1 Resting Complexes of the Persistent Yeast 20S RNA *Narnavirus* Consist Solely of the 20S
2 RNA Viral Genome and its RNA Polymerase p91*
3

4 **Lorena Vega[1,2], Laura Sevillano[1], Rosa Esteban[1] and Tsutomu Fujimura[1,3]**
5 [1]From the Instituto de Biología Funcional y Genómica. CSIC/Universidad de Salamanca, Spain
6 [2] Present address: VLP The Vaccine's Company. Avda. Francisco Vallés 8, 47151 Tech. Park,
7 Boecillo, Valladolid, Spain.
8

9 **Running title**: Yeast 20S RNA ribonucleoprotein complexes
10

11 [3]To whom correspondence should be addressed: Dr. Tsutomu Fujimura, Instituto de Biología
12 Functional y Genómica Lab P2.1. Zacarías González 2, Salamanca 37007, Spain. Tel. 34 923294901,
13 Fax. 34 923224876, E-mail: tfujimura@usal.es
14

15 **Keywords**: Ribonucleoprotein, yeast RNA virus, positive-strand RNA virus, virus persistence, RNA-
16 dependent RNA polymerase.
17

17 **Summary**
18
19 **The positive strand 20S RNA narnavirus persistently infects *Saccharomyces cerevisiae*.**
20 **The 20S RNA genome has a single gene that encodes the RNA-dependent RNA polymerase**
21 **(p91). 20S RNA forms ribonucleoprotein resting complexes (RNPs) with p91 and resides in the**
22 **cytoplasm. Here we found no host proteins stoichiometrically associated with the RNP by pull-**
23 **down experiments. Furthermore, 20S RNA, when expressed from a vector in *Escherichia coli*,**
24 **formed RNPs with p91 in the absence of yeast proteins. This interaction required the 3' *cis***
25 **signal for complex formation. Moreover, when 23S RNA, the genome of another narnavirus, was**
26 **expressed in *E. coli*, it also formed RNPs with its RNA polymerase p104. Finally, when both**
27 **RNAs are expressed in the same *E. coli* cell, they formed RNPs only with their cognate RNA**
28 **polymerases. These results altogether indicate that narnaviruses RNPs consist of only the viral**
29 **genomes and their cognate RNA polymerases. Because the copy number of the RNPs can be**
30 **induced almost equivalent to those of rRNAs in some yeast strains, the absence of host proteins**
31 **may alleviate the burden on the host by not sequestering proteins into the RNPs. It may also**
32 **contribute to the persistent infection of narnaviruses by decreasing their visibility.**
33
34 **Introduction**
35 20S RNA virus belongs to the genus *Narnavirus* and is among the simplest viruses in nature.
36 The virus has a single small positive strand genome (2514 nucleotides (nt)) called 20S RNA that
37 encodes a single protein of 91 kDa (p91), the RNA-dependent RNA polymerase (Wickner *et al.,*
38 2013). Because the virus has no capsid gene, 20S RNA is not encapsidated into a conventional virion
39 structure (Widner *et al.,* 1991; García-Cuéllar *et al.,* 1995). Instead, 20S RNA forms a
40 ribonucleoprotein (RNP) complexed with p91, and the virus, in the form of RNP, resides in the
41 cytoplasm of the yeast *Saccharomyces cerevisiae***.** Typical of fungal viruses, 20S RNA virus has no
42 extracellular transmission pathway. The virus is stably transmitted from mother to daughter cells, or
43 horizontally through mating. The virus does not kill the host nor render phenotypic changes to the
44 host.

45 20S RNA was originally discovered as an RNA species that accumulates under nitrogen
46 starvation (Kadowaki and Halvorson, 1971), a condition commonly used to induce sporulation in
47 yeast. In some strains, the copy number of 20S RNA becomes almost equivalent to those of rRNAs.
48 The high dosage may help the virus to distribute to meiotic progenies, although haploid cells can also
49 accumulate 20S RNA (Wejksnora and Haber, 1978). The majority of 20S RNA in induced cells is the
50 positive strand and it forms RNPs with p91 in a 1:1 stoichiometry (resting complexes) (Solórzano *et*
51 *al.,* 2000). The negative strands account only a few percent of the total 20S RNA population. Lysates
52 from induced cells also contain a minor amount of replication intermediates. These intermediates
53 contain p91 and synthesize 20S RNA positive strands *in vitro* (García-Cuéllar *et al.,* 1997). The
54 intermediates consist of a negative strand and a positive strand with less than unit length loosely
55 associated, perhaps through p91 (Fujimura *et al.,* 2005). The RNA backbone of the intermediates is
56 largely single-stranded but denaturation with phenol converts it to double-stranded. Upon completion
57 of positive strand synthesis, the product is released from the negative strand template. The released
58 product is still associated with protein and is indistinguishable from resting complexes. Because the
59 majority of negative strands in lysates are found in replication intermediates, the negative strand
60 template appears to be immediately recruited for another round of positive strand synthesis *in vivo*
61 (Fujimura *et al.,* 2005).

62 Most laboratory strains of yeast harbor 20S RNA virus. Fewer strains also contain another
63 narnavirus called 23S RNA. The 23S RNA genome (2891 nt) possesses a single gene that encodes its
64 RNA polymerase (p104) (Esteban *et al.,* 1992). 23S RNA also forms resting complexes with p104.
65 20S and 23S RNA viruses are independent and compatible in the same host. When co-habiting in the
66 same cell, these viruses form resting complexes containing the RNA genomes and their cognate RNA
67 polymerases and do not form hybrid complexes (García-Cuéllar *et al.,* 1995).

68 A launching system of 20S RNA virus from a yeast expression vector has been established
69 (Esteban *et al.,* 2005). The vector contains the full-length cDNA of 20S RNA under the constitutive
70 *PGK1* promoter. The ribozyme sequence from hepatitis delta virus (HDV) is directly attached to the 3'
71 end of the viral genome so that the precise 20S RNA 3' end can be generated *in vivo.* 20S RNA can be
72 launched efficiently from the vector and this system has been used to investigate *cis*-acting signals for

73 replication. Moreover, in the absence of an active ribozyme sequence, the transcripts from the vector,
74 without generating the virus, can form ribonucleoprotein complexes *in vivo* with p91 translated there
75 from. By combining with a pull-down assay, the latter system has served as a useful tool to investigate
76 *cis* acting signals for 20S RNA complex formation (Fujimura and Esteban, 2007). A similar launching
77 system (Esteban and Fujimura, 2003) and an assay system for complex formation (Fujimura and
78 Esteban, 2004) for 23S RNA virus have also been developed.

79 In resting complexes, p91 interacts with 20S RNA at three *cis* sites: the 5' and 3' end sites
80 and, to a lesser extent, an internal site (Fig. 1) (Fujimura and Esteban, 2007). The 3' site is located at
81 the 3rd and 4th C residues from the 3' end and the adjacent stem structure. The 3' site largely overlaps
82 (if not identical) with the 3' *cis* site for replication. The 5' site is located at the second stem structure
83 from the 5' end. Mutations at this site that destabilized complex formation also failed to generate the
84 virus from the launching vector. The tight relationship between complex formation and replication at
85 the 5' and 3' *cis* sites underlines the importance of stable resting complex formation in the virus life
86 cycle. The internal site is located somewhere between nt 1253 to 1515, but its precise location or
87 extent is not known. Mutations at the 5' or 3' *cis* site reduced complex formation to a basal level (10-
88 20% of the wild type level). The effect of a double mutation at both sites is not cumulative. We have
89 suggested that the interactions of p91 at the 5' and 3' *cis* sites are coordinated and that the internal *cis*
90 site is responsible for the basal level of complex formation observed (Fujimura and Esteban, 2007).

91 In spite of the small genome and its simple organization, 20S RNA virus establishes a
92 persistent infection in yeast. Because the majority of the viral genomes exist *in vivo* as resting
93 complexes, we have been investigating these complexes to understand the mechanism of viral
94 persistency. In this work we addressed the inquiry of whether the resting complex contains host
95 proteins. We found no host proteins stoichiometrically associated with metabolically labeled resting
96 complexes. Furthermore, 20S RNA and p91 formed complexes in *E. coli* with the same specificity as
97 in yeast, indicating that yeast proteins are not needed for complex formation. These results indicate
98 that the resting complexes consist of only 20S RNA and p91. The lack of host proteins in the resting
99 complexes may decrease the visibility of the virus in the cell. Moreover, when the virus accumulates
100 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,

129 RNPs were immunoprecipitated by partially purified anti-p91 antibodies. The pellet (fraction P2) and
130 the immunoprecipitate contained approximately 30% and 0.2%, respectively, of radioactivity of the
131 original cell lysate. Proteins in the immunoprecipitate were separated by SDS-PAGE and visualized
132 by fluorography (Fig. 3). We found no prominent yeast proteins pulled down specifically along with
133 p91. For example, the intensities of band ***a*** (68 KDa) and band ***b*** (25 kDa) proteins relative to that of
134 p91 correspond to 13.5 and 5.2%, respectively. These proteins appear to be more abundant in the
135 immunoprecipitate from 20S RNA-carrying cells than from the 20S RNA-negative strain. If we
136 assume that these proteins were labeled with ^{35}S with the same specific activity as p91, then we
137 calculated that only 0.18 and 0.19 molecules of ***a*** and ***b*** proteins, respectively, were pulled down along
138 with each molecule of p91. p91 (829 amino acids) contains 16 Met (1.93%) and 10 Cys (1.20%)
139 residues, while average *S. cerevisiae* proteins contain 2.08% Met and 1.31% Cys, and thus can be
140 labeled with ^{35}S slightly better than p91. These results strongly suggest that there is no host proteins
141 stoichiometrically associated with resting complexes. Because the P2 fraction also contains a small
142 amount of replication complexes (García-Cuéllar *et al.,* 1997), however, we cannot rule out the
143 possibility that minor proteins, such as ***a*** and ***b***, might be part of replication complexes. This
144 experimental approach underestimates proteins with lower Met and Cys contents. Furthermore we
145 cannot eliminate the possibility that the antibodies, upon binding to p91, may displace host protein(s)
146 from resting complexes.
147
148 *Expression of 20S RNA and p91 in E. coli*
149 If p91 does not require host proteins to form a resting complex, then the complex may be
150 formed even in a heterologous system in the absence of yeast proteins. We tested this possibility by
151 expressing p91 and 20S RNA in *Escherichia coli*. Two plasmids were constructed (Fig. 4, A and B).
152 One plasmid (pLOR91) contains the 20S RNA cDNA sequence under the T7 promoter. The Shine-
153 Dalgarno (SD) sequence AAGGAG was inserted between the promoter and the cDNA. The 3' end of
154 the 20S RNA genome was directly attached to the HDV ribozyme sequence. The second plasmid
155 (pLOR92) is the same as pLOR91 except that the 20S RNA cDNA was directly attached to the T7
156 promoter. Both plasmids expressed high amounts of 20S RNA in *E. coli* (Fig. 4C). As expected, p91

157 was expressed in cells containing pLOR91, while 20S RNA transcribed from pLOR92 was not
158 decoded to p91 because of the lack of the Shine-Dalgarno sequence. *E. coli* cells did not generate
159 autonomously propagating 20S RNA virus (Fig 4D). Once pLOR91 was cured, the cells did not
160 produce 20S RNA transcripts any longer. In yeast cells, launching of 20S RNA virus from a vector
161 required removal of non-viral sequences from the transcripts at both termini. If the proper viral
162 sequence is generated by removal of the Shine-Dalgarno sequence, then p91 cannot be translated from
163 it in *E. coli* cells. It is also possible that the virus needs yeast proteins for replication.

164 p91 expressed in *E. coli* has an extra amino acid sequence (MGADP) at the N-terminus. To
165 demonstrate that the extra sequence does not impair the activity of p91, we did the following *in vivo*
166 experiments in yeast. Previously we have shown that 20S RNA virus can be generated from 20S RNA
167 negative strands transcribed from a vector, provided an active p91 is supplied from a second vector
168 (Esteban *et al.,* 2005). p91 cannot be translated from the negative strands, thus in the absence of the
169 second vector there is no virus generation. It implies that the first round of positive strand synthesis
170 catalyzed by p91 expressed from the second vector is critical for virus generation. As expected, the
171 negative strand-expressing vector alone did not generate 20S RNA virus (Fig. 5, lane 4). However, if
172 intact p91 or p91 with MGADP was expressed from a second vector, 20S RNA virus was generated
173 with similar efficiency (Fig. 5, lanes 5 and 6.). The second vector alone (either with or without
174 MGADP) did not launch the virus because it contained the C4A mutation (numbered from the 3' end
175 of the 20S RNA genome) that abolishes 20S RNA replication (Fig. 5, lanes 2 and 3) (Esteban *et al.,*
176 2005). These results indicate that the extra amino acids at the N-terminus do not compromise the RNA
177 polymerase activity of p91.

178

179 *Complex Formation in E. coli*

180 To examine whether p91 forms a complex with 20S RNA in *E. coli* cells, two experimental
181 approaches were taken: pull-down assays and glycerol gradient sedimentation. In the first approach, a
182 lysate from *E. coli* cells transformed with pLOR91 was subjected to immunoprecipitation with anti-
183 p91 antiserum. As shown in Fig. 6 lane 2, 20S RNA was pulled down specifically with anti-p91
184 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

213 forms a RNP with its RNA polymerase p104. 20S RNA and 23S RNA viruses can reside and
214 propagate stably in the same yeast host. Even residing together in the same cell, they do not form
215 hybrid RNPs. We constructed a plasmid to express 23S RNA and p104 in *E. coli* and introduced it into
216 bacterial cells together with the 20S RNA-expressing plasmid. These two plasmids have different
217 antibiotic markers. The concentrations of antibiotics were adjusted so that similar amounts of 20S
218 RNA and 23S RNA were produced in the same cell (Fig. 9A, the far right column). Lysates were
219 prepared and subjected to pull-down assays using anti-p91 and anti-p104 antisera. 20S and 23S RNAs
220 in the immunoprecipitates were detected with specific probes. As shown in Fig. 9B, anti-p91
221 antiserum pulled down 20S RNA but not 23S RNA. It indicates that p91, even in *E. coli* cells,
222 specifically interacts with 20S RNA to form RNPs and discriminates 23S RNA from the reaction.
223 Thus p91 does not require yeast proteins for proper RNP formation. Similarly, the anti-p104 antiserum
224 pulled down 23S RNA but not 20S RNA (Fig. 9C). It indicates that p104 and 23S RNA can also form
225 an RNP in *E. coli* in the absence of yeast proteins and that p104 correctly chooses 23S RNA as a
226 partner to form its own RNPs.
227

228 **Discussion**

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

239 Although 20S RNA/p91 RNPs were assembled in *E. coli*, they did not produce autonomously
240 propagating 20S RNA virus. Launching of 20S RNA from a vector in yeast requires removal of

241 extraneous terminal sequences from the transcripts to expose the mature 5' and 3' viral termini. The
242 expression of p91 in *E. coli* required the Shine-Dalgarno sequence and the removal of the ribosome-
243 binding site would make the generated viral RNA a poor template for translation. Furthermore, we did
244 not observed 20S RNA negative strand synthesis by p91 in *E. coli* cells nor RNA polymerase activity
245 of p91 in bacterial cell lysates (unpublished results). Unlike resting complex formation, replication of
246 20S RNA may require yeast proteins.

247 Since there is no host protein in resting complexes, p91 is solely responsible for the
248 interactions at the three *cis* sites in forming the complexes. The 3' *cis* site is located close to the 3'
249 end. Because exonucleases play the major role in mRNA degradation in eukaryotes, it suggests that
250 p91 protect the 20S RNA genome from degradation by binding to the 3' end. mRNA decay usually
251 begins with shortening the 3' poly(A) tail followed by decapping at the 5' end (Wilusz *et al.,* 2001;
252 Parker and Song, 2004). Then decapped RNA is degraded by the *SKI1/XRN1* 5' exonuclease.
253 Alternatively, deadenylated RNA is digested by a 3' exonuclease complex called the exosome
254 (Mitchell *et al.,* 1997; Jacobs Anderson and Parker, 1998). The exosome is present in both the nucleus
255 and the cytoplasm (Mitchell *et al.,* 1997; Allmang *et al.,* 1999) and has compartment-specific auxiliary
256 factors. In the cytoplasm, *SKI*2, *SKI3*, and *SKI8* form the so-called SKI complex (Jacobs Anderson
257 and Parker, 1998; Brown *et al.,* 2000,) and the complex is physically linked to the exosome through
258 *SKI7* (Araki *et al.,* 2001). An RNA substrate is channeled from the SKI complex to the exosome for
259 degradation (Halbach *et al.,* 2013). 20S RNA has no 3' poly(A) tail. Although it is not known whether
260 the 5' end is capped, it resembles intermediates of mRNA degradation. In *ski2*, *ski3, ski7* or *ski8*
261 mutants, the copy number of 20S RNA (and 23S RNA) greatly increases (Matsumoto *et al.,* 1990;
262 Ramírez-Garratacho and Esteban, 2011), indicating that there is a stage in the virus life cycle
263 vulnerable to the exosome. 20S RNA has a strong secondary structure at the 5' end and the first four
264 consecutive Gs are buried at the bottom of the stem structure. These features confer on 20S RNA fully
265 resistance to the *SKI1/XRN1* 5' exonuclease. Destabilizing the 5' secondary structure makes 20S RNA
266 vulnerable to *SKI1* suppression (Esteban *et al.,* 2008). Although 20S RNA is not encapsidated into a
267 protective capsid structure, formation of resting complexes may protect 20S RNA from exonucleases
268 in the host cytoplasm. It is also possible that the formation of a resting complex with 20S RNA

269 stabilizes p91. So far we have been unable to dissociate p91 from the RNPs in an active form. It is
270 well known that in growing conditions both rRNAs and ribosomal proteins become very stable once
271 assembled into RNP particles (Deutscher, 2003). It has been observed that expression of active
272 hepatitis B reverse transcriptase requires the presence of the template RNA binding site (Wang *et al.,*
273 1994; Tavis and Ganem, 1996). The reverse transcriptase LtrA of group II intron is also stabilized by
274 forming RNP particles complexed with excised intron RNA (Saldanha *et al.,* 1999). *In silico*,
275 intramolecular long distance interactions bring the three *cis* sites of 20S RNA close together (Fujimura
276 *et al.,* 2007). Perhaps it is a prerequisite for a single p91 molecule to interact with the three sites
277 simultaneously. The molecular mass of p91 is one eighth of that of 20S RNA (736 kDa). A large area
278 of 20S RNA molecule would remain uncovered in the complex and directly exposed to the cytoplasm.
279 20S RNA/p91 RNPs may be well adapted to the challenge of exonucleases, however, they may be
280 vulnerable to endonucleolytic cleavages. Recently, it has been shown that *S. cerevisiae* can support the
281 RNAi system if Dicer and Argonaute are imported from *S. castellii* (Drinnenberg *et al.,* 2009). The
282 constructed strains lost the double-stranded RNA (dsRNA) killer viruses M and L-A (Drinnenberg *et*
283 *al.,* 2011). Curiously, however, L-BC dsRNA virus (Drinnenberg *et al.,* 2011) and L-A variants
284 (Rodríguez-Cousiño *et al.,* 2013) were not eliminated by the extraneous RNAi system. Their dsRNA
285 genomes are encapsidated into protective capsids, while 20S RNA is not. As expected, 20S RNA virus
286 is much more sensitive to RNA interference than these encapsidated dsRNA viruses (R.E., P.G. and
287 N.R, manuscript in preparation).

288 The absence of host proteins in the stable resting complexes suggests that 20S RNA viruses
289 keep the dependency to host proteins at minimum in its life cycle. In sporulation conditions some
290 yeast strains accumulate 20S RNA to an amount almost equivalent to those of rRNAs. Since extensive
291 degradation of ribosomes as well as vegetative proteins occurs during sporulation (Esposito and
292 Klapholz, 1981), it may not be much burden for the cell to provide precursors for synthesis of 20S
293 RNA and p91. The high copy number of resting complexes may help the virus to be stably transmitted
294 to the meiotic descendants. However, if host proteins were constituents of a resting complex, then a
295 great increase of the complex during sporulation might exhaust the proteins and thus be harmful for
296 the host. 20S RNA virus is a persistent virus and has no extracellular transmission pathway. Since

297 there is no opportunity of escaping from the cell, to inflict too much damage to the host against non-
298 infected cells is also undesirable for the virus. Furthermore, if such proteins were essential part of the
299 complex, then it would provide a measure for the host to antagonize the virus through regulating the
300 proteins. Thus the absence of host protein in the resting complex may contribute to the persistent
301 infection by decreasing the visibility of 20S RNA virus in the cell.
302
303 **Experimental procedures**
304 *In vivo Labeling*
305 Yeast strain 924 (*a ura3 his3 leu2 ski2Δ*, L-A-o, 20S RNA) or 913 (isogenic to 924 but 20S
306 RNA-o) was grown in complete synthetic medium H (Wickner, 1980) (5 ml) supplemented with a
307 mixture of ^{35}S-Met and ^{35}S-Cys (0.13 mCi ml^{-1}, Perkin Elmer). The concentrations of both amino acids
308 were adjusted to 20 μg ml^{-1}. The cells were grown at 28 ºC for three days, transferred to 1% K acetate
309 and then kept another 16 h to induce 20S RNA.
310
311 *Differential Centrifugation*
312 Cells were harvested, washed once with H_2O and then suspended in lysis buffer (50 mM Tris-
313 HCl pH 8.0, 100 mM NaCl). The cells were broken with glass beads (0.40-0.60 mm in diameter,
314 Sartorius) using Fast Prep P120 (Bio101Sarvant) with two pulses of 15 s at speed 4.5. After removing
315 cell debris and unbroken cells, the lysates were centrifuged at 55000 rpm (120000 xg) for 30 min with
316 the Beckman-Coulter rotor TLA-100.2 to remove ribosomes. The supernatant was re-centrifuged at
317 75000 rpm (250000 xg) for 2 h in a TLA-100.3 rotor to separate 20S RNA/p91 complexes from the
318 bulk of soluble proteins. The pellet was suspended in the lysis buffer and subjected to pull-down
319 experiments or glycerol gradient centrifugation.
320
321 *Antibody Purification*
322 Anti-p91 antibodies were partially purified using a column containing protein A-conjugated
323 Sepharose CL-4B (GE Healthcare). After extensive washing with PBS buffer (20 mM Na phosphate,

324 pH 7.0, 0.15 M NaCl), the antibodies bound were eluted with 0.1 M glycine-HCl pH 3.0. The pH of
325 the fractions was immediately adjusted to neutral by the addition of 1 M Tris-HCl pH 9.0.
326
327 *Preparation of E. coli Lysates*
328 *E. coli* BL21 cells transformed with 20S RNA- and/or 23S RNA-expressing plasmids were
329 grown at 37 ºC for 4 h in 30 ml of LB medium supplemented with 100 μg ml^{-1} ampicillin and/or 50 μg
330 ml^{-1} kanamycin. Isopropyl β-D-1-thiogalactopyranoside (1 mM) was added to the culture and the cells
331 were kept at 28 ºC for another 5 h to express the viral genome(s). Cells were harvested, suspended in
332 the lysis buffer supplemented with 0.1% bentonite, 1 mg ml^{-1} lysozyme, and 1x protease inhibitor
333 mixture (GE Healthcare), and broken with glass beads (0.25-0.30 mm in diameter) using Fast Prep
334 P120 (one pulse of 15 s with speed 4.5). The lysates were diluted three times with the lysis buffer,
335 centrifuged to remove cell debris and unbroken cells, and then subjected to pull-down experiments.
336
337 *Glycerol Gradients*
338 Glycerol gradient centrifugation was done following the procedure described previously for
339 sucrose gradient centrifugation (Wejksnora and Haber, 1978; Widner, *et al.,* 1991) by simply
340 substituting 10-40% sucrose with 10-40% glycerol.
341
342 *Pull-down Assay*
343 To 10-160 μl of the lysate prepared from yeast or *E. coli* as described above, 1 ml of Tris-
344 buffered saline-Tween 20 (10 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.05% Tween 20), 1 mM
345 DTT, 40 units of RNasin (Promega), 20 μg of yeast tRNA (Invitrogen) and 2 μl of anti-p91 or anti-
346 p104 antiserum or 0.5 μl of partially purified anti-p91 antibodies (16 mg ml^{-1}) were added and the
347 mixture was incubated at 4 ºC for 30 min. 25 μl (wet volume) of protein A conjugated Sepharose CL-
348 4B was added to the mixture and it was incubated at 4 ºC for another 30 min. The sepharose was
349 washed 5 times with 1 ml Tris-buffered saline-Tween 20 and 1 mM DTT. RNA was extracted from
350 the sepharose, slot-blotted and detected by hybridization as described in (Fujimura and Esteban,

351 2004). The probes used to detect 20S RNA, 23S RNA, and *E. coli* 23S rRNA are complementary to nt
352 1262-2514 of 20S RNA, nt 1-2891 of 23S RNA, and nt 435-1005 of *E. coli* 23S rRNA, respectively.
353 Alternatively, protein bound to sepharose was eluted with loading buffer for SDS acrylamide gels and
354 separated in a 7.5% or 14% SDS gel. After electrophoresis, the gel was soaked with the Amersham
355 amplify fluorographic reagent and protein bands were detected by fluorography. Quantification of
356 bands was done using a PMI^{TM} Personal Molecular Imager (Biorad).
357

358 *Plasmids*

359 For expression of 20S and 23S RNA in *E. coli* we used the following vectors. 20S RNA:
360 pLOR84 is a derivative of pT7-7 (Tabor and Richardson, 1985) that contains the complete 20S RNA
361 cDNA (2514 nt) downstream of the Shine-Dalgarno sequence with the HDV ribozyme fused at its 3'
362 end. p91 expressed from pLOR84 has 5 extra amino acids at its N-terminus (MGADP). pLOR91 was
363 constructed from pLOR84 by inserting a 1.4 kb DNA fragment containing the kanamycin resistance
364 gene into the ampicillin resistance gene. pLOR92 was made from pLOR91 by eliminating the
365 sequence between the T7 promoter and the 20S RNA 5' end. 23S RNA: 23S RNA and p104 were
366 expressed from pRE1048. This plasmid is identical to pLOR91, except that the 20S RNA cDNA was
367 substituted by the full 23S cDNA sequence. p104 expressed from pRE1048 has 3 extra amino acids at
368 its N-terminus (MGA). Yeast 20S RNA launching vectors from genomic or antigenomic strands have
369 been described previously (Esteban *et al.,* 2005). Mutations in the vectors were introduced by site
370 directed mutagenesis (Esteban *et al.,* 1989).
371

372 **References**

373 Allmang, C., Kufel, J., Chanfreau, G., Mitchell, P., Petfalski, E., and Tollervey, D. (1999) Functions
374 of the exosome in rRNA, snoRNA and snRNA synthesis. *EMBO J.* **18**, 5399-5410.
375

376 Araki, Y., Takahashi, S., Kobayashi, T., Kajiho, H., Hoshino, S., and Katada, T. (2001) Ski7p G
377 protein interacts with the exosome and the Ski complex for 3'-to-5' mRNA decay in yeast. *EMBO J.*
378 **20**, 4684-4693.
379

380 Brown, J.T., Bai, X., and Johnson, A.W. (2000) The yeast antiviral proteins Ski2p, Ski3p, and Ski8p
381 exist as a complex *in vivo*. *RNA* **6**, 449-457.
382

383 Deutscher, M.P. (2003) Degradation of stable RNA in bacteria. *J. Biol. Chem.* **278**, 45041-45044.

384
385 Drinnenberg, I.A., Fink, G.R., and Bartel, D.P. (2011) Compatibility with killer explains the rise of
386 RNAi-deficient fungi. *Science* **333**, 1592
387
388 Drinnenberg, I.A., Weinberg, D.E., Xie, K.T., Mower, J.P., Wolfe, K.H., Fink, G.R., and Bartel, D.P.
389 (2009) RNAi in budding yeast. *Science* **326**, 544-550
390
391 Esposito, R.E., and Klaplolz, S. (1981) Meiosis and ascospore development. In *The molecular biology*
392 *of the yeast Saccharomyces cerevisiae. Life cycle and inheritance*. Strathern, J.N., Jones, E.W., and
393 Broach, J.R. (eds). New York: Cold Spring Harbor laboratory. pp. 211-288.
394
395 Esteban, L.M., Rodriguez-Cousiño, N., and Esteban, R. (1992) T double-stranded RNA (dsRNA)
396 sequence reveals that T and W dsRNAs form a new RNA family in *Saccharomyces cerevisiae*.
397 Identification of 23 S RNA as the single-stranded form of T dsRNA. *J. Biol. Chem.* **267**, 10874-
398 10881.
399
400 Esteban, R., and Fujimura, T. (2003) Launching the yeast 23S RNA Narnavirus shows 5' and 3' *cis*-
401 acting signals for replication. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 2568-2573.
402
403 Esteban, R., Fujimura, T., and Wickner, R.B. (1989) Internal and terminal *cis*-acting sites are
404 necessary for *in vitro* replication of the L-A double-stranded RNA virus of yeast. *EMBO J.* **8**, 947-
405 954.
406
407 Esteban, R., Vega, L., and Fujimura, T. (2005) Launching of the yeast 20S RNA narnavirus by
408 expressing the genomic or antigenomic viral RNA *in vivo*. *J. Biol. Chem.* **280**, 33725-33734.
409
410 Esteban, R., Vega, L., and Fujimura, T. (2008) 20S RNA narnavirus defies the antiviral activity of
411 *SKI1/XRN1* in *Saccharomyces cerevisiae*. *J. Biol. Chem*. **283**, 25812-25820.
412
413 Fujimura, T., and Esteban, R. (2004) The bipartite 3'-*cis*-acting signal for replication is required for
414 formation of a ribonucleoprotein complex *in vivo* between the viral genome and its RNA polymerase
415 in yeast 23S RNA virus. *J. Biol. Chem*. **279**, 44219-44228.
416
417 Fujimura, T., and Esteban, R. (2007) Interactions of the RNA polymerase with the viral genome at the
418 5'- and 3'-ends contribute to 20S RNA narnavirus persistence in yeast. *J. Biol. Chem.* **282**, 19011-
419 19019.
420
421 Fujimura, T., Solórzano, A., and Esteban, R. (2005) Native replication intermediates of the yeast 20S
422 RNA virus have a single-stranded RNA backbone. *J. Biol. Chem.* **280**, 7398-7406.
423
424 García-Cuéllar, M.P., Esteban, L.M., Fujimura, T., Rodríguez-Cousiño, N., and Esteban, R. (1995)
425 Yeast viral 20 S RNA is associated with its cognate RNA-dependent RNA polymerase. *J. Biol. Chem.*
426 **270**, 20084-20089.
427
428 García-Cuéllar, M.P., Esteban, R., and Fujimura, T. (1997) RNA-dependent RNA polymerase activity
429 associated with the yeast viral p91/20S RNA ribonucleoprotein complex. *RNA* **3**, 27-36.
430
431 Halbach, F., Reichelt, P., Rode, M., and Conti, E. (2013) The yeast ski complex: crystal structure and
432 RNA channeling to the exosome complex. *Cell* **154**, 814-826.
433
434 Jacobs Anderson, J.S., and Parker, R. (1998) The 3' to 5' degradation of yeast mRNAs is a general
435 mechanism for mRNA turnover that requires the *SKI2* DEVH box protein and 3' to 5' exonucleases of
436 the exosome complex. *EMBO J.* **17**, 1497-1506.
437

Kadowaki, K., and Halvorson, H.O. (1971) Appearance of a new species of ribonucleic acid during sporulation in *Saccharomyces cerevisiae*. *J. Bacteriol.* **105**, 826-830.

Matsumoto, Y., Fishel, R., and Wickner, R.B. (1990) Circular single-stranded RNA replicon in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 7628-7632.

Mitchell, P., Petfalski, E., Shevchenko, A., Mann, M., and Tollervey, D. (1997) The exosome: a conserved eukaryotic RNA processing complex containing multiple 3'->5' exoribonucleases. *Cell* **91**, 457-466.

Parker, R., and Song, H. (2004) The enzymes and control of eukaryotic mRNA turnover. *Nat. Struct. Mol. Biol.* **11**, 121-127.

Ramírez-Garrastacho, M., and Esteban, R. (2011) Yeast RNA viruses as indicators of exosome activity: human exosome hCsl4p participates in RNA degradation in *Saccharomyces cerevisiae. Yeast* **28**, 821-832.

Rodríguez-Cousiño, N., Gómez, P., and Esteban, R. (2013) L-A-lus, a new variant of the L-A totivirus found in wine yeasts with Klus killer toxin-encoding Mlus double-stranded RNA: possible role of killer toxin-encoding satellite RNAs in the evolution of their helper viruses. *Appl. Environ. Microbiol.* **79**, 4661-4674

Saldanha, R., Chen, B., Wank, H., Matsuura, M., Edwards, J., and Lambowitz, A.M. (1999) RNA and protein catalysis in group II intron splicing and mobility reactions using purified components. *Biochemistry* **38**, 9069-9083.

Solórzano, A., Rodríguez-Cousiño, N., Esteban, R., and Fujimura, T. (2000) Persistent yeast single-stranded RNA viruses exist *in vivo* as genomic RNA/RNA polymerase complexes in 1:1stoichiometry. *J. Biol. Chem.* **275**, 26428-26435.

Tabor, S., and Richardson, C.C. (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. U. S. A*. **82**, 1074-1078.

Tavis, J.E, and Ganem, D. (1996) Evidence for activation of the hepatitis B virus polymerase by binding of its RNA template. *J. Virol.* **170**, 5741-5750.

Wang, G.H., Zoulim, F., Leber, E.H., Kitson, J., and Seeger, C. (1994) Role of RNA in enzymatic activity of the reverse transcriptase of hepatitis B viruses. *J. Virol.* **68**, 8437-8442.

Wejksnora, P.J., and Haber, J.E. (1978) Ribonucleoprotein particle appearing during sporulation in yeast. *J. Bacteriol.* **134**, 246-260.

Wickner, R.B. (1980) Plasmids controlled exclusion of the K2 killer double-stranded RNA plasmid of yeast. *Cell* **21**, 217-226.

Wickner, R.B., Fujimura, T., and Esteban, R. (2013) Viruses and prions of *Saccharomyces cerevisiae*. *Adv. Virus Res*. **86**, 1-16.

Widner, W.R., Matsumoto, Y., and Wickner, R.B. (1991) Is 20S RNA naked? *Mol. Cell. Biol.* **11**, 2905-2908.

Wilusz, C.J., Wormington, M., and Peltz, S.W. (2001) The cap-to-tail guide to mRNA turnover. *Nat. Rev. Mol. Cell Biol.* **2**, 237-246.

493 **Aknowledgments**
494
495 This work was supported by Grant BFU2010-15768 from The Spanish Ministry of Education
496 and Science. We wish to thank "Fundación Ramón Areces" for financial support of our Institute and to
497 Pilar Gómez Jiménez for her invaluable technical assistance.
498
499 **Legends to figures**
500
501 Figure 1 *A*. **Diagram of the 5' and 3' end regions of the 20S RNA genome**. The 5' and 3' end *cis*
502 sites for complex formation and replication are indicated. *B*. Nucleotide sequences and secondary
503 structures at the 5' end (left panel) and 3' end (right panel) regions. The initiation (*start*) and
504 termination (*stop*) codons of p91 are indicated.
505
506 Figure 2. **Partial purification of 20S RNA/p91 complexes by differential centrifugation**. *A*. A cell
507 lysate was separated into pellet (*P*) and supernatant (*S*) fractions by two sequential centrifugations (*1*
508 and *2*) at different speeds. RNA was extracted, separated on an agarose gel and visualized by ethidium
509 bromide staining. *T*, initial cell lysate. *B*. 20S RNA was immunoprecipitated from the P2 fraction
510 shown in *A* in the presence (*lane 2*) or absence (*lane 3*) of anti-p91 antiserum, separated in an agarose
511 gel, and detected by Northern hybridization using a 20S RNA-specific probe. As control, total RNA
512 from the initial lysate (*T* shown in *A*) was processed in parallel but without immunoprecipitation (*lane*
513 *1*). *C*. Fraction P2 was subjected to 10-40% glycerol gradient centrifugation. RNA from gradient
514 fractions was separated in an agarose gel and visualized by ethidium bromide staining (*upper panel*).
515 20S RNA and p91 were visualized by Northern hybridization (*middle panel*) and Western blotting
516 (*lower panel*), respectively.
517
518 Figure 3. **No yeast proteins are stoichiometrically associated with 20S RNA/p91 resting**
519 **complexes**. Lysates were prepared from 20S RNA-negative (*lane 1*) and positive (*lane 2*) strains
520 metabolically labeled with ^{35}S. After differential centrifugation, the pellet fractions (*P2*) were
521 subjected to immunoprecipitation with partially purified anti-p91 antibodies (*anti-p91*). The

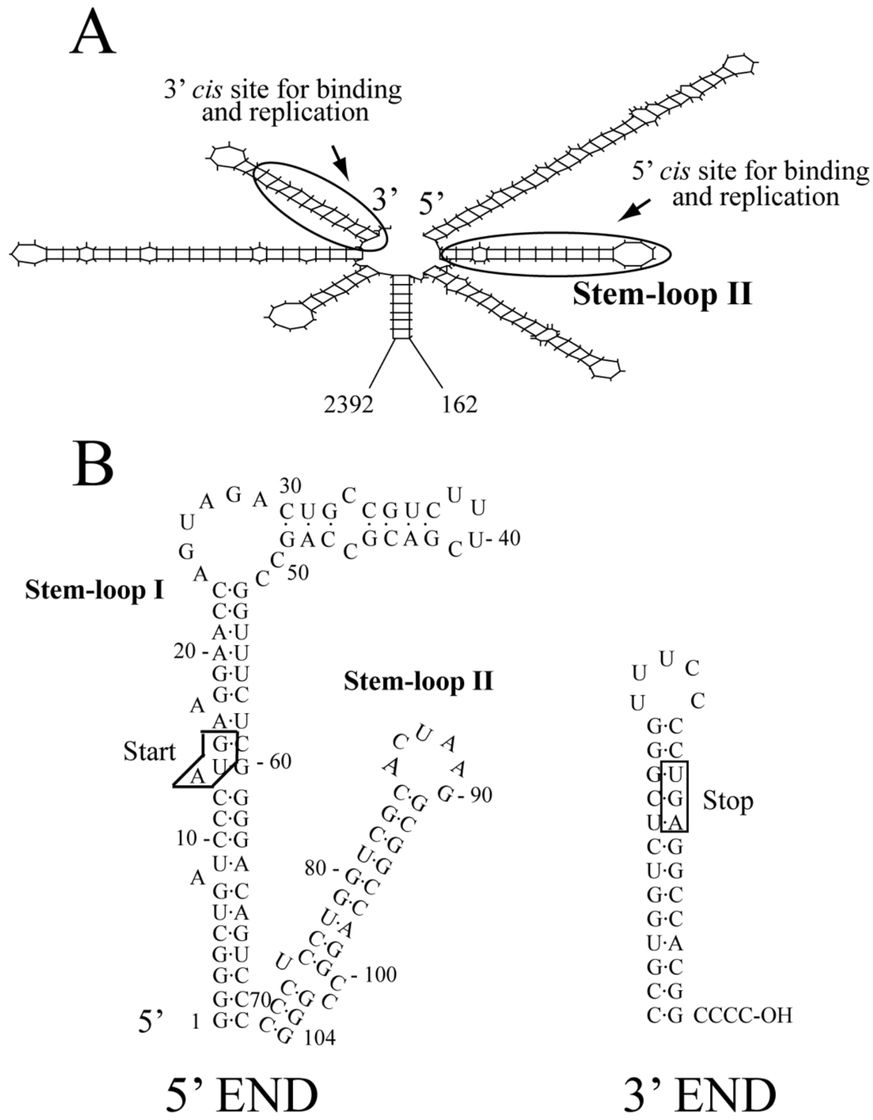
522 immunoprecipitates were separated in 7.5% (*A*) and 14% (*B*) acrylamide/SDS gels. Proteins were
523 visualized by fluorography. Scanning of protein bands are shown on the right of the panels. Black
524 color, strain with 20S RNA; pale gray, control strain without 20S RNA. The pellet fractions without
525 immunoprecipitation (*Total*) were also analyzed in a 7.5% gel as shown in *A*. *a* and *b* band proteins:
526 see the explanation in the text. M, molecular standards (*kDa*).
527
528 Figure 4. **The expression of p91 *in E. coli* requires the Shine-Dalgarno sequence**. *A*. The nucleotide
529 sequence at the 5' end of 20S RNA transcript expressed from pLOR91. The 5' terminal nucleotide of
530 the 20S RNA genome is numbered (*1*) and the initiation codon of p91 is marked by *start*. The bars
531 separate the codons of p91. The transcript contains an extra 66 nt upstream sequence derived from the
532 *E. coli* T7-7 expression plasmid, including the Shine-Dalgarno (*SD*) sequence and a new initiation
533 codon *Met*. Thus p91 expressed from this transcript has 5 extra amino acids (MGADP) at the N
534 terminus. *B*. Diagrams of pLOR91 and the control plasmid pLOR92. 20S RNA cDNA sequences are
535 indicated by thick black lines. *T7*; T7 promoter. *R*; HDV ribozyme. In pLOR92 the 20S RNA cDNA is
536 directly fused to the T7 promoter. *C*. Expression of 20S RNA and p91 in *E. coli* from the Shine-
537 Dalgarno-containing pLOR91 (+) or non-containing pLOR92 (-) plasmid. The expression of 20S RNA
538 was detected by ethidium bromide staining of an agarose gel (*EtBr*) and confirmed by Northern
539 hybridization using a 20S RNA-specific probe (*Northern*). The expression of p91 was monitored by
540 Western blotting using anti-p91 antiserum (*anti-p91*). *D*. 20S RNA virus is not generated in *E. coli*.
541 After expression of 20S RNA transcripts from pLOR91, *E. coli* cells were grown in the absence of
542 kanamycin to cure the plasmid. The expression of 20S RNA and p91 in pLOR91-containing (+) or
543 pLOR91-cured (-) cells was examined as described in *C*. Note that rRNAs in *E. coli* have smaller sizes
544 compared to the yeast counterparts (23S *versus* 25S, and 16S *versus* 18S).
545
546 Figure 5. **MGADP-p91 is active in yeast to generate 20S RNA in a two-vector system**. *A*. A yeast
547 strain free of 20S RNA was transformed with the 20S RNA negative strand-expressing vector pRE762
548 alone (*lane 4*) or along with p91 expressing vector pRE760 or pLS025 (*lane 5 or 6*). pRE760
549 expresses wild type p91, while p91 expressed from pLS025 has 5 extra amino acids at the N-terminus

550 (*MGADP-p91*). Both p91-expressing vectors have a mutation at the 3' terminus of the viral genome
551 (C4A) so that each vector alone cannot generate 20S RNA virus *in vivo*. RNA was extracted from the
552 cells and analyzed in an agarose gel. Ethidium bromide staining (*EtBr*) and Northern hybridization
553 with a 20S RNA probe (*20S RNA probe*) of the gel are shown. As controls, untransformed cells (*lane*
554 *1*) and cells transformed with p91-expressing vectors alone (*lanes 2 and 3*) were processed in parallel.
555 *B*. Diagrams of the p91 expressing vectors and 20S RNA negative strand expressing vector used in *A*.
556 *PGK1*: the constitutive *PGK1* promoter. The C4A mutation at the 3' end of 20S RNA genome on the
557 p91 expressing vectors is indicated by the asterisks.
558
559 Figure 6. **Pull down of 20S RNA expressed in *E. coli* with anti-p91 antiserum**. A lysate was
560 prepared from *E. coli* cells harboring the 20S RNA-expressing plasmid with Shine-Dalgarno sequence
561 (*pLOR91*) or a vector alone (*Vector*). 20S RNA was immunoprecipitated in the presence (+) or in the
562 absence (-) of anti-p91 antiserum. As positive control, a lysate prepared from yeast cells carrying
563 endogenous 20S RNA virus was processed in parallel. 20S RNA in the immunoprecipitates was
564 detected with a specific probe.
565
566 Figure 7. **20S RNA and p91 expressed *in E. coli* co-sediment through glycerol gradient**
567 **centrifugation**. A lysate prepared from pLOR91-containing cells was applied to 10-40% glycerol
568 gradient centrifugation (*left panel, RNase A* -). As a control, the lysate was pre-digested with RNase A
569 and then subjected to centrifugation (*right panel, RNAse A* +). After sedimentation the gradients were
570 fractionated. The top and bottom of the gradients are indicated. 20S RNA and p91 in the fractions
571 were detected as described in the legend to Figure 2. The main peaks of 20S RNA and p91 in the
572 gradients are indicated by the arrows. *L*, the lysate before loading onto the gradients.
573
574 Figure 8. **20S RNA/p91 RNP formation in *E. coli* requires the 3' *cis* site for full activity**. *A*.
575 Diagrams of the 3' end regions of 20S RNA WT and 3' *cis* mutants. Nucleotides changed are
576 underlined and in bold face. In the 5-bp stem mutant, the wild type sequence 12-GGCCACGG-5 was
577 replaced with 12-CAGGAGGC-5 (numbered from the 3' end). *B*. The expression of WT and mutant

578 20S RNA in *E. coli (upper panel).* p91 expressed from the plasmids is shown in the lower panel. Note
579 that the mutations analyzed do not change the amino acid sequence of p91. *C*. Pull-down assay. A
580 lysate containing WT or mutant 20S RNA was incubated in the presence (+) or absence (-) of anti-p91
581 antiserum to immunoprecipitate 20S RNA. The RNA was detected with a specific probe for 20S RNA.
582 Phenol-extracted lysates without immunoprecipitation were also analyzed as loading controls (*Total*).
583
584 Figure 9. **20S RNA and 23S RNA form complexes only with their respective cognate RNA**
585 **polymerases when expressed in the same *E. coli* cell**. *A*. Lysates prepared from *E. coli* cells
586 containing no plasmid (-), or harboring a 20S RNA (*20S*)- or a 23S RNA (*23S*)-expressing plasmid, or
587 both together (*20S+23S*) were analyzed in an agarose gel and RNA was visualized by ethidium
588 bromide staining (*EtBr*). *B* and *C*. Lysates were incubated in the presence (+) or absence (-) of anti-
589 p91 (*B*) or anti-p104 (*C*) antiserum. A set of two blots was made and each one was hybridized with
590 either 20S RNA (*20S RNA probe*), or 23S RNA (*23S RNA probe*)-specific probe. As a loading control,
591 phenol extracted lysates were blotted and the membranes were hybridized with a specific probe for *E.*
592 *coli* 23S ribosomal RNA (*23S rRNA*).
593

593
594 **Figure 1**

595

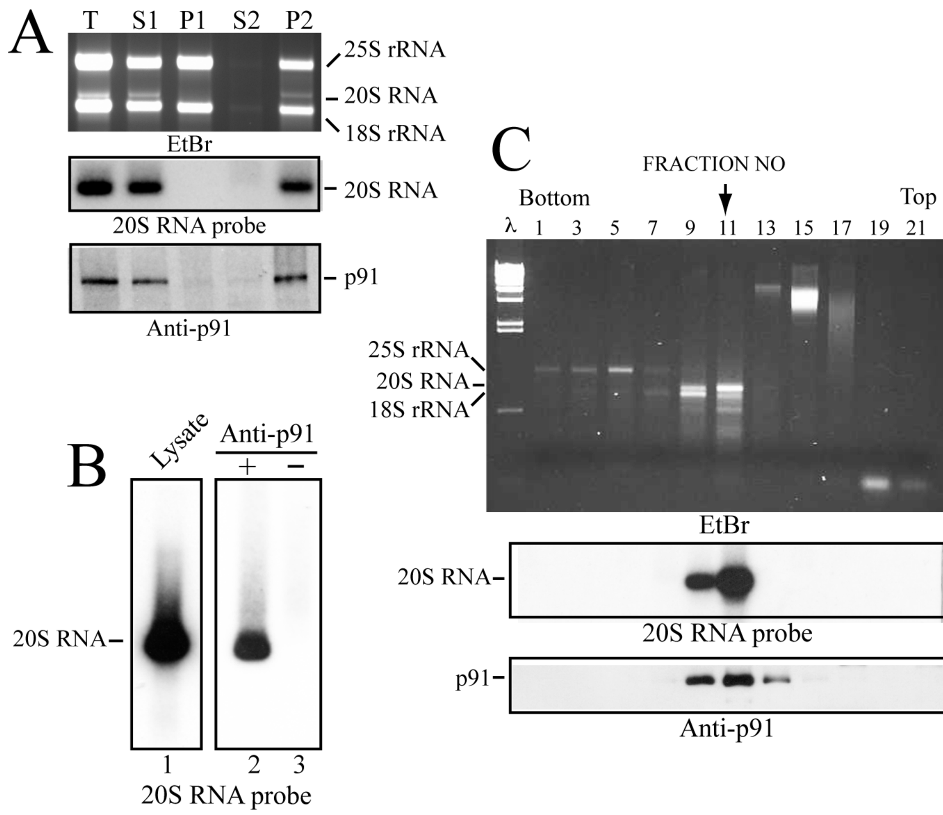


596
597

597 **Figure 2**

598

599



600

601

602

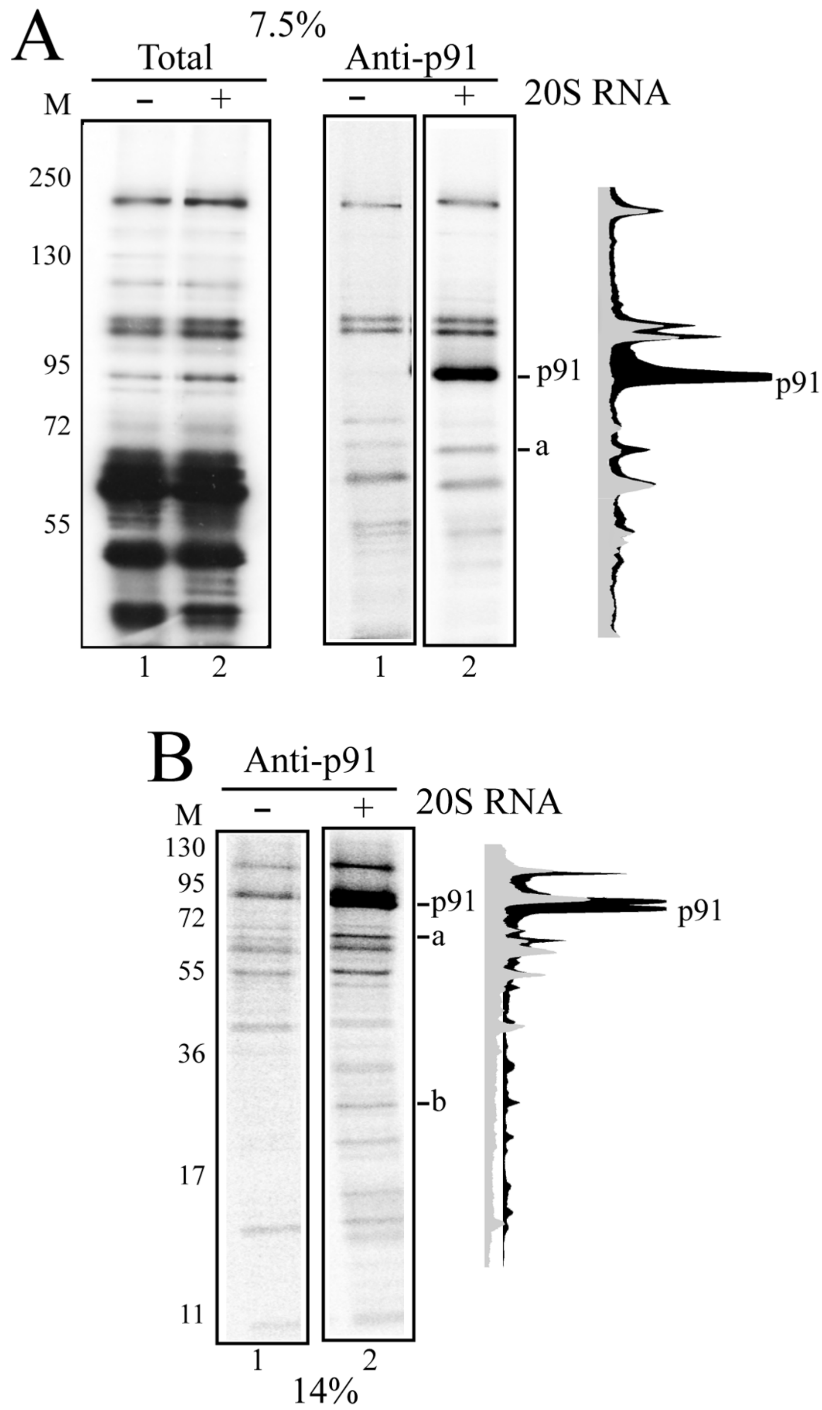
602

603 **Figure 3**

604

605

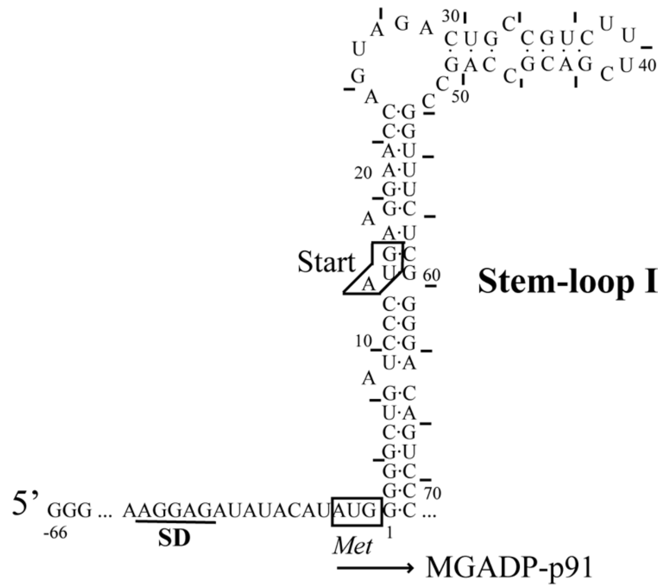
606

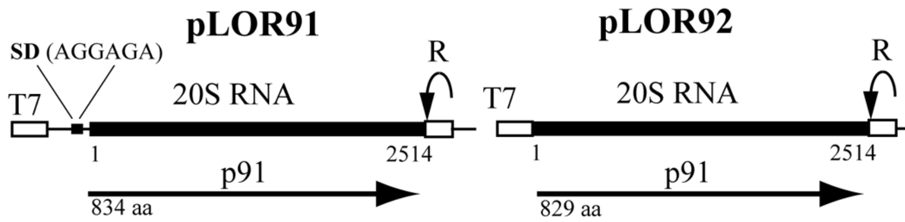


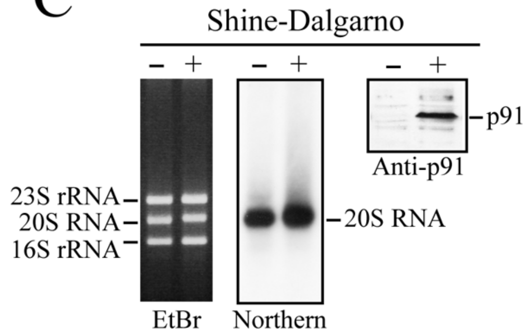
607

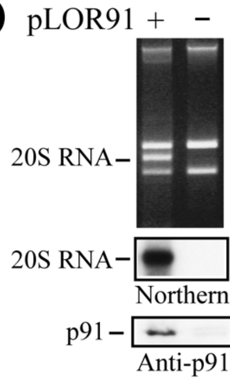
608

608
609
610 **Figure 4**





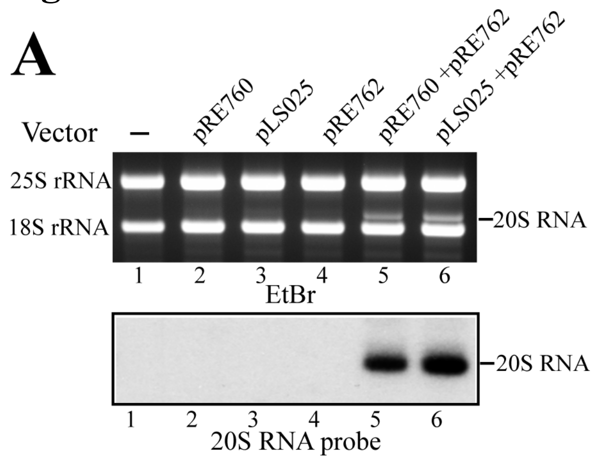


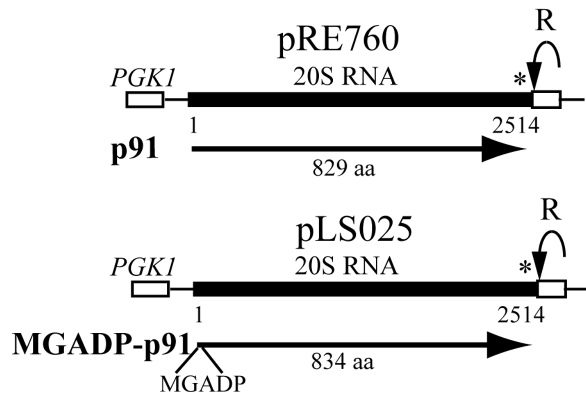


611
612
613
614
615
616
617
618

618

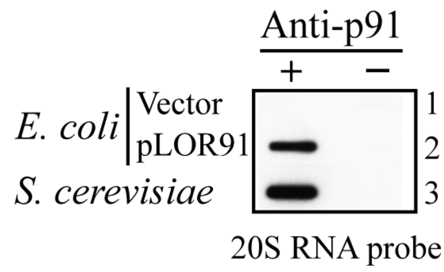
619 **Figure 5**



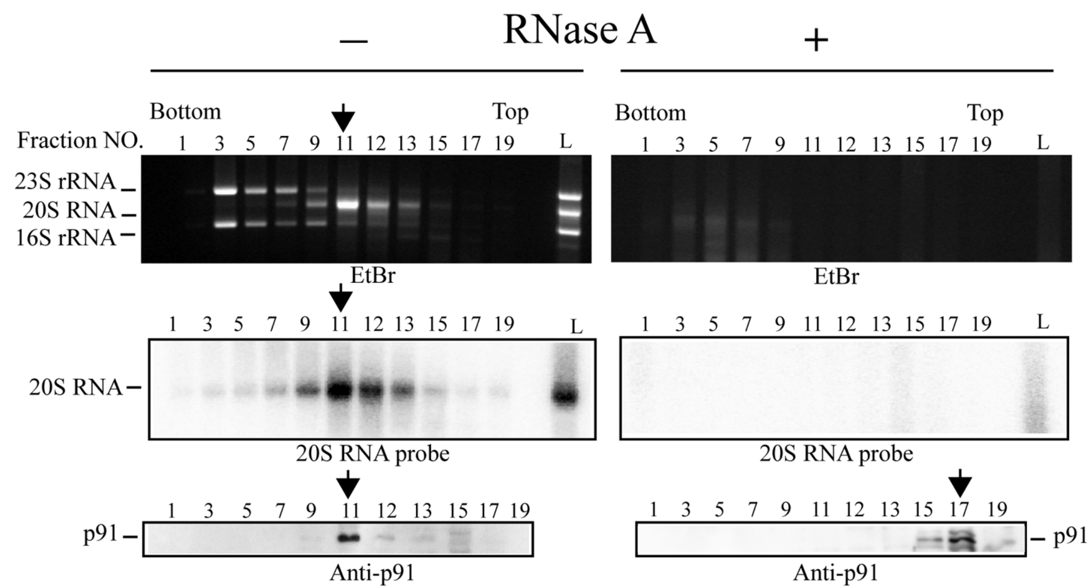


620

621 **Figure 6**
622



623
624
625
626
627
628 **Figure 7**
629

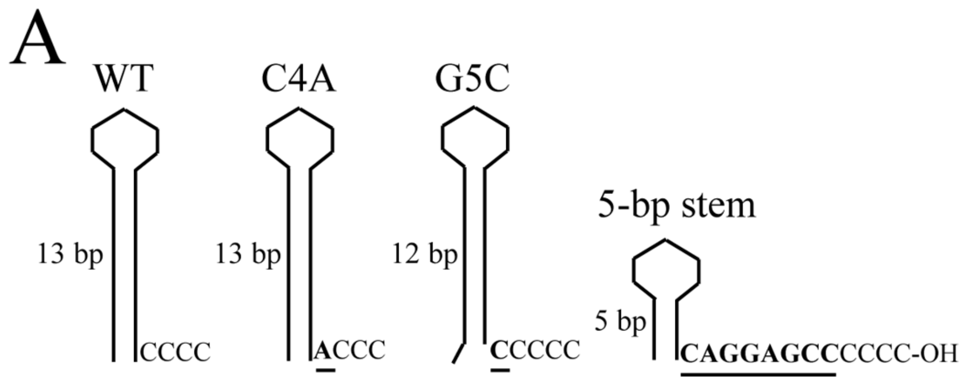


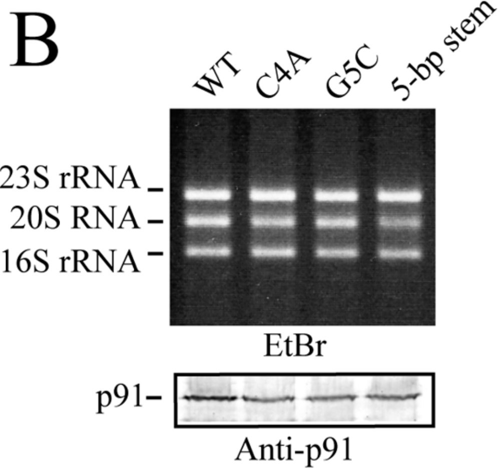
630
631
632
633
634
635
636
637
638

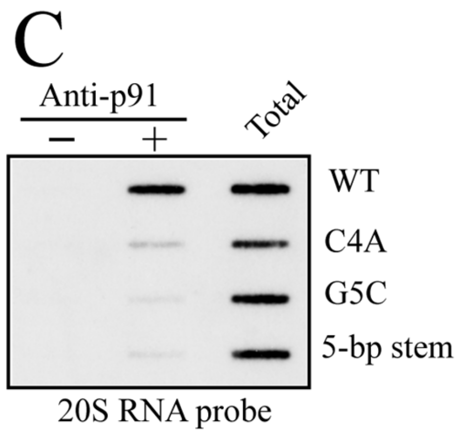
638

639 **Figure 8**

640



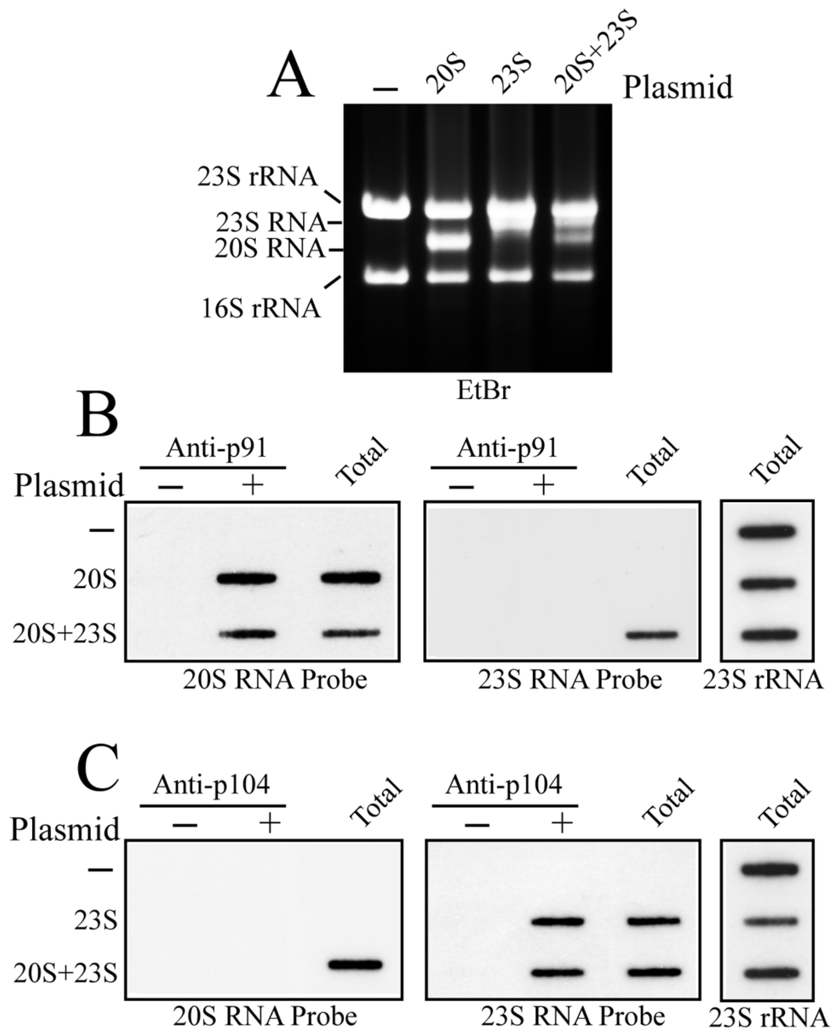




641

642 **Figure 9**

643
644
645



646
647