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.

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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; Teigel kamp et al. 1995a,b; Reyes et al. 1996; Sha et al. 1998). Between the first and second transesterification, Prp8 contacts the 3’ splice site (Teigel kamp 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; Sie tecka 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. Suppres sion 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 Snpl (S. W. Ruby, personal communication). Intriguingly, Snpl 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 (MATα snr14::TRP1 prp8::ADE2 trp1 ura3 lys2 his3 ade2 [pRS317U4-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 (MATα prp24Δ::ADE2 his3 leu2 trp1 ura3 md2 can1 ade2 lys2 [PUN50-PRP24]; Vidaver et al. 1999) and YKSL (MATα 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-prp24R1585 or prs313-prp24F2571, respectively. ANK201, the strain containing trp1-21 at the chromosomal locus and a chromosomal disruption of SNR14, was constructed from YSN405 (MATα trp1 ura3 lys2 his3 ade2 leu2; Nobl e and Guthrie 1996) and YKSL (MATα 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 ANK201. To test for genetic interactions between mutant prp38 alleles and snr14-cs1, ts192 (MATα prp38-1 trp1 ura3 leu2 his3 [Bla nton et al. 1992] and JXY6 (MATα prp38::LEU2 trp1 ura3 leu2 his3 ade2 [YCPlac22 (TRP1 prp38-2)]; Xie et al. 1998) were mated with YKSL and YKSL, 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 PJE94-A (MATα trp1 leu2 ura3 his3 galα gal80α lys2::GAL1-HIS3 GAL2-ADE2 md2::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(Sacl)1, which was used for the suppressor screen, was constructed by first subcloning the PRP8-containing XhoI/SnaBI fragment of YCp50-PRP8 (Kuhn et al. 1999) into XhoI/Ed316I-cut pRS313 (Sikorski and Hieter 1989), resulting in pRS313-PRP8. Subsequently, a Sacl 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 ClaI-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 SnaBI-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 mutagen of Prp8 (P303R/E304K and V1862Y, respectively). The clones used for the yeast two-hybrid analysis are based on pGBUD-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 (Ulmen 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 XhoI (717 base pairs upstream of the coding region), Sacl (position 1715 of the coding region), SphI (position 3354 of the coding region), SaclI (introduced by silent mutation at position 5096 of the coding region), and SphI (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 50 μl each. The products of each reaction were then cotransformed with 20 μg of appropriately gapped pRS313-PRP8(SaclI) 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 haploidal PRP8 alleles were then tested for suppression of the cold sensitivity caused by U4-cs1 by replica plating to YEPD and incubation at 18 °C 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 °C.

Mapping of the suppressor mutations. Selected regions of PRP8 containing potential suppressor mutations were PCR amplified using T7 DNA polymerase (Epigenic 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 °C was tested for six transformants for each suppressor strain analyzed after selection against the wild-type PRP8 plasmid. PCR fragments that confer viability to at least three of the six transformants when grown at 18 °C 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 XhoI/SnaBI or SnaBI/SalI, respectively, was used for the cotransformation. Suppression was assigned to the overlapping part if both fragments gave colonies growing at 18 °C, 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(SacI) was digested with SacI/SalI or SacI/SalI/SphI, 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(SacI) linearized with SalI. 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 Clal 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(SaclI) linearized with SphI.

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 °C 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 × 104 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 °C. 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 counter-selectable 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 haplovable PRP8 alleles were then tested for suppression of the cold sensitivity caused by U4-cs1. Altogether, 435 colonies that grew at 18°C were collected, corresponding to about 0.1% of the haplovable 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°C 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 suppression at 18°C 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-
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**TABLE 1**

<table>
<thead>
<tr>
<th>Mutagenized PRP8 interval</th>
<th>Subregion</th>
<th>No. of strains for which suppression was linked to subregion</th>
<th>Fraction of strains tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-253</td>
<td>6</td>
<td>10.2</td>
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<tr>
<td></td>
<td>254-355</td>
<td>4</td>
<td>6.8</td>
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<tr>
<td></td>
<td>356-660</td>
<td>27</td>
<td>45.8</td>
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<tr>
<td></td>
<td>479-660</td>
<td>3</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td>661-753</td>
<td>1</td>
<td>8.3</td>
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<td>2</td>
<td>479-761</td>
<td>6</td>
<td>13.3</td>
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<td>1597-1942</td>
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<td>55.8</td>
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<tr>
<td>4</td>
<td>1597-1942</td>
<td>41</td>
<td>82.0</td>
</tr>
</tbody>
</table>

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

- Given in amino acids encoded by the amplified fragment.
- Given in percentages per interval.
- 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.
- These numbers were obtained from a subpopulation of only 12 strains tested for linkage to amino acids 479–753.
- Eight strains, for which suppression was not linked to amino acids 1022–1213, were not tested for linkage to amino acids 1597–1942.

Sequence of suppression of the U4-cs1 growth defect. In addition to T1861P, only the adjacent change V1860D gives very slow growth at 37°C. 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 mutations at position 1860 (V1860N) or with the same change at position 1862 (V1862D) do not 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 1834–2042, 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.
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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°C (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°C, but also at 18°C and 37°C (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°C or 37°C (Figure 2 and data not shown).

However, a more complex pattern can be observed at 18°C. 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°C. Second, P1688L is able to partially suppress the slow growth defect conferred by H659P at 18°C, although not as well as L1634F (Figure 2). Third, a strain with Prp8-H659P/P1688L grows slower at 18°C 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 and Region e. Cells with these double-mutant alleles and U4-cs1 RNA do not grow at 18°C, indicating reversion of suppression, since cells with these PRP8 alleles are viable at 18°C 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°C growth defect caused by V1860D or T1861P (Figure 3B and data not shown). U4-cs1 also modestly enhances the 37°C 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

<table>
<thead>
<tr>
<th>Mutations</th>
<th>U4-wt</th>
<th>U4-cs1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prp8-wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H659P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H659P/L1634F</td>
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<td>H659P/P1688L</td>
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<td>Prp8-wt</td>
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<td>H659P/P1688L</td>
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</table>

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°C for 7 days (top), at 30°C for 3 days (middle), and at 37°C for 2 days (bottom).
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°C 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-
Role of Prp8 in Spliceosome Activation

TABLE 2
Proteins isolated more than once in yeast two-hybrid screens with Prp8 fragments

<table>
<thead>
<tr>
<th>Region(s) used as bait</th>
<th>Protein isolated</th>
<th>Codons included in fusion</th>
<th>Reported characteristics of the protein</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>a/b</td>
<td>Ygr021w</td>
<td>45–290 (290)</td>
<td>Involved in mitochondrial protein synthesis</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DnaJ-like protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Part of exocyst complex</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Homologues of 14–3–3 proteins</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phosphoglycerate mutase</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Regulatory role in sterol uptake and esterification</td>
<td>3</td>
</tr>
</tbody>
</table>

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.

1 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.

2 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).

3 Bmh1 and Bmh2 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°C, 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°C 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°C, 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-
rearrangements during spliceosome activation. V1862D, which does not confer temperature sensitivity, support Prp8’s function in controlling RNA-RNA splice site mutations. However, the adjacent substitution 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; Lucke 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). 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
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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 Snpl 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 and a number of proteins whose functions involve binding to nucleic acids or nucleotides were found using 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 protein data bases. Indeed, multiple weak similarities between Prp8 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 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.

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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 \textit{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 E362D) 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 human Prp8 that corresponds to amino acids 1966–1970 in \textit{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 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 Snpl (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 (Br2/Rss1/Slt22/Snu246), a component of the U5 snRNP in close association with Prp8 (Achse1 et al. 1998), was also shown to interact with Snpl 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 by 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 Abel son 1992; Wassarman and Stelitz 1992; Kandel et al. 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 by 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 Hennisch 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 phosphoethreonine 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 (Br2/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 RRM s, 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 DEAD/H-box family helicase, like Prp44, and eIF4B is an RRN-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 sn14-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 (Stauss and Guthrie 1991). Furthermore, prp28-1 is synthetically lethal with a mutation in PRP24 (Stauss and Guthrie 1991). Together these results suggest that prp28-1 might be synthetically enhanced by sn14-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, 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 (Tar n 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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