The Lsm1-7-Pat1 complex promotes viral RNA translation and replication by differential mechanisms

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ABSTRACT

The Lsm1-7-Pat1 complex binds to the 3' end of cellular mRNAs and promotes 3' end protection and 5'–3' decay. Interestingly, this complex also specifically binds to *cis*-acting regulatory sequences of viral positive-strand RNA genomes promoting their translation and subsequent recruitment from translation to replication. Yet, how the Lsm1-7-Pat1 complex regulates these two processes remains elusive. Here, we show that Lsm1-7-Pat1 complex acts differentially in these processes. By using a collection of well-characterized *Lsm1* mutant alleles and a system that allows the replication of Brome mosaic virus (BMV) in yeast we show that the Lsm1-7-Pat1 complex integrity is essential for both, translation and recruitment. However, the intrinsic RNA-binding ability of the complex is only required for translation. Consistent with an RNA-binding-independent function of the Lsm1-7-Pat1 complex on BMV RNA recruitment, we show that the BMV 1a protein, the sole viral protein required for recruitment, interacts with this complex in an RNA-independent manner. Together, these results support a model wherein Lsm1-7-Pat1 complex binds consecutively to BMV RNA regulatory sequences and the 1a protein to promote viral RNA translation and later recruitment out of the host translation machinery to the viral replication complexes.

Keywords: Lsm1-7; Pat1; Lsm1-7-Pat1 complex; decapping activators; viral RNA; BMV; RNA virus; Lsm proteins; translation; replication

INTRODUCTION

The highly conserved Lsm1-7-Pat1 complex plays a critical role in mRNA decay via the 5'–3' pathway. In this pathway, shortening of the poly(A) tail to oligo(A) length triggers decapping by Dcp1/Dcp2 holoenzyme and subsequent 5'–3' degradation of the body of the message by the exonuclease Xrn1 (Chen and Shyu 2011; Parker 2012; Schoenberg and Maquat 2012). Several factors facilitate decapping and they include Dhh1, the Lsm1-7-Pat1 complex, Edc1, Edc2, Edc3 (Kshirsagar and Parker 2004; Nissan et al. 2010; Borja et al. 2011; Fromm et al. 2012; Sweet et al. 2012; Arribas-Layton et al. 2013) and in metazoans Edc4 (Chang et al. 2014). The Lsm1-7-Pat1 complex is made up of the Pat1 protein, which is implicated in translational repression (Coller and Parker 2005; Marnef and Standart 2010), and the seven Sm-like proteins, Lsm1 through Lsm7 (Bouveret et al. 2000; Tharun 2009a; Tharun et al. 2000; Totaro et al. 2011). The Sm-like proteins form a large family of proteins whose founding members are the Sm proteins. They are ubiquitous in na-

ture, typically exist as hexa or heptameric RNA-binding complexes and carry out RNA-related functions (Beggs 2005; Tharun 2009b; Wilusz and Wilusz 2013). They are characterized by the presence of the Sm domain (Cooper et al. 1995; Hermann et al. 1995; Seraphin 1995; Salgado-Garrido et al. 1999). The Lsm1-7-Pat1 complex shares six of its seven Lsm subunits (Lsm2 through Lsm7) with the nuclear Lsm2-8 complex which plays a role in splicing but not in cytoplasmic mRNA decay (Mayes et al. 1999; Bouveret et al. 2000; Tharun et al. 2000; Ingelfinger et al. 2002; Tharun 2009b). Thus, Lsm1 is a key subunit that distinguishes these two complexes. Both Lsm1-7 and Lsm2-8 complexes have a ring shaped quaternary structure similar to that of the Sm complex wherein the Lsm subunits are arranged relative to each other in an analogous fashion (Kambach et al. 1999; Sharif and Conti 2013; Zhou et al. 2014). The Lsm1-7-Pat1 complex associates preferentially *in vivo* and *in vitro* with the 3' end of oligoadenylated mRNAs targeted for decay rather than polyadenylated translating messages (Tharun et al. 2000; Tharun and Parker

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2001; Chowdhury et al. 2007; Mitchell et al. 2013) and promotes decapping via an unknown mechanism. This complex also protects the 3' ends of mRNAs from trimming *in vivo* (Boeck et al. 1998; He and Parker 2001; Tharun et al. 2005). Mutagenic analysis of Lsm1 has revealed that the RNA-binding properties and the mRNA decay and 3' end protection functions of this complex are critically dependent on the Sm domain and the C-terminal domain of Lsm1 (Tharun et al. 2005; Chowdhury and Tharun 2008, 2009; Chowdhury et al. 2012).

Interestingly, in contrast to its decay function on cellular mRNAs, we and others have previously shown that the Lsm1-7-Pat1 complex plays a fundamental role in the life cycle of a wide range of positive-strand RNA [(+)RNA] viruses including the plant Brome mosaic virus (BMV), the animal Flock House virus, the human Hepatitis C virus (HCV) and the emerging West Nile virus (WNV) (Diez et al. 2000; Noueiry et al. 2003; Mas et al. 2006; Scheller et al. 2009; Chahar et al. 2013; Gimenez-Barcons et al. 2013). After these viruses enter the host cell, their single-stranded RNA genomes act as mRNAs and are directly translated to express the viral proteins. Then, once the viral proteins accumulate to sufficient levels, translation is repressed and viral genomes are specifically recruited out of the cellular translation machinery and into the viral replication complexes, where they act as templates for replication. Because these two functions are mutually exclusive, they need to be tightly controlled. The Lsm1-7-Pat1 complex plays a key role in such control since it promotes both translation and recruitment to replication of the viral RNA genomes (Diez et al. 2000; Noueiry and Ahlquist 2003; Mas et al. 2006; Scheller et al. 2009).

The replication of the plant BMV in the yeast *Saccharomyces cerevisiae* is a fruitful model system for studying common and fundamental steps of (+)RNA virus biology in a relatively simple background (Alves-Rodrigues et al. 2006; Galao et al. 2007). The BMV genome consists of three RNAs (RNA1, RNA2, and RNA3) that are 5' capped (Fig. 1A). At their 3' ends, the BMV RNAs carry instead of a poly(A) tail a conserved tRNA like structure (TLS). The nondefined 3'-UTR sequence element that precedes the TLS is referred to as non-TLS sequence (NTLS). Both 5' and 3' UTRs contain overlapping sequences that control translation and initiation of negative-strand synthesis (for review, see Noueiry and Ahlquist 2003). The TLS element is required for efficient translation, initiation of replication, and encapsidation of viral genomes. The recruitment element, at the 5' terminal ends of RNA1 and RNA2 and at the intergenic region (IR) of RNA3, is necessary for recruitment of the viral RNAs to replication complexes. RNA1 encodes the helicase 1a, which is the only BMV protein required for recruitment, while RNA2 codifies the polymerase 2a. RNA3 encodes the movement protein 3a and, through a subgenomic RNA generated during replication, also the coat protein.

By using the BMV/yeast system and *in vitro* band shift assays with reconstituted recombinant human Lsm1-7 com-

FIGURE 1. (A) Schematic representation of the BMV genome. (B) Schematic representation of the Lsm1 protein. The locations of the generated mutations are indicated.

plexes we previously reported that the capacity of the Lsm1-7 ring to directly bind key BMV RNA *cis*-acting regulatory elements is critical for the viral functions of this complex *in vivo*. (Galao et al. 2010). Yet, how Lsm1-7-Pat1 facilitates both translation and recruitment of viral genomes remains unclear. In this study, we have analyzed a set of *lsm1* mutant alleles having different effects on the Lsm1-7-Pat1 complex to address this issue experimentally. By combining functional *in vivo* studies with *in vitro* binding assays using Lsm1-7-Pat1 complex purified from yeast, we show that the Lsm1-7-Pat1 complex acts differentially in promoting viral RNA translation and recruitment. While *lsm1* mutations affecting Lsm1-7-Pat1 complex integrity inhibit both translation and recruitment, *lsm1* mutations affecting RNA-binding inhibit only BMV RNA translation. Consistent with an RNA-binding-independent function of Lsm1-7-Pat1 on BMV RNA recruitment, we show that the BMV 1a protein interacts with this complex in an RNA-independent manner. Furthermore, our results suggest that, like mRNA decay, viral RNA translation may need both Lsm1-7-Pat1 binding to the RNA and subsequent facilitation of post-binding steps. Together, these results open up new perspectives in the functionality of this versatile complex.

RESULTS

BMV RNA2 translation is impaired in mRNA decay-defective *lsm1* mutants

Lsm1-7-Pat1 promotes both viral RNA translation and cellular mRNA 5'–3' decay. To analyze whether these apparently antagonistic Lsm1-7-Pat1 functions occur by similar mechanisms, we analyzed BMV RNA translation in a set of *lsm1*

mutants whose mRNA decay defects are well-characterized (Tharun et al. 2005; Chowdhury and Tharun 2008, 2009). This includes the *lsm1-6*, *lsm1-13*, *lsm1-9*, *lsm1-14*, *lsm1-9,14*, and *lsm1-8* mutants all of which are defective in mRNA decay and 3' end protection (Fig. 1B) although the defects are milder in the *lsm1-6* mutant compared with the other mutants. The residues targeted in *lsm1-13* and *lsm1-6* alleles are implicated in intersubunit interactions (Sharif and Conti 2013; Zhou et al. 2014) although complex assembly is abolished only in the *lsm1-13* mutant (Chowdhury and Tharun 2009; A Chowdhury and S Tharun, unpubl.). The milder phenotype of the *lsm1-6* mutant can be suppressed upon overexpressing the *lsm1-6* allele in the mutant strain (Tharun et al. 2005; Chowdhury and Tharun 2009). The residues targeted in the *lsm1-9* and *lsm1-14* alleles are implicated in RNA binding (Tharun et al. 2005; Chowdhury and Tharun 2008). The mutant complexes assembled in *lsm1-9* and *lsm1-14* mutants are moderately affected in the RNA-binding activity but lack the ability to recognize the presence of a 3'-oligo(A) tail on the RNA (Chowdhury and Tharun 2008). Finally, the *lsm1-9,14* double mutant (generated by combining the mutations of *lsm1-9* and *lsm1-14*) and the *lsm1-8* mutant are severely defective in the RNA-binding activity of the Lsm1-7-Pat1 complex (Chowdhury and Tharun 2009).

To test the effect of these *lsm1* mutations on BMV RNA translation we focused on RNA2 since it is highly dependent on Lsm1-7 for translation (Noueiry et al. 2003). *lsm1Δ* yeast cells were transformed with *CEN* plasmids expressing RNA2 from a *GAL* promoter and WT or mutant *LSM1* alleles expressed from their native promoters. Since helicase 1a was not expressed, RNA2 will be translated but not recruited to replication. After induction of RNA2 expression, total protein and RNA were extracted from the cells and analyzed by Western and Northern blot analysis, respectively. Translation efficiency was determined by normalizing the 2a protein levels with the respective RNA2 levels (Fig. 2). As previously reported, RNA2 translation was strongly inhibited in the *lsm1Δ* strain relative to the *LSM1* strain. In all the analyzed *lsm1* mutants RNA2 translation was inhibited ~90% relative to the *LSM1* strain, except in the *lsm1-6* mutant, which is known to exhibit only a moderate defect in mRNA decay, where the inhibition was 50%. The observed differences are not caused by insufficient expression of the mutant Lsm1 proteins since Western blot analyses showed that the expression levels of the Lsm1 mutant proteins were comparable with those of Lsm1 WT protein (Fig. 2). Thus, the *lsm1* mutants that are defective in mRNA decay and 3' end protection are also defective in RNA2 translation suggesting the requirement of common Lsm1-7-Pat1 features for these processes.

Prior studies revealed that protection of the 3' end of mRNAs by the Lsm1-7-Pat1 complex depends on the ability of this complex to bind the mRNA. However, activation of decapping by the Lsm1-7-Pat1 complex depends not only on this binding but also on the ability of the complex to

FIGURE 2. BMV RNA2 translation is strongly inhibited by mutations in *LSM1* affecting RNA-binding or intersubunit contacts. *lsm1Δ* yeast strain was transformed with a plasmid transcribing BMV RNA2 together with an empty plasmid or a plasmid expressing *LSM1* or the different *lsm1* mutant alleles. (Upper panels) Northern blot (NB) analyses of RNA2 accumulation. Detection of the 18S ribosomal RNA was used to assure equal loading and sample quality. (Lower panels) Western blot analyses (WB) of 2a and Lsm1 expression. As a control for equal loading of total protein, expression of the phosphoglycerate kinase protein (PGK) was also analyzed. Histograms show the average and standard error of the mean of the relative accumulation of 2a protein relative to the amount of RNA2 for at least three independent colonies. The average value obtained in cells expressing *LSM1* gene was set to 100.

facilitate some unknown post-binding events that ultimately lead to decapping (Chowdhury and Tharun 2009). The *lsm1-9* and *lsm1-14* mutants are mainly affected in the post-binding events and hence, in these mutants the 3' end protection defect but not the mRNA decay defect can be suppressed by overexpression of the corresponding *lsm1* alleles (Chowdhury and Tharun 2009). On the other hand, overexpression of *lsm1-9* or *lsm1-14* in WT cells results in dominant inhibition of mRNA decay. This might be due to the sequestration of the mRNA by the mutant Lsm1-7-Pat1 complexes into mRNP structures that fail to proceed through the post-binding events. Therefore, in order to gain more insight into how BMV RNA translation is affected in these mutants, we first compared the BMV RNA2 translation efficiency of *lsm1Δ* cells that express these alleles from *CEN* and multicopy 2μ vectors (Fig. 3). These experiments revealed that, as in mRNA decay, RNA2 translation was not significantly enhanced upon overexpression of *lsm1-9* or *lsm1-14* alleles. Next, we asked if, like in mRNA decay, BMV RNA translation gets inhibited in a dominant manner upon overexpression of *lsm1-9* or *lsm1-14* alleles in WT cells. In these analyses, we also included the *lsm1-9,14* allele as a negative control because the *lsm1-9,14* mutant is almost completely defective in RNA-binding activity of the Lsm1-7-Pat1 complex

FIGURE 3. BMV RNA2 translation defect in *lsm1-9* and *lsm1-14* mutants cannot be suppressed by overexpressing the corresponding *lsm1* alleles. *lsm1Δ* strain was transformed with a plasmid transcribing BMV RNA2 and *CEN* or 2μ plasmids expressing *LSM1*, *lsm1-9*, or *lsm1-14* alleles (indicated on top). Accumulations of RNA2, 2a and Lsm1 protein were analyzed as in Figure 2. Relative accumulation of 2a protein relative to the amount of RNA2 represented in histogram as described for Figure 2.

and therefore overexpression of this allele does not cause dominant inhibition of decay in WT cells (Chowdhury and Tharun 2009). As seen in Figure 4, these studies revealed that overexpression of *lsm1-9* or *lsm1-14* alleles in wild-type cells did not significantly affect BMV RNA2 translation.

Thus, although mRNA decay, mRNA 3' end protection and BMV RNA2 translation are all impaired in *lsm1-9* and *lsm1-14* mutants, only the 3' end protection defect can be suppressed by overexpression of the respective *lsm1* alleles in those mutants.

Lsm1 promotes BMV RNA translation and recruitment by differential mechanisms

In early stages of the BMV infection cycle, the RNA genome after being translated has to be recruited in a 1a-dependent manner to a membrane bound complex where it serves as template for replication. This process protects the viral RNA from degradation and hence strongly enhances its accumulation (Janda and Ahlquist 1993, 1998; Sullivan and Ahlquist 1999; Diez et al. 2000; Mas et al. 2006). Therefore, the extent to which the BMV RNA levels are elevated upon expressing the 1a protein reflects the efficiency of recruitment (Janda and Ahlquist 1998). The Lsm1-7-Pat1 complex does not only promote translation but also the recruitment out of the translation machinery of the BMV RNA genomes. To get further insight into the Lsm1-7-Pat1 complex features required for this function we focused on RNA3, the BMV RNA

whose regulation of recruitment is characterized the most. Two separate plasmids that express BMV RNA3 and an allele (mutant or WT) of *LSM1*, respectively, were introduced into the *lsm1Δ* strain with or without an additional third plasmid expressing the 1a protein. In the absence of protein 1a, RNA3 accumulates to similar levels in all *lsm1* mutants assayed (data not shown) indicating that, as previously shown (Mas et al. 2006; Galao et al. 2010), Lsm1 does not affect BMV RNA3 steady state. When 1a was coexpressed, the recruitment of RNA3 was inhibited in *lsm1Δ* cells as evident from the poor accumulation of RNA3 in those cells compared with the WT cells (Fig. 5; Mas et al. 2006). Unexpectedly, none of the *lsm1* mutations that are known to impair the RNA-binding activity of the Lsm1-7-Pat1 complex (*lsm1-9*, *lsm1-14*, *lsm1-9,14*, and *lsm1-8*) significantly affected recruitment (Fig. 5). It is important to note that the mutant Lsm1-7-Pat1 complexes purified from two of these mutants, *lsm1-9,14* and *lsm1-8*, almost completely lack RNA-binding activity although they are normal with regard to complex integrity (Chowdhury and Tharun 2009). On the other hand, mutants like *lsm1-6* and *lsm1-13*, wherein the intersubunit contact

FIGURE 4. *lsm1-9*, *lsm1-14*, and *lsm1-9,14* alleles do not cause dominant inhibition of BMV RNA2 translation when overexpressed in WT yeast cells. WT yeast strain was transformed with a plasmid transcribing BMV RNA2 and 2μ plasmids expressing *LSM1*, *lsm1-9*, *lsm1-14*, or *lsm1-9,14* alleles (indicated on top). Accumulations of RNA2, 2a and Lsm1 protein were analyzed by Northern (NB) and Western (WB) blot. Histogram shows the average and standard error of the mean of the relative accumulation of 2a protein relative to the amount of RNA2 for at least three independent colonies. Relative accumulation of 2a protein relative to the amount of RNA2 represented in histogram as described for Figure 2. The average value obtained in WT cells expressing an empty plasmid was set to 100.

FIGURE 5. Mutations in *LSM1* that inhibit BMV RNA2 translation do not affect BMV RNA3 recruitment. *lsm1Δ* strain was transformed with a plasmid transcribing BMV RNA3, a plasmid expressing protein 1a plus an empty plasmid or plasmids expressing *LSM1* and the different *lsm1* mutant alleles. Similar protein 1a levels in the presence of *LSM1* and mutant alleles were achieved by using different strength promoters (*GAL1* or *ADHI*). RNA3 accumulation was analyzed by Northern blot and detection of SCR1 was used as the loading control. The accumulation of 1a protein and Lsm1 was analyzed by Western blotting. Histogram shows average and standard errors of the mean of the relative accumulation of RNA3 from three or more independent colonies. The average RNA3 accumulation in *lsm1Δ* yeast expressing WT *LSM1* gene in the presence of 1a protein was set to 100 ([*] $P < 0.05$, relative to strain expressing *LSM1*).

residues are targeted, exhibited moderate (but reproducible), and strong defects in RNA3 recruitment, respectively. Thus, as for mRNA decay and viral RNA translation, Lsm1-7-Pat1 complex integrity is also important for viral RNA recruitment. However, mutations that impair RNA binding do not seem to affect the ability of this complex to support RNA3 recruitment.

These results suggest that a direct binding of the viral RNA by the Lsm1-7-Pat1 complex is not necessary for recruitment and probably involves an indirect interaction between the Lsm1-7-Pat1 complex and RNA3 through some bridging protein(s). Since the helicase 1a is the sole BMV protein required for recruitment (Janda and Ahlquist 1998; Sullivan and Ahlquist 1999), we reasoned that it would be a good candidate for mediating such interaction. To test this idea we conducted anti-flag antibody immunoprecipitation assays in WT cells expressing 1a-flag or flag in the presence of RNA3. As a negative control we used PGK, a protein not expected to interact with 1a. As shown in Figure 6, both Lsm1 and Pat1 specifically coimmunoprecipitated with protein 1a (sevenfold and 25-fold enrichment compared with “Flag only” controls). To test whether this interaction depends on RNA we treated the samples with RNaseA. This eliminated any

visible signal of total RNA, as observed in a formaldehyde gel and also decreased 99% of the BMV RNA3 levels as measured by qPCR (data not shown). Interestingly, the RNase treatment decreased but did not abolish Lsm1 and Pat1 coimmunoprecipitation (fold enrichment compared with “Flag only” control was down to twofold and 4.5-fold, respectively). Thus, the Lsm1-7-Pat1 complex interacts with protein 1a in a manner that is not dependent on but likely facilitated by RNA.

Purified yeast Lsm1-7-Pat1 complex binds to the 3' UTR of BMV RNA1, RNA2, and RNA3 and to the intergenic region of RNA3

In order to further understand how the interaction of the Lsm1-7-Pat1 complex with the BMV RNAs affects the BMV life cycle, we carried out RNA-binding assays using uniformly radiolabeled in vitro transcripts carrying sequences from different parts of the BMV RNAs 1, 2, and 3 and the Lsm1-7-Pat1 complex purified from yeast. In these assays, the in vitro transcript was first incubated with the purified Lsm1-7-Pat1 complex. The complex with the bound RNA was then pulled down using anti-Flag antibody matrix followed by washing of the matrix and extraction of the co-precipitated RNA which is then separated on denaturing acrylamide gel and visualized and quantitated by phosphorimaging. As shown in Figure 7, the purified Lsm1-7-Pat1 complex binds to the 3'- and 5'-UTR sequences of all the three BMV RNAs although the binding of the 5'-UTR sequences is weaker than that of the 3'-UTR sequences. We also observed that the Lsm1-7-Pat1 complex binds to the intergenic region of RNA3. Finally, binding assays carried out using truncated RNA substrates carrying only the TLS or NTLS parts of the 3' UTR of RNA3 revealed that both of these parts are critical for binding of the 3' UTR of

FIGURE 6. Lsm1-7-Pat1 complex interacts with 1a protein. Western blot analysis of immunoprecipitation assays carried out in WT cells expressing BMV RNA3 and flag or 1a-flag. The blot was probed with anti-1a, anti-Lsm1, and anti-Pat1 antibodies. Input corresponds to 100 μ g of total protein present in lysates prior to precipitation. Output corresponds to the corresponding eluates from anti-flag matrix following precipitation. Extracts were either treated (+) or not treated (-) with RNaseA prior to the washing steps.

FIGURE 7. Lsm1-7-Pat1 complex purified from *lsm1-14* mutant is weaker than the wild-type complex in binding to the UTR sequences of the BMV RNAs. Uniformly labeled in vitro transcripts carrying different parts (as indicated in the figure) of BMV RNA1 (panel A), RNA2 (panel B), and RNA3 (panel C) were incubated with increasing concentrations of the Lsm1-7-Pat1 complex purified from wild type (WT) or *lsm1-14* cells (lanes spanned by black triangles on top) or just BSA as indicated in the figure. The RNA bound to the Lsm1-7-Pat1 complex was then pulled down and visualized by denaturing gel run and phosphorimaging as described in Materials and Methods. Untreated RNA (20% of the input amount) run alongside the bound RNA is also shown. Plot of the percentage of RNA bound (quantitated using the phosphorimager) versus the concentration of the complex used is also shown in each panel.

RNA3 with the Lsm1-7-Pat1 complex (Fig. 7C). These results show that the yeast complex is similar to the human complex with regard to its relative binding affinities for the different regions of the BMV RNAs (Galao et al. 2010).

Cis elements determining the Lsm1-7-Pat1 complex dependence of BMV RNA translation bind less efficiently to the mutant complex purified from *lsm1-14* cells

Our results presented above have shown that the purified Lsm1-7-Pat1 complex has different affinities for different parts of the BMV RNAs. The data presented in Figures 2, 3, and 5 also show that the *lsm1-14* mutant is impaired in BMV RNA translation but is unaffected in BMV RNA recruitment to the replication complex. Earlier studies are consistent with the idea that the *lsm1-14* mutant complexes are able to bind cellular mRNA but unable to support the post-binding steps required for decay (Chowdhury and Tharun 2009). These studies also revealed that this mutant complex fails to exhibit enhanced affinity for oligoadenylated RNA (Chowdhury and Tharun 2008). We showed earlier that the BMV RNA 3' UTR is a key determinant of the

dependence on the Lsm1-7-Pat1 complex for translation in vivo of the BMV RNAs (Galao et al. 2010). Therefore, in order to determine if the mutant complex assembled in the *lsm1-14* cells is defective in binding to the 3'-UTR sequences, we purified the Lsm1-7-Pat1 complex from the *lsm1-14* mutant cells. Analysis of the purified complex revealed that it has a subunit composition similar to that of wild-type complex (Supplemental Fig. S1) as expected and shown earlier (Chowdhury and Tharun 2008). RNA-binding assays carried out with transcripts derived from RNA2 and RNA3 using the mutant (Lsm1-14 containing) complex revealed that indeed the mutant complex binds to the 3'-UTR and 5'-UTR sequences of these RNAs with lesser affinity compared with the wild-type complex (Fig. 7B,C). This result is consistent with our observation that the *lsm1-14* mutant is defective in supporting the translation of BMV RNA2 in vivo because it is known that both UTRs contribute to the dependence of BMV RNA translation on the Lsm1-7-Pat1 complex. Finally the RNA-binding assays revealed that the mutant complex interacted with less affinity (compared with the wild-type complex) to the intergenic region of RNA3 as well.

DISCUSSION

The Lsm1-7-Pat1 complex binds to the 3' end of cellular mRNAs and promotes their decay. Intriguingly, Lsm1-7-Pat1 complex also specifically binds to *cis*-acting regulatory sequences in the BMV RNA genomes and promotes their translation and replication (Scheller et al. 2009; Galao et al. 2010). Like mRNA decay, translation, and recruitment of the viral genome to replication are multistep processes that require profound rearrangements of the viral ribonucleoprotein (RNP) structure. How the Lsm1-7-Pat1 complex promotes both translation and recruitment, two antagonistic processes, remains unclear. By using a collection of well-characterized *lsm1* mutant alleles here we show using functional *in vivo* analyses that Lsm1-7-Pat1 promotes these two processes by different mechanisms. While Lsm1-7-Pat1 complex integrity was essential for both processes, the intrinsic ability of Lsm1-7-Pat1 to bind RNA was only required for its function in translation. Our results support the idea that, as in mRNA decay, the Lsm1-7-Pat1 complex functions in viral RNA translation also by facilitating RNA-binding and post-binding steps. However such post-binding steps might be different in mRNA decay and viral RNA translation. Moreover, the RNA-binding-independent function of Lsm1-7-Pat1 complex in recruitment is likely mediated through binding to the viral 1a helicase, the sole viral protein needed for recruitment. The obtained results support a model in which Lsm1-7-Pat1 complex sequentially regulates key steps required for expression and replication of (+)RNA viral genomes by subsequent binding to viral RNA *cis*-signals and the 1a protein (see below).

The functions of Lsm1-7-Pat1 complex in cellular mRNA 3' end protection, 5'-3' decay, and BMV RNA translation share some common features. Mutations in the *LSM1* gene that affected complex integrity or RNA-binding capacity inhibited all three processes. Moreover, these functions are affected to similar relative degrees in the different *lsm1* mutants. For example, *lsm1-6* mutant has a weak phenotype while *lsm1-9,14*, *lsm1-8*, and *lsm1-13* have a strong phenotype in all three functions indicating that these three processes depend on Lsm1-7-Pat1 complex integrity and its capacity to bind RNA. Consistent with this idea, Pat1 is also important for viral RNA translation and recruitment (Noueiry et al. 2003) and Pat1 is essential for the normal RNA-binding ability of the Lsm1-7-Pat1 complex (Chowdhury et al. 2014). Our studies show that the Lsm1-7-Pat1 complex is quite selective in binding to the different regulatory sequences in the BMV RNA genome. Purified yeast Lsm1-7-Pat1 complexes bound much more strongly to the 3' UTR than the 5' UTR of the three BMV RNAs. They also bound to the intergenic region of the RNA3 (Fig. 7). Although both the TLS and NTLS regions within the 3' UTR of RNA3 were bound, a stronger binding was observed when both regions were present. These results recapitulate the ones obtained with human reconstituted recombinant Lsm1-7 rings (Galao et al. 2010), pointing out the high conservation of the Lsm1-7 capacities throughout evolution.

Since studies with the human complex were carried out using a reconstituted complex lacking Pat1, our results also suggest that in the case of viral RNAs, the role of Pat1 as part of the Lsm1-7-Pat1 complex is primarily to enhance the strength of binding rather than to dictate the specificity of binding. In contrast to the results obtained with the human rings, we observed that the purified yeast Lsm1-7-Pat1 complex bound, although weakly, to the BMV RNA 5' UTRs. The presence of Pat1 could be directing this recognition.

We also found some differences in the way Lsm1-7-Pat1 complexes affect viral RNA translation compared with the way they affect mRNA 3' end protection and decay. While all the three functions are impaired in *lsm1-9* and *lsm1-14* mutants, only the 3' end protection defect can be suppressed by overexpression of the respective *lsm1* alleles in those mutants (Fig. 3; Chowdhury and Tharun 2009). These observations suggest that viral RNA translation requires, as decay, binding, and facilitation of post-binding events by the Lsm1-7-Pat1 complex. On the other hand, overexpression of the *lsm1-9* or *lsm1-14* allele in wild-type cells causes dominant inhibition of mRNA decay but not viral RNA translation (Fig. 4; Chowdhury and Tharun 2009). This is consistent with the observation that the mutant complex purified from *lsm1-14* cells has reduced affinity for the viral RNA sequences than the wild-type complex (Fig. 7).

The mechanism by which the Lsm1-7-Pat1 complex promotes recruitment of BMV RNA3 to replication is different from that by which it promotes BMV RNA translation and 3' end protection and 5'-3' decay of host mRNAs. While *lsm1-13*, a mutation that abolishes complex formation, inhibited all four processes, all mutations shown to alter or to completely abolish the RNA-binding capacity of the complex without abolishing complex integrity (*lsm1-9*, *lsm1-14*, *lsm1-9, 14*, and *lsm1-8*) did not affect BMV RNA recruitment to replication.

Therefore, counter intuitively, the ability of the Lsm1-7-Pat1 complex to directly bind to the intergenic region, which contains the *cis*-acting sequences necessary and sufficient for 1a-mediated recruitment, seems not to be necessary for recruitment. Thus, either this binding is involved in other functions, such as regulation of BMV RNA translation, or does not occur *in vivo*, probably because of the presence of competing binding sequences in the same RNA molecule. Consequently, the involvement of the Lsm1-7-Pat1 complex in recruitment needs to be mediated by another protein. Indeed, our results suggest that the Lsm1-7-Pat1 complex function in recruitment is mediated by an interaction of that complex with the BMV 1a protein (Fig. 6).

Based on the above results, we propose the following model. In the absence of protein 1a, Lsm1-7-Pat1 complex binds to the 3'- and 5'-UTR sequences and for RNA3 to the intergenic region as well. This somehow triggers the interaction between the ends and therefore circularization of the BMV RNA so that translation is facilitated. When the helicase 1a is coexpressed with RNA3, 1a interacts with the replication

enhancer, a sequence within the intergenic region required and sufficient to promote 1a-dependent recruitment. Next, 1a would bind to the Lsm1-7-Pat1 complex located at the 3' UTR. This would then disrupt the Lsm1-7-Pat1 mediated 3'-5' circularization, and possibly the RNA contacts of the Lsm1-7-Pat1 complex, repressing translation while allowing subsequent recruitment of the viral RNA to the replication complex. This view is suggested by the following observations. First, mutations that affect the intrinsic RNA-binding capacities of the Lsm1-7-Pat1 complex inhibit BMV RNA translation. Second, the replacement of the native 3' and 5' UTRs of the BMV RNAs with other sequences impacts the dependence of the BMV RNAs on Lsm1-7-Pat1 for translation (Noueiry et al. 2003; Galao et al. 2010). Third, the mutant Lsm1-7-Pat1 complex purified from *lsm1-14* mutant which is defective in supporting BMV RNA2 translation, is impaired in binding to the 5'- and 3'-UTR sequences of RNA2 and RNA3. Fourth, all *lsm1* mutations affecting RNA binding had no effect on BMV RNA3 recruitment indicating that recruitment can happen independently from translation. Lastly, coimmunoprecipitation analyses showed that 1a can interact with Lsm1-7-Pat1 complexes in the absence of RNA. This is consistent with the observation that RNA-binding activity of the Lsm1-7-Pat1 complex is not needed for recruitment. Nevertheless, it seems likely that binding of 1a and WT Lsm1-7-Pat1 complexes to the same RNA molecule will favor their interaction.

A growing body of evidence indicates the existence of a complex net of interactions between the cellular mRNA decay machinery and viral replication (Moon and Wilusz 2013; Narayanan and Makino 2013). Our data show that the decapping activator Lsm1-7-Pat1 complex is exploited by BMV to sequentially regulate translation and replication of the viral genomes. Given the common replication strategies of (+)RNA viruses, the dependence on Lsm1-7-Pat1 complex for replication of at least four (+)RNA viruses infecting plants, insects, and humans and the dependence on Hfq, a homolog of Lsm1 in bacteria, for replication of the bacteriophage Q β (Franze de Fernandez et al. 1968; Diez et al. 2000; Noueiry et al. 2003; Mas et al. 2006; Scheller et al. 2009; Chahar et al. 2013; Gimenez-Barcons et al. 2013), it is likely that these complexes are broadly used within this viral group by similar mechanisms as the ones described here. Moreover, the obtained results uncover functionalities of these versatile complexes that might provide novel insights into their essential role in cellular mRNA decay, since it also requires sequential binding and post-binding steps that are still not understood.

MATERIALS AND METHODS

Yeast cells

WT *S. cerevisiae* strain YPH500 (Mata *ura3-52 lys2-801 ade2-101 trp1 Δ 63 his3- Δ 200 leu2- Δ 1*) and the derivative *lsm1 Δ* strain were

used (Diez et al. 2000). Yeast cells were grown in standard media selective for the desired plasmids with 2% galactose at 30°C until the doubling time between triplicates was similar and an OD₆₀₀ of ~0.6 was reached.

Plasmids

RNA2 was expressed from pB2NR3, a centromeric plasmid with 2a linked to the *GAL* promoter (Chen et al. 2001) and BMV RNA3 was expressed from the centromeric pB3RQ39 (Ishikawa et al. 1997) under control of the *GAL* promoter. Recruitment was studied by expressing BMV RNA3 from the *Cup* promoter from plasmid pJDSal1 (Diez et al. 2000) induced with 0.5 mM CuSO₄. Additionally protein 1a was expressed from either pB1CT19 or pB1YT3H by using the *ADH* or *GAL* promoter, respectively (Janda and Ahlquist 1993; Ahola et al. 2000). To test the effect of the different *lsm1* mutants on translation or recruitment the *lsm1 Δ* strain was transformed with plasmids expressing *LSM1* WT (pIR15), *lsm1-6* (pIR16), *lsm1-8* (pJJ14), *lsm1-9* (pIR17), *lsm1-14* (pIR19), *lsm1-9,14* (pJJ13), or *lsm1-13* (pIR18) mutant proteins. pIR15, pIR16, pIR17, pIR18, and pIR19 were generated by excising the corresponding *lsm1* mutant alleles from pST11, pST26, pST29, pST33, and pST34, respectively using *Xma*I and *Sac*I and inserting it in pRS414 also digested with *Xma*I and *Sac*I (Tharun et al. 2005). pJJ13 and pJJ14 were generated by excising the corresponding *lsm1* mutant alleles from pST155 and pST28 (Tharun et al. 2005; Chowdhury and Tharun 2009), respectively, using *Sac*I and *Spe*I and inserting them in pRS414 digested with *Sac*I and *Spe*I. To study the dominant negative effect of *LSM1* WT, *lsm1-9*, *lsm1-14*, and *lsm1-9,14* on RNA2 translation these mutants were expressed from the 2 μ plasmids pST70, pST57, pST59, and pST188, respectively (Chowdhury and Tharun 2009).

The effect of overexpressing *lsm1-9* or *lsm1-14* on RNA2 translation was studied by using the centromeric plasmids pIR17 and pIR19 and the 2 μ plasmids pJJ11 and pJJ12 expressing *lsm1-9* or *lsm1-14* from its own promoter, respectively. pIR17 and pIR19 were obtained by excising the corresponding *lsm1* mutant from plasmids pST29 and pST34 by using *Xma*/S*ac*I and inserting them in pRS414. pJJ11 and pJJ12 were obtained by excising the corresponding fragment from pST57 and pST59 using *Sac*I/S*ma*I and inserting it in pRS424 using the same enzymes. To study the interaction of protein 1a with Lsm1 protein a plasmid expressing a carboxy-terminal flag-tagged version of protein 1a (gift from P. Ahlquist) was used. As negative control a flag-tagged version of USA1 protein which also localizes to the ER, was expressed from plasmid pC289 (Carvalho et al. 2010). To enable growth in the same selective media centromeric plasmids YCplac111 (Gietz and Sugino 1988) and pRS313 (Sikorski and Hieter 1989) were used.

Translation and recruitment assays

To evaluate BMV 2a protein translation, yeast cells were transformed with the corresponding plasmids and grown in 2% galactose at 30°C until midlog phase. Total protein was extracted from the same number of yeast cells, separated by SDS-gel electrophoresis and immunoblotted as previously described (Ishikawa et al. 1997). Detection was done with the infrared imaging system Odyssey (LI-COR Biosciences) or in the case of BMV 2a with FUJIFILM Luminescent Image Analyzer LAS-1000.

Antibodies against 1a (Restrepo-Hartwig and Ahlquist 1999), 2a (Noueiry et al. 2003), and PGK (Molecular Probes), and Lsm1 and Pat1 specific antisera (Rodriguez-Cousino et al. 1995; Bonnerot et al. 2000) were used for Western blot. Total RNA2 was extracted and RNA2 accumulation was measured as explained below.

To analyze recruitment, yeast cells were transformed with the corresponding plasmids and grown in 2% galactose at 30°C until mid-log phase. Total RNA from yeast cells was isolated by a hot phenol method and analyzed by Northern blot as previously described (Janda and Ahlquist 1993). Specific probes to detect positive-strand RNA2 and RNA3 and 18S have been described previously (Ishikawa et al. 1997; Alves-Rodrigues et al. 2007) and were generated using the MAXIScript in vitro transcription kit (Ambion). To probe RNAs for *SCR1* an oligonucleotide antisense to *SCR1* (oRP100; 5'-GTCTAGCCGCGAGGAAGG-3') was used. Northern blots were exposed to PhosphorImager screens and imaged on a Typhoon 8600 instrument (Amersham). Band intensities were quantified using the ImageQuant software (Molecular Dynamics).

Coimmunoprecipitation

One hundred to 200 mL yeast cultures were grown until OD₆₀₀ ~0.6 and harvested by centrifugation and lysed by vortex with glass beads in 1xNET buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% IGEPAL CA-630 [from Sigma], 1 mM EDTA) containing a protease inhibitor cocktail (Roche), 10 mM of pepstatin, leupeptin, aprotinin (Sigma), and 80 U/mL RNasin (Promega). After extraction, the total protein amount was quantified and 100 µg of each sample were used to control the input by Western blot. Equal amounts of at least 1 mg total protein were used for immunoprecipitations and incubated with anti-flag matrix (Sigma) over night at 4°C. After extensive washes in 1xNET buffer containing the described inhibitors and RNasin, 20% of each sample was used for protein analysis by Western blot. Immunoprecipitates were eluted by boiling samples in SDS loading buffer for 5 min at 95°C.

Purification of Lsm1-7-Pat1 complex and RNA-binding assays

Purification of Lsm1-7-Pat1 complex from yeast, gel shift assays and preparation of radiolabeled in vitro transcripts for gel shift assays were carried out as previously described (Chowdhury et al. 2007; Tharun 2008). For pull down based RNA-binding assays, RNA-binding reaction was carried out as for gel shift assays but in 40 µL volume. Radiolabeled RNA was used at a final concentration of 1 nM in the binding reaction. The binding reaction mixture was then incubated with the anti-Flag antibody matrix for 1 h. The matrix was then washed thrice with 50 mM Tris pH 7.5; 50 mM NaCl; 0.5 mM MgCl₂ followed by phenol:chloroform extraction of the RNA bound to the matrix. The extracted RNA was ethanol precipitated and then subjected to urea polyacrylamide gel electrophoresis and phosphorimaging. Viral RNA derived in vitro transcripts were used for RNA-binding assays without prior denaturation. Plasmids used for making the transcripts carrying sequences from 5' UTR and 3' UTR of BMV RNAs 1, 2, and 3 and the intergenic region of BMV RNA3 (via in vitro run on transcription) are described in Galao et al. (2010).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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