Multiple Functional Interactions Between Components of the Lsm2–Lsm8 Complex, U6 snRNA, and the Yeast La Protein

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ABSTRACT

The U6 small nuclear ribonucleoprotein is a critical component of the eukaryotic spliceosome. The first protein that binds the U6 snRNA is the La protein, an abundant phosphoprotein that binds the 3′ end of many nascent small RNAs. A complex of seven Sm-like proteins, Lsm2–Lsm8, also binds the 3′ end of U6 snRNA. A mutation within the Sm motif of Lsm8p causes *Saccharomyces cerevisiae* cells to require the La protein Lhp1p to stabilize nascent U6 snRNA. Here we describe functional interactions between Lhp1p, the Lsm proteins, and U6 snRNA. *LSM2* and *LSM4*, but not other *LSM* genes, act as allele-specific, low-copy suppressors of mutations in Lsm8p. Overexpression of *LSM2* in the *lsm8* mutant strain increases the levels of both Lsm8p and U6 snRNPs. In the presence of extra U6 snRNA genes, *LSM8* becomes dispensable for growth, suggesting that the only essential function of *LSM8* is in U6 RNA biogenesis or function. Furthermore, deletions of *LSM5*, *LSM6*, or *LSM7* cause *LHP1* to become required for growth. Our experiments are consistent with a model in which Lsm2p and Lsm4p contact Lsm8p in the Lsm2–Lsm8 ring and suggest that Lhp1p acts redundantly with the entire Lsm2–Lsm8 complex to stabilize nascent U6 snRNA.

THE process of pre-mRNA splicing requires five small ribonucleoprotein particles, the U1, U2, U4, U5, and U6 snRNPs. These small RNPs associate with each other, with the mRNA, and with a large number of splicing factors to form the spliceosome (reviewed by Burge *et al.* 1998). The U1, U2, U4, and U5 snRNPs each consist of an RNA molecule, seven common proteins known as Sm proteins, and additional snRNP-specific proteins. In vertebrates, binding of the Sm proteins to the snRNA occurs in the cytoplasm and is required for hypermethylation of the 5′ cap structure and reimport of the snRNPs into the nucleus (Kambach *et al.* 1999a). All Sm proteins share a conserved Sm motif consisting of two short submotifs, Sm1 and Sm2. The crystal structures of two Sm heterodimers have revealed that the Sm motif folds into an N-terminal α-helix, followed by a five-stranded antiparallel β sheet (Kambach *et al.* 1999b). From these structures, a model has been proposed in which the seven Sm proteins interact via their Sm motifs to form a heptameric ring around the snRNA (Kambach *et al.* 1999b).

In addition to the canonical Sm proteins, other Sm motif-containing proteins have been identified in many eukaryotes and certain archaebacteria (Cooper *et al.* 1995; Hermann *et al.* 1995; Seraphin 1995; Fromont-Racine *et al.* 1997; Achsel *et al.* 1999; Mayes *et al.* 1999; Salgado-Garrido *et al.* 1999). In yeast, there are nine of these Sm-like proteins (named Lsm1–Lsm9 for *L*ike *Sm*). Two distinct heptameric complexes of these Sm-like proteins have been described. One complex, consisting of the Lsm2–Lsm8 proteins, binds to the 3′ end of the U6 snRNA and is required for the stable accumulation of U6 snRNPs (Pannone *et al.* 1998; Achsel *et al.* 1999; Mayes *et al.* 1999; Salgado-Garrido *et al.* 1999; Vidal *et al.* 1999). A second complex, consisting of the Lsm1–Lsm7 proteins, functions in mRNA degradation, most likely at the decapping step (Boeck *et al.* 1998; Bouveret *et al.* 2000; Tharun *et al.* 2000). The Lsm2–Lsm8 complex was recently purified from human cells and found to resemble a doughnut, similar in size and shape to the core Sm snRNPs (Achsel *et al.* 1999). This finding, coupled with the fact that each of the Lsm2–Lsm8 proteins can be specifically aligned with one of the *bona fide* Sm proteins (Fromont-Racine *et al.* 1997; Salgado-Garrido *et al.* 1999), suggests that the Lsm proteins assemble into analogous heptameric ring structures. The order of the Lsm2–Lsm8 proteins within the ring is unknown.

Although the Lsm2–Lsm8 complex binds the 3′ end of U6 RNA and is required for the stable accumulation of U6 snRNPs, the precise role of this complex in U6 biogenesis and function is unknown. The first protein known to bind U6 RNA is the La protein, an abundant nuclear phosphoprotein that binds and stabilizes many newly synthesized small RNAs (Rinke and Steitz 1982, 1985; Pannone *et al.* 1998; Kufel *et al.* 2000; Xue *et al.*

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TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference
CYY0	*MAT***a**/α *ura3/ura3 lys2/lys2 ade2/ade2 trp1/trp1 his3/his3 leu2/leu2 LHP1/lhp1::LEU2*	Yoo and Wolin (1994)
CY2	*MAT*α *ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2*	Yoo and Wolin (1997)
CY3	*MAT***a** *ura3 lys2 ade2 trp1 his3 leu2 LHP1*	Yoo and Wolin (1994)
CY4	*MAT***a** *ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2*	Yoo and Wolin (1997)
BP1	*MAT***a** *lsm8-1 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pATL	Pannone *et al.* (1998)
BP2	*MAT*α *lsm8-1 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pATL	Pannone *et al.* (1998)
BP4	*MAT***a** *lsm8-1 ura3 lys2 ade2 trp1 his3 leu2 LHP1*	Pannone *et al.* (1998)
BP5	*MAT*α *lsm8-1 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pMETLHP1	Pannone *et al.* (1998)
BP8	*MAT*α *lsm8-2 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pMETLHP1	This study
BP10	*MAT*α *lsm8::HIS3 ura3 lys2 ade2 trp1 his3 leu2* p22myc	Pannone *et al.* (1998)
BP24	*MAT***a** *lsm8-1 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pLSM2	This study
BP25	*MAT***a** *lsm8-1 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pLSM8	This study
BP26a	*MAT***a** *lsm8-2 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pATL	This study
BP26α	*MAT*α *lsm8-2 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pATL	This study
BP34	*MAT***a** *lsm8-1 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pLSM3-PrA	This study
BP36	*MAT***a** *lsm8-1 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2* pRS426-SNR6	This study
DNY4	*MAT***a**/*MAT*α *ura3/ura3 lys2/lys2 ade2/ade2 trp1/trp1 his3/his3 leu2/leu2 LHP1/lhp1::LEU2 LSM6/lsm6::URA3*	This study
DNY8	*MAT***a**/*MAT*α *ura3/ura3 lys2/lys2 ade2/ade2 trp1/trp1 his3/his3 leu2/leu2 LHP1/lhp1::LEU2 LSM5/lsm5::HIS3*	This study
DNY9	*MAT***a**/*MAT*α *ura3/ura3 lys2/lys2 ade2/ade2 trp1/trp1 his3/his3 leu2/leu2 LHP1/lhp1::LEU2 LSM7/lsm7::TRP1*	This study
LSM1Δ	*MAT*α *ura3 lys2 ade2 trp1 his3 leu2 lsm1::URA3*	Diez *et al.* (2000)

2000). The La protein binds the UUU_{OH} at the 3′ end of these RNAs (Stefano 1984), which, for U6 RNA, overlaps the binding site of the Lsm2–Lsm8 complex (Achsel *et al.* 1999; Vidal *et al.* 1999). Thus, binding by the Lsm2–Lsm8 complex to the 3′ end of U6 RNA may displace the La protein. Consistent with this idea, yeast cells containing a mutation in *LSM8* require Lhp1p, the yeast La protein homologue, to stabilize newly synthesized U6 RNA (Pannone *et al.* 1998). However, it is unclear whether mutations in other Lsm proteins cause cells to require Lhp1p.

To better understand the functions of individual members of the Lsm2–Lsm8 complex, we examined the genetic interactions between Lhp1p, U6 RNA, and the Lsm proteins in the yeast *Saccharomyces cerevisiae*. We report that *LSM2* and *LSM4* function as allele-specific suppressors of two mutations in *LSM8*, consistent with the speculation (He and Parker 2000; Pannone and Wolin 2000) that Lsm2p and Lsm4p directly contact Lsm8p in the Lsm2–Lsm8 ring. Consistent with a direct interaction, overexpression of *LSM2* in the *lsm8-1* mutant strain increases the levels of the mutant Lsm8 protein. In the presence of high levels of U6 RNA, the normally essential *LSM8* becomes dispensable for growth, indicating that the only essential function of LSM8 is in U6 RNA biogenesis and/or stability. Furthermore, deletions of *LSM5*, *LSM6*, or *LSM7*, but not *LSM1*, are synthetically lethal with deletions of *LHP1*. Our experiments support the idea (Achsel *et al.* 1999) that Lhp1p acts redundantly with the assembled Lsm2–Lsm8 complex to stabilize newly synthesized U6 RNA.

MATERIALS AND METHODS

Yeast media, strains, and plasmids: Yeast media and manipulations were as described in Sherman (1991). The strains used in this study are listed in Table 1. The *lsm1*Δ strain (Diez *et al.* 2000) was a gift of P. Ahlquist (University of Wisconsin).

Synthetic lethal screen: The synthetic lethal screen was performed as described by Pannone *et al.* (1998) with the following modifications. CY2 cells carrying pATL, a centromeric plasmid containing *LHP1*, *TRP1*, and *ADE2* (Pannone *et al.* 1998), were mutagenized with ethylmethane sulfonate to 15% survival and plated on synthetic complete media containing limiting amounts of adenine (SCiade). Colonies were screened at 25° for the inability to lose pATL and form red sectors. Of 185,000 colonies screened, 68 did not form red sectors. These candidates were transformed with a second plasmid, pSLL28 (Yoo and Wolin 1997), which contains *LHP1*, *URA3*, and *LYS2*, and tested for the ability to lose pATL. Forty-nine candidates were able to form sectoring colonies. Of the 49 that sectored, 15 were dead on media containing 1 μg/ml 5-fluoroorotic acid, indicating they could not lose pSLL28. Backcrossing to CY4 revealed that five strains carried a single recessive mutation that caused them to require pATL. Complementation analysis with BP2 demonstrated that one strain failed to complement the *lsm8-1* mutation. This strain was backcrossed three times to CY2 to generate BP26a and BP26α. The characterization of the other strains will be presented elsewhere (S. D. Kim and S. L. Wolin, unpublished results).

Subcloning *LSM* genes into pRS316: Each *LSM* gene was

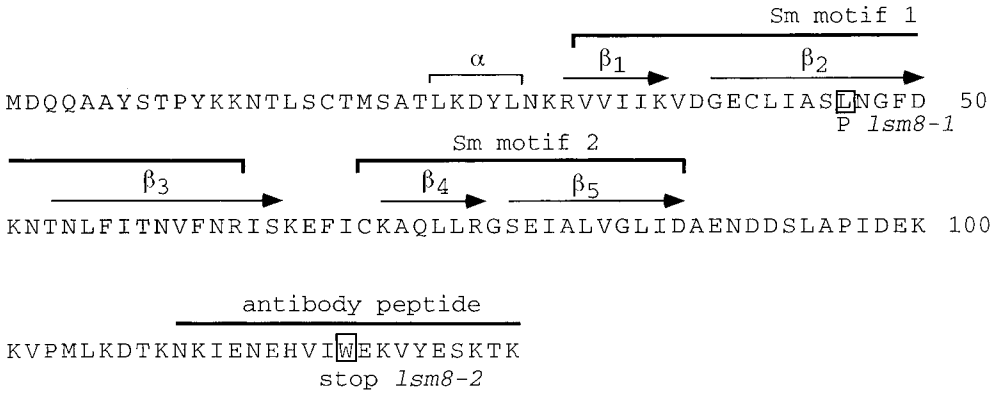

FIGURE 1.—Sequence of Lsm-8p. The sites of the *lsm8-1* and *lsm8-2* mutations are indicated, as is the sequence of the peptide used to generate anti-Lsm8p antibodies. The positions of the α-helix and five β-strands that make up the Sm motif are modeled on the crystal structures of the SmD_3B and SmD_1D_2 heterodimers (KAMBACH *et al.* 1999b).

subcloned into pRS316 (*CEN*, *URA3*; SIKORSKI and HIETER 1989) as described below. Plasmids carrying the *LSM3*, *LSM5*, *LSM6*, and *LSM7* genes fused to two IgG-binding domains of *Staphylococcus aureus* protein A (PrA) were gifts of B. Seraphin (SERAPHIN 1995; SALGADO-GARRIDO *et al.* 1999). The plasmid pBS959 was digested with *Bam*HI and *Hin*dIII and the *LSM5-PrA*-containing fragment was subcloned into the same sites of pRS316 to create pLSM5-PrA. The plasmid pBS1296 was digested with *Bam*HI and *Sac*I and the fragment containing *LSM6-PrA* was ligated into the *Bam*HI-*Sac*I sites of pRS316 to create pLSM6-PrA. The plasmid pBS957 was digested with *Bam*HI and *Hin*dIII and the fragment containing *LSM7-ProtA* was subcloned into the *Bam*HI-*Hin*dIII sites of pRS316 to create pLSM7-PrA. The plasmid pBS867 contains *LSM3-PrA* in vector pRS316. To create untagged versions, PCR was used to amplify the *LSM* genes from plasmids pLSM5-PrA, pLSM6-PrA, pLSM7-PrA, and pBS867. In each case, the oligonucleotide used to amplify the 3′ portion of each *LSM* gene introduced a stop codon at the end of the *LSM* coding sequence. The resulting DNAs were cloned into the *Bam*HI-*Eco*RI sites of pRS316 to create pLSM3, pLSM5, pLSM6, and pLSM7. Plasmid pSDB23-1 (COOPER *et al.* 1995; a gift from J. Beggs) was digested with *Hin*dIII and the 1.4-kb fragment containing *LSM4* was subcloned into the *Hin*dIII site in pRS316 to create pLSM4. Plasmid pJD09 (a gift from P. Ahlquist, University of Wisconsin) was digested with *Hin*dIII and *Eco*RI and the 1.5-kb *LSM1* fragment was subcloned into the *Hin*dIII-*Eco*RI sites of pRS316 to create pLSM1. Plasmids p22U and pSNPU (PANNONE *et al.* 1998) contain the *LSM8* and *LSM2* genes, respectively, in pRS316. pLSM1, pSNPU, pBS867, pLSM4, pLSM5, pLSM6, pLSM7, p22U, pRS426-SNR6 (a gift from D. Brow, University of Wisconsin), and pRS316 were transformed individually into strains BP5 and BP8.

Anti-Lsm8p antibody synthesis: Anti-Lsm8p peptide antibodies were generated by AnaSpec. A C-terminal Lsm8p peptide (H-Cys-Asn-Lys-Ile-Glu-Asn-Glu-His-Val-Ile-Trp-Glu-Lys-Val-Tyr-Glu-Ser-Lys-Thr-Lys-OH) was conjugated to BSA and injected into rabbits. The antisera was affinity purified against the peptide following the fifth bleed.

Native gels, Northern blots, and oligonucleotides: Extracts were prepared by vortexing 5 OD_{600} units of cells in 4 volumes of buffer A (50 mM Tris HCl pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$) with 0.25 μM phenylmethylsulfonyl fluoride in the presence of glass beads. The extract was sedimented at 100,000 × *g* in a Beckman TLA100 rotor for 20 min. A total of 0.1 OD_{260} units of each supernatant was mixed with an equal volume of buffer A containing 8% glycerol and loaded on a 4% polyacrylamide gel (80:1 acrylamide:bis) in 25 mM Tris, 25 mM boric acid, and 1 mM EDTA that had been prerun at 250 V for 30 min at 4°. Gels were run at 300 V until the bromophenol blue dye reached the bottom. RNA was transferred to ZetaProbe GT membranes (Bio-Rad) in 0.5× TBE at 150 mA for 16 hr. Hybridization with [γ-^{32}P]ATP-labeled oligonucleotides was done as described by TARN *et al.* (1995). Oligonucleotides used were U4: 5′-AGGTATTCCAAAAATTC CCTAC-3′ and U6D2: 5′-CGAAATAAATCTCTTTGTAAAA CGG-3′.

Disruption of the *LSM5*, *LSM6*, and *LSM7* genes: To disrupt *LSM5*, the *HIS3* gene from pRS313 (SIKORSKI and HIETER 1989) was amplified using forward and reverse primers that contained 45 and 49 nucleotides (nt) of *LSM5* sequence at their 5′ ends, respectively. This PCR product was transformed into CYY0, where it replaced one allele of the *LSM5* open reading frame with *HIS3*, leaving only 11 nt of *LSM5* coding sequence at the 5′ terminus and 13 nt at the 3′ terminus. This generated strain DNY8. To disrupt *LSM6*, the *URA3* gene from pRS316 (SIKORSKI and HIETER 1989) was amplified using forward and reverse primers that contained 39 and 53 nt of *LSM6* sequence at their 5′ ends, respectively. This PCR product was transformed into CYY0 where it replaced one allele of *LSM6* with *URA3*, leaving only 17 nt of *LSM6* coding sequence at the 5′ terminus and 15 nt at the 3′ terminus. This generated strain DNY4. To disrupt *LSM7*, the *TRP1* gene from pRS314 (SIKORSKI and HIETER 1989) was amplified using forward and reverse primers that contained 48 and 50 nt of *LSM7* sequence at their 5′ ends, respectively. This PCR product was transformed into CYY0 where it replaced one allele of *LSM7* with *TRP1*, leaving 10 nt of *LSM7* coding sequence at both the 5′ and 3′ termini. This generated strain DNY9.

RESULTS

Allele-specific suppression of *lsm8* mutations by Lsm proteins: To identify additional mutations that cause cells to require *LHP1*, we performed a genetic screen using a previously described strategy (PANNONE *et al.* 1998; XUE *et al.* 2000). An *ade2* strain lacking *LHP1* in the genome, but containing *LHP1*, *ADE2*, and *TRP1* on a centromeric plasmid, was mutagenized with ethylmethane sulfonate and screened for the inability to lose the plasmid. As a red pigment accumulates in *ade2* strains, cells that lose the plasmid are red, while cells that retain it are white. To determine if any mutants were allelic to previously isolated mutations (Yoo and WOLIN 1997; PANNONE *et al.* 1998; XUE *et al.* 2000), we performed complementation analyses. Each mutant strain was mated to our previously isolated mutant strains, and the diploids were tested for the ability to

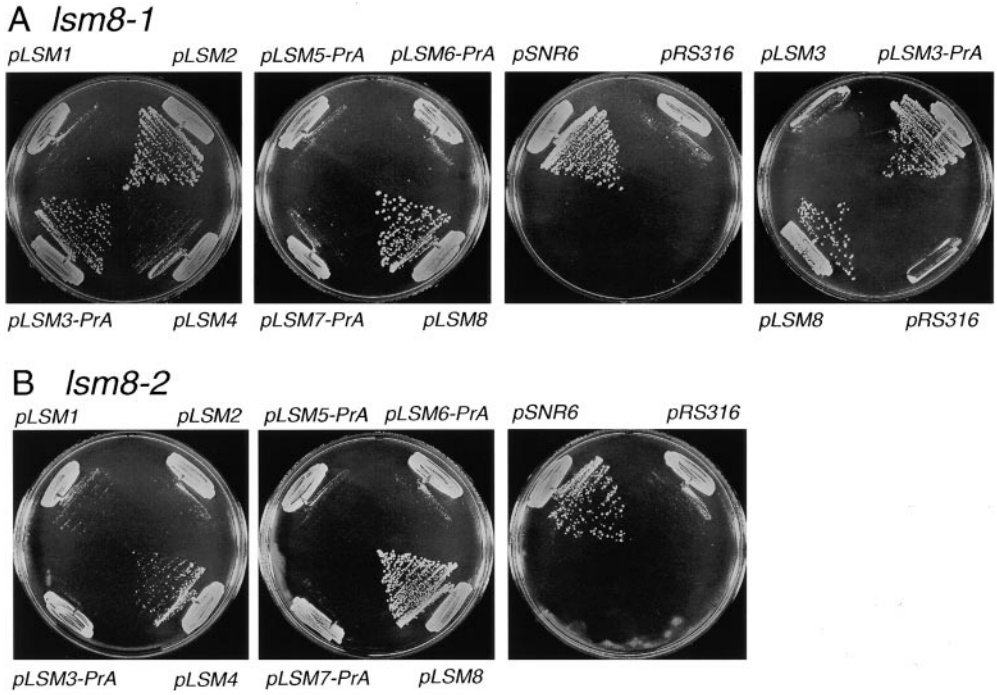

FIGURE 2.—Extra copies of certain *LSM* genes or U6 snRNA eliminate the requirement for *LHP1* in *lsm8* mutants. Plasmids containing the indicated *LSM* or *LSM-PrA* genes in the low-copy plasmid pRS316 were introduced into *lhp1::LEU2* strains carrying the *lsm8-1* (A) or *lsm8-2* (B) mutation. The strains also contained the pMETLHP1 plasmid (PANNONE *et al.* 1998) in which *LHP1* is under the control of the *MET3* promoter. Cells were streaked to single colonies on medium containing 2 mM methionine, which represses the *MET3* promoter (CHEREST *et al.* 1987), and grown at 25°. As controls, strains were transformed with the pRS316 vector. To determine whether multiple U6 RNA genes would eliminate the requirement for *LHP1*, the *lsm8* strains were transformed with *SNR6* in the high-copy plasmid pRS426. Introduction of pRS426 had no effect on the requirement for *LHP1* in *lsm8-1* (PANNONE *et al.* 1998) or *lsm8-2* cells (data not shown).

lose the *LHP1*-containing plasmid. One strain, which we refer to as *lsm8-2*, failed to complement the *lsm8-1* strain. Sequencing of *LSM8* in the strain revealed a single base change that converted the tryptophan at position 119 to a stop codon. Because the requirement for *LHP1* in this strain could be complemented by a low-copy plasmid containing *LSM8*, we conclude that the requirement for *LHP1* is due to truncation of Lsm8p. The positions of the *lsm8-1* and *lsm8-2* mutations are shown in Figure 1. Similar to the *lsm8-1* strain (PANNONE *et al.* 1998), the *lsm8-2* strain was slightly cold sensitive for growth (data not shown).

Previously, we identified *LSM2*, which encodes another Sm-like U6 snRNP protein, as a low-copy suppressor of the *lsm8-1* mutation (PANNONE *et al.* 1998). To determine whether other Sm-like proteins were able to eliminate the requirement for *LHP1*, we performed suppression analyses. Each of the *LSM1–LSM8* genes was subcloned into the low-copy plasmid pRS316 and tested for the ability to eliminate the requirement for *LHP1* in the *lsm8* strains. For these experiments, we used *lsm8* mutant strains that contained *LHP1* under control of the *MET3* promoter. Because the *MET3* promoter is repressed by high concentrations of methionine (CHEREST *et al.* 1987), the *lsm8* strains are unable to grow on plates containing 2 mM methionine (not shown, but see Figure 2, A and B). In our initial experiments, *LSM3*, *LSM5*, *LSM6*, and *LSM7* were tested as fusion proteins, as we used plasmids in which the C terminus of each coding sequence was fused to two IgG-binding domains of the *S. aureus* protein A (PrA; SALGADO-GARRIDO *et al.* 1999). For the *lsm8-1* mutant strain, introduction of either *LSM2*, *LSM3-PrA*, or *LSM8* on the low-copy plasmid eliminated the requirement for *LHP1* (Figure 2A), as these strains were able to grow on high methionine-containing media. Overexpression of *LSM1*, *LSM4*, *LSM5-PrA*, *LSM6-PrA*, *LSM7-PrA*, or the parent vector failed to eliminate the requirement for *LHP1*, although the background growth was reproducibly higher in the presence of extra copies of *LSM4* (Figure 2A).

To determine whether the protein A tags affected the ability of *LSM3* and *LSM5-7* to suppress the *lsm8* mutations, each of these genes was cloned without the tag into pRS316 and tested in the mutant strains. This revealed that, while the *LSM3-PrA* fusion suppressed the requirement for *LHP1*, wild-type *LSM3* did not (Figure 2A, fourth panel). Similar to the result obtained with the PrA fusions, the wild-type *LSM5-LSM7* failed to suppress the requirement for *LHP1* in the mutant strain (data not shown).

Interestingly, the second *lsm8* mutant allele, *lsm8-2*, exhibited a different pattern of suppression by *LSM* genes. Neither *LSM2*, *LSM3*, or *LSM3-PrA*, when present

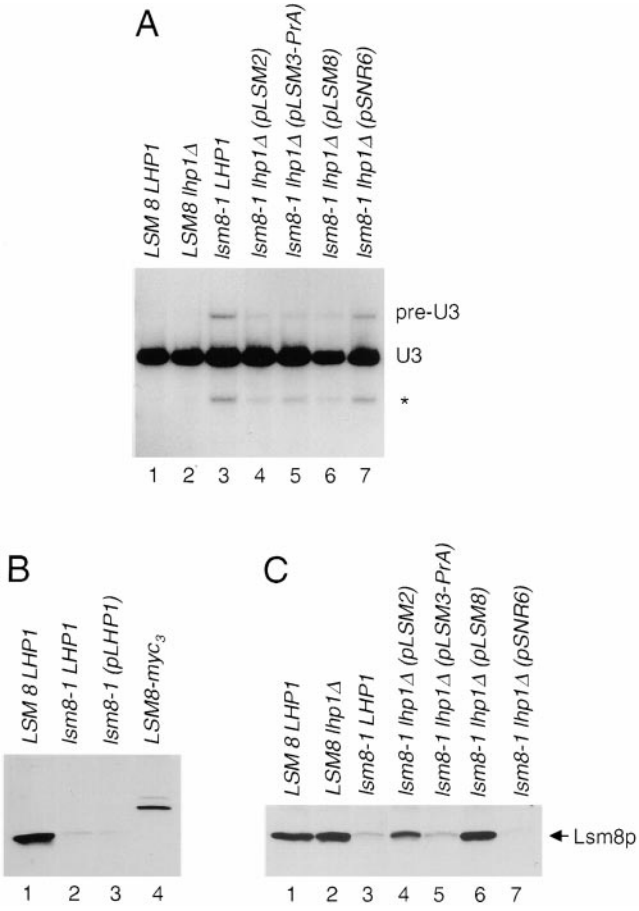

FIGURE 3.—In the presence of extra copies of LSM2, pre-mRNA splicing in the *lsm8-1* strain is more efficient and the levels of Lsm8p increase. (A) Total RNAs isolated from wild-type cells (lane 1), *lhp1::LEU2* cells (lane 2), *lsm8-1* cells containing chromosomal *LHP1* (lane 3), *lsm8-1 lhp1::LEU2* cells containing the indicated *LSM* genes in the low-copy plasmid pRS316 (lanes 4–6) or *SNR6* in the high-copy plasmid pRS426 (lane 7) were subjected to Northern analysis and probed to detect U3 RNA. The asterisk indicates a degradation product of pre-U3 RNA (HUGHES and ARES 1991). (B) Extracts of wild-type (lane 1), *lsm8-1* cells containing chromosomal *LHP1* (lane 2), and *lsm8-1* cells containing *LHP1* on a centromeric plasmid (lane 3) were analyzed for the presence of Lsm8p by Western blotting with anti-Lsm8p antibodies. To verify that the detected band was Lsm8p, lane 4 contains extract from a strain in which three copies of the human c-myc epitope (KOLODZIEJ and YOUNG 1991) were fused to the C terminus of Lsm8p. (C) Extracts of wild-type cells (lane 1), *lhp1::LEU2* cells (lane 2), *lsm8-1* cells containing chromosomal *LHP1* (lane 3), and *lsm8-1 lhp1::LEU2* cells containing either the indicated *LSM* genes in the low-copy plasmid pRS316 (lanes 4–6) or *SNR6* in the high-copy plasmid pRS426 (lane 7) were subjected to Western blotting to detect Lsm8p.

on the low-copy plasmid, allowed the mutant cells to grow on the 2-mM methionine medium (Figure 2B, also data not shown). In contrast, expression of *LSM4* allowed some growth, although less than when *LSM8* was expressed in the mutant cells (Figure 2B). Thus, while both *LSM2* and *LSM3-PrA* are able to suppress the requirement for *LHP1* in the *lsm8-1* mutant cells, only *LSM4* partially suppresses the *LHP1* requirement in the *lsm8-2* cells. However, as previously reported for the *lsm8-1* allele (PANNONE *et al.* 1998), expression of U6 snRNA on the high-copy plasmid pRS426 eliminates the *LHP1* requirement of the *lsm8-2* strain (Figure 2B, *pSNR6* sector). Thus, despite the differences in suppression of the two alleles by *LSM* genes, at least part of the requirement for *LHP1* in both strains is likely due to defects in U6 snRNP biogenesis or stability.

Extra copies of *LSM2* and *LSM3-PrA* increase pre-mRNA splicing efficiency in the *lsm8-1* mutant strain: To explore the mechanism by which extra copies of *LSM* and U6 genes eliminated the requirement for Lhp1p, we created *lsm8* strains in which *LHP1* was replaced by extra copies of these genes. We started with *lsm8 ade2* strains in which the only copy of *LHP1* was supplied on an *ADE2*-containing plasmid. These strains are white as long as the *ADE2* plasmid is present. Following transformation with plasmids containing *LSM* or U6 RNA genes, the strains were screened for the ability to lose the *LHP1* and *ADE2*-containing plasmid and form colonies with red sectors. In the presence of either *LSM2* or *LSM3-PrA* on a centromeric plasmid, or *SNR6* on a high-copy plasmid, the *lsm8-1* strains were able to lose the *LHP1*-containing plasmid. However, when *LSM4* was expressed in the *lsm8-2* strain, the strain was unable to lose the *LHP1* plasmid and remained white, suggesting that at least a low level of *LHP1* expression was required for efficient growth. Thus, while expression of *LSM4* allows *lsm8-2* strains to grow under conditions where *LHP1* transcription is repressed (Figure 2B), it may not completely eliminate the requirement for *LHP1*. Even though the *MET3* promoter is tightly regulated by methionine (CHEREST *et al.* 1987), a small amount of Lhp1p may still be synthesized under repressive conditions.

Since the *lsm8-1* mutation results in a pre-mRNA splicing defect (PANNONE *et al.* 1998), we determined whether extra copies of *LSM2* and *LSM3-PrA* increased the efficiency of pre-mRNA splicing in the mutant strains. Total RNA was extracted from each strain and subjected to Northern analysis to detect the U3 small nucleolar RNA, since this RNA contains an mRNA-type intron (MYSLINSKI *et al.* 1990). As previously described (PANNONE *et al.* 1998), cells containing the *lsm8-1* mutant allele accumulate unspliced pre-U3 RNA (Figure 3A, lane 3). In the presence of extra copies of *LSM2* or *LSM3-PrA*, the splicing defect was less evident (lanes 4 and 5), consistent with the finding that these genes function as low-copy suppressors of the *lsm8-1* mutation. However, while extra copies of *SNR6* also allow *lsm8-1* cells to grow in the absence of *LHP1*, only a slight decrease in pre-U3 RNA was detected in this strain (lane 7). Thus, while extra copies of *LSM2*, *LSM3-PrA*, and *SNR6* all allow *lsm8-1* cells to grow in the absence of *LHP1*, *LSM2* and *LSM3-PrA* are more efficient at suppressing the pre-mRNA splicing defect than is *SNR6*.

The level of the *lsm8-1* protein increases in the pres-

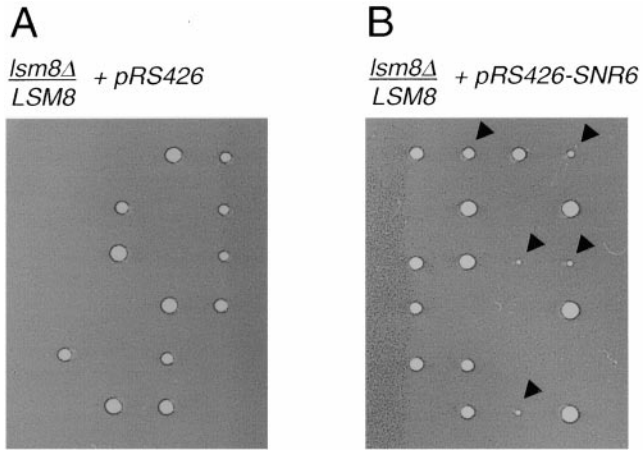

FIGURE 4.—*LSM8* is dispensable in cells containing multiple U6 snRNA genes. Either the high-copy vector pRS426 (A) or *SNR6* cloned into pRS426 (B) was introduced into *lsm8::HIS3/LSM8* diploids. Following sporulation at 25°, the resulting tetrads were dissected and incubated at 30°. Arrowheads indicate segregants carrying the *lsm8::HIS3* allele.

ence of extra copies of *LSM2*: To understand how extra copies of *LSM2, LSM3-PrA*, and *SNR6* suppress the requirement for Lhp1p in *lsm8-1* cells, we examined the levels of Lsm8p in the various strains. To this end, we prepared antibodies against the C-terminal 19 amino acids of Lsm8p and performed protein immunoblots (Figure 3B). The antibodies detected a polypeptide of 14 kD in wild-type extracts (lane 1), consistent with the predicted molecular weight of 14.5 kD. This band was greatly reduced in extracts from *lsm8-1* mutant cells (lanes 2 and 3). To confirm the identification of the 14-kD protein as Lsm8p, we examined extracts from a strain in which three copies of the human c-myc epitope were fused to the C terminus of Lsm8p (PANNONE *et al.* 1998). As expected, the 14-kD band was replaced by a band of ~20 kD (lane 4). Because the mutation in *lsm8-2* cells truncates Lsm8p, we were unable to detect Lsm8p in these cells with the antibody against the C terminus (data not shown).

We next determined the levels of Lsm8p in the *lsm8-1* mutant strains. In the presence of the *LSM2*-containing plasmid, the levels of the mutant Lsm8p increased, although not to wild-type levels (Figure 3C, compare lanes 3 and 4). Expression of *LSM3-PrA* on the centromeric plasmid did not result in a significant increase in the levels of Lsm8p (lane 5), while *LSM8* restored Lsm8p to wild-type levels (lane 6), as expected. Curiously, in the presence of extra copies of *SNR6*, the mutant Lsm8p was further reduced (lane 7). Thus, expression of *LSM2*, but not *LSM3-PrA* or *SNR6*, in the *lsm8-1* mutant strain results in an increase in the steady-state level of the mutant protein. Although we have not established the molecular mechanism by which this occurs, we note that Lsm8p and Lsm2p interact strongly in two-hybrid analyses (FROMONT-RACINE *et al.* 1997, 2000; MAYES *et al.* 1999; UETZ *et al.* 2000) and that these proteins have been proposed to be adjacent in the Lsm2–Lsm8 complex (HE and PARKER 2000; PANNONE and WOLIN 2000). Thus, binding of Lsm2p to Lsm8p may stabilize the mutant Lsm8 protein.

***LSM8* is not essential in the presence of multiple U6 RNA genes:** The observation that the levels of the mutant Lsm8p were drastically reduced in the presence of extra copies of *SNR6* was surprising, since *LSM8* is essential for yeast viability (FROMONT-RACINE *et al.* 1997; PANNONE *et al.* 1998). We thus determined whether *LSM8* becomes dispensable for growth in the presence of multiple copies of *SNR6*. We transformed the high-copy plasmid containing *SNR6*, or the empty vector alone, into a diploid strain in which one allele of *LSM8* was replaced with *HIS3* (PANNONE *et al.* 1998). In the presence of the empty vector, sporulation of the diploid and tetrad dissection yielded only two viable segregants per tetrad (Figure 4A), all of which required histidine for growth (data not shown). However, when *SNR6* was present on the high-copy plasmid, tetrad dissection yielded two, three, or four viable segregants per tetrad (Figure 4B). A total of 26 tetrads were dissected. In all cases, two segregants per tetrad were auxotrophic for histidine, indicating that they contained the wild-type *LSM8* gene. In addition, 12 tetrads contained one or two additional viable progeny, all of which were His^+. The His^+ segregants were variable in size (arrowheads, Figure 4B), but tended to be smaller than the segregants containing *LSM8*. One explanation for the size variation may be that the *lsm8::HIS3* progeny contain different numbers of the *SNR6* plasmid, since the copy number of these plasmids ranges from 10 to 40 per cell (SHERMAN 1997). Consistent with this, in all tetrads that contained only two viable segregants, the cells required both histidine and uracil for growth, revealing that they also lacked the *SNR6*-containing plasmid. Thus, those diploid cells that gave rise to only two his^- ura^- progeny may have lost the *SNR6* plasmid during sporulation. Nonetheless, the fact that a large fraction of the tetrads yielded viable His^+ progeny reveals that *LSM8* is dispensable for growth in the presence of multiple copies of *SNR6*. This result strongly suggests that the only essential function of Lsm8p is in U6 snRNP biogenesis or stability.

U6 snRNP levels in *lsm8-1* cells are increased by extra copies of *LSM2*: To further examine the mechanism by which extra copies of *LSM2, LSM3-PrA*, and *SNR6* suppress the requirement for Lhp1p in *lsm8-1* cells, we examined the various U6 RNA-containing particles. We fractionated whole-cell extracts from wild-type and *lsm8-1* strains using native gel electrophoresis and detected the U6 and U4 RNA-containing particles using Northern hybridization (Figure 5). Four distinct U6 RNA-containing complexes are present in wild-type extracts: the U4/U6.U5 tri-snRNP, the U4/U6 snRNP, the free U6 snRNP, and the Lhp1p/U6 RNA complex (Figure 5A, lane 1). As previously described (PANNONE *et al.* 1998),

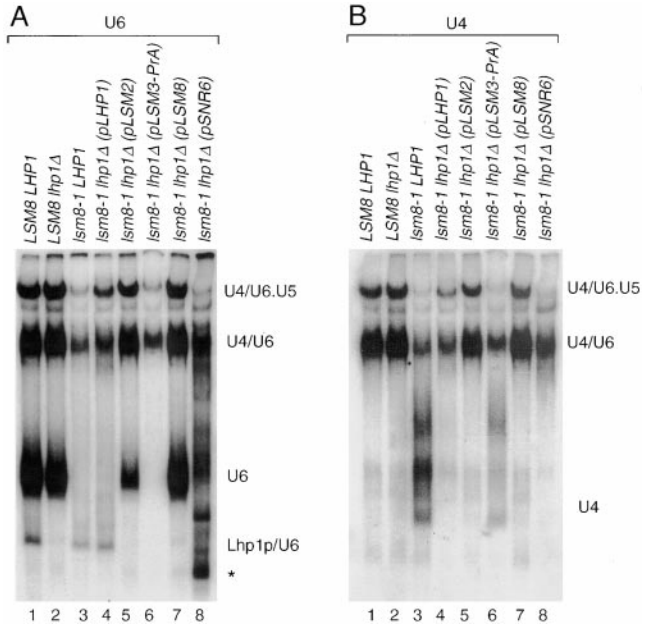

FIGURE 5.—The levels of the free U6 snRNP increase when *LSM2* is overexpressed in the *lsm8-1* strain. Extracts from wild-type cells (lane 1), *lhp1::LEU2* cells (lane 2), *lsm8-1* cells containing chromosomal *LHP1* (lane 3), *lsm8-1* cells containing *LHP1* on a centromeric plasmid (lane 4), and *lsm8-1 lhp1::LEU2* cells containing either *LSM2* (lane 5), *LSM3-PrA* (lane 6), or *LSM8* (lane 7) in the centromeric plasmid pRS316 were fractionated in 4% polyacrylamide gels and subjected to Northern analysis. The blot was probed with an oligonucleotide complementary to U6 snRNA (A) or U4 snRNA (B). Lane 8 contains extract from *lsm8-1 lhp1::LEU2* cells containing *SNR6* in the high-copy plasmid pRS426. The asterisk denotes the position at which naked U6 RNA migrates on these gels (data not shown).

the free U6 snRNP is undetectable in *lsm8-1* cells carrying chromosomal *LHP1*, and both the U4/U6 complex and U4/U6.U5 tri-snRNP are drastically reduced (lane 3). In addition, free U4 snRNPs accumulate in the mutant strain, consistent with a defect in U6 snRNP assembly (Figure 5B, lane 3; also PANNONE *et al.* 1998). When the sole copy of *LHP1* is supplied on a centromeric plasmid (which raises the levels of Lhp1p two- to threefold), the levels of the U4/U6.U5 tri-snRNP increase, consistent with the presence of more functional U6 snRNPs in this strain (Figure 5A, lane 4; also PANNONE *et al.* 1998). Interestingly, expression of extra copies of *LSM2* (lane 5), but not *LSM3-PrA* (lane 6), in the *lsm8-1* strain resulted in a large increase in the levels of U6-containing snRNPs and a decrease in the levels of free U4 snRNPs. In the presence of the *LSM2* plasmid, both the tri-snRNP and U4/U6 snRNP levels increased to ~90% of wild-type levels and mature U6 snRNPs became detectable (Figure 5A, lane 5). However, while the *LSM3-PrA* plasmid also eliminated the requirement of *lsm8-1* cells for *LHP1*, the only detectable change from the *lsm8-1 LHP1* extracts was a small increase in the levels of the U4/U6 snRNPs (Figure 5A; compare lanes 3 and 6), with a concomitant decrease in the free U4 snRNPs (Figure 5B, lane 6). Thus, while both *LSM2* and *LSM3-PrA* suppress the requirement for *LHP1*, only *LSM2* restores the levels of U6-containing snRNPs in *lsm8-1* cells to near wild-type levels.

Expression of the high-copy plasmid containing *SNR6* in the *lsm8-1* strain also did not significantly increase the level of the U4/U6.U5 tri-snRNP (Figure 5, A and B, lane 8). Instead, a heterogeneous smear of U6 RNA-containing particles migrated both with and ahead of the U4/U6 snRNP (lane 8). The smallest detectable band (asterisk, lane 8) comigrated with free U6 snRNA (data not shown). However, probing the blot to detect U4 RNA revealed that U4/U6 snRNP levels were restored to near wild-type levels (Figure 5B, lane 8). Thus, in the presence of additional copies of *SNR6*, U6 RNA assembles with U4 RNA to form the U4/U6 snRNP. However, assembly with the U5 snRNP remains impaired in the mutant strain.

***LHP1* is essential in strains lacking *LSM5*, *LSM6*, or *LSM7*:** Since our synthetic lethal screens identified two *lsm8* alleles that cause yeast to require *LHP1*, but did not reveal mutations in other *LSM* genes, we asked whether mutations in other components of the Lsm2–Lsm8 complex would cause a requirement for Lhp1p. *LSM2*, *LSM3*, and *LSM4* are all essential for viability (COOPER *et al.* 1995; MAYES *et al.* 1999; SALGADO-GARRIDO *et al.* 1999), and nonlethal mutations in these genes have not been described. However, *LSM6* and *LSM7* are both nonessential genes (MAYES *et al.* 1999; SALGADO-GARRIDO *et al.* 1999), and *LSM5* has been reported to be both essential (MAYES *et al.* 1999) and nonessential (SALGADO-GARRIDO *et al.* 1999). To determine whether *LHP1* becomes essential in cells lacking one of these genes, we disrupted the genes encoding *LSM5*, *LSM6*, and *LSM7* in a *LHP1/lhp1::LEU2* diploid strain. When our diploid strain (*lsm5::HIS3/LSM5, lhp1::LEU2/LHP1*) was sporulated at 25°, followed by incubation of the dissected spores at 30°, we obtained two viable segregants per tetrad (Figure 6A, left), consistent with the report that *LSM5* is essential (MAYES *et al.* 1999). However, when the dissected spores were incubated at 25°, we obtained tetrads containing either two, three, or four viable progeny (Figure 6A, right). In all cases, two segregants were large and lacked the *LSM5* disruption marker. The remaining progeny were all small (Figure 6A) and His^+, indicating that they contained the disrupted *LSM5* gene. Thus, at 25°, segregants lacking *LSM5* are viable in our strain background. Examination of the leucine requirement revealed that all His^+ progeny required leucine for growth, indicating that they contained the wild-type *LHP1* allele. Furthermore, all double mutants containing both the *lhp1::LEU2* and *lsm5::HIS3* alleles were dead (as deduced from the genotypes of the live segregants in each tetrad). Thus, *LHP1* is required for viability in strains lacking *LSM5*. Similarly, sporulation of the *lsm6::URA3/LSM6 lhp1::LEU2/*

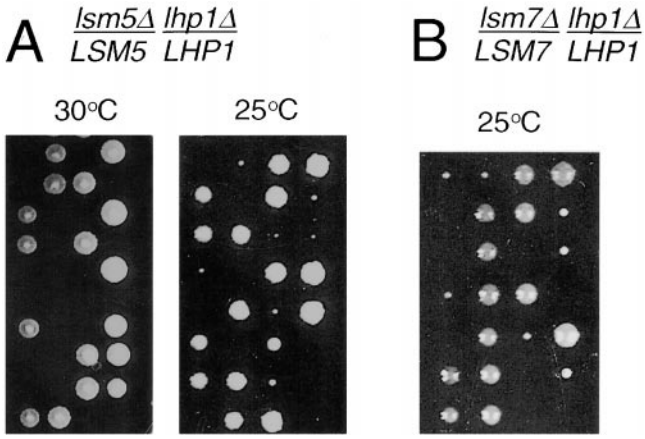

FIGURE 6.—Mutations in other components of the Lsm2p–Lsm8p complex cause yeast cells to require *LHP1*. (A) An *lsm5::HIS3/LSM5, lhp1::LEU2/ LHP1* diploid was sporulated at 25°, and the resulting tetrads dissected and incubated at either 30° (left) or 25° (right). At 30°, only two viable progeny were recovered per tetrad, all of which were auxotrophic for histidine. At 25°, tetrads contained two, three, or four viable progeny. In all cases, two haploid spores gave rise to two large colonies that were auxotrophic for histidine. The additional colonies were all small, His^+, and required leucine for growth. (B) An *lsm7::TRP1/LSM7, lhp1::LEU2/LHP1* diploid was sporulated at 25°, and the resulting tetrads dissected and incubated at 25°. All tetrads gave rise to two large colonies that required tryptophan for growth. In addition, some tetrads also gave rise to small colonies, all of which were Trp^+ and required leucine for growth.

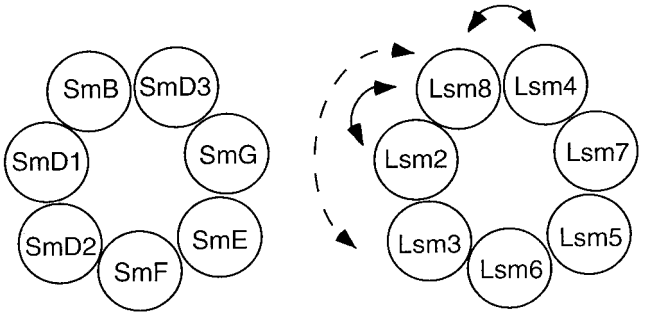

FIGURE 7.—Functional interactions detected between *LSM8* and other *LSM* genes. The model for the order of the Sm proteins within the heptameric ring is from KAMBACH *et al.* (1999b). The order of the Lsm2–Lsm8 proteins in this heptameric ring is unknown, but has been speculated to be similar to the proposed order for the Sm ring (HE and PARKER 2000; PANNONE and WOLIN 2000). Arrows joined by solid lines indicate functional interactions detected between *LSM8*, *LSM2*, and *LSM4*. The genetic interaction between *LSM3PrA* and *LSM8* is indicated by a dashed line.

LHP1 (data not shown) and *lsm7::TRP1/LSM7 lhp1::LEU2/LHP1* (Figure 6B) diploids and tetrad dissection revealed that *LHP1* was also required for viability in strains lacking either *LSM6* or *LSM7*.

Another nonessential *LSM* protein, Lsm1p, associates with Lsm2–Lsm7 to form a complex that participates in mRNA degradation (BOECK *et al.* 1998; BOUVERET *et al.* 2000; THARUN *et al.* 2000). To determine whether *LHP1* is required in strains lacking functional Lsm1p, we mated our *lhp1::LEU2* strain to a temperature-sensitive strain carrying a large disruption within *LSM1* (DIEZ *et al.* 2000). Sporulation of the diploid and dissection of the resulting tetrads revealed that *lsm1* mutant progeny lacking *LHP1* were able to grow at 30° (data not shown). Thus, *LHP1* only exhibits genetic interactions with the *LSM* genes that encode components of the U6 snRNP.

DISCUSSION

In yeast, newly synthesized U6 RNA is bound and stabilized by Lhp1p, the yeast La protein. Since both Lhp1p and the Lsm2–Lsm8 complex bind the 3′ end of U6 RNA (ACHSEL *et al.* 1999; VIDAL *et al.* 1999), Lhp1p and the Lsm2–Lsm8 complex may bind consecutively to U6 RNA during U6 snRNP assembly (PANNONE *et al.* 1998; ACHSEL *et al.* 1999). However, while Lhp1p and other La proteins preferentially bind RNAs terminating with UUU_{OH} (STEFANO 1984; TERNS *et al.* 1992; YOO and WOLIN 1994), mature U6 RNA ends with either a 2′,3′-cyclic phosphate (in mammals) or a 3′ monophosphate (in yeast; LUND and DAHLBERG 1992). Thus, while either Lhp1p or the Lsm2–Lsm8 complex can bind the nascent transcript, mature U6 RNA will only be bound by the Lsm2–Lsm8 complex. Consistent with the idea that either Lhp1p or the Lsm2–Lsm8 complex can bind nascent U6 RNA, Lhp1p is not required for U6 RNA biogenesis in otherwise wild-type yeast. However, Lhp1p becomes required to stabilize newly synthesized U6 RNA when cells contain a mutation in Lsm8p (PANNONE *et al.* 1998). Our experiments reveal that mutations in at least four *LSM* genes (*LSM5*, *LSM6*, *LSM7*, and *LSM8*) cause cells to require Lhp1p. Thus, the requirement for Lhp1p may be caused by any mutation that slows or impairs binding of the Lsm2–Lsm8 complex to U6 RNA. In this situation, Lhp1p may be required to stabilize newly synthesized U6 RNA, thus allowing assembly of the U6 snRNP.

Our result that *LSM8* becomes dispensable for growth in the presence of excess U6 RNA genes strongly suggests that the only essential function of the Lsm2–Lsm8 complex is in U6 snRNA metabolism. Consistent with these results, it was previously demonstrated that U6 snRNA overproduction suppressed the growth defect caused when a strain harboring a *GAL1*-regulated copy of *LSM8* was grown on glucose-containing medium (MAYES *et al.* 1999). However, in these experiments, it could not be ruled out that a small amount of Lsm8p (due to incomplete depletion and/or leakiness of the *GAL1* promoter) was required for viability. The fact that *LSM8* can be deleted in the presence of extra U6 snRNA genes establishes that the requirement for Lsm8p in U6 biogenesis and function can be bypassed as long as sufficient U6 RNA is present. Furthermore, the fact that

Lsm8p becomes undetectable in the presence of excess U6 snRNA (Figure 3) suggests that cells may possess mechanisms for downregulating *LSM8* expression in response to increased U6 RNA levels.

Although the human Lsm2–Lsm8 complex forms a ring of similar size and shape to the core Sm snRNPs, the order of the individual subunits around the rings is unknown. From the crystal structures of two Sm protein heterodimers, together with data from biochemical fractionation and two hybrid experiments, a model has been proposed in which specific Sm proteins interact with one another through their Sm motifs to form a heptameric ring (Kambach *et al.* 1999b). Because each of the Lsm2–Lsm8 proteins can be aligned with one of the Sm proteins (Fromont-Racine *et al.* 1997; Salgado-Garrido *et al.* 1999), Lsm proteins may interact in an analogous fashion (He and Parker 2000; Pannone and Wolin 2000; diagrammed in Figure 7). In this model, Lsm8p contacts Lsm2p and Lsm4p. We have demonstrated that both Lsm8p and U6 snRNP levels increase when Lsm2p is overexpressed in cells carrying a mutation in the Sm motif of Lsm8p. These findings are consistent with the specific interaction of these proteins within the Lsm2–Lsm8 complex. Our result that Lsm4p, when overexpressed in yeast carrying a truncation of Lsm8p, partially suppresses the requirement for Lhp1p is consistent with the idea that Lsm4p and Lsm8p directly interact within the complex. Furthermore, the fact that *LSM2* does not suppress the requirement for *LHP1* in the *lsm8-2* truncation mutant suggests that Lsm2p may contact the C terminus of Lsm8p (in addition to the Sm motif).

Curiously, while *LSM3-PrA* is also a low-copy suppressor of the *lsm8-1* mutation, Lsm3-PrAp overexpression did not increase Lsm8p and only moderately increased U4/U6 snRNP levels. Since *LSM3-PrA* overexpression can substitute for *LHP1* in the *lsm8-1* strain, one possibility is that the excess Lsm3-PrA protein functions similar to Lhp1p in binding and stabilizing newly synthesized U6 RNA. However, since we did not detect a Lsm3-PrA/U6 complex when the U6-containing particles were fractionated on native gels (Figure 5), this complex would have to be considerably less stable than the Lhp1p/U6 RNA complex. Since *LSM3-PrA*, but not wild-type *LSM3*, suppresses the requirement for *LHP1*, another possibility is that the extra C-terminal sequences in the Lsm3-PrA fusion interact with the Lsm8p tail to stabilize or enhance the function of the mutant Lsm2–Lsm8 complex. This enhancement of Lsm2–Lsm8 function could occur at a later stage of the U6 snRNP cycle, such as U4/U6 snRNP assembly or recycling of the U6 snRNP following splicing. Consistent with an interaction with the Lsm8p C terminus, excess Lsm3-PrA did not suppress the requirement for Lhp1p in the *lsm8-2* truncation mutant.

Interestingly, the genetic interactions that we have identified are far more restricted than those identified using two-hybrid analyses. In two-hybrid screens, Lsm8p interacts with each of the other six members of the Lsm2–Lsm7 complex (Fromont-Racine *et al.* 1997, 2000; Mayes *et al.* 1999; Uetz *et al.* 2000). We note that, in two-hybrid analyses, the two proteins being tested are expressed as fusion proteins. Thus, it is possible that if the fusion proteins are incorporated into the Lsm2–Lsm8 ring, the additional sequences appended to one or both proteins could result in interactions that do not occur between the wild-type proteins. This scenario would be compatible with the observation that Lsm3p only suppresses the requirement for *LHP1* when extra sequences are appended to the C terminus.

Because a small fraction of yeast pre-RNase P RNA is bound by six Sm-like proteins (Lsm2–Lsm7; Salgado-Garrido *et al.* 1999; B. K. Pannone and S. L. Wolin, unpublished data) and small changes in the levels of certain RNA polymerase III RNAs have been observed at late times after depletion of Lsm proteins (Mayes *et al.* 1999), it has been suggested that Sm-like proteins may function in the biogenesis of other small RNAs. Our result that *LSM8* becomes dispensable when *SNR6* is overexpressed reveals that these other possible functions are not essential roles of Lsm8p. In this regard, we note that Lsm8p, unlike Lsm2p–Lsm7p, is not detected bound to pre-RNase P RNA (Salgado-Garrido *et al.* 1999; B. K. Pannone and S. L. Wolin, unpublished data). Thus, there may be yet another complex of Lsm proteins, distinct from the Lsm2–Lsm8 and Lsm1–Lsm7 complexes, that functions in these other processes.

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