Suppressors of a Cold-Sensitive Mutation in Yeast U4 RNA Define Five Domains in the Splicing Factor Prp8 That Influence Spliceosome Activation

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ABSTRACT

The highly conserved splicing factor Prp8 has been implicated in multiple stages of the splicing reaction. However, assignment of a specific function to any part of the 280-kD U5 snRNP protein has been difficult, in part because Prp8 lacks recognizable functional or structural motifs. We have used a large-scale screen for *Saccharomyces cerevisiae PRP8* alleles that suppress the cold sensitivity caused by U4-cs1, a mutant U4 RNA that blocks U4/U6 unwinding, to identify with high resolution five distinct regions of *PRP8* involved in the control of spliceosome activation. Genetic interactions between two of these regions reveal a potential long-range intramolecular fold. Identification of a yeast two-hybrid interaction, together with previously reported results, implicates two other regions in direct and indirect contacts to the U1 snRNP. In contrast to the suppressor mutations in *PRP8*, loss-of-function mutations in the genes for two other splicing factors implicated in U4/U6 unwinding, Prp44 (Brr2/Rss1/Slt22/Snu246) and Prp24, show synthetic enhancement with U4-cs1. On the basis of these results we propose a model in which allosteric changes in Prp8 initiate spliceosome activation by (1) disrupting contacts between the U1 snRNP and the U4/U6-U5 tri-snRNP and (2) orchestrating the activities of Prp44 and Prp24.

NUCLEAR pre-mRNA splicing, the process by which introns are removed from primary transcripts via a two-step transesterification mechanism, is performed by the spliceosome, a complex of five small nuclear RNAs (U1, U2, U4, U5, and U6) and more than 60 proteins (Will and Lührmann 1997; Burge *et al.* 1999). On the basis of the discovery of self-splicing by group II introns (Peebles *et al.* 1986; Van der Veen *et al.* 1986) it has been proposed that the RNA components of the spliceosome are reponsible for the catalysis of pre-mRNA splicing, since a similar chemical mechanism is used in both reactions (Sharp 1985; Cech 1986). The spliceosome forms anew on each intron in an ordered manner. Initially, the 5′ splice site is recognized by the U1 snRNP (U1 RNA and associated proteins). Next, the U2 snRNP binds to the intron branchpoint. Finally, the U4/U6-U5 tri-snRNP is stably incorporated to form the complete spliceosome. Activation of the spliceosome for the first transesterification reaction requires structural rearrangements, including unwinding of U1/pre-mRNA and U4/U6 RNA duplexes and formation of a U2/U6/pre-mRNA structure (Nilsen 1998; Staley and Guthrie 1998). Although much is known about the RNA-RNA rearrangements that take place during formation of the catalytic spliceosome, the mechanism by which proteins control the dynamics and timing of RNA-RNA interactions during splicing is not well understood.

We previously identified a mutation in the *Saccharomyces cerevisiae* gene for U4 RNA, *SNR14*, that confers a cold-sensitive growth phenotype by blocking the splicing reaction after assembly of a complete spliceosome, but prior to U1/pre-mRNA and U4/U6 RNA unwinding (Li and Brow 1996; Kuhn *et al.* 1999). This mutation, called *snr14-cs1*, leads to a triple nucleotide substitution immediately adjacent to the sequences in U4 RNA that base-pair with U6 RNA. The mutant U4-cs1 RNA apparently inhibits splicing by masking the ACAGA-box, the U6 sequence element known to interact with the 5′ splice site (Sawa and Shimura 1992; Sawa and Abelson 1992; Wassarman and Steitz 1992; Kandels-Lewis and Séraphin 1993; Lesser and Guthrie 1993). This aberrant secondary structure leads to formation of an assembled spliceosome that cannot undergo activation at low temperature. The block of both U1/pre-mRNA and U4/U6 RNA unwinding in U4-cs1 splicing complexes suggests that these two events are coupled during spliceosome activation, a hypothesis supported by the fact that hyperstabilization of the U1/pre-mRNA base pairing also prevents U4/U6 unwinding (Staley and Guthrie 1999).

Isolation of extragenic suppressors of the cold-sensitive growth defect caused by U4-cs1 identified a novel allele of *PRP8*, named *prp8-201*. *PRP8* encodes an evolutionarily conserved 280-kD splicing factor that is a component of the U5 snRNP (Lossky *et al.* 1987; Brown

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and Beggs 1992). Like U5 RNA (Newman and Norman 1992; Sontheimer and Steitz 1993), Prp8 interacts with the pre-mRNA adjacent to both splice sites. Prior to the first transesterification, Prp8 can be crosslinked to 5′ exon sequences and the GU intron dinucleotide at the 5′ splice site (Wyatt *et al.* 1992; Teigelkamp *et al.* 1995a,b; Reyes *et al.* 1996; Sha *et al.* 1998). Between the first and second transesterification, Prp8 contacts the 3′ splice site (Teigelkamp *et al.* 1995a,b; Umen and Guthrie 1995). It has been proposed that Prp8 helps in the aligning of 5′ and 3′ splice sites by loop I of U5 RNA for the second step (Newman 1997). This notion is supported by crosslinking of Prp8 to loop I of U5 RNA in the U5 snRNP (Dix *et al.* 1998) and by the identification of mutations in Prp8 that suppress both 3′ splice site mutations and 5′ splice site mutations that block the second transesterification (Umen and Guthrie 1995, 1996; Collins and Guthrie 1999; Siatecka *et al.* 1999). The *prp8-201* mutation is the first mutation in *PRP8* that appears to specifically affect a function prior to the first transesterification. Suppression of the U4-cs1 splicing defect by *prp8-201* suggests a model in which Prp8 proofreads recognition of the 5′ splice site by the U6 ACAGA-box (Kuhn *et al.* 1999). For example, binding of the U6/5′ splice site helix by Prp8 may cause an allosteric change that releases repression of splicing factors that execute spliceosome activation. The *prp8-201* mutation may relax control of U4/U6 unwinding by (1) directly influencing recognition of the U6/5′ splice site helix, (2) inducing the allosteric change in Prp8 conformation in the absence of U6/5′ splice site helix recognition, or (3) altering protein-protein interactions with other splicing factors, thereby uncoupling their activities from Prp8.

In the work described here, we have carried out an extensive screen for mutations in the *PRP8* gene that suppress the growth defect caused by U4-cs1 and thus define residues important for Prp8's function in governing U4/U6 unwinding. Forty-six different mutations were obtained that mapped to five discrete regions of Prp8. Interestingly, one of the regions overlaps with the part of Prp8 shown to interact with the U1 snRNP protein Prp40 (Abovich and Rosbash 1997) and shares weak sequence similarities with eIF4E, the cap-binding subunit of the translation initiation complex. Certain pairwise combinations of *PRP8* mutations from the different regions exhibit either mutual suppression or enhancement, suggestive of functional intramolecular interactions, including a potential α-helical coiled-coil structure. Two-hybrid screens with the five regions of Prp8 identified an interaction with Exo84, which has recently been implicated in splicing based on protein-protein interactions with the U1 snRNP proteins Prp40 (P. G. Siliciano, personal communication) and Snp1 (S. W. Ruby, personal communication). Intriguingly, Snp1 has also been shown to interact with Prp44 (Brr2/Rss1/Slt22/Snu246; Fromont-Racine *et al.* 1997), the putative U4/U6 RNA helicase (Laggerbauer *et al.* 1998; Raghunathan and Guthrie 1998; Kim and Rossi 1999) whose human homologue is in close association with Prp8 in the U5 snRNP (Achsel *et al.* 1998). We find that loss-of-function mutations in the genes for Prp44 or Prp24, another splicing factor implicated in U4/U6 unwinding (Shannon and Guthrie 1991; Vidaver *et al.* 1999), enhance the growth defect caused by U4-cs1. On the basis of these findings we propose a model in which tri-snRNP incorporation into the spliceosome is accompanied by the formation of contacts between the U5 and U1 snRNPs. Upon proper positioning of the tri-snRNP, in part evidenced by formation of a correct U6/5′ splice site helix, the interactions between the U1 and U5 snRNPs are disrupted, and Prp8 initiates spliceosome activation by orchestrating the activities of Prp44 and Prp24.

MATERIALS AND METHODS

Yeast strains, plasmids, and oligonucleotides: The screen for *PRP8* alleles that suppress the cold sensitivity caused by U4-cs1 and characterization of the *PRP8* alleles obtained was performed with ZRL102 (*MAT***a** *snr14::TRP1 prp8*Δ*::ADE2 trp1 ura3 lys2 his3 ade2* [pRS317-U4-cs1] [YCp50-PRP8]), which was created from ZRL103 (Kuhn *et al.* 1999) by substituting the plasmid pJDY13 (*GAL1-PRP8 HIS3 CEN6 ARSH4*) with YCp50-PRP8 (*PRP8 URA3 CEN4 ARS1*). Strains ANK800 and ANK814 are isogenic to ZRL102, except that pRS317-U4-cs1 (*snr14-cs1 LYS2 CEN6 ARSH4*) is replaced with pRS317-U4-wt or pRS317-U4-G14C, respectively. To create the *SNR14/PRP24* double disruption strain ANK240, strains LL101 (*MAT***a** *prp24*Δ*::ADE2 his3 leu2 trp1 ura3 met2 can1 ade2 lys2* [pUN50-PRP24]; Vidaver *et al.* 1999) and YKS1 (*MAT*α *snr14::TRP1 trp1 ura3 lys2 his3 ade2* [YCp50-SNR14]; Shannon and Guthrie 1991) were mated. Diploids were streaked to medium containing 0.75 mg/ml 5-fluoroorotic acid (5-FOA) to select against the *URA3*-marked plasmids. The resulting diploid strain was transformed with YCp50-SNR14 and pRS313-PRP24 (Vidaver *et al.* 1999) and then sporulated. An Ade^+ Trp^+ spore was propagated and named ANK240. Strains ANK241 and ANK242 are isogenic to ANK240, except that pRS313-PRP24 is replaced with pRS313-prp24-R158S or pRS313-prp24-F257I, respectively. ANK021, the strain containing *brr2-1* at the chromosomal locus and a chromosomal disruption of *SNR14*, was constructed from YSN405 (*MAT*α *brr2-1 ura3 lys2 his3 ade2 leu2*; Noble and Guthrie 1996) and YKS2 (*MAT***a** *snr14::TRP1 trp1 ura3 lys2 his3 ade2* [YCp50-SNR14]; Shannon and Guthrie 1991). The two strains were mated, and the diploid was then sporulated. A Trp^+, cold-sensitive, and 5-FOA-sensitive spore was propagated and named ANK021. To test for genetic interactions between mutant *prp38* alleles and *snr14-cs1*, ts192 (*MAT*α *prp38-1 trp1 ura3 leu2 his3*; Blanton *et al.* 1992) and JXY6 (*MAT***a** *prp38::LEU2 trp1 ura3 leu2 his3 ade2* [YCplac22 (*TRP1 prp38-2*)]; Xie *et al.* 1998) were mated with YKS2 and YKS1, respectively. After sporulation of each of the two diploids, resulting spores were screened for Trp^+ Ura^+, temperature-sensitive, and 5-FOA-sensitive phenotypes. A positive spore with *snr14::TRP1* and either *prp38-1* or *prp38-2* was propagated and named ANK381 and ANK382, respectively. The two-hybrid screens were performed with PJ69-4A (*MAT***a** *trp1 leu2 ura3 his3 gal4*Δ *gal80*Δ *LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*; James *et al.* 1996). All methods for manipulation of yeasts, *e.g.*, transformation, plasmid recovery, and selec-

tion on medium containing 5-FOA, were performed according to standard procedures (Guthrie and Fink 1991).

Plasmid pRS313-PRP8(SacII), which was used for the suppressor screen, was constructed by first subcloning the *PRP8*-containing *Xho*I/*Sna*BI fragment of YCp50-PRP8 (Kuhn *et al.* 1999) into *Xho*I/*Ecl*136II-cut pRS313 (Sikorski and Hieter 1989), resulting in pRS313-PRP8. Subsequently, a *Sac*II restriction site was introduced at position 5096 of the *PRP8*-coding sequence by silent mutation using site-directed mutagenesis (Kunkel *et al.* 1987). Similarly, pRS313-PRP8(ΔClaI) was obtained from pRS313-PRP8 by destroying the *Cla*I-restriction site at position 7226 of the *PRP8*-coding region by silent mutation. Plasmids pRS313-PRP8(SnaBI-1) and pRS313-PRP8(SnaBI-3/4) were similarly constructed by creating a *Sna*BI-restriction site at position 916 or 5582 of the *PRP8*-coding region, respectively. In these two plasmids the introduction of the restriction site leads to mutation of Prp8 (P303R/E304K and V1862Y, respectively). The clones used for the yeast two-hybrid analysis are based on pGBDU-C(x) (James *et al.* 1996). They contain fragments of *PRP8* coding for amino acids 80–777 (pY2H-a/b), 756–931 (pY2H-c), 1015–1220 (pY2H-d), and 1591–1923 (pY2H-e) fused to the Gal4 DNA-binding domain. General cloning methods were carried out as described in Sambrook *et al.* (1989).

Sequences of oligonucleotides used for PCR amplification, sequencing, and mutagenesis are available upon request.

Screen for mutations in *PRP8* that suppress *snr14-cs1*: The screen was based on a previously described procedure (Umen and Guthrie 1996). *PRP8* was PCR mutagenized in four intervals (named 1 to 4 starting from the N terminus) that encompass pairwise combinations of the unique restriction sites *Xho*I (717 base pairs upstream of the coding region), *Sal*I (position 1715 of the coding region), *Spe*I (position 3354 of the coding region), *Sac*II (introduced by silent mutation at position 5096 of the coding region), and *Sph*I (position 7009 of the coding region). Primers for the PCR amplification lie about 250 bp outside of each restriction site.

Mutagenesis was done under standard PCR conditions using only the natural error rate of Taq DNA polymerase (United States Biochemical, Cleveland). For each of the four *PRP8* intervals, at least two independent PCR amplifications were performed in a total volume of 500 μl each. The products of each reaction were then cotransformed with 20 μg of appropriately gapped pRS313-PRP8(SacII) into ZRL102, and transformants were selected on medium lacking histidine. We usually obtained about 50,000 His^+ colonies for each transformation, whereas <5000 colonies were obtained when the PCR product was omitted from the transformation. The His^+ colonies were replica plated to medium containing 5-FOA to select against the *URA3*-marked plasmid with the wild-type *PRP8* gene. Strains with haploviable *PRP8* alleles were then tested for suppression of the cold sensitivity caused by U4-cs1 by replica plating to YEPD and incubation at 18° for 7 days. Altogether, 435 colonies were obtained, 143, 152, 63, and 77 from mutagenizing intervals 1, 2, 3, and 4, respectively. The suppression phenotype was confirmed by streaking strains to YEPD and testing for growth at 18°.

Mapping of the suppressor mutations: Selected regions of *PRP8* containing potential suppressor mutations were PCR amplified using Tfl DNA polymerase (Epicentre Technologies, Madison, WI) and DNA was isolated from suppressor strains by the method described by Ling *et al.* (1995). Linkage of the suppression phenotype to the amplified region of *PRP8* was analyzed essentially as described for the initial screening. Growth at 18° was tested for six transformants for each suppressor strain analyzed after selection against the wild-type *PRP8* plasmid. PCR fragments that conferred viability to at least three of the six transformants when grown at 18° were sequenced to identify the suppressor mutations.

For interval 1, 59 strains were tested for linkage of suppression to two overlapping fragments comprising the coding region for amino acids 1–355 and 254–660. Plasmid pRS313-PRP8(SnaBI-1) digested with *Xho*I/*Sna*BI or *Sna*BI/*Sal*I, respectively, was used for the cotransformation. Suppression was assigned to the overlapping part if both fragments gave colonies growing at 18°, or to the region specific to one fragment if only that fragment conferred suppression. Similarly, 40 strains from interval 2 were tested for linkage to the two overlapping fragments coding for amino acids 479–913 and 762–1213. Plasmid pRS313-PRP8(SacII) digested with *Sal*I/*Sac*I or *Sac*I/*Spe*I, respectively, was used for the cotransformation. In addition, 12 and 5 more strains from intervals 1 and 2, respectively, were tested for linkage to a fragment shared by both intervals (for technical reasons slightly extended so that it spans the coding region of amino acids 479–753) using pRS313-PRP8(SacII) linearized with *Sal*I. For intervals 3 and 4, we tested linkage to the fragment coding for amino acids 1597–1942 for 36 and 50 strains, respectively. Plasmid pRS313-PRP8(ΔClaI) linearized with *Cla*I was used for the cotransformation. The 12 strains of interval 3, for which suppression could not be linked to amino acids 1597–1942, were tested for linkage to amino acids 1022–1213 together with 15 more strains from interval 3 using pRS313-PRP8(SacII) linearized with *Spe*I.

Yeast two-hybrid screen: The yeast two-hybrid screen was performed using the improved yeast two-hybrid system established by James *et al.* (1996). The four bait plasmids pY2H-a/b, pY2H-c, pY2H-d, and pY2H-e were separately transformed into PJ69-4A. Subsequently, the four strains obtained were transformed with the libraries Y2HL-C1, Y2HL-C2, and Y2HL-C3, which have yeast genomic fragments fused to the Gal4 activation domain in all three reading frames (James *et al.* 1996). Transformation efficiency was examined by plating a portion of the transformation to −Ura/−Leu medium. The rest was plated to −His medium to select for transformants that activate the *HIS3* reporter gene. After growth at 30° for 2 wk, colonies were replica plated to −Ade medium to select for transformants that also activate the more stringent *ADE2* reporter gene. For all colonies growing on medium lacking adenine after 7 days, dependence of the activation of the reporter genes on the bait plasmid was verified after selection against pY2H-a/b, pY2H-c, pY2H-d, or pY2H-e on medium containing 5-FOA. The genomic fragment fused to the Gal4 activation domain was identified by PCR amplification and sequencing for strains that showed bait-dependent activation of the reporter genes. Altogether, about 3×10^6 transformants were screened for each of the four bait plasmids.

RESULTS

Screen for *PRP8* alleles that suppress the cold sensitivity caused by U4-cs1: We previously identified a mutant allele of *PRP8*, called *prp8-201*, in a genome-wide selection for spontaneous suppressors of the cold-sensitive lethality conferred by U4-cs1. On the basis of this result and our biochemical characterization of the U4-cs1 splicing defect, we proposed that Prp8 controls the timing of U4/U6 unwinding during spliceosome activation (Kuhn *et al.* 1999). To characterize the region(s) of Prp8 involved in this process, we performed a screen to identify additional alleles of *PRP8* that allow yeast cells with U4-cs1 to grow at 18°. A gap-repair strategy was used to introduce a library of PCR-mutagenized *PRP8* alleles into the recipient strain, ZRL102. This

strain has U4-cs1 as its sole copy of U4 RNA, a chromosomal deletion of *PRP8*, and wild-type *PRP8* on a counterselectable *URA3*-marked plasmid.

As the coding region of *PRP8* spans more than 7 kb, four separate screens were done using overlapping 2-kb PCR products and a *PRP8* plasmid gapped at unique restriction sites (see materials and methods for details). Division of *PRP8* into four intervals reduces the risk of generating null alleles due to multiple mutations and facilitates the identification of the suppressor mutations (Umen and Guthrie 1996). For each interval, ~100,000 transformants were screened, which originated from at least two independent PCR reactions/transformations. When replica plated to medium with 5-FOA to select against the wild-type *PRP8* gene on the *URA3*-marked plasmid, ~90% of the transformants survived, showing that most of the introduced *PRP8* alleles are functional. The haploviable *PRP8* alleles were then tested for suppression of the cold sensitivity caused by U4-cs1. Altogether, 435 colonies that grew at 18° were collected, corresponding to about 0.1% of the haploviable transformants. The number of cold-resistant strains obtained from each of the four intervals used in the screen ranged from 63 to 152. Five strains did not grow when retested at 18° and thus were discarded.

To confirm that suppression of the U4-cs1 cold sensitivity is due to mutation of *PRP8*, we rescued the plasmid carrying the *PRP8* gene from 48 strains. When transformed into the starting strain, 46 plasmids conferred suppression of the U4-cs1 cold sensitivity, indicating that indeed most of the strains have suppressor mutations in *PRP8*. For 17 of the 48 strains, we also tested for suppression of the U4-cs1 cold sensitivity in the presence of wild-type Prp8. All of the *PRP8* alleles tested confer growth at 18° in the presence of wild-type Prp8, suggesting that most of the isolated *PRP8* alleles exhibit dominant suppression.

Suppressor mutations of the U4-cs1 cold sensitivity localize to five discrete regions of *PRP8*: The suppressor mutations were fine mapped prior to sequencing. This was done by a second gap-repair step (see materials and methods for details). Briefly, selected regions of *PRP8* DNA from the suppressor strains were PCR amplified using a high-fidelity DNA polymerase to preserve the original mutation(s). The PCR products were then cotransformed into the starting strain with an appropriately gapped or linearized vector and the resulting *PRP8* alleles tested for suppression of the cold sensitivity conferred by U4-cs1, as described above.

Altogether, we analyzed 217 of the cold-resistant strains, and for most of these the location of the suppressor mutation(s) could be narrowed down to a small fragment of *PRP8* (Table 1). Sequencing of the fragments sufficient for suppression from 152 plasmids revealed that 99 of these contain a mutation that changes only a single amino acid in the fragment. However, as most of the changes were identified multiple times, this corresponds to only 44 different single-site substitutions (Figure 1). Two more alleles have two mutations that affect two closely spaced amino acids encoded by the fragment sufficient for suppression (M1095T·I1104M and N1099K·R1105L; Figure 1); in neither case do we know whether just one or both substitutions are required for suppression. An additional 46 alleles contain two or more mutations, at least one of which was isolated as a single mutation sufficient for suppression. Interestingly, 6 of these 46 alleles contain two mutations that were each shown to be sufficient for suppression on their own (E624G·D651G obtained three times, E624G·D651N, L1624M·L1634F, and L1624F·I1875T). We have not determined if the suppressive effects of these mutations are additive. The remaining 5 alleles with multiple mutations give rise to at least one substitution closely adjacent to or in the same amino acid as a change known to confer suppression on its own (F367L, F1092I, P1191Q, D1192G, and T1872I).

All of the single-site mutations and the two double mutations sufficient for suppression can be grouped into five regions of *PRP8* that collectively span a large part of the gene (Figure 1). These regions encode amino acids 236–362 (Region a), 611–684 (Region b), 788–861 (Region c), 1094–1197 (Region d), and 1624–1875 (Region e). Region e includes the T1861P substitution originally identified as *prp8-201* (Kuhn *et al.* 1999). One of the newly found alleles also has the T1861P change. Another suppressor substitution in Region e, V1862Y, resulted from a mutation introduced to create a restriction site.

Strikingly, almost half of the identified suppressor mutations were isolated in two or more independent screens, suggesting that these screens identified most, if not all, of the regions of *PRP8* involved in suppression of the cold sensitivity caused by U4-cs1. This conclusion is also supported by the identification of several different suppressor mutations affecting the same amino acid, as for example the proline at position 1191, where changes to leucine, serine, or threonine confer suppression (Figure 1B). Furthermore, for >75% of the 217 alleles that were fine mapped, the mutation(s) responsible for suppression could be linked to one of the Regions a–e (see above). As the remaining 25% include strains that fortuitously acquired a genomic suppressor mutation outside of *PRP8* (see above and data not shown), the overall linkage of *PRP8* mutations to the five regions is even higher than 75%. Therefore we conclude that the five discrete regions identified in our screen represent the main parts of Prp8 involved in suppression of the growth defect conferred by U4-cs1 and, thus, in governing U4/U6 unwinding.

General features of *PRP8* suppressor mutations: In contrast to the previously identified *prp8-201* mutation, most of the newly isolated *PRP8* suppressor mutations do not confer a temperature-sensitive growth defect. Thus temperature sensitivity is not a necessary conse-

TABLE 1

Linkage of suppression of U4-cs1 cold sensitivity to fragments of mutagenized *PRP8*

Mutagenized *PRP8* interval	Subregion[a]	No. of strains for which suppression was linked to subregion	Fraction of strains tested (%)[b]
1	1–253	6	10.2
	254–355	4	6.8
	356–660	27	45.8
	479–660[c]	3	25.0[d]
	661–753[c]	1	8.3[d]
2	479–761	6	13.3
	762–913	25	55.6
	914–1213	8	17.7
3	1022–1213	18	35.3
	1597–1942	24[e]	55.8[e]
4	1597–1942	41	82.0

Linkage of the suppression phenotype to small fragments of Prp8 was tested for selected strains that originated from mutagenizing the four intervals of *PRP8*.

[a] Given in amino acids encoded by the amplified fragment.

[b] Given in percentages per interval.

[c] To be comparable with the subregion comprising amino acids 356–660, the subregion coding for amino acids 479–753 was divided, and the strains were assigned on the basis of the sequencing result.

[d] These numbers were obtained from a subpopulation of only 12 strains tested for linkage to amino acids 479–753.

[e] Eight strains, for which suppression was not linked to amino acids 1022–1213, were not tested for linkage to amino acids 1597–1942.

quence of suppression of the U4-cs1 growth defect. In addition to T1861P, only the adjacent change V1860D gives very slow growth at 37°. Suppressor strains with a different substitution at position 1860 (V1860N) or with the same change at position 1862 (V1862D) do not exhibit any observable temperature-sensitive phenotype. A strain with *prp8-H659P* grows slower than wild-type cells at all temperatures (see Figure 2).

Detailed inspection of the site and kind of mutations yielded some interesting observations. First, most of the single-site suppressor mutations affect residues that are conserved in at least 8 of the 9 known Prp8 orthologues (27 of 44; Figure 1B). For 6 of the mutations affecting less conserved amino acids, the change between orthologues is generally conservative, whereas the suppressor mutation introduces a nonconservative change (*e.g.*, D1192Y, where the corresponding position in *Trichomonas vaginalis* is an asparagine and in *Trypanosoma brucei* is a glutamine). The same trend can also be seen for the alleles with double or multiple mutations (Figure 1B and data not shown). Second, almost half (21 of 44) of the mutations lead to a change of charge. Third, there are 3 mutations that introduce an amino acid that is present at that position in a Prp8 orthologue from another organism (L261P, K611R, and P1191S; Figure 1B and data not shown). One more mutation introduces an arginine, where *T. brucei* has a lysine at the corresponding position (W856R; Figure 1B). However, as no information about the functionality of Prp8 orthologues from other organisms in *S. cerevisiae* is available, the significance of this observation remains unclear.

Relation of suppressor Regions a–e to previously identified functions of Prp8: The largest group of suppressor mutations falls into Region e, with 16 different single-site substitutions identified. Fourteen of these cluster in two smaller subregions of 64 and 25 amino acids at the boundaries of the region (Figure 1). Interestingly, Region e overlaps mutations in *PRP8* that suppress mutations in the 5′ and 3′ splice sites or that alter selection of splice sites with a mutated pyrimidine-rich tract (Umen and Guthrie 1995, 1996; Collins and Guthrie 1999; Siatecka *et al.* 1999). These regions span amino acids 1399–1982 and amino acids 1834–1960, respectively (see also Figure 5A). However, each of seven different *PRP8* alleles isolated by Umen and Guthrie (1996) or Collins and Guthrie (1999) failed to suppress the cold sensitivity of U4-cs1 (data not shown). Similarly, all but one of six *PRP8* suppressor alleles of the cold sensitivity caused by U4-cs1 tested for suppression of splice site mutations failed to do so (C. A. Collins and C. Guthrie, personal communication). Only the *prp8-201* (T1861P) allele confers suppression of 5′ and 3′ splice site mutations. The basis of the cross-suppression phenotype of this mutation remains to be determined. The C-terminal border of Region e is also close to the portion of Prp8 that crosslinks to the 5′ splice site, which has been mapped to amino acids 1894–1898 in human Prp8 (Reyes *et al.* 1999). This corresponds to amino acids 1966–1970 in *S. cerevisiae* Prp8 (see also Figure 5A).

No information about mutations, RNA-protein interactions, or protein-protein interactions involving Re-

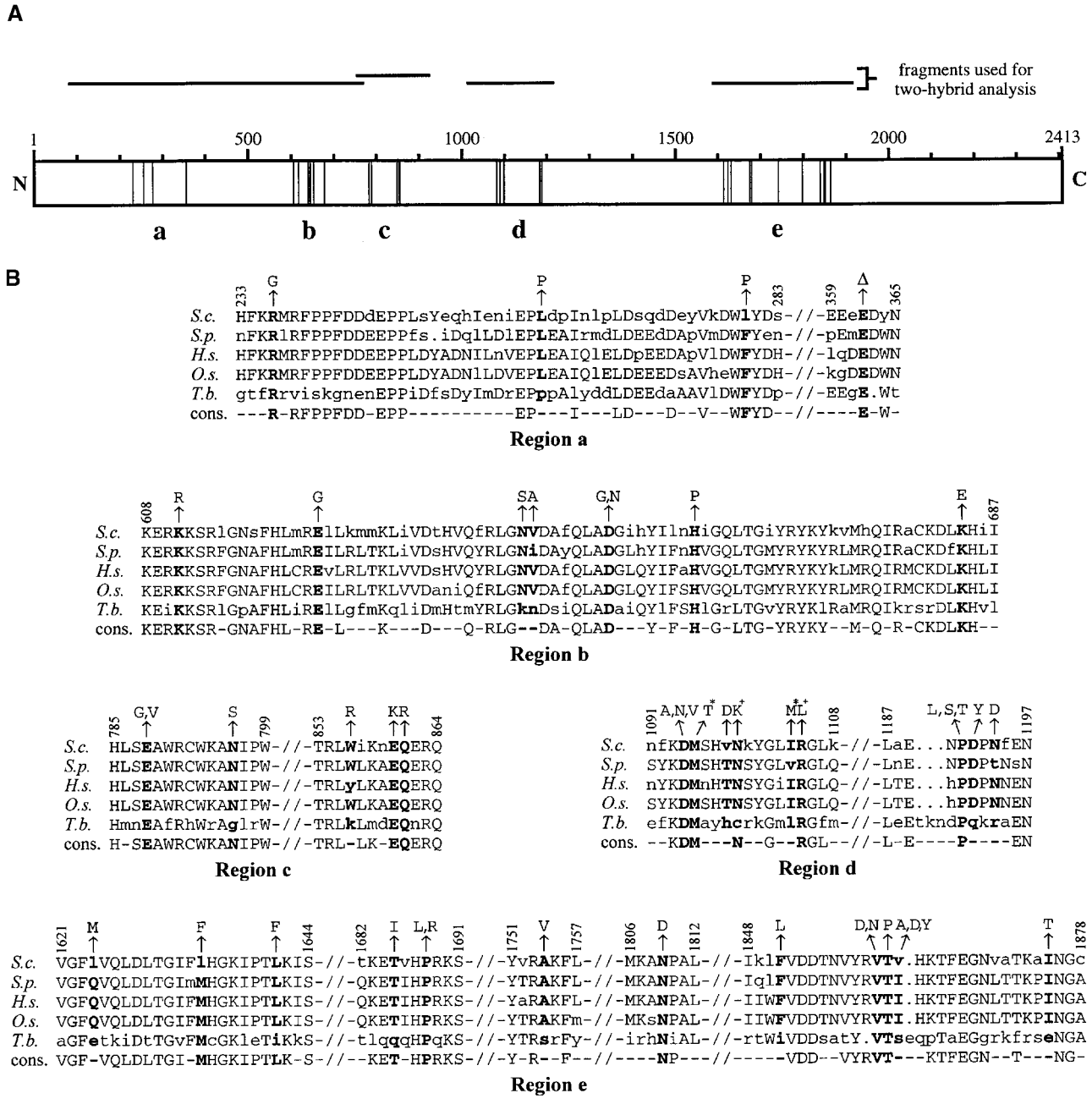


Figure 1.—The *PRP8* suppressor mutations of the cold sensitivity caused by U4-cs1 fall into five discrete, highly conserved regions of Prp8. (A) Shown is a schematic of the primary structure of Prp8 with the locations of each of the suppressor mutations indicated by a thin vertical line. Regions of suppressor mutations are labeled a–e. The horizontal lines above the protein represent the fragments of Prp8 used in our two-hybrid analysis. (B) Alignment of Regions a–e of *S. cerevisiae* Prp8 (*S.c.*) with Prp8 orthologues from *Schizosaccharomyces pombe* (*S.p.*), *Homo sapiens* (*H.s.*), *Oryza sativa* (*O.s.*), and *T. brucei* (*T.b.*). The consensus (cons.) lists residues that are identical in at least eight of nine known Prp8 orthologues. (For simplicity, the sequences of *Caenorhabditis elegans* and *Arabidopsis thaliana* Prp8, which are nearly identical in the five regions to *H.s.* and *O.s.*, respectively, are not shown. *Plasmodium falciparum* and *T. vaginalis* Prp8s, which are less divergent than *T.b.*, are also omitted from the alignment.) Numbers above the most N- and C-terminal amino acids give the position of each cluster in the *S.c.* Prp8 protein. Shown in bold are amino acids mutated in *PRP8* alleles that suppress the U4-cs1 growth defect. The changes are listed above the alignment with arrows pointing to the mutant amino acid. The two double mutations mentioned in the text are labeled with * and +, respectively.

gions b, c, or d of Prp8 has been reported. Region a overlaps with amino acids 1–349, the part of Prp8 that has previously been shown to interact with the U1 snRNP protein Prp40 in a yeast two-hybrid assay (Abovich and Rosbash 1997). However, it was proposed that this interaction is mediated by a proline-rich region at the very N terminus of Prp8, a part not included in Region a. Nevertheless, the close proximity of Region

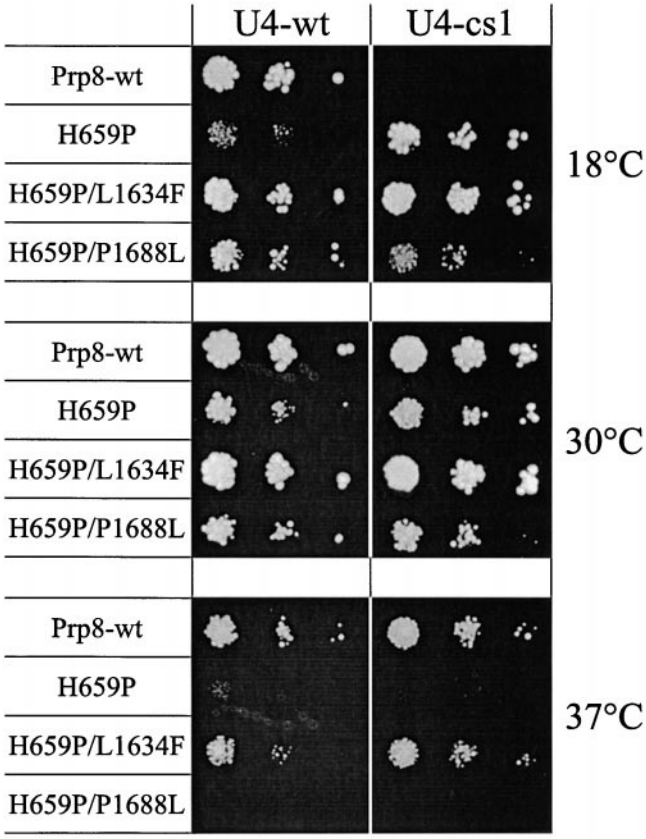


Figure 2.—The growth defect of *prp8-H659P*, a mutation in Region b, is suppressed by a second suppressor of the U4-cs1 cold sensitivity, L1634F from the N-terminal cluster of Region e, without affecting suppression of *snr14-cs1*. Yeast strains containing either wild-type U4 or U4-cs1 RNA and *PRP8* on a *URA3*-marked plasmid were transformed with different single- and double-mutant *prp8* alleles. Tenfold dilutions were plated to medium containing 5-FOA, and growth of the strains was tested at 18° for 7 days (top), at 30° for 3 days (middle), and at 37° for 2 days (bottom).

a with the part of Prp8 that binds to Prp40 suggests that there might be a functional relationship between suppression of the U4-cs1 cold sensitivity by Prp8 and physical contacts to the U1 snRNP.

Genetic interactions between mutations in Regions a–e: Localization of the *PRP8* suppressor mutations of the growth defect conferred by U4-cs1 in five distinct regions of *PRP8* indicated that more than one part of Prp8 is involved in governing U4/U6 unwinding. To get a better understanding of the relationship of Regions a–e to each other, we combined mutations from different regions to create double-mutant alleles. Each double mutant was tested for viability, conditional growth defects, and suppression of the U4-cs1 cold sensitivity. The mutations chosen for this analysis were L280P and E362Δ from Region a, E624G·D651G (an allele with 2 mutations, each sufficient for suppression) and H659P from Region b, E788G from Region c, D1094A and V1098D from Region d, and 4 mutations from Region e: L1634F and P1688L from the N-terminal cluster and V1860D and T1861P/*prp8-201* from the C-terminal cluster. These 11 mutations include all 3 that confer a temperature-sensitive or slow growth defect.

When analyzed for haploviability, all of the combinations tested display growth at 30° (data not shown). Interestingly, the slow growth phenotype caused by H659P, a mutation in region b, is actually suppressed by L1634F, a mutation in the N-terminal cluster of Region e. This is true not only at 30°, but also at 18° and 37° (Figure 2). However, the double mutation is still able to suppress the cold sensitivity caused by U4-cs1 (Figure 2). Suppression of the H659P growth defect at higher temperatures seems to be specific for mutation L1634F, as neither the nearby P1688L mutation nor any other mutation tested shows suppression of the growth defect at 30° or 37° (Figure 2 and data not shown). However, a more complex pattern can be observed at 18°. First, a strain with Prp8-H659P grows better in the presence of U4-cs1 RNA compared to wild-type U4 RNA (Figure 2), indicating that not only does *prp8-H659P* suppress the U4-cs1 cold sensitivity, but U4-cs1 also suppresses the slow growth defect of *prp8-H659P* at 18°. Second, P1688L is able to partially suppress the slow growth defect conferred by H659P at 18°, although not as well as L1634F (Figure 2). Third, a strain with Prp8-H659P/P1688L grows slower at 18° with U4-cs1 RNA than with wild-type U4 RNA (Figure 2), which could be due to either a synthetic enhancement of *prp8-H659P/P1688L* by *snr14-cs1* or weaker suppression of the U4-cs1 cold sensitivity by *prp8-H659P/P1688L* than by each single-site mutation alone. This indicates a highly specific interaction between H659P and L1634F, which could be explained by close proximity of the two amino acids in an intramolecular structure (see discussion).

Another set of genetic interactions is observed when either a Region c mutation (E788G) or a Region d mutation (either D1094A or V1098D) is combined with either V1860D or T1861P from the C-terminal cluster of Region e. Cells with these double-mutant alleles and U4-cs1 RNA do not grow at 18°, indicating reversion of suppression, since cells with these *PRP8* alleles are viable at 18° in the presence of wild-type U4 RNA (Figure 3 and data not shown). Furthermore, the temperature-sensitive growth defect caused by V1860D or T1861P is clearly enhanced by the presence of the Region b mutation E788G (Figure 3A and data not shown). In contrast, the Region d mutations D1094A or V1098D exhibit only a modest enhancement of the 37° growth defect caused by V1860D or T1861P (Figure 3B and data not shown). U4-cs1 also modestly enhances the 37° growth defect caused by V1860D and T1861P (Figure 3 and data not shown). The genetic interactions of E788G from Region c and D1094A and V1098D from Region d with the two mutations from the C-terminal cluster of Region e are specific. The two mutations analyzed from the N-terminal cluster of Region e (L1634F or P1688L) show no observable temperature-sensitive

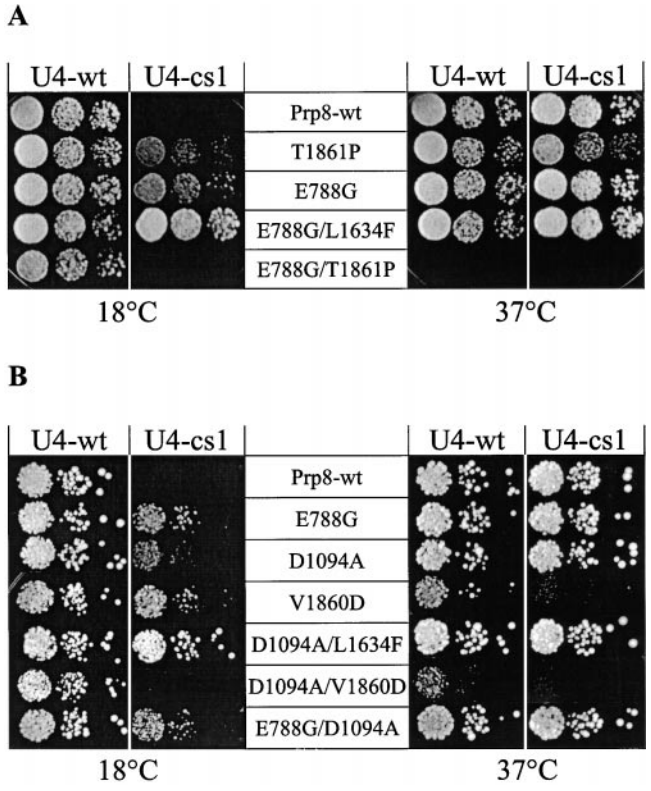


Figure 3.—Suppressor mutations from *PRP8* Regions c (E788G) and d (D1094A or V1098D) interact genetically with mutations V1860D and T1861P from the C-terminal cluster of Region e. (A) E788G specifically enhances the temperature sensitivity of mutations from the C-terminal cluster of Region e and reverses suppression by these mutations of the growth defect caused by U4-cs1. Tenfold dilutions of yeast strains containing either wild-type U4 or U4-cs1 RNA and the *PRP8* allele depicted were plated to YEPD medium, and growth of the strains was tested at 18° for 7 days (left) and at 37° for 2 days (right). For simplicity, only the combinations of E788G with T1861P are shown. Furthermore, only L1634F from the N-terminal cluster of Region e is shown as control. The results with P1688L and V1860D are identical to these with L1634F and T1861P, respectively. Note that the temperature sensitivity of T1861P/*prp8-201* is less pronounced when this allele is present on a plasmid. (B) Mutations D1094A or V1098D from Region d reverse suppression of the U4-cs1 cold sensitivity by V1860D or T1861P, without influencing the growth defect resulting from these mutations at 37°. Cells were plated and incubated as in A. Only the results for combinations using one of the mutations from Region d and the N- and C-terminal clusters of Region e are shown for simplicity. The effect observed is similar for the combinations not shown.

growth defect in combination with E788G, D1094A, or V1098D, nor do these double mutations reverse suppression of the growth defect caused by U4-cs1 (Figure 3 and data not shown).

For all other combinations of mutations that were tested, no effect on growth or suppression of the U4-cs1 cold sensitivity was observed. All of the intragenic interactions that were observed are specific for either one allele, or two alleles with adjacent mutations. The specificity of the interactions is further supported by the fact that a combination of E788G from Region c with either D1094A or V1098D from Region d has no effect on growth at 37° or on the suppression of the U4-cs1 growth defect (Figure 3B and data not shown), although each single mutation in combination with either V1860D or T1861P from the N-terminal cluster of Region e reverses suppression of the cold sensitivity caused by U4-cs1. Thus the genetic interactions observed do not appear to be due simply to additive effects of mutations.

Two-hybrid screens with the five suppressor regions of Prp8: The proposed model of Prp8 as a central factor controlling different activities during spliceosome activation presumes that Prp8 interacts with the proteins performing these functions, either directly or via bridging factors. To identify candidate proteins that may interact with Regions a–e, we carried out extensive yeast two-hybrid screens with all five regions. For technical reasons, Regions a and b were combined into one bait clone. All other regions were analyzed individually (Figure 1A). Several million transformants were analyzed for each bait clone, giving an expected sampling of >95% of the yeast genome. The screen was performed in a fairly stringent manner to avoid a high background of nonspecific interactions (see materials and methods). Altogether, 18 genes coding for potentially interacting proteins were identified, but only genes isolated more than once and therefore most likely to be authentic positive clones are reported (Table 2).

One or two genes were obtained multiple times with each bait used. Of the six genes, only one, *EXO84*, has previously been implicated in splicing. Exo84 is an essential protein of 84 kD that was identified in yeast two-hybrid screens with the U1 snRNP proteins Prp40 (P. G. Siliciano, personal communication) and Snp1 (S. W. Ruby, personal communication). As mentioned above, Prp40 also interacts with Prp8 (Abovich and Rosbash 1997). Furthermore, Snp1 has been shown to interact in the yeast two-hybrid assay with Prp44 (Brr2/Rss1/Slt22/Snu246; Fromont-Racine *et al.* 1997), whose human homologue is closely associated with Prp8 in the U5 snRNP (Achsel *et al.* 1998). Altogether, this network of interactions indicates the existence of multiple direct and indirect contacts between the U5 and U1 snRNPs. However, Exo84 has also been identified as a component of the exocyst complex, which mediates exocytosis (Guo *et al.* 1999). In this study it was shown that Exo84 is primarily localized in the cytoplasm. Nevertheless, it cannot be excluded that a smaller fraction of the protein localizes to the nucleus and functions in splicing.

Surprisingly, our yeast two-hybrid screen did not identify contacts between Regions a–e of Prp8 and any canonical splicing factors. A plausible explanation for this could be that more than a small region of Prp8, as used in our yeast two-hybrid screens, is necessary for a strong interaction of Prp8 with other splicing factors. The possi-

TABLE 2

Proteins isolated more than once in yeast two-hybrid screens with Prp8 fragments

Region(s) used as bait	Protein isolated	Codons included in fusion[a]	Reported characteristics of the protein	No. of isolates
a/b	Ygr021w	45–290 (290)	Involved in mitochondrial protein synthesis	2
c	Djp1	65–432 (432)	DnaJ–like protein	3
	Exo84	615–753[b] (753)	Part of exocyst complex	3
d	Bmh1/2[c]	87–267 (267)	Homologues of 14–3–3 proteins	3
	Gpm1	37–110 (247)	Phosphoglycerate mutase	2
e	Upc2	503–729 (913)	Regulatory role in sterol uptake and esterification	3

Listed are all proteins that were isolated at least twice as fusion proteins in the yeast two-hybrid screen with one of regions a–e as bait.

[a] Listed is the smallest fragment isolated that interacts with Prp8. Given in parentheses is the number of amino acids in the respective full-length protein.

[b] A frameshift is required to translate these codons (+1 in two clones and −1 in the third). It has previously been reported that frameshift events are selected for in the yeast two-hybrid screen, when viability depends on it (Fromont-Racine *et al.* 1997).

[c] Bmh1 and Bmh 2 are >90% identical.

ble physiological relevance of the interactions that were observed is considered in the discussion.

The U4-cs1 cold-sensitive growth defect is enhanced by mutations in *PRP44* and *PRP24*: In addition to Prp8, several other splicing factors have previously been implicated in U4 RNA release during spliceosome activation. These include Prp44, a putative RNA helicase also known as Brr2, Rss1, Slt22, and Snu246, which is proposed to unwind U4/U6 during spliceosome activation (Laggerbauer *et al.* 1998; Raghunathan and Guthrie 1998; Kim and Rossi 1999; Kuhn *et al.* 1999), Prp38, a tri-snRNP protein, whose depletion or inactivation leads to accumulation of arrested spliceosomes in which U4 RNA is still paired with U6 RNA (Xie *et al.* 1998), and Prp24, an RNA-binding protein that has recently been implicated in the structural rearrangements of U6 RNA during spliceosome activation (Vidaver *et al.* 1999). To test if one or more of these three splicing factors act together with Prp8 in regulating and/or executing spliceosome activation, genetic interactions of mutant alleles of the genes for these proteins with *snr14-cs1* were analyzed.

No genetic interactions could be observed when two different mutations in *PRP38* that both inhibit spliceosome activation, *prp38-1* or *prp38-2* (Xie *et al.* 1998), were combined with *snr14-cs1* (data not shown). Presence of U4-cs1 did not suppress the temperature sensitivity of *prp38-1* or *prp38-2*, nor did one of the mutations in Prp38 suppress the cold sensitivity caused by U4-cs1, as cells with U4-cs1 and a mutant Prp38 protein were both cold and temperature sensitive (data not shown). Furthermore, cells with *snr14-cs1* and *prp38-1* or *prp38-2* grow well at 30°, the permissive temperatures for all mutations (data not shown), indicating that these mutations do not enhance each other. In contrast, combination of *snr14-cs1* with *brr2-1*, a cold-sensitive mutation that decreases the unwinding activity of Prp44 (Raghunathan and Guthrie 1998), proved to be lethal at all temperatures, although each single mutation is able to grow well at temperatures of 27° and higher (Figure 4A and data not shown). The synthetic enhancement of the U4-cs1 growth defect by *brr2-1* is in good agreement with our hypothesis that *PRP8* suppressor mutations of the growth defect conferred by U4-cs1 relax negative control of U4/U6 unwinding, because this predicts that a mutation that decreases U4/U6 unwinding activity would aggravate the phenotype caused by U4-cs1.

Similarly, mutations in *PRP24* that have been proposed to weaken RNA binding, *PRP24-R158S* and *PRP24-F257I* (Vidaver *et al.* 1999), synthetically enhance the growth defect of *snr14-cs1* at 27°, a temperature at which strains with each individual mutation grow comparably to a wild-type strain (Figure 4B). Consistent with results from an earlier study (Shannon and Guthrie 1991), mutations in *PRP24* suppress the severe cold sensitivity caused by U4-G14C, a mutant U4 RNA that destabilizes the U4/U6 interaction (Figure 4B). The mutations in *PRP24* therefore have effects on mutant U4 RNAs exactly opposite to the effects of the *prp8-201* mutation (Kuhn *et al.* 1999), supporting our model that wild-type Prp8 negatively regulates U4/U6 unwinding (Figure 4C). The synthetic lethality of *snr14-cs1* with the loss-of-function mutations in *PRP44* and *PRP24* clearly substantiates the active role of Prp44 and Prp24 in U4/U6 unwinding (Figure 4C). In addition, the genetic interactions of U4-cs1 with a putative RNA heli-

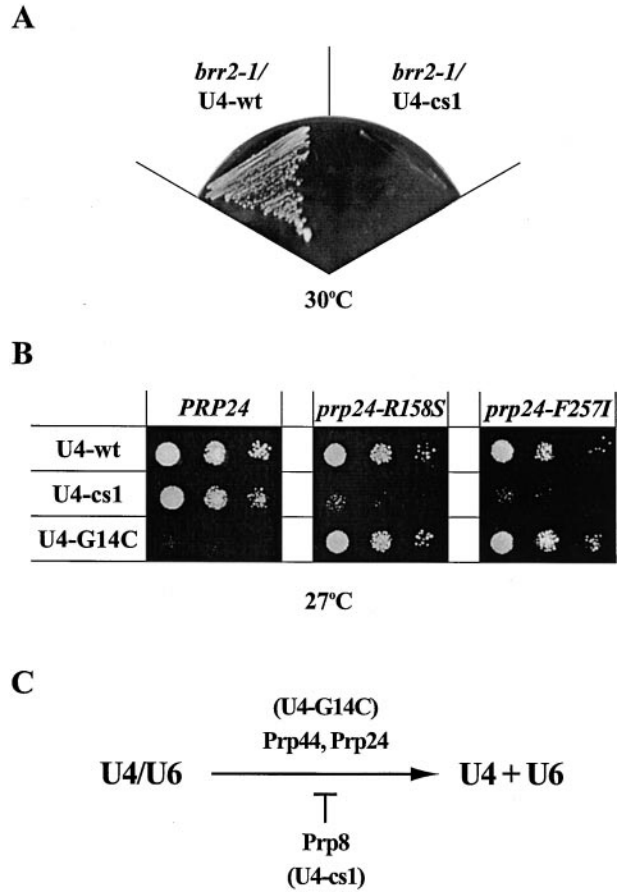


Figure 4.—U4-cs1 effects are synthetically enhanced by loss-of-function mutations in *PRP44* (*BRR2*/*RSS1*/*SLT22*/*SNU246*) or *PRP24*. (A) *snr14-cs1* is synthetically lethal with *brr2-1*. Plasmids with the genes coding for wild-type U4 RNA or U4-cs1 RNA were introduced into a strain (ANK021) with *brr2-1*, a chromosomal disruption of the gene for U4 RNA, and a copy of the gene for wild-type U4 RNA on a counterselectable *URA3*-marked plasmid. Shown are the resulting strains grown on medium containing 5-FOA at 30° for 3 days. (B) *prp24-R158S* and *prp24-F257I* each synthetically enhance *snr14-cs1* and suppress *snr14-G14C*. Strains with chromosomal disruptions of *PRP24* and the U4 RNA gene, the *PRP24* alleles indicated, and a copy of the gene for wild-type U4 RNA on a counterselectable *URA3*-marked plasmid were transformed with a plasmid encoding wild-type U4 RNA, U4-cs1, or U4-G14C. Shown are the resulting strains grown on medium containing 5-FOA at 27° for 4 days. (C) Schematic of the U4/U6 unwinding reaction. Shown are Prp44 and Prp24, the two factors proposed to help U4/U6 unwinding, and Prp8, which is thought to repress U4/U6 unwinding until the spliceosome is competent for activation. The two mutant U4 RNAs are also depicted as either favoring (U4-G14C) or repressing (U4-cs1) U4/U6 unwinding.

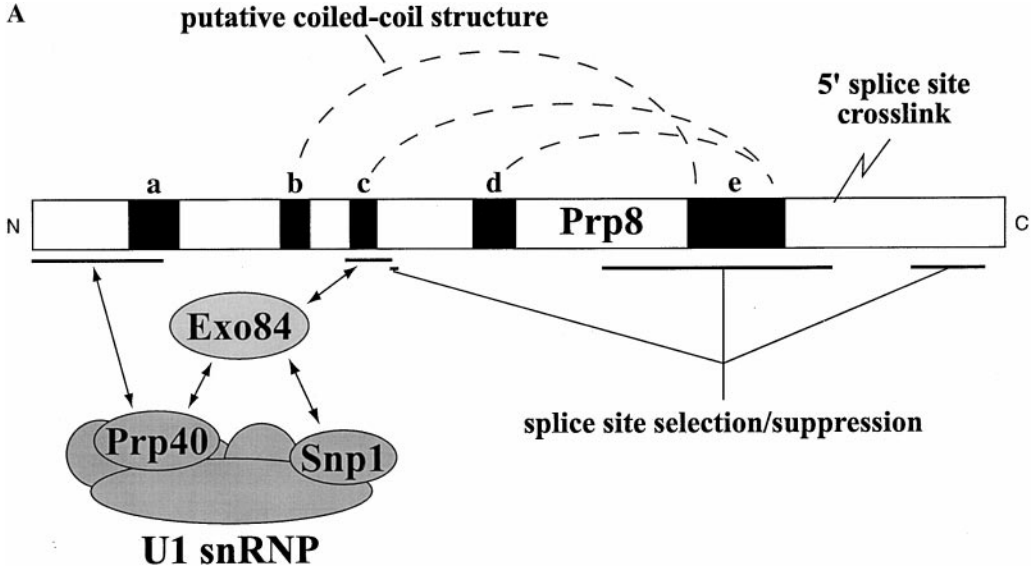
case, Prp44, and an RNA-binding protein, Prp24, further support Prp8's function in controlling RNA-RNA rearrangements during spliceosome activation.

DISCUSSION

Suppressor mutations of the U4-cs1 cold sensitivity define candidate functional domains of Prp8: We have identified five regions of *PRP8* in which mutations suppress the cold sensitivity caused by U4-cs1. Results from the biochemical analysis of U4-cs1-arrested spliceosomes indicate that these five regions of Prp8 are involved in the control of U4/U6 unwinding (Kuhn *et al.* 1999). The identification of novel functional domains in Prp8 is highly significant, because, despite its high evolutionary conservation, the 2413-residue protein contains no recognizable motifs (Hodges *et al.* 1995; Lücke *et al.* 1997; Luo *et al.* 1999). The collection of 46 different alleles described in this study more than doubles the number of alleles reported in all previous studies on *PRP8*. The extent of our screen allows us to define functional domains with high resolution.

The mutations we have identified affect a function of Prp8 distinct from the previously defined role in the second catalytic step (Umen and Guthrie 1996; Collins and Guthrie 1999; Siatecka *et al.* 1999; Ben-Yehuda *et al.* 2000). The Prp8 suppressor Regions a–d do not overlap with regions of Prp8 implicated in the second step. The most C-terminal Region of suppressor mutations, Region e, is encompassed by the region that contains most of the mutations that suppress 5′ and 3′ splice site mutations (Umen and Guthrie 1996; Collins and Guthrie 1999; Siatecka *et al.* 1999; Figure 5A). However, only one of the alleles tested, *prp8-201/T1861P*, confers suppression of both the U4-cs1 cold sensitivity and splice site mutations. The *PRP8* suppressor mutations clearly influence the splicing reaction prior to the first transesterification, as U4-cs1 causes a block to the activation of the spliceosome (Kuhn *et al.* 1999). In contrast, the *PRP8* alleles that suppress both 5′ and 3′ splice site mutations do so by enhancing the efficiency of the second transesterification (Umen and Guthrie 1996; Collins and Guthrie 1999; Siatecka *et al.* 1999). The participation of the splice site suppressor mutations in *PRP8* in the second transesterification is further supported by their genetic interaction with *PRP17*, the gene for a second step splicing factor (Ben-Yehuda *et al.* 2000). The most compelling explanation for these results is that two distinct functions of Prp8, regulation of U4/U6 unwinding and splice site recognition, utilize residues that partially overlap in the primary structure of the protein, but are fulfilled at different points during the splicing reaction. Cross-suppression by *prp8-201/T1861P* might then be explained by a severe distortion of the local Prp8 structure that fortuitously influences both processes and confers temperature sensitivity. The adjacent temperature-sensitive *PRP8* suppressor mutation (changing the valine at position 1860 to aspartate) has not been tested for suppression of splice site mutations. However, the adjacent substitution V1862D, which does not confer temperature sensitivity, and *prp8-H659P*, the allele that causes a growth defect at all temperatures, do not suppress splice site mutations (C. A. Collins and C. Guthrie, personal communication).

It seems likely that the *PRP8* suppressor mutations



B

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Prp8       KYLPHAILKLLENMPHPWEQAKEVKVLYHTSGAITFVNETPRVIEPVYTA 215
S.c. eIF4E MSVEEVSKKFEENVSVDDTTATPKTVLSDSA--------HFDVKHPLNT-  41

                *  *           G                        P
Prp8       QWSATWIAMRREKRDRTHFKRMRFPPFDDDEPPLSYEQHIENIEPLDPIN 265
S.c. eIF4E KWTL-WY--TKPAVDKSESWSDLLRPVTSFQTVEEFWAIQNI-P-EPHE  86

                      P     *           *
Prp8       LPLDSQDDEYVKDWLYDSRPLEEDSKKVNGTSYKKWSFDL----PEMSNL 311
S.c. eIF4E LPLKS--DYHV--FRNDVRPEWEDEANAKGG---KWSFQLRGKGADIDEL 129

           *                                          *
Prp8       YRLSTPLRDEVTDKNYYYLFDKKSFFNGKALNNAIPGGPKFEPLYPREE 360
S.c. eIF4E WLRTLLAVIGETIDED-------DSQINGVVLSIR-KGGNKFA-LWTKSE 170

           Δ
Prp8       EEDYNEFNSIDRVIFRVPIRSEYKVAFPPHLY-NSR-PRSVRIP       401
S.c. eIF4E DKEPLLRIGGKFKQVLKLTDDGHLEFFPHSSANGRHPQPSITL       213
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Figure 5.—(A) Overview of intra- and intermolecular interactions of Prp8. Shown is a schematic of the primary structure of Prp8 with the locations of the five regions involved in suppression of the cold sensitivity caused by U4-cs1 (solid boxes labeled a–e), the locations of the mutations involved in selection or suppression of mutant 5′ and 3′ splice sites (solid lines below Prp8), the locations of Prp8 mutations that interact genetically (connected by dashed lines above the protein), and the position of the crosslink to the 5′ splice site (zigzag line). The yeast two-hybrid interactions between Prp8, Exo84, and the U1 snRNP proteins Prp40 and Snp1 are depicted by double arrows. See text for references. (B) Region a may have a nucleic acid-binding function. Shown is the alignment of amino acids 166–401 of Prp8, which include Region a, with *S. cerevisiae* eIF4E, a subunit of translation initiation factor eIF4F, which binds the cap structure of mRNAs. Residues that are identical or conserved between Prp8 and eIF4E are shown on solid or shaded backgrounds, respectively. Tryptophan residues that are important for cap binding by eIF4E are labeled with asterisks. Surface residues of eIF4E important for protein-protein interactions with eIF4G are underlined. The *PRP8* mutations that suppress the growth defect caused by U4-cs1 in Region a are shown above the sequence.

that we have isolated are loss-of-function mutations. This conclusion is supported both by the large number of different alleles obtained and by the identification of structurally different substitutions in the same amino acid residue. Nevertheless, all *PRP8* alleles that were tested as heterozygous diploids did confer semidominant or dominant suppression of the U4-cs1 growth defect, indicating that the mutant proteins are able to efficiently compete with wild-type Prp8 for incorporation into the U5 snRNP and into the spliceosome. Therefore each mutation seems to affect only a very specific function of Prp8 important for spliceosome activation.

Region a possesses features suited for nucleic acid binding: Computational analysis of the full Prp8 sequence has provided no clear hints regarding domain(s) or function(s) of the protein. We reasoned that weak sequence similarities might be revealed by comparing specific segments of Prp8 with protein databases. Indeed, multiple weak similarities between Region a and a number of proteins whose functions involve binding to nucleic acids or nucleotides were found using FASTA and BLAST search algorithms (Pearson and Lipman 1988; Altschul *et al.* 1997). These include the large subunit of ribonucleotide reductase, a tRNA synthetase, a reverse transcriptase, the RNA recognition motif (RRM)-containing sex-lethal protein from Drosophila, and eIF4E, a subunit of the eukaryotic initiation factor eIF4F, which binds at the cap structure of mRNAs to promote translation.

The strongest match is with eIF4E (Figure 5B), which contains six conserved tryptophan residues that are essential for cap binding (Altmann *et al.* 1988; Rom *et al.* 1998; labeled with asterisks in Figure 5B). Intriguingly, five of these six residues are either tryptophan or tyrosine in Prp8. Altogether, the part of eIF4E surrounding

the conserved tryptophans is ~32% identical and >57% similar to Prp8 Region a, although several gaps must be introduced to achieve this alignment. About 65% of the amino acids identical or similar between *S. cerevisiae* Prp8 and eIF4E are identical or conserved between at least eight of the nine Prp8 orthologues known, including the five conserved tryptophan residues essential for RNA binding by eIF4E (see Figure 1). Thus, Region a of Prp8 may have a nucleic acid-binding function.

The region of eIF4E similar to Prp8 also contains several surface residues that have been shown to be important for protein-protein interactions with eIF4G (amino acids 58–63 and 71–75, underlined in Figure 5B; Matsuo *et al.* 1997; Ptushkina *et al.* 1998). These residues are in one of the least conserved parts of the eIF4E/Prp8 alignment, suggesting that the corresponding residues in Prp8 might be important for interaction with a different protein. Interestingly, when compared to the corresponding residues in the known structure of eIF4E bound to the cap nucleotide (Matsuo *et al.* 1997), the *PRP8* suppressor mutations in this region appear most likely to influence either the specificity of RNA binding (L280P and E362Δ) or the interaction with other proteins (R236G and L261P).

The similarity to the cap-binding protein eIF4E might suggest that Prp8 binds to the cap of the pre-mRNA or of one of the spliceosomal RNAs. The pre-mRNA cap is bound by the cap-binding complex (Cbp20/Cbp80) during spliceosome assembly (Colot *et al.* 1996; Lewis *et al.* 1996), so the latter seems more likely. The proximity of Region a to the Prp40-binding region suggests U1 RNA as a target. However, the weak similarity to known RNA-binding proteins can also be explained if this part of Prp8 is important for recognition of an intermediate RNA structure, for example, the U6 RNA/5′ splice site interaction. The reported crosslink of the 5′ splice site to the part of human Prp8 that corresponds to amino acids 1966–1970 in *S. cerevisiae* Prp8 (Reyes *et al.* 1999; see also Figure 5A) would require that these residues are juxtaposed with Region a in the folded protein to form an RNA-recognition domain. The influence of the suppressor mutations in Region a on RNA recognition or protein-protein interaction by Prp8 might allow spliceosome activation to occur even in the presence of an impaired U6 RNA/5′ splice site interaction.

Intramolecular interactions in Prp8: The synthetic intragenic interactions that we identified for specific combinations of *PRP8* suppressor mutations provide the first insight into intramolecular interactions in Prp8. Suppression of the slow growth phenotype of the H659P mutation from Region b by the L1634F mutation from the N-terminal cluster of Region e is especially interesting, because the sequences surrounding these two substitutions contain a pattern of leucine and isoleucine residues suggestive of a leucine-zipper motif (Landschulz *et al.* 1988). Furthermore, the computational method developed by Frishman and Argos (1997) predicts that

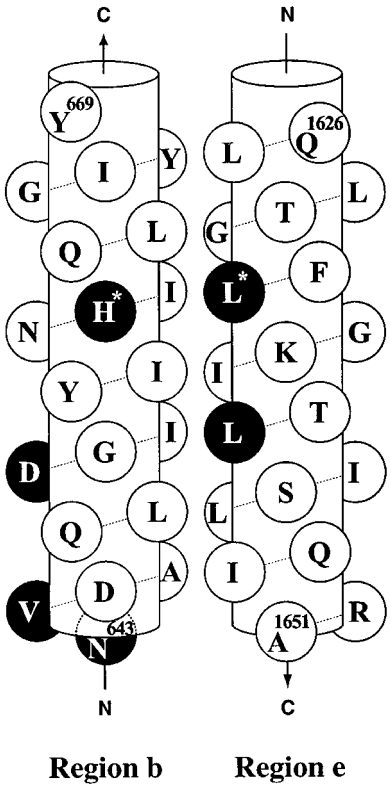

Figure 6.—Amino acids 643–669 from Region b and amino acids 1626–1651 from Region e form a potential coiled-coil structure. Portions of Region b (left) and Region e (right) are modeled on α-helical structures and arranged in an antiparallel coiled-coil. The two amino acids that show genetic interaction with each other, H659 and L1634, are labeled with asterisks. All residues in which suppressor mutations of the U4-cs1 cold sensitivity were identified are shown with a solid background.

these two subregions form α-helical secondary structures. Modeling of these two subregions as α-helices showed that in both cases the hydrophobic amino acids cluster on one face of the helix (Figure 6). Intriguingly, when the two helices are arranged in an antiparallel coiled-coil conformation with the two hydrophobic faces apposed, amino acids H659 and L1634 are in close proximity (labeled with asterisks in Figure 6).

In total, four of the suppressor mutations from Region b and two from the N-terminal cluster from Region e, shown as solid globes in Figure 6, co-localize in the α-helical structures. Three of these, H659, L1634, and L1641, lie in or near the interface and thus most likely influence the interaction of the two helices. Introduction of a proline residue into an α-helical structure, as in H659P, is thought to cause a local helix distortion, which might be the basis for the slow growth phenotype caused by this mutation. The substitution of leucine at position 1634 by phenylalanine might enable new molecular contacts that stabilize the coiled-coil structure, thereby restoring normal growth, while still changing the overall structure so that suppression of the

growth defect conferred by U4-cs1 occurs. In contrast, the three substitutions on the external face of the coiled-coil structure (N643S, V644A, and D651G/N) more likely influence interactions with other parts of Prp8 or with other proteins. Formation of the coiled-coil structure or changes in its overall structure might be important to trigger a signal for spliceosome activation, which is possibly mimicked by the *PRP8* suppressor mutations, either by influencing the structure or stability of the coiled-coil structure, or by changing molecular contacts of the helices with other proteins. Therefore we propose that the structure diagrammed in Figure 6 is part of an intramolecular interaction between Region b and the N-terminal part of Region e and that alterations in this structure or in its contacts to other proteins facilitate spliceosome activation in the presence of U4-cs1.

Interactions between Prp8 and U1 snRNP components: The fact that Prp8 region a defined by our suppressor screen overlaps with the part of Prp8 found to interact with the U1 snRNP protein Prp40 (Abovich and Rosbash 1997) and the finding that Region c interacts with Exo84, which was also identified in yeast two-hybrid screens with the U1 snRNP proteins Prp40 (P. G. Siliciano, personal communication) and Snp1 (S. W. Ruby, personal communication), suggest that contacts between Prp8 and the U1 snRNP influence spliceosome activation (Figure 5A). The putative U4/U6 helicase Prp44 (Brr2/Rss1/Slt22/Snu246), a component of the U5 snRNP in close association with Prp8 (Achsel *et al.* 1998), was also shown to interact with Snp1 in the yeast two-hybrid assay (Fromont-Racine *et al.* 1997). Additionally, a crosslinking interaction between U1 and U5 has been reported during an early step of the splicing reaction (Ast and Weiner 1997), further indicating that contacts between components of the U1 and U5 snRNPs are established during tri-snRNP incorporation into the spliceosome. This model is also consistent with a native gel analysis of U1 snRNP-containing splicing complexes during spliceosome assembly, in which a complex that contains all five spliceosomal RNAs could be detected (Ruby 1997). The contacts between components of the U1 and U5 snRNPs need to be disrupted for the U1 snRNP to leave the spliceosome during activation for catalysis. Work by Staley and Guthrie (1999) and our own biochemical analysis of the U4-cs1 splicing defect (Kuhn *et al.* 1999) indicates that U4/U6 unwinding is coupled to disruption of the U1/5′ splice site interaction. Together these observations suggest a model in which the contacts between components of the U1 and U5 snRNPs help to guide the tri-snRNP into the spliceosome. After correct positioning, the tri-snRNP is anchored by other molecular interactions, including base pairing of U6 RNA with the 5′ splice site (Sawa and Shimura 1992; Sawa and Abelson 1992; Wassarman and Steitz 1992; Kandels-Lewis and Séraphin 1993; Lesser and Guthrie 1993), and the contacts between the tri-snRNP and U1 snRNP are loosened so that the U1 snRNP can leave the spliceosome. Disruption of interactions between U1 and U5 snRNPs could be a prerequisite for spliceosome activation, and the *PRP8* mutations in Regions a and c that suppress the cold sensitivity caused by U4-cs1 might work by weakening interactions of Prp8 with the U1 snRNP, thereby allowing spliceosome activation to proceed in the presence of U4-cs1.

Proteins that interact with Prp8 in the yeast two-hybrid assay: Besides Exo84, our yeast two-hybrid screens with the *PRP8* suppressor regions identified five other proteins more than once: Ygr102w, Djp1, Bmh1/2, Gpm1, and Upc2. While further experiments are needed to analyze the physiological significance of these two-hybrid interactions, the identification of Djp1 and Bmh1/2 is interesting. Djp1, isolated using Region c, is a nonessential DnaJ-like protein (Hettema *et al.* 1998). DnaJ-like proteins are accessory factors for the Hsp70 family of chaperones and are believed to mediate substrate specificity (Cyr *et al.* 1994). Three other DnaJ-like proteins have previously been identified in yeast two-hybrid screens with different splicing factors from the U1 and U2 snRNPs (Fromont-Racine *et al.* 1997). Recent work from Bracken and Bond (1999) has shown that U snRNPs are the primary target for Hsp70 chaperones that confer "splicing thermotolerance," by which splicing can be protected from thermal inactivation if cells are first subjected to a mild heat treatment. Thus, Prp8 might be the target for an Hsp70/Djp1 complex.

BMH1 and *BMH2*, identified with Region d, code for 14-3-3 like proteins, a family of proteins that bind to phosphoserine residues in target proteins whose activity they regulate (Muslin *et al.* 1996; Yaffe *et al.* 1997). Reported target proteins include factors involved in cell cycle control, stress response, and differentiation (Aitken *et al.* 1995; Piwnica-Worms 1999). There are 10 serine residues in the part of Prp8 that was used for the yeast two-hybrid screen with Region d. One or more of these serine residues in a phosphorylated form might be the binding site for Bmh1 and Bmh2. Interestingly, Gpm1, a phosphoglycerate mutase (Rodicio and Heinisch 1987), was also isolated with Region d as bait. This enzyme catalyzes the interconversion of 2-phosphoglycerate and 3-phosphoglycerate, which are similar in structure to phosphothreonine and phosphoserine, respectively. This finding further supports the presence of phosphorylated serines in Region d of Prp8.

Prp44 and Prp24 as targets of Prp8 control: We find that loss-of-function mutations in the genes encoding two factors previously implicated in aiding conformational RNA rearrangements necessary for spliceosome activation, Prp44 (Brr2/Rss1/Slt22/Snu246) and Prp24, are synthetically enhanced when U4-cs1 is the only U4 RNA present. The involvement of Prp24, a protein with three RRMs, in spliceosome activation is based on the identification of mutant *PRP24* alleles that suppress the cold sensitivity of mutations in U6 RNA (A62G or A62U/

C85A) that are thought to block splicing shortly after U4/U6 unwinding (Vidaver *et al.* 1999). This block is apparently due to hyperstabilization of an intermediate U6 RNA structure that is incapable of forming a catalytically active structure with U2 RNA. Prp24 seems to be important for the formation of this intermediate U6 RNA structure, which might help to unwind U4 and U6 RNAs. Furthermore, by analogy to the translation initiation factors eIF4A and eIF4B, Prp24 may be important for Prp44 helicase activity. eIF4A is a DExD/H-box family helicase, like Prp44, and eIF4B is an RRM-containing protein that stimulates eIF4A activity (Niederberger *et al.* 1998; Rogers *et al.* 1999). Likewise, a direct interaction of Prp24 with Prp44 might be a prerequisite for activation of the U4/U6 RNA helicase, and Prp8 might control this interaction by limiting the access of Prp24 to Prp44 based on the status of the U6 RNA/5′ splice site interaction. Thereby Prp8 would repress U4/U6 unwinding until a competent spliceosome is assembled (Figure 4C).

Given the evidence that U4/U6 unwinding is coupled to disruption of the U1/5′ splice site interaction, one might expect genetic interactions between *snr14-cs1* and mutations affecting Prp28, the helicase involved in U1/5′ splice site unwinding (Staley and Guthrie 1999). Interestingly, a mutant allele of *PRP28*, *prp28-1*, which has a mutation in its helicase domain and is thus proposed to be weakened in unwinding activity, is suppressed by a mutation in *PRP8* (Strauss and Guthrie 1991). Furthermore, *prp28-1* is synthetically lethal with a mutation in *PRP24* (Strauss and Guthrie 1991). Together these results suggest that *prp28-1* might be synthetically enhanced by *snr14-cs1*, similar to the mutations in *PRP44* and *PRP24*. Unfortunately, no information about the location of the *PRP8* mutation(s) that suppresses *prp28-1* is available for comparison to Regions a to e. In addition to Prp8, Prp24, Prp28, Prp38, and Prp44, two other splicing factors have been implicated in U4 RNA release during spliceosome activation: the U4/U6 snRNP protein Prp4 (Ayadi *et al.* 1997), and the non-snRNP protein Prp19 (Tarn *et al.* 1993). Characterization of genetic interactions between *PRP8* alleles that suppress the cold sensitivity caused by U4-cs1 and the splicing factors mentioned above should help us to further understand the function of the five regions of Prp8 defined in this study and to unravel the intricate network of factors involved in spliceosome activation.

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