An Lsm2–Lsm7 Complex in *Saccharomyces cerevisiae* Associates with the Small Nucleolar RNA snR5

Cesar F. Fernandez, Barbara K. Pannone, Xinguo Chen, Gabriele Fuchs, and Sandra L. Wolin*

Departments of Cell Biology and Molecular Biophysics and Biochemistry, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06536

Submitted February 10, 2004; Revised March 30, 2004; Accepted March 31, 2004

Monitoring Editor: Joseph Gall

Sm-like (Lsm) proteins function in a variety of RNA-processing events. In yeast, the Lsm2–Lsm8 complex binds and stabilizes the spliceosomal U6 snRNA, whereas the Lsm1–Lsm7 complex functions in mRNA decay. Here we report that a third Lsm complex, consisting of Lsm2–Lsm7 proteins, associates with snR5, a box H/ACA snoRNA that functions to guide site-specific pseudouridylation of rRNA. Experiments in which the binding of Lsm proteins to snR5 was reconstituted *in vitro* reveal that the 3' end of snR5 is critical for Lsm protein recognition. Glycerol gradient sedimentation and sequential immunoprecipitation experiments suggest that the Lsm protein–snR5 complex is partly distinct from the complex formed by snR5 RNA with the box H/ACA proteins Gar1p and Nhp2p. Consistent with a separate complex, Lsm proteins are not required for the function of snR5 in pseudouridylation of rRNA. We demonstrate that in addition to their known nuclear and cytoplasmic locations, Lsm proteins are present in nucleoli. Taken together with previous findings that a small fraction of pre-RNase P RNA associates with Lsm2–Lsm7, our experiments suggest that an Lsm2–Lsm7 protein complex resides in nucleoli, contributing to the biogenesis or function of specific snoRNAs.

INTRODUCTION

Several six- or seven-membered ring complexes, composed of Sm and Sm-like proteins, function in critical aspects of RNA metabolism. The first family members characterized, the Sm proteins, are essential components of the spliceosomal U1, U2, U4, and U5 snRNPs. These Sm proteins share a conserved sequence known as the Sm motif. From the crystal structure of two Sm protein heterodimers, the seven distinct Sm proteins are proposed to interact via their Sm motifs to form a ring that surrounds and contacts the Sm site, a uridine-rich sequence found in each snRNA. Binding of Sm proteins is important for several steps in snRNP biogenesis, including 5' cap hypermethylation, nuclear import of the assembled snRNP, and stability of the snRNA (Will and Luhrmann, 2001). Consistent with a general role in snRNP biogenesis or function, Sm proteins also associate with the U7 snRNP, which functions in histone 3' end formation, and the yeast telomerase RNA (Seto *et al.*, 1999; Pillai *et al.*, 2003).

In addition to the bona fide Sm proteins, there are other proteins that contain the Sm motif. Sm-like proteins have been found in all sequenced eukaryotic genomes as well as certain archaeobacteria (Achse *et al.*, 1999; Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999) and eubacteria (Moller *et al.*, 2002; Zhang *et al.*, 2002). A bacterial Sm-like protein, Hfq, forms a homohexameric ring that associates with many small RNAs, facilitating RNA–RNA interactions (Moller *et al.*, 2002; Zhang *et al.*, 2002) and protecting the associated small RNAs from nucleases (reviewed by Masse *et al.*, 2003).

In eukaryotic cells, Sm-like proteins are present in several distinct complexes. One complex, consisting of the seven proteins Lsm2–Lsm8 (Lsm stands for Like Sm), binds and stabilizes the 3' end of the spliceosomal U6 snRNA (Pannone *et al.*, 1998; Achse *et al.*, 1999; Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999; Vidal *et al.*, 1999). Lsm proteins facilitate the annealing of U6 snRNA with U4 snRNA *in vitro* and interact with the U4/U6 annealing factor Prp24, suggesting that Lsm proteins function in U4/U6 snRNP formation *in vivo* (Achse *et al.*, 1999; Rader and Guthrie, 2002). Using electron microscopy, the Lsm2–Lsm8 complex appears as a doughnut, similar in size and shape to the core Sm snRNPs. Thus, the Lsm2–Lsm8 proteins likely form a heteroheptameric ring (Achse *et al.*, 1999).

A second complex of Sm-like proteins, Lsm1–Lsm7, functions in mRNA decay (Bouveret *et al.*, 2000; Tharun *et al.*, 2000). Capped mRNA degradation intermediates accumulate in yeast containing mutations in these proteins, indicating the Lsm1–Lsm7 complex functions in mRNA decapping (Bouveret *et al.*, 2000; Tharun *et al.*, 2000). As 3'-shortened mRNAs accumulate in mutant strains, the Lsm1–Lsm7 complex may protect 3' ends of deadenylated mRNAs from nucleases (Bouveret *et al.*, 2000; He and Parker, 2001). Immunofluorescence experiments reveal that Lsm1p is mostly cytoplasmic, whereas Lsm7p, a component of both complexes, is nuclear and cytoplasmic (Diez *et al.*, 2000; Tharun *et al.*, 2000). Within the cytoplasm, Lsm1p localizes to structures called P-bodies, which represent sites of mRNA degradation (Ingelfinger *et al.*, 2002; Sheth and Parker, 2003). Thus, the Lsm2–Lsm8 complex may be nuclear, consistent with its role as a U6 snRNP component, whereas the Lsm1–Lsm7 complex appears largely cytoplasmic.

Experiments in vertebrate cells have identified two additional Lsm protein-containing complexes. The U7 snRNA, which functions in histone 3' end formation, is bound by

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E04-02-0116. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-02-0116.

* Corresponding author. E-mail address: sandra.wolin@yale.edu.

five Sm proteins and two Sm-like proteins (Pillai *et al.*, 2003). One Sm-like protein, Lsm11, contributes to histone 3' end processing (Pillai *et al.*, 2003). In *Xenopus* oocytes, an Lsm complex binds the U8 small nucleolar RNA (snoRNA), a member of the box C/D class of snoRNAs (Tomasevic and Peculis, 2002). Six Lsm proteins (Lsm2–4 and Lsm6–8) were identified in this complex, making it unclear whether the Lsm2–Lsm8 complex has a second function or whether an unidentified Lsm protein confers specificity for U8 (Tomasevic and Peculis, 2002). The role played by Lsm proteins in the biogenesis and/or function of U8 snoRNA is unknown.

The total number of Lsm-containing complexes in eukaryotic cells as well as the roles they play, is unknown. In yeast, a small fraction of the precursor to RNase P RNA, which functions in pre-tRNA processing, is bound by Lsm2–Lsm7 proteins (Salgado-Garrido *et al.*, 1999). However, as the amount of pre-RNase P bound is very low (1–2%), the significance of this association for RNase P biogenesis is unclear. As depletion of essential Lsm proteins results in accumulation of certain pre-tRNAs and tRNA degradation products, defects in rRNA processing, and loss of certain U3 snoRNA precursors, Lsm proteins have been proposed to contribute to the maturation of all these RNAs (Kufel *et al.*, 2002, 2003a, 2003b).

Here we report that a novel Lsm complex resides in yeast nucleoli. We demonstrate that six Lsm proteins, Lsm2–Lsm7, associate with the small nucleolar RNA (snoRNA) snR5. This RNA is a member of the box H/ACA class of snoRNAs that function in pseudouridylation of rRNA. There are >20 box H/ACA snoRNAs in yeast, all of which are bound by four core proteins: Gar1p, Nhp2p, Nop10p, and the pseudouridine synthase Cbf5p (reviewed by Kiss, 2001). Approximately half the snR5 RNA in cells is bound by Lsm proteins. The Lsm complex bound to snR5 is distinct from the Lsm2–Lsm8 and Lsm1–Lsm7 complexes, because neither Lsm1p nor Lsm8p are associated with the snoRNA. Experiments in which binding of Lsm proteins to snR5 was reconstituted *in vitro* reveal that the 3' end of snR5 is required for Lsm protein recognition. We demonstrate that components of the Lsm2–Lsm7 complex are present in nucleoli. Interestingly, biochemical fractionation and immunoprecipitation experiments suggest that at least some of the Lsm2–Lsm7/snR5 complex is distinct from the fraction of snR5 bound by Gar1p and Nhp2p. Consistent with a separate complex, Lsm proteins are not required for the function of snR5 in pseudouridylation. Our experiments suggest that in addition to the Lsm1–Lsm7 and Lsm2–Lsm8 complexes, an Lsm2–Lsm7 complex resides in nucleoli, contributing to the biogenesis or function of certain snoRNAs.

MATERIALS AND METHODS

Yeast Media and Strains

Yeast media and manipulations were as described (Sherman, 1991). Our wild-type strain was CY3 (*MATa ura3 lys2 ade2 trp1 his3 leu2*) (Yoo and Wolin, 1997). BP10 (*MATa lsm8::HIS3 ura3 lys2 ade2 trp1 his3 leu2* carrying p22myc) contains myc-tagged Lsm8p (Pannone *et al.*, 1998). Strains BMA64 (*MATa trp1Δ1 his3-11-15 ura3-1 leu2-3-112 ade2-1 can1-100*), AEMY31 (*MATa trp1Δ1 his3-11-15, ura3-1 leu2-3-112 ade2-1 can1-100 lsm3Δ::TRP1 pBM125-HA:Lsm3*), AEMY33 (*MATa trp1Δ1 his3Δ200–15 ura3-1 leu2-3-112 ade2-1 can1-100 lsm2Δ::HIS3 pBM125-LSM2-HA*), and AEMY46 (*MATa trp1Δ1 his3-11-15 ura3-1 leu2-3-112 ade2-1 can1-100 lsm8Δ::TRP1 pBM125-HA:Lsm8*) were gifts of J. Beggs (University of Edinburgh). Strains described in this work are BP17 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM2::MYC₃*), BP18 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM3::MYC₃*), BP19 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM6::MYC₃*), BP22 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM5::MYC₃*), BP23 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM4::MYC₃*), BP27 (*MATa ura3 lys2 ade2 trp1 his3 leu2 SMB1::MYC₃,URA3*), BP28 (*MATa ura3 lys2 ade2 trp1 his3 leu2*

snr5::URA3), BP29 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM1::MYC₃,URA3*), BP30 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM7::MYC₃,URA3*), BP31 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM9::MYC₃,URA3*), BP32 (*MATa ura3 lys2 ade2 trp1 his3 leu2 SMD2::MYC₃,URA3*), DNY7 (*MATa ura3 lys2 ade2 trp1 his3 leu2 lsm6::URA3*), DNY10 (*MATa ura3 lys2 ade2 trp1 his3 leu2 lsm7::TRP1*), DNY11 (*MATa ura3 lys2 ade2 trp1 his3 leu2 lsm5::HIS3*), CF1 (*MATa ura3 lys2 ade2 trp1 his3 leu2 lsm6::HIS3*), CF2 (*MATa ura3 lys2 ade2 trp1 his3 leu2 SMX3::MYC₃*), CF3 (*MATa ura3 lys2 ade2 trp1 his3 leu2 SMX2::MYC₃,URA3*), CF4 (*MATa ura3 lys2 ade2 trp1 his3 leu2 SME1::MYC₃*), CF38 (*MATa ura3 lys2 ade2 trp1 his3 leu2 GAR1::HA₃,KANMX6*), CF44 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM4::MYC₃*), CF46 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM5::MYC₃*), CF49 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM8::MYC₃,URA3 GAR1::HA₃,KANMX6*), CF126 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM8::yEGFP,KANMX6 pRS314-DSRED-NOP1*), CF128 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM5::yEGFP,KANMX6 pRS314-DSRED-NOP1*), CF132 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM4::yEGFP,KANMX6 pRS314-DSRED-NOP1*), and CF169 (*MATa ura3 lys2 ade2 trp1 his3 leu2 LSM4::MYC₃ GAL1::HA₃GAR1, KANMX6*).

Construction of Epitope-tagged Proteins, Immunoprecipitations, and Gradients

Three copies of the *c-myc* epitope were integrated at the C-terminus of Lsm and Sm proteins. For each protein, the forward primer contained the last 60 nt of the ORF followed by AGGGAACAAAGCTGG, which is complementary to sequences in pMPY-3xMYC (Schneider *et al.*, 1995). The reverse primer contained 60 nt downstream of the ORF up to and including the stop codon, followed by CTATAGGCGAATTGG, which is complementary to pMPY-3xMYC. Each amplified DNA contained the last 60 base pairs of Lsm or Sm coding sequence, three copies of the *c-myc* epitope, *URA3*, a second copy of the 3X-myc epitope, and 60 base pairs of downstream sequence. DNA was transformed into CY3 or a wild-type diploid strain and transformants selected on synthetic complete (SC) media lacking uracil. For epitope-tagged LSM2, LSM3, LSM4, LSM5, LSM6, SME1, and SMX3, the *URA3* gene and the second copy of the 3X-myc epitope were eliminated by growth on 5-fluoroorotic acid. For LSM7, SMD2, SMD3, and SMX2, *URA3* was not removed. Tagging with yEGFP and 3xHA was as described (Knop *et al.*, 1999) using pYM12 for yEGFP and pYM1 for 3xHA. Forward primers contained the last 45–60 nt of the ORF followed by CGTACGCTGAGGTCGAC, which is complementary to pYM1 and pYM12. Reverse primers contained 45–60 nt downstream of the ORF followed by ATCGATGAATTCGAGCTCG, which is complementary to pYM1 and pYM12. Amplified DNA was transformed into CY1, plated on YPD overnight and replica-plated onto YPD-G418 (200 μg/ml). Integration of the *GAL1* promoter and (*HA*)₃ epitope upstream of *GAR1* was as described (Longtine *et al.*, 1998) using pFA6a-kanMX6-PGAL1-3HA. All tagged strains exhibited wild-type growth at 16, 30, and 37°C.

Immunoprecipitations were performed as described (Pannone *et al.*, 1998) using anti-myc antibodies (Evan *et al.*, 1985), anti-HA antibodies (BAbCO, Berkeley, CA) and anti-Nhp2p antibodies. Anti-Nhp2p was the gift of Y. Henry (CNRS, Toulouse, France). RNAs in immunoprecipitates were labeled at the 3' end with [³²P]pCp (England *et al.*, 1980). Synthesis of cDNA was as described (O'Brien and Wolin, 1994). Glycerol gradients (10–36%) were performed as described (Yang *et al.*, 2002).

Depletion of Lsm Proteins and Temperature Shift Experiments

A high copy *SNR6* plasmid was constructed by inserting the *Bam*HI fragment from pRS426-SNR6 (a gift of D. Brow, University of Wisconsin) into the same site of YEplac181. For depletions, strains BMA64 and AEMY31 were transformed with YEplac181 or YEplac181-SNR6. Cells were grown in SC-leu containing 2% galactose, grown to OD₆₀₀ = 0.1–0.3 at 25°C, spun down, washed twice with water, and transferred to SC-leu media containing 2% glucose at 25°C. Cultures were diluted in SC-leu containing 2% glucose to keep cells in log phase.

For temperature shifts, strains CY1, BP28, DNY7, DNY10, and DNY11 were grown at 30°C to OD₆₀₀ = 0.3. After shifting to 37°C, cultures were kept in log phase by diluting with YPD. After 7 h, cells were collected and stored at –80°C.

Northern, Pseudouridylation Assays, and Oligonucleotides

RNA was extracted from yeast using hot phenol and SDS (Ausubel *et al.*, 1998), fractionated in 5% polyacrylamide/8.3 M urea gels, and transferred to Zetaprobe GT nylon membranes (Bio-Rad, Hercules, CA) in 0.5× TBE at 150 mA for 16 h. Probes were U1: 5' GACCAAGGAGTTTGCAGC 3', U6: 5' AAAACGAAATAAATCTCTTTG 3', RP1: 5' GACGCTCAACGATTGCAC 3', snR5: 5' ATAGACATATGGAGCGCTG 3', U3: 5' TTCGGTTTCTCACTCTG 3', snR3: 5' TCGATCTTCGTACTGTCT 3', snR8: 5' GCACAGTGAATAGAAGGCATG 3', snR10: 5' CATGGGTCAAGAAGCCCCCGGAGGGG 3', snR13: 5' AGTAAAAAAGGTAGCTTGGAG 3', snR30: 5' CCGAAGCGCCATCTAGATGAATACGTGAGGCC 3', snR31: 5' GTA-GAACGAATCATGACC 3', snR37: 5' GATAGTATTAACCACTACTG 3',

snR44: 5' GAGGTGTAATCCATAACCGTG 3', snR45: 5' CCTCA-GATCGCTCCGAGAAGA 3', tRNA^{Leu}: 5' ATCCTTGCTTAAGCAAAT-GCGC 3', tRNA^{Glu}: 5' AAGATTTCGTAGTGATAA 3', pre-5.8S: 5' TGAGAAGGAAATGACGCT 3', U3-3': 5' GTGGTAACTTGCTCA 3'. Quantitation was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Pseudouridylation was assayed by primer extension (Bakin and Ofengand, 1993) using oligonucleotides 25S1G (site Ψ1003) 5' AATAGTCAAGGTCATTT 3' and 25S2G (Ψ1123) 5' TACGTTTCGGTTCATCCCGC 3'.

In Vitro Assembly

The DNA fragment used to transcribe U5_L was described (Xue *et al.*, 2000). To transcribe U6 snRNA, the plasmid pU62G (gift of Y. Shamoo, Rice University) was cut with *Dra*I. For snR5, a DNA fragment containing snR5 behind the T7 promoter was synthesized using overlapping oligonucleotides. One oligonucleotide contained a T7 promoter, followed by nt 1–96 of snR5 sequence. The second oligonucleotide encoded the antisense strand of snR5 from nucleotides 197–77. After annealing, oligonucleotides were extended using Pfu polymerase. In this construct, the first nt of snR5 was replaced by GG to allow initiation by T7 RNA polymerase. Transcription was performed using 50 μCi of either [α -³²P]UTP (for U5_L), [α -³²P]ATP (for U6), or [α -³²P]CTP (for snR5) as described (Yisraeli and Melton, 1989). DNAs allowing transcription of snR5 RNA fragments were synthesized from overlapping oligonucleotides and transcribed with [α -³²P]CTP.

Extracts were prepared from strains CY1, BP17, BP19, BP22, and BP10 as described (Xue *et al.*, 2000). To examine Lsm binding, 50,000 cpm (1–5 fmol) of each RNA was incubated with 4 μl extract for 30 min at 23°C in a volume of 10 μl under splicing conditions (Xue *et al.*, 2000).

In Situ Hybridization and Live Cell Imaging

To create an antisense snR5 probe, the snR5 coding sequence was amplified from genomic DNA and cloned into the *Eco*RI/*Bam*HI sites of pSP64 to create pSP64 SNR5. To allow transcription of sense RNA, the oligonucleotide used to amplify the snR5 5' end contained a T7 promoter. For antisense snR5 RNA, pSP64 SNR5 was digested with *Eco*RI, transcribed and labeled with digoxigenin-UTP using the DIG RNA kit (Roche, Indianapolis, IN). For in situ hybridization, cells carrying a Nop1-GFP plasmid (a gift of E. Hurt) were grown to OD₆₀₀ ~ 0.4–0.8 and fixed with 4% formaldehyde for 1 h. Cells were spheroplasted with 40 μg/ml zymolase 100T and 30 mM β-mercaptoethanol in 100 mM potassium phosphate, pH 7.4, 1.2 M sorbitol for 30 min at 37°C. After plating for 10 min on poly-L-lysine-coated coverslips, cells were rinsed in 0.2× SSC and incubated with hybridization solution (50% formamide, 5× SSC, 1 mg/ml *Escherichia coli* tRNA, 100 μg/ml heparin, 1× Denhardt's solution, 0.1% Tween-20, 0.1% Triton X-100, 5 mM EDTA, pH 8.0) for 1 h at 22°C, followed by incubation overnight with 5 ng/μl snR5 probe in hybridization solution at 37°C. Coverslips were rinsed in 0.2× SSC, washed three times for 20 min in 0.2× SSC at 37°C, and rinsed in 0.2× SSC. Cells were incubated in blocking buffer (0.1 M Tris-Cl, pH 7.5, 0.15 M NaCl, 5.0% fetal bovine serum) for 5 min and incubated with antidigoxin (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1.3 μg/ml in blocking buffer for 30 min at 37°C. Coverslips were rinsed in wash buffer (0.1 M Tris-Cl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20), washed three times for 10 min at 22°C, incubated with goat anti-mouse AlexaFluor 546 (1:500 in blocking buffer) at 22°C for 1 h, and washed three times for 10 min at 22°C. Coverslips were mounted with VectaShield (Vector Laboratories, Burlingame, CA) containing 1.25 μg/ml DAPI and viewed with an Axioplan2 Zeiss microscope (Thornwood, NY). Images were taken with a Hamamatsu CCD camera (Bridgewater, NJ) using MetaMorph software (Universal Imaging, Downingtown, PA).

Strains carrying Lsm-GFP fusions were transformed with pRS314 DsRed-Nop1 (a gift of E. Hurt). After growth in SC-trp to OD₆₀₀ = 0.3–0.6, 1 ml of culture was spun down and resuspended in 30–60 μl of SC-TRP. Cells were placed on a microscope slide and mixed with 2% low melting point agarose in PBS (precooled to 42°C). A coverslip was placed on top, and live cells viewed with a Zeiss LSM 510 confocal laser scanning microscope equipped with krypton/argon laser.

RESULTS

Six Sm-like Proteins Associate with the Small Nucleolar RNA snR5

To examine the small RNAs bound by yeast Lsm proteins, we generated strains in which the C-terminus of each Lsm protein was fused to three copies of the *c-myc* epitope (Evan *et al.*, 1985). Using a monoclonal anti-*c-myc* antibody, we performed immunoprecipitations from *LSM3(myc)*₃, *LSM5(myc)*₃, and *LSM8(myc)*₃ strains. RNAs in the immunoprecipitates were labeled at the 3' end with [³²P]pCp. As described (Pannone *et al.*, 1998; Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999), all three immunoprecipitations contained U4 and U5 RNAs (Figure 1, lanes 4, 6, and 7). Because

Figure 1. snR5 RNA associates with certain Lsm proteins. Lysates from the indicated strains were subjected to immunoprecipitation with anti-*c-myc* antibodies. RNAs in immunoprecipitates (lanes 3–7) were labeled with [³²P]pCp and fractionated in a 5% polyacrylamide, 8.3 M urea gel. Lanes 1 and 2, total RNA from *LSM3(myc)*₃ and *LSM3(myc)*₃ *lhp1Δ* lysates. The band labeled with the asterisk was identified as snR5. The identities of the U4, U5, and U6 snRNA bands in the immunoprecipitates were confirmed by Northern analyses.

yeast U6 terminates in a 3' phosphate (Lund and Dahlberg, 1992), it is a poor substrate for the labeling reaction; nonetheless, some U6 RNA was visible in the *LSM8(myc)*₃ immunoprecipitate (lane 7). Interestingly, an additional band of ~200 nts was detected in *LSM3(myc)*₃ and *LSM5(myc)*₃ immunoprecipitates (lanes 4 and 6, asterisk), but not in immunoprecipitates from the untagged and *LSM8(myc)*₃ strains (lanes 3 and 7).

Because both the La protein and the Lsm2–Lsm8 complex bind the uridylylates at the 3' end of U6 snRNA (Achsel *et al.*,

1999; Vidal *et al.*, 1999; Wolin and Cedervall, 2002), it was suggested that binding by the more abundant La to small RNAs ending in uridylates prevents Lsm protein binding (Achsel *et al.*, 1999). To test this possibility, we performed immunoprecipitations from a *LSM3(myc)*₃ strain lacking the yeast La protein Lhp1p. Although the novel band was present in the immunoprecipitate, no additional major species were detected (lane 5).

The identity of the small RNA in the *LSM3(myc)*₃ and *LSM5(myc)*₃ immunoprecipitates was determined by sequencing cDNAs. The RNA is snR5, a member of the box H/ACA class of snoRNAs (Tollervey, 1987; Balakin *et al.*, 1996). To determine whether snR5 associated with other Lsm proteins, we performed immunoprecipitations from strains containing tagged versions of each of the nine Lsm proteins. RNAs in immunoprecipitates were subjected to Northern analysis. snR5 RNA was present in immunoprecipitates from myc-tagged *LSM2*–*LSM7* strains (Figure 2A, lanes 4–9), but was undetectable in immunoprecipitates from myc-tagged *LSM1*, *LSM8* and *LSM9* strains (lanes 3, 10, and 11). Reprobing for U1 and U6 RNAs revealed that while U1 was undetectable, U6 RNA was present in tagged *LSM2*–*LSM8* immunoprecipitates, as expected (lanes 3–11, bottom and middle panels).

As most characterized Sm-like protein complexes contain seven distinct proteins, we tested whether an Sm protein was the seventh member of the complex. Anti-*c-myc* immunoprecipitations were performed from strains containing tagged versions of six of the seven Sm proteins (SmB1, SmD2, SmD3, SmE, SmF, and SmG). Although U1 and U6 RNA were present in all immunoprecipitates, snR5 RNA was undetectable (Figure 2A, lane 12 and unpublished data). Immunoprecipitations using an antibody against the seventh protein, Smd1p, also did not reveal snR5 in the immunoprecipitate (lane 13). Thus, only six Lsm proteins associate with snR5 RNA.

To determine the fraction of snR5 bound by Lsm proteins, we compared the portion of snR5 in the immunoprecipitates with that in the supernatants. PhosphorImager quantitation revealed that 56% of the snR5 and 86% of the U6 RNA were immunoprecipitated from the tagged *LSM5* strain (Figure 2B, lane 5). As described (Salgado-Garrido *et al.*, 1999), pre-RNase P RNA, but not the mature RNA, was present at low levels (~1–2%) in *Lsm2*–*Lsm7* immunoprecipitates (lane 5 and unpublished data). In contrast, while pre-tRNA^{Lys}_{UUU} and several U3 RNA precursors were also reported to associate with Lsm proteins at low levels (Kufel *et al.*, 2002; Kufel *et al.*, 2003b), we did not detect these RNAs in our immunoprecipitates (Figure 2B, lane 5).

We also examined whether other snoRNAs associate with Lsm proteins. Probing to detect the box H/ACA snoRNAs snR3, snR8, snR10, snR30, snR31, snR37, and snR44 failed to reveal these RNAs in the immunoprecipitates (Figure 2B, see also Figure 5A and unpublished data). Similarly, the box C/D snoRNAs U3, snR13, and snR45 were undetectable (Figure 2B and unpublished data). Thus, although a substantial fraction of snR5 associates with *Lsm2*–*Lsm7* proteins, we have not detected significant levels of other mature snoRNAs associated with this complex.

An Intact 3' End of snR5 RNA Is Critical for Lsm Protein Binding

To examine the features of snR5 required for *Lsm2*–*Lsm7* binding, we determined whether these proteins associate with snR5 in vitro. Using T7 RNA polymerase, we synthesized ³²P-labeled snR5 RNA and incubated the RNA in extracts prepared from *c-myc*-tagged *LSM2*, *LSM5*, *LSM6*,

Figure 2. A substantial fraction of snR5 RNA associates with *Lsm2*–*Lsm7* proteins. (A) Anti-*c-myc* immunoprecipitations were performed from an untagged strain (lane 2), or the indicated *c-myc*-tagged strains (lanes 3–12). Equal amounts of lysates (as measured by OD₂₆₀) were used for immunoprecipitations. RNAs within immunoprecipitates were subjected to Northern analyses to detect U1, snR5, and U6 RNAs. Anti-Smd1p antibodies (Xue *et al.*, 2000) was used to immunoprecipitate Smd1p (lane 13). The low level of U6 RNA in the *LSM7(myc)*₃ immunoprecipitate is not reproducible. Lane 1, RNA from an equivalent amount of untagged lysate. (B) Lysates from untagged (lanes 1, 4, and 7), *LSM5(myc)*₃ (lanes 2, 5, and 8), and *LSM8(myc)*₃ (lanes 3, 6, and 9) strains were divided in equal aliquots and extracted with phenol (lanes 1–3) or incubated with anti-*c-myc* antibodies (lanes 4–6). RNAs in totals (lanes 1–3), immunoprecipitates (4–6), and supernatants (7–9) were subjected to Northern analyses to detect U1, snR5, U6, snR3, RNase P, U3, and pre-tRNA^{Lys}_{UUU}.

and *LSM8* strains. As controls, we included U5_L, the longer of two mature forms of U5 snRNA, and U6 snRNA. Immunoprecipitation revealed that snR5 was bound by *c-myc*-tagged *Lsm2*, *Lsm5*, and *Lsm6* proteins (Figure 3, lanes 7–9), but did not associate with tagged *Lsm8* (lane 10). In contrast, U6 snRNA was bound by all four proteins (lanes 7–10).

To determine the region of snR5 RNA that was recognized by Lsm proteins, we examined binding to truncated forms of snR5 consisting of nts 1–86, nts 60–140, and nts 110–197

Figure 3. Lsm2p, Lsm5p, and Lsm6p associate with snR5 RNA in vitro. 32 P-labeled snR5, U6, and U5_L snRNAs were incubated in untagged (lanes 1 and 6), *LSM2(myc)*₃ (lanes 2 and 7), *LSM5(myc)*₃ (lanes 3 and 8), *LSM6(myc)*₃ (lanes 4 and 9), or *LSM8(myc)*₃ (lanes 5 and 10) extracts. After incubation, samples were divided in half and either extracted with phenol (lanes 1–5) or subjected to immunoprecipitation with anti-*c-myc* antibodies (lanes 6–10).

(Figure 4A). The 110–197 fragment, and to a lesser extent the 60–140 fragment, were present in *LSM2(myc)*₃, *LSM5(myc)*₃, and *LSM6(myc)*₃ immunoprecipitates (Figure 4B, lanes 22–23 and 16–17; also unpublished data). These fragments were not detected in *LSM8(myc)*₃ immunoprecipitates (lanes 18 and 24).

To further characterize the binding site, we examined additional mutant RNAs. An snR5 RNA lacking the stem loop between nts 131–178 (snR5 1–131,178–197; Figure 4A), was bound by *c-myc*-tagged Lsm2, Lsm5, and Lsm6 proteins but was not bound by tagged Lsm8 (Figure 4C, lanes 10–12). However, snR5 lacking the last 20 nts (snR5 1–177, Figure 4A) failed to associate with Lsm(myc)₃ proteins, suggesting the 3' end was required (unpublished data). Similarly, variants of snR5 110–197, in which the last 9 nts were deleted (snR5 110–188) or converted to Cs (snR5 110–188, C9) were not bound by Lsm(myc)₃ proteins (Figure 4C, lanes 16–18, 22–24). As snR5 60–140, which lacks the 3' end, associated with Lsm proteins, there may be other determinants for binding that we have not uncovered. For example, nts 132–140 in the snR5 60–140 construct, which are basepaired in full-length snR5 (Figure 4A), may contain a cryptic binding site that becomes accessible when these nts are single-stranded. Nonetheless, the result that full-length and truncated snR5 RNAs lacking the last 9 nts do not associate with Lsm proteins reveals that these nts are critical for binding.

A Fraction of Lsm-containing snR5 snoRNPs May Lack Box H/ACA Core Proteins

As a box H/ACA snoRNA, snR5 associates with the proteins Gar1, Nhp2, Cbf5, and Nop10 (Kiss, 2001). We asked whether Lsm proteins and the box H/ACA proteins are found in a single complex with snR5. In initial experiments,

immunoprecipitations from *LSM4(myc)*₃, *LSM5(myc)*₃, and *LSM8(myc)*₃ strains were assayed for Nhp2p by Western blotting. Nhp2p was undetectable in the immunoprecipitates, either because it was not associated with Lsm proteins, or was of too low abundance to be detected. Similar experiments using *LSM4(myc)*₃, *LSM5(myc)*₃, and *LSM8(myc)*₃ strains containing influenza hemagglutinin (HA) epitope-tagged Gar1p also failed to detect Gar1p in the immunoprecipitates (unpublished data). In a second approach, we performed sequential immunoprecipitations. First, we established that two sequential immunoprecipitations with anti-*c-myc* were sufficient to remove the majority of the Lsm5(myc)₃-containing snR5 from the extract (Figure 5A, lanes 1–3). However, ~42% of the remaining snR5 could be immunoprecipitated with anti-Nhp2p (Figure 5A, lane 7), suggesting that a population of Nhp2p-containing snR5 lacks Lsm proteins. Similarly, two immunoprecipitations with anti-Nhp2p removed the majority of the immunoprecipitable snR5 from the extract (lanes 9–12). The fraction of snR5 that could be immunoprecipitated with anti-Nhp2p was 46%, whereas 72% of snR10, another H/ACA RNA, was immunoprecipitated (lanes 9, 10 and 13, 14). Subsequent immunoprecipitation with anti-*c-myc* revealed that ~35% of the remaining snR5 RNA was bound by Lsm proteins (lane 15). Similar results were obtained using a strain containing *c-myc*-tagged *LSM5* and HA-tagged *GAR1* (unpublished data). In these experiments, we cannot exclude the possibility that our failure to quantitatively immunoprecipitate snR5 with anti-Nhp2p, anti-HA or anti-*c-myc* antibodies results from masking of the epitopes, rather than absence of these proteins in the snoRNP. Nonetheless, our data suggest that a fraction of the Lsm-associated snR5 RNA may be distinct from the snR5 RNA bound to box H/ACA proteins.

To confirm that the Lsm2–Lsm7-associated snR5 RNA and the box H/ACA core protein-associated snR5 represent partly distinct populations, we ran glycerol gradients. As only limited quantities of the anti-Nhp2p antibody were available, we used a strain containing *c-myc*-tagged *LSM5* and HA-tagged *GAR1*. After gradient fractionation, samples were divided into three aliquots. Total RNA was extracted from one aliquot, and the other aliquots were subjected to immunoprecipitation with either anti-*c-myc* or anti-HA antibodies. For size markers, we examined the sedimentation of the U1 snRNP, (~18S), the U4/U6.U5 tri-snRNP (~25S), and the U3 snoRNP (~12S) (Fabrizio *et al.*, 1994). Analysis of total RNA revealed that most snR5 was found in the heaviest fractions, 1–5, with some snR5 also in fractions 7–11 (Figure 5B, third panel). The majority of snR5 sediments as >25S in gradients, which is larger than the 14–17S estimated for two purified box H/ACA snoRNPs, snR42 and snR30 (Watkins *et al.*, 1998). Thus, in addition to the box H/ACA proteins, other components may associate with snR5 snoRNPs. Lsm5(myc)₃-associated snR5 RNA was detected in two peaks, one corresponding to the heaviest fraction 1 and the remainder in fractions 5–9 (Figure 5B, top panel), between 18S and 25S. In contrast, Gar1-associated snR5 RNA peaked in fraction 3 (>25S) (second panel). The finding that Lsm-associated snR5 sediments differently in gradients from Gar1p-bound snR5 suggests that these may represent separate populations of snR5 RNA.

Localization of snR5 and Lsm Proteins to Nucleoli

As at least part of the Lsm2–Lsm7-associated snR5 may be distinct from the snR5 associated with box H/ACA proteins, we used in situ hybridization to determine whether a fraction of snR5 localized outside nucleoli. Using Nop1p-GFP as a nucleolar marker, snR5 was nucleolar in wild-type strains (Figure 6A). The localization of snR5 was unchanged in

Figure 4. Association of Lsm proteins with snR5 RNA requires an intact 3' end. (A) Top: A secondary structure for snR5 RNA, based on models for H/ACA RNAs (Kiss, 2001), was derived using MFOLD (Mathews *et al.*, 1999). Box H (nts 87–92) and ACA (nts 192–194) sequences are bold. Bottom: snR5 RNA derivatives used in binding. (B) ³²P-labeled snR5 1–197 RNA (lanes 1–6) and truncation mutants snR5 1–86 (lanes 7–12), snR5 60–140 (lanes 13–18), and snR5 110–197 (lanes 19–24) were incubated with extracts from untagged (lanes 3, 9, 15, and 21), *LSM5(myc)*₃ (lanes 4, 10, 16, and 22), *LSM6(myc)*₃ (lanes 5, 11, 17, and 23) and *LSM8(myc)*₃ (lanes 6, 12, 18, and 24) strains. U5_L and U6 snRNAs were included in each reaction. After incubation, reactions were divided in half and extracted with phenol (lanes 2, 8, 14, 20; shown for *LSM5(myc)*₃ extract) or subjected to immunoprecipitation. Lanes 1, 7, 13, and 19 contain the equivalent amount of labeled RNAs as was added to extracts. Asterisks denote snR5 RNAs. For unknown reasons, less snR5 was bound by Lsm proteins in this experiment compared with Figure 3. (C) ³²P-labeled snR5 mutants, along with U5_L and U6 snRNAs, were incubated with extracts of untagged (lanes 3, 9, 15, and 21), *LSM5(myc)*₃ (lanes 4, 10, 16, and 22), *LSM6(myc)*₃ (lanes 5, 11, 17, and 23) and *LSM8(myc)*₃ (lanes 6, 12, 18, and 24) strains. Each reaction was divided in half and extracted with phenol (lanes 2, 8, 14, 20, shown for *LSM5(myc)*₃ extract) or subjected to immunoprecipitation. Lanes 1, 7, 13, and 19 contain the equivalent amount of labeled RNAs as was added to the extracts. Asterisks denote snR5 RNAs.

strains lacking the nonessential Lsm proteins *LSM5*, *LSM6*, and *LSM7* (Figure 6A and unpublished data), revealing that these proteins are not required for snR5 localization.

Previously, yeast Lsm proteins were localized to the nucleus and cytoplasm (Diez *et al.*, 2000; Tharun *et al.*, 2000; Sheth and Parker, 2003). Our finding that snR5 associates with Lsm2–Lsm7 suggested these proteins might also be nucleolar, as described for *Xenopus* Lsm4p (Tomasevic and Peculis, 2002). We used GFP-tagged *LSM4*, *LSM5*, and *LSM8* strains and examined their distribution by confocal microscopy. For a nucleolar marker, cells were transformed with a plasmid carrying DsRed-Nop1p. In addition to being distributed throughout the nucleoplasm and cytoplasm, Lsm5p-GFP and Lsm4p-GFP were present in nucleoli (Figure 6B, a–c and d–f, respectively). As described (Sheth and Parker, 2003), these proteins were also detected in punctate cytoplasmic structures proposed to be sites of mRNA decay.

Lsm8p-GFP was concentrated in nuclei although some staining was detected throughout the cell (Figure 6B, g-i). Some colocalization of Lsm8p with the DsRed-Nop1 nucleolar marker was observed, mostly in areas where there is a transition from the nucleus to the nucleolus. Thus, in addition to their nuclear and cytoplasmic locations, two Lsm proteins that associate with snR5 are found in nucleoli.

Lsm Proteins Are Not Required for snR5 RNA Accumulation or Its Function in Pseudouridylation

To examine the role of Lsm proteins in snR5 function, we used strains in which *LSM2*, *LSM3*, and *LSM8* were under control of the glucose-repressible *GAL* promoter (Mayes *et al.*, 1999) to deplete the proteins. To distinguish direct effects of depleting Lsm proteins from indirect effects due to lowering U6 levels, we transformed each strain with either a high copy plasmid containing *SNR6* or with the vector

Figure 5. The Lsm-associated snR5 appears partly distinct from the box H/ACA protein-associated snR5. (A) An *LSM5(myc)₃* strain was subjected to sequential anti-*c-myc* and anti-Nhp2p immunoprecipitations. In Set A (lanes 1–3), the lysate was incubated three times with anti-*c-myc* antibodies. For Set B (lanes 5–7), two anti-*c-myc* immunoprecipitations were followed by an anti-Nhp2p immunoprecipitation. Set C (lanes 9–11) shows three anti-Nhp2p immunoprecipitations. In Set D (lanes 13–15) two anti-Nhp2p immunoprecipitations were followed by an anti-*c-myc* immunoprecipitation. Lanes 4, 8, 12, and 16 show the supernatants after the third immunoprecipitation. RNAs from immunoprecipitates and supernatants were subjected to Northern analysis to detect snR5, U6, U1, and snR10 RNAs. Lane 17, RNA from an equivalent amount of lysate. (B) *LSM5(myc)₃ GAR1(HA)₃* lysates were fractionated in a 10–36% glycerol gradient. Odd-numbered fractions were divided into three parts and either extracted with phenol (third panel) or incubated with anti-*c-myc* (top panel) or anti-HA antibodies (second panel). RNAs from immunoprecipitates and totals were subjected to Northern analysis to detect snR5 RNA (top three panels). The third panel was reprobed to detect U1, U5, U4, U6, and U3 snRNAs (bottom four panels).

alone. As reported (Mayes *et al.*, 1999), *GAL::LSM* cells slow in growth within 12 h in glucose-containing media. However, in the presence of extra U6 genes, growth was near normal (Figure 7A). Western blotting revealed that Lsm proteins became undetectable in both strains within 8 h after the switch to glucose media. Thus, as described (Mayes *et al.*, 1999; Pannone *et al.*, 2001), these Lsm proteins become dispensable for growth in the presence of extra U6 genes.

At intervals after the shift to glucose media, RNA was extracted and subjected to Northern analyses. As expected, U6 RNA levels declined during Lsm2, Lsm3, or Lsm8p depletion (Figure 7B, lanes 15–21, and unpublished data). However, in cells carrying high copy *SNR6*, U6 levels were elevated compared with wild-type cells, and remained stable during Lsm protein depletion (Figure 7B, lanes 22–28). Examination of snR5 revealed that levels of this RNA were unaffected by Lsm protein depletion (Figure 7B, lanes 15–21, and unpublished data). Thus, Lsm2p, Lsm3p, and Lsm8p are not required for snR5 accumulation.

Unexpectedly, we found that certain RNA processing defects previously detected during Lsm protein depletion (Kufel *et al.*, 2002, 2003a, 2003b) were suppressed by U6 RNA overexpression. Specifically, pre-tRNAs accumulate during depletion of essential Lsm proteins, as do intron-containing pre-U3 RNAs (Kufel *et al.*, 2002, 2003b). Also, both spliced pre-U3 RNAs and certain rRNA processing intermediates decline during Lsm protein depletion (Kufel *et al.*, 2003a). As reported, pre-tRNA^{Tyr}_{CUA} and intron-containing pre-U3 RNA increase during Lsm3p depletion, whereas spliced pre-U3 RNA decreases (Figure 7B, lanes 15–21). Similarly, two pre-5.8S rRNA processing intermediates (7S and 6S) decreased (Figure 7B, 15–21). However, all these effects were ameliorated when U6 was overexpressed during Lsm3p depletion (Figure 7B, lanes 22–28), suggesting these processing anomalies may be indirect effects of lowering U6 levels and inhibiting splicing.

We examined whether Lsm proteins were required for the function of snR5 in pseudouridylation. Modification of 25S rRNA at two sites, ψ 1003 and ψ 1123, requires snR5 (Ganot *et al.*, 1997). Total RNA from strains depleted of Lsm3p was analyzed for pseudouridines (Figure 7C). After treatment of the RNA with *N*-cyclohexyl-*N*- β -(4-methylmorpholinum)-ethylcarbodiimide *p*-tosylate (CMC), which reacts irreversibly with pseudouridines, sites of modification were detected by primer extension (Bakin and Ofengand, 1993). Pseudouridylation was efficient at both positions 1003 and 1123 in strains depleted of Lsm3p (Figure 7C).

As snR5 associates with both Lsm proteins and box H/ACA specific proteins, we determined whether depleting essential Lsm proteins resulted in increased binding of box H/ACA proteins to snR5 RNA. At intervals during the shift to glucose media, we performed immunoprecipitations with anti-Nhp2p. No increase in the levels of snR5 bound by Nhp2p was detected during Lsm3p depletion. Similarly, depletion of Gar1p using a *GAL::GAR1 LSM4(myc)₃* strain did not result in increased levels of snR5 in anti-*c-myc* immunoprecipitates (unpublished data).

Although *LSM2–LSM4* are essential, strains lacking *LSM5*, *LSM6*, or *LSM7* are viable but temperature sensitive for growth (Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999). We examined whether *LSM5*, *LSM6*, or *LSM7* are required for accumulation of snR5 RNA or for its role in pseudouridylation. Cells lacking each of these proteins were grown either at 30°C or examined during growth at the nonpermissive temperature of 37°C. As expected, U6 snRNA levels were slightly reduced at 30°C in strains lacking *LSM* proteins and were drastically reduced at 37°C. However, snR5 levels were unaffected, as was pseudouridylation at positions 1003 and 1123 in 25S rRNA (unpublished data). Thus, the Lsm2–Lsm7 complex does not appear to be required for the accumulation of snR5 RNA or for its role in pseudouridylation.

Figure 6. snR5 and Lsm proteins are present in nucleoli. (A) Wild-type, *lsm6Δ*, and *snR5Δ* strains were subjected to in situ hybridization using an antisense snR5 probe (a, d, and g, respectively). Nop1p-GFP was used as a nucleolar marker (b, e, and h). DNA was stained with DAPI and is shown in blue in c, f, and i. (B) Live cell imaging of Lsm5p-GFP, Lsm4p-GFP, and Lsm8p-GFP using confocal microscopy. DsRed-Nop1p was used as a nucleolar marker. Only a fraction of cells express detectable DsRed-Nop1.

Figure 7. Lsm proteins are not required for snR5 stability or its function in pseudouridylation. (A) The growth of wild-type and *GAL::LSM3* strains is compared during growth in glucose. Strains contain either *SNR6* in the high copy plasmid YEplac181 (pSNR6) or YEplac181 (vector). At time 0, strains were switched to medium containing glucose. (B) At intervals after the switch to glucose, RNA was extracted and subjected to Northern analysis to detect snR5, U6, and U1 snRNAs (top three panels). We also examined levels of two 3' extended U3 precursors (the intron-containing U3-3' int and the spliced U3-3') (Kufel *et al.*, 2003b), pre-tRNA^{Tyr}_{GUA}, and the 7S and 6S precursors to 5.8S rRNA. Although Lsm2–Lsm8 proteins are normally required for stable accumulation of U6 snRNA (Pannone *et al.*, 1998; Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999), the overexpressed U6 snRNA is stable. One possibility is that other RNA-binding proteins, whose affinity for U6 is low when U6 is present at wild-type levels, bind and stabilize U6 when the RNA is overexpressed. (C) Total RNA was extracted from *GAL::LSM3* strains carrying pSNR6 at intervals after the switch to glucose and assayed for pseudouridines by primer extension. RNA from wild-type and *snr5Δ* strains was also examined.

DISCUSSION

Two complexes of Lsm proteins, Lsm1–Lsm7 and Lsm2–Lsm8, have been described in yeast cells. The Lsm1–Lsm7 complex functions in mRNA decay, whereas the Lsm2–Lsm8 complex is important for U6 snRNA stability and function. We found that a third Lsm complex, composed of Lsm2–Lsm7, associates with about half of the box H/ACA snoRNA snR5 in *Saccharomyces cerevisiae*. Using live cell imaging, we determined that Lsm proteins are present in nucleoli. Together with previous data that Lsm2–Lsm7 proteins bind pre-RNase P RNA, our data suggest that an Lsm2–Lsm7 complex plays a role in the biogenesis or function of a subset of nucleolus-associated small RNAs.

Although all well-characterized eukaryotic Sm and Lsm protein complexes contain seven subunits, only six Lsm proteins associate with snR5 and pre-RNase P RNA. One possibility is that an unidentified yeast protein, whose homology to Lsm proteins has escaped detection, forms the seventh member of the complex. Alternatively, one Lsm protein could be present in two copies, resulting in a seven-membered ring. Finally, as described for Hfq (Moller *et al.*, 2002; Zhang *et al.*, 2002), the snR5-bound Lsm complex may be a hexamer. Consistent with a hexamer, only six proteins were identified in the *Xenopus* Lsm complex that binds U8 snoRNA (Tomasevic and Peculis, 2002).

The finding that both RNAs that bind Lsm2–Lsm7, snR5, and pre-RNase P RNA, are nucleolar (Srisawat *et al.*, 2002; see also Figure 6) suggests that the Lsm2–Lsm7 complex functions in nucleoli. For the Lsm1–Lsm7 and Lsm2–Lsm8 complexes, Lsm1p and Lsm8p are likely responsible for the different subcellular locations of their respective complexes. Similarly, Lsm10, a member of the Sm complex that binds U7 snRNA, may govern localization of U7 to coiled bodies (Pillai *et al.*, 2001). Thus, an unidentified seventh Lsm protein could confer nucleolar localization of the Lsm2–Lsm7 complex. Alternatively, the default subcellular location for Lsm proteins may be nucleoli, with Lsm1p or Lsm8p required to localize the complex elsewhere in cells. As a different set of six Lsm proteins (Lsm2–Lsm4 and Lsm6–Lsm8) were identified in the complex that binds *Xenopus* U8 RNA (Tomasevic and Peculis, 2002), another possibility is that any complex containing only six Lsm proteins will localize to nucleoli.

Our finding that binding of Lsm proteins to snR5 requires the RNA 3' end is consistent with the fact that Lsm2–Lsm8 and Lsm1–Lsm7 likely associate with the 3' ends of their target RNAs. The 3' end of U6 snRNA is protected by Lsm2–Lsm8 proteins from nucleases (Achsel *et al.*, 1999) and is required for Lsm protein binding in vitro (Vidal *et al.*, 1999). Similarly, the presence of 3' trimmed mRNAs in *lsm* mutant strains suggests that Lsm1–Lsm7 stabilizes the 3' ends of deadenylated mRNAs from exonucleases (He and Parker, 2001). To date, the sole exception is the Lsm complex that associates with U8 snoRNA, which requires an internal sequence for efficient binding in vitro (Tomasevic and Peculis, 2002). One possibility is that Lsm complexes play a general role in stabilizing associated RNAs from nucleases. Our finding that snR5 is stable during Lsm depletion does not negate this possibility, because other proteins may redundantly stabilize this RNA. A role in stabilizing 3' ends would be reminiscent of the La protein, which stabilizes the 3' ends of nascent RNAs ending in UUU_{OH} (Wolin and Cedervall, 2002). However, our finding that additional RNAs do not become bound by Lsm proteins in strains lacking Lhp1p (Figure 1) indicates that binding of these proteins to RNA 3' ends is not interchangeable.

How many roles do Lsm complexes play in eukaryotic cells? In bacteria, the Sm-like protein Hfq binds a myriad of small

RNAs, stabilizing them from degradation, and facilitating basepairing between many of these RNAs and their targets. Hfq is also implicated in the translation and degradation of several mRNAs (reviewed by Masse *et al.*, 2003). In yeast, Lsm2–Lsm8 and Lsm1–Lsm7 function in U6 metabolism and mRNA decay, respectively. Because abnormalities in pre-tRNA processing, rRNA processing and U3 snoRNA maturation occur on depletion of essential Lsm proteins, Lsm proteins have also been proposed to function in these processes (Kufel *et al.*, 2002, 2003a, 2003b). However, as overexpression of U6 snRNA suppresses many of the detected changes in processing (Figure 7), these defects may be secondary to the decline in U6 snRNA levels. Nonetheless, our finding that Lsm2–Lsm7 associate with about half the snR5 in yeast, coupled with the finding that *Xenopus* Lsm proteins bind U8 RNA (Tomasevic and Peculis, 2002), implies an additional function for these proteins in the biogenesis or function of at least a subset of nucleolar RNAs.

What role might Lsm proteins play in snoRNA metabolism? One possibility is that Lsm proteins assist specific snoRNAs in basepairing with their rRNA targets. This role would be consistent with the finding that Hfq assists RNA-RNA pairing (Moller *et al.*, 2002; Zhang *et al.*, 2002) and the fact that most small RNAs bound by Sm family members function by basepairing with other RNAs. In this scenario, the fact that rRNA pseudouridylation is unaffected by Lsm protein depletion may reflect redundancy between box H/ACA-specific proteins and Lsm proteins in assisting basepairing. However, although snR5 and U8 snoRNAs base pair with rRNA (Ganot *et al.*, 1997; Tomasevic and Peculis, 2002), basepairing has not been described between RNase P and pre-tRNAs. It is also unclear why snR5 and U8, and not the many other snoRNAs that base pair with rRNA, would require Lsm-assisted basepairing. One possibility is that the Lsm2–Lsm7 complex interacts transiently with many snoRNAs to facilitate basepairing, but associates stably with snR5, perhaps because the 3' end of snR5 contains a high-affinity site for Lsm protein binding.

A second possibility is that the Lsm2–Lsm7 complex assists in the biogenesis of snR5, RNase P, and perhaps additional snoRNAs. A role in snoRNA biogenesis is suggested by the finding that *LSM5* has genetic interactions with *SRP40*, a gene implicated in both box H/ACA and box C/D snoRNA biogenesis (Yang and Meier, 2003). Possible roles include localization or retention of these snoRNAs in nucleoli and facilitation of RNA maturation or RNP assembly.

Finally, the Lsm2–Lsm7 complex may assist snR5, and perhaps pre-RNase P, in functions that are distinct from their roles in rRNA pseudouridylation and pre-tRNA maturation. Consistent with this possibility, our experiments suggest that the Lsm-associated snR5 is at least partly nonoverlapping with the complex formed by snR5 RNA with the box H/ACA-specific proteins Gar1 and Nhp2. Moreover, the fact that Lsm proteins require the last 9 nt of snR5, which contain the conserved ACA box, hints that the binding of box H/ACA snoRNP proteins and Lsm proteins may be mutually exclusive. Interestingly, our gradient fractionations reveal that the Lsm-associated snR5 RNA sediments in complexes ranging from ~18S to greater than 25S, suggesting that additional proteins or RNAs are present. Identification of these other components may give insights into the role of the Lsm2–Lsm7/snR5 RNA complex in cell function.

ACKNOWLEDGMENTS

We thank Peter Takizawa for teaching us *in situ* hybridization; James Smith for technical assistance; Jean Beggs, David Brow, Jennifer Gallagher, Yves

Henry, Ed Hurt, and Yousif Shamoo for providing reagents; and Lara Sze-wczak and Kazio Tycowski for comments on the manuscript. This work was supported by National Institutes of Health Grant R01-GM48410. S.L.W. is an Associate Investigator of the Howard Hughes Medical Institute.

REFERENCES

- Achse, T., Brahms, H., Kastner, B., Bachi, A., Wilm, M., and Luhrmann, R. (1999). A doughnut-shaped heteromer of human Sm-like proteins binds to the 3'-end of U6 snRNA, thereby facilitating U4/U6 duplex formation in vitro. *EMBO J.* 18, 5789–5802.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1998). *Current Protocols in Molecular Biology*. New York: John Wiley & Sons.
- Bakin, A., and Ofengand, J. (1993). Four newly located pseudouridylate residues in *Escherichia coli* 23S ribosomal RNA are all at the peptidyltransferase center: analysis by the application of a new sequencing technique. *Biochemistry* 32, 9754–9762.
- Balakin, A.G., Smith, L., and Fournier, M.J. (1996). The RNA world of the nucleolus: two major families of small RNAs defined by different box elements with related functions. *Cell* 86, 823–834.
- Bouveret, E., Rigaut, G., Shevchenko, A., Wilm, M., and Seraphin, B. (2000). A Sm-like protein complex that participates in mRNA degradation. *EMBO J.* 19, 1661–1671.
- Diez, J., Ishikawa, M., Kaido, M., and Ahlquist, P. (2000). Identification and characterization of a host protein required for efficient template selection in viral RNA replication. *Proc. Natl. Acad. Sci. USA* 97, 3913–3918.
- England, T.E., Bruce, A.G., and Uhlenbeck, O.C. (1980). Specific labeling of 3' termini of RNA with T4 RNA ligase. *Methods Enzymol.* 65, 65–74.
- Evan, G.I., Lewis, G.K., Ramsay, G., and Bishop, J.M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* 5, 3610–3616.
- Fabrizio, P., Esser, S., Kastner, B., and Luhrmann, R. (1994). Isolation of *S. cerevisiae* snRNPs: comparison of U1 and U4/U6.U5 to their human counterparts. *Science* 264, 261–265.
- Ganot, P., Bortolin, M.L., and Kiss, T. (1997). Site-specific pseudouridine formation in preribosomal RNA is guided by small nucleolar RNAs. *Cell* 89, 799–809.
- He, W., and Parker, R. (2001). The yeast cytoplasmic Lsm1/Pat1p complex protects mRNA 3' termini from partial degradation. *Genetics* 158, 1445–1455.
- Ingelfinger, D., Arndt-Jovin, D.J., Luhrmann, R., and Achse, T. (2002). The human Lsm1–7 proteins colocalize with the mRNA-degrading enzymes Dcp1/2 and Xrn1 in distinct cytoplasmic foci. *RNA* 8, 1489–1501.
- Kiss, T. (2001). Small nucleolar RNA-guided post-transcriptional modification of cellular RNAs. *EMBO J.* 20, 3617–3622.
- Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K., and Schiebel, E. (1999). Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast* 15, 963–972.
- Kufel, J., Allmang, C., Petfalski, E., Beggs, J., and Tollervey, D. (2003a). Lsm Proteins are required for normal processing and stability of ribosomal RNAs. *J. Biol. Chem.* 278, 2147–2156.
- Kufel, J., Allmang, C., Verdone, L., Beggs, J., and Tollervey, D. (2003b). A complex pathway for 3' processing of the yeast U3 snoRNA. *Nucleic Acids Res.* 31, 6788–6797.
- Kufel, J., Allmang, C., Verdone, L., Beggs, J.D., and Tollervey, D. (2002). Lsm proteins are required for normal processing of pre-tRNAs and their efficient association with La-homologous protein Lhp1p. *Mol. Cell. Biol.* 22, 5248–5256.
- Longtine, M.S., McKenzie III, A., Demarini, D.J., Shah, N.G., Wach, A., Brachaet, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953–961.
- Lund, E., and Dahlberg, J.E. (1992). Cyclic 2',3' phosphates and nontemplated nucleotides at the 3' end of spliceosomal U6 small nuclear RNA's. *Science* 255, 327–330.
- Masse, E., Majdalani, N., and Gottesman, S. (2003). Regulatory roles for small RNAs in bacteria. *Curr. Opin. Microbiol.* 6, 120–124.
- Mathews, D.H., Sabina, J., Zuker, M., and Turner, D.H. (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J. Mol. Biol.* 288, 911–940.
- Mayes, A.E., Verdone, L., Legrain, P., and Beggs, J.D. (1999). Characterization of Sm-like proteins in yeast and their association with U6 snRNA. *EMBO J.* 18, 4321–4331.
- Moller, T., Franch, T., Hojrup, P., Keene, D.R., Bachinger, H.P., Brennan, R.G., and Valentin-Hansen, P. (2002). Hfq: a bacterial Sm-like protein that mediates RNA-RNA interaction. *Mol. Cell* 9, 23–30.
- O'Brien, C.A., and Wolin, S.L. (1994). A possible role for the 60 kd Ro autoantigen in a discard pathway for defective 5S ribosomal RNA precursors. *Genes Dev.* 8, 2891–2903.
- Pannone, B.K., Kim, S.D., Noe, D.A., and Wolin, S.L. (2001). Multiple functional interactions between components of the Lsm2-Lsm8 complex, U6 snRNA, and the yeast La protein. *Genetics* 158, 187–196.
- Pannone, B.K., Xue, D., and Wolin, S.L. (1998). 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. *EMBO J.* 17, 7442–7453.
- Pillai, R.S., Grimmer, M., Meister, G., Will, C.L., Luhrmann, R., Fischer, U., and Schumperli, D. (2003). Unique Sm core structure of U7 snRNPs: assembly by a specialized SMN complex and the role of a new component, Lsm11, in histone RNA processing. *Genes Dev.* 17, 2321–2333.
- Pillai, R.S., Will, C.L., Luhrmann, R., Schumperli, D., and Muller, B. (2001). Purified U7 snRNPs lack the Sm proteins D1 and D2 but contain Lsm10, a new 14 kDa Sm D1-like protein. *EMBO J.* 20, 5470–5479.
- Rader, S.D., and Guthrie, C. (2002). A conserved Lsm-interaction motif in Prp24 required for efficient U4/U6 di-snRNP formation. *RNA* 8, 1378–1392.
- Salgado-Garrido, J., Bragado-Nilsson, E., Kandels-Lewis, S., and Seraphin, B. (1999). Sm and Sm-like proteins assemble in two related complexes of deep evolutionary origin. *EMBO J.* 18, 3451–3462.
- Schneider, B.L., Seufert, W., Steiner, B., Yang, Q.H., and Fletcher, A.B. (1995). Use of polymerase chain reaction epitope tagging for protein tagging in *Saccharomyces cerevisiae*. *Yeast* 11, 1265–1274.
- Seto, A.G., Zaug, A.J., Sobel, S.G., Wolin, S.L., and Cech, T.R. (1999). *Saccharomyces cerevisiae* telomerase is an Sm small nuclear ribonucleoprotein particle. *Nature* 401, 177–180.
- Sherman, F. (1991). Getting started with yeast. *Methods Enzymol.* 194, 3–21.
- Sheth, U., and Parker, R. (2003). Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. *Science* 300, 805–808.
- Srisawat, C., Houser-Scott, F., Bertrand, E., Xiao, S., Singer, R.H., and Engelke, D.R. (2002). An active precursor in assembly of yeast nuclear ribonuclease P. *RNA* 8, 1348–1360.
- Tharun, S., He, W., Mayes, A.E., Lennertz, P., Beggs, J.D., and Parker, R. (2000). Yeast Sm-like proteins function in mRNA decapping and decay. *Nature* 404, 515–518.
- Tollervey, D. (1987). A yeast small nuclear RNA is required for normal processing of pre-ribosomal RNA. *EMBO J.* 6, 4169–4175.
- Tomasevic, N., and Peculis, B.A. (2002). *Xenopus* LSm proteins bind U8 snoRNA via an internal evolutionarily conserved octamer sequence. *Mol. Cell. Biol.* 22, 4101–4112.
- Vidal, V.P., Verdone, L., Mayes, A.E., and Beggs, J.D. (1999). Characterization of U6 snRNA-protein interactions. *RNA* 5, 1470–1481.
- Watkins, N.J., Gottschalk, A., Neubauer, G., Kastner, B., Fabrizio, P., Mann, M., and Luhrmann, R. (1998). Cbf5p, a potential pseudouridine synthase, and Nhp2p, a putative RNA-binding protein, are present together with Gar1p in all H BOX/ACA-motif snoRNPs and constitute a common bipartite structure. *RNA* 4, 1549–1568.
- Will, C.L., and Luhrmann, R. (2001). Spliceosomal UsnRNP biogenesis, structure and function. *Curr. Opin. Cell Biol.* 13, 290–301.
- Wolin, S.L., and Cedervall, T. (2002). The La protein. *Annu. Rev. Biochem.* 71, 375–402.
- Xue, D., Robinson, D.A., Pannone, B.K., Yoo, C.J., and Wolin, S.L. (2000). U snRNP assembly in yeast involves the La protein. *EMBO J.* 19, 1650–1660.
- Yang, P.K., Rotondo, G., Porras, T., Legrain, P., and Chanfreau, G. (2002). The Shq1p-Naf1p complex is required for box H/ACA small nucleolar ribonucleoprotein particle biogenesis. *J. Biol. Chem.* 277, 45235–45242.
- Yang, Y., and Meier, U.T. (2003). Genetic interaction between a chaperone of small nucleolar ribonucleoprotein particles and cytosolic serine hydroxymethyltransferase. *J. Biol. Chem.* 278, 23553–23560.
- Yisraeli, J.K., and Melton, D.A. (1989). Synthesis of long, capped transcripts *in vitro* by SP6 and T7 RNA polymerases. *Methods Enzymol.* 180, 42–50.
- Yoo, C.J., and Wolin, S.L. (1997). The yeast La protein is required for the 3' endonucleolytic cleavage that matures tRNA precursors. *Cell* 89, 393–402.
- Zhang, A., Wassarman, K.M., Ortega, J., Steven, A.C., and Storz, G. (2002). The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. *Mol. Cell* 9, 11–22.