Requirements for nuclear localization of the Lsm2-8p complex and competition between nuclear and cytoplasmic Lsm complexes

Michael P. Spiller*,†, Martin A. M. Reijns* and Jean D. Beggs§

Wellcome Trust Centre for Cell Biology, University of Edinburgh, King’s Buildings, Mayfield Road, Edinburgh, EH9 3JR, UK

*These authors contributed equally to this work
†Present address: Faculty of Life Sciences, Michael Smith Building, University of Manchester, Oxford Road, Manchester, M13 9PT, UK
§Author for correspondence (e-mail: jbeggs@ed.ac.uk)

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Summary
Sm-like (Lsm) proteins are ubiquitous, multifunctional proteins that are involved in the processing and/or turnover of many RNAs. In eukaryotes, a hetero-heptameric complex of seven Lsm proteins (Lsm2-8) affects the processing of small stable RNAs and pre-mRNAs in the nucleus, whereas a different hetero-heptameric complex of Lsm proteins (Lsm1-7) promotes mRNA decapping and decay in the cytoplasm. These two complexes have six constituent proteins in common, yet localize to separate cellular compartments and perform apparently disparate functions. Little is known about the biogenesis of the Lsm complexes, or how they are recruited to different cellular compartments. We show that, in yeast, the nuclear accumulation of Lsm proteins depends on complex formation and that the Lsm8p subunit plays a crucial role. The nuclear localization of Lsm8p is itself most strongly influenced by Lsm2p and Lsm4p, its presumed neighbours in the Lsm2-8p complex. Furthermore, overexpression and depletion experiments imply that Lsm1p and Lsm8p act competitively with respect to the localization of the two complexes, suggesting a potential mechanism for co-regulation of nuclear and cytoplasmic RNA processing. A shift of Lsm proteins from the nucleus to the cytoplasm under stress conditions indicates that this competition is biologically significant.

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Key words: Lsm proteins, Nuclear localization, Yeast

Introduction
The Sm-like (Lsm) proteins were identified in Saccharomyces cerevisiae by their sequence similarity to the canonical Sm proteins (Cooper et al., 1995; Fromont-Racine et al., 1997; Séraphin, 1995). They are found throughout eukaryotes and related proteins are also present in archaeabacteria (Achsel et al., 1999; Mayes et al., 1999; Salgado-Garrido et al., 1999) and in eubacteria (Moller et al., 2002; Zhang et al., 2002). Eight Lsm proteins have been identified in S. cerevisiae. A ring-shaped hetero-heptameric complex of Lsm proteins 1-7 (Lsm1-7p) is involved in mRNA decapping and decay in the cytoplasm (Boeck et al., 1998; Bouveret et al., 2000; Tharun et al., 2000), whereas a different hetero-heptameric complex comprised of Lsm proteins 2-8 (Lsm2-8p) binds to the 3′ end of U6 snRNA, and is required for its stability (Achsel et al., 1999; Mayes et al., 1999; Pannone et al., 1998; Salgado-Garrido et al., 1999) and nuclear accumulation in yeast (Spiller et al., 2007). In addition, the Lsm2-8p complex facilitates incorporation of U6 snRNPs into U4/U6 snRNPs and U4/U6.U5 tri-snRNPs, and has been proposed to have a chaperone-like function in remodelling RNP particles (Verdone et al., 2004). The nuclear Lsm proteins were found to contribute to other RNA processing events, including the processing of pre-tRNAs, pre-snoRNAs and pre-rRNAs, and the degradation of pre-mRNAs (Kufel et al., 2003b; Kufel et al., 2004; Kufel et al., 2002; Kufel et al., 2003a; Watkins et al., 2004) (reviewed in Beggs, 2005). Furthermore, various nuclear Lsm proteins have been shown to interact with the U8 snoRNA in Xenopus laevis (Tomasevic and Peculis, 2002) and the snR5 snoRNA in S. cerevisiae (Fernandez et al., 2004). Because these RNAs are all nuclear and, with the exception of the U6 snRNA, their associations with the Lsm proteins are highly transient, this suggests that at least some of the Lsm proteins enter the nucleus separately from their target RNAs.

The mechanism of nuclear import of the Lsm2-Lsm8 proteins has not been systematically studied, although we recently showed that nuclear accumulation of Lsm8p requires Kap95p (Spiller et al., 2007). Production of recombinant human Lsm proteins in bacteria, followed by injection of these proteins into HeLa cells, showed that the pre-assembled LSM2-8 complex localized to the nucleus, whereas LSM8 injected by itself accumulated in the cytoplasm (Zaric et al., 2005). These results suggest that LSM8 nuclear import involves an unidentified nuclear-import signal that is only present when LSM8 interacts with other LSM2-8 subunits. In the case of the Sm proteins, the adjacent subunits, SmB, SmD1 and SmD3, are predicted to form a basic protuberance that might act as a nuclear-localization signal in the yeast and human Sm complexes (Bordone, 2000; Girard et al., 2004). Because the three paralogous yeast Lsm proteins, Lsm8p, Lsm2p and Lsm4p, also contain basic C-termini, they might form a nuclear-localization signal in a similar fashion. Genetic evidence supports an interaction between these three
components, because mutations in yeast LSM8 are suppressed by overexpression of LSM2 or LSM4 (Pannone et al., 2001).

Lsm1p-7p differs from Lsm2-8p by just one polypeptide, but is located in the cytoplasm (He and Parker, 2000). In S. cerevisiae, Lsm1p-Lsm7 proteins and a wide range of RNA decapping and decay factors have been shown to accumulate in cytoplasmic foci, named processing or P-bodies, but only under certain conditions (Sheth and Parker, 2003; Teixeira et al., 2005). In log-phase yeast cells, P-body components, including Lsm1-7p, are spread diffusely throughout the cytoplasm, but localize to foci that increase in number and size with increased cell density (Teixeira et al., 2005). In addition, P-body formation is increased by glucose deprivation, osmotic stress and ultra-violet radiation, suggesting that they form in response to stress. In these circumstances, the transcriptome of the cell changes to cope with the new conditions, and increased mRNA degradation occurs. P-bodies appear to be sites of RNA decapping and degradation (Sheth and Parker, 2003; Teixeira et al., 2005), and they are probably also sites of nonsense-mediated decay and translational repression (Bruno and Wilkinson, 2006; Sheth and Parker, 2006; Teixeira et al., 2005). Studies of lsm1Δ yeast indicate that Lsm1p is needed for accumulation of the other Lsm proteins in P-bodies (Tharun et al., 2005), but is not required for P-body formation (Sheth and Parker, 2003).

The existence of two Lsm complexes that differ by just one member, but which have different, even apparently opposing, functions (promoting RNA stability versus degradation), is remarkable, especially because the protein sequences of Lsm1p and Lsm8p are highly similar (Salgado-Garrido et al., 1999). The different functions of the complexes might, therefore, be determined by their localization, as was suggested by Tharun et al. (Tharun et al., 2005). It is not currently known what determines the cellular localization of Lsm complexes, although it has been suggested that both the nuclear and the P-body localizations require Lsm-complex formation (Ingelfinger et al., 2002; Tharun et al., 2005; Zarin et al., 2005). In the absence of Lsm1p, nuclear levels of Lsm2p and Lsm7p are slightly elevated (Tharun et al., 2005), indicating that Lsm1p might compete with Lsm8p for complex formation.

Here, we show that sequences within Lsm8p as well as formation of a heteromeric complex are important for the nuclear accumulation of the Lsm2-8p complex. Furthermore, we investigated competition between Lsm1p and Lsm8p by overexpression and depletions of these proteins. Imbalance between these two factors affects localization of other Lsm-complex members and, under some conditions, even shows deleterious effects on cell viability. Under normal physiological conditions, competition between Lsm1p and Lsm8p might provide a link between RNA processing events in the nucleus and mRNA degradation in the cytoplasm. We therefore investigated the effects of stress on nuclear localization of the Lsm7 and Lsm8 proteins. We observed a small shift of Lsm7p to the cytoplasm upon glucose deprivation, and a rapid and almost complete depletion of Lsm7p and Lsm8p from the nucleus after hyperosmotic shock. This effect occurred independently from Lsm1p; however, deletion of LSM1 inhibited subsequent recovery of nuclear Lsm protein levels. Taken together, our findings suggest that co-regulation of cytoplasmic and nuclear RNA processing events could be mediated through changes in sub-cellular Lsm protein levels.

Results

Lsm localizations

Yeast strains were constructed that produce 13myc-tagged Lsm1p, Lsm7p or Lsm8p (Fig. 1A), and immunofluorescence-microscopy results clearly show specific localization patterns for the Lsm proteins: Lsm1p was predominantly cytoplasmic (Fig. 1B, note the ‘holes’ in the immunofluorescence, highlighted by arrows, and the lack of colocalization of immunofluorescence with DAPI staining of nuclear DNA in the merged image), Lsm8p localized exclusively to the cell nucleus and Lsm7p was present throughout the cells. These results are consistent with the proposed existence of two Lsm complexes, a nuclear Lsm2-8p complex and a cytoplasmic Lsm1-7p complex.

Lsm proteins are actively imported through the nuclear pore

To investigate the mechanism of nuclear accumulation of Lsm proteins, a nup49-313 strain (a nuclear-pore mutant that affects protein import) (Doye et al., 1994) was constructed that produces 13myc-tagged Lsm1p, Lsm7p or Lsm8p. Because this mutation causes temperature-sensitive growth, the cultures were grown at 23°C and then shifted to 37°C (restrictive temperature) for 5 hours prior to processing for microscopic

Fig. 1. Localization of Lsm1p, Lsm7p and Lsm8p. (A) Western blot showing 13myc-tagged Lsm1p, Lsm7p and Lsm8p from yeast strains MPS1, MPS2 and MPS3, respectively. (B) Localization of Lsm1-13myc, Lsm7-13myc and Lsm8-13myc in strains MPS1, MPS2 and MPS3, respectively. Fixed cells were stained with anti-Myc antibodies followed by Cy3-conjugated secondary antibody (red in merge). Nuclei were stained with DAPI (green in merge). For clarity, single channel images of DAPI and of immunofluorescence are shown in greyscale. Arrows indicate ‘holes’ in the immunofluorescence at the positions of nuclei. Bars, 10 μm.
Following the shift to 37°C, Lsm7-13myc displayed a reduced signal in the nuclei of the nup49-313 cells compared with wild-type cells (Fig. 2A). This effect was clearer for Lsm8-13myc, which was no longer restricted to the nucleus in the nup49-313 cells at 37°C (Fig. 2B). To control for a non-specific effect of the nup49-313 mutation on the integrity of the nuclear membrane, U1 snRNA localization was investigated and showed no difference between wild-type and nup49-313 cells (Fig. 2C). In combination with our previous report showing that the nuclear accumulation of Lsm8p is importin β/Kap95p-dependent (Spiller et al., 2007), these results suggest that, although individual Lsm proteins are small (10-21 kDa), their nuclear accumulation probably depends on active import through the nuclear pores. This is in agreement with the report that the human LSM2-8 complex is actively imported into rat fibroblast nuclei (Zaric et al., 2005). In contrast to the effect of the nup49-313 mutation on Lsm protein localization, the temperature-sensitive xpo1-1 nuclear-export mutation showed no effect on the localization of Lsm1p, Lsm7p or Lsm8p at the restrictive temperature in otherwise wild-type cells (Fig. 2D). Thus, it seems that the cytoplasmic localization of Lsm1p is not a consequence of nuclear exclusion by continual active export from the nucleus, at least not through this export pathway. By contrast, the xpo1-1 mutation did show a clear effect on the localization of a control protein (NLS-NES-GFP), both under permissive and restrictive conditions (Fig. S1 in supplementary material) (Stade et al., 1997).

Nuclear localization of Lsm7p requires other Lsm proteins

As the only member of the Lsm2-8p complex that is exclusively nuclear, Lsm8p might be required for nuclear uptake or retention of the Lsm2-Lsm7 proteins. To investigate this, yeast cells that produce 13myc-tagged Lsm7p and in which LSM8 was under control of a galactose-inducible promoter (PGAL1) were grown to log phase in galactose then switched to glucose medium for 12 hours (long enough to deplete Lsm8p but not long enough to significantly deplete U6 snRNA) (Mayes et al., 1999), and Lsm7p localization was examined. In cells depleted of Lsm8p, Lsm7p levels were decreased in the nuclei (Fig. 3A, large arrow) and increased in cytoplasmic foci (probably P-bodies; small arrows). To test for the dependence of Lsm7p nuclear accumulation on the presence of other Lsm proteins, yeast strains were tested in which LSM3, LSM4 or LSM5 were expressed from the PGAL1 promoter (for unknown reasons it proved difficult or impossible to 13myc-tag Lsm7p in the PGAL-LSM2 strain) and these were shifted from galactose to glucose medium for 12 hours to metabolically deplete these proteins (Mayes et al., 1999), whereas lsm1/H9004 and lsm6/H9004 cells, which are heat-sensitive, were grown in glucose at the permissive temperature of 30°C. The nuclear localization of Lsm7-13myc was unaffected by the absence of Lsm1p (Fig. 3B). Depletion of
Lsm3p, Lsm4p, Lsm5p or Lsm6p appeared to reduce the nuclear Lsm7p signal slightly, as seen by decreased colocalization of Lsm7p with the DAPI stain, with depletion of the essential Lsm4 protein having the strongest effect, as seen by the hole in the immunofluorescence (Fig. 3B, arrow). Thus, nuclear localization of Lsm7p requires the presence of Lsm8p and is more efficient when most or all of the other components of the Lsm2-8p complex are present, but it is not affected by the absence of cytoplasmic Lsm1p.

Depletion of Lsm2p or Lsm4p disrupts Lsm8p localization

Because Lsm8p is normally nuclear, a defect in its localization is more readily detected. Therefore, to confirm that complex formation is required for Lsm2-8p nuclear localization, Lsm8p localization was investigated in strains in which Lsm2p and Lsm4p, its proposed neighbours in the Lsm2-8p ring, were depleted. This assignment is based on the organization of the Sm protein complex, in which SmB (closest in structure to Lsm8p) is flanked by SmD1 and SmD3 (most similar to Lsm2p and Lsm4p, respectively) (Kambach et al., 1999), and on genetic and yeast two-hybrid interactions between LSM2, LSM4 and LSM8 (Lehner and Sanderson, 2004; Pannone et al., 2001). 13myc-tagged Lsm8p localization was examined in wild-type (WT), PGAL-LSM2 and PGAL-LSM4 cells (Fig. 4).

Following depletion of Lsm2p or Lsm4p by growth in glucose medium for 12 hours (Mayes et al., 1999), there was still some nuclear signal for Lsm8p, but there was a dramatic increase in the level of signal in the cytoplasm. By contrast, absence of Lsm6p caused no visible change in Lsm8p localization. Overall, these results demonstrate that Lsm8p by itself does not accumulate in the nucleus, because loss of either of its proposed partners in the Lsm2-8p ring results in its delocalization. However, it seems that a complete Lsm2-8p complex is not essential, because lack of Lsm6p has no effect, which might be expected because Lsm6p is a non-essential protein (Mayes et al., 1999).

Lsm7p and Lsm8p delocalization is not caused by defective splicing

Depletion of Lsm2-Lsm8 proteins leads to decreased levels of U6 snRNA and an accumulation of pre-mRNA (Mayes et al., 1999). To investigate whether delocalization of Lsm7p and Lsm8p from the nucleus upon depletion of other Lsm proteins might be due to an indirect effect on pre-mRNA splicing, cells were depleted of SmD1 and localization of myc-tagged Lsm7p and Lsm8p was investigated (supplementary material Fig. S2A). Depletion of SmD1 had no effect on nuclear localization of either of these Lsm proteins, whereas northern blot analysis
showed decreased levels of U1 snRNA and an accumulation of pre-U3 RNA (supplementary material Fig. S2B).

**Lsm8p truncations affect its nuclear localization**

Because Lsm8p is important for the nuclear accumulation of other Lsm proteins, we performed deletion analysis on N-terminally GFP-tagged Lsm8 protein to investigate sequences in Lsm8p that might be responsible for its nuclear localization. Plasmids encoding the GFP-Lsm8 variants (Fig. 5A) were tested for their ability to support the growth of strain MPS11 (PGAL1-LSM8) on glucose (Table 1) and for localization (Fig. 5B). SmB, the member of the Sm protein family that is structurally most similar to Lsm8p, contains a functional nuclear-localization signal in its C-terminus (Bordonne, 2000). The C-termini of both proteins are basic in nature, containing a high number of lysine residues, which, in SmB as well as SmD1 and SmD3, are part of a nuclear-localization signal (NLS)-like motif (Bordonne, 2000). The effect of C-terminal truncations of Lsm8p on its nuclear localization was therefore tested. GFP–Lsm8-2 protein, an Lsm8 construct that lacks the extreme C-terminal 10 amino acids of Lsm8 (analogous to the lsm8-2 mutation) (Pannone et al., 1998), showed normal nuclear accumulation (Fig. 5Bi,ii), whereas Lsm8-313p, which lacks a further 14 residues at the C-terminus, was mislocalized (Fig. 5Biii). However, the Lsm8 C-terminus (aa65-109) alone, when fused to GFP, failed to accumulate in the nucleus (Lsm8C; Fig. 5Biv). Thus, unlike the C-terminus of SmBp (Bordonne, 2000), the C-terminus of Lsm8p, although apparently required for nuclear localization, is not by itself sufficient to determine nuclear localization.

Although the region of Lsm8p that is missing in Lsm8-313p contains six lysine residues, simultaneous mutagenesis of all six lysines to alanine (Lsm8-6A3; Fig. 5Bv) did not significantly affect nuclear accumulation of GFP-Lsm8p, supporting the conclusion that the C-terminus of Lsm8p does not behave like a classical NLS or a functional NLS-like motif. Truncation of 11 amino acids at the N-terminus (Lsm8/H9004N; Fig. 5Bvi), or endogenous Lsm8p plus 1-109 was detected with anti-myc antibodies, and Lsm7p was detected with anti-myc antibodies.

All the GFP-Lsm8 constructs were stably expressed, as shown by western blotting (and data not shown) using anti-GFP antibodies.

Table 1. Effects of various mutations on Lsm8p localization and function

| Construct       | Lsm8p residues | Localization   | Supports viability |
|-----------------|----------------|---------------|-------------------|
| GFP alone       | –              | Non-specific  | No                |
| GFP-Lsm8        | 1-109          | Mostly nuclear| Yes               |
| GFP-Lsm8-2      | 1-99           | Mostly nuclear| Yes               |
| GFP-Lsm8-313    | 1-85           | As GFP        | No                |
| GFP-Lsm8C       | 65-109         | As GFP        | No                |
| GFP-Lsm8-6A3    | K87,90,92,102,107,109A | Mostly nuclear| Yes               |
| GFP-Lsm8ΔN      | 12-109         | Weakly nuclear| No                |
| GFP-Lsm8ΔSm     | 1-51/65-109    | As GFP        | No                |
| GFP-Lsm8C       | 65-109         | As GFP        | No                |
| GFP-Lsm8 plus   | 1-109          | Weakly nuclear| N/A               |
deletion of residues 52-64, corresponding to the Sm2 motif that is required for Lsm-complex formation (Lsm8/H9004Sm; Fig. 5Bvii), caused these mutant proteins to localize throughout the cell, similar to GFP alone (Fig. 5Bix). This latter result emphasizes the importance of complex formation for nuclear localization of Lsm8p, because the Sm motif is crucial for inter-subunit interactions within the Lsm complexes.

To determine the effects of some of these Lsm8 mutations on complex formation, tagged proteins were precipitated with anti-GFP antibodies and co-precipitation of 13myc-Lsm7 (as a representative of the other members of the Lsm2-8p complex) was analyzed by western blotting. Lsm7-13myc co-precipitated with GFP–Lsm8-2p, albeit less efficiently than with full-length GFP-Lsm8p, whereas GFP-Lsm8ΔN and GFP–Lsm8-313 did not pull-down any Lsm7p (Fig. 5C), although the GFP-Lsm8 proteins were present in similar amounts in the respective extracts (Fig. 5D). Also, overexpression of the Lsm8ΔN and Lsm8-313 proteins (which do not by themselves support growth; Table 1) did not affect growth of cells containing wild-type Lsm8p (data not shown), a finding that is compatible with these mutant proteins being incapable of competing with wild-type Lsm8p for assembly into Lsm2-8p complexes. Thus, the nuclear accumulation of the GFP-Lsm8 (mutant) proteins correlated with their ability

![Fig. 6. Over-production of Lsm1p or Lsm8p has opposing effects on Lsm7p localization. (A) HA-tagged Lsm1p or Lsm8p was over-produced from plasmid pAEM80 or pAEM76, respectively, in strain MPS2 (Lsm7-13myc) grown in SDGal-Ura. Lsm7-13myc localization (red in merge) in wild type (WT; MPS2 carrying pBM125 empty vector), or with LSM1 or LSM8 overexpression (o/e LSM1 and o/e LSM8, respectively). Over-production of Lsm1p caused reduction of nuclear Lsm7p (large arrow) and its accumulation in foci (small arrows). Bar, 10 μm. (B) Lsm8p over-production does not affect Lsm1p levels. MPS1 was grown with pBM125 or pBM125-HA-LSM8 in SD-Ura (Glu) or SDGal-Ura (Gal). Total protein was separated by SDS-PAGE and the western blot was probed for Lsm1-13myc, HA-Lsm8 and α-Tubulin.](image)

![Fig. 7. Overexpression of LSM1 in an lsm8-1 background results in a severe slow-growth phenotype. (A) Growth curve of wild-type (WT; MPS2) and lsm8-1 (MPS17) strains with or without over-production of Lsm1p (1oe). Wild-type yeast with Lsm7p myc-tagged (MPS2) or lsm8-1 yeast with Lsm7p myc-tagged (MPS17) were transformed with either pBM125 (empty vector) or pAEM80 (PGAL1-HA-LSM1). All strains were grown in galactose-based media. (B) Northern blot showing the levels of U1 and U6 snRNAs in wild-type or lsm8-1 cells combined with over-production of Lsm1p. (C) Localization of Lsm7-13myc in wild-type and lsm8-1 strains, with or without over-production of Lsm1p. All strains were grown in galactose and the localization of Lsm7p was detected with anti-Myc antibodies. Representative cells are shown. Arrows indicate ‘holes’ in the immunofluorescence at the positions of nuclei. Bars, 10 μm.](image)
to associate with Lsm7p, which probably reflects their incorporation into a complete Lsm2-8p complex. Furthermore, in the presence of chromosomally encoded wild-type Lsm8p, plasmid-encoded GFP-Lsm8p shows weaker nuclear accumulation (Fig. 5Bviii). This is probably due to the Lsm8 protein being in excess over other members of the Lsm2-8p complex, and indicates that GFP-Lsm8p by itself does not accumulate in the nucleus. Taken together, these results strongly support the conclusion that the Lsm8 protein needs to interact with other Lsm proteins for its nuclear accumulation.

**Over-production of Lsm1p or Lsm8p has opposing effects on Lsm7p localization**

To investigate the effect of overproducing Lsm1p or Lsm8p, HA-tagged *LSM1* or *LSM8* was overexpressed from the *PGAL1* promoter in yeast cells in which *LSM7* was myc-tagged. Over-production of Lsm8p (confirmed by western blot; data not shown) caused an increase in the nuclear fraction of Lsm7p compared with control cells (Fig. 6A and Fig. 3A), whereas over-production of Lsm1p had the opposite effect, namely a reduced level of nuclear Lsm7p and accumulation of Lsm7p in cytoplasmic foci (Fig. 6A), similar to depletion of Lsm8p (Fig. 3A). The level of Lsm1p did not change with *LSM8* overexpression compared to no overexpression, as shown by western blot analysis (Fig. 6B).

Although Lsm8p is essential for viability, the *lsm8-1* mutation, which causes dramatically decreased levels of Lsm8p, is not lethal, resulting only in a weak growth phenotype (Pannone et al., 1998). Intriguingly, Pannone et al. (Pannone et al., 1998) showed that the *lsm8-1* mutation is synthetic lethal with deletion of *LHP1* (which encodes the yeast homolog of La, another U6 RNA-binding protein), and that the requirement for Lhp1p in an *lsm8-1* strain can be suppressed by low-copy overexpression of *LSM2* (Pannone et al., 2001). In contrast to the effect of *LSM2* overexpression, we found that overexpression of *LSM1* in *lsm8-1* cells (strain MPS17) resulted in a severe slow-growth phenotype, but had only a minor effect with wild-type cells (Fig. 7A). These results further indicate that Lsm1p and Lsm8p act in an antagonistic fashion. Because the Lsm2-8p complex is required for stability of the U6 snRNA, the level of...
U6 was examined in these yeast strains. When LSM1 was overexpressed in the lsm8-1 strain the U6 RNA level was reduced to only 10% of the wild-type level (normalized to the level of U1 snRNA; Fig. 7B), indicating that a high level of Lsm1p interferes directly or indirectly with the function of Lsm8p (in this case Lsm8-1p). Lsm7p localization was examined in the lsm8-1/LSM1 overexpression strain and was found to be almost entirely cytoplasmic, much of it in foci, and it was undetectable in the nucleus (Fig. 7C). Thus, the effect on the level of U6 snRNA of over-producing Lsm1p was most likely a consequence of reduced nuclear accumulation of the Lsm2-8p complex.

Lsm7p and Lsm8p are de-localized upon hyperosmotic shock

The existence of an apparent equilibrium between the Lsm1-7p and Lsm2-8p complexes raises the possibility that this balance might be altered in response to changing physiological conditions. Therefore, Lsm7-13myc localization was examined after glucose deprivation (galactose or YP medium) or osmotic shock (exposure to 1 M KCl). As expected, Lsm7p accumulated in P-bodies at 5-15 minutes after glucose deprivation or hyperosmotic shock (Fig. 8A). There was also an apparent reduction in nuclear Lsm7p, which was most striking following hyperosmotic shock. Hyperosmotic shock also led to quick and almost complete depletion of Lsm8p from the nucleus (Fig. 8B). To determine whether these effects were due to active export of Lsm proteins from the nucleus, their localization was examined after hyperosmotic shock in the temperature-sensitive xpo1-1 strain (Fig. 8A,B). The xpo1-1 strains showed an identical shift of Lsm7-13myc and Lsm8-13myc to the cytoplasm, indicating that these proteins are not actively exported by Xpo1p after osmotic shock. Because the half-life of Lsm7p is more than 2 hours (Fig. 8C; tested in a PGAL1-LSM7 strain, AEMY35) (Mayes et al., 1999), the reduced nuclear Lsm7p signal within 15 minutes of stress most probably represents movement of Lsm7p from the nucleus to the cytoplasm rather than failure of newly synthesized Lsm7p to enter the nucleus.

To rule out a non-specific effect of osmotic stress on nuclear protein localization, GFP-Lhp1 localization was examined before and after exposure to 1 M KCl (Fig. 8D). GFP-Lhp1 localizes exclusively to the nucleus, and concentrates in the nucleolus in some cells. No increase in cytoplasmic localization was detected 15 minutes after exposure to 1 M KCl. It did appear to localize to a smaller, more compact area, just like the DAPI-stained nuclear DNA. This is presumably due to an effect of osmotic stress on nuclear morphology and recovers after longer periods of time. By contrast, Lsm8-GFP showed delocalization similar to Lsm8-13myc, although somewhat less severe; interestingly, some cells showed accumulation of Lsm8-GFP at the nuclear periphery (Fig. S3 in supplementary material).

Lsm delocalization does not depend on Lsm1p, whereas relocation does

Delocalization of Lsm7 and Lsm8 proteins is reversible because nuclear levels return to normal after longer periods of incubation (Fig. 9A; Fig. S3 in supplementary material and data not shown). Recovery was more complete for lower salt concentrations and started around 30 minutes after first exposure (Fig. 9A). Exposure to 1 M sorbitol showed a similar effect on Lsm8 and Lsm7-13myc localization (data not shown). Other effects of hyperosmotic stress, including those on translation initiation, have previously been shown to occur...
within a similar time-frame and recovery was shown to depend on the Hog1p protein kinase (Uesono and Toh, 2002). We therefore investigated Lsm8 and Lsm7-13myc localization after hyperosmotic shock in hog1Δ strains (Fig. S4 in supplementary material and data not shown). Both delocalization and recovery were identical to that seen for the isogenic HOG1 strains, indicating that these effects are independent of this particular protein kinase. Because P-body localization of Lsm1-7p depends on Lsm1p (Tharun et al., 2005) and osmotic shock leads to accumulation of Lsm proteins in these cytoplasmic foci, we investigated whether nuclear delocalization of Lsm proteins is dependent on Lsm1p (Fig. 9B). Surprisingly, nuclear localization of Lsm8-13myc was slightly reduced in an lsm1Δ strain under normal growth conditions. This effect was even more apparent for GFP-Lsm8 expressed in the same strain background (data not shown). Delocalization of Lsm8-13myc from the nucleus was even stronger after hyperosmotic shock and failed to recover after up to 2 hours in YPD with 0.6 M NaCl, whereas some (incomplete) recovery occurred when cells were shifted back to YPD. These results might indicate that, in the lsm1Δ strain, Lsm8p might move to the cytoplasm to replace Lsm1p.

**Discussion**

In this work, factors that affect the localization of the Lsm7 and Lsm8 proteins were investigated in order to try to understand what determines the nuclear localization of the Lsm2-8p complex. The results show that Lsm8p is a determinant for the nuclear accumulation of Lsm7p, although they do not clearly distinguish between nuclear import and nuclear retention. However, considering the effect of the **nup49-313** determinant for the nuclear accumulation of Lsm7p, although Lsm2-8p complex formation or complex stability. 

Unexpectedly, a relatively small N-terminal deletion outside the Sm core motif also affected complex formation. In principal, this could be due to a direct or indirect effect on complex formation or complex stability.

These results strongly suggest that Lsm8p must interact with other Lsm proteins to form a nuclear-localization signal that is unlikely to be of the basic SV40 type of NLS. Whereas depletion of Lsm2p and Lsm4p, the presumed neighbours of Lsm8p in the Lsm2-8p complex, resulted in delocalization of Lsm8p, lack of the non-essential Lsm6p had no apparent effect. By contrast, the complete Lsm2-8p complex is required for nuclear accumulation of U6 snRNA (Spiller et al., 2007). Lsm6p and Lsm7p are non-essential; thus, in the absence of Lsm6p or Lsm7p, alternative Lsm complexes must form in which there are fewer subunits or in which Lsm6p and Lsm7p are replaced by other proteins (Verdone et al., 2004). However, because U6 snRNA is unstable and mislocalized in lsm6Δ and lsm7Δ strains, these alternative complexes are probably less-stably associated with U6 snRNA. Thus, nuclear retention of U6 snRNA requires its stable association with a complete Lsm2-8p complex. The normal occurrence of nuclear Lsm complexes that have different subunit compositions and that associate with other RNAs is possible. For example, the processing of pre-tRNAs, pre-snRNAs and pre-rRNAs is unaffected by lack of Lsm6p or Lsm7p, but depends on Lsm2, Lsm3, Lsm4, Lsm5 and Lsm8 (Kufel et al., 2003b; Kufel et al., 2002; Kufel et al., 2003a). The proposed existence of an Lsm2-7p complex that associates with snR5 in the nucleolus (Fernandez et al., 2004) is in apparent contradiction with our finding of Lsm8p requirement for nuclear localization. It seems possible that the full Lsm2-8p complex might interact with snR5, but that the Lsm8p epitope tag might be masked in the snR5 RNP.

Our results are compatible with the observation of Zaric et al. (Zaric et al., 2005) that an RNA-free LSM2-8 complex, which had been pre-assembled from recombinant human LSM proteins and injected into the cytoplasm of rat fibroblast cells, accumulated in nuclei, whereas LSM8 alone did not. Thus, the Lsm-2-8 assembly pathway might be conserved throughout eukaryotes.

Because all the Lsm proteins are small enough to diffuse into the nucleus, there might be a mechanism to retain the Lsm1-7p complex in the cytoplasm. In this scenario, if Lsm1p is present in excess, Lsm2-Lsm7 proteins will be cytoplasmic unless incorporated into the Lsm2-8p complex. This might explain why depletion of Lsm8p had the most-dramatic effect on the nuclear localization of Lsm7p, because lack of any of the other Lsm proteins probably affects formation of the cytoplasmic Lsm1-7p complex as well, thereby allowing some diffusion of free Lsm7p into the nucleus. This can also explain the strong antagonistic effect of over-producing either Lsm1p or Lsm8p, if each is competing for the same pool of cytoplasmic Lsm proteins rather than interacting with the available pools of cytoplasmic or nuclear Lsm proteins, respectively.

Although no increased nuclear accumulation of Lsm7p was observed in an lsm1 deletion strain, nuclear accumulation of Lsm7p might be limited by the level of Lsm8p. One of the major functions of Lsm2-8p is in U6 snRNP biogenesis and stability, and repression of **Lsm8** transcription stops cell growth (Mayes et al., 1999). However, the **lsm8-1** mutant strain, which has a low level of Lsm8 protein, has only a moderately reduced U6 snRNA level and no growth defect at 30°C, suggesting that the yeast cell requires only a low level of Lsm8p for survival. This could explain why overexpression of Lsm1p does not cause a significant growth defect despite the effect on Lsm7p localization. The fact that it requires both mutation of **Lsm8** and overexpression of **Lsm1** to see a severe effect on cell growth indicates that the yeast cell can absorb large reductions in Lsm2-8p complex without significant deleterious effects. Thus, the ability to survive severe alterations in the levels of Lsm complexes in these mutant strains suggests that wild-type cells might significantly alter the equilibrium in response to various stimuli and stresses without detrimental effects.

As shown here, causing stress by hyperosmotic shock, or to a lesser extent by glucose deprivation, leads to a rapid shift of Lsm proteins from the nucleus to the cytoplasm and localization of Lsm1-7p to P-bodies. However, this delocalization depends neither on P-body formation (no effect of hog1Δ), nor on the presence or action of Lsm1-7p in these foci (no effect of lsm1Δ). By contrast, Lsm1p appears to be essential for relocalization when cells recover from...
hyperosmotic shock, indicating that an intact Lsm1-7p complex is required. The activity of Lsm1-7p in RNA turnover might be required for cells to efficiently re-programme to cope with a sudden change in conditions.

Because the Lsm1 and Lsm8 proteins are rather similar in sequence, it is remarkable that they effect the different localizations of their constituent complexes. A more-extensive analysis of the mechanism of Lsm-complex localization will require a detailed examination of the roles of individual amino acids in these and other Lsm proteins, and is a focus of our ongoing investigations. In addition, there is the interesting question of why has the family of Lsm proteins evolved to form two competing complexes? The multiple functions of the Lsm proteins and their conservation throughout all organisms suggests that they evolved as general RNA/RNP chaperones, and that gene amplification produced the many Sm and Lsm proteins now found in eukaryotes, with their more specialized functions possibly being determined by their localization in the cell. Interestingly, no Lsm1 gene has been identified in the genomes of Trypanosoma brucei or Trypanosoma cruzi, and RNA-silencing experiments in T. brucei indicate that Lsm8 functions in both U6 stabilization and mRNA decay, suggesting that a single Lsm protein performs the roles of Lsm1 and Lsm8 (Liu et al., 2004). Thus, it seems likely that Lsm1 and Lsm8 have evolved from a common ancestral protein by gene duplication, and developed separate intracellular locations and functions. Having two or more Lsm complexes, with overlapping composition, involved in different metabolic processes, such as pre-mRNA splicing and degradation in the nucleus and mRNA degradation in the cytoplasm, allows scope for a regulatory link between these processes. For example, by transiently varying the relative levels of each Lsm complex, nuclear RNA degradation could be reduced while cytoplasmic RNA turnover is enhanced. This would allow a quick and efficient change in genome expression in response to a sudden change in environmental conditions. Our overexpression experiments show that such effects can be artificially created, and similar effects in cells under stress show that cells are able to respond by changing sub-cellular localizations of the Lsm1-7p and Lsm2-8p complexes, thus allowing co-regulation of nuclear and cytoplasmic RNA-processing events.

Materials and Methods

Yeast media, plasmids and strains

Yeast media and manipulations were as described previously (Sherman, 1991). YPGalA isYPD with 2% glucose replaced by 2% galactose. For overexpression in yeast and for GFP fusions, complete synthetic drop-out medium (SD) was used. A list of plasmids used is given in Table S1 in supplementary material, and yeast inositol requirements for GFP fusions, complete synthetic drop-out medium (SD) was used. For overexpression in yeast and for GFP fusions, complete synthetic drop-out medium (SD) was used.

Construction of epitope-tagged proteins

Thirteen copies of coding sequence for the c-myc epitope were integrated at the end of the LSm genes by homologous recombination in BMA38a or a mutant derivative, using PCR product amplified from pFA6a-13MyccamMX6 (Longtine et al., 1998) or pFA6a-13MyccamHphMX6 (derived from pFA6a-13MyccamMX6, our laboratory). Each PCR-amplified DNA contained the last 45 base pairs of LSm coding sequence, thirteen copies of the c-myc epitope, hygromycin- or kanamycin-resistance sequence, and 45 base pairs of downstream sequence. All wild-type tagged strains exhibited wild-type growth at 14, 23, 30 and 37°C.

GFP-tagged constructs

GFP-tagged genes were constructed by PCR amplification of the relevant part of LSm9, using primers containing XbaI and Clfl sites at their 5’ ends. The PCR products were cut with XbaI and Clfl and ligated into pGFP-N-FUS or pGFP-C-FUS (Niedenthal et al., 1996). Site-directed mutagenesis was performed (Costa et al., 1996) to delete the part of the LSm8 gene encoding the N-terminal 591 motif. Details of the GFP-Lsm8p fusions are given in Table 1.

Immunofluorescence and fluorescent in situ hybridization

For immunofluorescence, yeast cells grown to logarithmic phase in liquid medium were fixed for 40 minutes in 3% formaldehyde and spheroplasted for 30 minutes with Lyticase. Cells were incubated with anti-myc antibody (Abcam) overnight in 5% milk, and then for 2 hours with Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch). Mounting media containing DAPI (Vectashield) was added and cells were viewed using a Leica FV400 microscope.

For in situ hybridization, yeast cells in logarithmic growth phase in liquid culture were fixed, probed with fluorescently labelled oligodeoxynucleotides, stained with DAPI and imaged as described previously (Long et al., 1995; Samarsky et al., 1998).

Imaging was performed essentially as described (Carder et al., 1993; Samarsky et al., 1998) and images were captured using Leica FV4000 software (Scanalytics, Fairfax, VA) with a CH-250 16-bit, cooled CCD camera (Photometrics, Tucson, AZ) mounted on a Leica FV4000 fluorescence microscope (Leica). When images were restored, a three-dimensional data set, composed of 20 images separated by 200 nm in the axial direction, was acquired and deconvolved with an acquired point spread function using Leica Deblur software (Leica). Oligonucleotide probes for the U1 snRNA were labelled with a single fluorescent attached to the 5’ end and had the sequences 5’-ACACAAATTTAAGGGGTGTTCAACCTTCCTCCAGGCA-GAGAAGAAAAAGGCCAAAAAATGTTTA-3’ and 5’-AATCCTCGCT-AAAACATTAAGGCGCATGAGAAAATGTACAAAAGAATGCTC-TACAAAAG-3’.

Yeasts extracts, immunoprecipitation and western blotting

Whole-cell yeast extracts were prepared as described (Liu et al., 1995) from strain MPS111 carrying plasmid pMP58, pMP82-53 or pMP82-ΔN after growth in SD-Ura-Met plus 2% glucose for 12 hours. Immunoprecipitations were performed as described (Teigelkamp et al., 1995) using anti-GFP antibodies (Invitrogen). Pellets were resuspended in 20 μl loading buffer and run on a denaturing 4-12% SDS-PAGE gel, alongside 10% of the supernatants. Proteins were transferred to a nitrocellulose membrane and detected with HRP-conjugated mouse anti-Myc or anti-HA (Santa Cruz Biotechnology), rabbit anti-GFP (Invitrogen), or anti-α-tubulin antibodies (Sigma).

RNA extraction and northern blotting

Total RNA was extracted (Schmitt et al., 1990) from BMA38a and lsm8-1 cells carrying either pEMB0 or an empty vector that had been grown in SDGal-Ura medium to OD600 0.5. Purified RNA was fractionated in an 8% (19:1) denaturing acrylamide urea gel. After transfer to nitrocellulose, the membrane was probed with labelled DNA complementary to the U1 and U6 snRNAs (Cooper et al., 1995). Total RNA was similarly extracted from MPS7, MRY76 and MRY77 cells before and after growth in YPDA. The same probes for U1 and U6 snRNAs were used, as well as probes against U3A (5’-GTTTAGGGAGCTCATC-3’) and seR1 (5’-ATCCC-GCCGCCCTCCATAC-3’).

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References

Afzelius, B. A., Brans, H., Kastner, B., Bach, A., Wilm, M. and Lührmann, R. (1999). A donut-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.

Beggs, J. D. (2005). RNA processing and the Lsm proteins. Novartis Medal Lecture. Biochem. Soc. Trans. 33, 433-438.

Boeck, R., Lapeyre, B., Brown, C. E. and Sachs, A. B. (1998). Capped mRNA degradation intermediates accumulate in the yeast spb8-2 mutant. Mol. Cell. Biol. 18, 5062-5075.

Borner, T. (2000). Functional characterization of nuclear localization signals in yeast Sm proteins. Mol. Cell. Biol. 20, 7943-7954.

Bouveret, E., Rigaut, G., Shevchenko, A., Wilm, M. and Séraphin, B. (2000). An Sm-like protein complex that participates in mRNA degradation. EMBO J. 19, 1661-1671.

Bruno, I. and Wilkinson, M. F. (2006). P-bodies react to stress and nonsense. Cell 125, 1036-1038.

Carter, K. C., Bowman, D., Carrington, W., Fogarty, K., McNeil, J. A., Fay, F. S. and Lawrence, J. B. (1993). A three-dimensional view of pre-messenger RNA metabolism within the mammalian nucleus. Science 259, 1330-1335.

Nuclear localization of the Lsm2-8p complex 4319
Cooper, M., Parkes, V., Johnston, L. H. and Beggs, J. D. (1995). Identification and characterisation of U31p (Sdh23p): a novel U6 snRNA-associated protein with significant similarity to core proteins of small nuclear ribonucleoproteins. EMBO J. 14, 2066-2075.

Costa, G. L., Bauer, J. C., Mcgowan, B., Angert, M. and Weiner, M. P. (1996). Site-directed mutagenesis using a rapid PCR-based method. Methods Mol. Biol. 57, 239-248.

Doye, V., Weft, R. and Hurt, E. C. (1994). A novel nuclear pore protein Nup133p with distinct roles in poly(A)+ RNA transport and nuclear pore distribution. EMBO J. 13, 6062-6075.

Fernandez, C. F., Pannone, B. K., Chen, X., Fuchs, G. and Wolin, S. L. (2004). An Sm-like protein in Saccharomyces cerevisiae associates with the U6 small nuclear RNA. EMBO J. 23, 16066-16075.

Hoffman, M., A. and Li, X.-h., Uliel, S., Belahcen, M., Unger, R. and Michaeli, S. (2004). Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast. Yeast 12, 773-786.

Panone, B. K., Xue, D. and Wolin, S. L. (1998). A role for the yeast La protein in U6 snRNA assembly: evidence that the La protein is a molecular chaperone for RNA polymerase III transcripts. EMBO J. 17, 7442-7453.

Panone, 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.

Roy, J., Zheng, B. H., Rymond, B. C., and Woolford, Jr. J. L. (1995). Structurally related but functionally distinct yeast Sm core small nuclear ribonucleoprotein particle proteins. Mol. Cell. Biol. 15, 445-455.

Salgado-Garrido, J., Bragado-Nilsson, E., Kanadelos-Lewis, S. and Séraphin, B. (1999). Sm and Sm-like proteins assemble in two related complexes of deep evolutionary origin. EMBO J. 18, 3451-3462.

Samarsky, D. A., Fournier, M. J., Singer, R. H. and Bertrand, E. (1998). The snoRNA box C/D motif directs nuclear targeting and also couples snoRNA synthesis and localization. EMBO J. 17, 3747-3757.

Schmitt, M. E., Brown, T. A. and Trumpower, B. L. (1990). A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. Nucleic Acids Res. 18, 3901-3902.

Séraphin, B. (1995). Sm and Sm-like proteins belong to a large family: identification of proteins of the U6 as well as the U1, U2, U4 and U5 snRNPs. EMBO J. 14, 2089-2098.

Sherman, F. (1991). Getting started with yeast. Meth. Enzymol. 194, 3-21.

Sheth, U. and Parker, R. (2003). Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. Science 300, 753-755.

Sheth, U. and Parker, R. (2006). Targeting of aberrant mRNAs to cytoplasmic processing bodies. Cell 125, 1095-1109.

Spiller, M. P., Boon, K.-L., Reijns, M. A. M. and Beggs, J. D. (2007). The Lsm2-8 complex determines nuclear localization of the splicosomal U6 snRNA. Nucleic Acids Res. 35, 923-929.

Stade, K., Ford, C. S., Guthrie, C. and Weiss, K. (1997). Exportin-1 (Crn1p) is an essential nuclear export factor. J. Cell Biol. 139, 1041-1050.

Treigekamp, S., Newman, A. J. and Beggs, J. D. (1995). Extensive interactions of PRP8 protein with the 5' and 3' splice sites during splicing suggest a role in stabilization of exon alignment by U5 snRNA. EMBO J. 14, 2602-2612.

Triveira, D., Seth, U., Valencia-Sanchez, M. A., Brenques, M. and Parker, R. (2005). Processing bodies require RNA for assembly and contain nontranslating mRNAs. RNA 11, 371-382.

Thorun, S., He, W., Mayes, A. E., Lennerz, P., Beggs, J. D. and Parker, R. (2000). Yeast Sm-like proteins function in mRNA decapping and decay. Nature 404, 515-518.

Thorun, S., Muhlrud, D., Chowdhury, A. and Parker, R. (2005). Mutations in the Saccharomyces cerevisiae LSM1 gene that affect mRNA decapping and 3' end protection. Genetics 167, 33-46.

Tomasevic, N. and Peculis, B. A. (2002). Xenopus LSm proteins bind U8 snRNA via an internal evolutionarily conserved octamer sequence. Mol. Cell. Biol. 22, 4101-4112.

Uesono, Y. and Toh, E. (2002). Transient inhibition of translation initiation by osmotic stress. J. Biol. Chem. 277, 13483-13485.

Verdone, L., Galardi, S., Page, D. and Beggs, J. D. (2004). Lsm proteins promote regeneration of pre-mRNA splicing activity. Curr. Biol. 14, 1487-1491.

Watson, N. J., Lommel, I., Ingelfinger, D., Schneider, C., Hossbach, M., Urbahl, H. and Lührmann, R. (2004). Assembly and maturation of the U3 snRNP in the nucleolus in a large dynamic multiprotein complex. Mol. Cell 16, 789-798.

Zaric, B., Chami, M., Remigy, H., Engel, A., Ballmer-Hofer, K., Winkler, F. K. and Kambach, C. (2003). Reconstitution of two recombinant LSm protein complexes reveals aspects of their architecture, assembly, and function. J. Biol. Chem. 280, 16066-16075.

Zhang, A., Wassarman, K. M., Ortega, J., Steven, A. C. and Storz, G. (2002). The Sm-like Hfq protein increases OxysR RNA interaction with target mRNAs. Mol. Cell. 9, 11-22.