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Both the Prp18 protein and the U5 snRNA function in the second step of pre-mRNA splicing. We identified suppressors of mutant prp18 alleles in the gene for the U5 snRNA (SNR7). The suppressors’ U5 snRNAs have either a U4-to-A or an A8-to-C mutation in the evolutionarily invariant loop 1 of U5. Suppression is specific for prp18 alleles that encode proteins with mutations in a highly conserved region of Prp18 which forms an unstructured loop in crystals of Prp18. The snr7 suppressors partly restored the pr-mRNA splicing activity that was lost in the prp18 mutants. The close functional relationship of Prp18 and U5 is emphasized by the finding that two snr7 alleles, U5A and U64, are dominant synthetic lethal with prp18 alleles. Our results support the idea that Prp18 and the U5 snRNA act in concert during the second step of pre-mRNA splicing and suggest a model in which the conserved loop of Prp18 acts to stabilize the interaction of loop 1 of the U5 snRNA with the splicing intermediates.

Pre-mRNA is spliced in two sequential transesterification reactions within the spliceosome (5, 7, 34, 54). The active spliceosome is composed of the U2, U5, and U6 snRNPs together with a dynamic cast of proteins. The U2 and U6 snRNAs appear to form the catalytic core of the spliceosome, while the U5 snRNP is thought to hold the substrate RNA and to align the exons for splicing. We focus here on the second step of splicing, in which the exons are joined to form the product mRNA. The U2, U5, and U6 snRNAs play key roles in the second step, and mutations in each specifically block the second step (17, 35, 43). Six proteins, namely, Prp16, Prp17, Prp18, Prp22, Slu7, and Prp8, function specifically in the second step.

The second step of splicing can be divided into stages based on the different protein and ATP requirements of each stage. After the first transesterification reaction, the DEAH-box RNA helicase Prp16 catalyzes an ATP-dependent rearrangement of the spliceosome (49, 50). Prp17 acts at this stage as well, although its function is unknown (33, 47). The Prp16-catalyzed conformational change permits the binding of Slu7, Prp18, and Prp22 to the spliceosome (6, 31); addition of these proteins allows the ATP-independent transesterification reaction to proceed (26, 33, 48). How these three proteins facilitate the second reaction is not known; however, the observations that none of the three is needed for splicing substrates with short branch point-to-3′ splice site distances and that Slu7 affects 3′ splice site choice suggest that the proteins may form a bridge between the branch site and the 3′ splice site (6, 19, 48, 61). Following exon ligation, Prp22, another DEAH family member, catalyzes an ATP-dependent conformational change that releases the mRNA (14). The Prp8 protein, which is also required for the first step, appears to have multiple functions during the second step (45; reviewed in references 3 and 13).

The U5 snRNA plays a central role in the second step of splicing, in which it is hypothesized to align the exons for joining (42; reviewed in references 38 and 57). Other proteins cross-link at the 5′ end of exon 1 to the pre-mRNA or intermediates and that this pairing can determine splice site selection in some instances (15, 37, 39). Promiscuous base-pairing by uridine residues has been suggested as one mechanism for allowing the interaction of loop 1 bases with the substrate RNAs (39), and proteins are likely to be involved as well. Interaction of exon 1 with loop 1 is established during the first step of splicing and the interaction persists through the second step, while the interaction with exon 2 is not present until the second step (1, 40, 53). In studies using model substrates in vitro, loop 1 was dispensable for the first step (despite the fact that it can alter 5′ splice site choice) and required for the second in yeast extracts (43), but it was dispensable for both steps of splicing in HeLa cell extracts.

Proteins are likely to play a role in stabilizing the interaction of loop 1 of U5 with the substrate. The involvement of Prp8 is perhaps the best supported by the evidence. Prp8, a component of the U5 snRNP, cross-links to loop 1 in free U5 snRNP as well as to the ends of both exons during splicing, with kinetics that parallel those of U5 snRNA cross-linking (16, 55; reviewed in references 3 and 57). Other proteins cross-link at or near the 3′ splice site during the second step, but it is not known whether they interact with the exonic sequences or with
McKay, and the face of Prp18 opposite the conserved loop binds to Slu7 (2, 61). This interaction is necessary for both proteins to bind stably to the spliceosome (31). Mutant Prp18 proteins lacking their carboxyl-terminal third of yeast Prp18 is conserved in human Prp18, yet yeast Prp18 can function in human splicing, showing the importance of this region to Prp18 action (28). Mutational analysis based on the structure implies that Prp18 has at least two separable functions. Prp18 interacts with Slu7, and the face of Prp18 opposite the conserved loop binds to Slu7 (2, 61). This interaction is necessary for both proteins to bind stably to the spliceosome (31). Mutant Prp18 proteins lacking their conserved region are partly functional and apparently bind Slu7 and enter the spliceosome normally (2). However, these mutant Prp18 proteins do not support wild-type growth at any temperature. Prp18 is physically associated with the U5 snRNP, although its binding is not tight (22, 27), and we had previously speculated that the conserved loop of Prp18 could interact with U5.

We devised a genetic test to look for a functional interaction between Prp18 and loop 1 of the U5 snRNA. Suppressors of prp18 alleles that encode a mutant Prp18 protein lacking its conserved loop were found in the gene for U5 snRNA. The results imply a functional connection between the invariant loop 1 of U5 and the conserved loop of Prp18 and suggest that Prp18 could stabilize the interaction of loop 1 with the splicing intermediates.

MATERIALS AND METHODS

Plasmids. All PRP18 mutants and plasmids are described in reference 2. The Prp18 mutants used in this study are shown in Fig. 1. A wild-type SNR7 plasmid was made by genomic PCR and cloning of the ClaI-HindIII fragment (44) into pRS316 (52). The snR7 library of U5-loop 1 mutants was obtained from Andrew Newman (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom) (37). The six additional point mutations in loop 1 (see Fig. 6, below) were made with the QuikChange mutagenesis kit (Stratagene).

Strains. The yeast strains W303-1A (MATa leu2-3,112 his3-11,15 prp1-1 can1-100 ade2-1 ura3-1), W303-1B (MATa), and W303 (diploid) were used (56). The W303-1A prp18::HIS3 strain was made as described previously (27). The W303-1A prp18::KAN mutant strain was made by PCR with pFA6a-kanMX6 (60) replacing the coding sequence for amino acids 117 to 234 in Prp18. These two strains transformed with a pRS314-prp18 plasmid were used as mutant prp18 yeast in all U5 experiments. The W303-1A prp18::Ura4 strain described previously (2) was used for making RNA.

SNR7 disruptions were made in diploid W303 by PCR with pFA6a-kanMX6 (41). W303 SNR7::kanMX4 was transformed with pRS316-SNR7 or pRS316-347 and dissected, giving the haploid W303-1B snr7::kanMX4 (or SNR7) strains. prp18::HIS3 strains were made by crossing W303-1A prp18::HIS3 with W303-1B snr7::kanMX4 and selecting for amino acids 117 to 234 in Prp18. These two strains transformed with a pRS314-prp18 plasmid were used as mutant prp18 yeast in all U5 experiments. The W303-1A prp18::Ura4 strain described previously (2) was used for making RNA.

Yeast screens and manipulations. W303-1A prp18::HIS3 yeast were transformed (24) with the pRS314-derived plasmid bearing the prp18::h23-1, prp18::ΔCR, or prp18::h5-Δ5 alleles. These yeast plus the parent strain were then transformed with the snr7 loop 1 library (in pRS316). Two methods were used to find suppressors. In the first, the transformed yeast were grown at 23°C and replica plated to restrictive temperature. In the second, the transformed yeast were grown for 1 day at 23°C and then shifted to nonpermissive temperature. For the prp18 knockout strain, no 34°C survivors were found from 35,000 transformants (only replica plating was used). For the prp18::h23-1 mutant, 25,000 (replica plating) and 0 of 9,000 (shifting) colonies grew at 34°C; all 25 of these grew at 37°C on 5-fluoroorotic acid (5-FOA); which selects for yeast cured of pRS316-sn7 and presumably identifies chromosome-plasmid recombinants that regenerated a wild-type PRP18 and were not considered further. For the prp18::h5-Δ5 mutant, 36 of 40,000 colonies (replica plating) grew at 34°C; all of these grew at 37°C on 5-FOA and were not considered further. For prp18::ΔCR yeast, 34 of 6,000 (replica plating) and 20 of 2,000 (shifting) transformants grew at 37°C. Three of these 54 initial candidates grew at 37°C on 5-FOA, and 51 were evaluated further. The U5 library plasmid was isolated from 47 candidates and was restested for suppression of prp18::ΔCR. The U5 alleles from the best-scoring 26 candidates were sequenced, and all of these were mutant Yeast (2). Dominant synthetic lethality of the six mutants shown in Fig. 6 was determined by transformation. W303-1A prp18::KAN yeast bearing either the pRS314-prp18::ΔCR or pRS314-prp18::KAN plasmid were transformed with the pRS316-sn7 plasmids. Several hundred to a couple of thousand transformants were obtained from each plasmid with yeast bearing a wild-type allele for PRP18 and from four of the plasmids (pRS316-sn7-C3U, U46G, A86G, and C94A) in prp18::ΔCR yeast. For sn7-U5A and sn7-U6a in prp18::ΔCR yeast, no viable colonies were obtained at 26, 30, or 34°C. In some attempts an occasional transformation survivor was seen; the pRS316-sn7 plasmid was recovered from some of these yeast and the
**RESULTS**

**Experimental rationale and design.** We surmised that Prp18 played a role in stabilizing the interaction of loop 1 of U5 with the splicing intermediates or products. This conjecture was based on the association of Prp18 with the U5 snRNP (27), the specific requirement of loop 1 of U5 for the second step of splicing (43), and the synthetic lethal interaction of the prp18-1 allele with mutations in the part of the U5 snRNA gene (SNR7) that corresponds to loop 1 (20). Cross-linking results suggest that Prp8 is involved in this stabilization (16, 55), and we have used a complementary genetic approach to look for evidence of a functional interaction between Prp18 and loop 1 of U5. We reasoned that splicing of some pre-mRNAs in prp18 yeast could be enhanced by mutations in loop 1 of U5 that strengthened its base-pairing with these substrate RNAs. Thus, we sought suppressors of four prp18 alleles in yeast bearing a wild-type copy of the U5 snRNA gene (SNR7) together with a copy in which the bases corresponding to loop 1 had been randomly mutated (37).

We used three functionally distinct mutants of Prp18 plus a PRP18 knockout strain for our suppressor search (2). Two of these are multiple point mutants (shown in Fig. 1): the Prp18-hlx2-1 protein has four mutations in helix 2 that disrupt its interaction with Slu7, and the Prp18-hlx5 protein has two mutations in helix 5 that may interfere with the interaction of Prp18 with another, unidentified splicing factor. In the third mutant protein, Prp18ΔCR, 28 of the 36 amino acids that comprise the conserved loop between helices 4 and 5 have been deleted. Previous work provided strong evidence that the three mutant proteins fold properly (2). In particular, prp18ΔCR is dominant negative at all temperatures when highly expressed; the Prp18ΔCR protein apparently enters the spliceosome but is not fully functional.

**Isolation of suppressors.** Suppressors of the three prp18 alleles described above and of a prp18 knockout allele were sought in the snr7 library. Yeast with a disrupted SNR7 gene were transformed with a prp18-bearing plasmid followed by a plasmid with an snr7 allele from the library of loop 1 mutants (37). A total of 8,000 to 40,000 candidates for each prp18 allele (depending on the transformation efficiency) from the randomly mutagenized library of U5 mutants were screened by replica plating or by temperature shifting of the plates to the lowest reliably nonpermissive temperature for each prp18 allele. For three of the prp18 alleles (prp18-hlx2-1, prp18-hlx5, and a prp18 knockout), no suppressors were found. For prp18ΔCR, 54 high-temperature (37°C) suppressors were found, of which the best-scoring 26 were analyzed further. Figure 2 shows the mutations in loop 1 of the U5 snRNA from the snr7 suppressors projected on a secondary structure diagram (panel A) and a tabulation of the sequences (panel B). Figure 3 shows the growth of prp18ΔCR and wild-type yeast with the isolated suppressors over a range of temperatures. The suppressors, which were isolated in yeast with both a wild-type and a mutant allele of SNR7, appear to be dominant.

The suppressors fall into two classes. We named the snr7 suppressors by appending the loop 1 mutation(s) to snr7:

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**References:**

1. W303-1A prp18::URA3 yeast transformed with pRS314, pRS314-PRP18, or one of the pRS314-prp18 plasmids were grown in SD-Trp at 26°C to an A_{600} of 0.5 for harvesting or at 26°C to an A_{600} of 0.25 and then shifted to 37°C for 2 h before harvesting. Cell pellets were frozen at −70°C. For the U5 suppressors, overnight cultures of W303-1A prp18::KAN yeast bearing either pRS314-PRP18 or pRS314-prp18ΔCR and pRS316-SNR7 or one of the pRS316-sn7 plasmids were grown in SD-Trp-Ura and were used to inoculate cultures in yeast extract-peptone-dextrose which were grown at 30 or 34°C to an A_{600} of 0.5 for harvesting or at 30°C to an A_{600} of 0.25 and then for 30 min or 2 h at 37°C before harvesting (11). Alternatively, W303-1A prp18::HIS3 yeast were used as above, except that SD-Trp-Ura was used throughout.

RNA was prepared by hot phenol extraction, essentially according to the method described in reference 12. Six micrograms of total RNA per lane was run in agarose-formaldehyde gels (46), and blots were probed according to the method of Cheng and Abelson (9). DNA for making probes was obtained by cloning appropriate PCR products of yeast genomic DNA into Bluescript KS−. Probes for mRNAs were made by random priming of gel-purified restriction fragments (38), and the oligonucleotide probe for SCR1 RNA was obtained from Thuram Sundaresan (Uniformed Services University of the Health Sciences, Bethesda, Md.). Probes specific for introns were made by 35 cycles of reactions of 2 ng of intronic DNA fragment, 2 pmol of antisense primer, 50 μCi of [α-32P]dCTP, and Taq DNA polymerase in 20 μl to generate single-stranded, full-length probe. Blots were quantitated with a Molecular Dynamics Phosphor-Imager.
hence, for example, snr7-A8C. The major class has a mutation at position A8 (24 of 26 suppressors). snr7-A8C itself accounts for 18 of 26 suppressors and appears to be the strongest of the suppressors. Three suppressors have mutations at C2 in addition to A8C, but these were not found without A8C and did not improve suppression. snr7-A8U was also found, but it is the weakest of the six suppressors (Fig. 3). The second class of suppressor has the mutation U4A, which was found by itself and with A8C. The suppressors all have one or two mutations in loop 1, in contrast to the multiply mutant sequences found by Newman and Norman (37), who had selected for splicing of a specific mutant message in a wild-type strain, using the same library.

The U5-loop 1 suppressors have a clear salutary effect on growth of prp18ΔCR yeast, but they do not fully restore wild-type growth (Fig. 3). The suppressors are graded in their effect, with snr7-A8C being the strongest and snr7-A8U the weakest. Yeast bearing the prp18ΔCR allele grow more slowly than wild-type yeast at 23 to 34°C (Fig. 3 and reference 2), and snr7-A8C suppressed the prp18ΔCR phenotype at all temperatures. In contrast, both suppressor alleles with a U4A mutation exacerbated the slow-growth phenotype of prp18ΔCR yeast at 23 and 26°C. None of the suppressors had a discernible effect when expressed in wild-type cells (Fig. 3).

**Genetic characterization of the suppressors.** The U5-loop 1 suppressors are specific for prp18 alleles that encode Prp18 proteins with mutations in their conserved regions. We tested two representative alleles, snr7-A8C and snr7-U4A, for suppression of seven different prp18 mutant alleles plus the original conserved-region deletion allele (Fig. 4). A second conserved-region deletion mutant, prp18ΔCR-2, which is very similar to prp18ΔCR, was suppressed by both snr7-A8C and snr7-U4A. Three point-mutant alleles that encode Prp18 proteins in which three consecutive invariant amino acids in the conserved region have been replaced by alanines (Fig. 1) were suppressed well by snr7-A8C, but only one of these alleles, prp18-CR-a, was clearly suppressed by snr7-U4A (prp18-CR-a and prp18-CR-b are shown in Fig. 4; prp18-CR-c is not). Three other prp18 alleles, including the prp18-hlx2-1 and prp18-hlx5 alleles that we had used in the initial suppressor screen plus the allele prp18-hlx2-2 (Fig. 1) that is less temperature sensitive than prp18-hlx2-1, were not detectably suppressed by either of the snr7 alleles. We conclude that suppression is specific for mutations within the conserved loop of Prp18 and that snr7-A8C is a more general suppressor than snr7-U4A.

Only the suppressors that have the A8C mutation are effective when there is no wild-type copy of the U5 snRNA gene. We tested the U5-loop 1 suppressors, which had been isolated in strains with a chromosomal, wild-type SNR7 gene, in yeast that had only a mutant copy of SNR7 (Fig. 5). The suppression of the prp18ΔCR growth phenotype by snr7-A8C was independent of the presence of SNR7 (Fig. 5, compare A8C with wild type plus A8C). Likewise, suppression by the snr7 alleles with A8C combined with C2U and C2A or by A8U did not depend on the wild-type SNR7. In contrast, the snr7-U4A allele and, more dramatically, the snr7-U4A A8C allele, slowed the growth of prp18ΔCR strains at 23 to 34°C (below the already slow growth rate of the prp18ΔCR strain) and did not suppress the temperature sensitivity conferred by prp18ΔCR in the absence of a wild-type SNR7. This finding implies that in a prp18ΔCR strain some pre-mRNAs are spliced more efficiently with the U4A U5 snRNAs, while the rest are more efficiently spliced with wild-type U5 snRNA. In an snr7 knockout strain that was otherwise wild type, five of the six snr7 suppressor alleles had no apparent effect on growth, whereas the snr7-U4A A8C double mutant conferred a slow-growth phenotype at high temperature (Fig. 5).

To determine whether other SNR7 point mutations that we
did not find in our screen would also suppress prp18ΔCR, we made six additional single mutations in SNR7 in bases corresponding to loop 1 of U5 and assayed them for suppression of the temperature sensitivity of a prp18/H9004 CR SNR7 strain. Three of the snr7 alleles, A8G, C9A, and U4G (Fig. 6), had no effect; snr7-C3U was a weak suppressor at 37°C (slightly weaker than snr7-A8U). Unexpectedly, both the snr7-U5A and snr7-U6A mutations were dominant synthetic lethal at all temperatures in a prp18/H9004 CR SNR7 strain (that is, they killed the yeast despite the presence of a wild-type gene for U5) (data not shown). None of the six snr7 alleles had a discernible effect on the growth of wild-type yeast.

Effect of mutations in PRP18 on splicing in vivo. As a prerequisite to understanding the effect of the snr7 suppressors on splicing, we evaluated the splicing defects of representative prp18 yeast. Four spliced mRNAs, ACT1, CYH2, POP8, and RPS1A, were assayed using Northern blotting, but splicing intermediates could be reliably quantitated only for ACT1 (Fig. 7A). Three intronless RNAs, the TDH1 and SEC4 mRNAs and the SCR1 RNA, were also assayed by blotting. Samples were loaded by comparison of A260 values, which reliably agreed with rRNA levels measured by staining (Fig. 7A).

We examined the amounts of splicing intermediates and products in prp18 mutant yeast (Fig. 7A). At 26°C, a permissive temperature for all the mutants, ACT1 splicing intermediates accumulated in amounts well-correlated with the severity of the prp18 mutation. After a 2-h shift to 37°C, the amounts of accumulated intermediates could not be as readily interpreted: some amounts declined (perhaps from a decline in transcription), and the highest levels were found in yeast with the weakest ts alleles (e.g., prp18-hlx2-2). The amounts of the spliced ACT1 and CYH2 mRNAs were reduced less than 2-fold by prp18 mutations at 26°C and were sharply reduced (up to 15-fold) following a 2-h shift to 37°C. The behavior of the RPS1A and POP8 mRNAs was similar to that of the ACT1 and CYH2 mRNAs (data not shown). The amount of accumulated intermediate was only a small fraction of the amount of mRNA; presumably, the intermediates that are not spliced rapidly are degraded (4, 25).

The amounts of the intronless mRNAs for TDH1 and SEC4 varied in the prp18 strains (Fig. 7A). At 26°C, the level of SEC4 mRNA is relatively constant (11), but the level of TDH1 mRNA changed in parallel with the levels of ACT1 and CYH2. At 37°C, the level of SEC4 mRNA varied up to 2.5-fold and
that of TDH1 mRNA varied up to 4-fold. The SCR1 RNA, a polymerase III transcript that has been used as a standard (8), varied about 1.6-fold and was higher in prp18 yeast than in wild-type yeast. These variations in the levels of intronless mRNAs, even at permissive temperature, indicate that transcription and/or RNA stability is affected in the prp18 mutants and they complicate the interpretation of the levels of the spliced mRNAs. The four spliced mRNAs we examined were affected substantially more than the intronless mRNAs at 37°C, but not at 26°C.

The ratio of splicing intermediates to mRNA (termed the i/m ratio) may provide the best relative measure of severity of Prp18 mutation (Fig. 7B). If the yeast are at steady state, then the i/m ratio is directly proportional to the rate constant for the second step (using the kinetic formulation of Frank and Guthrie [19]). Comparison of different prp18 strains using the i/m ratio is independent of transcription rates and of the decay rates of intermediates, but it is sensitive to mRNA decay rates. Using the i/m ratio for comparison has the practical advantage that normalized comparisons of absolute amounts of RNA are avoided. The prp18 knockout strain had the highest i/m ratio at both assay temperatures (Fig. 7B); the sixfold increase in the ratio between 26 and 37°C shows that the second step is slowing at high temperature, as expected from in vitro results (27). In the three prp18-hlx2 strains that are temperature sensitive to different extents, the i/m ratio tracked the severity of the alleles at both 26 and 37°C (Fig. 7B). The prp18-hlx2 mutant gave an i/m ratio at 26°C that was apparently in good accord with its severity (between that of prp18-hlx2-1 and prp18-hlx2-2), but the ratio increased only half as much at 37°C as those of the prp18-hlx2 mutants.

The prp18ΔCR allele behaved differently from the other prp18 alleles. At 26°C its i/m ratio was comparable to those of the other strains; however, on shifting to 37°C the ratio increased only 1.6-fold, apparently as a result of a decrease in mRNA, not an increase in intermediates (based on the A260 normalization). In addition, at 37°C the level of the intronless TDH1 mRNA declined almost as much as that of ACT1 mRNA. The chemistry of the Prp18ΔCR protein may explain the differences between its behavior and that of the other Prp18 mutants. The Prp18ΔCR protein binds to the spliceosome at all temperatures but does not function correctly, and its functional defects may be exacerbated at high temperature (2). The mutations in the other Prp18 proteins interfere with interactions of Prp18 with the spliceosome, perhaps blocking or inhibiting its entry into the spliceosome at nonpermissive temperature, leading to a more pronounced effect on splicing.
The results support the idea that the i/m ratio is a reliable method for comparing splicing defects. Its utility appears to extend to yeast that have been shifted to nonpermissive temperature, although these cannot be strictly at steady state. We were only able to measure the ratio for ACT1 RNAs, and we cannot tell whether splicing of ACT1 pre-mRNA is directly affected or is reporting the status of splicing in general, as might be the case if splicing factors are sequestered in inactive complexes.

**Effect of suppressors on splicing in prp18ΔCR mutants.** Assays of splicing in a prp18ΔCR strain showed that the snr7 suppressor alleles do suppress the prp18ΔCR splicing phenotype, although the suppression effects were relatively modest. As described above, the ratio of intermediates to products is a kinetically interpretable measurement of splicing efficiency and is well-suited to measuring small differences because it does not depend on absolute comparisons between samples. Levels of ACT1 splicing intermediates and mRNA were measured at 30 to 37°C in prp18ΔCR snr7 strains bearing a plasmid with either the SNR7, snr7-A8C, or snr7-U4A allele.

The effects of the two snr7 alleles tested were not the same. The snr7-A8C allele reduced the i/m ratio by 1.6-fold ± 0.2-fold at 37°C and had a smaller effect at 34 and 30°C (Fig. 8). The change in the ratio appeared to result primarily from an increase in the amount of ACT1 mRNA, not from a decrease in splicing intermediates, based on the A200 normalization. The level of the intronless TDH1 mRNA also increased in the suppressed strains, so that there was no relative change in the ratio of ACT1 to TDH1 mRNAs at any temperature. The snr7-U4A allele caused a small decrease, 1.3-fold ± 0.2-fold, in the intermediates to products ratio at 37°C but caused a 1.4-fold ± 0.1-fold increase in the ratio at 30°C, consistent with the deleterious effect of snr7-U4A on growth of prp18ΔCR yeast at 30°C (Fig. 5). It is not clear why the suppression effects of both snr7 alleles were not identical. The suppression effects of both snr7 alleles as quantified by their effects on the intermediates to products ratio were small—only 1.6-fold at their largest. The magnitudes of these effects are similar to the change seen on shifting prp18ΔCR yeast from permissive to restrictive temperature. The observed suppression effect is consistent with the idea that the snr7 suppressors reverse the splicing phenotype of the prp18ΔCR strain, although the size of the effects does not allow us to draw a definitive conclusion. Models in which other steps of splicing are affected to suppress indirectly are disfavored by our results.

**DISCUSSION**

We report the isolation and characterization of suppressors of prp18 alleles in the SNR7 (U5 snRNA) gene. Based on previous genetic and biochemical evidence that connected Prp18 with the U5 snRNP, we specifically sought suppressors of mutant PRP18 alleles in a library of snr7 alleles with mutations in bases corresponding to loop 1 of the U5 snRNA, as explicated at the beginning of Results. Two U5 suppressors of the prp18ΔCR allele, snr7-U4A and snr7-A8C, were identified. The snr7-A8C allele is the stronger of the two suppressors and is dominant, whereas snr7-U4A only suppresses in the presence
of a wild-type SNR7 allele. Suppression is specific to prp18 alleles that encode Prp18 proteins with mutations in their conserved regions, suggesting that the evolutionarily invariant loop 1 of U5 and conserved loop of Prp18 function together during the second step of splicing. The interdependence of the functions of Prp18 and U5 is emphasized by the finding of a dominant synthetic lethal interaction between two snr7 loop 1 mutations and the prp18ΔCR allele. Analysis of mRNAs from prp18ΔCR strains showed that the splicing defect of prp18ΔCR strains was partly compensated by both of the snr7 suppressor alleles, consistent with the notion that the suppressors restore the lost function(s) to the spliceosome. Our results show that the conserved loop of Prp18 interacts genetically with loop 1 of U5 and suggest a direct functional interaction between them. We suggest that Prp18 acts to stabilize the interactions between loop 1 and the splicing intermediates during the second step of splicing. Previous structural and mutational studies of Prp18 showed that the face of Prp18 that is opposite the conserved loop interacts with the Slu7 protein, and the results here suggest that Prp18 forms a bridge between U5 and Slu7.

The suppression results provide important information about the roles of Prp18 and the U5 snRNA in splicing. The suppressors display some allele specificity in that they only suppress mutations within the conserved loop of Prp18, but because they suppress a deletion of the conserved region they do not imply a direct physical interaction in the way that a true allele-specific suppressor would. The suppressors’ specificity instead appears to be for one function of Prp18, and the suppressors must then replace that function, essentially acting as bypass suppressors. The measurement of the effect of the suppressors on splicing, which is described in more detail in Results, supports the idea that the defect of Prp18ΔCR in splicing has been overcome by the suppressors, but the specific mechanism cannot be inferred. We envision two general types of mechanistic models for the suppression. In the first type, the suppressors restore the Prp18ΔCR-affected process, implying a close functional connection between the conserved loop of Prp18 and loop 1 of U5 snRNA. In the second type, the suppressors bypass the need for the process affected by Prp18ΔCR, perhaps by interfering with a checkpoint or proofreading step that would slow or halt splicing in prp18ΔCR strains; no proofreading steps are known at or after the second step. On balance, we think that the evidence favors a mechanism in which the Prp18ΔCR-affected process is restored by the snr7 suppressors.

Our conclusions considerably extend earlier results concerning the interaction of Prp18 and U5. Two previous studies addressed the connection of Prp18 and U5. Frank et al. (20) found a synthetic lethal interaction between prp18-1 and two snr7 alleles with mutations in the loop 1 region. Horowitz and Abdel (27) found that Prp18 is associated with the U5 snRNP by coimmunoprecipitation. Our study provides new information in two ways. First, our results imply that the interaction of Prp18 with U5 specifically involves the conserved loop of Prp18 and, second, our suppression results suggest a specific functional relationship between Prp18 and loop 1 of U5. Suppressors provide much stronger evidence of a close functional connection than synthetic lethals (20, 23, 30).

The roles and interactions of the bases in loop 1 of U5 have been investigated, and the current model suggests how our suppressor U5 snRNAs could function. The snr7-U4A allele, our weaker suppressor, is easier to interpret within the framework of known U5 actions. Base U4 of loop 1 interacts with the 3′-terminal base in exon 1 as well as the 5′-terminal base in exon 2; these interactions can occur by base-pairing, although strict base complementarity cannot be required (39). The 3′-terminal base of exon 1 interacts with U4 during both steps of splicing while interaction with exon 2 occurs after the first step of splicing, but it is not known whether U4 could interact with both exons simultaneously (40, 53). We imagine that mutating U4 to A could strengthen the interaction(s), perhaps through base-pairing, with some transcripts, thereby facilitating their splicing. Obviously, interactions with other transcripts would be weakened, inhibiting their splicing. This view is sustained by the observation that in a prp18ΔCR strain the snr7-U4A allele only works well in the presence of a wild-type SNR7 allele. That is, in the SNR7/snr7-U4A prp18ΔCR strains, there is a mandatory division of labor, with each U5 snRNA splicing a subset of the pre-mRNAs optimally. In a PRP18 wild-type strain, snr7-U4A works fine (i.e., an snr7-U4A strain grows normally), consistent with the idea that Prp18 acts to stabilize
interactions of loop 1 with the substrate RNA. Our results on the effect of the snr7-U4A allele on ACT1 splicing do not lead to an unequivocal conclusion, with splicing inhibited at 30°C and improved at 37°C. A simple base-pairing model would not explain improvement of splicing of ACT1 pre-mRNA (exon 1 ends with TCTG-3' and exon 2 begins with 5'-AGG), suggesting either that there is a different type of interaction or that ACT1 is reporting the status of splicing in general, as described in Results.

The snr7-A8C suppressor is difficult to interpret mechanistically because of the paucity of data about the function of A8. Newman and Norman (39) found that an A8C mutation within a multiply mutant loop 1 had complex effects on splicing of a model pre-mRNA with a disabling G-to-A mutation at the first base of the intron, but A8C did not have a determinative role in splice site choice. snr7-A8C is a stronger suppressor of prp18ΔCR than snr7-U4A. snr7-A8C is dominant, and it suppresses the splicing phenotype of prp18ΔCR more persuasively than does the snr7-U4A allele. snr7-U8A is a weak suppressor of prp18ΔCR, but snr7-A8G is not. We suggest three possible mechanisms for snr7-A8C suppression. First, A8 could interact with some pre-mRNAs, and A8C could restore splicing by base-pairing interaction. No evidence suggests that A8 interacts with substrate RNA in the pre-mRNAs that have been studied, although base-pairing has been suggested for the adjacent base U7 (39), and there may be some flexibility in the way that loop 1 interacts with pre-mRNAs (15, 42). Second, A8C could change the conformation of loop 1, indirectly affecting or enhancing the ability of loop 1 to interact with the substrate RNA. We view both of these mechanisms as restoration-of-function suppressors. Third, A8C could bypass the role of the conserved loop of Prp18, perhaps by disabling a checkpoint, as described above. From on our results we do not favor any one of these models over another.

We found that the snr7-U5A and snr7-U6A alleles were both dominant synthetic lethal with prp18ΔCR, killing the yeast despite the presence of a wild-type allele of SNR7. Both the U5 and U6 bases pair with the 3' end of exon 1, and mutation at either position can activate cryptic 5' splice sites. Yeast with snr7-U6A as their only version of SNR7 are temperature sensitive at 37°C, and snr7-U6A is synthetic lethal with the prp18-1 mutant (20, 41). The dominant synthetic lethality that we observe can be explained either as a general inhibition of splicing or as a specific effect on a small number of transcripts. In the SNR7/snr7 prp18ΔCR strains, half the spliceosomes (those with the mutant U5 snRNA) could be inactive. The remaining splicing activity could be insufficient, or those spliceosomes could sequester splicing factors, ultimately blocking splicing more completely. However, spliceosomes blocked at the second step are rapidly degraded (4, 25); in addition, yeast tolerate equal amounts of wild-type and ATPase-defective versions of the RNA helicase Prp16 (29), suggesting that blocking half the spliceosomes is not lethal. Alternatively, the U5A or U6A versions of the U5 snRNA could be preferentially recruited to some transcripts by base pairing, inhibiting or affecting the splicing of selected pre-mRNAs. Wild-type yeast tolerate considerable variation in the sequence of loop 1 (41). Our finding that prp18ΔCR yeast cannot cope with some loop 1 sequences even in the presence of a wild-type U5 underscores the close functional relationship of the conserved loop of Prp18 and loop 1 of U5.

We analyzed pre-mRNA splicing in six prp18 strains that represented the three classes of Prp18 mutant that we had identified previously (2). All the prp18 strains showed defects in the second step of splicing; our best quantitative assessment of the splicing defects, using the ratio of splicing intermediates to mRNAs, showed a good correlation between the severities of the temperature sensitivities and of the second-step splicing defects. While this result does not rule out other functions for Prp18, it is consistent with the idea that the only function of Prp18 is in splicing. Using microarrays, Clark et al. (11) found that the levels of the vast majority of spliced mRNAs are not significantly changed compared to intronless mRNAs in a prp18 knockout strain at 26°C (the levels of less than 10% of the spliced mRNAs changed by more than 50% compared to reference intronless mRNAs). We observed parallel declines in spliced and intronless mRNAs, in substantial agreement with the microarray results.

The studies we report here imply a close functional relationship between the conserved loop of Prp18 and loop 1 of the U5 snRNA, and we suggest that Prp18 may act to stabilize the complex interaction of loop 1 with the splicing intermediates. Previous work has suggested a role for Prp8 in this stabilization (16, 55), and the two proteins could act together in this function. Combining earlier structural and mutational analysis of Prp18 with the work presented here yields a picture of Prp18 in which Prp18 is bound to the spliceosome by interaction of helices 1 and 2 with Slu7, and perhaps by interaction of helix 5 with another component of the spliceosome, positioning the conserved loop to interact with loop 1 of U5. Understanding the precise mechanism by which Prp18 acts will require biochemical analysis of these processes.

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