Mutational analysis identifies two separable roles of the *Saccharomyces cerevisiae* splicing factor Prp18

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ABSTRACT

Prp18 functions in the second step of pre-mRNA splicing, joining the spliceosome just prior to the transesterification reaction that creates the mature mRNA. Prp18 interacts with Slu7, and the functions of the two proteins are intertwined. Using the X-ray structure of Prp18, we have designed mutants in Prp18 that imply that Prp18 has two distinct roles in splicing. Deletion mutations were used to delineate the surface of Prp18 that interacts with Slu7, and point mutations in Prp18 were used to define amino acids that contact Slu7. Experiments in which Slu7 and mutant Prp18 proteins were expressed at different levels support a model in which interaction between the proteins is needed for stable binding of both proteins to the spliceosome. Mutations in an evolutionarily conserved region show that it is critical for Prp18 function but is not involved in binding Slu7. Alleles with mutations in the conserved region are dominant negative, suggesting that the resulting mutant prp18 proteins make proper contacts with the spliceosome, but fail to carry out a Prp18-specific function. Prp18 thus appears to have two separable roles in splicing, one in stabilizing interaction of Slu7 with the spliceosome, and a second that requires the conserved loop.

Keywords: pre-mRNA splicing; second-step factor; Slu7

INTRODUCTION

The second step of splicing takes place within an assembled spliceosome composed of the U2, U5, and U6 snRNPs together with a number of proteins (reviewed in Umen & Guthrie, 1995; Burge et al., 1999; Hastings & Krainer, 2001). The U2 and U6 snRNAs are proposed to form the catalytic core of the spliceosome, whereas the U5 snRNP holds the exons in position for the transesterification that yields the spliced mRNA (Newman, 1997; Nilsen, 1998; Will & Lührmann, 2001; Villa et al., 2002).

Stages within the second step have been delineated by their requirements for ATP and for different proteins. In the first identified stage following the first transesterification reaction, the DExH-box RNA helicase Prp16 drives an ATP-dependent rearrangement of the spliceosome (Schwer & Guthrie, 1991, 1992). Prp17 acts at this stage as well, although its function is unknown (Jones et al., 1995). The hydrolysis of ATP plays a role in ensuring the fidelity of the first step (Burgess & Guth-

rie, 1993), and the conformational change of the spliceosome sets the stage for the actions of the Slu7, Prp18, and Prp22 proteins (Brys & Schwer, 1996). The entry of these three proteins into the spliceosome allows the ATP-independent exon ligation reaction to proceed (Horowitz & Abelson, 1993a; Jones et al., 1995; Schwer & Gross, 1998). *PRP18* is not essential in *Saccharomyces cerevisiae*, although yeast without it grow slowly and are temperature sensitive; both *SLU7* and *PRP22* are essential (Company et al., 1991; Frank & Guthrie, 1992; Horowitz & Abelson, 1993b). However, none of the three proteins is necessary for exon ligation in vitro if the distance from the branchpoint to the 3' splice site is sufficiently short (Brys & Schwer, 1996; Zhang & Schwer, 1997; Schwer & Gross, 1998). A network of genetic and physical interactions connects these splicing factors. Biochemical experiments show that Prp18 is associated with the U5 snRNP, and *PRP18* interacts genetically with the U5 snRNA gene *SNR7* (Frank et al., 1992; Horowitz & Abelson, 1993b). *SLU7* also interacts genetically with *SNR7*. High levels of Slu7 can partly compensate for the absence of Prp18 both in vivo and in vitro, and two-hybrid studies show that the two proteins interact (Jones et al., 1995; Zhang & Schwer, 1997). Although additional interactions of Prp18 are not

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known, interactions of Slu7 with Prp22, Brr2, and Slt11 have been reported (van Nues & Beggs, 2001; Xu & Friesen, 2001). How Prp18, Slu7, and Prp22 function during the second reaction is not well understood. Slu7 plays a role in the selection of 3' splice sites (Frank & Guthrie, 1992), and the distance dependence of their action suggests that the three proteins may act as a bridge between the 3' splice site and the branchpoint site (Schwer & Gross, 1998). The role of Prp18 has been investigated using microarrays (Clark et al., 2002). In *PRP18*-knockout yeast, splicing of the vast majority of intron-containing pre-mRNAs is significantly affected even at a permissive temperature, suggesting an important role for Prp18 in splicing of most pre-mRNAs.

Following the transesterification reaction, Prp22, which is a member of the DExH-box family, catalyzes an ATP-dependent conformational change of the spliceosome that releases the mRNA (Company et al., 1991). This second role for Prp22 is distinct from its role before exon ligation (Schwer & Gross, 1998). The Prp8 protein also functions during the second step (reviewed in Collins & Guthrie, 2000). Prp8 stabilizes several interactions involving both the snRNAs and the pre-mRNA or splicing intermediates; these interactions may be important through all of the stages of the second step.

The Prp18 proteins from *S. cerevisiae* and *Homo sapiens* have been characterized (Horowitz & Abelson, 1993a, 1993b; Horowitz & Krainer, 1997). These two Prp18s are strongly homologous only in the carboxyl-terminal thirds of the proteins (starting around position 140 in the yeast sequence). Nevertheless, the proteins are true functional homologs. Homologs from many species are found in the databases, and representative examples are aligned in Figure 1A. All display strong homology at the carboxyl end, including a nearly invariant block of 19 amino acids (positions 187–205). In the amino-terminal halves of the proteins, there are two regions of homology: limited homology extends about 35 amino acids in from the amino termini of the proteins, and a more conserved block, which is absent from *S. cerevisiae*, is centered at amino acid 95 of human Prp18 and is the binding site for the cyclophilin USA-CyP (Horowitz et al., 2002). The Prp18 sequences are not homologous to other proteins and do not display any motifs.

The structure of a large fragment of yeast Prp18 was determined by X-ray crystallography (Jiang et al., 2000). The fragment lacks the amino-terminal 80 amino acids, but is fully functional in vitro. The structure consists of five tightly packed α -helices (Fig. 1B,C). The first two helices form one face of the protein, whereas helices 4 and 5 are on the opposite face and helix 3 runs through the middle of the protein. The highly conserved block falls in a large unstructured loop between helices 4 and 5, and was only partly visible in the crystal structure.

In these studies, we designed and tested a large set of mutant alleles of *PRP18*. We combined the informa-

tion from the X-ray structure with that from the homology to design mutants that would allow us to address several questions. We were particularly interested in evaluating the role of the conserved region of Prp18, identifying contacts between Prp18 and Slu7, and determining how their interaction affects their functions in splicing.

RESULTS

$\Delta PRP18$ yeast strains

We constructed two yeast strains in which *PRP18* was disrupted. The knockout strains grew slowly at 23 °C, very slowly or not at all at 30 °C, and not at all at 34 °C and 37 °C, in agreement with previous results (Horowitz & Abelson, 1993b). For assay of mutant alleles of *PRP18*, the 2.5-kb *PRP18* gene was placed on the single-copy plasmid pRS314 to obtain wild-type levels of Prp18. The strains gave very similar results in complementation assays with mutant alleles of *PRP18*.

Deletion mutations

We constructed a series of amino- and carboxyl-terminal deletions in Prp18, as shown in Figure 2. The deletions encompassed entire helices or loops. All the deletions at the carboxyl terminus abolished the function of Prp18. Deletion of the first 79 amino acids of Prp18 had no effect on activity, consistent with in vitro results (Jiang et al., 2000). Further deletion of the 25-amino-acid loop preceding helix 1 gave a protein that was functional up to 34 °C, but additional deletion of helices 1 and 2 gave an inactive protein.

Interaction between Slu7 and the Prp18 deletion proteins was assayed using the yeast two-hybrid system (Figs. 2 and 3). The strength of these interactions was estimated from comparing the growth of indicator yeast bearing wild-type and mutant alleles of *PRP18* on media lacking histidine and/or adenine, and containing different concentrations of 3-aminotriazole (Amberg & Botstein, 1997). Example results are shown in Figure 3B, and the scale we used is detailed in the legend and in the Materials and Methods section. Carboxyl-terminal deletions through helix 4 or amino-terminal deletion of the first 79 amino acids did not substantially affect the strength of interaction with Slu7, although the prp18 in which only helix 5 had been deleted interacted less strongly with Slu7 than did proteins with longer deletions. Further deletion of helix 3 or of helices 1 and 2 abolished interaction. (We were unable to evaluate the $\Delta 6$ -104 deletion mutant because the protein gave a weakly positive result in combination with a blank control vector.) The 98-amino-acid polypeptide consisting of loop 1 and helices 1–3 interacted with Slu7, although very weakly. The results indicate that helices 1 and 2 are important for interaction of Prp18 with Slu7. Helix 3

FIGURE 1. A: Alignment of sequences of representative Prp18 proteins. Positions at which at least four of the six sequences (three of the five in the long section with no *S. cerevisiae* sequence) are identical are shown yellow on dark blue, and positions at which 3 amino acids are identical or at least 4 are similar are shown black on cyan. Similarities used were D ~ E, R ~ K ~ H, I ~ L ~ V ~ M, N ~ Q, and F ~ W ~ Y. The positions of helices and loops in the structure are indicated above the sequence with regions for which the structure was not resolved shown with dashed lines. The short helical region at the beginning of the structure was not assigned a number (Jiang et al., 2000). The amino acids SLLAEERFFGGNREQV SEERLRLMPSQSES are not shown at the carboxyl terminus of the *Arabidopsis thaliana* Prp18. **B, C:** Structure of Prp18 from two angles. Helices and the conserved loop are shown in various colors, as indicated in the figure, and other loops are shown in cyan. **D:** The conserved loop of Prp18. The structurally resolved residues of the loop are shown with stick figures with the rest of Prp18 as a gray ribbon drawing. Amino acids 190, 191, 218, and 219, which were used for the generation of deletion mutations, are highlighted to show their proximity.

is mostly buried inside Prp18 and contacts helices 1 and 2 extensively (Fig. 1); in its absence (as in the $\Delta 153$ –251 mutant) helices 1 and 2 may not fold properly.

Mutations within helices 1 and 2

To examine the role of helices 1 and 2 in the interaction of Prp18 with Slu7, we made a set of point mutations in the helices. The most conserved amino acids in this

region are hydrophobic ones that are in the interior of Prp18 (Fig. 1). We wished to focus on surface residues that might contact Slu7 directly. Zhang and Schwer (1997) defined a 25-amino-acid stretch of Slu7 that is necessary for its interaction with Prp18. Nine of these 25 amino acids are acidic, and the region has a charge of -6 . The Slu7-interacting portion of Prp18 defined by the deletions includes eight solvent-exposed positively charged residues (Jiang et al., 2000). We mutated these

FIGURE 2. Schematic diagram of deletion mutations in Prp18. Each deletion is represented as a gray box with numbers indicating the extent of the deletion. The positions of helices 1 through 5 are shown along the top. The growth at different temperatures of *PRP18*-knockout yeast bearing the mutant alleles of *PRP18* is shown. The strength of interaction of each mutant prp18 with Slu7, measured in the two-hybrid system, is shown using the scale described in the Materials and Methods section and in the legend to Figure 3. The Δ6-104 mutant, indicated as “not determined,” gave a strong positive result in the two-hybrid assay; however, it also gave a weak positive signal with a DNA-binding domain control vector, suggesting that this result is not reliable.

residues singly or in pairs, changing the positively charged amino acids to glutamic acid, and tested the interactions of the resulting mutant Prp18 proteins with Slu7 (Fig. 4A). The double mutant R151E|R152E did not interact with Slu7, and the H118E protein interacted only weakly, implying that these amino acids are important contacts for Slu7. R151 and R152 occur in the loop between helices 2 and 3, and this loop is positively charged in all the aligned Prp18s except for that from *Schizosaccharomyces pombe* (Fig. 1A).

To clarify the contributions of R151 and R152, we made the mutations R151A, R152A, and R151A|R152A (Fig. 4B). Only the double mutation had a discernable effect, suggesting that both residues are important for interaction with Slu7. We combined R151A or R152A with other mutations both to assess the relative contributions of R151 and R152 and to identify additional amino acids that interact with Slu7. The combination of R151A with K140E|K141E greatly reduced the interaction of the mutant Prp18 with Slu7, suggesting that K140 and K141 contribute to binding of Slu7 (Fig. 4B). The R152A combination had no effect; this pattern was repeated with other combination mutants described below, suggesting that R151 is more important than R152 in Slu7 interaction. We used R151A in combination with other mutations elsewhere in Prp18. Combination of R151A with R80, K89, or K125 suggested a secondary role for R80 in Slu7 binding and no role for the other amino acids. H118 and R151 are juxtaposed in the

structure of Prp18, raising the possibility that substitution of glutamic acid for histidine at position 118 interfered with R151. However, the double mutants H118A|R151A and H118E|R151A were both more severe than the R151A mutant, consistent with distinct roles for H118 and R151 in binding Slu7.

The temperature sensitivities of these *PRP18* mutants correlate with the strengths of their interactions with Slu7 in the two-hybrid assay (Fig. 4). Mutants that do not interact with Slu7 do not support growth at 37 °C, but do support it at 34 °C; conversely, mutants that interact well with Slu7 support growth at 37 °C. We constructed some *PRP18*s with multiple mutations in the Slu7-binding helices that have more severe temperature-sensitive (ts) phenotypes. Alleles in which codons for several basic amino acids had been changed to acidic ones (Fig. 4C) conferred stronger ts phenotypes. These phenotypes apparently result from the added repulsion of the mutant Prp18 for Slu7, and we suggest that, in the spliceosome, other interactions hold Prp18 and Slu7 together against small repulsive forces, but that sufficient repulsive interactions can drive them apart.

A possible role for the amino terminal 80 amino acids in interaction with Slu7 was evaluated by making combination mutations of this deletion with R151 and R152 (Fig. 4D). The Prp18 protein bearing the R151A mutation and the Δ6-79 deletion interacted only weakly with Slu7 and did not support growth at 37 °C, suggesting that the first 80 amino acids of Prp18 contribute to its

FIGURE 3. Complementation of $\Delta PRP18$ yeast and two-hybrid assays. **A:** $\Delta PRP18$ yeast (from the parent strain TDY2037) bearing the indicated *prp18* alleles on single-copy plasmids were streaked and grown at the temperatures indicated along the top. The GVT $\rightarrow$ AAA mutant is at positions 196 to 198, and $\Delta CR\#1$ is the conserved region deletion mutant ign-GG-nim. **B:** The two-hybrid indicator strain AH109 was transformed with plasmids that direct the expression of the Slu7 and Prp18 fusion proteins described in detail in the Materials and Methods section. The yeast were grown on the synthetic media indicated above each plate; all plates lacked tryptophan and leucine.

interaction with Slu7. (Consistent with a lesser role for R152, the R152A mutant had no effect in combination with $\Delta 6-79$.) The longer amino-terminal deletion $\Delta 6-104$ in combination with R151A could not be tested because the protein gave a positive result with a blank control vector.

Mutations in helix 5

In contrast to the case in helices 1 and 2, surface residues in helix 5 are well conserved (Fig. 1), and we made mutations in many of the conserved amino acids (Fig. 5A). The mutation D223K|E224A, which changes two invariant residues, had no evident effect on the function of Prp18 or on its interaction with Slu7 (Fig. 5A). Slu7 interaction was also tested in combination with R151A, and no effect was seen. Likewise, the mutation R225E|R227E, which includes the invariant R227, had no discernable effects. (Note that R225 is on the Slu7-interacting face of Prp18.) However, the mutation of the

invariant K234 and R235 to A234|E235 had a significant effect; this mutant allele conferred a strong ts phenotype on the yeast, which grew only up to 30 °C. This mutant interacted normally with Slu7, even in combination with R151A.

Mutations in the highly conserved region

We made two types of mutations in the conserved region between amino acids 187 and 205 (Fig. 1). First, we mutated many of the residues to alanine in groups of three (Fig. 5B). All of these mutant alleles were temperature sensitive to some degree, with the most severe supporting growth only up to 30 °C. In addition, some of these mutants grew more poorly at 23 °C than the *PRP18*-knockout strain itself. The G196A|V197A|T198A and G201A|I202A|H203A proteins showed a weakened interaction with Slu7 in the two-hybrid assay, but the combination G196A|V197A|T198A with R151A or R152A restored its two-hybrid interaction to

FIGURE 4. Mutations in helices 1 and 2. The positions of the point mutations (A–C) and deletions (D) in Prp18 are shown schematically. The sequence and secondary structure of Prp18 are shown at the top, with the amino acids of interest in larger type. The growth at different temperatures of $\Delta PRP18$ yeast transformed with each mutant and strength of interaction of Slu7 with each mutant are shown at the right. The scoring is detailed in the Materials and Methods section and displayed in Figure 3. The interaction between Slu7 and the $\Delta 6$ –104 mutant (shown in D) could not be reliably assayed, as described in the text and in the legend to Figure 2.

normal strength, a finding for which we provide an explanation in the Discussion.

The second type of mutation that we made was deletion of the conserved region. The ends of the conserved loop run close together and antiparallel to each other (Fig. 1D). Gly189 and Val191 appear to contact Ile220 and Ala218. We designed four deletion mutants in which the 26 or 28 amino acids between the spatially close amino acids highlighted in Figure 1D were replaced by a two- or three-amino-acid bridge. Their sequences are shown in Figure 5C. Despite deletion of almost all of the most conserved part of the protein, these mutants retained considerable function. They supported nearly wild-type growth at 30 °C and slow growth at 34 °C, but did not support growth at 37 °C. Additionally, they all slowed the growth of *PRP18*-knockout yeast at 23 °C. The proteins all appeared to interact with Slu7 at slightly less than wild-type strength, but, as for the

G196A|V197A|T198A mutant, combination with R151A restored wild-type interaction.

Several arguments imply that these deletion-mutant proteins are folding correctly. First, the ts phenotype of these mutants is less severe than that of deletion of helix 5 or of the larger deletion including the conserved region (Fig. 2). Second, we constructed a double mutant in which a conserved-loop deletion was combined with the K234A|R235E mutation in helix 5 (Fig. 5C). If helix 5 were not properly folded in the presence of the deletion, then the point mutations in helix 5 would be expected to have no effect; instead, this double mutant is much less active than the conserved-loop deletion, strongly suggesting that helix 5 is properly folded in the deletion mutants. Similarly, the phenotype of the combination of a conserved loop deletion with R151A suggests that helix 2 is properly folded, although the effects are smaller (Fig. 5C). Finally, we show below that the

FIGURE 5. Mutations in helix 5 and in the conserved loop. The positions of point mutations in helix 5 (A) and of point mutations (B) and deletion mutations (C) in the conserved loop are shown. Reference sequences and/or secondary structures are shown at the top of each panel. The growth at different temperatures of $\Delta PRP18$ yeast transformed with each mutant and strength of interaction of Slu7 with each mutant are shown at the right.

deletion mutants are dominant in high copy, suggesting that they retain substantial structural similarity to the wild-type protein.

In vitro assay of interaction with Slu7

To look directly at the binding of Prp18 to Slu7, we made an affinity column using the 25-amino-acid peptide from Slu7 that Zhang and Schwer (1997) had shown to be necessary for binding to Prp18. This peptide is a part of the 80-amino-acid portion of Slu7 shown by van Nues and Beggs (2001) to be sufficient for binding. Mutant Prp18 proteins were synthesized in *Escherichia coli* for testing. As shown in Figure 6A, Prp18 bound tightly to this column and could not be eluted with NaCl (up to 1 M). SDS was required for elution (Fig. 6A,

lane 8). A peptide of identical composition in which the order of the amino acids had been changed did not bind to Prp18, indicating that the interaction was specific (data not shown).

Binding of several mutant Prp18s to the Slu7-peptide column was tested (Fig. 6B). In general, binding to the column was abolished by mutation of any amino acid that had been implicated in Slu7 binding by the two-hybrid tests, and no gradation of affinities was observed. No binding was seen for mutant proteins like R151E|R152E, which was negative in the two-hybrid test. In addition, binding was abolished by the mutations R151A or K125E|K140E|K141E, which gave positive two-hybrid results, but were implicated in Slu7 binding in combination with other mutations (Figs. 4B and 6B). The Slu7 peptide may be too short to fold by

FIGURE 6. Binding of Prp18 to a Slu7-peptide column. **A:** A lysate of *E. coli* that overproduced Prp18 was applied to beads to which a 25-amino-acid peptide from Slu7 had been covalently attached. The column load (lane 1), unbound fraction (lane 2), wash fractions with the concentrations of NaCl shown at the top of each lane (lanes 3–7), and SDS elution fraction (lane 8) are indicated. **B:** The indicated mutant prp18s were applied to the resin and treated as in **A**. Only the section of gel with prp18 is shown. The mutant proteins that were tested and bound normally to the column were G201A|I202A|H203A, K234A|R235E, $\Delta 6$ –79, and $\Delta 173$ –251, and the internal deletions ign-GAG-nim and ignv-GAG-anim. The proteins that failed to bind the column were H118E, R151E|R152E, K125E|K140E|K141E, K140E|K141E|R151E|R152E, R151A|R152A, R80A|R151A, and R151A.

itself, and therefore requires all the interactions with Prp18 to adopt its proper conformation and bind to Prp18. In contrast to the helix 1 and helix 2 mutants, the conserved region mutants, which interacted weakly with Slu7 in the two-hybrid assay, bound normally to the column, as did the helix 5 mutant K234A|R235E and the amino-terminal $\Delta 6$ –79 deletion mutant. A complete list of mutants assayed appears in the legend to Figure 6. The results show that the 25-amino-acid region from Slu7 is sufficient for binding Prp18, and strongly support a picture in which the 25-amino-acid

region from Slu7 interacts directly with the surface formed by helix 1 and helix 2.

Overexpression of Slu7 in *prp18* yeast

To understand the interplay of Slu7 and Prp18, we studied the effect of overexpression of Slu7 in yeast bearing mutant alleles of *PRP18*. Overexpression of Slu7 has been shown to partly suppress the *prp18-1* ts allele (Jones et al., 1995), and a high concentration of Slu7 can compensate for the absence of Prp18 in vitro (Zhang & Schwer, 1997). We found that expression of Slu7 from multicopy plasmids partly suppressed our *PRP18*-knockout alleles. These yeast grew moderately well at 30°C, but not at higher temperatures (Figs. 7 and 8A), similar to the suppression reported previously (Jones et al., 1995). No better suppression was seen when Slu7 was more highly expressed using the *TPI1* promoter (not shown).

The ts phenotype of alleles with conserved loop deletion mutations was very similar to that of overexpressed Slu7 in *PRP18*-knockout strains. We surmised that the similarity reflected an underlying functional equivalence. In the conserved loop deletion mutants, Slu7 may be able both to bind normally to the spliceosome and to function; likewise, in the *PRP18*-knockout strains, overexpression of Slu7 allows it to bind to the spliceosome and function. However, in neither case does Prp18 function properly. The hypothesis predicts that overexpression of Slu7 in yeast strains whose Prp18 has mutations in the conserved loop would have no effect but that overexpression of Slu7 in yeast whose Prp18 has mutations in its Slu7-interacting surface could restore wild-type growth.

To test the model, we initially constructed additional mutants in Prp18. Most of the mutants that we had used for identifying contacts between Prp18 and Slu7 had changes of basic residues to acidic ones, presumably creating repulsive interactions between the two proteins. For this experiment, we made neutral changes that we believed would allow Slu7 and Prp18 to be present in the spliceosome simultaneously (Fig. 7). We tested the growth of yeast bearing these mutant alleles of *PRP18* with normal or high levels of Slu7 (Figs. 7A,B, and 8A). Overexpression of Slu7 (using its own promoter and a multicopy plasmid) suppressed all neutral or weakly repulsive mutations in *PRP18*. We observed a roughly 4°C increase in the permissive temperature in every case. *prp18* alleles that included R151E|R152E were suppressed less well or not at all. In a few cases tested, expression of Slu7 at higher levels using the *TPI1* promoter did not afford additional suppression (data not shown).

We also examined the effect of Slu7 overexpression on three amino-terminal deletion mutants (Fig. 7B). Slu7 overexpression partly suppressed the two shorter deletions but had no effect on the longer $\Delta 6$ –152 deletion.

FIGURE 7. Chart of results of overexpression of Slu7 and of *prp18*. A schematic placing all the mutations is shown at the left. Most of the mutations are shown in more detail in Figures 2, 4, and 5. **A:** Growth of the mutants expressed at wild-type levels in *PRP18*-knockout yeast. **B:** Growth of the mutants expressed in *PRP18*-knockout yeast at wild-type levels when Slu7 was expressed from a multicopy (2μ) plasmid. Overexpression of Slu7 suppresses a *prp18* allele if the overexpression allowed better growth than wild-type expression of Slu7 and better growth than Slu7 overexpression in the absence of Prp18 (shown in the lowest line). Thus, neither the $\Delta 6-152$ nor the H118E/K140E/K141E/R151E/R152E allele was suppressed by overexpression of Slu7. The $\Delta 6-79 + R151E/R152E$ allele was also not suppressed, but if this *prp18* was expressed at a higher level, then overexpression of Slu7 did suppress it. **C:** Growth of *PRP18*-knockout yeast in which the mutant *prp18* was overexpressed, either at medium level (M) from a multicopy plasmid or at high level (H) using the *TP11* promoter. For some mutants, both M and H phenotypes are shown. **D:** Growth at different temperatures of wild type yeast that expressed mutant Prp18 proteins at high levels. "nd" indicates "not determined."

The suppression of the shorter deletion alleles is consistent with a role for the amino-terminal domain in interacting with Slu7, as suggested by the two-hybrid results.

Overexpression of Slu7 in yeast bearing *prp18* alleles with mutations in the conserved loop or in helix 5 had no effect on the growth of yeast (Figs. 7B and 8A). The results are all consistent with, but cannot prove, our hypothesis. The failure of Slu7 overexpression to suppress the conserved region deletions suggests that Slu7 is not limiting in these yeast and is properly binding to the spliceosome. The suppression of the *prp18* alleles that are defective in their Slu7 interaction is consistent with our hypothesis or with any hypothesis in which this interaction is important.

Overexpression of mutant *prp18* proteins

We examined the effect of overexpression of some mutant *prp18* proteins in *PRP18*-knockout yeast (Figs. 7C and 8A). Overexpression of Prp18 proteins with mutations in the conserved region or in helix 5 had little effect on their growth phenotypes. In contrast, overexpression of proteins with mutations in helices 1 or 2 (or combined with amino-terminal deletions) reduced their severity, increasing the permissive temperature for growth by roughly the same amount as overexpression of Slu7. In these cases, very high levels of the mutant *prp18* protein (expressed from the *TP11* promoter) or co-overexpression of Slu7 (on a multicopy

FIGURE 8. Overexpression of *prp18* and *Slu7* in $\Delta PRP18$ and in wild-type yeast. **A:** *PRP18*-knockout yeast bearing the mutant *prp18* alleles indicated in the figure guide and a multicopy plasmid either including *SLU7* (indicated as +*SLU7* in the outer ring) or without *SLU7* (-*SLU7*) were streaked and grown at the temperature indicated at the top. In the upper panel, the *prp18* was on a single-copy (*CEN*) plasmid. In the lower half-panel, the mutant H118A|K140A|K141A|R151A|R152A (shown in Fig. 7) was expressed from a single-copy (*CEN*) plasmid or from a multicopy (2μ) plasmid, as indicated in the figure guide. $\Delta CR\#1$ is the ign-GG-nim conserved region deletion mutant. **B:** Mutant *prp18* proteins were expressed in wild-type yeast that have a normal chromosomal copy of *PRP18*. The *prp18*s were expressed at high levels from the *TPI1* promoter on a multicopy plasmid, and the yeast were grown at the indicated temperatures. The mutants used are indicated in the schematic at the left, where $\Delta CR\#1$ is the ign-GG-nim allele and $\Delta CR\#2$ is the ign-GAG-nim allele (both shown in Fig. 5C) and KR $\rightarrow$ AE represents K234A|R235E.

plasmid) further reduced the severity of the phenotype (Figs. 7C and 8A). Mutations in *PRP18* that could be suppressed by overexpression of *Slu7* could be similarly suppressed by overexpression of the mutant *prp18* protein, and, conversely, *prp18* alleles whose ts phenotypes were unaffected by high levels of *Slu7* did not benefit significantly from overexpression.

We assayed some of our mutant alleles of *PRP18* to determine whether any were dominant negative. When expressed at wild-type levels in yeast bearing a normal, chromosomal copy of *PRP18*, none of the tested alleles had a significant effect (data not shown). However, when expressed at high levels, every mutant in the conserved region was dominant. Figure 7D tabulates the effects of overexpression of several conserved-region mutants in wild-type yeast, and Figure 8B shows the dominance of one of the conserved region alleles. The growth phenotypes of the conserved region alleles overexpressed in wild-type yeast were similar to those seen in *PRP18*-knockout yeast (either overexpressed or expressed at wild-type levels), although in the wild-type strains, the deleterious effects of the mutant alleles at 23 and 30 °C were more evident. Additional

mutation of the conserved region-deletion *prp18*s, either with R151A (which has only a small effect on the growth phenotype of the mutant *prp18*) or K234A|R235E, abolished the dominant negative phenotype, presumably by interfering with other interactions of *prp18* and allowing the wild-type *Prp18* to compete effectively. Alleles of *PRP18* bearing mutations in the *Slu7*-interacting helices or in helix 5 were also tested for dominance, but none had any effect on wild-type cells (Fig. 7D).

DISCUSSION

We report the design and analysis of a set of mutant alleles of the yeast *Prp18* protein. Information from the homologies among *Prp18*s suggested regions of interest, and the information from the X-ray structure enabled us to pinpoint surface residues for mutation and to design specific deletions within the conserved loop. The mutations fall broadly into three classes. First, mutations of helices 1 and 2 affect the interaction of *Prp18* with *Slu7*, and are partly suppressed by overexpression of either protein. Second, mutations in the

evolutionarily conserved loop apparently abolish a Prp18-specific function; they are dominant negatives and are not affected by overexpression of Prp18 or Slu7. Third, one mutant in helix 5 is strongly temperature sensitive, is recessive, and is not affected by overexpression of Prp18 or Slu7, properties that suggest that helix 5 may interact with another molecule in the spliceosome.

Reliability of the experimental approach

We made single or multiple point mutations and deletions in *PRP18*. For us to interpret the phenotypes of the mutants, the mutant prp18 proteins must fold properly and be essentially identical to wild-type Prp18 except for the mutation(s). Several arguments lead us to believe that this condition is met. The changes are exclusively to surface amino acids, which are not expected to contribute significantly to the stability of Prp18 (Kyte, 1995). We designed the mutations to avoid generating unfavorable interactions between the new amino acids and existing surface charges or bulky groups. For many of the mutations, there is additional evidence that the mutant proteins are folding correctly. For the mutations in helices 1 and 2, the strength of interaction of Prp18 with Slu7 measured at a permissive temperature correlates well with the ts phenotype, strongly suggesting that the phenotype results from the loss of Slu7 binding rather than from a conformational change (or denaturation) of Prp18. The conserved region point and deletion mutations are almost all dominant when overexpressed, consistent with their folding properly, as argued more fully in the Results section.

Interaction of Prp18 with Slu7

Our results define many of the critical contacts between Prp18 and Slu7. In Prp18, the surface formed by helices 1 and 2 and the loop preceding helix 1 is necessary for binding to Slu7, and, together with helix 3, which may stabilize the conformation of the surface, is sufficient for binding Slu7. The amino terminal 80 amino acids of Prp18 also play a role in Slu7 binding, but it is not an essential one. Helices 1 and 2, and the loop preceding helix 1, were initially found by deletion mutations to be essential for interaction of Prp18 and Slu7. This part of Prp18 is positively charged, and previous studies had defined a 25-amino-acid, negatively charged region of Slu7 that is necessary for its interaction with Prp18 (Zhang & Schwer, 1997). Here we showed that three basic residues, H118, R151, and R152, play a critical role in Prp18 binding to Slu7, and three other residues, R80, K140, and K141, play supporting roles (Fig. 9). In vitro binding experiments imply that helices 1 and 2 interact directly with the 25-amino-acid region of Slu7. H118, R151, and R152 are juxtaposed in the structure of Prp18, and they appear to form one

FIGURE 9. Positions of basic residues that interact with Slu7. The portion of Prp18 needed for binding to Slu7 determined by deletion mutations is shown in light green. The key residues, H118, R151, and R152, are shown in orange. Residues that play a lesser role in the interaction are shown in magenta.

side of a groove in the protein (Fig. 9). R80 is positioned on the opposite side of the groove, and it is easy to imagine the negatively charged peptide from Slu7 threaded through this groove.

Contact between Slu7 and the amino-terminal portion of Prp18 is suggested by two-hybrid data from deletion mutations of *PRP18*. These contacts are not essential for interaction of Slu7 with Prp18 or for splicing, and they do not appear to involve the 25-amino-acid portion of Slu7 that interacts with helices 1 and 2 of Prp18.

Mutants in the conserved region displayed an apparently weak interaction in the two-hybrid assay. However, in combination with the R151A mutation, their two-hybrid phenotypes were restored to wild-type. Almost all of the alleles with mutations in the conserved region are dominant and slow growth, but not the R151A combinations with these alleles. We believe that the growth of the yeast in the two-hybrid assay is being slowed by the mutant *prp18* alleles, which are expressed from a strong promoter in the two-hybrid construction, and hence that the slow growth does not reflect anything about the interaction of Slu7 with the mutant prp18s.

The interaction between Prp18 and Slu7 appears to be important for the proper function of both proteins. Mutant Prp18s that fail to interact with Slu7 are invariably temperature sensitive. These mutations can be suppressed to a similar extent by overexpression of Slu7 or by overexpression of the mutant prp18. These

results are consistent with any interpretation in which Prp18 must bind to Slu7 for activity. We interpret the overexpression results to mean that neither protein binds tightly to the spliceosome in the absence of the other; if one protein, say Prp18, bound very tightly to the spliceosome and created the binding site for Slu7, then overexpression of Prp18 would have no effect. Two observations, that *prp18*s that repel Slu7 more strongly are less functional and that parts of Prp18 that do not interact with Slu7 are needed for dominance of conserved region mutations, suggest that Prp18 and Slu7 interact stably only within the spliceosome. This idea is consistent with the apparently individual immunodepletions of the proteins from extracts (Horowitz & Abelson, 1993a; Brys & Schwer, 1996; Zhang & Schwer, 1997), but the possibility that Prp18 and Slu7 form a stable complex outside the spliceosome is not excluded by our results. Our interpretation of the interactions of Prp18 and Slu7 is consistent with the recent work of James et al. (2002), who studied the binding of Slu7 and Prp18 to the spliceosome *in vitro*.

Mutations in the conserved loop of Prp18

We made both point and deletion mutations in Prp18's conserved loop (Fig. 1). The deletion mutants share several properties, which are seen to varying degrees in the point mutants: They are dominant negatives when overexpressed, they are not suppressed by high levels of Slu7, and their growth phenotypes are similar to that of the *PRP18*-knockout strain in which Slu7 is overexpressed. We think that conserved region mutants properly assist in the binding of Slu7 to the spliceosome; hence, overexpressing Slu7 does not have any effect. However, mutation of the conserved region prevents *prp18* from carrying out its function, which is helpful at 34 °C and essential at 37 °C. In our view, the temperature sensitivity of a *PRP18*-knockout strain has two causes; at 30 °C, it results from the failure of Slu7 to bind stably to the spliceosome, but at 37 °C, it results from Prp18 failing to function.

All of the conserved loop mutations are dominant negative. Two conserved loop-deletion mutants with additional mutations that weaken interaction either with Slu7 or with another spliceosomal component (a helix 5 mutation) are not dominant, suggesting that multiple interactions of the mutant *prp18* within the spliceosome are needed for dominance. We conclude that the mutant proteins enter the spliceosome but cannot carry out Prp18's function, which apparently requires the conserved loop. What this Prp18-specific function is is not addressed by our experiments, but the temperature sensitivity of the requirement for it suggests that Prp18 holds a spliceosomal protein or RNA in a conformation that is unstable at high temperature.

We identified two amino acids in helix 5 that appear to be critical for Prp18 function. The characteristics of this

prp18 allele are distinct from those of the other mutants: It is strongly temperature sensitive, interacts normally with Slu7, is not suppressed by overexpression of Slu7, and is not dominant when highly expressed. These properties suggest to us that helix 5 interacts with a component of the spliceosome other than Slu7. Other binding partners of Prp18 have not been identified in two-hybrid screens (van Nues & Beggs, 2001), and it is possible that our postulated interaction would be seen only within the spliceosome. We note that only a single representative of this class of mutants was found and that our interpretation relies on this mutant to fold properly at non-permissive temperatures.

CONCLUSION

Different classes of *PRP18* mutants that we report can be correlated with the structure of the protein to provide a picture of some interactions of Prp18 with spliceosomal components. The results provide molecular detail for a model in which both Prp18 and Slu7 are needed for their stable binding to the spliceosome. The characteristics of the conserved loop mutants imply that Prp18 has another function in splicing that is distinct from its role in stabilizing Slu7 in the spliceosome. The identification of these mutant alleles as dominant negatives will aid in the elucidation of this function of Prp18.

MATERIALS AND METHODS

Yeast strains and growth conditions

Two strains, TDY2037 (*Mat α lys2 ura3-52 trp1 Δ leu2 Δ* ; from T. Dunn; Haak et al., 1997), which is a derivative of S288c, and W303-1a (*MATa leu2-3,112 his3-11,15 trp1-1 can1-100 ade2-1 ura3-1*; Thomas & Rothstein, 1989), were used for complementation studies. *URA3* disruptions of *PRP18* were made in both strains by replacing the region between codons 119 and 214 in *PRP18* with the 1,172-bp *URA3* gene that was recovered by PCR from pRS316. Disruptions were verified by PCR and southern blotting. Both disrupted strains grew slowly at 23 °C and very slowly or not at all at 30 °C, and were fully rescued by single-copy plasmids bearing the *PRP18* gene. For assay of dominant negatives, TDY2037 was used.

For assay of growth, haploid, *PRP18*-disrupted yeast were grown at 23 °C in synthetic medium (Bio101 or Difco), transformed by standard methods (Hill et al., 1991), selected and restreaked at 23 °C, and tested for growth at 23, 30, 34, and 37 °C. Growth is shown for all mutants at 30–37 °C, and for conserved loop alleles, also at 23 °C. Conserved region mutant alleles were selected and maintained at 30 °C. W303-1a grew more rapidly than TDY2037, especially when multiple plasmids were required, but the relative growth rates of the two knockout strains with mutant *prp18*s were similar.

Generation of mutant alleles and plasmids for complementation

The 2,500-bp *EcoRI*-*Bam*HI fragment containing *PRP18*, its promoter, the *MUQ1* gene, and the polyadenylation site for

the principal *PRP18* transcript (which is downstream of *MUQ1*; Horowitz & Abelson, 1993b), was placed in pRS314 (Sikorski & Hieter, 1989). Amino-terminal deletions were made by PCR; deletions included the first five original codons to maintain the wild-type translation initiation context and a *Bgl*II site used for further cloning. Carboxyl-terminal deletions were made either by PCR or by introduction of consecutive stop codons with the QuikChange Mutagenesis Kit (Stratagene). Point mutations were made with the QuikChange Kit, which was used according to the manufacturer's instructions. The conserved-loop deletion mutations were made by introducing a silent *Bsp*HI site in the codon for I220 and making the deletions by PCR. The coding and promoter regions of *PRP18* were completely sequenced in every mutant.

Some mutant alleles were transferred to the 2 μ -derived plasmid pRS424 (Sikorski & Hieter, 1989) or to the plasmid pYX232 (Novagen), which is a 2 μ -derived plasmid in which the *TPI1* promoter drives protein expression. By western blot, we estimated that the ratio of chromosomal: single-copy plasmid: multicopy plasmid: pYX232 for wild-type Prp18 was 1:1:15:250.

The 2,087-bp *Xba*I-*Sal*I fragment containing the *SLU7* gene was excised from pYEP24-*SLU7* (obtained from B. Schwer) and placed in the 2 μ -derived pRS425. *SLU7* was also placed in pYX232 for some experiments.

Two-hybrid analysis

The 78-bp *Bgl*II-*Nco*I sequence of pGADT7 (Clontech) was replaced with the corresponding region from pACT2 except that the *Bgl*II site was not restored (resulting in change of a Cys to Ser in the linker region). The 2,250-bp *Nco*I-*Eco*RI fragment from pDH105 (Horowitz & Abelson, 1993b), bearing the *PRP18* coding region, was cloned into this slightly modified pGADT7, making pDB151. The *GAL4AD-PRP18* fusion gene complemented a *PRP18* knockout. For testing in the two-hybrid assay, the 2,240-bp *Bgl*II-*Eco*RI fragment bearing each mutant *prp18* was transferred into pDB151. The levels of expression of many of the mutants were determined by western blotting using anti-HA tag (16B12HA; Covance). All point mutants and internal deletions tested were expressed at high levels. Carboxyl-terminal deletions and longer amino-terminal deletions were expressed at lower levels, but their expression levels were not correlated with the strengths of their Slu7 interaction measured in the two-hybrid assay.

The AH109 (Clontech) strain was used for assay of two-hybrid interactions (James et al., 1996). The *SLU7-GAL4* DNA binding domain fusion was expressed from the plasmid pGBD-*SLU7* (obtained from B. Schwer; Zhang & Schwer, 1997). Following transformation and restreaking, the strength of interaction between Slu7 and Prp18 was determined by streaking on synthetic medium -leu-trp, -leu-trp-his, -leu-trp-his-ade, and -leu-trp-his with 5, 15, or 30 mM 3-aminotriazole (3-AT; Amberg & Botstein, 1997). 3-AT inhibits the activity of the His3 reporter protein, and growth at higher concentrations of 3-AT indicates that more His3 is being synthesized and implies that the interaction is stronger. The independent Ade2 reporter is more stringent than His3, and is used as a check on the results (James et al., 1996). The scoring scheme used in this report is + + +, wild-type; + +, no growth at high [3-AT]; +, no growth on -ade; $\pm$, no growth with any [3-AT]; -, no growth on -his.

Slu7-peptide binding assay

Zhang and Schwer (1997) defined a 25-amino-acid portion of Slu7, beginning at amino acid 200, that is necessary for Slu7 to bind Prp18. The peptide, CKDKSQDTLWDTDEEIELMKLELY, was synthesized chemically with a cysteine at the amino terminus and coupled to SulfoLink Coupling Gel (Pierce). The partly scrambled peptide CEDLSQTDKTEWD TDEEIKLMELKLY (altered residues underlined), was synthesized and coupled as a control.

The plasmid pDH111, which was made from pET3b and directs the overproduction of Prp18 (Horowitz & Abelson, 1993b), was modified by changing the *Bgl*II site in pET3b to a *Scal* site and adding a *Sma*I site after the *PRP18* coding region, yielding the plasmid pDB601. The 784-bp *Bgl*II-*Eco*RV fragment bearing the coding region of each mutant *prp18* was ligated into *Bgl*II- and *Sma*I-cut pDB601. Production of mutant *prp18* proteins in BL21(DE3) was usually good, but mutant proteins that had the R151A mutation gave lower yields, making the binding assay harder to interpret.

E. coli expressing mutant *prp18*s were lysed with lysozyme in 10% sucrose, 50 mM Tris, pH 7.5, 1 mM EDTA, by the addition of 0.5% NP-40 (Calbiochem). Chromosomal DNA was digested with DNase I, and NaCl was added to 0.1 M. The supernatant was diluted into 50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 0.2 mM EDTA, 1 mM β -mercaptoethanol, and protease inhibitors (Buffer A + 150), and mixed with peptide resin for 30 min at 23°C and then 30 min at 4°C. The resin was collected by centrifugation and was washed twice with Buffer A + 150, twice with Buffer A + 400, once with Buffer A + 1,000, and finally eluted at 55°C with 2% SDS.

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