Resting Complexes of the Persistent Yeast 20S RNA Narnavirus Consist Solely of the 20S RNA Viral Genome and its RNA Polymerase p91*

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Summary

The positive strand 20S RNA narnavirus persistently infects *Saccharomyces cerevisiae*. The 20S RNA genome has a single gene that encodes the RNA-dependent RNA polymerase (p91). 20S RNA forms ribonucleoprotein resting complexes (RNPs) with p91 and resides in the cytoplasm. Here we found no host proteins stoichiometrically associated with the RNP by pull-down experiments. Furthermore, 20S RNA, when expressed from a vector in *Escherichia coli*, formed RNPs with p91 in the absence of yeast proteins. This interaction required the 3’ *cis* signal for complex formation. Moreover, when 23S RNA, the genome of another narnavirus, was expressed in *E. coli*, it also formed RNPs with its RNA polymerase p104. Finally, when both RNAs are expressed in the same *E. coli* cell, they formed RNPs only with their cognate RNA polymerases. These results altogether indicate that narnaviruses RNPs consist of only the viral genomes and their cognate RNA polymerases. Because the copy number of the RNPs can be induced almost equivalent to those of rRNAs in some yeast strains, the absence of host proteins may alleviate the burden on the host by not sequestering proteins into the RNPs. It may also contribute to the persistent infection of narnaviruses by decreasing their visibility.

Introduction

20S RNA virus belongs to the genus *Narnavirus* and is among the simplest viruses in nature. The virus has a single small positive strand genome (2514 nucleotides (nt)) called 20S RNA that encodes a single protein of 91 kDa (p91), the RNA-dependent RNA polymerase (Wickner *et al.*, 2013). Because the virus has no capsid gene, 20S RNA is not encapsidated into a conventional virion structure (Widner *et al.*, 1991; García-Cuéllar *et al.*, 1995). Instead, 20S RNA forms a ribonucleoprotein (RNP) complexed with p91, and the virus, in the form of RNP, resides in the cytoplasm of the yeast *Saccharomyces cerevisiae*. Typical of fungal viruses, 20S RNA virus has no extracellular transmission pathway. The virus is stably transmitted from mother to daughter cells, or horizontally through mating. The virus does not kill the host nor render phenotypic changes to the host.
20S RNA was originally discovered as an RNA species that accumulates under nitrogen starvation (Kadowaki and Halvorson, 1971), a condition commonly used to induce sporulation in yeast. In some strains, the copy number of 20S RNA becomes almost equivalent to those of rRNAs. The high dosage may help the virus to distribute to meiotic progenies, although haploid cells can also accumulate 20S RNA (Wejsnora and Haber, 1978). The majority of 20S RNA in induced cells is the positive strand and it forms RNPs with p91 in a 1:1 stoichiometry (resting complexes) (Solórzano et al., 2000). The negative strands account only a few percent of the total 20S RNA population. Lysates from induced cells also contain a minor amount of replication intermediates. These intermediates contain p91 and synthesize 20S RNA positive strands \textit{in vitro} (García-Cuéllar et al., 1997). The intermediates consist of a negative strand and a positive strand with less than unit length loosely associated, perhaps through p91 (Fujimura et al., 2005). The RNA backbone of the intermediates is largely single-stranded but denaturation with phenol converts it to double-stranded. Upon completion of positive strand synthesis, the product is released from the negative strand template. The released product is still associated with protein and is indistinguishable from resting complexes. Because the majority of negative strands in lysates are found in replication intermediates, the negative strand template appears to be immediately recruited for another round of positive strand synthesis \textit{in vivo} (Fujimura et al., 2005).

Most laboratory strains of yeast harbor 20S RNA virus. Fewer strains also contain another narnavirus called 23S RNA. The 23S RNA genome (2891 nt) possesses a single gene that encodes its RNA polymerase (p104) (Esteban et al., 1992). 23S RNA also forms resting complexes with p104. 20S and 23S RNA viruses are independent and compatible in the same host. When co-habiting in the same cell, these viruses form resting complexes containing the RNA genomes and their cognate RNA polymerases and do not form hybrid complexes (García-Cuéllar et al., 1995).

A launching system of 20S RNA virus from a yeast expression vector has been established (Esteban et al., 2005). The vector contains the full-length cDNA of 20S RNA under the constitutive \textit{PGK1} promoter. The ribozyme sequence from hepatitis delta virus (HDV) is directly attached to the 3’ end of the viral genome so that the precise 20S RNA 3’ end can be generated \textit{in vivo}. 20S RNA can be launched efficiently from the vector and this system has been used to investigate \textit{cis}-acting signals for
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replication. Moreover, in the absence of an active ribozyme sequence, the transcripts from the vector, without generating the virus, can form ribonucleoprotein complexes in vivo with p91 translated there from. By combining with a pull-down assay, the latter system has served as a useful tool to investigate cis acting signals for 20S RNA complex formation (Fujimura and Esteban, 2007). A similar launching system (Esteban and Fujimura, 2003) and an assay system for complex formation (Fujimura and Esteban, 2004) for 23S RNA virus have also been developed.

In resting complexes, p91 interacts with 20S RNA at three cis sites: the 5' and 3' end sites and, to a lesser extent, an internal site (Fig. 1) (Fujimura and Esteban, 2007). The 3' site is located at the 3rd and 4th C residues from the 3' end and the adjacent stem structure. The 3' site largely overlaps (if not identical) with the 3' cis site for replication. The 5' site is located at the second stem structure from the 5' end. Mutations at this site that destabilized complex formation also failed to generate the virus from the launching vector. The tight relationship between complex formation and replication at the 5’ and 3’ cis sites underlines the importance of stable resting complex formation in the virus life cycle. The internal site is located somewhere between nt 1253 to 1515, but its precise location or extent is not known. Mutations at the 5’ or 3’ cis site reduced complex formation to a basal level (10-20% of the wild type level). The effect of a double mutation at both sites is not cumulative. We have suggested that the interactions of p91 at the 5’ and 3’ cis sites are coordinated and that the internal cis site is responsible for the basal level of complex formation observed (Fujimura and Esteban, 2007).

In spite of the small genome and its simple organization, 20S RNA virus establishes a persistent infection in yeast. Because the majority of the viral genomes exist in vivo as resting complexes, we have been investigating these complexes to understand the mechanism of viral persistency. In this work we addressed the inquiry of whether the resting complex contains host proteins. We found no host proteins stoichiometrically associated with metabolically labeled resting complexes. Furthermore, 20S RNA and p91 formed complexes in E. coli with the same specificity as in yeast, indicating that yeast proteins are not needed for complex formation. These results indicate that the resting complexes consist of only 20S RNA and p91. The lack of host proteins in the resting complexes may decrease the visibility of the virus in the cell. Moreover, when the virus accumulates at a high number, it will not hurt the cell by depleting vital host proteins.
Results

Partial Purification of 20S RNA/p91 RNPs

In induced conditions, the majority of 20S RNA virus exists in the form of a resting complex consisting of 20S RNA and p91 in a 1:1 stoichiometry (Solórzano et al., 2000). We decided to investigate whether a host protein(s) is involved in resting complex formation. Purification of RNP by affinity chromatography was unsuccessful. We attached p91 with a Histidine tag, the Flag peptide, or the TAP epitope, however, p91 with the appendix did not bind to the respective affinity column, perhaps due to the bulky structure of 20S RNA in the complex. Conventional column chromatography did not work either. It was difficult to keep 20S RNA intact during the purification. Since 20S RNA can be immunoprecipitated well with anti-p91 antisera, we metabolically labeled yeast proteins and performed pull-down experiments to see whether any host proteins were brought down along with the RNP. To avoid high background caused by non-specific pull-down of proteins, we took two measures. Firstly, anti-p91 antibodies were partially purified with protein A Sepharose and then used for immunoprecipitation. Secondly, 20S RNA/p91 RNP was partially purified through differential centrifugation and then subjected to pull-down experiments. During the first high-speed centrifugation (Fig. 2A), more than half of ribosomes were precipitated (P1), while 20S RNA/p91 remains in solution (S1). In the second centrifugation at higher speed, RNP can be pelleted (P2) while soluble proteins remained in the supernatant (S2). The majority of resting complexes were recovered in the pellet fraction. This fraction contained more than 80% of 20S RNA and p91 from the original lysate. Anti-p91 antiserum can immunoprecipitate intact 20S RNA from the pellet fraction (Fig. 2B). Furthermore, the co-sedimentation of 20S RNA and p91 in a glycerol gradient indicates that the 20S RNA/p91 RNP remains intact after the differential centrifugation (Fig. 2C).

In vivo Labeling

Yeast cells with or without 20S RNA virus were grown in the presence of a mixture of $^{35}$S-labeled Met and Cys and then transferred to 1% K acetate to induce 20S RNA virus. Cells were broken and an RNP-enriched pellet fraction (P2) was prepared by differential centrifugation. Finally,
RNP s were immunoprecipitated by partially purified anti-p91 antibodies. The pellet (fraction P2) and the immunoprecipitate contained approximately 30% and 0.2%, respectively, of radioactivity of the original cell lysate. Proteins in the immunoprecipitate were separated by SDS-PAGE and visualized by fluorography (Fig. 3). We found no prominent yeast proteins pulled down specifically along with p91. For example, the intensities of band a (68 KDa) and band b (25 kDa) proteins relative to that of p91 correspond to 13.5 and 5.2%, respectively. These proteins appear to be more abundant in the immunoprecipitate from 20S RNA-carrying cells than from the 20S RNA-negative strain. If we assume that these proteins were labeled with $^{35}$S with the same specific activity as p91, then we calculated that only 0.18 and 0.19 molecules of a and b proteins, respectively, were pulled down along with each molecule of p91. p91 (829 amino acids) contains 16 Met (1.93%) and 10 Cys (1.20%) residues, while average S. cerevisiae proteins contain 2.08% Met and 1.31% Cys, and thus can be labeled with $^{35}$S slightly better than p91. These results strongly suggest that there is no host proteins stoichiometrically associated with resting complexes. Because the P2 fraction also contains a small amount of replication complexes (García-Cuéllar et al., 1997), however, we cannot rule out the possibility that minor proteins, such as a and b, might be part of replication complexes. This experimental approach underestimates proteins with lower Met and Cys contents. Furthermore we cannot eliminate the possibility that the antibodies, upon binding to p91, may displace host protein(s) from resting complexes.

Expression of 20S RNA and p91 in E. coli

If p91 does not require host proteins to form a resting complex, then the complex may be formed even in a heterologous system in the absence of yeast proteins. We tested this possibility by expressing p91 and 20S RNA in Escherichia coli. Two plasmids were constructed (Fig. 4, A and B). One plasmid (pLOR91) contains the 20S RNA cDNA sequence under the T7 promoter. The Shine-Dalgarno (SD) sequence AAGGAG was inserted between the promoter and the cDNA. The 3' end of the 20S RNA genome was directly attached to the HDV ribozyme sequence. The second plasmid (pLOR92) is the same as pLOR91 except that the 20S RNA cDNA was directly attached to the T7 promoter. Both plasmids expressed high amounts of 20S RNA in E. coli (Fig. 4C). As expected, p91
was expressed in cells containing pLOR91, while 20S RNA transcribed from pLOR92 was not decoded to p91 because of the lack of the Shine-Dalgarno sequence. *E. coli* cells did not generate autonomously propagating 20S RNA virus (Fig 4D). Once pLOR91 was cured, the cells did not produce 20S RNA transcripts any longer. In yeast cells, launching of 20S RNA virus from a vector required removal of non-viral sequences from the transcripts at both termini. If the proper viral sequence is generated by removal of the Shine-Dalgarno sequence, then p91 cannot be translated from it in *E. coli* cells. It is also possible that the virus needs yeast proteins for replication.

p91 expressed in *E. coli* has an extra amino acid sequence (MGADP) at the N-terminus. To demonstrate that the extra sequence does not impair the activity of p91, we did the following *in vivo* experiments in yeast. Previously we have shown that 20S RNA virus can be generated from 20S RNA negative strands transcribed from a vector, provided an active p91 is supplied from a second vector (Esteban *et al.*, 2005). p91 cannot be translated from the negative strands, thus in the absence of the second vector there is no virus generation. It implies that the first round of positive strand synthesis catalyzed by p91 expressed from the second vector is critical for virus generation. As expected, the negative strand-expressing vector alone did not generate 20S RNA virus (Fig. 5, lane 4). However, if intact p91 or p91 with MGADP was expressed from a second vector, 20S RNA virus was generated with similar efficiency (Fig. 5, lanes 5 and 6.). The second vector alone (either with or without MGADP) did not launch the virus because it contained the C4A mutation (numbered from the 3’ end of the 20S RNA genome) that abolishes 20S RNA replication (Fig. 5, lanes 2 and 3) (Esteban *et al.*, 2005). These results indicate that the extra amino acids at the N-terminus do not compromise the RNA polymerase activity of p91.

**Complex Formation in E. coli**

To examine whether p91 forms a complex with 20S RNA in *E. coli* cells, two experimental approaches were taken: pull-down assays and glycerol gradient sedimentation. In the first approach, a lysate from *E. coli* cells transformed with pLOR91 was subjected to immunoprecipitation with anti-p91 antiserum. As shown in Fig. 6 lane 2, 20S RNA was pulled down specifically with anti-p91 antiserum. A lysate from yeast cells harboring 20S RNA virus was processed in parallel as positive
control. As expected, the probe detected 20S RNA in the immunoprecipitate (Fig. 6 lane 3). In the second approach we tried to visualize physical interactions between p91 and 20S RNA through glycerol gradient sedimentation. A lysate prepared from cells carrying pLOR91 was directly applied to a 10-40% glycerol gradient. As shown in Fig. 7, the majority of p91 co-migrated with 20S RNA in the gradient during centrifugation. As a control, the lysate was predigested with RNase A and then applied to the gradient. Now 20S RNA is not visible and p91 remains in the upper part of the gradient. These results indicate that most of p91 molecules expressed in E. coli cells are physically associated with 20S RNA. Both experimental approaches clearly indicate that p91 can form RNP in E. coli cells.

**Specificity of Complex Formation in E. coli**

We addressed the question of how faithfully the formation of 20S RNA/p91 RNP in E. coli reflects the reaction that occurs in the native yeast cells. To answer this question we examined the specificity of complex formation in E. coli. In the native host, p91 interacts with 20S RNA at the 5'-, internal-, and 3'-cis sites to form the RNP. The 3' cis site consists of the 3rd and 4th Cs at the 3' end and the adjacent stem structure. We constructed three plasmids to express 20S RNA modified at the 3' cis site in E. coli (Fig. 8A). These RNAs, when expressed in yeast, failed to generate 20S RNA virus and formed RNPs at the basal low level (Fujimura and Esteban, 2007). The first plasmid contains the C4A mutation (numbered from the 3’ end of the 20S RNA genome). In the second plasmid, a small disturbance in the stem was introduced by G5C. The third plasmid contains a substitution (5 bp-stem mutation) that destroys a large part of the stem structure. All these changes were introduced into the 3’ non-coding region of the viral genome. Therefore, p91 expressed from these plasmids has the same wild type amino acid sequence. The three mutant plasmids as well as the reference plasmid pLOR91 expressed similar amounts of 20S RNA and p91 in E. coli (Fig. 8B). Lysates prepared from these cells were subjected to pull-down experiments with anti-p91 antiserum. As shown in Fig. 8C, mutant RNAs formed reduced amounts of complexes compared with the WT RNA. Especially, G5C and 5-bp stem mutations severely affected complex formation. These results indicate that, even in E. coli cells, the 3’ cis site is required for full activity to form complexes. We also tried another approach to examine the specificity of complex formation in E. coli. Like 20S RNA virus, the genome of 23S RNA virus also
forms a RNP with its RNA polymerase p104. 20S RNA and 23S RNA viruses can reside and propagate stably in the same yeast host. Even residing together in the same cell, they do not form hybrid RNPs. We constructed a plasmid to express 23S RNA and p104 in *E. coli* and introduced it into bacterial cells together with the 20S RNA-expressing plasmid. These two plasmids have different antibiotic markers. The concentrations of antibiotics were adjusted so that similar amounts of 20S RNA and 23S RNA were produced in the same cell (Fig. 9A, the far right column). Lysates were prepared and subjected to pull-down assays using anti-p91 and anti-p104 antisera. 20S and 23S RNAs in the immunoprecipitates were detected with specific probes. As shown in Fig. 9B, anti-p91 antiserum pulled down 20S RNA but not 23S RNA. It indicates that p91, even in *E. coli* cells, specifically interacts with 20S RNA to form RNPs and discriminates 23S RNA from the reaction. Thus p91 does not require yeast proteins for proper RNP formation. Similarly, the anti-p104 antiserum pulled down 23S RNA but not 20S RNA (Fig. 9C). It indicates that p104 and 23S RNA can also form an RNP in *E. coli* in the absence of yeast proteins and that p104 correctly chooses 23S RNA as a partner to form its own RNPs.

Discussion

In this work we have investigated whether yeast proteins are involved in the formation of 20S RNA/p91 resting complexes. We found that no host proteins were stoichiometrically associated with metabolically labeled RNPs in pull-down experiments. This suggests that p91 and 20S RNA are capable of forming RNPs by themselves. In fact, when expressed in a heterologous organism, *E. coli*, they formed RNPs in the absence of yeast proteins. The authenticity of complex formation in the surrogate host was demonstrated by two specificity experiments. Mutations at the 3′ cis site of 20S RNA drastically reduced RNP formation as in yeast. Furthermore, p91 correctly chose 20S RNA as partner but not 23S RNA for RNP formation. The formation of RNPs in *E. coli*, thus, retains the same specificity as the reaction that occurs in its native host. In addition, we found that 23S RNA/p104 RNPs can be also formed in *E. coli*.

Although 20S RNA/p91 RNPs were assembled in *E. coli*, they did not produce autonomously propagating 20S RNA virus. Launching of 20S RNA from a vector in yeast requires removal of
extraneous terminal sequences from the transcripts to expose the mature 5’ and 3’ viral termini. The expression of p91 in *E. coli* required the Shine-Dalgaro sequence and the removal of the ribosome-binding site would make the generated viral RNA a poor template for translation. Furthermore, we did not observe 20S RNA negative strand synthesis by p91 in *E. coli* cells nor RNA polymerase activity of p91 in bacterial cell lysates (unpublished results). Unlike resting complex formation, replication of 20S RNA may require yeast proteins.

Since there is no host protein in resting complexes, p91 is solely responsible for the interactions at the three *cis* sites in forming the complexes. The 3’ *cis* site is located close to the 3’ end. Because exonucleases play the major role in mRNA degradation in eukaryotes, it suggests that p91 protect the 20S RNA genome from degradation by binding to the 3’ end. mRNA decay usually begins with shortening the 3’ poly(A) tail followed by decapping at the 5’ end (Wilusz *et al.*, 2001; Parker and Song, 2004). Then decapped RNA is degraded by the *SKII/XRN1* 5’ exonuclease. Alternatively, deadenylated RNA is digested by a 3’ exonuclease complex called the exosome (Mitchell *et al.*, 1997; Jacobs Anderson and Parker, 1998). The exosome is present in both the nucleus and the cytoplasm (Mitchell *et al.*, 1997; Allmang *et al.*, 1999) and has compartment-specific auxiliary factors. In the cytoplasm, *SKI2, SKI3*, and *SKI8* form the so-called SKI complex (Jacobs Anderson and Parker, 1998; Brown *et al.*, 2000,) and the complex is physically linked to the exosome through *SKI7* (Araki *et al.*, 2001). An RNA substrate is channeled from the SKI complex to the exosome for degradation (Halbach *et al.*, 2013). 20S RNA has no 3’ poly(A) tail. Although it is not known whether the 5’ end is capped, it resembles intermediates of mRNA degradation. In *ski2, ski3, ski7* or *ski8* mutants, the copy number of 20S RNA (and 23S RNA) greatly increases (Matsumoto *et al.*, 1990; Ramirez-Garratacho and Esteban, 2011), indicating that there is a stage in the virus life cycle vulnerable to the exosome. 20S RNA has a strong secondary structure at the 5’ end and the first four consecutive Gs are buried at the bottom of the stem structure. These features confer on 20S RNA fully resistance to the *SKII/XRN1* 5’ exonuclease. Destabilizing the 5’ secondary structure makes 20S RNA vulnerable to *SKII* suppression (Esteban *et al.*, 2008). Although 20S RNA is not encapsidated into a protective capsid structure, formation of resting complexes may protect 20S RNA from exonucleases in the host cytoplasm. It is also possible that the formation of a resting complex with 20S RNA
stabilizes p91. So far we have been unable to dissociate p91 from the RNPs in an active form. It is well known that in growing conditions both rRNAs and ribosomal proteins become very stable once assembled into RNP particles (Deutscher, 2003). It has been observed that expression of active hepatitis B reverse transcriptase requires the presence of the template RNA binding site (Wang et al., 1994; Tavis and Ganem, 1996). The reverse transcriptase LtrA of group II intron is also stabilized by forming RNP particles complexed with excised intron RNA (Saldanha et al., 1999). In silico, intramolecular long distance interactions bring the three cis sites of 20S RNA close together (Fujimura et al., 2007). Perhaps it is a prerequisite for a single p91 molecule to interact with the three sites simultaneously. The molecular mass of p91 is one eighth of that of 20S RNA (736 kDa). A large area of 20S RNA molecule would remain uncovered in the complex and directly exposed to the cytoplasm.

20S RNA/p91 RNPs may be well adapted to the challenge of exonucleases, however, they may be vulnerable to endonucleolytic cleavages. Recently, it has been shown that S. cerevisiae can support the RNAi system if Dicer and Argonaute are imported from S. castellii (Drinnenberg et al., 2009). The constructed strains lost the double-stranded RNA (dsRNA) killer viruses M and L-A (Drinnenberg et al., 2011). Curiously, however, L-BC dsRNA virus (Drinnenberg et al., 2011) and L-A variants (Rodríguez-Cousiño et al., 2013) were not eliminated by the extraneous RNAi system. Their dsRNA genomes are encapsidated into protective capsids, while 20S RNA is not. As expected, 20S RNA virus is much more sensitive to RNA interference than these encapsidated dsRNA viruses (R.E., P.G. and N.R, manuscript in preparation).

The absence of host proteins in the stable resting complexes suggests that 20S RNA viruses keep the dependency to host proteins at minimum in its life cycle. In sporulation conditions some yeast strains accumulate 20S RNA to an amount almost equivalent to those of rRNAs. Since extensive degradation of ribosomes as well as vegetative proteins occurs during sporulation (Esposito and Klapholz, 1981), it may not be much burden for the cell to provide precursors for synthesis of 20S RNA and p91. The high copy number of resting complexes may help the virus to be stably transmitted to the meiotic descendants. However, if host proteins were constituents of a resting complex, then a great increase of the complex during sporulation might exhaust the proteins and thus be harmful for the host. 20S RNA virus is a persistent virus and has no extracellular transmission pathway. Since
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there is no opportunity of escaping from the cell, to inflict too much damage to the host against non-infected cells is also undesirable for the virus. Furthermore, if such proteins were essential part of the complex, then it would provide a measure for the host to antagonize the virus through regulating the proteins. Thus the absence of host protein in the resting complex may contribute to the persistent infection by decreasing the visibility of 20S RNA virus in the cell.

Experimental procedures

In vivo Labeling

Yeast strain 924 (a ura3 his3 leu2 ski2Δ, L-A-α, 20S RNA) or 913 (isogenic to 924 but 20S RNA-α) was grown in complete synthetic medium H (Wickner, 1980) (5 ml) supplemented with a mixture of $^{35}$S-Met and $^{35}$S-Cys (0.13 mCi ml$^{-1}$, Perkin Elmer). The concentrations of both amino acids were adjusted to 20 µg ml$^{-1}$. The cells were grown at 28 ºC for three days, transferred to 1% K acetate and then kept another 16 h to induce 20S RNA.

Differential Centrifugation

Cells were harvested, washed once with H$_2$O and then suspended in lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl). The cells were broken with glass beads (0.40-0.60 mm in diameter, Sartorius) using Fast Prep P120 (Bio101Sarvant) with two pulses of 15 s at speed 4.5. After removing cell debris and unbroken cells, the lysates were centrifuged at 55000 rpm (120000 xg) for 30 min with the Beckman-Coulter rotor TLA-100.2 to remove ribosomes. The supernatant was re-centrifuged at 75000 rpm (250000 xg) for 2 h in a TLA-100.3 rotor to separate 20S RNA/p91 complexes from the bulk of soluble proteins. The pellet was suspended in the lysis buffer and subjected to pull-down experiments or glycerol gradient centrifugation.

Antibody Purification

Anti-p91 antibodies were partially purified using a column containing protein A-conjugated Sepharose CL-4B (GE Healthcare). After extensive washing with PBS buffer (20 mM Na phosphate,
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pH 7.0, 0.15 M NaCl), the antibodies bound were eluted with 0.1 M glycine-HCl pH 3.0. The pH of the fractions was immediately adjusted to neutral by the addition of 1 M Tris-HCl pH 9.0.

Preparation of E. coli Lysates

E. coli BL21 cells transformed with 20S RNA- and/or 23S RNA-expressing plasmids were grown at 37 °C for 4 h in 30 ml of LB medium supplemented with 100 µg ml⁻¹ ampicillin and/or 50 µg ml⁻¹ kanamycin. Isopropyl β-D-1-thiogalactopyranoside (1 mM) was added to the culture and the cells were kept at 28 °C for another 5 h to express the viral genome(s). Cells were harvested, suspended in the lysis buffer supplemented with 0.1% bentonite, 1 mg ml⁻¹ lysozyme, and 1x protease inhibitor mixture (GE Healthcare), and broken with glass beads (0.25-0.30 mm in diameter) using Fast Prep P120 (one pulse of 15 s with speed 4.5). The lysates were diluted three times with the lysis buffer, centrifuged to remove cell debris and unbroken cells, and then subjected to pull-down experiments.

Glycerol Gradients

Glycerol gradient centrifugation was done following the procedure described previously for sucrose gradient centrifugation (Wejksnora and Haber, 1978; Widner, et al., 1991) by simply substituting 10-40% sucrose with 10-40% glycerol.

Pull-down Assay

To 10-160 µl of the lysate prepared from yeast or E. coli as described above, 1 ml of Tris-buffered saline-Tween 20 (10 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.05% Tween 20), 1 mM DTT, 40 units of RNasin (Promega), 20 µg of yeast tRNA (Invitrogen) and 2 µl of anti-p91 or anti-p104 antiserum or 0.5 µl of partially purified anti-p91 antibodies (16 mg ml⁻¹) were added and the mixture was incubated at 4 °C for 30 min. 25 µl (wet volume) of protein A conjugated Sepharose CL-4B was added to the mixture and it was incubated at 4 °C for another 30 min. The sepharose was washed 5 times with 1 ml Tris-buffered saline-Tween 20 and 1 mM DTT. RNA was extracted from the sepharose, slot-blotted and detected by hybridization as described in (Fujimura and Esteban,
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2004). The probes used to detect 20S RNA, 23S RNA, and *E. coli* 23S rRNA are complementary to nt 1262-2514 of 20S RNA, nt 1-2891 of 23S RNA, and nt 435-1005 of *E. coli* 23S rRNA, respectively.

Alternatively, protein bound to sepharose was eluted with loading buffer for SDS acrylamide gels and separated in a 7.5% or 14% SDS gel. After electrophoresis, the gel was soaked with the Amersham amplify fluorographic reagent and protein bands were detected by fluorography. Quantification of bands was done using a PMI™ Personal Molecular Imager (Biorad).

**Plasmids**

For expression of 20S and 23S RNA in *E. coli* we used the following vectors. 20S RNA:

pLOR84 is a derivative of pT7-7 (Tabor and Richardson, 1985) that contains the complete 20S RNA cDNA (2514 nt) downstream of the Shine-Dalgarno sequence with the HDV ribozyme fused at its 3’ end. p91 expressed from pLOR84 has 5 extra amino acids at its N-terminus (MGADP). pLOR91 was constructed from pLOR84 by inserting a 1.4 kb DNA fragment containing the kanamycin resistance gene into the ampicillin resistance gene. pLOR92 was made from pLOR91 by eliminating the sequence between the T7 promoter and the 20S RNA 5’ end. 23S RNA: 23S RNA and p104 were expressed from pRE1048. This plasmid is identical to pLOR91, except that the 20S RNA cDNA was substituted by the full 23S cDNA sequence. p104 expressed from pRE1048 has 3 extra amino acids at its N-terminus (MGA). Yeast 20S RNA launching vectors from genomic or antigenomic strands have been described previously (Esteban et al., 2005). Mutations in the vectors were introduced by site directed mutagenesis (Esteban et al., 1989).

**References**


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Legends to figures

Figure 1. Diagram of the 5′ and 3′ end regions of the 20S RNA genome. The 5′ and 3′ end cis sites for complex formation and replication are indicated. B. Nucleotide sequences and secondary structures at the 5′ end (left panel) and 3′ end (right panel) regions. The initiation (start) and termination (stop) codons of p91 are indicated.

Figure 2. Partial purification of 20S RNA/p91 complexes by differential centrifugation. A. A cell lysate was separated into pellet (P) and supernatant (S) fractions by two sequential centrifugations (1 and 2) at different speeds. RNA was extracted, separated on an agarose gel and visualized by ethidium bromide staining. T, initial cell lysate. B. 20S RNA was immunoprecipitated from the P2 fraction shown in A in the presence (lane 2) or absence (lane 3) of anti-p91 antiserum, separated in an agarose gel, and detected by Northern hybridization using a 20S RNA-specific probe. As control, total RNA from the initial lysate (T shown in A) was processed in parallel but without immunoprecipitation (lane 1). C. Fraction P2 was subjected to 10-40% glycerol gradient centrifugation. RNA from gradient fractions was separated in an agarose gel and visualized by ethidium bromide staining (upper panel). 20S RNA and p91 were visualized by Northern hybridization (middle panel) and Western blotting (lower panel), respectively.

Figure 3. No yeast proteins are stoichiometrically associated with 20S RNA/p91 resting complexes. Lysates were prepared from 20S RNA-negative (lane 1) and positive (lane 2) strains metabolically labeled with $^{35}$S. After differential centrifugation, the pellet fractions (P2) were subjected to immunoprecipitation with partially purified anti-p91 antibodies (anti-p91). The
immunoprecipitates were separated in 7.5% (A) and 14% (B) acrylamide/SDS gels. Proteins were visualized by fluorography. Scanning of protein bands are shown on the right of the panels. Black color, strain with 20S RNA; pale gray, control strain without 20S RNA. The pellet fractions without immunoprecipitation (Total) were also analyzed in a 7.5% gel as shown in A. a and b band proteins: see the explanation in the text. M, molecular standards (kDa).

Figure 4. **The expression of p91 in E. coli requires the Shine-Dalgarno sequence.** A. The nucleotide sequence at the 5’ end of 20S RNA transcript expressed from pLOR91. The 5’ terminal nucleotide of the 20S RNA genome is numbered (1) and the initiation codon of p91 is marked by start. The bars separate the codons of p91. The transcript contains an extra 66 nt upstream sequence derived from the *E. coli* T7-7 expression plasmid, including the Shine-Dalgarno (SD) sequence and a new initiation codon Met. Thus p91 expressed from this transcript has 5 extra amino acids (MGADP) at the N terminus. B. Diagrams of pLOR91 and the control plasmid pLOR92. 20S RNA cDNA sequences are indicated by thick black lines. T7; T7 promoter. R; HDV ribozyme. In pLOR92 the 20S RNA cDNA is directly fused to the T7 promoter. C. Expression of 20S RNA and p91 in *E. coli* from the Shine-Dalgarno-containing pLOR91 (+) or non-containing pLOR92 (-) plasmid. The expression of 20S RNA was detected by ethidium bromide staining of an agarose gel (EtBr) and confirmed by Northern hybridization using a 20S RNA-specific probe (Northern). The expression of p91 was monitored by Western blotting using anti-p91 antiserum (anti-p91). D. 20S RNA virus is not generated in *E. coli*. After expression of 20S RNA transcripts from pLOR91, *E. coli* cells were grown in the absence of kanamycin to cure the plasmid. The expression of 20S RNA and p91 in pLOR91-containing (+) or pLOR91-cured (-) cells was examined as described in C. Note that rRNAs in *E. coli* have smaller sizes compared to the yeast counterparts (23S versus 25S, and 16S versus 18S).

Figure 5. **MGADP-p91 is active in yeast to generate 20S RNA in a two-vector system.** A. A yeast strain free of 20S RNA was transformed with the 20S RNA negative strand-expressing vector pRE762 alone (lane 4) or along with p91 expressing vector pRE760 or pLS025 (lane 5 or 6). pRE760 expresses wild type p91, while p91 expressed from pLS025 has 5 extra amino acids at the N-terminus.
Yeast 20S RNA ribonucleoprotein complexes

(MGADP-p91). Both p91-expressing vectors have a mutation at the 3’ terminus of the viral genome (C4A) so that each vector alone cannot generate 20S RNA virus in vivo. RNA was extracted from the cells and analyzed in an agarose gel. Ethidium bromide staining (EtBr) and Northern hybridization with a 20S RNA probe (20S RNA probe) of the gel are shown. As controls, untransformed cells (lane 1) and cells transformed with p91-expressing vectors alone (lanes 2 and 3) were processed in parallel.

B. Diagrams of the p91 expressing vectors and 20S RNA negative strand expressing vector used in A. *PGK1*: the constitutive *PGK1* promoter. The C4A mutation at the 3’ end of 20S RNA genome on the p91 expressing vectors is indicated by the asterisks.

Figure 6. **Pull down of 20S RNA expressed in E. coli with anti-p91 antiserum.** A lysate was prepared from *E. coli* cells harboring the 20S RNA-expressing plasmid with Shine-Dalgarno sequence (pLOR91) or a vector alone (Vector). 20S RNA was immunoprecipitated in the presence (+) or in the absence (-) of anti-p91 antiserum. As positive control, a lysate prepared from yeast cells carrying endogenous 20S RNA virus was processed in parallel. 20S RNA in the immunoprecipitates was detected with a specific probe.

Figure 7. **20S RNA and p91 expressed in E. coli co-sediment through glycerol gradient centrifugation.** A lysate prepared from pLOR91-containing cells was applied to 10-40% glycerol gradient centrifugation (left panel, RNase A -). As a control, the lysate was pre-digested with RNase A and then subjected to centrifugation (right panel, 
RNase A +). After sedimentation the gradients were fractionated. The top and bottom of the gradients are indicated. 20S RNA and p91 in the fractions were detected as described in the legend to Figure 2. The main peaks of 20S RNA and p91 in the gradients are indicated by the arrows. L, the lysate before loading onto the gradients.

Figure 8. **20S RNA/p91 RNP formation in E. coli requires the 3’ cis site for full activity.** A. Diagrams of the 3’ end regions of 20S RNA WT and 3’ cis mutants. Nucleotides changed are underlined and in bold face. In the 5-bp stem mutant, the wild type sequence 12-GGCCACGG-5 was replaced with 12-CAGGAGGC-5 (numbered from the 3’ end). B. The expression of WT and mutant
Yeast 20S RNA ribonucleoprotein complexes

20S RNA in *E. coli* (upper panel). p91 expressed from the plasmids is shown in the lower panel. Note that the mutations analyzed do not change the amino acid sequence of p91. *C*. Pull-down assay. A lysate containing WT or mutant 20S RNA was incubated in the presence (+) or absence (-) of anti-p91 antiserum to immunoprecipitate 20S RNA. The RNA was detected with a specific probe for 20S RNA. Phenol-extracted lysates without immunoprecipitation were also analyzed as loading controls (*Total*).

Figure 9. **20S RNA and 23S RNA form complexes only with their respective cognate RNA polymerases when expressed in the same *E. coli* cell.** *A*. Lysates prepared from *E. coli* cells containing no plasmid (-), or harboring a 20S RNA (20S)- or a 23S RNA (23S)-expressing plasmid, or both together (20S+23S) were analyzed in an agarose gel and RNA was visualized by ethidium bromide staining (*EtBr*). *B* and *C*. Lysates were incubated in the presence (+) or absence (-) of anti-p91 (*B*) or anti-p104 (*C*) antiserum. A set of two blots was made and each one was hybridized with either 20S RNA (20S RNA probe), or 23S RNA (23S RNA probe)-specific probe. As a loading control, phenol extracted lysates were blotted and the membranes were hybridized with a specific probe for *E. coli* 23S ribosomal RNA (23S rRNA).
Figure 1

A

3' cis site for binding and replication

5' cis site for binding and replication

Stem-loop II

2392 162

B

Start

Stem-loop I

Stem-loop II

Stop

5' END

3' END

Yeast 20S RNA ribonucleoprotein complexes
Figure 2

A

B

C

Yeast 20S RNA ribonucleoprotein complexes

Figure 2

A

B

C

Yeast 20S RNA ribonucleoprotein complexes

Figure 2

A

B

C

Yeast 20S RNA ribonucleoprotein complexes

Figure 2

A

B

C

Yeast 20S RNA ribonucleoprotein complexes

Figure 2

A

B

C

Yeast 20S RNA ribonucleoprotein complexes
Yeast 20S RNA ribonucleoprotein complexes

Figure 3

Panel A: 7.5% gel showing total and anti-p91 samples. Two lanes for each condition.

Panel B: 14% gel showing anti-p91 samples. Two lanes for each condition.
Yeast 20S RNA ribonucleoprotein complexes

Figure 4

A

5' GGG ... AGGAGAUAAUACATGCUUGG
SD
Met
MGADP-p91

20S RNA

B

SD (AGGAGA)
PLOP91

T7
20S RNA

R

p91
834 aa

B

SD (AGGAGA)
PLOP92

T7
20S RNA

R

p91
829 aa

C

Shine-Dalgarno

23S rRNA
20S RNA
16S rRNA

EtBr
Northern

D

pLOP91 + -

20S RNA

20S RNA

p91 + -

Northern

Anti-p91
Yeast 20S RNA ribonucleoprotein complexes

**Figure 5**

**A**

- Vector
- pRE760
- pLS025
- pRE762
- pRE760 + pRE762
- pLS025 + pRE762

**B**

**p91 expressing vectors**

- PGK1
- p91
- PGK1
- MGADP

**20S RNA (-) strand vector**

- PGK1
- 2514
- 1

- α18 20S
Yeast 20S RNA ribonucleoprotein complexes

Figure 6

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20S RNA probe

Figure 7

- RNase A +

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EtBr

20S RNA probe

p91

Anti-p91
Figure 8

A

WT

C4A

G5C

13 bp

13 bp

12 bp

5-bp stem

CCCC

ACC

CCCC

5 bp

CAGGAGCCCCC-OH

B

23S rRNA

20S RNA

16S rRNA

EtBr

p91

Anti-p91

C

Anti-p91

Total

WT

C4A

G5C

5-bp stem

20S RNA probe
Yeast 20S RNA ribonucleoprotein complexes

**Figure 9**

![Image of gel electrophoresis](image)

- **Panel A**: Gel showing bands of 23S rRNA, 23S RNA, 20S RNA, and 16S rRNA.
- **Panel B**: Western blots showing protein expression under different conditions.
- **Panel C**: Western blots showing protein expression under different conditions.