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A conserved Lsm-interaction motif in Prp24 required for efficient U4/U6 di-snRNP formation

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ABSTRACT

The assembly of the U4 and U6 snRNPs into the U4/U6 di-snRNP is necessary for pre-mRNA splicing, and in *Saccharomyces cerevisiae* requires the splicing factor Prp24. We have identified a family of Prp24 homologs that includes the human protein SART3/p110^{nrb}, which had been identified previously as a surface antigen in several cancers. Sequence conservation among the Prp24 homologs reveals the existence of a fourth previously unidentified RNA recognition motif (RRM) in Prp24, which we demonstrate is necessary for growth of budding yeast at 37 °C. The family is also characterized by a highly conserved 12-amino-acid motif at the extreme C terminus. Deletion of this motif in Prp24 causes a cold-sensitive growth phenotype and a decrease in base-paired U4/U6 levels in vivo. The mutant protein also has a reduced association with U6 snRNA in extract, and is unable to interact with the U6 Lsm proteins by two-hybrid assay. In vitro annealing assays demonstrate that deletion of the motif causes a defect in U4/U6 formation by reducing binding of Prp24 to its substrate. We conclude that the conserved C-terminal motif of Prp24 interacts with the Lsm proteins to promote U4/U6 formation.

Keywords: annealing; p110nrb; RRM; SART3; splicing; U4-U6

INTRODUCTION

Introns are removed from pre-mRNA by a complex ribonucleoprotein particle known as the spliceosome. The spliceosome consists of five snRNAs (U1, U2, U4, U5, U6), each of which is tightly associated with a number of proteins to form snRNPs, as well as a number of non-snRNP protein factors. A striking feature of the splicing process is the number of conformational changes that occur within the spliceosome (Staley & Guthrie, 1998). During assembly, catalysis, and disassembly of the spliceosome, the snRNAs dynamically associate with one another and with the spliced transcript, requiring extensive formation and dissolution of base-pairing interactions. The timing and regulation of these RNA conformational changes are thought to be controlled by spliceosomal protein factors.

U6 snRNA is found in multiple forms in cell extracts, including: (1) a U6 mono-snRNP particle; (2) a U4/U6 di-snRNP particle, in which U6 is base-paired to U4 snRNA by two intermolecular helices; and (3) a U4/

U6.U5 tri-snRNP particle with U5 snRNA. Additionally, in yeast extracts undergoing splicing, U2 snRNA associates with U6 snRNA by at least two interactions (Madhani & Guthrie, 1992). Notably, catalytic activation of the spliceosome requires unwinding of U4 from U6 to enable the mutually exclusive interaction of U6 with U2 snRNA (Ryan & Abelson, 2002).

In order for U4 and U6 to be reutilized for subsequent rounds of splicing, a mechanism must exist for their reassociation. A role for the RNA recognition motif (RRM)-containing protein Prp24 in this process was initially suggested by genetics: Mutations in the third of Prp24's three RNA recognition motifs were isolated that suppressed the cold-sensitive growth phenotype of U4/U6-destabilizing mutations in U4 snRNA (Shannon & Guthrie, 1991). Subsequent experiments have shown that mutations in RRM2 and RRM3 have U4/U6 assembly defects in vivo, and that a triple-alanine substitution in the conserved RNP1 motif of RRM2 is lethal (Vidaver et al., 1999). Furthermore, mutations in RRM2 and RRM3 that are predicted to reduce RNA binding suppress the cold-sensitive growth phenotype of hyperstabilized U6 mutants (Fabrizio et al., 1989; Fortner et al., 1994). Finally, footprinting experiments on U6 demonstrate that Prp24 binds to U6 nt 39–57 in the

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free mono-snRNP, as well as to one of the base-paired U4/U6 stems in the di-snRNP (Ghetti et al., 1995; Jan-drositz & Guthrie, 1995). These experiments establish the importance of Prp24's RRM for U4/U6 formation, and strongly suggest that the RRM functions by binding directly to U6 and U4 snRNAs.

It has subsequently been shown that Prp24 (recombinant or purified from yeast) greatly accelerates the formation of base-paired U4/U6 from in vitro-transcribed U4 and U6 RNAs (Ghetti et al., 1995), and furthermore that the stimulatory effect of Prp24 is 15-fold greater using protein-containing U4 and U6 snRNPs as the substrate (Raghu-nathan & Guthrie, 1998). Recent work from several laboratories has provided compelling evidence that the U6 Lsm proteins contribute to this difference (reviewed in He & Parker, 2000). Seven proteins (Lsm2–Lsm8) have been shown to associate with the extreme 3' end of U6 snRNA, probably in a ring-shaped complex (Ach-sel et al., 1999; Vidal et al., 1999; Ryan et al., 2002). Yeast two-hybrid analysis has demonstrated that a subset of Lsm proteins (Lsm2, 5, 6, 7, and 8) can interact with Prp24 (Fromont-Racine et al., 2000), and coimmunoprecipitation experiments have shown that both Prp24 and the Lsm2–Lsm8 proteins are associated with U6 in extract (Vidal et al., 1999; Bouveret et al., 2000). Indeed, Prp24 and Lsm2–Lsm8 are the only proteins detected by mass spectrometry analysis of purified free U6 snRNPs (Stevens et al., 2001). Furthermore, deletion of Lsm6 causes a dramatic decrease in base-paired U4/U6 snRNA levels, whereas deletion of Lsm6 or Lsm7 or the Lsm8-1 mutation each cause an accumulation of free U4 snRNA (Pannone et al., 1998; Mayes et al., 1999). These observations support a model in which Prp24 and the Lsm2–Lsm8 complex act cooperatively to promote association of U4 and U6 snRNPs.

The base-paired U4/U6 structure is highly conserved among metazoans, as is the presence of Lsm proteins, suggesting that the U4/U6 formation pathway is also conserved. Indeed, early studies revealed that a factor present in HeLa cell extract was required for efficient U4/U6 formation (Wolff & Bindereif, 1993). A likely candidate for a mammalian U4/U6 annealing factor has since been described: the protein p110^{nrb}, which was purified as a U6-associated protein localized primarily in the nucleus (Gu et al., 1998). The status of p110^{nrb} as the Prp24 ortholog has recently been confirmed by biochemical experiments (Bell et al., 2002). The identification of a functional homolog of Prp24 in humans stands to dramatically increase our understanding of the mechanism of U4/U6 formation by identifying the conserved elements of the system.

An important unanswered question is how Prp24 interacts with the Lsm complex. BLAST searches and sequence alignments revealed a family of previously undiscovered metazoan homologs of Prp24 that share a common domain structure and, in particular, a highly

conserved 12-amino-acid C-terminal motif. Strikingly, this family includes the human protein SART3, recently discovered in a screen for tumor antigens recognized by T cells (Yang et al., 1999), which also turns out to be p110^{nrb}. We show that the conserved C-terminal motif is necessary for the interaction of Prp24 with the Lsm proteins of U6 and that deletion of the motif causes a cold-sensitive growth phenotype and a defect in U4/U6 formation both in vivo and in vitro.

RESULTS

Domain structure of Prp24 and homologs

Because important Lsm-interacting portions of Prp24 might be evolutionarily conserved, we carried out BLAST searches and sequence alignments. These identified clear homologs in *Schizosaccharomyces pombe*, *Ophiostoma ulmi*, and *Candida albicans*, from which it became apparent that a stretch of 10 amino acids at the C terminus is very highly conserved relative to the rest of the protein. Using the PSI-BLAST and PHI-BLAST algorithms (Altschul et al., 1997; Zhang et al., 1998), we were able to identify the more distantly related metazoan homologs of Prp24 (also identified in Bell et al., 2002; see Discussion), including the human protein SART3, recently identified in a screen for tumor-rejection antigens recognized by T cells (Yang et al., 1999). The high degree of conservation of the C-terminal motif allowed us to confidently include distant Prp24 homologs (Fig. 1A) in spite of the overall weak homology (22% identity between Prp24 and SART3 over 183 amino acids in the RRM-containing domain).

Alignment of the Prp24 homologs (Fig. 1A) revealed that they have a common domain structure consisting of an N-terminal region (notably absent in Prp24 itself—see below) containing multiple half-a-tetratricopeptide repeats (half-TPR; Preker & Keller, 1998), a C-terminal region containing RNA-recognition motifs (RRM; Fig. 1B), and the highly conserved motif at the extreme C terminus, referred to herein as the C-terminal motif (Fig. 1C). Significantly, the half-TPR motif is found almost exclusively in proteins that are known or suspected to be involved in RNA metabolism (Preker & Keller, 1998). The number of half-TPRs varies from 4 in *O. ulmi* to 10 in several of the metazoans, although the identification of these motifs is somewhat ambiguous owing to their degeneracy. Similarly, the number of RRMs in the C-terminal domain varies from 1 in *Arabidopsis thaliana* to 4 in the fungal family members. Interestingly, the *Drosophila melanogaster* homolog, Rnp4F, appears to have a third RRM at its extreme N terminus. Finally, differences in the conserved C-terminal motif include the C-terminal extension in *O. ulmi* beyond the conserved motif, and the absence of the conserved motif in *C. albicans*.

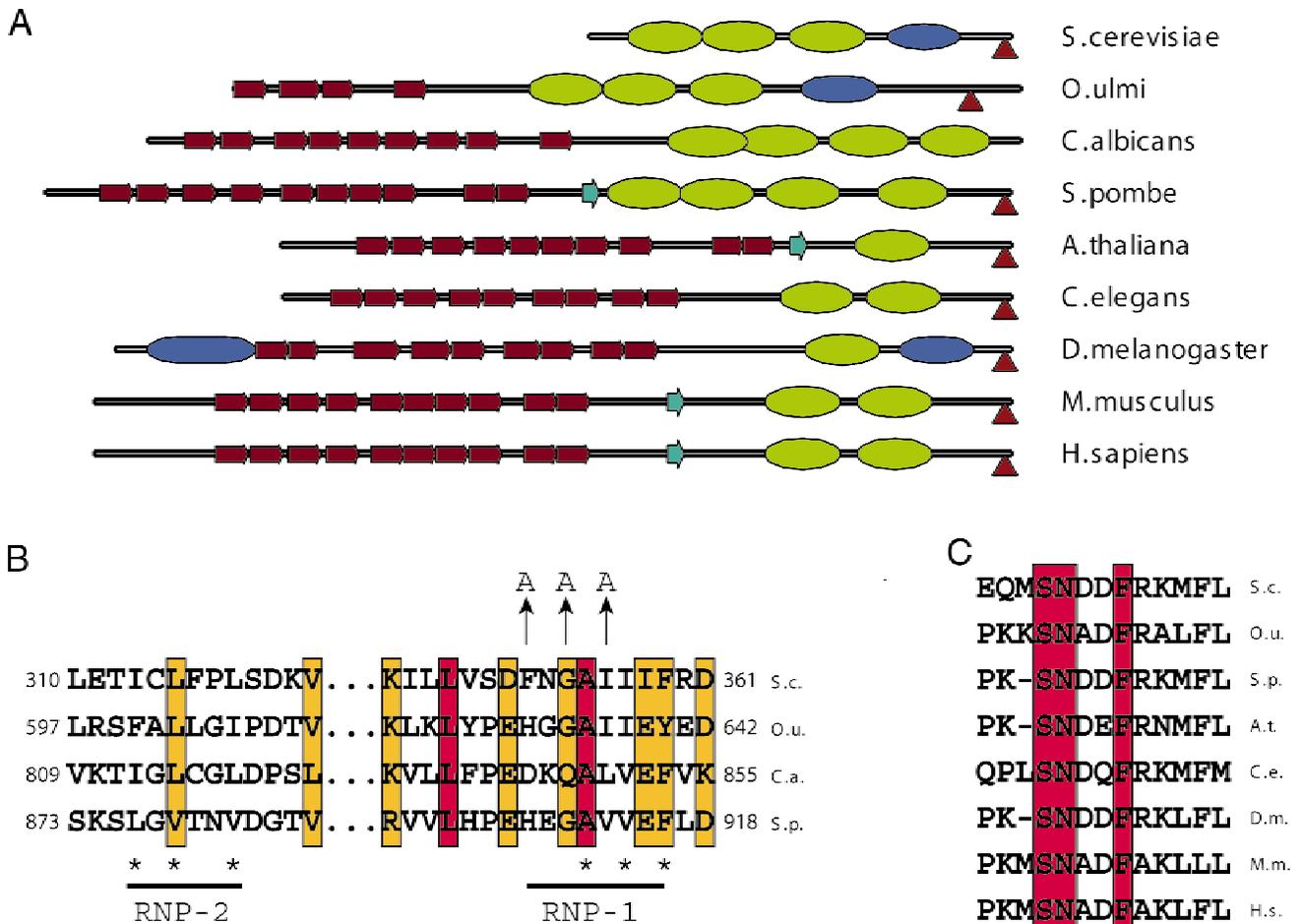


FIGURE 1. A family of homologs identifies new domains in Prp24. **A:** Domain structure of Prp24 and homologs. The half-TPR motif is indicated by red arrows, predicted nuclear localization signals (NLS) are indicated by green arrows, RNA-binding domains (RRMs) are indicated by green ovals, and the conserved C-terminal motif is denoted by red triangles. Blue ovals indicate putative RRMs that are not recognized by motif identification algorithms. **B:** Sequence alignment of the RNP-1 and -2 regions of RRM4. The predicted RNP-1 and -2 consensus sequences are underlined, and asterisks indicate the consensus positions of hydrophobic amino acids. Orange shading denotes conservation in three of the four sequences, and red shading denotes identity in all four sequences. The three alanine substitutions in RRM4sub are shown above the alignments. Amino acid positions are indicated relative to each sequence. **C:** Sequence alignment of the C-terminal motif. Absolutely conserved residues are shaded. Gaps were inserted manually.

Mutation of the fourth RRM in Prp24 causes a temperature-sensitive growth phenotype

Only three RRMs have previously been recognized in Prp24 (Shannon & Guthrie, 1991). The presence of a fourth RRM in the three fungal homologs suggested that Prp24 might also have a fourth RRM too divergent from the RRM consensus to have been detected by computational methods. Secondary structure predictions revealed a pattern of beta sheets and alpha helices consistent with the existence of a fourth RRM (data not shown), and sequence alignments supported this prediction, revealing conserved residues in the correct position for the conserved RNP-1 and RNP-2 consensus sequences (Fig. 1B; Kenan et al., 1991). To test whether RRM4 has an important biological function, we made a triple-alanine mutation (Prp24-RRM4sub,

indicated in Fig. 1B) of residues in the RNP-1 consensus that are predicted to interact with an RNA substrate. This mutation is similar to those that have been used previously to demonstrate the importance of RNP-1 residues in RRM2 and RRM3 (Vidaver et al., 1999).

Prp24-RRM4sub confers a strong growth defect at 37 °C but not at 30 °C or colder (Fig. 2A; data not shown). To test whether RRM4 function is redundant with any of the other RRMs, we made pair-wise combinations of RRM4sub with similar mutants in RRM1, RRM2, and RRM3 (termed RRM1sub, RRM2sub, and RRM3sub respectively; Vidaver et al., 1999), that is, 1sub/4sub, 2sub/4sub, and 3sub/4sub. Western blot analysis demonstrated that the viable combinations (1sub/4sub, 3sub/4sub) are expressed in yeast at wild-type levels or higher (data not shown). Strikingly, as shown in Fig-

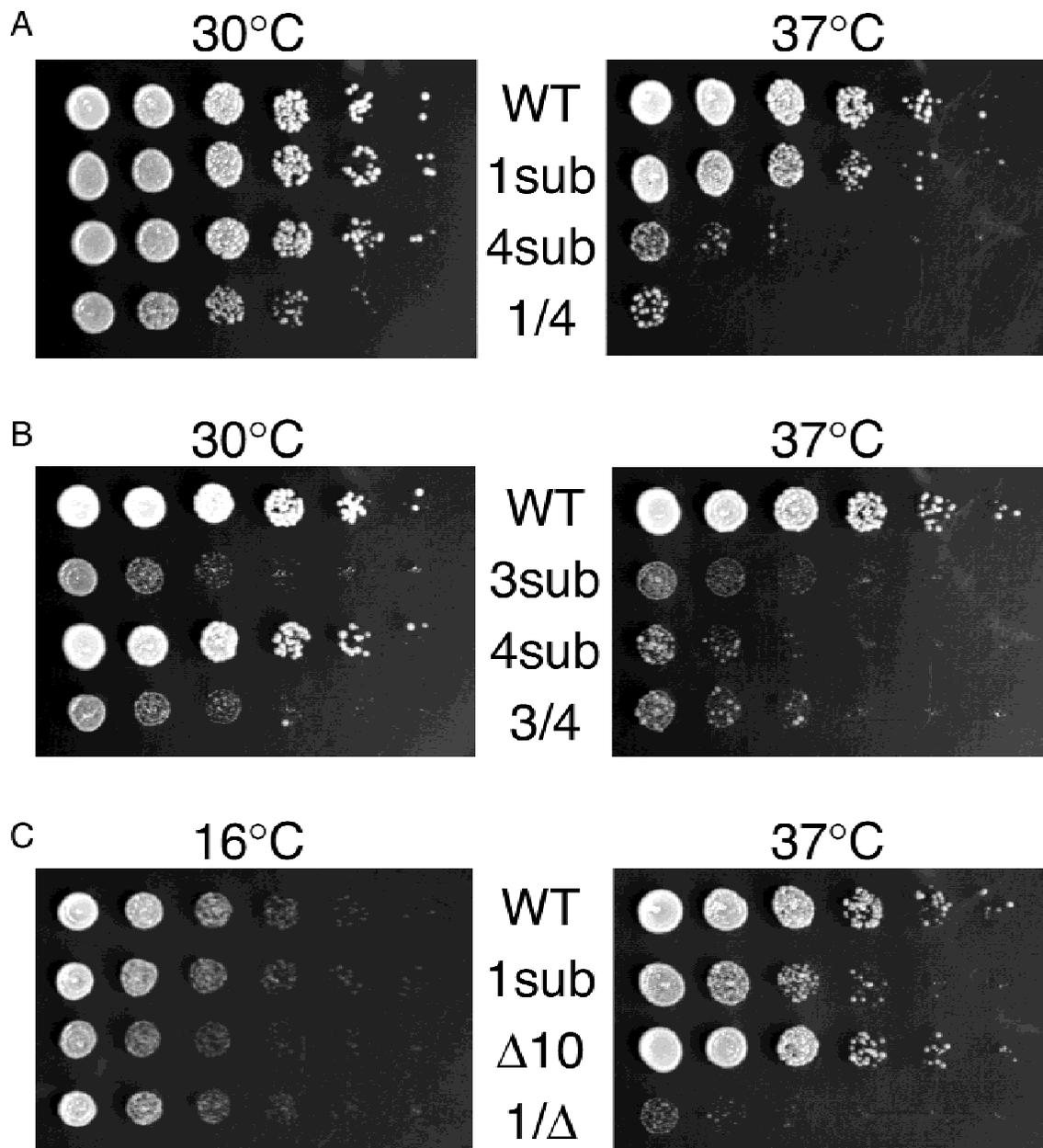


FIGURE 2. RRM4 and the C-terminal motif confer conditional growth phenotypes. Each strain contains only the allele of *Prp24* indicated. **A:** Growth of serial dilutions of yeast strains containing either *Prp24* (wild type), *Prp24*-RRM1sub, *Prp24*-RRM4sub, or *Prp24*-RRM1sub/4sub (denoted "1/4"). **B:** As in **A**, but with *Prp24*-RRM3sub and *Prp24*-RRM3sub/4sub (denoted "3/4"). **C:** As in **A**, but with *Prp24*Δ10 and *Prp24*-RRM1sub/Δ10 (denoted "1/Δ"). All strains have been grown on YPD for 2 days, except **C**, 16°C, which is after 5 days of growth.

ure 2, the RRM1sub/4sub combination was more temperature sensitive than RRM4sub, even though RRM1sub by itself has no phenotype (Fig. 2A). In contrast, the addition of RRM3sub (strongly temperature sensitive by itself) to RRM4sub does not exacerbate the temperature-sensitive phenotype at all (serial dilutions marked 3/4, Fig. 2B). These results are summarized in Table 1. As RRM2sub is lethal by itself, it was not surprising that the combination with RRM4sub was also lethal (data not shown). We conclude that RRM4

has an important biological function that is partially redundant with RRM1 and may be antagonistic to RRM3.

Deletion of the C-terminal motif causes a cold-sensitive growth phenotype in yeast

The high level of conservation of the C-terminal motif suggests that this motif may have an important biological function. To address this possibility, we made a mutant of *Prp24* in which the 10 most highly conserved

TABLE 1. Growth phenotypes of Prp24 allele combinations.

	WT	1sub	2sub	3sub	4sub
WT		viable	dead	ts	ts
1sub	viable				
2sub	dead				
3sub	ts				
4sub	ts	ts ^a	dead	ts ^b	
Δ10	cs	cs/ts	dead	dead	dead

WT: wild type; ts: temperature sensitive; cs: cold sensitive.

^aThis combination is more temperature sensitive than 4sub by itself (see Fig. 2B, 30 °C).

^bThis combination grows identically to 3sub.

amino acids in this region were deleted (Prp24Δ10). Growth of the mutant strain was clearly impaired relative to the wild type at 16 °C (Fig. 2C), whereas at 30 °C (not shown) or 37 °C (Fig. 2C) the mutation had no effect. This was confirmed by measuring the growth rates of the two strains, which revealed a reproducible 15% increase in the doubling time of Prp24Δ10 relative to wild type at 16 °C (data not shown).

To assess whether the C-terminal motif is involved in the same processes as the RRM, we made pair-wise combinations of the Δ10 deletion with the triple-Ala substitutions in the RRM as described above (Fig. 2C; Table 1). Notably, deletion of the C-terminal motif is lethal in the RRM3sub and RRM4sub backgrounds at all temperatures tested (20, 25, and 30 °C; not shown). Combining Δ10 with RRM1sub also resulted in a qualitatively different phenotype from either mutation alone: The combination is strongly temperature sensitive (Fig. 2C). As with RRM4sub, the combination of Δ10 with RRM2sub was lethal. These results demonstrate that the C-terminal motif also has an important biological function and suggest that it is partially redundant with the RRM.

Deletion of the C-terminal motif results in reduced U4/U6 levels in vivo

Because numerous experiments have implicated Prp24 in U4/U6 formation, we next determined whether Prp24Δ10 affects the equilibrium between U4/U6 and the unpaired snRNPs in vivo. Yeast strains harboring either Prp24 wild type or Δ10 were grown up at 30 °C and then shifted to 16 °C. Total RNA was isolated at 0, 13, and 28 h after the shift by cold phenol extraction so as not to disrupt U4/U6 base pairing (see Materials and Methods). Native (nondenaturing) northern analysis showed a marked decrease in the amount of base-paired U4/U6 relative to free U4 in the Prp24Δ10 strain (30% of U4 in the base-paired di-snRNP) compared to the wild type (80% of U4 in the di-snRNP; Fig. 3A). Because U6 is present in approximately fivefold ex-

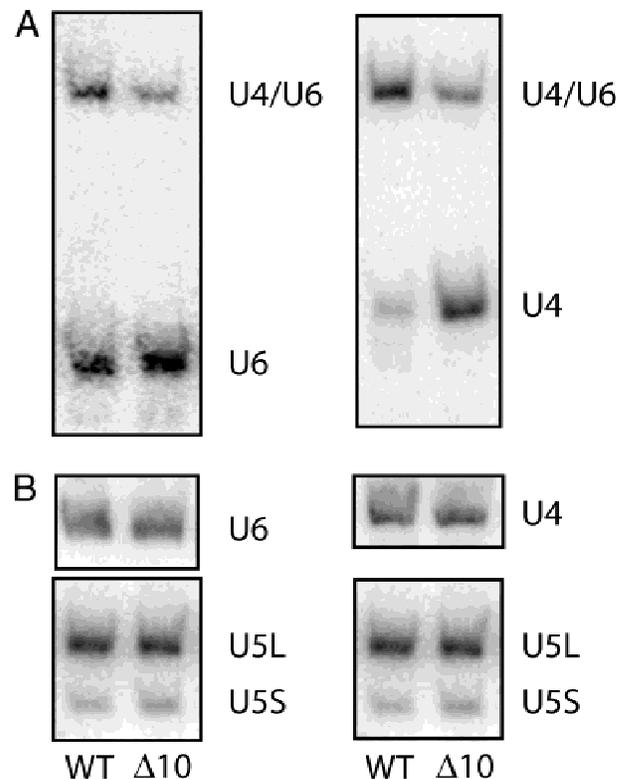


FIGURE 3. The Δ10 mutation causes a decrease in base-paired U4/U6 levels in vivo. **A:** Non-denaturing northern analysis of base-pairing status of U4 and U6 snRNAs from Prp24 wild-type and Δ10 strains after 13-h shift to 16 °C in YPD liquid medium. The same blot was probed for U6 (left) and U4 (right). **B:** Denaturing northern blot of the same samples as in **A**, showing total U6 (left) and U4 (right) snRNA levels. For comparison, U5 snRNA levels are also shown from the same samples (bottom panels).

cess over U4 (Li & Brow, 1993), we observed a less dramatic decrease of its fraction annealed, as expected. There was a concomitant increase in the amount of both free U4 and free U6, suggesting that the decrease in U4/U6 levels is due to decreased association of the free snRNPs, rather than reduction in snRNA levels. Denaturing northern blots confirmed that there was less than 10% change in total U4 and U6 snRNA levels between wild type and Δ10 (Fig. 3B). Although there was a slight exacerbation of the decrease in U4/U6 levels after the shift to 16 °C, most of the effect was already manifest at the permissive temperature (data not shown). We conclude that Prp24Δ10 causes a substantial decrease in U4/U6 formation in vivo.

The C-terminal motif is required for efficient coimmunoprecipitation of U6 snRNA

The reduced ability of Prp24Δ10 to promote U4/U6 formation in vivo could be due either to reduced binding to its substrate snRNPs (U4 and U6) or to reduced catalytic activity. As a first attempt to distinguish between these possibilities, we immunoprecipitated wild

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type and $\Delta 10$ Prp24 from whole-cell extract and analyzed by northern blotting the relative amounts of snRNAs that were coimmunoprecipitated. As a control for specificity, the pellets were washed in buffer containing either 50, 150, or 500 mM NaCl. In all cases, there was a clear decrease in the amount of both U4 and U6 snRNA in the $\Delta 10$ pellet compared to the wild type (Fig. 4A). As summarized in Table 2, quantitation of the northern blots showed a 2.0-fold reduction of U6 in $\Delta 10$ compared to wild type (averaged over all salt concentrations), and a 2.6-fold reduction of U4. At 500 mM NaCl, the U4 levels were reduced to background (compare preimmune lanes to 500 mM NaCl lanes), whereas the U6 coIP was only reduced approximately 2-fold relative to the lower salt concentrations. This is consistent with the stronger affinity of Prp24 for in vitro-transcribed U6 over U4 (Ghetti et al., 1995) and previous observations of salt-resistant association between Prp24 and U6 (Shannon & Guthrie, 1991). Western blotting against an HA epitope at the N terminus of the protein confirmed that the wild type and $\Delta 10$ proteins are immunoprecipitated with equal efficiency (Fig. 4B). In summary, these results indicate that Prp24 associates most strongly with the U6 snRNP and that the C-terminal motif contributes substantially to this interaction.

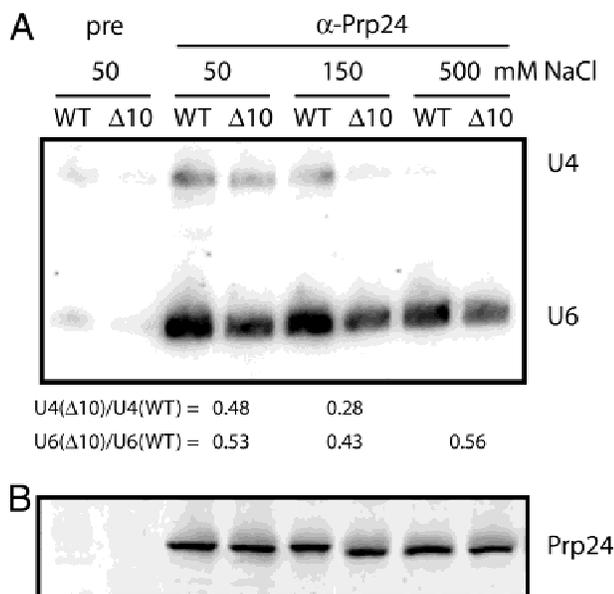


FIGURE 4. The C-terminal motif is required for normal association of Prp24 with U6. **A:** Denaturing northern blot of snRNA from α Prp24 immunoprecipitation pellets. Control immunoprecipitations were carried out using preimmune serum (lanes marked pre). Immune pellets (lanes marked α Prp24) were washed with buffer containing either 50, 150, or 500 mM NaCl as indicated. Immunoprecipitations were carried out from either wild-type (WT) or $\Delta 10$ extract as indicated above the lanes, and the snRNAs were detected with probes directed against U4 and U6 snRNA. The ratio of $\Delta 10$ snRNA to wild-type snRNA for this blot is shown at the bottom. **B:** Western blot of immunoprecipitation pellets probed with antibody to the HA epitope tag at the N terminus of Prp24.

TABLE 2. U6 and U4 snRNAs are reduced in $\Delta 10$ Prp24 immunoprecipitates.^a

	50 mM	150 mM	500 mM
U4	0.36 (0.11)	0.49 (0.05)	N/A
U6	0.51 (0.02)	0.49 (0.05)	0.52 (0.15)

^aValues shown are the mean ratio of the snRNA signal in the $\Delta 10$ pellet divided by the wild-type pellet ($(\text{RNA})_{\Delta 10}/(\text{RNA})_{\text{wild-type}}$). Numbers in parentheses are the standard deviation of three measurements.

The C-terminal motif is required for Prp24–Lsm interaction

The preceding results indicate that the C-terminal motif of Prp24 is involved in substrate binding and contributes to U4/U6 formation in vivo. As the C-terminal motif is not a known RNA-binding module, we investigated the possibility that it might mediate a protein–protein interaction with one or more of the Lsm proteins. The $\Delta 10$ mutation was therefore generated in appropriate two-hybrid constructs (Fromont-Racine et al., 2000), and its interaction tested in combination with each of the U6-associated Lsm proteins (Lsm2–Lsm8) as well as Lsm1. Because Lsm1, Lsm3, and Lsm4 did not interact with Prp24 in the two-hybrid screen (Fromont-Racine et al., 2000), we included them here as direct tests of the interaction with Prp24, expecting that Lsm1 would not interact, as it is not found in the U6 snRNP (Stevens et al., 2001).

The interaction was monitored by activity of a β -galactosidase reporter and a histidine reporter, as shown for the Prp24–Lsm5 interaction using a β -galactosidase filter assay (Fig. 5A) and growth on yeast plates lacking histidine (Fig. 5B). The wild-type Prp24 showed a clear interaction with most of the Lsm proteins, as reported previously (Fromont-Racine et al., 2000). In striking contrast, the $\Delta 10$ mutation almost completely abolished this interaction (Fig. 5A, B; Table 3), even though western analysis showed that Prp24 $\Delta 10$ was expressed at essentially the same levels as wild-type protein (Fig. 5C). The exceptions to the general trend are Lsm3, for which the interaction with wild-type Prp24 was no higher than the Lsm1 control despite a large effect of the $\Delta 10$ mutation, and Lsm6, for which the $\Delta 10$ mutation had almost no impact (Table 3). The disruption of two-hybrid interaction by the $\Delta 10$ mutation was also observed when the truncated Prp24 fragment (amino acids 354–444) was used in this assay (data not shown), confirming that the last 90 amino acids of Prp24 are sufficient for Lsm interaction and demonstrating that the C-terminal motif is necessary.

The C-terminal motif is required for efficient U4/U6 formation in vitro

To investigate the molecular basis of the $\Delta 10$ defect in more detail, we used an in vitro U4/U6 annealing assay

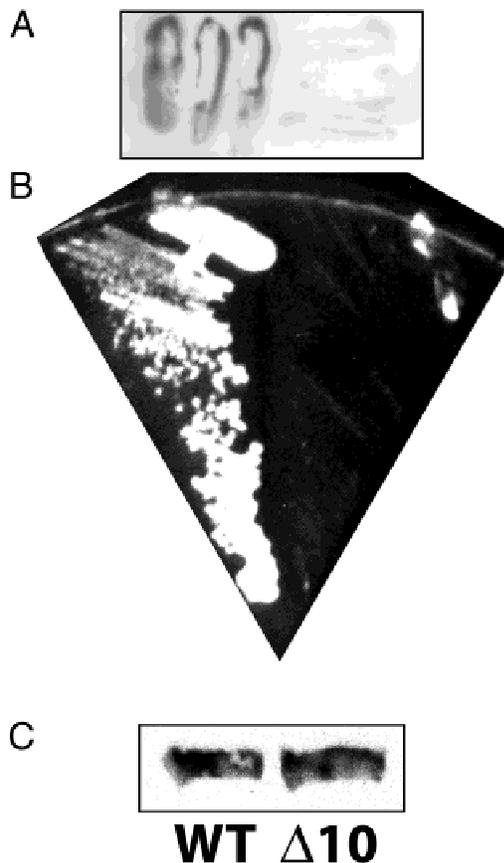


FIGURE 5. The C-terminal motif is necessary for Prp24–Lsm5 interaction by two-hybrid. For each panel wild-type (WT) is on the left and $\Delta 10$ on the right, as indicated below **C**. **A:** Filter-lift assay for β -galactosidase activity. **B:** Growth on SD–His plates to score for activity of the HIS3 reporter. **C:** Western blot of wild-type and $\Delta 10$ Prp24 from the two-hybrid strains, probed with an antibody against the N terminus of Prp24.

to test the effect of the mutation on the activity of recombinant protein. As previously described (Raghuathan & Guthrie, 1998), the substrates for this assay are generated by addition of ATP to an immunoprecipitated Brr2 particle (see Materials and Methods), which releases free U4 and U6 snRNPs into the supernatant.

Upon addition of Prp24 to the supernatant, the free snRNPs rapidly and quantitatively reanneal to form base-paired U4/U6. This protocol allowed us to compare the rate of U4/U6 formation for wild-type and $\Delta 10$ Prp24 under a variety of experimental conditions.

Annealing rates (expressed as moles of U4/U6 di-snRNP formed per second per unit volume) were measured by monitoring the conversion of free U4 snRNA to base-paired U4/U6 as a function of time under single turnover conditions (Fig. 6A; see Materials and Methods). This analysis revealed that Prp24 $\Delta 10$ had a clear and reproducible defect compared to wild type (1.6-fold at 10 nM and 3-fold at 1 nM for the data shown; Fig. 6B). A difference in the rate of U4/U6 formation was observed at all temperatures tested between 5 °C and 37 °C (data not shown) and at substrate concentrations up to 10-fold higher than those used in the experiments of Figure 6 (Fig. 7). At the highest Prp24 concentrations, however, the difference between wild type and $\Delta 10$ diminishes, with both reaching a plateau at ~ 160 fM/s. Although there is less than a 2-fold increase in rates between 15 °C (Fig. 7A) and 30 °C (Fig. 7B), the rates increase by a factor of approximately 10 between 15 °C and 37 °C (data not shown). We conclude that the C-terminal motif is required for efficient U4/U6 formation in vitro.

We fit curves to the rate data with three parameters, corresponding to the uncatalyzed rate (V_0), the maximum rate (V_{max}), and the apparent K_m (Fig. 7). The values for these parameters are given in Table 4. Notably, V_{max} is approximately 160 fM/s for both Prp24 and Prp24 $\Delta 10$, indicating that the C-terminal motif does not play a significant role in the catalytic function of Prp24. In contrast, most of the difference in annealing rates between wild-type and $\Delta 10$ Prp24 is explained by the difference in K_m values. At 15 °C, the apparent K_m for wild type is 22 nM, compared to 47 nM for $\Delta 10$. At 30 °C, where binding is weaker, the effect is exacerbated, with apparent K_m s of 77 and 260 nM for wild type and $\Delta 10$, respectively. The uncatalyzed rate (V_0) was not constrained in the curve fitting procedure, so the observation that this parameter is close to zero for

TABLE 3. The $\Delta 10$ mutation abolishes two-hybrid interaction between Prp24 and Lsm proteins.

	Lsm1 ^a	Lsm3 ^a	Lsm4 ^a	Lsm6 ^a	Lsm8 ^c	Lsm2 ^b	Lsm5 ^b	Lsm7 ^b	Lsm8 ^b
Wild type	0.19	0.18	0.29	1.3	0.77	0.30	21	7.1	10
$\Delta 10$	0.18	0.01	0.09	1.1	0.08	0.05	0.09	0.11	0.11
ratio ^c	1.1	16	3.2	1.2	9.6	6.0	230	65	94
error ^d	0.15	0.27	0.03	0.02	1.1	1.5	3.5	5.0	43

Values are the mean of at least three independent measurements from liquid β -galactosidase assay expressed in Miller units (see Materials and Methods).

^aLsm proteins expressed as a fusion with the Gal binding domain.

^bLsm proteins expressed as a fusion with the LexA binding domain.

^cRatio of wild-type Prp24 to Prp24 $\Delta 10$ activity.

^dStandard deviation of the wild-type/ $\Delta 10$ ratio.

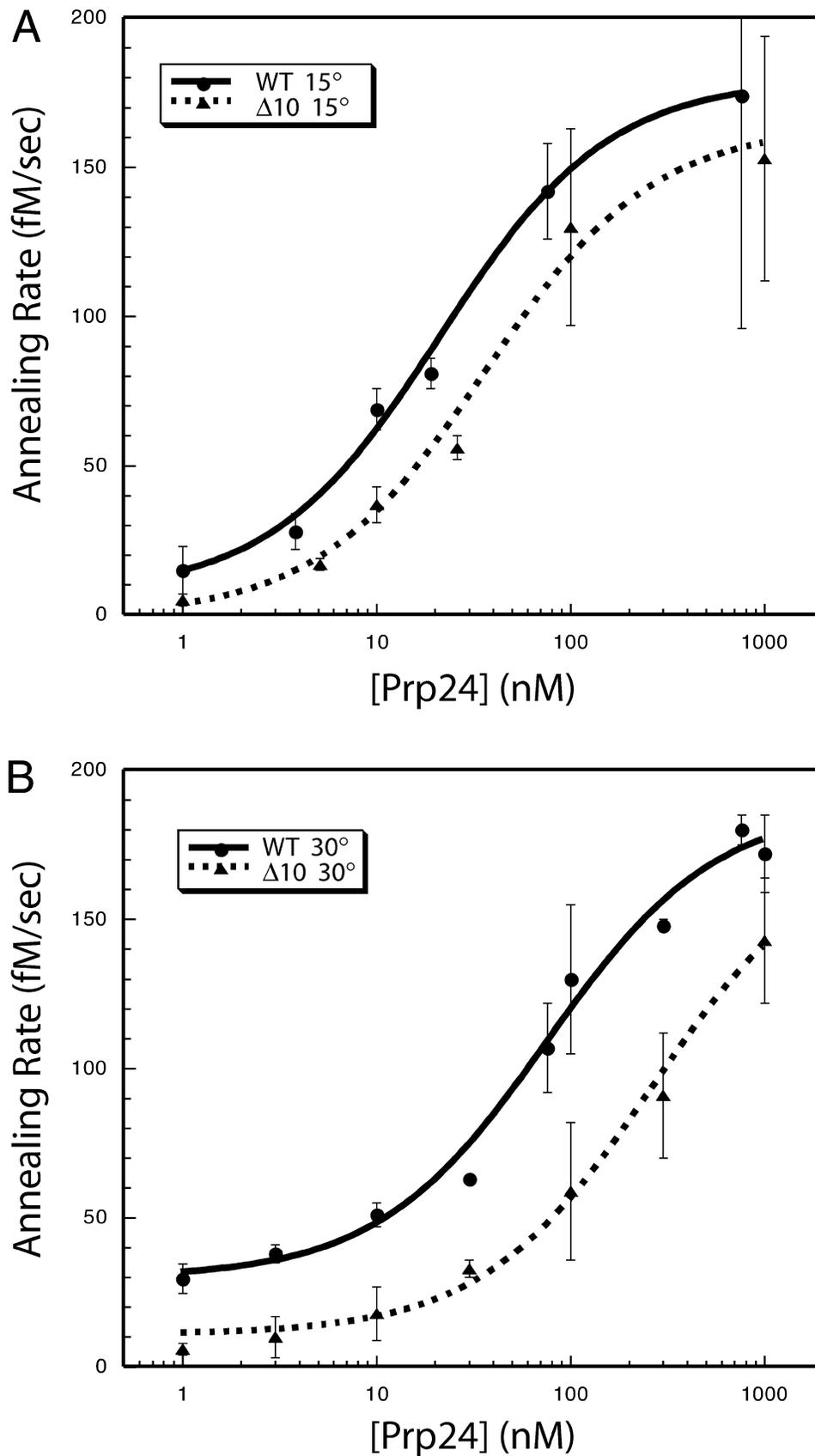


FIGURE 7. The C-terminal motif functions primarily in substrate binding. Annealing rates at 15°C (**A**) and 30°C (**B**) as a function of the concentration of Prp24. The data have been fit with curves describing the substrate binding equilibrium (see Table 4). Error bars are one standard deviation.

A conserved *Lsm*-interacting motif in *Prp24***TABLE 4.** The C-terminal motif functions primarily in substrate binding.

	K_m (nM) ^a	V_{max} (fM/s) ^b	V_0 (fM/s) ^c	R^d
15 °C wild type	22 (9)	170 (28)	7 (8)	.99
15 °C $\Delta 10$	47 (18)	160 (36)	2 (2)	.99
30 °C wild type	77 (16)	160 (8)	30 (6)	.99
30 °C $\Delta 10$	260 (62)	160 (12)	10 (3)	.99

Kinetic parameters are calculated from a fit of the data in Figure 7 to an equation describing the substrate binding equilibrium (see Materials and Methods). Numbers in parentheses are the calculated error in the fit ($n = 4-6$).

^aApparent K_m for Prp24 binding to substrate.

^bCalculated maximum annealing rate under the specified conditions ([U4] = 0.4 nM).

^cCalculated annealing rate in the absence of Prp24.

^d R value for the curve fit.

(2002). In spite of the overall low sequence homology, we were able to unambiguously identify family members by the presence of a highly conserved 12-amino-acid motif at their extreme C terminus. Notably, this comparison predicted that the human protein SART3 is a homolog of Prp24, an exciting discovery, as it implies that the mechanism of U4/U6 formation is probably conserved in humans. Indeed, this prediction has recently been confirmed (see below; Bell et al., 2002). In agreement with the results of Bell et al., examination of the family revealed a high degree of conservation in the domain structure, including the C-terminal motif and the RRM-containing domain. However, we also identified homologs in *Mus musculus* and *C. albicans*, and observed half-TPR repeats in the N-terminal domain of *O. ulmi*. A striking exception to the conserved domain structure is the absence in *Saccharomyces cerevisiae* Prp24 of an N-terminal domain containing multiple half-TPRs. The analogous N terminus for Prp24 may reside in a separate polypeptide. Alternatively, the function provided by the N-terminal domain may not be required in budding yeast.

A fourth RRM in Prp24

Pattern recognition algorithms have previously identified only three RRMs in Prp24. Sequence alignments, however, revealed that a conserved region in the C-terminal portion of the protein matched predicted RRMs in the *C. albicans* and *S. pombe* sequences, and that the *O. ulmi* sequence was also conserved in this region. These data, along with secondary structure predictions, strongly suggested that there is a fourth RRM in Prp24, and the results presented here have now confirmed that this domain plays an important biological role, at least at 37 °C. Interestingly, mutations in RRM1, which were previously reported to have no

effect on growth (Vidaver et al., 1999), strongly exacerbate the temperature-sensitive phenotype of RRM4 mutations, suggesting that RRM1 and RRM4 have at least partially redundant functions. RRM2 and RRM3 have been genetically implicated in binding U6 (Shannon & Guthrie, 1991; Vidaver et al., 1999), and, more specifically, in shifting the U4/U6 equilibrium towards free U6. Therefore, the observation that mutations in RRM3 and RRM4 partially suppress one another suggests that RRM4 may play a role in shifting the equilibrium towards the base-paired di-snRNP. One possible model is that RRM2 and RRM3 interact with U6, thereby stabilizing it, while RRM1 and RRM4 bind U4, which results in its being brought into close proximity with U6, thereby promoting U4/U6 formation. We are currently testing this hypothesis.

Genetic evidence for the role of the C-terminal motif in U4/U6 formation

The identification of a highly conserved motif at the C terminus of Prp24 suggests that this region has an important biological function. The data presented here confirm this prediction by demonstrating that a deletion of the motif causes a cold-sensitive growth phenotype. The discovery of a cold-sensitive mutation in Prp24 is intriguing because many mutations in U4 and U6 snRNAs that affect the U4/U6 equilibrium also confer cold-sensitive growth. In particular, U4 mutations that *destabilize* the di-snRNP are cold sensitive (Shannon & Guthrie, 1991), as are U6 mutations that *hyper-stabilize* free U6 (Fortner et al., 1994). In contrast, mutations in Prp24 that suppress the cold-sensitive phenotypes of these mutated snRNAs are themselves temperature sensitive (Shannon & Guthrie, 1991; Vidaver et al., 1999) and map to RRM2 and RRM3. These data are consistent with a model in which the wild-type snRNA structures promote U4/U6 formation, whereas the consequence of Prp24 binding to U6 via RRM2 and RRM3 is stabilization of the structure found in free U6 snRNP. In this scenario, the C-terminal motif acts similarly to the snRNA sequences in favoring the U4/U6 di-snRNP, as a loss-of-function mutation in this region ($\Delta 10$) is cold sensitive and results in decreased U4/U6 levels (see below).

One further genetic observation sheds light on the function of the C-terminal motif: the RRM1sub mutation (no phenotype on its own) and the $\Delta 10$ mutation (mildly cold sensitive) are strongly temperature sensitive in combination. Based on the RRM4sub phenotypes, we have suggested that RRM1 and RRM4 cooperatively promote U4/U6 formation by binding to U4 snRNA. The observation that the $\Delta 10$ mutation has a strong synthetic phenotype with RRM1sub suggests that RRM1 and the C-terminal motif have partially redundant functions, and that both of these regions may be involved in promoting U4/U6 formation.

Biochemical evidence for a role for the C-terminal motif in U4/U6 formation

Our genetic data, along with extensive previous work on Prp24 (Shannon & Guthrie, 1991; Ghetti et al., 1995; Jandrositz & Guthrie, 1995; Raghunathan & Guthrie, 1998; Vidaver et al., 1999), suggested that the $\Delta 10$ mutation might affect U4/U6 levels in vivo, and the biochemical experiments presented here demonstrate that this is indeed the case. The $\Delta 10$ mutation leads to a significant decrease (~ 3 times) in U4/U6 levels in vivo, consistent with Prp24's role in U4/U6 formation. Furthermore, Prp24 $\Delta 10$ coimmunoprecipitates only about half as much U6 as wild-type Prp24, even though U6 levels are approximately the same in the two extracts. To elucidate the role of the C-terminal motif in U6 snRNP binding, we turned our attention to the Lsm proteins of the U6 snRNP.

Several lines of evidence suggest that a subset of the Lsm proteins interact with Prp24 and help to promote U4/U6 formation (Cooper et al., 1995; Mayes et al., 1999; Vidal et al., 1999; Fromont-Racine et al., 2000; Ryan & Abelson, 2002). We used a two-hybrid assay to determine whether the C-terminal motif was important for this interaction. The original screen found Prp24 as an interacting partner for Lsm2, 5, 6, 7, and 8. We have now shown that Lsm4 also interacts with Prp24 by two-hybrid; however, we failed to see an interaction with Lsm3. In the absence of the C-terminal motif, the strength of the Prp24–Lsm interactions are reduced to the same level as the nonspecific control (Lsm1), except for the Prp24 $\Delta 10$ –Lsm6 interaction, which is still fivefold higher than the Prp24–Lsm1 interaction. Taken together with the effect of the $\Delta 10$ mutation on U4/U6 levels and on coimmunoprecipitation of U6, our data suggest that Prp24 binding to the Lsm proteins stabilizes its interaction with the U6 snRNP, and thereby promotes U4/U6 formation.

A cooperative model of Prp24–Lsm interaction does not exclude the possibility that either Prp24 or the Lsm2–Lsm8 complex can each promote U4/U6 formation without the other. This has been demonstrated for Prp24, which accelerates base-pair formation between in vitro-transcribed U4 and U6 snRNAs 25-fold over the uncatalyzed rate (Ghetti et al., 1995). It has also been demonstrated with the human Lsm proteins: Addition of biochemically purified human Lsm2–Lsm8 to in vitro-transcribed human U4 and U6 snRNAs stimulated the rate of base pairing approximately fivefold, apparently in the absence of any other proteins (Achsel et al., 1999). The molecular consequences of proteins binding to U6 snRNA remain to be determined, but will certainly be important for understanding the mechanism of U4/U6 di-snRNP formation.

To demonstrate a direct role for the C-terminal motif in U4/U6 formation, we turned to an in vitro annealing assay described previously (Raghunathan & Guthrie,

1998). Our annealing data demonstrate a clear defect in U4/U6 formation with Prp24 $\Delta 10$ at subsaturating protein concentrations. The observation that wild-type and $\Delta 10$ Prp24 have approximately the same maximum annealing rate under saturating conditions (high Prp24 concentration) indicates that the C-terminal motif does not contribute to the catalytic rate, but rather contributes to substrate binding. This is borne out by our estimates of the apparent K_m s, which show that deletion of the C-terminal motif causes a two- to threefold decrease in substrate binding. Apparently this is only rate limiting for growth at 16 °C, as the mutant has no phenotype at higher temperatures.

In conclusion, we propose the following model for the role of the C-terminal motif in the mechanism of annealing (shown in Fig. 8). Prp24 initially binds free U6 snRNP and subsequently recruits U4 snRNP. The Prp24–Lsm interaction mediated by the C-terminal motif promotes U4/U6 formation in vivo and in vitro by stabilizing the association of Prp24 with free U6 snRNPs. This model assumes that the direct substrate of Prp24 is the U6 snRNP, which is consistent with the known association of Prp24 with the free U6 snRNP in yeast whole-cell extracts (Raghunathan & Guthrie, 1998; Bouveret et al., 2000; Stevens et al., 2001) and with our observation that deletion of the C-terminal motif reduces the amount of U6 coimmunoprecipitated with Prp24. To address the possible contribution of U4 snRNP to this kinetic framework, we would need to be able to vary the concentrations of U4 and U6 snRNPs independently, which is not possible using our current protocol (see Materials and Methods).

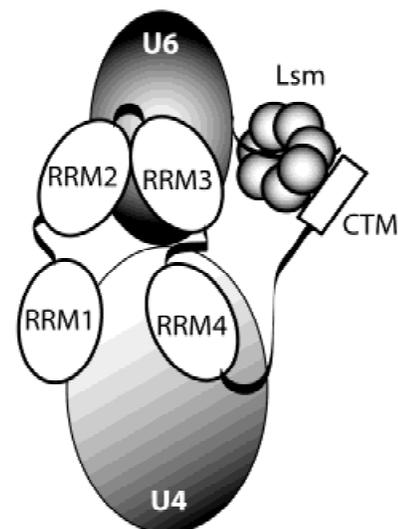


FIGURE 8. Model of the proposed interactions between Prp24 and the U4 and U6 snRNPs. RRM2 and RRM3 of Prp24 bind directly to U6 snRNA, and the C-terminal motif (CTM) interacts with the Lsm proteins (Lsm) of U6. RRM1 and RRM4 bind to U4 snRNA.

A human homolog and its implications

Given the low overall sequence similarity between Prp24 and SART3/p110^{nrB}, an obvious question is whether they have the same function. Notably, the existence of a mammalian ortholog of Prp24 was hinted at some time ago by the observation that an additional protein factor(s) is required for U4 snRNP-U6 snRNA interaction in HeLa cell extract (Wolff & Binder, 1993). We have used the two-hybrid system to demonstrate that SART3 interacts with hLsm7 (our unpubl. results). While this work was in progress, we learned that SART3 associates with human U6 and U4 snRNAs, and that it is required for human U4/U6 di-snRNP formation (Bell et al., 2002), strongly supporting the functional similarities of SART3 and Prp24. However, the difference in the number of RRM s (two in SART3 versus four in Prp24), and the failure of SART3 to complement mutations or deletion of Prp24 in yeast (our unpubl. results), indicate that there are functional differences.

The intriguing absence of the half-TPR domain in yeast Prp24 itself (Fig. 1A) may indicate that its function is not required in budding yeast. A more interesting possibility, supported by the presence of a half-TPR domain in the *C. albicans* Prp24 homolog, is that the function of the missing N-terminal domain is provided by a separate polypeptide. The most likely candidate, based on sequence homology, is Prp39, a U1 snRNP component consisting solely of half-TPRs (Lockhart & Rymond, 1994). Although a functional association of Prp24 with the U1 snRNP has not been reported to date, the proposed switch of U6 for U1 at the pre-mRNA 5' splice site (Staley & Guthrie, 1999) is one place where such an interaction might occur. Indeed, it has recently been reported that nucleotides in both U4 and U6 can be crosslinked to the 5' splice site in a *trans*-splicing assay (Johnson & Abelson, 2001). Consistent with this hypothesis, we have observed a weak but reproducible two-hybrid interaction between Prp24 and Prp39 (our unpubl. data). Further experiments in both the yeast and mammalian systems will be required to reveal the function of the enigmatic half-TPR domain.

MATERIALS AND METHODS

Homology searches and sequence alignments

Initial homology searches were performed with the BLAST algorithm on the NCBI web site (Altschul et al., 1990). Once the C-terminal motif was identified, PHI-BLAST was used in additional searches. The results from these searches were confirmed with PSI-BLAST (Altschul et al., 1997). Protein motifs were identified using the PROSITE server (Bucher & Bairoch, 1994) on the ExpASY web site, except for the

C-terminal motif, which was identified and aligned manually. Multiple sequence alignments were generated with CLUSTALW (Thompson et al., 1994) and displayed with BOXSHADE. Protein sequences are: Prp24 (*S. cerevisiae*, NC_001145.1), colony 1 (*O. ulmi*, U35661.1), unannotated ORF (*C. albicans*, from Contig6-2283), "similar to Prp24" (*S. pombe*, AL109834.1), hypothetical protein T22A6.100 (*A. thaliana*, NC_003075.1), hypothetical protein B0035.12 (*Caenorhabditis elegans*, T18650), Rnp4F (*D. melanogaster*, AE003433.1), mSART3 (*M. musculus*, NM_016926.1), and SART3/KIAA0156 (*Homo sapiens*, NM_014706.1).

Yeast strains and plasmids

Plasmid pPR113 was made by inserting a PCR product containing the entire Prp24 ORF flanked by *EcoRI* and *SalI* sites into pSE362 (HIS3, CEN; Elledge & Davis, 1988; P. Raghunathan & C. Guthrie, unpubl. results; see Table 5 for a list of all plasmids and yeast strains used in this work). The Prp24-RRM4sub triple-alanine substitution (F352A, G354A, I356A) was generated in pPR113 using the QuikChange mutagenesis kit (Stratagene) and oligonucleotides A and B, yielding plasmid pSR070. A Prp24 deletion strain was made by transforming Research Genetics strain #20854 (Winzeler et al., 1999) with pPR097 (pSE360-Prp24; P. Raghunathan, un-

TABLE 5. Plasmids and yeast strains.

Name	Description
pPR097	pSE360-Prp24 (wild type)—CEN, URA3
pPR113	pSE362-Prp24 (wild type)—CEN, HIS3
pSR070	pSE362-Prp24-RRM4sub
pSR086	pSE362-Prp24-RRM1sub
pSR087	pSE362-Prp24-RRM2sub
pSR053	pSE362-Prp24-RRM3sub
pSR093	pSE362-Prp24-RRM1sub/RRM4sub
pSR126	pSE362-Prp24-RRM2sub/RRM4sub
pSR105	pSE362-Prp24-RRM3sub/RRM4sub
pSR039	pSE362-Prp24Δ10
pSR151	pSE362-Prp24Δ10/RRM1sub
pSR152	pSE362-Prp24Δ10/RRM2sub
pSR153	pSE362-Prp24Δ10/RRM3sub
pSR154	pSE362-Prp24Δ10/RRM4sub
pPR127	pG1-Prp24 (wild type)
pSR044	pG1-Prp24Δ10
pSR177	pSE362-HA-Prp24
pSR178	pSE362-HA-Prp24Δ10
pSR108	pACT1Ist-Prp24 (wild type)
pSR109	pACT1Ist-Prp24Δ10
pSR112	pACT1Ist-Prp24 wild type (amino acids 354–444)
pSR113	pACT1Ist-Prp24Δ10 (amino acids 354–444)
pSR171	pET3a-Prp24 (wild type)
pSR172	pET3a-Prp24Δ10
Strains	
GLS616	MAT A haploid from BY4743 prp24::kanR Met-Lys-Ura-His-Leu- + pPR097
GLS618	MAT α haploid from BY4743 prp24::kanR Met-Lys-Ura-His-Leu- + pPR097
GLS619	GLS618 + pPR113
GLS620	GLS618 + pSR039

publ.) and sporulating the diploid. MAT A and MAT α met-lys-kanR spores (GLS616 and GLS618) were picked and used to test the growth phenotype of Prp24 alleles. Plasmid pSR070 was transformed into the Prp24 deletion strain GLS618, and the wild-type URA-marked plasmid removed by counterselection on 5-FOA (Boeke et al., 1987). The wild-type control strain containing pPR113 is GLS619. Yeast plasmids containing Prp24-RRM1sub, RRM2sub, and RRM3sub (a generous gift of D. Brow; referred to here as pSR086, pSR087, and pSR053, respectively) were similarly shuffled into GLS618. Allele combinations with RRM4sub were created by: subcloning an *ApaI*-*ClaI* fragment from pSR086 (1sub) into pSR070 to generate pSR093 (1/4sub); QuikChange mutagenesis of pSR087 with oligos A and B to generate pSR126 (2/4sub); and by QuikChange mutagenesis of pSR053 (3sub) with oligos A and B to generate pSR105 (3/4sub). These combinations were also shuffled into GLS618. All strains were tested for growth by streaking on YPD plates at the indicated temperatures.

A 10-amino-acid deletion (nt 1297–1326) was generated in the conserved C-terminal motif using oligonucleotides C and D. The resulting plasmid (pSR039) was transformed into GLS616 to generate GLS620. Combinations of the $\Delta 10$ mutation with the triple-Ala RRM substitutions were made by QuikChange using oligos C and D on plasmids pSR086, pSR087, pSR053, and pSR070. These were transformed into GLS618. The $\Delta 10$ mutation was also made in pPR127 (pG1-Prp24; P. Raghunathan, unpubl.) creating pSR044, providing overexpression of Prp24 under the constitutive GPD promoter. pPR127 and pSR044 were introduced into GLS618. To insert an HA epitope at the N terminus of Prp24, an *NcoI* site was introduced using QuikChange with oligos E and F on plasmids pPR113 (wild type) and pSR039 ($\Delta 10$), yielding plasmids pSR156 and pSR168, respectively. A cassette encoding a single HA epitope (oligos G and H) was inserted at the *NcoI* site, generating plasmids pSR177 and pSR178.

For two-hybrid analysis, full-length and truncated (amino acids 354–444) Prp24 were created by PCR using oligonucleotides I–K (containing *Bam*HI sites) as indicated below. Wild-type PCR products were generated using pPR113 as a template (for pSR108 and pSR112), whereas $\Delta 10$ products were made using pSR039 (for pSR109 and pSR113). The Prp24 PCR products were subcloned into plasmid pACT11st (Fromont-Racine et al., 1997) using the *Bam*HI restriction site, and screening for correct orientation by PCR.

Recombinant Prp24 was expressed from the pET3a vector (Novagen) as described previously (Ghetti et al., 1995). A cloning mistake that resulted in an additional 20 amino acids at the C terminus was corrected by subcloning a *ClaI*-*Sna*BI fragment from pPR113 into the *ClaI*-*Eco*RI (blunted) sites of pET3a-Prp24. A vector for recombinant Prp24 $\Delta 10$ was generated in the same way by subcloning the *ClaI*-*Sna*BI fragment from pSR039. This generated plasmids pSR171 and pSR172 for wild type and $\Delta 10$, respectively.

Oligonucleotides:

A: 5'-CTTCTCGTTAGCGACGCCAATGCCGCCGCTATTAT
ATTTAGAGATAG
B: 5'-CTATCTCTAAATATAATAGCGCGGCATTGGCGTCG
CTAACGAGAAG
C: 5'-CCTGACAAACAAGAGCAGATGGGTGAGTAGACTAA
CCGAACCG

D: 5'-CGGTTTCGGTTAGTCTACTCACCCATCTGCTCTTGT
TTGTCAGG
E: 5'-TAATCTGTGACCATGGAGTATGGACATCACGC
F: 5'-GCGTGATGTCCATACTCCATGGTCACAGATTA
G: 5'-CATGGGATACCCATACGATGTTCTGACTATGCGG
GATC
H: 5'-CATGGATCCCGCATAGTCAGGAACATCGTATGGGT
ATCC
I: (full-length forward) 5'-CTTCATAATGGATCCTAATGGAG
TATGGACATCACGC
J: (truncated forward) 5'-CGTTAGCGGGATCCATGGCGCC
ATTATTATTTAG
K: (universal reverse) 5'-CGTCTGGATCCGGTTCGGTTAG
TCTACTCACC.

Preparation of yeast RNA and northern analysis

For analysis of U4/U6 levels in vivo, strains GLS619 and GLS620 (Prp24 and Prp24 $\Delta 10$) were grown up in 10 mL of YPD medium at 30°C for 12 h. At the start of the shift, the cultures were spun down and resuspended in 100 mL of prechilled YPD medium and grown at 16°C. At the indicated times, 10-mL samples were removed, and total RNA was prepared using cold phenol extraction as described (Li & Brow, 1993). Northern analysis was performed as described (Raghunathan & Guthrie, 1998).

Coimmunoprecipitation

Splicing extracts were prepared from yeast strain GLS616 harboring pSR177 or pSR178 as described (Ansari & Schwer, 1995). One hundred microliters of splicing extract were incubated for 16 h at 4°C with 40 μ L of α Prp24Nt [a rabbit polyclonal antibody raised against an N-terminal peptide of Prp24 (amino acids 4–18, GHARPDSKRPLDEC; Sigma Genosys)] coupled to protein A sepharose (prepared as in Raghunathan & Guthrie, 1998). The resin was removed, washed four times with 1.5 mL NET buffer (1 mM EDTA, 0.05% NP-40, 50 mM TrisCl, pH 7.4) containing the appropriate concentration of NaCl, and split in two. Half of each sample was boiled in 30 μ L SDS sample buffer and run on a 4–20% acrylamide minigel (ICN). The gel was blotted onto nitrocellulose for 1 h at 200 mA in a semidry blotter (C.B.S. Scientific Co.). The blot was blocked for 30 min in TBS (10 mM TrisCl, pH 8, 50 mM NaCl) with 1% (w/v) milk, incubated for 1 h at room temperature with 12CA5 (monoclonal against the HA epitope; Roche) diluted 1:2,000 in TBS + milk, incubated for 1 h with a 1:2,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit secondary (BioRad), and visualized with ECF (Amersham) on a STORM 860 scanner (Molecular Dynamics). The other half of the pellet was cold phenol extracted and used for northern analysis as described above.

Yeast two-hybrid analysis

Strains and plasmids for two-hybrid analysis of the Prp24-Lsm interaction have been described (Fromont-Racine et al., 2000) and were generously provided to us by J. Beggs and P. Legrain. Lsm and Prp24 plasmids were cotransformed into

yeast strains L40ΔG, for Lsm-LexA-binding-domain fusions, or CG1945, for Lsm-Gal-binding-domain fusions, and transformants were selected on SD–Trp–Leu plates. The strength of the protein–protein interaction was assessed by growth on SD–His plates and by β-galactosidase activity as described (Fromont-Racine et al., 1997). Prp24 expression levels were checked by western blotting using αPrp24Nt (see above). Quantitation of the two-hybrids was performed by standard liquid β-galactosidase assay (Miller, 1972) using 1 mL (for LexA-binding-domain fusions) or 10 mL (for Gal-binding-domain fusions) of culture at an OD₆₀₀ of 0.3–1.0.

In vitro annealing

Recombinant Prp24 was expressed from pSR171 (wild type) or pSR172 (Δ10) in BL21* pLysS *Escherichia coli* (Novagen) and purified as described previously (Ghetti et al., 1995). Free U4 and U6 snRNPs were generated by adding ATP to a bead-bound Brr2 immunoprecipitate, as described (Raghu-nathan & Guthrie, 1998). The annealing substrate contained 4.0 nM U6 and 0.4 nM U4 snRNA, as determined by primer extension (data not shown).

Annealing was initiated by addition of Prp24, diluted appropriately in buffer D (Lin et al., 1985) with 0.1% Triton X-100. Following incubation, 4-μL aliquots were removed into 6-μL stop solution (300 mM NaOAc, pH 5.3, 10 mM EDTA, 0.5% SDS, 1 mg/mL proteinase K) and incubated 5 min at 37 °C to digest the proteins. ³²P-labeled oligo 14B complementary to U4 (Jandrositz & Guthrie, 1995) was added in 5-μL 3× hybridization buffer (Li & Brow, 1993) and incubated 15 min at 42 °C. Five microliters 4× native sample buffer (4× TBE, 60% glycerol) were added, and the samples loaded on a 4.5% nondenaturing polyacrylamide gel containing 5% glycerol in TBE. The precooled gels were run at 450 V for 1 h at 4 °C. Gels were dried onto Whatman (3M) paper and exposed to phosphor screens (Molecular Dynamics).

Band intensities were measured and corrected for background variation. The fraction annealed was calculated from the ratio of the annealed band to the sum of the annealed and free bands. Knowing the total concentration of U4 (0.4 nM), the fraction annealed was converted to concentration and plotted as a function of time. Line fits yielded the annealing rates, which were plotted as a function of protein concentration. Annealing data (averaged for each protein concentration) were fit to the equation

$$Rate = ((V_{max} * [Prp24]) / (K_m + [Prp24])) + V_0,$$

where V_{max} is the maximum rate at the given substrate concentration and temperature, V_0 is the uncatalyzed rate, and K_m is the apparent binding constant.

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