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A Sm-like protein complex that participates in mRNA degradation

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In eukaryotes, seven Sm proteins bind to the U1, U2, U4 and U5 spliceosomal snRNAs while seven Sm-like proteins (Lsm2p–Lsm8p) are associated with U6 snRNA. Another yeast Sm-like protein, Lsm1p, does not interact with U6 snRNA. Surprisingly, using the tandem affinity purification (TAP) method, we identified Lsm1p among the subunits associated with Lsm3p. Coprecipitation experiments demonstrated that Lsm1p, together with Lsm2p–Lsm7p, forms a new seven-subunit complex. We purified the two related Sm-like protein complexes and identified the proteins recovered in the purified preparations by mass spectrometry. This confirmed the association of the Lsm2p–Lsm8p complex with U6 snRNA. In contrast, the Lsm1p–Lsm7p complex is associated with Pat1p and Xrn1p exoribonuclease, suggesting a role in mRNA degradation. Deletions of *LSM1*, *6*, *7* and *PAT1* genes increased the half-life of reporter mRNAs. Interestingly, accumulating mRNAs were capped, suggesting a block in mRNA decay at the decapping step. These results indicate the involvement of a new conserved Sm-like protein complex and a new factor, Pat1p, in mRNA degradation and suggest a physical connection between decapping and exonuclease trimming.

Keywords: mRNA turnover/Pat1p/TAP/U6 snRNA/Xrn1p

Introduction

Sm and Sm-like proteins belong to a family of polypeptides present in eukaryotes and in archaebacteria (Séraphin, 1995; Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999). These small proteins (8–28 kDa) share a common domain called the Sm domain, sometimes followed by variable C-terminal extensions (Hermann *et al.*, 1995; Séraphin, 1995). The structures of two dimers of human Sm proteins (D_3B and D_1D_2), determined by X-ray crystallography (Kambach *et al.*, 1999), confirmed that the Sm domains from different proteins adopt the same fold.

Seven Sm proteins (B, D_1, D_2, D_3, E, F and G) from organisms as divergent as yeast and human are associated with a conserved sequence present in the U1, U2, U4 and U5 snRNAs (Branlant *et al.*, 1982). These proteins play a role in the biogenesis of the corresponding snRNPs. They associate with the U snRNAs in the cytoplasm, trigger snRNA cap hypermethylation and are part of the snRNP nuclear import signal (Mattaj *et al.*, 1993a; Plessel *et al.*, 1994). Yeast Sm proteins are also required for the stable accumulation of the associated U snRNAs *in vivo* (Rymond, 1993; Roy *et al.*, 1995; Bordonne and Tarassov, 1996). Additional snRNAs associated with Sm proteins include U7, U11, U12, $U4_{ATAC}$, X8, and virally encoded snRNAs in mammals (Lee *et al.*, 1988; Yu *et al.*, 1996; Tarn and Steitz, 1997), and the telomerase RNA in yeast (Seto *et al.*, 1999).

In yeast and in human, the seven Sm-like proteins Lsm2–Lsm8 have been shown to be associated with U6 snRNA in the free U6 snRNP, the U4/U6 complex and the U4/U6·U5 tri-snRNP (Achsel *et al.*, 1999; Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999). Analysis of mutant yeast strains revealed that Lsm2p–Lsm8p are required for U6 snRNA accumulation, supporting a role for these proteins in U6 snRNP biogenesis and/or U6 snRNA stability (Pannone *et al.*, 1998; Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999). Consistently, Lsm proteins have been found in the recently purified U4/U6·U5 tri-snRNP in yeast (Gottschalk *et al.*, 1999; Stevens and Abelson, 1999). In both yeast and mammals, the Lsm2p–Lsm8p complex appears to associate with the 3′ end of U6 snRNA (Achsel *et al.*, 1999; Vidal *et al.*, 1999).

The similarity between Lsm2p–Lsm8p and the canonical Sm proteins extends beyond the presence of seven different subunits in each complex. Indeed, based on sequence comparisons, each of the seven Lsm2–Lsm8 proteins can be paired specifically with one of the seven Sm proteins, suggesting a common evolutionary origin and related internal arrangement of the complexes (Salgado-Garrido *et al.*, 1999). This is supported by electron microscopy observations which revealed that the Sm and Lsm2p–Lsm8p complexes have similar shape and size (Kastner *et al.*, 1992; Achsel *et al.*, 1999). Modeling studies using structural X-ray data and known protein–protein interactions, mapped biochemically and by two-hybrid analyses (Raker *et al.*, 1996; Fury *et al.*, 1997; Camasses *et al.*, 1998), suggest that Sm and Sm-like complexes are constituted of a heptameric ring of proteins whose size would be consistent with electron microscopy data (Kambach *et al.*, 1999).

Two additional Sm-like proteins exist in yeast, Lsm1p and Lsm9p–SmX1p–Mak31p, but they do not interact with U6 snRNA (Séraphin, 1995; Salgado-Garrido *et al.*, 1999). Lsm9p has been found to be in complex with Mak10p and Mak3p, which are involved in maintenance of the yeast killer plasmid by mediating protein modification (Wickner, 1996; Rigaut *et al.*, 1999). Lsm1p was shown to interact with some other Sm-like proteins by the two-hybrid approach and with Lsm4p by coprecipitation experiments (Mayes *et al.*, 1999). However, consistent with the fact that it does not coprecipitate U6 snRNA,

Lsm1p was not found in two different U4/U6·U5 tri-snRNP purifications (Gottschalk *et al.*, 1999; Stevens and Abelson, 1999). Furthermore, *LSM1* deletion does not affect either the level of U6 snRNA or splicing efficiency (Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999). Similarly, the human LSM1 homolog, CaSm (for 'cancer-associated Sm'; Schweinfest *et al.*, 1997) was not found in the human U4/U6·U5 tri-snRNP purification (Achsel *et al.*, 1999). Finally, it has been shown in yeast that a *lsm1* mutation suppressed a deletion of the poly(A) binding protein (PAB1) and stabilized mRNAs, suggesting that Lsm1p could be involved in mRNA degradation (Boeck *et al.*, 1998).

The general degradation pathway of mRNAs in yeast involves deadenylation of mRNAs, followed by Dcp1p-mediated decapping and 5′–3′ exonucleolytic degradation by Xrn1p (reviewed in Caponigro and Parker, 1996). Moreover, Dcp1p needs the presence of Dcp2p in order to be active in decapping (Dunckley and Parker, 1999). Several genes have been shown to be involved in the regulation of this general pathway. For example, mutations in MRT1 and MRT3 (Hatfield *et al.*, 1996) and in VPS16 (Zhang *et al.*, 1999) have been shown to block the decapping step. Mutants for *MRT4*, *GRC5* and *SLA2* genes are defective at an early step of mRNA decay, in deadenylation or decapping (Zuk *et al.*, 1999). Furthermore, some mutants in translation factors stimulate mRNA degradation (Schwartz and Parker, 1999). LSM1 appears to belong to a growing family of genes whose mutation affects decapping. The *lsm1* mutant accumulates full-length capped transcripts, but does not stabilize mRNAs containing premature nonsense codons. Since this mutation also suppresses the lethality conferred by a *PAB1* deletion (Boeck *et al.*, 1998), its phenotype appears very similar to those generated by mutations in the *MRT1* and *MRT3* genes (Hatfield *et al.*, 1996).

We have now extended our previous analysis of the yeast Sm-like proteins using biochemical purification and genetic analyses. We show that Sm-like proteins assemble in two related but distinct complexes. Lsm1p and Lsm8p are specific to each complex whereas Lsm2p–Lsm7p are common to the two complexes. Furthermore, the Lsm1p–Lsm7p complex is associated with Xrn1p and Pat1p and is involved in mRNA degradation.

Results

Purification of Lsm3p-interacting proteins by the TAP method

In order to characterize further Sm-like proteins in yeast, we purified proteins associated with Lsm3p using the tandem affinity purification (TAP) procedure recently developed in our laboratory (Rigaut *et al.*, 1999). Lsm3p was fused at its C-terminus with the TAP tag. This construction was under the control of the wild-type *LSM3* promoter, which maintains expression of the fusion protein at its natural level. Lsm3p-associated proteins purified from 2 l of yeast culture were concentrated, fractionated by SDS–PAGE and detected by Coomassie Blue staining (Figure 1A). This revealed the presence of ~20 proteins ranging in size from 8 to 250 kDa. It is noteworthy that their relative levels varied significantly. The various bands were excised from the gel and the proteins were identified by MALDI peptide mapping or nano-electrospray tandem mass spectrometry (Shevchenko *et al.*, 1996a; Wilm *et al.*, 1996b). Including the tagged Lsm3p, the seven Lsm proteins interacting with U6 snRNA (Lsm2p–Lsm8p) were identified in the recovered proteins (Figure 1A). These proteins migrated according to their predicted molecular weight, except for Lsm6p, which had a much lower apparent molecular mass than the predicted 14 kDa. However, the mass of one of the tryptic fragments showed that the second methionine in the predicted protein corresponds to the initiator amino acid, indicating that the open reading frame was erroneously predicted to include an N-terminal extension (Jacq *et al.*, 1997). This is supported by comparison of the yeast *LSM6* sequence with homologs from other organisms (e.g. the human hLSM6 protein; Salgado-Garrido *et al.*, 1999). Therefore, Lsm6p has a molecular mass of 9.3 kDa, consistent with its apparent molecular mass determined by SDS–PAGE. Several proteins known to be present in the U6 snRNP,

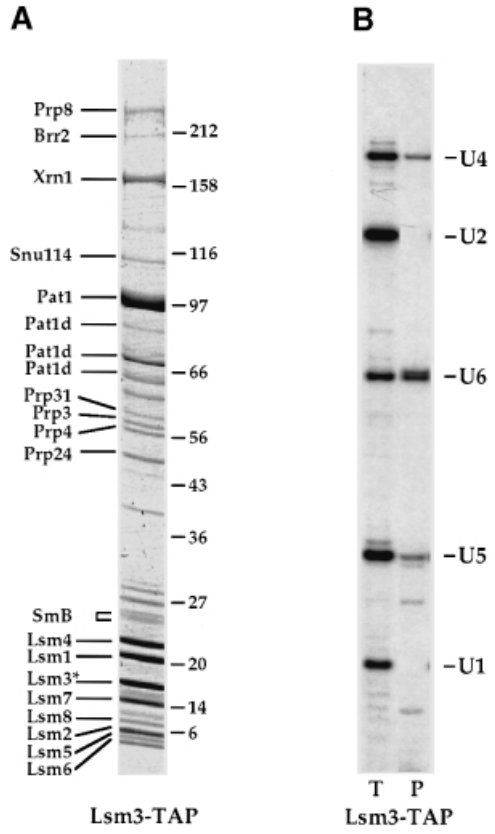

Fig. 1. Purification of the Lsm3p-interacting proteins. From 2 l of culture of Lsm3p–TAP-expressing strain, the Lsm3p-interacting proteins were purified by the TAP method (Rigaut *et al.*, 1999). (**A**) The purified material was fractionated on a 7–25% gradient SDS gel, which was then Coomassie Blue stained. Proteins identified either by MALDI or by nano-electrospray tandem mass spectrometry in this purification and/or similar purifications are indicated on the left. Some faint bands that were not reproducibly found in different purifications are not labeled. Molecular weight markers are indicated on the right. An asterisk after Lsm3 (Lsm3*) indicates that it still carries a part of the TAP tag. Pat1d stands for putative degradation products of Pat1. SmB was identified from a piece of gel containing the closely spaced bands indicated. (**B**) Total RNAs were recovered from the extract before purification (T) and from the purified fraction (P) and their U snRNAs content analyzed by primer extension. Signals corresponding to the different U snRNAs are indicated on the right. There is a 5-fold excess loaded for the purified fraction compared with the total RNAs.

U4/U6 di-snRNP or U4/U6·U5 tri-snRNP were identified: Prp8, Brr2, Snu114, Prp31, Prp3, Prp4, Prp24 and SmB (Figure 1A) (Lossky *et al.*, 1987; Banroques and Abelson, 1989; Bjorn *et al.*, 1989; Shannon and Guthrie, 1991; Lauber *et al.*, 1996; Lin and Rossi, 1996; Noble and Guthrie, 1996; Xu *et al.*, 1996; Anthony *et al.*, 1997; Fabrizio *et al.*, 1997; Weidenhammer *et al.*, 1997). This is consistent with the presence of Lsm3p in these three complexes (Salgado-Garrido *et al.*, 1999). We also used primer extension to analyze the levels of U snRNAs present in the purified fraction. The U4, U5 and U6 snRNAs were detected but not U1 and U2 snRNAs (Figure 1B), indicating that we recovered a mixture of U6 snRNP, U4/U6 di-snRNP and U4/U6·U5 tri-snRNP. Quantification of the signals present in the various bands indicated that 20% of total U6 snRNA was recovered compared with only 6% of U4 snRNA and 3% of U5 snRNA. Taken together with the distribution of the various snRNA in snRNP complexes (Séraphin, 1995), this reveals that we recovered an excess of U6 snRNP over multi-snRNP complexes.

Surprisingly, among the recovered proteins we also identified Xrn1p (= Sep1p, Kem1p), a major 5′–3′ exoribonuclease involved in mRNA decay (Hsu and Stevens, 1993), Pat1p, reported as a topoisomerase II-associated protein (Wang *et al.*, 1996) and Lsm1p, a Sm-like protein not associated with the U6 snRNA (Figure 1A). As judged from the Coomassie staining of the gel, these proteins were abundant and sometimes present at a higher level than the U6 snRNP-associated proteins, arguing that they were not contaminants. Pat1p was present in several bands that may represent degradation products or shorter forms of the protein. It is also noteworthy that the predicted molecular weight of Pat1p is 88 kDa, but that it runs aberrantly at ~97 kDa on SDS gels as described previously (Rodriguez-Cousino *et al.*, 1995). Interestingly, the level of the Lsm1–Lsm7 proteins appeared higher than the level of Lsm8p. Taken together with the proposed function of Lsm1p in mRNA decay (Boeck *et al.*, 1998) and the presence of Xrn1p, this suggested that Lsm3p was associated with a Lsm1p-containing complex involved in mRNA decay.

Lsm1p and Lsm8p belong to two distinct Lsm complexes that have six other Lsm proteins in common

The results presented above indicated that while Lsm1p was not associated with U6 snRNA, it was present in another complex containing at least one other Lsm protein: Lsm3p. The apparently higher levels of Lsm2–Lsm7 proteins compared with Lsm8p in the Lsm3p–TAP purified fraction suggested that additional Lsm proteins could be associated with Lsm1p. To address this possibility, we tested which of the Lsm1–Lsm8 proteins were coprecipitated with Lsm1p. For this purpose, we constructed all combinations of yeast strains expressing Lsm1p fused at its C-terminus with the calmodulin binding peptide (CBP) tag and a second Lsm protein fused to two IgG-binding modules from *Staphyloccoccus aureus* protein A (ProtA). Extracts were prepared from the various strains and Lsm1p-containing complexes were recovered following precipitation with calmodulin beads. The presence, or absence, of ProtA-tagged Sm-like proteins in the pellet

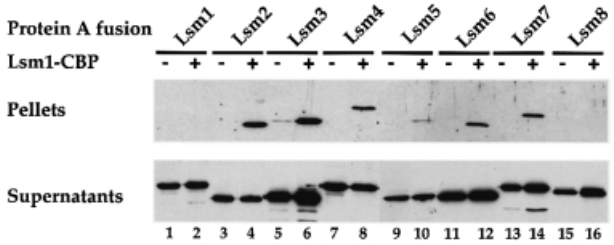

Fig. 2. Lsm1p is in a complex with the Sm-like proteins Lsm2p–Lsm7p. Extracts were prepared from strains expressing a CBP-tagged Lsm1 protein (even lanes) or wild-type Lsm1p (odd lanes) in addition to ProtA-tagged Sm-like proteins (see Materials and methods). After precipitation on calmodulin beads, the presence of coprecipitated ProtA-tagged Sm-like proteins with the Lsm1p–CBP fusion was assayed by 15% SDS–PAGE and Western blotting. Proteins present in the supernatants and in the pellets are shown. There is an 8-fold excess of pellet loaded compared with the supernatants.

was then assayed by Western blotting. To control for the specificity of the precipitation, we performed the same assay with cell extracts carrying ProtA-tagged proteins but expressing a wild-type (i.e. non-tagged) Lsm1p. We found that Lsm1p coprecipitated the Lsm2–Lsm7 proteins tagged with ProtA, but neither Lsm1p–ProtA nor Lsm8p–ProtA (Figure 2). In each case, the coprecipitation was specific, as no signals (or only a very weak signal for Lsm3p–ProtA) were detected in the control precipitations (Figure 2, odd lanes). We observed consistently that the Lsm5p–Lsm7p signals were weaker compared with the Lsm2p–Lsm4p signals (Figure 2, compare lanes 4, 6 and 8 with lanes 10, 12 and 14). This suggests that these proteins are not present in all complexes and/or are less tightly bound and therefore more easily lost during precipitation.

Using the same strategy with a Lsm8p–CBP fusion instead of Lsm1p–CBP, Salgado-Garrido *et al.* (1999) have shown that Lsm8p was in a complex with proteins Lsm2–Lsm7 but neither with itself nor with Lsm1p. These results, together with those in Figure 2, clearly demonstrate that Lsm1p and Lsm8p are part of two distinct complexes, each containing the six other Lsm2–Lsm7 proteins.

Purification of the two Sm-like protein complexes

To confirm that the Lsm1–Lsm8 proteins were part of two distinct complexes and to characterize their specific composition, we purified them using the TAP strategy. We fused the TAP tag at the C-terminus of Lsm1p and Lsm8p, as these were the predicted specific components of the two complexes, according to the coprecipitation results presented above. The strain expressing Lsm8p–TAP as the only source of Lsm8p grew like the wild-type parental strain (data not shown). The result of a TAP purification starting from 4 l of culture of this strain is shown in Figure 3A, lane 1. The proteins present in the purified fraction were identified by mass spectrometry. In addition to the proteins identified in the Lsm3p–TAP purification, we detected Snu66p and Prp6p. Prp6p was probably present in the previous purification but hidden by Pat1p with which it comigrates and which is much more abundant (Figure 1A). In the same vein, Snu66p is present at a low level (Figure 3A, lane 1) and was probably obscured by the shorter Pat1p degradation product in the Lsm3p–TAP purification (Figure 1A). These two proteins

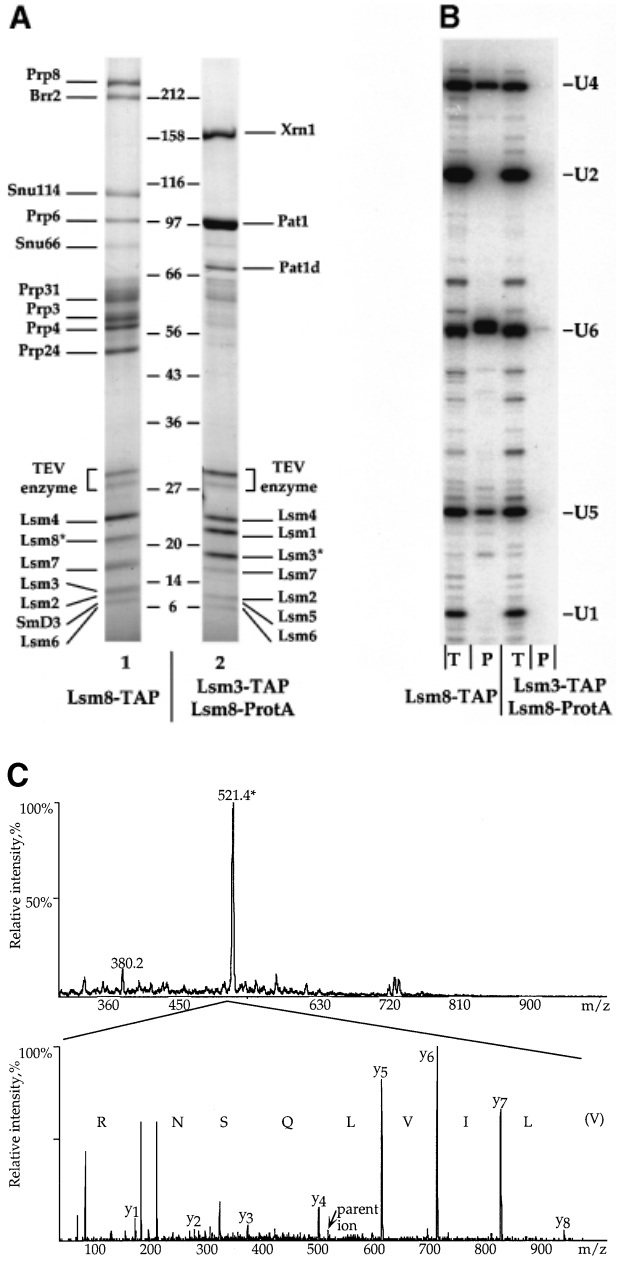

Fig. 3. Purifications of the Lsm1p- and Lsm8p-containing complexes. Extracts from strains expressing either Lsm8p–TAP or Lsm3–TAP/Lsm8–ProtA fusions were prepared from cultures of 4 l. The complexes were purified using the TAP method (Rigaut *et al.*, 1999). (**A**) Purified fractions were fractionated on a 7–25% gradient SDS gel. The figure shows the Coomassie Blue staining of this gel. The names of the proteins, which were identified either by MALDI or by nano-electrospray tandem mass spectrometry, are indicated on the sides. The molecular weight markers are indicated in the middle. The asterisk in Lsm8* and Lsm3* indicates that these proteins still carry part of the TAP tag. Pat1d stands for a putative degradation product of Pat1. Contaminants coming from the TEV preparation are indicated. (**B**) RNAs extracted from the extracts and the purifications were analyzed by primer extension for their U snRNA content. (T) Total RNAs in the extract before purification, (P) RNAs in the purified fraction. The different U snRNAs are indicated on the right. There is a 5-fold excess loaded for the purification fraction compared with the extract fraction. (**C**) Identification of Lsm5p in the Lsm1p-containing complex (A, lane 2) with tandem mass spectrometry. Two peptides of the protein were identified by comparison of the spectrum with a blank to distinguish them from autolysis products of the enzyme and common keratin peptides. Both peptides were fragmented and allowed independently the identification of Lsm5p (SwissProt P40089, hypothetical 10.4 kDa protein). One of the peptides identified and the corresponding spectra are shown.

were known, or have been shown while this work was in progress, to belong to the yeast U6, U4/U6 or U4/U6·U5 snRNPs (Abovich *et al.*, 1990; Gottschalk *et al.*, 1999; Stevens and Abelson, 1999). Importantly, Lsm1p was clearly absent.

The strain expressing Lsm1p–TAP had a slow growth phenotype and gave poor results in the purification experiment (data not shown). Therefore, we decided to use a TAP-based subtraction strategy to purify the Lsm1p complex. Briefly, we constructed a strain expressing simultaneously Lsm3p–TAP and Lsm8p–ProtA. Using extracts from this strain, the two Lsm complexes are specifically retained on the IgG beads since Lsm3p is common to both complexes (as demonstrated in Figure 1A). However, after cleavage of the TAP tag by the TEV protease, only the complex lacking Lsm8p–ProtA is released from the IgG beads, resulting in specific purification of the Lsm1p-containing complex. The purified material obtained from 4 l of yeast culture is shown in Figure 3A, lane 2. Protein subunits of the Lsm1p-associated complex were identified by mass spectrometry. The Lsm1–Lsm7 proteins were present, while Lsm8p was absent. This confirmed the existence of a second complex of seven Sm-like proteins. The Xrn1 and Pat1 proteins were specifically associated with this new Sm-like complex.

Interestingly, all the proteins that were identified in the original Lsm3p–TAP purification (Figure 1A) were found subsequently in the Lsm8p complex or in the Lsm1p complex (Figure 3A), confirming that the Lsm3p–TAP purified fraction contained a mixture of the two complexes. Both complexes presented no detectable cross-contaminations, showing that they are truly independent entities. However, co-immunoprecipitation and purification data indicate that they share the Lsm2–Lsm7 proteins.

We analyzed the presence of U snRNAs in both the Lsm8p–TAP and Lsm3p–TAP minus Lsm8p–ProtA purified fractions by primer extension. U4, U5 and U6 snRNAs were detected in the Lsm8p–TAP purified fraction in amounts and ratios identical to those of the Lsm3p–TAP purification (Figure 3B). In contrast, none of these RNAs were found in the Lsm1p-containing complex (Figure 3B).

Altogether, these results demonstrate that the yeast Sm-like proteins Lsm1–Lsm8 assemble in two complexes, one associated with the U6 snRNP and a second one, which contains Pat1p and Xrn1p, which is not tightly associated with spliceosomal U snRNAs.

Pat1p is associated with the Lsm1p complex and with Xrn1p

To confirm the association of Pat1p with Sm-like proteins Lsm1–Lsm7, we used the same approach as described above for Lsm1p. In this case, we used a strain expressing a Pat1–TAP protein, taking advantage of the CBP moiety of the TAP tag to assay for coprecipitation of ProtA-tagged Lsm proteins (Materials and methods). Lsm–ProtA fusion proteins that specifically coprecipitated with Pat1p–TAP on calmodulin beads were detected by Western blotting (Figure 4). Consistent with the purification results, ProtA-tagged Lsm1p–Lsm7p but not Lsm8p–ProtA were coprecipitated with Pat1p–TAP. As in the Lsm1p–CBP coprecipitation experiments, lower levels of Lsm5–Lsm7 than of Lsm1–Lsm4 fusion proteins were recovered (Figure 4, compare lanes 10, 12 and 14 with lanes 2, 4,

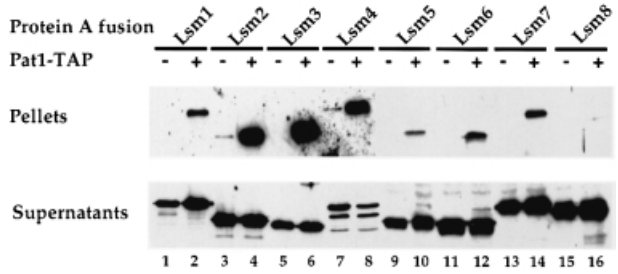

Fig. 4. Pat1p is in complex with the Sm-like proteins Lsm1p–Lsm7p. Extracts were prepared from strains expressing a TAP-tagged Pat1 protein together with ProtA-tagged Sm-like proteins (see Materials and methods). After precipitation of Pat1p–TAP on calmodulin beads, the presence of a coprecipitated ProtA-tagged Sm-like protein was assayed by 15% SDS–PAGE and Western blotting. Proteins present in the supernatants and the pellets are shown. There is an 8-fold excess of pellet loaded compared with the supernatant.

6 and 8). In all cases, the interactions were clearly specific (Figure 4, compare odd and even lanes).

To confirm interaction of Xrn1p with Pat1p, we tagged Xrn1p at its C-terminus with the TAP sequence and purified associated proteins using the TAP procedure. Pat1p was identified by mass spectrometry in a purified material recovered from 2 l of yeast culture, confirming the association between Xrn1p and Pat1p (data not shown).

Reporter mRNAs are stabilized in lsm1, 6, 7 and pat1 deletion mutants

The *LSM1* gene has been shown to be required for mRNA decapping while Xrn1p is the major exoribonuclease responsible for 5′–3′ degradation of mRNAs. Therefore, the presence of Xrn1p suggested strongly that the whole Lsm1p-containing complex was involved in mRNA degradation. To test this possibility and to determine which step of mRNA decay was affected, we decided to analyze mRNA decay in *lsm* and *pat1* mutants. Because the *lsm2–lsm4* disruptions are not viable (Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999), we selected *lsm1*, *lsm5*, *lsm6*, *lsm7* and *pat1* for further analysis. Disruptions were constructed using a *TRP1* marker from *Kluyveromyces lactis*. Consistent with our previous results (Salgado-Garrido *et al.*, 1999), deletion of *LSM1* or *LSM5–7* caused a slow growth phenotype that was exacerbated at 37°C (data not shown). Comparable results have been reported by others (Mayes *et al.*, 1999), except that the *LSM5* gene appeared essential in another strain background. Our *pat1*Δ strain was viable but with a slow growth phenotype at 30°C and lethality at 37°C, a phenotype consistent with previous reports (Rodriguez-Cousino *et al.*, 1995; Wang *et al.*, 1996).

To follow the effect of *lsm* or *pat1* deletion on mRNA degradation, we introduced the well-characterized reporter MFA2pG into the mutant strains and wild-type control. This reporter is under the control of the tightly regulated *GAL* promoter, which can be turned off by addition of glucose to the medium (Decker and Parker, 1993). The corresponding mRNA is rapidly degraded in wild-type yeast (half-lives from 3.5 to 6 min have been reported; Beelman *et al.*, 1996; Hatfield *et al.*, 1996; Boeck *et al.*, 1998). Furthermore, insertion of a poly(G) sequence in the mRNA 3′ UTR allows the detection of degradation intermediates because it blocks progression of the exoribonucleases. We followed the kinetics of decay of the reporter RNA *in vivo* in the wild-type strain, and in *pat1*, *lsm1* and *lsm5–7* mutant strains incubated at their highest permissive temperature. RNA extracted at various timepoints after blocking the reporter transcription by addition of glucose was fractionated on denaturing polyacrylamide gels and detected by Northern blotting (Figure 5). Quantitative analysis of the signals allowed the determination of the reporter mRNA half-life in the various mutant backgrounds. Compared with the wild-type strain, the reporter was greatly stabilized in *pat1*Δ, *lsm1*Δ, *lsm6*Δ and *lsm7*Δ strains but not in the *lsm5*Δ strain. Half-lives were increased by factors of 2.5–4.5 in these mutants. The result obtained for *LSM1* (half-life increased 3-fold) is in agreement with the result reported previously (Boeck *et al.*, 1998). We noticed that the amount of the major degradation intermediates, resulting from block of the 5′–3′ exonucleolytic trimming of the mRNA by the poly(G) sequence, was greatly reduced in all mutants except *lsm5*Δ, which behaved like the wild-type strain (Figure 5A). Interestingly, a shorter degradation intermediate, which has been described to result from a 3′–5′ degradation blocked by the poly(G) sequence (Boeck *et al.*, 1998), was detected in the mutants and wild-type strains at 37°C (Figure 5A). The level of this species did not appear to be affected by the *lsm* mutations. Another RNA species is detected in *lsm1*Δ, *lsm6*Δ and *lsm7*Δ mutants, which migrates slightly more quickly than the full-length transcript. This was already observed in *lsm1*Δ mutant and was proposed to result from a 3′–5′ degradation (Boeck *et al.*, 1998). In summary, the MFA2pG decay was affected in an identical manner in the *lsm1*Δ, *lsm6*Δ, *lsm7*Δ and *pat1*Δ strains. Overall, the results reveal a defect in degradation of mRNA by the 5′–3′ pathway but not by the 3′–5′ pathway.

To test whether this effect was substrate specific, we analyzed the degradation of another reporter (PGK1pG; Muhlrad *et al.*, 1995) in the *pat1*Δ and wild-type strains. The PGK1pG reporter was significantly stabilized in the *pat1*Δ mutant (data not shown). Our experiments show, therefore, that PAT1 was required for the normal degradation of both MFA2pG and PGK1pG mRNAs.

The involvement of Pat1p, Lsm6p and Lsm7p in the degradation of MFA2pG and the involvement of Pat1p in the degradation of PGK1pG confirmed that the Lsm1p–Lsm7p complex and associated Pat1 and Xrn1 proteins are involved in mRNA degradation.

mRNAs accumulating in the pat1Δ mutant are capped

It has been shown previously that Lsm1p is required for mRNA decapping (Boeck *et al.*, 1998). To test the presence of a cap on the accumulating MFA2pG transcript, we performed immunoprecipitation experiments using an antibody directed against the 7-methyl cap. We analyzed the *pat1*Δ mutant because the data reported above indicate that Lsm1p, Lsm6p, Lsm7p and Pat1p have highly similar, if not identical, phenotypes due to their presence in a multi-subunit complex involved in mRNA degradation. RNAs present in the input fraction as well as in the pellet and supernatant were fractionated by denaturing gel electrophoresis and detected by Northern blotting (Figure 6). As in the wild-type strain, full-length MFA2pG

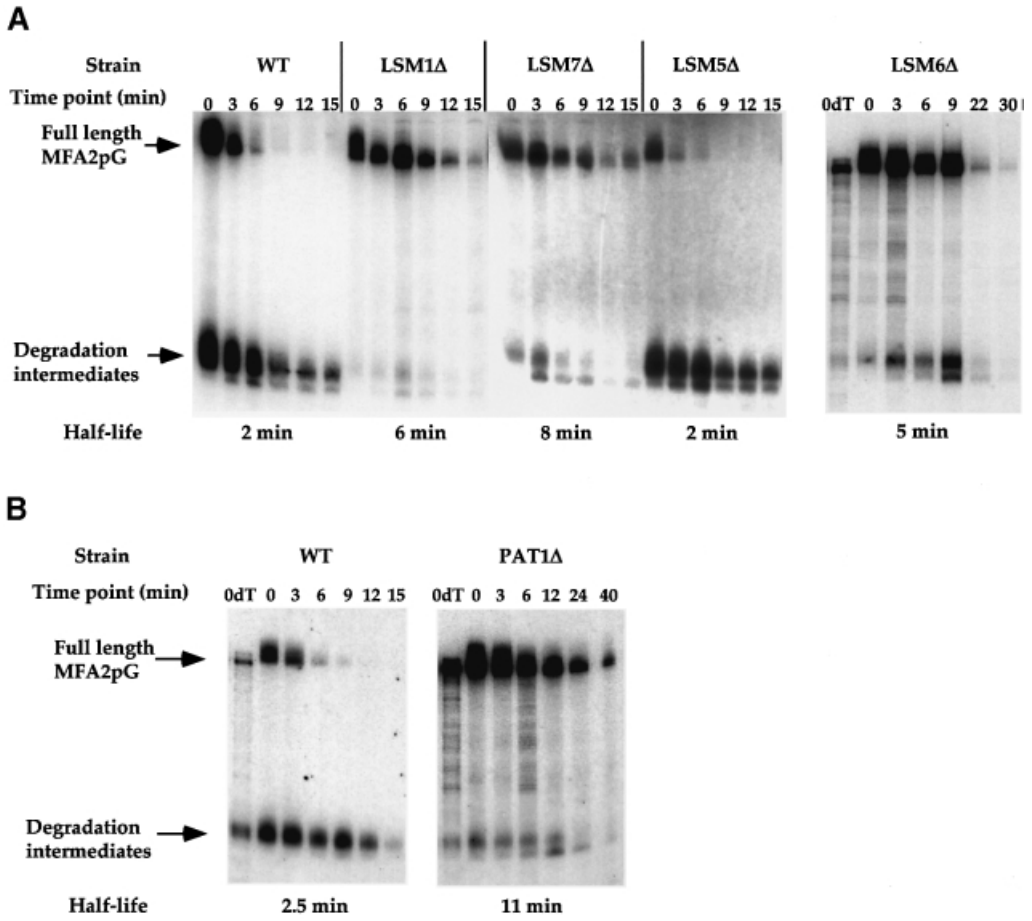

Fig. 5. MFA2pG mRNA is stabilized in *lsm1*, *6*, *7* and *pat1* null mutants. Wild-type *lsm1*, *5*, *6*, *7* and *pat1* null mutant yeast strains carrying the GAL1:MFA2pG reporter (RP485; Decker and Parker, 1993) were grown in 2% galactose-containing minimal medium to an OD_{600} of 0.4. Transcription was then repressed with 2% glucose at time 0. At the indicated time-points, cells were harvested, RNAs extracted and then analyzed on a 6% denaturing acrylamide gel and by Northern blotting using the oligo(C) probe bo29. As an internal loading standard, we used the oligonucleotide bo36 to detect the scR1 transcript (not shown). For the *lsm6*Δ, *pat1*Δ and wild-type strains, the samples from zero time-point were deadenylated (0dT) to serve as size marker for deadenylated species. The calculated half-life of the MFA2pG reporter in each mutant is indicated below the gels. (**A**) Wild-type, *lsm1*Δ, *lsm6*Δ, *lsm7*Δ and *lsm5*Δ strains grown at 37°C. (**B**) Wild-type and *pat1*Δ strains grown at 30°C.

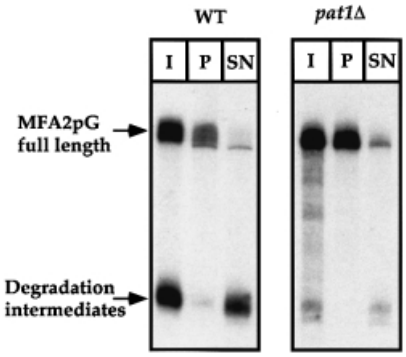

Fig. 6. mRNAs that accumulate in *pat1*Δ mutant are capped. RNAs from time-points 0 min for the wild-type strain and 6 min for the *pat1*Δ strain corresponding to Figure 5 were used. These RNAs were immunoprecipitated with an antiserum directed against the 7-methyl cap structure as described in Materials and methods and then analyzed by Northern blotting as described in Figure 5. I, input; P, pellet; SN, supernatant. The relative amounts loaded for each fraction are identical.

transcripts accumulating in the *pat1*Δ mutant 6 min after addition of glucose were recovered in the pellet fraction, indicating that they were capped. As a control, the major degradation intermediates were found in the supernatant, consistent with these degradation intermediates resulting from a 5′–3′ degradation abutting on the poly(G) sequence, and therefore lacking a cap (Decker and Parker, 1993). This demonstrates the specificity of the precipitation of full-length transcript. We conclude that full-length transcripts accumulating in the absence of Pat1p are capped. Furthermore, preliminary semi-quantitative studies indicate that in the *lsm1*Δ and *lsm6*Δ mutants a significant fraction of accumulating mRNAs was also capped (data not shown).

The nonsense-mediated decay pathway is not affected in pat1Δ and lsm7Δ strains

It was shown that decapping is separately (independently) regulated by Upf factors in the nonsense-mediated decay pathway and by the *LSM1* or *MRT1* gene in the normal degradation pathway (Beelman *et al.*, 1996; Boeck *et al.*, 1998). We examined whether *pat1*Δ and *lsm7*Δ mutations were also affecting the degradation of mRNAs containing a premature stop codon. We used the $PGK1^{NS}$pG reporter, which has been described to undergo rapid nonsense-mediated degradation (Muhlrad and Parker, 1994). We observed that the rate of degradation of the $PGK1^{NS}$pG reporter was the same in *pat1*Δ and *lsm7*Δ strains and in

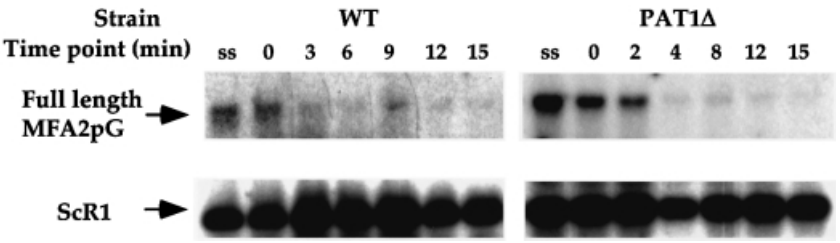

Fig. 7. The *pat1* null mutant is not affected in PGK1^{NS}pG mRNA degradation. The wild-type and *pat1*Δ strains carrying the GAL1:PGK1^{NS}pG reporter (RP611; Muhlrad and Parker, 1994) were grown in 2% galactose-containing minimal medium to an OD_{600} of 0.4 (lanes labeled ss for steady state). Transcription was then repressed with 2% glucose at time 0. At the indicated time-points, cells were harvested, RNAs extracted and then analyzed on a 1% formaldehyde–agarose gel and by Northern blotting using the oligo(C) probe bo29. As an internal loading standard, we used the oligonucleotide bo36 to detect the scR1 transcript.

the wild-type strain (Figure 7 and data not shown). This suggests that the Lsm1p-containing complex is not involved in the decapping of mRNAs in the nonsense-mediated decay pathway but is involved in the decapping of mRNAs in the general degradation pathway.

Discussion

In this study, we have shown that the yeast Sm-like proteins Lsm1–Lsm8 form two distinct complexes. One complex contains Lsm2p–Lsm8p, is present in the U6, U4/U6 and U4/U6·U5 snRNPs, and therefore associates directly or indirectly with several splicing factors. More importantly, we identified a second Sm-like complex containing the seven Lsm1–Lsm7 proteins, which is associated with Pat1p and Xrn1p. This organization of Sm-like proteins explains why Lsm1p was reported to be coprecipitated with Lsm4p but did not coprecipitate the U6 snRNA (Mayes *et al.*, 1999). Lsm2p–Lsm7p are thus participating in two distinct complexes and are therefore involved in two different cellular pathways, splicing and mRNA degradation. Given that these proteins have two nearly opposite functions, namely stabilization of the U6 spliceosomal snRNA (Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999) and mRNA degradation (Boeck *et al.*, 1998; this study), it will be of interest to determine which was their original primary role. It is noteworthy, however, that the number of different functions played by proteins from the Sm and Sm-like family is constantly growing. These proteins are also involved in maintenance of the killer plasmid in yeast (Lsm9p), probably by affecting protein modification (Rigaut *et al.*, 1999), in telomerase function (Sm proteins; Seto *et al.*, 1999), histone mRNA processing (Mattaj *et al.*, 1993b) and in mRNA decay (Lsm1p–Lsm7p; Boeck *et al.*, 1998; this study).

In our purifications, we identified essentially the same set of proteins as was recently reported from U4/U6·U5 tri-snRNP purification experiments (Gottschalk *et al.*, 1999; Stevens and Abelson, 1999; Figure 8). Like these two other groups, we did not identify Prp18p by mass spectrometry indicating that, at most, it is loosely associated with U6 snRNP-containing complexes. Because we purified a mixture of U6, U4/U6 and U4/U6·U5 snRNPs, with free U6 snRNP being by far the most abundant particle, some other proteins present specifically in multi-snRNPs could not be detected due to their low abundance and/or their comigration with more abundant proteins such as U6 snRNA-associated subunits (or the TEV protease). In contrast, we identified Prp24p. Its absence in the purified tri-snRNP (Gottschalk *et al.*, 1999; Stevens and Abelson, 1999) and its presence in our purified fraction is consistent with its specific association with the U6 snRNP (Ghetti *et al.*, 1995; Jandrositz and Guthrie, 1995). Interestingly, even though we used small volumes of yeast cultures, we identified Lsm7p and Lsm3p in the Lsm8p–TAP purification. While the first one was identified by one group (Gottschalk *et al.*, 1999), Lsm3p was missing in both reported large-scale tri-snRNP purifications (Gottschalk *et al.*, 1999; Stevens and Abelson, 1999). Taken together, the purification data confirm the presence of a heptameric Sm-like protein complex associated with the U6 snRNP (Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999).

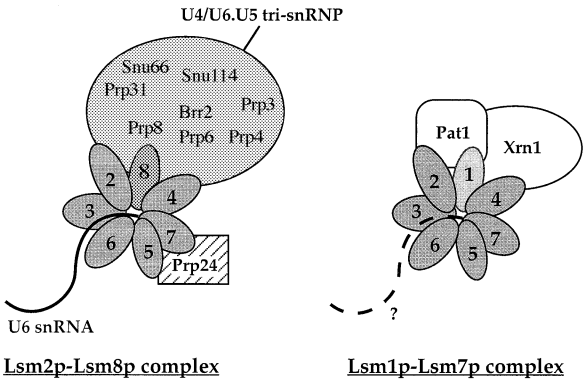

Fig. 8. Composition of the two Lsm complexes in yeast. Lsm protein organization characterized in the present study is summarized. Names of proteins are indicated, except for Lsm proteins, which are only indicated by their number. The order of Lsm proteins in the putative heptameric rings is based on similarity between Sm and Sm-like proteins (Salgado-Garrido *et al.*, 1999) and the model proposed for Sm proteins by Kambach *et al.* (1999). Interactions of non Sm-like proteins with the Sm-like complexes may be direct or indirect and are not known precisely (see Discussion). Prp24p is not a component of U4/U6·U5 tri-snRNP and therefore is shown separately.

Our study has shown the existence of a second complex of Sm-like proteins containing Lsm1p–Lsm7p and associated with Pat1p and Xrn1p. This complex was purified by a subtraction variant of the TAP procedure. The protein pattern differs significantly from the one observed as a result of the Lsm8p–TAP purification (Figure 3A). This indicates the effectiveness of the subtraction of the Lsm8p complex from the mixture of two complexes obtained with the Lsm3p–TAP purification. The Lsm1p–Lsm7p–Xrn1p–Pat1p complex is very likely to be cytoplasmic. Indeed, Xrn1p was described as a cytoplasmic protein, contrasting with the nuclear localiz-

ation of the related exoribonuclease Rat1p (Heyer *et al.*, 1995). Furthermore, Xrn1p was described to be associated with polysomes, consistent with a cytoplasmic localization (Mangus and Jacobson, 1999). Pat1p was also described as a cytoplasmic protein (Rodriguez-Cousino *et al.*, 1995). It remains possible that a fraction of the Lsm1p–Lsm7p complex could be localized in the cell nucleus, possibly not associated with Pat1p and/or Xrn1p. The Lsm1p–Lsm7p complex is likely to be organized like the Sm and Lsm2p–Lsm8p complexes, i.e. as a heptameric structure, with each subunit present in one copy according to the current model (Figure 8). This model is strengthened by prior phylogenetic analyses that indicated that Lsm1p was, like Lsm8p, related to SmB (Salgado-Garrido *et al.*, 1999). Our current results explain this situation as the two related Sm-like protein complexes differ only by the presence of Lsm1p or Lsm8p but are otherwise likely to assemble in an identical manner. In these complexes, Lsm1p and Lsm8p would therefore correspond to the SmB-related subunit. It is interesting to note that it is also SmB that is replaced by the SmN variant in canonical Sm complex found in neural tissue (McAllister *et al.*, 1989). However, the function of this specific substitution is currently unknown.

Our results indicate that the Lsm1p–Lsm7p complex is involved in mRNA degradation. However, several pathways of mRNA degradation exist and the Lsm1p-associated complex does not appear to be involved in the degradation of mRNA-containing premature nonsense codon (Boeck *et al.*, 1998 and this study). The function of the Lsm1p–Lsm7p complex in mRNA degradation is likely to be dispensable for the cell. Indeed, the proteins specifically present in the complex, Lsm1p and Pat1p, are not required for vegetative cell growth. This is not unexpected given the multiplicity of redundant mRNA degradation pathway and the non-essentiality of many factors involved in this process (e.g. Beelman *et al.*, 1996; Hatfield *et al.*, 1996; Jacobs *et al.*, 1998). In contrast, the related Lsm2p–Lsm8p complex required for pre-mRNA splicing by stabilizing U6 snRNA is probably essential given that Lsm8p is essential (Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999). It is therefore likely that Lsm2p, Lsm3p and Lsm4p are essential for the function of the U6 snRNA-associated complex but not for their role in the Lsm1p–Lsm7p complex involved in mRNA degradation. However, Lsm5p–Lsm7p are not required for yeast viability and are thus dispensable for the function of the U6 snRNA-associated complex (Salgado-Garrido *et al.*, 1999). This allowed us to test the phenotypes of these mutants together with *lsm1* and *pat1* mutants. This confirmed that Lsm1p was required for turnover of the MFA2pG reporter transcript (Boeck *et al.*, 1998) and demonstrated further that Lsm6p and Lsm7p were required for the same function (Figure 5A). However, the *lsm5* null mutant displayed no phenotype regarding mRNA decay. Similarly to the situation observed for the U6-associated complex, it is possible that all the Lsm proteins of the complex are not equally important or required for the mRNA decay function. Furthermore, the fact that Lsm5p was recovered in small amounts in coprecipitates with Lsm1p–CBP and Pat1p–TAP (Figures 2 and 4, lane 10) suggests that it may not be present in all complexes. However, this issue is complicated because of the involvement of Lsm5p in two complexes and because its phenotype appears dependent upon the strain background (Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999). Further studies will be required to clarify the involvement of Lsm5p in mRNA degradation.

We have identified Pat1p as an abundant subunit associated with the Lsm1p–Lsm7p complex and demonstrated that it is a new factor involved in mRNA decay. Our data do not prove that Pat1p interacts directly with any of the Lsm1–Lsm7 proteins. It could as well interact indirectly via Xrn1p. However, inspection of the Coomassie Blue-stained gels suggests that Pat1p is present in stoichiometric amount with Lsm proteins (Figure 3A, lane 2). Furthermore, given that Pat1p interacts with the Lsm1p–Lsm7p complex but not with the Lsm2p–Lsm8p complex (Figure 3A), it is likely that Pat1p interacts at least with Lsm1p as it is the only Lsm subunit specifically present in the interacting complex. Alternatively, Lsm8p may prevent interaction of Pat1p with the second complex. The *pat1* null mutant shows a slow growth phenotype, which is more marked at 37°C, consistent with published data (Rodriguez-Cousino *et al.*, 1995; Wang *et al.*, 1996). In the *pat1* null mutant, the MFA2pG reporter is greatly stabilized and remains in a capped state. Therefore, PAT1 has to be added to the growing list of genes whose mutation induces stabilization of capped mRNAs, which already includes the *MRT1*, *MRT3*, *LSM1* and *VPS16* genes (see Introduction). Pat1p was first described as a topoisomerase II-interacting protein, based on a two-hybrid assay. The phenotypes displayed by the *pat1*Δ strain were similar to those of a topoisomerase mutant (Wang *et al.*, 1996, 1999). However, it remains possible that the involvement of Pat1p in topoisomerase function is indirect and results from its role in mRNA decay.

What could be the function of the complex containing Lsm1p–Lsm7p, Pat1p and Xrn1p in mRNA degradation? Given that the two other related complexes (Sm proteins and Lsm2p–Lsm8p) interact with RNA (Lührmann *et al.*, 1990; Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999), it is likely that the Lsm1p–Lsm7p complex also interacts with RNA. It was previously proposed that Lsm1p could be an activator of decapping. Consistently, we found that degradation of mRNA in the *pat1* mutant was also blocked before decapping. Recently, it was shown that mutations in MRT1 or VPS16 block the decapping step by activating an inhibitor of Dcp1p or Dcp2p (Zhang *et al.*, 1999). Lsm1p and Pat1p may act in the same way. It is noteworthy that we did not find Dcp1p or Dcp2p in our purifications. Moreover, to test for a potential physical link between the Lsm1p–Lsm7p–Pat1p–Xrn1p complex and Dcp1p, we assayed for coprecipitation of Lsm proteins fused to ProtA with Dcp1p–TAP. These experiments failed to reveal a direct interaction between Dcp1p–TAP and the Sm-like protein complex (E.Bouveret and B.Séraphin, unpublished results). These results strengthen the hypothesis that the Lsm1p–Lsm7p–Pat1p–Xrn1p complex would not be necessary *per se* for the decapping activity but would more likely have a regulatory function. This is consistent with the observation that *lsm1* and *pat1* mutants are not affected in the nonsense-mediated decay. The presence of a nonsense codon would trigger decapping by a separate regulatory mechanism, which involves Upf factors (Ruiz *et al.*, 1996).

The presence of Xrn1p associated with a complex required for mRNA decapping is somewhat surprising. Xrn1p is a 5′–3′ exoribonuclease that functions after the decapping step. Indeed, in a *xrn1* null mutant, full-length mRNAs are stabilized, but these mRNAs are not capped (Hsu and Stevens, 1993). Therefore, Xrn1p cannot be strictly required for decapping. However, at least a fraction of the highly abundant Xrn1p (Heyer *et al.*, 1995) is (directly or indirectly) associated with Pat1p, since we identified Pat1p in the purified material from a Xrn1p–TAP-expressing strain (data not shown). What could be the function of a physical link between proteins acting before and after the decapping? An interesting model is that the Lsm1p–Lsm7p protein complex associated with Pat1p could recognize some features of mRNA [e.g. lack or short poly(A) tail] allowing their binding. The bound mRNA would then be directly or indirectly targeted for decapping. Through their association with Xrn1p, the Sm-like protein–Pat1p complex would ensure that the target RNA is immediately degraded following the decapping step. Experiments to test the validity of this model are in progress in our laboratory.

It is very likely that Sm-like protein–Pat1p–Xrn1p complexes involved in mRNA degradation similar to the one that we have observed in yeast will be found in other eukaryotes. Indeed, each of the Sm-like proteins from Lsm1p to Lsm8p have a homolog in human (Salgado-Garrido *et al.*, 1999). Consistent with the yeast data, the homolog of Lsm1p, CaSm, was not found in the human U4/U6·U5 purification (Achsel *et al.*, 1999), which suggests that it must have a distinct function. Moreover, proteins related to yeast Pat1p are also present in other species (Rother *et al.*, 1992; data not shown). Similarly, putative Xrn1p homologs are present in mouse (Bashkirov *et al.*, 1997) and other species (data not shown). MmXrn1p was demonstrated to be localized in the cytoplasm and involved in mRNA turnover. Furthermore, it complemented a *xrn1* yeast mutant (Bashkirov *et al.*, 1997). As potential homologs of all components of the yeast Lsm1p-associated complex are present in other eukaryotes, it is therefore likely that this complex, named LSM-1, has a conserved function in eukaryotic mRNA decay.

Materials and methods

Yeast plasmids and strains

Yeast strains were transformed with the Li-acetate method (Soni *et al.*, 1993). The 2μ plasmid encoding Lsm1p–CBP (pBS1751) was obtained by transferring the sequence of *LSM1* from pBS1298 (Salgado-Garrido *et al.*, 1999) in pRS425 (Christianson *et al.*, 1992) together with a C-terminal CBP tag (Stratagene). The centromeric plasmid encoding Lsm8p–TAP (pBS1608) was obtained by transferring the TAP tag sequence from pBS1479 (Rigaut *et al.*, 1999) and the Lsm8p sequence from pBS1302 (Salgado-Garrido *et al.*, 1999) in pRS415 (Christianson *et al.*, 1992). The centromeric plasmid encoding Lsm3p–TAP was obtained by transferring the TAP tag sequence and the Lsm3p sequence from pBS809 (Séraphin, 1995) in pRS415. The series of centromeric plasmids encoding the ProtA-tagged Lsm proteins was obtained as described in Salgado-Garrido *et al.* (1999) for 2μ plasmids but using the pRS415 backbone instead of pRS425 vector. pBS1783, pBS1788, pBS1786, pBS1784, pBS1785, pBS1782 and pBS1789 plasmids code, respectively, for Lsm1, Lsm3, Lsm4, Lsm5, Lsm6, Lsm7 and Lsm8 fusion proteins.

Gene disruption and tagging on the chromosome were performed using PCR fragments following a published strategy (Puig *et al.*, 1998). Integrations were checked by three different PCRs. The wild-type haploid strain used in all experiments is MGD453-13D (*MAT***a**, *ade2*, *arg4*, *leu2-3 112*, *trp1-289*, *ura3-52*). *PAT1*, *LSM1*, *LSM5*, *LSM6* and *LSM7* genes were disrupted with the *K.lactis TRP1* marker from plasmid pBS1408 (Caspary *et al.*, 1999) giving, respectively, strains BSY791, BSY845, BSY846, BSY847 and BSY844. Lsm1p, Pat1p and Xrn1p were tagged by inserting the sequence coding for the TAP tag downstream of the *LSM1*, *PAT1* or *XRN1* gene together with the *K.lactis TRP1* marker using plasmid pBS1479 as template (Rigaut *et al.*, 1999), giving, respectively, strains BSY745, BSY741 and BSY793. Lsm1p was also tagged with CBP using the *K.lactis TRP1*-containing plasmid pBS1512 as template (Caspary *et al.*, 1999), giving the strain BSY912. Lsm2p was tagged with ProtA using plasmid pBS1365 as template (Puig *et al.*, 1998), giving strain BSY629. The strain expressing Lsm3p–TAP (BSY785) contains a disrupted chromosomal copy of the LSM3 gene (Séraphin, 1995) complemented by the centromeric plasmid expressing Lsm3p–TAP. The strains expressing Lsm8p–TAP (BSY752) and Lsm8p–ProtA (BSY838) were obtained by transforming, respectively, pBS1608 and pBS1789 in BSY699 (diploid strain carrying a disrupted LSM8 allele; Salgado-Garrido *et al.*, 1999) followed by dissection. The strain expressing Lsm3p–TAP and Lsm8p–ProtA (BSY851) was obtained by inserting the sequence coding for the TAP tag downstream of the *LSM3* gene in the BSY838 strain.

Coprecipitations

Extracts were prepared as described previously (Séraphin, 1995). Coprecipitations of Lsm proteins with either Lsm1p–CBP or Pat1p–TAP were performed as described previously (Salgado-Garrido *et al.*, 1999) except that for the coprecipitation of Lsm2p–ProtA with Lsm1p–CBP, we used the strain BSY629 (carrying a chromosomal LSM2–ProtA fusion) transformed with pBS1751 (LSM1–CBP inserted in a 2μ plasmid). For the coprecipitation of Lsm2p–ProtA with Pat1–TAP, we used pBS826 instead (LSM2–ProtA inserted in a 2μ plasmid; Salgado-Garrido *et al.*, 1999).

Purifications

Lsm3p–TAP, Lsm8p–TAP and Xrn1p–TAP complexes were purified using the standard TAP procedure (Rigaut *et al.*, 1999). The Lsm1p-containing complex was purified by a subtractive method. Briefly, BSY851 strain expresses Lsm3p–TAP and Lsm8p–ProtA fusion proteins. In the first affinity step, all Lsm3p-containing complexes are purified. However, the Lsm8p complex is trapped on the IgG beads and is not released by the TEV cleavage. Thereby, only the Lsm1p-containing complex is recovered.

Purified proteins were TCA precipitated, the whole samples were loaded on a 7–25% exponential gradient SDS gel using PROTEAN II xi cell from Bio-Rad, and finally the gels were Coomassie Blue stained.

Protein bands were excised from the gel, reduced, alkylated and digested overnight with trypsin (Shevchenko *et al.*, 1996b; Wilm *et al.*, 1996a). The proteins were identified either by MALDI peptide mapping or nano-electrospray tandem mass spectrometry (Shevchenko *et al.*, 1996a; Wilm *et al.*, 1996b). The electrospray identification was required when too few peptides were present after the digest due to the small size of the proteins. MALDI analysis was done on a Bruker Daltonik REFLEX time-of-flight mass spectrometer (Bremen, Germany) equipped with delayed extraction and detector bias gating for the discrimination of low molecular weight ions. For the MALDI analysis, 0.3 μl of the supernatant was used. For the electrospray analysis the peptide mixture was extracted, desalted on a self assembled 100 ml PorosTM R2 column, eluted in a total volume of 1 μl of 60% methanol, 5% formic acid into a gold-coated nano-electrospray needle and investigated on a quadrupole mass spectrometer (AOI III, PE-Sciex, Ontario, Canada) (Shevchenko *et al.*, 1996b). Peptides were detected using a precursor ion scan for the immonium ion of leucine/isoleucine (86 Da). Proteins were identified by searching a comprehensive non-redundant protein database using the program PeptideSearch (Mann *et al.*, 1993).

mRNA in vivo degradation and mRNA immunoprecipitation

The RP485, RP602 and RP611 plasmids (Decker and Parker, 1993; Muhlrad and Parker, 1994; Muhlrad *et al.*, 1995) encoding, respectively, the MFA2pG, $_{B55T}$PGK1pG and $_{B55T}$PGK1$_{N103}$pG reporter mRNAs under the control of the *GAL1* promoter were transformed in the *lsm1*Δ, *lsm5*Δ, *lsm6*Δ, *lsm7*Δ or *pat1*Δ strains as well as in the isogenic wild-type strain. The degradation of these reporters was assayed as described (Boeck *et al.*, 1998). *lsm1*Δ, *lsm5*Δ, *lsm6*Δ and *lsm7*Δ strains as well as the isogenic wild-type strain were first grown at 30°C until OD_{600} = 0.3, then shifted at 37°C for 2 h. Transcription of the reporter was then blocked by addition of glucose and cells incubated at 37°C

during the time course experiment. *pat1*Δ and wild-type strains were incubated at 30°C during all the experiment. Total RNA extraction and deadenylation with RNase H–oligo(dT) were performed as described (Pikielny and Rosbash, 1985; Boeck *et al.*, 1998). The different reporters were detected by Northern blotting using the poly(C) oligonucleotide bo29, identical to oRP121 (Boeck *et al.*, 1998). The scR1 transcript detected using oligonucleotide bo36, identical to o77 (Felici *et al.*, 1989), was used as an internal loading control. Membranes were quantified using a Phosphorimager (FLA2000, Fujifilm). Half-lives were calculated using the linear regression of the plot ln(band intensity) function of time.

For mRNA immunoprecipitation, 1.5 µg of total RNA from the degradation experiments described above were immunoprecipitated using an anti-7-methyl cap antibody as described (Munns *et al.*, 1982; Dunckley and Parker, 1999).

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