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A role for the yeast La protein in U6 snRNP assembly: evidence that the La protein is a molecular chaperone for RNA polymerase III transcripts

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The first protein that binds to all newly synthesized RNA polymerase III transcripts is a highly conserved phosphoprotein known as the La autoantigen. Although binding by the yeast La protein Lhp1p to pre-tRNAs is required for the normal pathway of tRNA maturation, the role of the La protein in the biogenesis of other polymerase III transcripts has been unclear. We identified a mutation in a novel component of the U6 snRNP that causes yeast cells to require Lhp1p for growth. This protein, Lsm8p, is a member of a family of proteins, known as Sm-like proteins, that shares two conserved motifs with the core Sm proteins of the U1, U2, U4 and U5 snRNPs. The *lsm8-1* cells have drastically reduced levels of the mature U6 snRNP, consistent with a defect in U6 snRNP assembly. In these cells, Lhp1p stabilizes newly synthesized U6 RNA, thus facilitating assembly of the RNA into the U6 snRNP. These results provide evidence that Lhp1p is a molecular chaperone for polymerase III-transcribed RNAs and implicate Lsm8p as a key component in the very early steps of U6 snRNP assembly.

Keywords: La autoantigen/*Saccharomyces cerevisiae*/Sm-like proteins/snRNP assembly/U6 snRNA

Introduction

The many conserved and abundant small RNAs transcribed by RNA polymerase III play critical roles in eukaryotic cell metabolism. In the nucleus, the best characterized of these RNAs are the U6 RNA, which is essential for pre-mRNA splicing, and the RNase P RNA, which processes the 5′ trailer sequence from pre-tRNAs. In the cytoplasm, tRNA and 5S ribosomal RNA are crucial elements of the protein synthesis machinery. Another cytoplasmic RNA, the RNA component of the signal recognition particle (SRP RNA), is important for targeting nascent secretory proteins to the rough endoplasmic reticulum (reviewed by Baserga and Steitz, 1993). Although much is known about the transcription of class III genes, far less is known about how these RNAs are folded, assembled with proteins and sorted within cells. For example, it is not known whether these RNAs fold spontaneously *in vivo*, or if molecular chaperones assist in their folding and assembly into ribonucleoprotein particles (RNPs). It is also not known why some RNAs, such as U6 RNA, are retained in the nucleus (Terns *et al*., 1993; Boelens *et al*., 1995), while other RNAs, such as SRP RNA, are exported to the cytoplasm.

The first protein that binds all newly synthesized polymerase III transcripts is the La autoantigen. Originally identified as a target of the autoimmune response in patients suffering from the rheumatic diseases systemic lupus erythematosus and Sjogren's syndrome, the La protein binds precursors to tRNA, 5S rRNA, U6 snRNA, SRP RNA and the cytoplasmic Y RNAs (Hendrick *et al*., 1981; Rinke and Steitz, 1982, 1985; Chambers *et al*., 1983). The La protein is able to bind all these different RNAs because it recognizes the sequence UUU_{OH}, which is at the 3′-terminus of all newly synthesized polymerase III transcripts (Stefano, 1984). Since the La protein binds these nascent transcripts immediately after transcription termination, an analysis of its function should provide insights into the earliest events in the biogenesis of these RNAs.

Using a combination of genetics and biochemistry in the yeast *Saccharomyces cerevisiae*, we demonstrated that the yeast La protein Lhp1p (La homologous protein 1) is required for the normal pathway of tRNA maturation (Yoo and Wolin, 1997). All tRNAs are transcribed as precursors containing 5′ and 3′ extensions that are removed to generate mature tRNA. In wild-type yeast cells and extracts, an endonuclease removes the 3′ trailer from the majority of tRNA precursors. In the absence of Lhp1p, the 3′ ends of these tRNAs are trimmed by an exonuclease(s) (Yoo and Wolin, 1997). Interestingly, while yeast cells lacking Lhp1p are viable (Yoo and Wolin, 1994; Lin-Marq and Clarkson, 1995), cells containing a mutation that disrupts the anticodon stem of an essential $tRNA^{Ser}$ require *LHP1* for growth. As introduction of a second mutation that restores basepairing in the anticodon stem alleviates the requirement for *LHP1*, binding by Lhp1p may stabilize formation of the correctly folded structure (Yoo and Wolin, 1997).

To determine the role played by Lhp1p in the biogenesis of the other RNAs transcribed by RNA polymerase III, we have carried out a genetic screen to identify additional mutations that cause yeast cells to require *LHP1* for growth. We report that yeast cells containing a mutation in a novel protein component of the U6 small nuclear ribonucleoprotein particle (snRNP) require Lhp1p for growth. This protein, Lsm8p, is a member of a family of proteins, known as Sm-like proteins, that share conserved motifs with the core proteins ('Sm' proteins) that are components of the U1, U2, U4 and U5 snRNPs (Cooper *et al*., 1995; Hermann *et al*., 1995; Seraphin, 1995). These four U snRNPs, like the U6 snRNP, are critical constituents of the pre-mRNA splicing machinery (reviewed by Madhani and Guthrie, 1994). We demonstrate that cells carrying a mutation in Lsm8p have reduced levels of the

U6 snRNA and are defective in U6 snRNP assembly. In these mutant cells, Lhp1p functions to stabilize the unassembled U6 snRNA, thus facilitating the assembly of the RNA into the mature U6 snRNP. Our data implicate Lsm8p as a key component in U6 snRNP assembly and provide evidence that the La protein functions as a molecular chaperone for RNA polymerase III-transcribed small RNAs.

Results

A mutation in an essential Sm-like protein causes yeast cells to require Lhp1p for growth

To identify additional roles of the La protein, we carried out a genetic screen to find mutations that cause cells to require *LHP1*. An *ade2* strain carrying a disruption in *LHP1* (*lhp1::LEU2*) was transformed with a plasmid carrying the *LHP1*, *ADE2* and *TRP1* genes. Cells that lose the plasmid turn red due to the accumulation of a red pigment in *ade2* strains (Hieter *et al*., 1985). Thus colonies that lose the plasmid contain red sectors, while colonies that retain the plasmid are white due to the *ADE2* gene. Following mutagenesis with ultraviolet light to 40% survival, colonies were screened for the inability to lose the plasmid. From 63 000 colonies, a single mutant strain was identified that required *LHP1* for growth. In addition to the requirement for *LHP1*, this strain grew slowly relative to wild-type cells at lower temperatures (~60% of the wild-type rate at 16°C).

The mutant gene was cloned based on complementation of the *LHP1* requirement and the cold-sensitive growth phenotype. Two different genomic DNA clones rescued both phenotypes. Subcloning of the DNAs in a low-copy vector revealed that both the open reading frame (ORF) *YJR022w* and the gene *LSM2/SMX5* complemented the requirement for *LHP1* (Figure 1A) and the slow growth at 16°C. Both genes are members of a newly described family of proteins known as Sm-like proteins. These proteins share two motifs with the Sm proteins which are the core components of the U1, U2, U4 and U5 snRNPs (Cooper *et al*., 1995; Hermann *et al*., 1995; Seraphin, 1995; Fromont-Racine *et al*., 1997). At least two Sm-like proteins, Lsm3p/SmX4p and Lsm4p/Uss1p, are associated with the U6 snRNP (Cooper *et al*., 1995; Seraphin, 1995). As the nine yeast members of this family were recently named *LSM1–9* (like Sm; J.Beggs, personal communication), we refer to *YJR022w* as *LSM8*. An alignment of Lsm8p and Lsm2p with the Sm motifs from several members of the Sm and Sm-like protein families is shown in Figure 1B.

Because only *LSM8* fully complemented the requirement for *LHP1*, we suspected that the mutation resided in *LSM8* and that *LSM2* was a suppressor. This was confirmed by integrating a *URA3* gene adjacent to *LSM8* in an *lhp1::LEU2* strain. By analyzing the segregants of a cross between the *URA3*-marked strain and the mutant strain, we determined that *URA3* segregated with the ability to form sectoring colonies. Sequencing of *LSM8* in the mutant strain revealed a point mutation (T137C) that changes a conserved leucine at position 46 to proline (Figure 1B). We designate the mutant allele *lsm8-1*.

To determine the phenotype of cells lacking *LSM8*, we created a null allele in which the entire coding sequence of *LSM8* was replaced with *HIS3*. The *lsm8::HIS3* allele was used to displace one allele of *LSM8* in a diploid strain. Sporulation of the diploid and dissection of the resulting tetrads yielded two viable spores per tetrad, none of which were auxotrophic for histidine (Figure 1C). Thus, *LSM8* is an essential gene.

Lsm8p is a component of the U6 snRNP and the U4/U6 snRNP

As two Sm-like proteins are components of the U6 snRNP (Cooper *et al*., 1995; Seraphin, 1995), we investigated whether Lsm8p associated with U6 RNA. For these experiments, three copies of the human c-*myc* epitope (Kolodziej and Young, 1991) were fused to the C-terminus of Lsm8p. The epitope-tagged *LSM8(myc)*$_3$ was functional, as it complemented the *lsm8::HIS3* disruption as well as the cold sensitivity and requirement for *LHP1* in *lsm8-1* cells (data not shown). We used a monoclonal antibody against the c-*myc* epitope to perform immunoprecipitations. RNAs within the immunoprecipitates were labeled with [^{32}P]pCp and fractionated in a denaturing gel. Four RNAs that migrated with mobilities characteristic of the yeast U4, U5 and U6 RNAs were present in the immunoprecipitate from *LSM8(myc)*$_3$ cells (Figure 2A, lane 3). These RNAs were absent when the immunoprecipitations were performed on an untagged strain (Figure 2A, lane 4). However, antibodies against the 2,2,7-trimethylguanosine (TMG) cap common to the polymerase II-transcribed snRNAs precipitated the same RNAs in both strains (Figure 2A, lanes 5 and 6).

To confirm the identities of the immunoprecipitated RNAs and to quantitate the amounts present, we performed Northern blots. RNAs present in the immunoprecipitate, the supernatant and an equivalent amount of extract were subjected to Northern hybridization with oligonucleotides complementary to U4, U5 and U6 RNAs. At least half of the total U4 and U6 RNAs were associated with the *myc*-tagged Lsm8p, but only a small fraction of the two forms of U5 RNA was bound (Figure 2B, lanes 2 and 3). Two control RNAs, the U1 and U3 RNAs, were not detected in the immunoprecipitate (Figure 2B, lane 2). We repeated the experiment using increasing amounts of the anti-*myc* antibody and quantitated the amounts of U4, U5 and U6 RNAs present. At the highest concentration of antibody, we immunoprecipitated 70% of the U6 RNA and 50% of the U4 RNA, but <20% of the U5 RNA (data not shown, but see Figure 2C).

U6 snRNA exists in several particles in the cell: as a free U6 snRNP; base-paired with U4 snRNA in the U4/U6 snRNP; and associated with the U5 snRNP to form the U4/U6.U5 tri-snRNP. As our immunoprecipitates contained large amounts of the U4 and U6 snRNPs, it was likely that Lsm8p was a component of the U4/U6 snRNP. In addition, the presence of U5 RNA in the immunoprecipitates suggested that Lsm8p also associated with the U4/U6.U5 tri-snRNP. To determine whether Lsm8p was a component of the free U6 snRNP, we fractionated the immunoprecipitated RNAs in a native gel under conditions that preserve base-pairing between U4 and U6 RNAs. Probing of the Northern blot for U4 and U6 RNAs revealed that Lsm8p is a component of both the U4/U6 snRNP and the free U6 snRNP (Figure 2C, lane 2). Quantitation of the RNAs revealed that 65% of the U4/U6 snRNP and

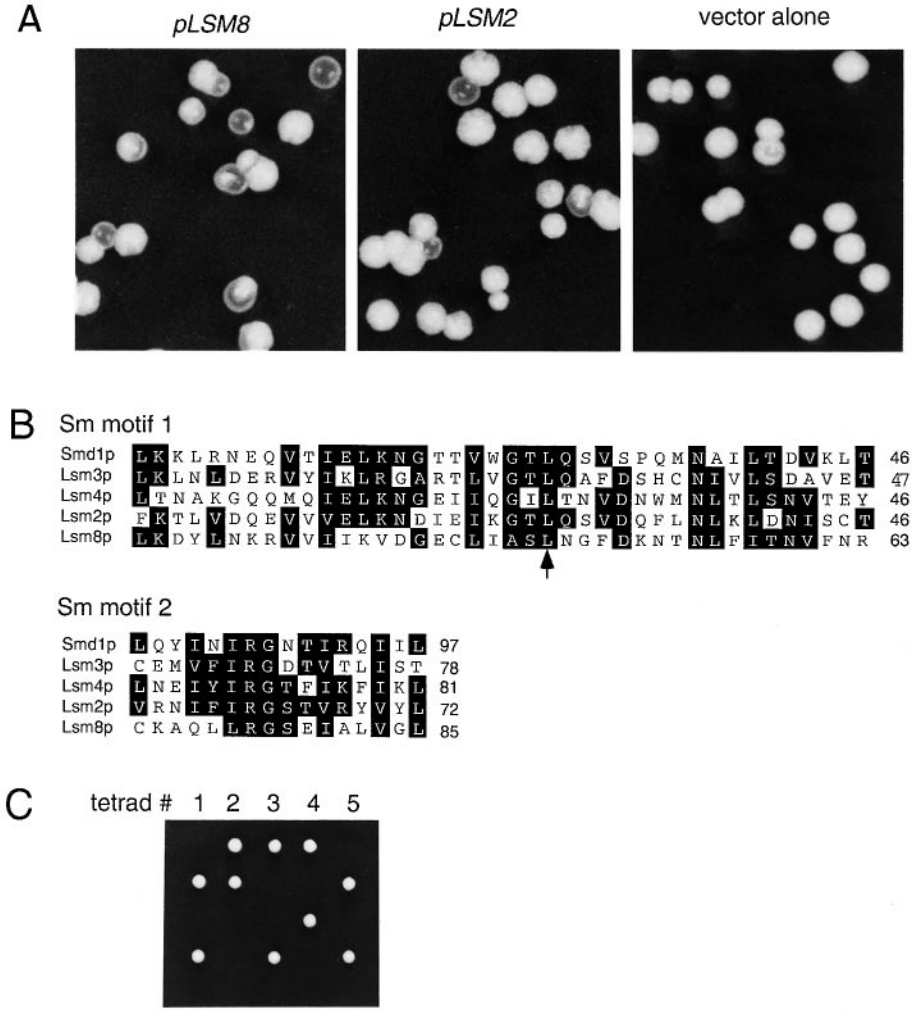

Fig. 1. Two Sm-like proteins rescue the requirement for *LHP1* in cells carrying the *lsm8-1* mutation. (**A**) *LSM8* and *LSM2* were cloned into pRS316 and the plasmids introduced into the *lsm8-1 lhp1::LEU2* strain carrying pATL. Strains were streaked to single colonies on synthetic complete media containing limiting amounts of adenine to enhance red color formation and grown at 30°C. Red colonies and sectors appear gray. (**B**) The Sm motifs 1 and 2 from several *S.cerevisiae* Sm and Sm-like proteins are aligned with those of Lsm8p. Conserved residues are highlighted. The arrow indicates the leucine that is changed to a proline in the *lsm8-1* mutation. Smd1p is an Sm protein, whereas Lsm3p, Lsm4p and Lsm2p are Sm-like proteins. (**C**) One allele of *LSM8* was replaced by *lsm8::HIS3* in a wild-type diploid strain. After sporulation, tetrads were dissected on YPD. All colonies that grew on YPD failed to grow on medium lacking histidine (data not shown).

65% of the free U6 snRNP were immunoprecipitated (Figure 2C, compare lanes 2 and 4). As expected, anti-TMG antibodies immunoprecipitated the U4/U6 snRNP, but not the free U6 snRNP, since U6 RNA lacks this cap (lane 3). These data demonstrate that Lsm8p is a component of the U4/U6 snRNP and the free U6 snRNP.

The levels of U6 RNA in lsm8-1 cells are increased by extra copies of LHP1

To determine whether Lhp1p was required for the biogenesis of U6 RNA in the *lsm8-1* strain, we compared the levels of U4 and U6 RNAs in wild-type cells, cells lacking Lhp1p (*lhp1::LEU2*) and *lsm8-1* cells. Total RNA was isolated from cells grown at 30°C and subjected to Northern analysis. Probing of the blots for U4 and U6 RNA revealed that the levels of these RNAs in *lhp1::LEU2* cells were indistinguishable from wild-type cells (Figure 3A, lanes 1 and 2). However, in *lsm8-1* cells containing chromosomal *LHP1*, the levels of U6 RNA were reproducibly decreased to ~50% of wild-type levels (Figure 3A, lane 3). Interestingly, when RNA was extracted from *lsm8-1* cells carrying the sole copy of *LHP1* on a low-copy plasmid, the level of U6 RNA increased (Figure 3A, lane 4) but was not restored to wild-type levels (Figure 3A, lane 1). Western blotting with antibodies to Lhp1p revealed that the amount of protein produced when *LHP1* was supplied on the plasmid was 2- to 3-fold higher than when the gene was present in the chromosome (data not shown). Thus, although the chromosomal copy of *LHP1* is sufficient to suppress lethality in *lsm8-1* cells, *LHP1* also acts as a multicopy suppressor of the U6 RNA biogenesis defects associated with the *lsm8-1* mutation.

Since the levels of U6 RNA are reduced in the *lsm8-1* strain, we suspected that these cells might be defective in RNA splicing. We therefore reprobed the blot in Figure 3A to detect the U3 small nucleolar RNA, since yeast U3 RNA contains an intron that is removed by the pre-mRNA splicing machinery (Myslinski *et al*., 1990). Pre-U3 RNA was undetectable in both wild-type and *lhp1::LEU2* cells (Figure 3B, lanes 1 and 2). However, cells containing the *lsm8-1* mutation and a chromosomal copy of *LHP1* accumulated unspliced pre-U3 RNA (Figure 3B, lane 3),

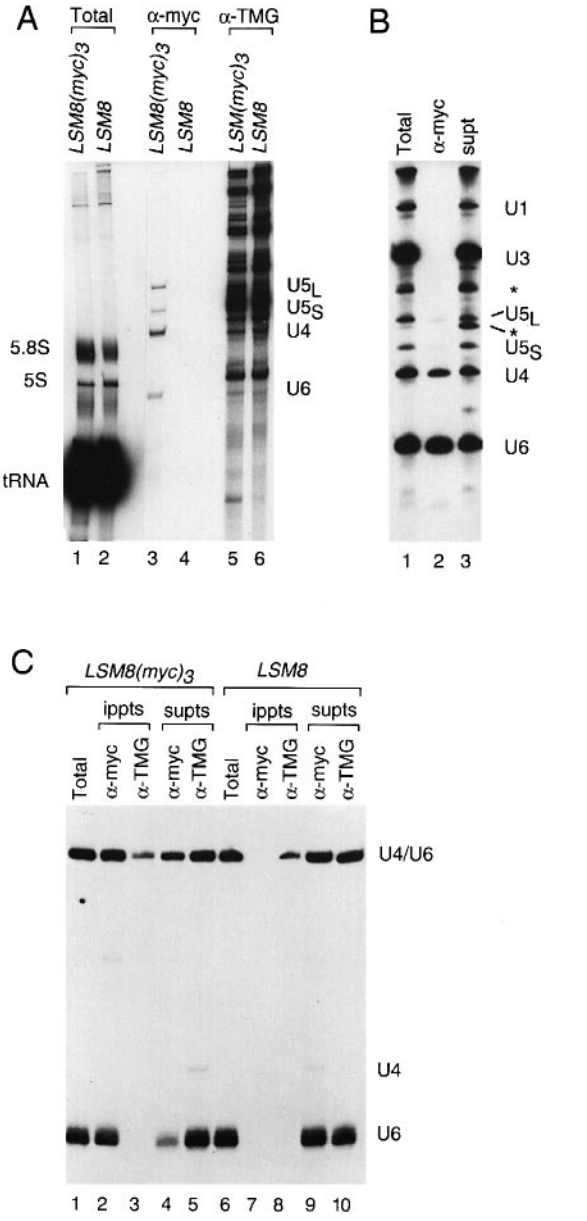

Fig. 2. Lsm8p is a component of both the free U6 snRNP and the U4/U6 snRNP. (**A**) Extracts from *LSM8(myc)*$_3$ (lanes 1, 3 and 5) or untagged (lanes 2, 4 and 6) cells were divided into equal aliquots and phenol extracted (lanes 1 and 2), mixed with anti-*myc* antibodies (lanes 3 and 4), or mixed with anti-TMG cap antibodies (lanes 5 and 6). RNAs in the immunoprecipitates and 3% of the total RNA were labeled with [^{32}P]pCp and fractionated in 5% polyacrylamide/8 M urea gels. (**B**) *LSM8(myc)*$_3$ extracts were subjected to immunoprecipitation with anti-*myc* antibodies as above. RNAs from the immunoprecipitate (lane 2), the supernatant (lane 3) and an equivalent amount of total extract (lane 1) were subjected to Northern analysis using oligonucleotide probes complementary to U1, U3, U4, U5 and U6 RNAs. The asterisks indicate degradation products of U3 and U1 RNAs. (**C**) Extracts prepared from *LSM8(myc)*$_3$ (lanes 1–5) or untagged (lanes 6–10) strains were either phenol extracted (lanes 1 and 6), incubated with anti-*myc* antibodies (lanes 2, 4, 7 and 9) or incubated with anti-TMG antibodies (lanes 3, 5, 8 and 10). Total RNA (lanes 1 and 6), immunoprecipitated RNAs (lanes 2, 3, 7 and 8) and RNAs in the supernatant (lanes 4, 5, 9 and 10) were fractionated in 9% polyacrylamide native gels to resolve free U6 RNA from base-paired U4 and U6 RNAs. RNAs were analyzed by Northern hybridization using oligonucleotides complementary to U4 and U6 RNAs.

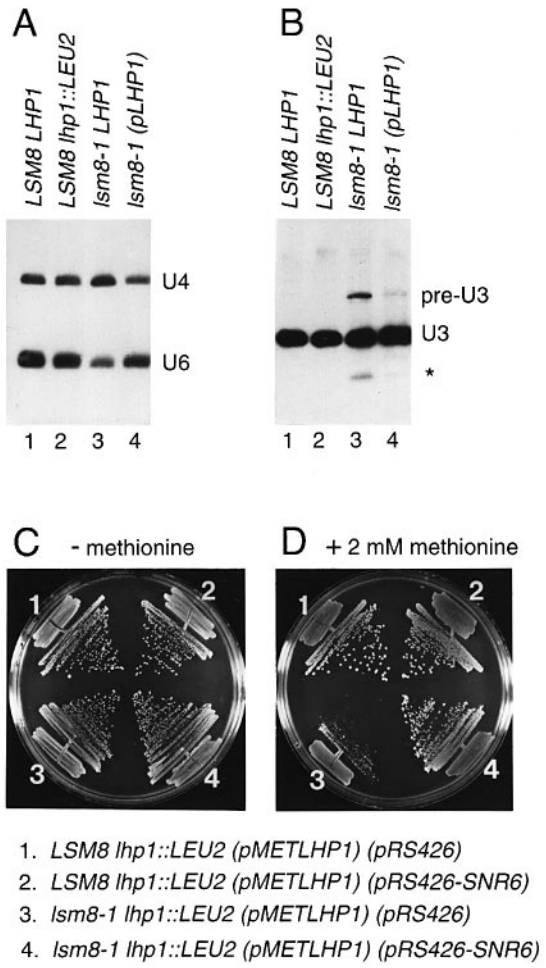

Fig. 3. *lsm8-1* cells have reduced levels of U6 RNA and are defective in splicing, but do not require *LHP1* when multiple U6 RNA genes are present. (**A** and **B**) RNAs isolated from wild-type cells (lane 1), *lhp1::LEU2* cells (lane 2), cells containing the *lsm8-1* mutation and chromosomal *LHP1* (lane 3) and cells carrying the *lsm8-1* mutation and *LHP1* on a low-copy plasmid (pATL) (lane 4) were subjected to Northern analysis and probed for either U4 and U6 RNAs (A) or U3 RNA (B). The asterisk denotes a stable degradation product of pre-U3 RNA (Hughes and Ares, 1991). (**C** and **D**) A plasmid containing *SNR6* in the high-copy vector pRS426 was introduced into *lhp1::LEU2* strains that were either wild-type for *LSM8* (sector 2) or carried the *lsm8-1* mutation (sector 4). Both strains also carried a plasmid with *LHP1* under the control of the *MET3* promoter (pMETLHP1). As controls, these strains were also transformed with pRS426 (sectors 1 and 3). Cells were streaked to single colonies on medium lacking methionine to allow *LHP1* to be expressed (C), or on medium containing 2 mM methionine which represses the *MET3* promoter (D). Relevant genotypes of each strain are listed.

indicating that splicing was inefficient. Consistent with the idea that *LHP1* is also a multicopy suppressor of the *lsm8-1* mutation, the levels of pre-U3 RNA were reduced when *LHP1* was supplied on the low-copy plasmid (lane 4).

We determined whether the requirement for Lhp1p in *lsm8-1* cells could be eliminated by raising the number of U6 RNA genes in the mutant strain. For these experiments, we placed *LHP1* under control of the *MET3* promoter, which is repressed by high concentrations of methionine (Cherest *et al*., 1987). The resulting *lsm8-1* strain grew well on media lacking methionine, but was unable to grow on plates containing 2 mM methionine (data not shown, but see Figure 3C and D, sector 3). Introduction of a high-

copy plasmid containing *SNR6*, which encodes U6 RNA, into the *lsm8-1* cells eliminated the requirement for Lhp1p, as these cells were now able to grow on medium containing 2 mM methionine (Figure 3D, sector 4). As expected, introduction of the high-copy plasmid alone had no effect on the requirement for Lhp1p in these cells (Figure 3D, sector 3).

lsm8-1 cells contain an Lhp1p/U6 RNP but have reduced levels of the mature U6 snRNP

To determine why the levels of U6 RNA were reduced in *lsm8-1* cells, we examined the various U6 RNA-containing particles. For these experiments, whole cell extracts were subjected to electrophoresis in nondenaturing polyacrylamide gels, and the U6 RNA-containing RNPs were visualized by Northern hybridization. In wild-type cells, we observed four U6 RNA-containing complexes (Figure 4A, lane 1). By performing sequential hybridizations with oligonucleotides complementary to U4 (Figure 4D) and U5 snRNAs (data not shown), we established that the three most slowly migrating complexes corresponded to the U4/U6.U5 tri-snRNP, the U4/U6 snRNP and the free U6 snRNP.

Interestingly, we also observed a complex that migrated faster than the free U6 snRNP (Figure 4A, lane 1). As this complex was not detected in *lhp1::LEU2* extracts (lane 2), we suspected that it represented a complex between Lhp1p and U6 RNA. We therefore compared the mobility of the complex with that of an Lhp1p/U6 complex formed *in vitro*. For these experiments, purified Lhp1p was incubated with *in vitro*-transcribed U6 snRNA, and the complexes subjected to native gel electrophoresis and Northern hybridization. The *in vitro*-assembled Lhp1p/U6 RNA complex migrated to the same position as the smallest complex detected in wild-type extracts (Figure 4A, lanes 1, 4 and 5). Both complexes migrated more slowly than naked U6 RNA (Figure 4A, lane 3). We confirmed that Lhp1p was in the complex by performing antibody supershift experiments. The majority of the complex disappeared when anti-Lhp1p antibodies were added to wild-type extracts (Figure 4B, compare lanes 1 and 2), presumably because the shifted complex either comigrates with the mature U6 snRNP or is disrupted by the antibodies.

There were several changes in the levels of the U6 RNA-containing particles in *lsm8-1* mutant cells that carried a chromosomal copy of *LHP1*. All four complexes were reproducibly reduced in abundance compared with wild-type cells (Figure 4C, lanes 1 and 3). However, the levels of the Lhp1p/U6 complex were only moderately affected by the *lsm8-1* mutation, as this complex was present at 50% of wild-type levels. In contrast, the levels of the U4/U6 and U4/U6.U5 complexes were more drastically reduced (~15 and ~10% of wild-type, respectively) and the free U6 snRNP was undetectable. Reprobing of the blot to detect U4 revealed that the *lsm8-1* mutant cells contained significant amounts of the free U4 snRNP, which is not observed in wild-type cells (Figure 4D, lanes 1 and 3). These data suggest that the *lsm8-1* mutation results in a defect in U6 snRNP assembly. Since the Lhp1p/U6 complex is only slightly reduced in *lsm8-1* mutant cells, while the levels of the mature U6 snRNP are drastically reduced, it is likely that the block in assembly occurs after binding of Lhp1p to the newly transcribed RNA.

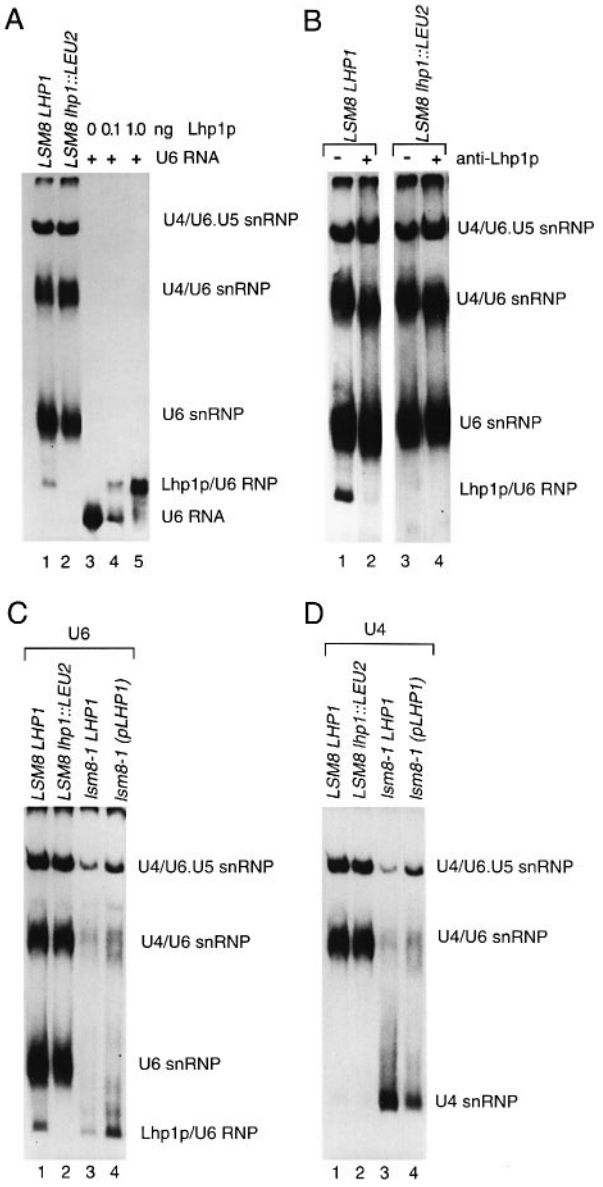

Fig. 4. *lsm8-1* cells contain a Lhp1p/U6 RNP but are defective in U6 snRNP assembly. (**A**) Extracts from wild-type (lane 1) and *lhp1::LEU2* (lane 2) cells were loaded onto a 4% polyacrylamide native gel and subjected to Northern analysis. For comparison, an Lhp1p/U6 RNP was assembled by incubating 27 fmol of *in vitro*-transcribed U6 RNA with the indicated amounts of Lhp1p (lanes 3–5), and the complexes resolved in the native gel. U6 RNA-containing complexes were detected using an oligonucleotide probe. The bands at the top of the gel correspond to the well. (**B**) Extracts prepared from wild-type (lanes 1 and 2) and *lhp1::LEU2* (lanes 3 and 4) cells were incubated with (lanes 2 and 4) or without (lanes 1 and 3) anti-Lhp1p antibodies. U6 RNA-containing complexes were resolved and detected as in (A). (**C** and **D**) Extracts prepared from wild-type cells (lane 1), *lhp1::LEU2* cells (lane 2), *lsm8-1* cells containing the chromosomal *LHP1* (lane 3) and *lsm8-1 lhp1::LEU2* cells carrying *LHP1* on the low-copy plasmid pATL (lane 4) were subjected to Northern analysis using oligonucleotides complementary to U6 RNA (C) and U4 RNA (D). As a control for loading, the blot was reprobed to detect U1 RNA. The levels of the U1 snRNP were approximately the same in all four lanes (not shown).

In *lsm8-1* cells carrying *LHP1* on a low-copy plasmid, the levels of the Lhp1p/U6 complex increased ~3-fold.

Likewise, the levels of the U4/U6 and U4/U6.U5 snRNPs increased ~1.5-fold (Figure 4C, lane 4), consistent with the observation that the U6 RNA biogenesis and splicing defects are less severe when *LHP1* is present in multiple copies. In addition, the band corresponding to the U4/U6 complex became more diffuse and we detected several faint bands migrating above the Lhp1p/U6 RNP and the U4/U6 snRNPs (Figure 4C, lane 4). Although we have not determined the identity of these bands, they could represent partially assembled complexes and/or mature particles bound by the overexpressed Lhp1p. Reprobing of the blot for U4 revealed that in the presence of extra copies of *LHP1*, the levels of the free U4 snRNP were reduced relative to *lsm8-1* cells containing a single copy of *LHP1* (Figure 4D, lane 4). Thus, in *lsm8-1* cells, overexpression of *LHP1* results in increased levels of the mature U6 snRNP.

lsm8-1 cells require LHP1 for the stable accumulation of the U6 snRNP

To determine why *lsm8-1* cells require *LHP1* for growth, we used our strain containing *LHP1* under control of the *MET3* promoter to deplete Lhp1p from the mutant cells. When this strain was grown in medium lacking methionine, the levels of Lhp1p were 2-fold higher than in *lsm8-1* cells carrying *LHP1* chromosomally (Figure 5B, lanes 2 and 3). Upon addition of 2 mM methionine, the levels of Lhp1p declined, becoming undetectable within 12 h (Figure 5B, lanes 3–6). At the same time, growth of the *lsm8-1* mutant cells slowed slightly (Figure 5A). The fact that the *lsm8-1* cells continued to divide in the absence of detectable Lhp1p yet were dependent on Lhp1p for growth on agar plates (see Figure 3) might be due to different requirements for Lhp1p under these growth conditions. Alternatively, although we cannot detect Lhp1p by Western blotting, sufficient Lhp1p may be synthesized under repressive conditions to allow viability.

We examined the fate of the U6 snRNP and its associated particles during Lhp1p depletion from *lsm8-1* cells. First, RNA was extracted from the cells at intervals, fractionated in denaturing gels and analyzed by Northern hybridization. This analysis revealed that the levels of U6 snRNA were reduced ~7-fold during Lhp1p depletion (Figure 5C, lanes 3–8), whereas the levels of U4 snRNA were unaffected. Probing of the blot with an oligonucleotide complementary to U3 RNA revealed that the levels of unspliced U3 RNA increased nearly 10-fold during Lhp1p depletion (Figure 5C, lanes 3–8), confirming that the splicing defect in the *lsm8-1* mutant cells is exacerbated by the loss of Lhp1p.

We determined which of the U6 RNA-containing particles was most affected by Lhp1p depletion. Fractionation of the RNA in a native gel to preserve base-pairing between U4 and U6 RNAs revealed that the level of the U4/U6 complex decreased slightly as Lhp1p was depleted, while the levels of free U4 RNA increased (Figure 6A, lanes 3–8). However, the free U6 RNA was most dramatically affected by Lhp1p depletion, declining >4-fold. To determine whether the decrease in free U6 snRNA was due to loss of the Lhp1p/U6 RNP or the U6 snRNP, cell extracts were fractionated on native gels. Northern analysis revealed that the Lhp1p/U6 RNP became undetectable during the experiment (Figure 6B, lanes 3–4). This observation, coupled with the observed decrease

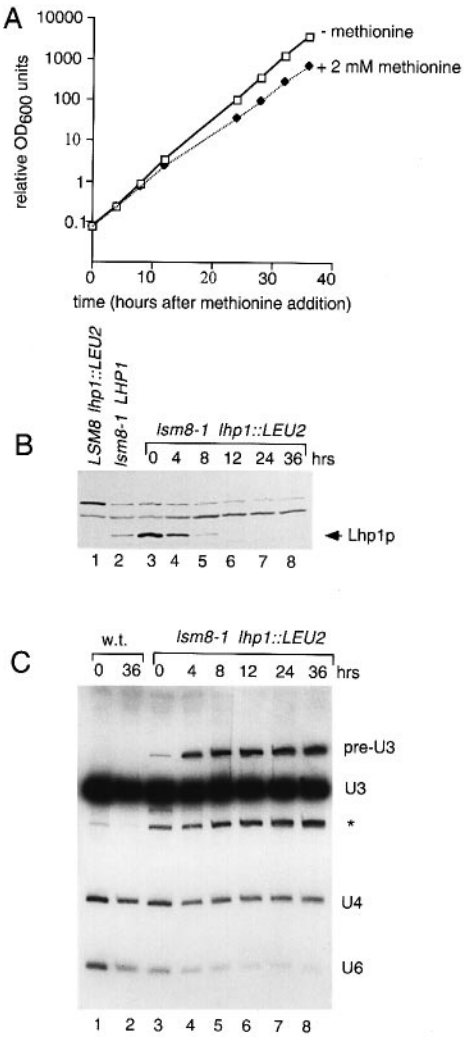

Fig. 5. Depletion of Lhp1p from cells carrying the *lsm8-1* mutation. (**A**) The growth of *lsm8-1 lhp1::LEU2* cells carrying pMETLHP1 is compared in media lacking methionine versus media containing 2 mM methionine. At time 0, cells were either shifted to 2 mM methionine or left to continue growing in media lacking methionine. (**B**) Aliquots of *lsm8-1 lhp1::LEU2* cells carrying pMETLHP1 were removed at intervals after the switch to 2 mM methionine media (lanes 3–8) and subjected to Western blot analysis to detect Lhp1p. The levels of Lhp1p in *lhp1::LEU2* and wild-type cells are shown (lanes 1 and 2). (**C**) RNA was extracted from wild-type cells (lanes 1 and 2) and *lsm8-1 lhp1::LEU2* cells (lanes 3–8) at intervals after the switch to 2 mM methionine media and subjected to Northern analysis using oligonucleotides complementary to U3, U4 and U6 RNAs. The asterisk denotes a stable degradation product of pre-U3 RNA (Hughes and Ares, 1991). The decreases in U3, U4, and U6 RNAs in lane 2 are due to underloading.

in the U4/U6 complex and the increase in free U4 RNA, allows us to conclude that Lhp1p is required for the stable accumulation of the U6 snRNP in *lsm8-1* cells.

Lhp1p stabilizes newly synthesized U6 RNA from degradation

The decrease in U6 snRNP accumulation as Lhp1p was depleted from *lsm8-1* cells could be due to a requirement for Lhp1p to stabilize U6 RNA prior to assembly into the U6 snRNP. Alternatively, Lhp1p could be required for efficient U6 RNA transcription. To distinguish between these possibilities, we examined the synthesis of U6 RNA during Lhp1p depletion. *lsm8-1* cells containing *LHP1*

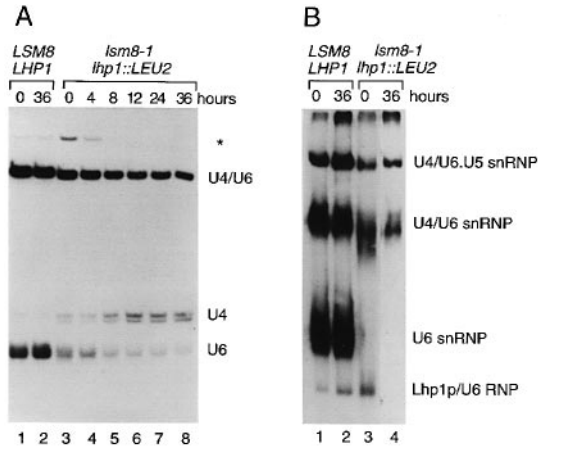

Fig. 6. Lhp1p is required for stable accumulation of the U6 snRNP in *lsm8-1* cells. (**A**) RNA was extracted from wild-type cells (lanes 1, 2) and *lsm8-1 lhp1::LEU2* cells carrying pMETLHP1 (lanes 3–8) at intervals after the switch to media containing 2 mM methionine and resolved on 9% polyacrylamide native gels. The resulting Northern blot was probed with oligonucleotides complementary to U4 and U6 RNAs. The band denoted by the asterisk is a complex between a 3′ extended form of U4 RNA and U6 RNA. This complex is stabilized when Lhp1p is overexpressed (unpublished data). (**B**) Wild-type cells (lanes 1 and 2) and *lsm8-1 lhp1::LEU2* cells carrying pMETLHP1 (lanes 3 and 4) were harvested after 0 or 36 h of growth in 2 mM methionine media. Extracts were fractionated on a 4% polyacrylamide gel and the U6 RNA-containing complexes detected by Northern hybridization using an oligonucleotide complementary to U6 RNA.

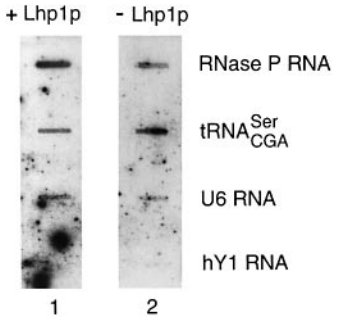

Fig. 7. U6 RNA continues to be synthesized in the absence of Lhp1p. *lsm8-1* cells containing the chromosomal *LHP1* (lane 1) and *lsm8-1 lhp1::LEU2* cells carrying pMETLHP1 (lane 2) were grown in 2 mM methionine media for 13 h to deplete Lhp1p. After a 10 min pulse with $^{32}PO_4$, RNA was extracted from the strains and hybridized to DNA slot blots. The immobilized DNAs consisted of the coding sequences for the *S.cerevisiae* RNase P RNA, tRNA$^{Ser}_{CGA}$ and U6 RNA. As a negative control, the coding sequence of the human Y1 RNA (Wolin and Steitz, 1983) was included. Although there are slight differences in the amounts of RNase P RNA, tRNA$^{Ser}_{CGA}$ and U6 RNA that hybridized to the immobilized DNAs (compare lanes 1 and 2), these differences were not reproducible when identical RNA samples were used in a second hybridization experiment.

under control of the *MET3* promoter were grown for 13 h in low phosphate medium containing 2 mM methionine to deplete Lhp1p. For comparison, *lsm8-1* cells containing chromosomal *LHP1* were grown in the same medium. After a 10 min pulse with $^{32}PO_4$, RNA was extracted from both strains and hybridized to U6 DNA-containing slot blots. Similar amounts of newly synthesized U6 RNA transcripts were detected in the presence and absence of Lhp1p (Figure 7, lanes 1 and 2). Thus, although the steady-state levels of U6 RNA decline during Lhp1p depletion, U6 RNA continues to be transcribed. Thus, the most likely explanation for the decline in U6 RNA is that Lhp1p stabilizes newly synthesized RNA from degradation.

We next examined whether Lhp1p was required for U6 RNA transcription *in vitro*. First, we compared the transcription of *SNR6* in whole-cell extracts prepared from wild-type cells with *lhp1::LEU2* extracts. Although the U6 gene is poorly transcribed in extracts compared with 5S and tRNA genes (Brow and Guthrie, 1990; Eschenlauer *et al*., 1993), incubation of *SNR6* in the wild-type extract yielded the two major species that have been described previously (Margottin *et al*., 1991; Eschenlauer *et al*., 1993) (Figure 8A, lane 3, indicated by dots). The larger band is the full-length U6 RNA, while the 90 nt species has been described to be a 3′ truncated form of U6 RNA generated by exonucleolytic digestion of the full-length RNA (Margottin *et al*., 1991; Eschenlauer *et al*., 1993). Interestingly, when *SNR6* was transcribed in the *lhp1::LEU2* extract, the major product was the 90 nt RNA (Figure 8A, lane 5). Addition of purified Lhp1p to the *lhp1::LEU2* extract largely restored the ratio of the two species to that of the wild-type extract (lane 6).

It was demonstrated previously that incubation of gel-purified full-length U6 RNA with cell extracts results in the generation of the 90 nt species (Eschenlauer *et al*., 1993). To confirm that the U6 transcripts synthesized in our *lhp1::LEU2* extracts were initially full length, we examined the transcription products as a function of time. After 10 min, the *SNR6* products made in both extracts consisted largely of the full-length species (Figure 8B, compare lanes 4 and 11). However by 60 min, nearly all the transcripts in the *lhp1::LEU2* extracts corresponded to the 90 nt RNA (lane 14). Because sequences downstream of the U6 RNA coding region (but within the B box of the *SNR6* promoter) give rise to transcripts that are larger than U6 RNA (Eschenlauer *et al*., 1993), it is possible that some of the 90 nt species is generated from the larger transcripts. Nonetheless, as addition of Lhp1p both decreased the amount of the 90 nt RNA and increased the amount of the full-length U6 RNA, we conclude that Lhp1p stabilizes newly synthesized U6 RNA from nucleolytic digestion.

We also examined the role of Lhp1p in extracts prepared from the *lsm8-1* mutant cells. To deplete Lhp1p, *lsm8-1* cells containing *LHP1* under control of the *MET3* promoter were grown for 14 h in medium containing 2 mM methionine. As a control, *lsm8-1* cells containing chromosomal *LHP1* were grown in the same medium. Transcription of *SNR6* in extracts prepared from these strains is shown in Figure 8C. As observed for wild-type extracts, transcription of *SNR6* in *lsm8-1* cells containing Lhp1p yielded primarily full-length U6 RNA (Figure 8C, lane 3). Although extracts from Lhp1p-depleted cells yielded only the 90 nt product (Figure 8C, lane 5), addition of purified Lhp1p restored the ratio of transcripts to that of the *LHP1* extracts (lane 6). In the presence of increased amounts of Lhp1p (equivalent to 2- and 3-fold the amount in wild-type extracts) the levels of the full-length U6 RNA were further increased while the 90 nt product decreased (Figure 8C, lanes 7 and 8). These results, together with the observation that U6 RNA continues to be transcribed in *lsm8-1* cells that have been depleted of Lhp1p, allow

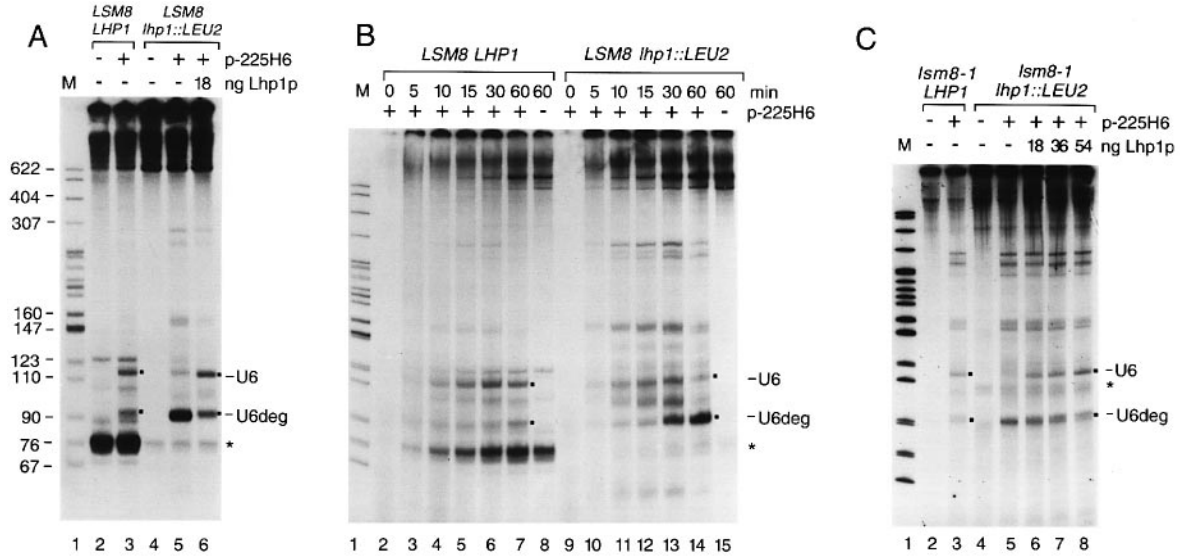

Fig. 8. Lhp1p protects newly synthesized U6 RNA from degradation *in vitro*. (**A**) Either no plasmid (lanes 2 and 4) or plasmid p-225H6 (lanes 3, 5 and 6) encoding U6 RNA was incubated with wild-type (lanes 2 and 3) or *lhp1::LEU2* extracts (lanes 4–6) for 1 h in the presence of [α-^{32}P]rUTP. In lane 6, 18 ng of Lhp1p (an amount equivalent to that present in the wild-type extract) was included in the reaction. Lane 1, molecular size markers. The asterisk denotes a product of transcription in the absence of plasmid. (**B**) p-225H6 was transcribed in wild-type (lanes 2–8) and *lhp1::LEU2* (lanes 9–15) extracts in the presence of [α-^{32}P]rUTP. Aliquots were removed at the times indicated. In lanes 8 and 15, no plasmid was included in the reaction. The full-length U6 RNA and the 90 nt product are indicated by dots. Lane 1, molecular size markers. The asterisk denotes a product of transcription in the absence of plasmid. The transcripts migrating above U6 RNA probably correspond to the downstream transcripts described by Eschenlauer *et al*. (1993). (**C**) Either no plasmid (lanes 2 and 4) or plasmid p-225H6 (lanes 3, 5, 6, 7 and 8) was incubated with *lsm8-1 LHP1* (lanes 2 and 3) or *lsm8-1 lhp1::LEU2* extracts (lanes 4–8) for 1 h in the presence of [α-^{32}P]rUTP. In the indicated lanes, either 18 (lane 6), 36 (lane 7) or 54 ng (lane 8) of Lhp1p was included in the reaction. Lane 1, molecular size markers. The asterisk denotes a product of transcription in the absence of plasmid.

us to conclude that Lhp1p stabilizes newly synthesized U6 RNA from degradation.

Discussion

We have identified a mutation in a novel U6 snRNP protein, Lsm8p, that causes yeast cells to require the La protein Lhp1p for growth. While both wild-type and mutant cells contain Lhp1p/U6 RNA complexes similar to the La/U6 RNA complexes that have been detected in vertebrates, the mutant cells are defective in U6 snRNP assembly. In these cells, Lhp1p stabilizes newly synthesized U6 RNA, thus facilitating assembly of the RNA into the U6 snRNP. This finding, together with the result that Lhp1p is required for the normal pathway of tRNA maturation (Yoo and Wolin, 1997), suggests that the La protein functions as a molecular chaperone to ensure that nascent RNA polymerase III transcripts are correctly folded, processed and assembled into functional RNA–protein complexes.

An Lhp1p/U6 RNA complex in S.cerevisiae

Approximately 10% of the U6 RNA is bound by the La protein in both mammalian cells and *Xenopus laevis* oocytes (Rinke and Steitz, 1985; Terns *et al*., 1992). As the fraction of U6 RNA bound by La terminates with UUU_{OH}, while the majority of U6 RNAs in vertebrate cells contain 2′,3′ cyclic phosphates at their 3′ ends, the La-bound U6 RNA is considered to represent newly synthesized RNA (Lund and Dahlberg, 1992; Terns *et al*., 1992). Furthermore, the U6 RNA bound by the human La protein contains fewer modified nucleotides than either total U6 RNA or U6 RNA isolated from the U4/U6 complex (Rinke and Steitz, 1985). Similar to the situation in vertebrates, we can immunoprecipitate ~10% of the U6 RNA in *S.cerevisiae* with our anti-Lhp1p antibodies (unpublished data). This is consistent with the finding that most of the U6 RNA in *S.cerevisiae* contains a 3′-monophosphate at the 3′ end (Lund and Dahlberg, 1992), which is not efficiently bound by vertebrate or yeast La proteins (Stefano, 1984; Terns *et al*., 1992; Yoo and Wolin, 1994).

Although other groups have fractionated yeast snRNPs on native gels (Cheng and Abelson, 1987; Raghunathan and Guthrie, 1998), the complex between U6 RNA and Lhp1p has not been detected previously. In these earlier experiments, whole-cell splicing extracts were prepared from cells in the late logarithmic phase of growth and analyzed on native gels. Since RNA polymerase III transcription is downregulated as yeast cells approach stationary phase (Sethy *et al*., 1995), it is possible that these extracts were lacking in nascent U6 RNA transcripts bound by Lhp1p.

Although we have shown that one function of the La/U6 RNP is to stabilize nascent U6 RNA, binding by Lhp1p may directly facilitate U6 snRNP assembly. For example, Lhp1p binding may stabilize U6 RNA in a conformation that favors incorporation into the U6 snRNP or may retain unassembled U6 RNA in the nucleus [as has been proposed for the vertebrate protein (Boelens *et al*., 1995)]. In addition, Lhp1p may interact with U6 snRNP proteins, such as Lsm8p, during snRNP assembly. If Lsm8p functions redundantly with Lhp1p to stabilize U6 RNA structure and/or recruit U6-specific proteins to the nascent RNA (see below), then cells containing wild-

type Lsm8p would be able to efficiently assemble the U6 snRNP in the absence of Lhp1p.

Despite the fact that wild-type yeast cells do not require *LHP1* for growth, there may be situations in which Lhp1p becomes required for U6 snRNP assembly. Our finding that Lhp1p stabilizes unassembled U6 snRNA when snRNP assembly is inefficient implies that growth conditions that slow assembly of RNA–protein complexes could cause a requirement for Lhp1p. Possible scenarios include conditions which slow the rate of forming the correctly folded U6 RNA structure, such as growth in media containing low amounts of divalent cations such as magnesium, or conditions that result in a decreased rate of protein synthesis, such as amino acid starvation (reviewed by Hinnebusch, 1994).

A molecular chaperone for polymerase III-transcribed small RNAs

The function of the vertebrate La protein is controversial. Although the human protein is proposed to facilitate transcription termination and reinitiation by RNA polymerase III (Gottlieb and Steitz, 1989; Maraia, 1996), *X.laevis* cell extracts that have been immunodepleted of the La protein are active in RNA polymerase III transcription (Lin-Marq and Clarkson, 1998). In addition, *S.cerevisiae* extracts that have been genetically depleted of Lhp1p are indistinguishable from wild-type extracts in their ability to transcribe genes encoding tRNAs (Yoo and Wolin, 1997) and U6 RNA (Figure 8). Other proposed functions for the La protein include retaining RNA polymerase III transcripts in the nucleus (Boelens *et al*., 1995; Simons *et al*., 1996), facilitating nuclear import of RNA (Grimm *et al*., 1997), and facilitating translation initiation for both viral and cellular mRNAs (Meerovitch *et al*., 1993; McBratney and Sarnow, 1996).

Using genetics in *S.cerevisiae*, we have uncovered two roles played by the La protein *in vivo*. Binding by Lhp1p is required for the endonucleolytic cleavage that removes the 3′ trailer from many pre-tRNAs. In the absence of Lhp1p, the 3′ trailer is removed by exonucleases (Yoo and Wolin, 1997). Evidence that this is a conserved function of the La protein was provided by studies in *Schizosaccharomyces pombe*, where cells depleted of the La protein homologue Sla1 show similar alterations in the mechanism of tRNA maturation (Van Horn *et al*., 1997). As expression of the human protein in *S.pombe* restored the normal pathway of tRNA processing, all structural features required for the La protein to function in pre-tRNA processing are conserved in humans. Furthermore, binding by the human and *Xenopus* La proteins stabilizes pre-tRNAs from exonucleolytic degradation (Fan *et al*., 1998; Lin-Marq and Clarkson, 1998).

Our finding that Lhp1p stabilizes newly synthesized U6 RNA to facilitate its assembly into a functional snRNP suggests that the La protein functions as a molecular chaperone for nascent RNA polymerase III transcripts. Hendrick and Hartl (1993) defined a molecular chaperone as 'a protein that binds to and stabilizes an otherwise unstable conformer of another protein, and by controlled binding and release of the substrate protein, facilitates its correct fate *in vivo*.' As binding by Lhp1p stabilizes unassembled U6 RNA from degradation and is required for the normal pathway of tRNA maturation, Lhp1p meets the definition of a molecular chaperone for these RNAs. Whether binding by Lhp1p also aids in the formation of the correctly folded RNA structure, as has been observed for many protein chaperones (Hendrick and Hartl, 1993), is under investigation.

We do not yet know the role played by Lhp1p in the biogenesis of other polymerase III transcripts, such as the SRP RNA or RNase P RNA. By analogy with the demonstrated roles of Lhp1p in tRNA maturation and U6 snRNP assembly, binding by Lhp1p may facilitate the normal pathway by which these RNAs are folded, processed or assembled into RNA–protein complexes. Analysis of additional mutations that cause cells to require Lhp1p for growth is likely to reveal other roles of this protein in RNA biogenesis.

Assembly of the U6 snRNP

For the polymerase II-transcribed U1, U2, U4 and U5 snRNAs, binding by the Sm proteins is required for hypermethylation of the snRNA cap, import of newly assembled snRNPs into the nucleus and binding of snRNP-specific proteins (Mattaj, 1988; Nelissen *et al*., 1994). At least three Sm-like proteins [Lsm3p/SmX4p (Seraphin, 1995), Lsm4p/Uss1p (Cooper *et al.*, 1995) and Lsm8p] are components of the U6 snRNP, suggesting that an analogous complex may function in U6 snRNP biogenesis. As a fourth Sm-like protein, Lsm2p, when present on a low-copy vector, suppressed both the requirement for Lhp1p and the cold sensitivity associated with the *lsm8-1* mutation, this protein may also associate with the U6 snRNP. Interestingly, seven of the nine yeast Sm-like proteins, including Lsm3p/SmX4p, Lsm4p/Uss1p and Lsm2p/SmX5p, can be aligned with one of the seven distinct Sm protein subfamilies (Fromont-Racine *et al*., 1997). Although Lsm8p cannot be aligned with any particular Sm subfamily, it interacts with both Lsm4p/Uss1p and Lsm2p/SmX5p in two-hybrid screens (Fromont-Racine *et al*., 1997), consistent with our finding that the protein is a component of the U6 snRNP.

The fact that *LSM8* exhibits genetic interactions with Lhp1p suggests that this protein is important for an early step of U6 snRNP assembly. In four genetic screens, in which we screened nearly 500 000 yeast colonies for synthetic lethality with *LHP1*, we identified two alleles of *LSM8* but did not obtain mutations in other U6 snRNP proteins (B.K.Pannone, D.Rubinson, C.Yoo, S.Kim and S.L.Wolin, unpublished data). Thus, Lsm8p may be the first U6-specific protein that binds to U6 RNA. Similar to Lhp1p, binding of Lsm8p to U6 RNA may stabilize the newly synthesized RNA from degradation, thus explaining the synthetic lethality of the two proteins. As Lsm8p interacts with Lsm4p/Uss1p and Lsm2p/SmX5p in two-hybrid screens (Fromont-Racine *et al*., 1997), the bound Lsm8p might recruit these, and perhaps other Sm-like proteins, to the U6 RNA. As cells that contain wild-type *LSM8* do not require Lhp1p, the assembly process must be sufficiently rapid to prevent unassembled, naked RNA from accumulating in *lhp1::LEU2* cells.

Interestingly, the U6 snRNP biogenesis defects due to the *lsm8-1* mutation are less severe when *LHP1* is present on a low-copy plasmid. If binding by Lhp1p to newly synthesized U6 RNA is transient, overexpression of Lhp1p in *lsm8-1* cells could increase the level of U6 RNA that

is protected from degradation and available for assembly into the U6 snRNP. Consistent with this possibility, overexpression of U6 RNA in *lsm8-1* cells eliminated the requirement for Lhp1p in these cells (Figure 3). Alternatively, binding of Lhp1p to U6 RNA may slow the rate of assembly of U6 RNA with Lsm8p and other Sm-like proteins. In this scenario, overexpression of Lhp1p would increase the time-window for the productive interaction of the mutant Lsm8p with U6 RNA and these other proteins.

While association of the core Sm proteins with the U1, U2, U4 and U5 snRNAs is critical for several steps in the biogenesis of these snRNPs, little is known about the functions of the individual proteins. For example, it is unclear whether specific Sm proteins are required for recognition of the snRNPs by the cap methylation or nuclear import machinery. Although biochemical studies using purified proteins have begun to unravel the pathway by which these proteins assemble with each other and with RNA to form mature snRNPs (Raker *et al*., 1996), genetic screens in *S.cerevisiae* have thus far failed to isolate mutations in any of the core Sm proteins. The mutation that we have described in *LSM8* is the first mutation to be identified in an essential member of the Sm motif family of proteins. As nothing is known about the role of the Sm-like proteins in U6 snRNP biogenesis, our *lsm8-1* strain will be a valuable tool for elucidating the contributions of these proteins to U6 RNA maturation, snRNP assembly and function. Furthermore, information about the role of Sm-like proteins in U6 snRNP biogenesis should be helpful in deciphering the functions of the canonical Sm proteins in the assembly of the U1, U2, U4 and U5 snRNPs.

Materials and methods

Yeast media and strains

Yeast media and manipulations were as described in Sherman (1991). Wild type and *lhp1::LEU2* strains were CY1(*MAT*α *ura3 lys2 ade2 trp1 his3 leu2 LHP1*), CY3 (*MAT***a** *ura3 lys2 ade2 trp1 his3 leu2 LHP1*), CY2 (*MAT*α *ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2*) and CY4 (*MAT***a** *ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2*) (Yoo and Wolin, 1994). Mutant strains described below are BP1 (*MAT***a** *lsm8-1 lhp1::LEU2 ura3 lys2 ade2 trp1 his3 leu2* carrying pATL), BP2 (*MAT*α *lsm8-1 lhp1::LEU2 ura3 lys2 ade2 trp1 his3 leu2* carrying pATL), BP4 (*MAT***a** *lsm8-1 LHP1 ura3 lys2 ade2 trp1 his3 leu2*), BP9 (*MAT***a**/α *lsm8::HIS3/LSM8 ura3/ura3 lys2/lys2 ade2/ade2 trp1/trp1 his3/his3 leu2/leu2*), BP10 (*MAT*α *lsm8::HIS3 ura3 lys2 ade2 trp1 his3 leu2 carrying p22myc*), BP11 (*MAT*α *lsm8::HIS3 ura3 lys2 ade2 trp1 his3 leu2 carrying pAD12-22w*) and BP5 (*MAT*α *lsm8-1 lhp1::LEU2 ura3 lys2 ade2 trp1 his3 leu2* carrying pMETLHP1).

Synthetic lethal screen

For the synthetic lethal screen, the plasmid containing *LHP1* in pBluescript KSII (Yoo and Wolin, 1994) was digested with *Sac*I and *Cla*I, and the 1.8 kb fragment cloned into the *Sac*I–*Cla*I sites of pAD12 (a gift of R.Padmanabha and M.Snyder, Yale University, CT) to create pATL. pAD12 is pRS314 (Sikorski and Hieter, 1989) containing a 3.6 kb fragment of the *ADE2* gene inserted at the *Xho*I site. The synthetic lethal screen was performed as described by Costigan *et al*. (1992). Briefly, CY2 cells carrying pATL were grown in synthetic complete media (SC) lacking tryptophan to mid-log phase. After mutagenesis with ultraviolet light to 40% survival, cells were plated on SC containing limiting amounts of adenine (SC iade) (16 μg/ml) to enhance red color formation. Of 63 000 colonies screened, 48 did not form red sectors. The 48 candidates were transformed with a second plasmid, pSLL28 (Yoo and Wolin, 1997), which contains *LHP1*, *URA3* and *LYS2*, and tested on SC iade lacking uracil for the ability to lose pATL. This step eliminated mutants that required the *ADE2* or *TRP1* genes. Of the 14 that sectored, 11 were dead on media containing 1 μg/ml 5-fluoroorotic acid (5-FOA), indicating that they could not lose pSLL28. These were transformed with pAD12 and tested on 5-FOA to eliminate mutants that required any plasmid for growth. Only three mutants were unable to grow on 5-FOA. Backcrossing these mutants to CY2 revealed that each contained a single nuclear mutation that caused the cells to require pATL. To confirm that the mutation was lethal in combination with *lhp1::LEU2*, each strain was mated to CY1. Following loss of pATL from the diploids, segregants were analyzed. Only one mutation, *lsm8-1*, was lethal in combination with *lhp1::LEU2*. A single copy of *LHP1* suppressed the lethality. In addition to the requirement for *LHP1*, the mutant strain grew slowly at 16°C. This strain was backcrossed three times to CY4, resulting in strains BP1 (*MAT***a**) and BP2 (*MAT*α).

Cloning and disruption of the LSM8 gene

A yeast genomic library in YCp50 (Rose *et al*., 1987) was transformed into BP1, and the transformants screened on SC iade lacking uracil for the ability to lose pATL. Two different genomic clones were identified, and fragments from each were subcloned into pRS316 (Sikorski and Hieter, 1989). A 1.5 kb *Xba*I–*Cla*I insert containing *LSM8* from chromosome X (p22U) and a 1.1 kb *Dra*I insert containing *LSM2/SMX5* from chromosome II (pSNPU) rescued the requirement for *LHP1* and restored growth at 16°C. To determine that *LSM8* was the mutated gene, a *URA3* marker was integrated near the *LSM8* locus in strain CY4. After verifying integration by PCR, the strain was crossed to BP2, and segregants were analyzed. Analysis of 16 tetrads revealed that the requirement for *LHP1* segregated with the uracil auxotrophic phenotype. To identify the mutation, genomic DNA from BP1 was used as the template for PCR amplification.

To disrupt *LSM8*, the *HIS3* gene from pAD11 (R.Padmanabha and M.Snyder, Yale University) was amplified using primers that contained 60 nt of *LSM8* sequences at their 5′ ends. This 1.4 kb PCR product was transformed into a wild-type diploid strain, where it replaced one allele of the *LSM8* ORF with *HIS3*, leaving only 45 nt of *LSM8* coding sequence at the 5′-terminus and 32 nt at the 3′-terminus. After integration at the *LSM8* locus was verified by PCR, the diploid was sporulated and the tetrads analyzed. Because the ORF *YJR023c* overlaps with the last 311 nt of *LSM8*, it was possible that disruption of *YJR023c* caused the observed lethality. We therefore verified that the *LSM8(myc)*$_3$ allele (see below), which lacks an intact *YJR023c* ORF, rescued the lethality.

Construction of epitope-tagged Lsm8p and immunoprecipitations

PCR was used to fuse three copies of the human c-*myc* epitope (Kolodziej and Young, 1991) to the C-terminus of Lsm8p. The resulting plasmid, p22myc, consisted of 387 nt of 5′ flanking sequence, the entire *LSM8* coding sequence, three copies of the c-*myc* epitope and a stop codon cloned into the *Sma*I–*Cla*I sites of pAD12. p22myc was transformed into BP9 and the diploid sporulated to create strain BP10. To construct the control strain BP11, p22U was digested with *Sac*I and *Cla*I, and the resulting *LSM8*-containing fragment was cloned into the *Sac*I–*Cla*I sites of pAD12 to create pAD12-22w. This plasmid was transformed into BP9 and the diploid sporulated as above.

To immunoprecipitate Lsm8p(myc)$_3$, strains BP10 and BP11 were grown in YPD at 30°C to OD_{600} between 0.4 and 0.75, washed once with H_2O, and resuspended in 400 μl NET-2 (40 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40) and 0.25 mM phenylmethylsulfonyl fluoride (PMSF). Cells were disrupted by vortexing in the presence of glass beads, followed by sedimentation at 2000 r.p.m. in a microcentrifuge. The supernatants were sedimented at 100 000 *g* for 30 min in a Beckman TLA100.2 rotor, and subjected to immunoprecipitation (Yoo and Wolin, 1994) using anti-*myc* antibodies (Kolodziej and Young, 1991). RNAs in the immunoprecipitates were fractionated in 5% polyacrylamide–8 M urea gels. For the native gel analysis, RNA was mixed with loading buffer to obtain a final concentration of 6% glycerol, 0.025% bromophenol blue (BPB) and 0.025% xylene cyanole FF (XC), and fractionated in a 9% polyacrylamide (37.5:1 acrylamide:bis) native gel at 4°C at 250–300 V until XC reached the bottom of the gel.

Northern blots and oligonucleotides

For analysis on denaturing gels, total RNA was extracted by the hot phenol method (Ausubel *et al*., 1998). Five micrograms total RNA was fractionated in 5% polyacrylamide/8 M urea gels and transferred to ZetaProbe GT membranes (Bio-Rad) in 0.5× TBE at 150 mA for 16 h. Hybridization with [γ-^{32}P]ATP-labeled oligonucleotides was done as described previously (Tarn *et al*., 1995). For native gel analysis, RNA was extracted by vortexing in the presence of phenol and glass beads (Ausubel *et al*., 1998). Five micrograms RNA was fractionated in

9% polyacrylamide gels, and the blots hybridized with radiolabeled oligonucleotides. Oligonucleotides used were: U6, 5′-AAAACGAAAT-AAATCTCTTTG-3′; U4: 5′-AGGTATTCCAAAAATTCCCTAC-3′; U5, 5′-CCACAGTTCTTGATGTTGACC-3′; U5B, 5′-AAGTTCCAAA-AAATATGGCAAGC-3′; U1, 5′-GACCAAGGAGTTTGCAGC-3′; and U3, 5′-TTCGGTTTCTCACTCTG-3′. The amounts of RNAs detected were quantitated with a PhosphorImager (Molecular Dynamics).

U6 particle gels, reconstitution and supershifts

Extracts were prepared by vortexing 5–25 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 PMSF in the presence of glass beads. The extract was sedimented at 100 000 *g* in a Beckman TLA100 rotor for 20 min. 0.1 OD_{260} units of each supernatant were 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, 1 mM EDTA that had been pre-run at 250 V for 30 min at 4°C. Gels were run at 275 V until BPB reached the bottom. To supershift the Lhp1p/U6 complex, anti-Lhp1p antibodies (Yoo and Wolin, 1994) were incubated with extracts for 1 h on ice. To reconstitute the Lhp1p/U6 complex, yeast U6 RNA was transcribed using T7 polymerase from a clone provided by K.S.Long (Yale University). Purified Lhp1p (Yoo and Wolin, 1997) was incubated with 27 fmol of U6 RNA in binding buffer (20 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.01% NP-40, 0.05 M KCl, 0.25 μg polyC/ml) in a final volume of 10 μl. After incubating at room temperature for 30 min and 4°C for 45 min, 1 μl 10× loading buffer (0.5 M Tris pH 7.5, 0.25 M NaCl, 5 mM $MgCl_2$, 40% glycerol) was added. The amounts of the different snRNP complexes detected were quantitated using a PhosphorImager (Molecular Dynamics).

Depletion of Lhp1p and in vivo labeling

To place *LHP1* under the control of the *MET3* promoter, the plasmid pART1-LHP1 (Van Horn *et al*., 1997) was digested with *Bam*HI and *Sac*I, and the 1 kb fragment containing *LHP1* was cloned into the *Bam*HI–*Sac*I sites of pRS313-METp, a pRS313 derivative containing the *MET3* promoter (a gift of B.Andrews, University of Toronto) to create pMETLHP1. This plasmid was transformed into BP2 to create strain BP5. To deplete Lhp1p, BP5 was grown in SC lacking methionine at 30°C to OD_{600} 0.3, diluted into SC + 2 mM methionine and grown for 36 h. Cultures were diluted to keep the OD_{600} below 0.3. Aliquots were collected at various time points, and RNA prepared as described above. Protein extracts were prepared for Western analysis as described (Yoo and Wolin, 1994). For *in vivo* labeling, strains BP4 and BP5 were grown in phosphate-depleted YPD (Warner, 1991) containing 2 mM methionine for 13 h (OD_{600} = 0.25). Carrier-free [^{32}P]phosphoric acid (New England Nuclear) was added to each culture at 100 μCi/ml, and 10 ml aliquots were removed at intervals. After RNA was extracted using the hot phenol method, 20 μg from each sample was hybridized to nitrocellulose filters containing immobilized DNAs. The DNAs were prepared by using PCR to amplify the coding sequences of yeast RNase P RNA, $tRNA^{Ser}_{CGA}$ and U6 RNA and the human Y1 RNA. Because the coding sequences of $tRNA^{Ser}_{CGA}$, U6 RNA and hY1 RNA were too short to be retained on nitrocellulose, *Eco*RI sites were built into the oligonucleotides used for amplification. The PCR products were digested with *Eco*RI, ligated to concatemers, and immobilized on nitrocellulose as described (Ullu and Tschudi, 1990). The hybridization and washes were performed as described (Ullu and Tschudi, 1990), except that after the final wash, the filters were washed in 0.1× SSC, 0.1% SDS for 1 h at 65°C.

In vitro transcriptions and plasmids

Whole-cell extracts were prepared as described by Nichols *et al*. (1990). Transcription reactions were performed as described (Yoo and Wolin, 1997). The p-225H6 plasmid (Brow and Guthrie, 1990) and the pRS426-SNR6 plasmid were gifts of D.Brow (University of Wisconsin).

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