNA 62/85 base pair occurs at a stage after assembly of the U4/U6 complex: U6-A62U/C85A confers little or no U4/U6 complex assembly defect, yet its cold-sensitive growth defect is efficiently suppressed by *prp24-R158S* and *prp24-F257I*. Thus, these Prp24 mutations alleviate the effect of hyperstabilization of the U6 RNA 62/85 base pair during more than one of U6 RNA's conformational changes.

A potential point of action for suppressors of U6-A62G cold sensitivity: If, as our results indicate, suppression of the U6-A62G mutation is not due to correction of the U4/U6 assembly defect, then at what point in the splicing cycle do the suppressors act? One intriguing possibility is that there is a free U6 RNA intermediate containing the 3′ stem that forms after U4 RNA dissociates from U6 RNA but before U6 RNA base-pairs with U2 RNA. Alteration or premature formation of this structure by hyperstabilization of the 62/85 base pair of U6 RNA could have detrimental effects on the progression of spliceosome activation, and suppressor mutations may counteract these effects by destabilizing the structure.

The yeast U6 RNA telestem/Prp24 complex might form immediately prior to unwinding of the U4/U6 complex during spliceosome activation, promoting both formation of the central and 3′ stem loops in U6 RNA and concomitant disruption of U4/U6 base-pairing (see Figures 1 and 3). U6 RNA thus freed from U4 RNA then base-pairs to U2 RNA to form the active catalytic core. In the presence of the U6-A62G mutation, the free U6 RNA intermediate may form prematurely and thus be unable to pair properly with U2 RNA. Disruption of base-pairing elsewhere in the extended intramolecular structure, for example, in the telestem, may suppress the effect of U6-A62G by destabilizing the U6 RNA intermediate, thus delaying its formation until the appropriate point in spliceosome activation.

Several lines of evidence point to the yeast protein Brr2/Rss1/Slt22/Snu246, recently renamed Prp44, as the helicase responsible for unwinding the U4/U6 complex during spliceosome activation (Laggerbauer *et al.* 1998; Raghunathan and Guthrie 1998a; Kim and Rossi 1999; Kuhn *et al.* 1999). However, because this protein is a component of the U4/U6.U5 tri-snRNP (Lauber *et al.* 1996; Stevens and Abelson 1999), there must be a regulatory mechanism to prevent premature dissociation of the U4/U6 complex on the spliceosome. Perhaps formation of the U6 RNA telestem/Prp24 complex is required for efficient progression of U4/U6 complex dissociation on the fully assembled yeast spliceosome. Examples of RRM-containing proteins influencing the action of a helicase exist, notably the interaction between eukaryotic initiation factor 4B (eIF4B) and eIF4A. eIF4A, a helicase, and eIF4B, an RRM-containing protein, act in a concerted manner to unwind structured 5′ untranslated regions of mRNAs to allow initiation of translation (reviewed in Sonenberg 1998). Thus, it seems reasonable to hypothesize that, akin to the eIF4A/eIF4B complex, Prp44 and Prp24 act in a concerted fashion to dissociate the yeast U4/U6 complex.

A model for Prp24 function: Our data support a model for Prp24 action in the spliceosome, shown in Figure 10, that also incorporates previously defined interactions of Prp24 with other splicing factors. In the fully assembled precatalytic spliceosome, U1 RNA is paired with the 5′ splice site and U4 RNA is paired with U6 RNA. Recent findings indicate that Prp28 unwinds U1 RNA from the pre-mRNA 5′ splice site during spliceosome activation (Staley and Guthrie 1999), and the *prp24-1* allele exhibits a synthetic lethal phenotype when combined with the *prp28-1* allele (Strauss and Guthrie 1991). Thus, it seems likely that Prp24 is present when U6 RNA replaces U1 RNA at the 5′ splice site. The telestem/Prp24 complex could form while U4/U6 base-pairing is intact, either before or after U6 RNA base-pairs to the 5′ splice site. Formation of the telestem would not interfere with interaction of the essential ACAGA sequence with the 5′ splice site (reviewed in Nilsen 1998; see Figure 1), nor with base-pairing of the adjacent U46 with intron position 7 as predicted for the second most abundant class of *S. cerevisiae* introns (Spingola *et al.* 1999). It was previously proposed that unwinding of U1 RNA from the 5′ splice site and U4/U6 unwinding are coupled (Laggerbauer *et al.* 1998; Kuhn *et al.* 1999; Staley and Guthrie 1999). The *prp28-1*/*prp24-1* synthetic interaction can thus be explained if telestem/Prp24 complex formation is required for U4/U6 unwinding: reduction in function of both Prp24 and Prp28 would synergistically inhibit spliceosome activation. Because the U4-cs1 mutation in U4 RNA inhibits U4/U6 complex disassembly in the spliceosome (Li and Brow 1996; Kuhn *et al.* 1999), we predicted that this mutation would also enhance mutations in Prp24 if the telestem/Prp24 complex functions as proposed. Indeed, a synthetic lethal interaction is observed between U4-cs1 and either *prp24-R158S* or *prp24-F257I* (A. N. Kuhn and D. A. Brow, unpublished

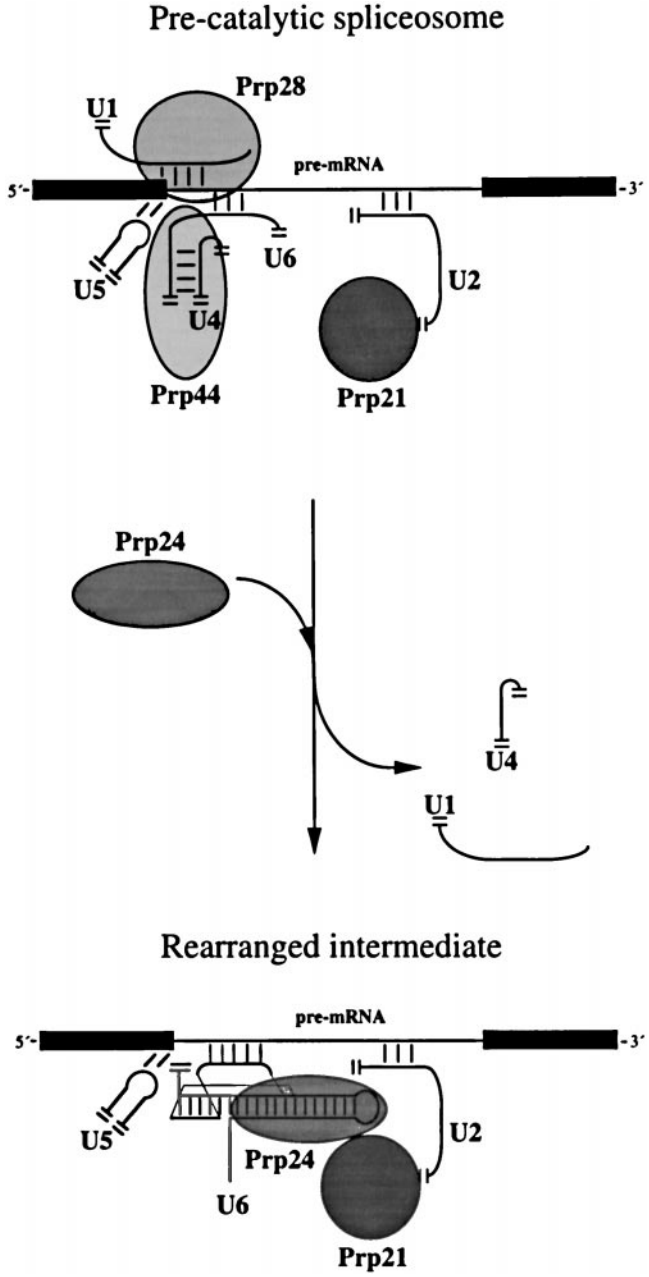


Figure 10.—Model of Prp24/U6 RNA telestem action in the spliceosome. Exons are shown as black boxes, and all other RNAs are shown as lines. Approximate sites of base-pairing interactions are shown as short lines. Proteins are depicted as filled ovals. See text for a full description.

results), strengthening the notion that Prp24 is involved in U4/U6 complex disassembly.

Further evidence for a spliceosomal function of Prp24 comes from the isolation of a mutation in Prp21, a U2 snRNP protein, that suppresses *prp24-1* temperature sensitivity (Vaidya *et al.* 1996). This result raises the possibility of an interaction between Prp24 and Prp21 prior to or concomitant with formation of the U6/U2/pre-mRNA complex, before catalysis. Taken together, the genetic interaction results suggest the following series of events whose order is not clear: Prp24 binds U6 RNA and forms the telestem, Prp28 unwinds the U1/5′ splice site helix, U6 RNA base-pairs to the 5′ splice site, Prp44 unwinds the U4/U6 complex, and the central and 3′ stem loops of U6 RNA form. The rearranged intermediate thus formed (Figure 10) is not yet active for splicing, because U6 RNA has not yet paired to U2 RNA. We propose that mutations that hyperstabilize the 62/85 base pair of the U6 RNA 3′ stem block spliceosome activation at this point.

The model presented in Figure 10 does not include all of the proteins likely involved in rearrangements of U6 RNA on the spliceosome. We recently showed that Prp8 influences U4/U6 complex dissociation and perhaps unwinding of U1 RNA from the 5′ splice site as well (Kuhn *et al.* 1999). Because Prp8 has been crosslinked to the GU dinucleotide of the 5′ splice site (Reyes *et al.* 1996), and our model predicts the U6 RNA telestem would form adjacent to the 5′ splice site, there is potential for an interaction between Prp8 and the U6 RNA telestem/Prp24 complex. Other factors, including Prp4, Prp19, and Prp38 (Tarn *et al.* 1993; Ayadi *et al.* 1997; Xie *et al.* 1998), are also thought to function in U4 release during spliceosome activation, but it is not clear how or if they might interact with Prp24.

The U6 RNA/Prp24 interaction appears to be a dynamic one. It is possible that different RRMs of Prp24 function at different times during the splicing cycle, perhaps binding to different sites on U6 RNA. Genetic interactions described in this work suggest that RRM2, but not RRM3, binds directly to the U6 RNA telestem, which we predict is involved in dissociation of the U4/U6 complex. *In vitro* binding studies with individual Prp24 RRMs and mutant U6 RNAs may help determine which residues and/or structures are recognized by Prp24. We expect that studies on Prp24/U6 RNA dynamics will reveal general mechanisms by which RNA-binding proteins direct conformational changes in RNAs.

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