Persistent Yeast Single-stranded RNA Viruses Exist in Vivo as Genomic RNA-RNA Polymerase Complexes in 1:1 Stoichiometry* 

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Yeast narnavirus 20 S and 23 S RNAs encode RNA-dependent RNA polymerases p91 and p104, respectively, but do not encode coat proteins. Both RNAs form ribonucleoprotein complexes with their cognate polymerases. Here we show that these complexes are not localized in mitochondria, unlike the closely related mitoviruses, which reside in these organelles. Cytoplasmic localization of these polymerases was demonstrated by immunofluorescence and by fluorescence emitted from green fluorescent protein-fused polymerases. These fusion proteins were able to form ribonucleoprotein complexes as did the wild-type polymerases. Fluorescent observations and cell fractionation experiments suggested that the polymerases were stabilized by complex formation with their viral RNA genomes. Immunoprecipitation experiments with anti-green fluorescent protein antibodies demonstrated that a single polymerase molecule binds to a single viral RNA genome in the complex. Moreover, the majority (if not all) of 20 S and 23 S RNA molecules were found to form complexes with their cognate RNA polymerases. Since these viral RNAs were not encapsidated, ribonucleoprotein complex formation with their cognate RNA polymerases appears to be their strategy to survive in the host as persistent viruses.

RNAs are involved in many fundamental biological processes and also serve as genetic entities. Although some RNAs have catalytic activities by themselves, most of their tasks are accomplished through interactions with proteins. Because of their versatility, RNA-protein interactions not only serve catalytic purposes, but can also affect the fate of the RNAs themselves. For example, the stability of mRNA is governed by complex interactions between cis-elements on the RNA and various proteins (1, 2). It is also well known that tRNAs, once assembled into ribosomal subunits, become extremely stable in vivo.

(±) Single-stranded 20 S RNA (ScNV-20 S) and 23 S RNA (ScNV-23 S) viruses belong to the family Narnaviridae (3) and infect many Saccharomyces cerevisiae laboratory strains. Unlike other fungal viruses, they are not encapsidated in vivo into viral particles. Then, how can they survive stably without protective coats in the host cell as persistent viruses? This is one of the interesting questions to be answered.

20 S and 23 S RNA viruses have no extracellular transmission pathway and do not render any phenotypic changes in the host. They show 4+0 segregation in meiosis and can be efficiently transmitted during cytoduction using karyogamy-deficient mutants, thus suggesting their non-nuclear localization. 20 S and 23 S RNAs are induced under nitrogen starvation conditions, and their copy numbers reach up to 100,000 copies/cell. They are stably maintained; and at present, there are no known procedures to eliminate these cryptic viruses from the host cell.

The complete nucleotide sequences of 20 S and 23 S RNAs have been determined: 2514 bases for 20 S RNA and 2891 bases for 23 S RNA (4). These RNAs do not encode coat proteins, but do encode their RNA-dependent RNA polymerases, p91 and p104, respectively (see Fig. 1) (5, 6). 20 S and 23 S RNAs are linear molecules (7), but they have 5'-nucleotide inverted sequences at their 5'- and 3'-ends that can potentially form panhandle structures. There are no poly(A) tails at their 3'-ends (4). Yeast strains also contain double-stranded RNAs called W and T (8). The (+)-strands of W and T double-stranded RNAs are identical to 20 S and 23 S RNAs, respectively (4–6).

Since 20 S and 23 S RNAs do not encode coat proteins, they are not encapsidated into viral particles. Both RNAs instead form ribonucleoprotein complexes with their cognate RNA polymerases (9, 10). p91 and p104 have consensus sequences for RNA-dependent RNA polymerases that are closely related to those of the replicases of RNA coliphages. Recently, several RNAs of viral origin have been isolated from plant pathogenic fungi (11, 12). When the mitochondrial genetic code of fungi was applied, these RNAs showed only one open reading frame with consensus sequences for RNA-dependent RNA polymerases. These consensus sequences are most closely related to those of the RNA-dependent RNA polymerases encoded by 20 S and 23 S RNAs. Furthermore, they are reported to reside in mitochondria, thus are called mitoviruses, and belong to the same Narnaviridae family as the yeast single-stranded RNA viruses.

In this work, we have determined the subcellular localization of 20 S and 23 S RNA ribonucleoprotein complexes. Unlike mitoviruses, they do not reside in mitochondria, but in the cytoplasm. Immunofluorescence microscopy and cell fractionation experiments suggest that the viral RNA polymerases form aggregates in the absence (or low amounts) of the cognate viral RNAs, but are stabilized by the formation of complexes with them. When the green fluorescent protein (GFP)1 was
fused to the C termini of the polymerases, the fusion proteins were active in complex formation with their cognate viral RNAs. These complexes co-sedimented with the authentic, endogenous viral RNA-RNA polymerase complexes in sucrose gradients. When p91-GFP was immunoprecipitated with anti-GFP antibodies, only the fusion protein and the viral RNA (but not the endogenous polymerase) were precipitated, thus suggesting that a single RNA polymerase molecule binds to the viral RNA in the complex. Finally, we found that virtually all of the 20 S and 23 S RNA molecules form complexes with their cognate RNA polymerases. Thus, the yeast single-stranded RNA viruses exist in the host cytoplasm as ribonucleoprotein complexes that contain the viral RNA genome and their cognate RNA polymerases in a 1:1 stoichiometry.

EXPERIMENTAL PROCEDURES

Yeast Strains and Induction Conditions—The yeast strains used are listed in Table I. Induction of 20 S and 23 S RNAs by nitrogen starvation was done as described elsewhere (5). Briefly, yeast cells were grown in 1% yeast extract, 2% peptone, 0.04% adenine sulfate, and 2% glucose at 30 °C for 2 days and then shifted into 1% potassium acetate (pH 7.0) at 30 °C for 16 h.

Expression Plasmids—Plasmid pMBC31 contains the GAL4 DNA-binding domain fused in frame to GFP and has been described elsewhere (13). pALI40 and pALI59 were used to express p91-GFP and p104-GFP, respectively. pALI59 was constructed as follows. The complete 23 S RNA cDNA fragment (2891 base pairs) was inserted downstream of the PGK1 promoter of p2T (14). Then the termination codon of p104 was modified by in vitro mutagenesis. A polymerase chain reaction-cloned 0.7-kilobase fragment from pMCB15 containing the GFP gene was then fused in frame to p104 (Fig. 1B). pALI40 was constructed similarly using p2T, but contained an additional 1-kilobase UR3 DNA fragment inserted into the EcoRV site of the TRP1 gene. The p91-GFP expressed from pALI40 consists of full-length p91 and GFP at the C terminus (Fig. 1B). pALI2 was made by subcloning a fragment of 0.7 kilobases containing the 20 S RNA cDNA sequence from nucleotides 1885 to 2506 into the SalI site of pML62, a derivative of the pTT7-7 vector (9, 15).

Northern Hybridization Probes—pT39 contains a 251-base pair cDNA fragment from yeast mitochondrial 21 S ribosomal RNA (from nucleotides 1192 to 1442) cloned into the unique BstNI site of the Bluescript SK– vector and was used to make a mitochondrial rRNA-specific probe. pNR15 contains almost the entire 20 S RNA cDNA nucleotide sequence (from nucleotides 13 to 2504) (10) and was used to make 20 S RNA-specific probes. pALI38 contains the complete 23 S RNA cDNA nucleotide sequence (2991 base pairs) between the T3 RNA polymerase promoter and the Smal site of the Bluescript SK– vector. It was used to synthesize 23 S RNA-specific probes.

Antiserum—Antiserum against p104 and purified antibodies have been described elsewhere (9). Antiserum against p91 was raised using p2T overproduced in Escherichia coli BL21(DE3) cells from plasmid pALI2. p2T has the last 205 amino acids from the C terminus of p91 and 11 extra amino acids from a vector at the N terminus. Antibodies against GFP were purified from preimmune sera) were used at dilutions of 1:100 for 16 h at 4 °C. Cy3-conjugated anti-rabbit secondary antibody (Sigma) was used at a 1:300 dilution for 45 min at 25 °C. After mounting, images were captured with the confocal microscope and processed as described above.

Immunoprecipitation—Immunoprecipitation with specific antibodies was done using protein A-Sepharose CL-4B, and proteins in the immunoprecipitates were then separated on SDS-acrylamide gels and detected by Western blot analysis. The presence of 20 S or 23 S RNAs in the immunoprecipitates was tested directly by dot blot analysis (9).

Analysis of Ribonucleoprotein Complexes on Native Agarose Gels—Yeast crude cell lysates enriched in 20 S RNA-p91 complexes were prepared as described (18). Briefly, yeast cells grown under nitrogen starvation conditions were broken with glass beads by Vortex mixing at the maximum speed for 15 ± 10 times in buffer A (100 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). Cell debris was removed by centrifugation in an Eppendorf centrifuge at the maximum speed for 4 °C for 3 min. The supernatant was centrifuged again at 80,000 rpm (228,000 × g) in a Beckman TLA 100.2 rotor for 1 h at 4 °C. All of the 20 S RNA and p91 were recovered in the precipitate (18). The precipitate was suspended in the same buffer and used immediately. The lysate was applied directly onto a 1% native agarose gel or preincubated with the anti-p91 or anti-p104 antisera or their preimmune sera before loading. As a control, the lysate was extracted with phenol, and protein-striped RNAs were loaded onto the gel. Electrophoresis was done at 4.5 V/cm for 2.5 h at 4 °C in TAE buffer (40 mM Tris-acetate and 1 mM EDTA (pH 8.0)) containing 0.5 μg/ml ethidium bromide. Then RNAs on the gel were blotted onto nitrocellulose or positively charged nylon membranes in TAE buffer at 270 mA for 1 h at 4 °C using an electrottransfer unit from the Mini-Protean II series (Bio-Rad). 20 S RNA on the membranes was detected by Northern hybridization using a 20 S RNA-specific probe and visualized by autoradiography.

Due to the instability of 23 S RNA-p104 complexes in buffer A, we substituted it with buffer B (25 mM Tris-Cl (pH 7.5), 100 mM NaCl, 30
Fig. 2. 20 S RNA:p91 and 23 S RNA:p104 ribonucleoprotein complexes are not located in mitochondria. A yeast cell lysate from strain 37-4C was separated by differential centrifugation into two fractions: a pellet fraction (P) that contains mitochondria and a soluble fraction (S) that contains the rest of the cytoplasmic components. A, proteins in both fractions were separated on SDS-acrylamide gels and analyzed by immunoblotting with specific antisera. B, RNAs in the fractions were separated on a native agarose gel, blotted onto a nylon membrane, and detected by Northern blot hybridization with $^{32}$P-labeled specific probes. mt rRNA, mitochondrial rRNA.

mM MgCl$_2$, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) to prepare the lysates and analyzed 23 S RNA:p104 complexes as described above. 20 S RNA:p91 complexes in cell lysates, whether prepared in buffer A or B, gave the same results, as shown in Fig. 7 (A and B).

Miscellaneous—Sucrose gradient centrifugation was carried out as described previously (9). Northern (19) and Western (9) analyses were done according to the methods described. Yeast mitochondria were prepared according to the standard method (20).

RESULTS

20 S and 23 S RNAs and Their RNA Polymerases Are Not Localized in Mitochondria—Fungal mitochondria have been reported as the organelles in which linear DNA or RNA extrachromosomal genetic elements are frequently located (21–23). Among them are the mitovirus NB631 naked RNA (CpMV1-NB631) from the chestnut blight fungus *Cryphonectria parasitica*, which is closely related to 20 S and 23 S RNAs (11), and multiple RNA viruses found in an isolate of the Dutch elm disease fungus *Ophiostoma novo-ulmi*. (12). This prompted us to study the localization of 20 S and 23 S RNAs and their polymerases within the cell.

Yeast cells that harbor 20 S and 23 S RNAs were grown under nitrogen starvation conditions to induce high copy numbers of the viral RNAs. Then the cells were gently broken, and the mitochondrial pellet and the post-mitochondrial soluble fraction were prepared according to the standard method of Daum et al. (20).

By Northern hybridization, we detected 20 S and 23 S RNAs in the soluble fraction, but not in the mitochondrial pellet (Fig. 2B). Similarly, their RNA polymerases (p91 and p104, respectively) were detected in the soluble fraction by Western blotting (Fig. 2A). Thus, these data suggest the non-mitochondrial localization of 20 S and 23 S RNAs and their RNA polymerases. However, if the mitochondria had been damaged during the preparation, their contents could have leaked into the soluble fraction. Therefore, we did several control experiments to see if the organelles in the pellet were intact. We used two marker proteins, the mitochondrial outer membrane porin (24) and the matrix protein hsp60 (25). Both proteins were found in the pellet fraction, but not in the soluble fraction, indicating that the soluble fraction was not contaminated with mitochondria (Fig. 2A). Furthermore, the confinement of the soluble matrix protein hsp60 in the pellet indicates the intactness of the organelles. The other control was the 21 S large mitochondrial rRNA. This RNA was found only in the pellet, but not in the soluble fraction (Fig. 2B). Again, this result indicates the intactness of the mitochondria and shows no contamination of the soluble fraction with mitochondria and their contents. Therefore, these results clearly established that 20 S and 23 S RNAs and their RNA polymerases do not reside in mitochondria.

Cytoplasmic Localization of the RNA Polymerases p91 and p104—We also examined the nuclear localization of 20 S and 23 S RNAs. When intact nuclei were isolated by a Percoll density gradient (26), 2 µm DNA remained associated with the nuclei, whereas 20 S and 23 S RNAs and their polymerases distributed evenly throughout the gradient (data not shown). Furthermore, we also found that p91 and 20 S RNA were not associated with the endoplasmic reticulum or plasma membranes by sucrose density gradient centrifugation (data not shown). We used Sec36p and Pma1p as protein markers for the endoplasmic reticulum and plasma membranes, respectively (27). Therefore, these results indirectly suggest that 20 S and 23 S RNAs and their polymerases localize in the cytoplasm. Since 20 S and 23 S RNAs form ribonucleoprotein complexes in vivo with their cognate RNA polymerases (Refs. 9 and 10; see also Fig. 7) and these polymerases are responsible for the viral replication (18), we tried to locate p91 and p104 within the cell by immunofluorescent microscopy.

Nitrogen-starved cells were fixed with formaldehyde and then converted to spheroplasts. The spheroplasts were treated with the anti-p91 or anti-p104 antisera. The immunocomplexes were detected with a secondary antibody conjugated with the fluorochrome Cy3 and visualized with a laser confocal microscope. As shown in Fig. 3 (A and D), p91 and p104 were present throughout the cytoplasm and were apparently absent from vacuoles. When the preimmune sera were used, only background fluorescence was detected (Fig. 3, C and F). Similarly, 20 S RNA$^+$, 23 S RNA$^-$ cells gave only background fluorescence (data not shown).

We tried another approach to confirm their cytoplasmic localization. For this purpose, we constructed yeast vectors that can express GFP fused to the C termini of the complete amino acid sequences of p91 and p104. The fusion proteins were expressed in nitrogen-starved cells that harbored endogenous 20 S and 23 S RNA viruses. As shown in Fig. 4A, the fluorescence emitted directly from p91-GFP was observed throughout the cytoplasm, with a distribution very similar to that of p91 detected by indirect immunofluorescence (Fig. 3A). Although p104-GFP was expressed at a much lower level, it likewise distributed throughout the cytoplasm (Fig. 4E). As a control, GAL4BD-GFP fusion protein was expressed. Since GAL4BD has a nuclear localization signal (28), this fusion protein was confined within the nuclei (Fig. 4I), quite different from the localization of p91-GFP and p104-GFP. The non-nuclear localization of p91 and p104 is consistent with the results of isolation of intact nuclei and also with genetic data: their 4+0 segregation in meiosis and efficient transmission during cytokinesis (6). Therefore, these data altogether indicate that p91 and p104 are localized in the cytoplasm.

p91 and p104, When Alone, Form Aggregates—During the course of the fluorescent microscopy experiments, we noticed that p91 and p104 formed clusters in the cytoplasm of log-phase cells (Fig. 3, B and E). If these cells were starved for nitrogen, a condition that induced a high copy number of the viral RNAs (Fig. 3, G–I), the polymerases were more evenly distributed throughout the cytoplasm (Fig. 3, A and D). When the p91-GFP fusion protein was observed in log-phase cells, the cluster formation of the fusion protein became more evident,
irrespective of the presence or absence of 20 S RNA viruses (Fig. 4, B and D). They appear to be localized near the cell periphery. If these cells were starved for nitrogen, however, the fusion protein spread throughout the cytoplasm in the 20 S RNA-positive strain, while remaining in clusters in the 20 S RNA-negative strain (Fig. 4, A and C). The dispersion of fluorescence in the nitrogen-starved cells was not caused by proteolytic release of GFP fragments from aggregates of the fusion protein since we detected only full-length p91-GFP under these conditions (Figs. 5A and 6B). We also observed a similar cluster formation/dispersion pattern of p104-GFP in the cytoplasm, and the dispersion was again evident in endogenous 23 S RNA virus (Fig. 4E). Thus, these observations suggest that p91 and p104 form aggregates when the cognate viral RNA is absent or scarce for ribonucleoprotein complex formation. Consistently, when nitrogen-starved cells were broken and centrifuged at a low speed (13,200 rpm in an Eppendorf centrifuge for 5 min), most of p91-GFP was found in the pellet (60%) in 20 S RNA-negative cells, but remained soluble (>80%) in 20 S RNA-positive cells. If these extracts were further subjected to sucrose gradient centrifugation, p91-GFP from 20 S RNA-positive cells co-sedimented with the endogenous 20 S RNA-p91 complex in the gradient (Fig. 5A), but p91-GFP from 20 S RNA-negative cells ran much faster and was distributed more broadly in the gradient (Fig. 5B). These results strongly suggest that the viral RNA polymerases form aggregates when the cognate viral RNA is absent or in insufficient amounts to form the ribonucleoprotein complexes.

A Single p91 Molecule Binds to 20 S RNA in the Ribonucleoprotein Complex—As shown above, p91-GFP, when expressed in 20 S RNA-positive cells, coprecipitated with the endogenous 20 S RNA-p91 complexes in sucrose gradients. Similarly, p104-GFP, although much less expressed, coprecipitated with the endogenous 20 S RNA-p104 complexes in these gradients (data not shown). In contrast, both fusion proteins expressed in the absence of the endogenous cognate RNAs ran much faster in the gradients (Fig. 5B and data not shown). These data suggest that p91-GFP and p104-GFP can form complexes with endogenous 20 S and 23 S RNAs, respectively.

To prove the formation of a complex between p91-GFP and 20 S RNA, we immunoprecipitated p91-GFP with anti-GFP antibodies and tested whether 20 S RNA is also coprecipitated along with p91-GFP. Since p104-GFP was expressed much less, we focused our attention on the 20 S RNA-p91-GFP complex. The peak fraction of the sucrose gradient (fraction 13 in Fig.
with p91 or 20 S RNA z p91 complexes. In the second control
bility that the anti-GFP antibodies accidentally cross-reacted
failed to do so. Thus, these results clearly eliminate the possi-
oprecipitated 20 S RNA, whereas the anti-GFP antibodies
bodies. As shown in Fig. 6 A
and 20 S RNA. To rule out the possibility that the anti-GFP
results suggest the formation of complexes between p91-GFP
signals in these experiments (data not shown). Therefore, these
produce by the anti-p104 antiserum. When anti-GFP antibod-
ycles were used, 20 S RNA was also precipitated (Fig. 6A). More-
has brought down p91 along with p91-GFP to the immuno-
considering the ratio of p91 over p91-GFP (7:3) in the sucrose
bind to a single 20 S RNA molecule to form the complex, and
nature of the complex. If two molecules of p91 independently
require the mediation
experiment, we directly examined the presence of p91 and
GFP in the immunoprecipitates. The sucrose gradient frac-
tion 13 (Fig. 5A), which contained both p91 and p91-GFP, was
incubated with the anti-p91 antiserum, and then the immuno-
precipitates were analyzed by Western blotting using the anti-
p91 antiserum. As shown in Fig. 6B, the immunoprecipitates
contained both p91 and p91-GFP, and their ratio was almost
equivalent to that in the original gradient fraction (Fig. 5A).
When the anti-GFP antibodies were used, however, only p91-
GFP but not p91 was detected in the precipitates. These results
again clearly eliminated the possibility that the anti-GFP an-
tibodies cross-reacted with p91. From these results, we con-
clude that p91-GFP can form ribonucleoprotein complexes with
20 S RNA by its own virtue and does not require the mediation
of p91 for complex formation.

Moreover, these results provide further information on the
nature of the complex. If two molecules of p91 independently
bind to a single 20 S RNA molecule to form the complex, and
considering the ratio of p91 over p91-GFP (7:3) in the sucrose
gradient sample (fraction 13), the anti-GFP antibodies should
have brought down p91 along with p91-GFP to the immuno-
precipitates. As shown in Fig. 6B, however, p91 was not found
in the precipitates. Since 20 S RNA-p91 complexes were quite
intact under the immunoprecipitation conditions used (see Fig.
7, A and B), we eliminated the possibility that RNase in the
sera might have cleaved 20 S RNA and separated multiple
p91-binding sites in a single 20 S RNA molecule. These results
thus imply that the majority of the complexes in the gradient
consist of a 1:1 ratio of 20 S RNA and a single molecule (or a
single homo-oligomer) of p91 or p91-GFP. Similarly, if an oli-

FIG. 5. p91-GFP co-sediments with 20 S RNA-p91 complexes in
sucrose gradients. A, cells from strain TF395 containing endogenous
20 S RNA viruses and plasmid pALI40 expressing p91-GFP were grown
under induction conditions for 20 S RNA accumulation. A cell lysate
was prepared and separated in a 10–40% sucrose gradient. Proteins
and RNAs in the gradient were analyzed by Western blotting using
anti-p91 or anti-GFP antibodies and by Northern hybridization with a
20 S RNA-specific probe. Fraction 1 corresponds to the bottom of the
gradient. B, the same p91-GFP expression plasmid (pALI40) was used
to transform a 20 S RNA-negative strain, and a lysate was prepared and
analyzed as described for A. Note that only p91-GFP was detected,
which sedimented faster than the 20 S RNA-p91 complexes detected in
A.

FIG. 6. Immunoprecipitation of 20 S RNA by anti-GFP antibod-
ies. A, an aliquot of fraction 13 from the gradient shown in Fig. 5A
(prepared from strain TF395/pALI40) that contained p91, p91-GFP,
and 20 S RNA was incubated with the anti-p91 antiserum (blot 5), its
preimmune serum (blot 4), or anti-GFP antibodies (blot 6). Immunopre-
cipitates were extracted with phenol and then applied to a nylon mem-
brane. The membrane was hybridized with a 20 S RNA-specific probe.
As a negative control (blots 1–3), fraction 13 from a sucrose gradient
prepared from strain 37-4C containing only p91 and 20 S RNA was
processed similarly: precipitated with the anti-p91 antiserum (blot 2),
its preimmune serum (blot 1), or anti-GFP antibodies (blot 3) and
hybridized as described above. B, an aliquot of fraction 13 containing
p91, p91-GFP, and 20 S RNA was incubated with the anti-p91 anti-
sers (prepared from strain TF395/pALI40) that contained p91, p91-GFP,
and RNAs in the gradient were analyzed by Western blotting using a
20 S RNA-specific probe.

5A) that contained both p91 and p91-GFP was incubated with the
anti-p91 antiserum or its preimmune serum. Then the
immunoprecipitates were analyzed by dot blot hybridization
using a 20 S RNA-specific probe. As shown in Fig. 6A, the
anti-p91 antiserum, but not its preimmune serum, precipitated
20 S RNA. We have shown (10) that this hybridization is 20 S
RNA-specific since a 23 S RNA-specific probe did not hybridize
to the immunoprecipitates produced by the anti-p91 anti-
sers, and a 20 S RNA-specific probe did not hybridize to those
produced by the anti-p104 antiserum. When anti-GFP antibod-
ies were used, 20 S RNA was also precipitated (Fig. 6A). More-
over, none of these antisera precipitated the p91-GFP mRNA
since a specific probe for the GFP RNA failed to detect positive
signals in these experiments (data not shown). Therefore, these
results suggest the formation of complexes between p91-GFP
and 20 S RNA. To rule out the possibility that the anti-GFP
antibodies cross-reacted with p91 or the 20 S RNA-p91 com-
plex, two control experiments were carried out. A sucrose gra-
dient fraction that contained only the 20 S RNA-p91 complex
was incubated with the anti-p91 antiserum or anti-GFP antibod-
ies. As shown in Fig. 6A, the anti-p91 antiserum co-immu-
noprecipitated 20 S RNA, whereas the anti-GFP antibodies
failed to do so. Thus, these results clearly eliminate the possi-
ability that the anti-GFP antibodies accidentally cross-reacted
with p91 or 20 S RNA-p91 complexes. In the second control
Dp91 complexes, and the supercomplex are indicated. RNA, 20 S RNA hybridization using a 20 S RNA-specific probe. The mobilities of 20 S nylon membrane (lane 5); or after being nitrocellulose membrane (lane 2), or the anti-p104 antiserum (lane 3); or after being protein-striped by phenol extraction (lane 5). Two sets of experiments were run in parallel on the same gel. One set was electroblotted onto a nitrocellulose membrane (A), and the other onto a positively charged nylon membrane (B). 20 S RNA on the membranes was detected by hybridization using a 20 S RNA-specific probe. The mobilities of 20 S RNA, 20 S RNA-p91 complexes, and the supercomplex are indicated. C and D, a crude lysate from the same strain was applied directly onto a native agarose gel (lane 1); after pretreatment with the anti-p91 antiserum (lane 4), its preimmune serum (lane 2), or the anti-p91 antiserum (lane 3); or after being protein-striped by phenol (lane 5). Two sets of experiments were done as described above, and 23 S RNA on the nitrocellulose (C) or positively charged nylon (D) membrane was detected using a 23 S RNA-specific probe. The mobilities of some λ HindIII markers are shown to the right of the panels. kb, kilobases.

FIG. 7. All of the 20 S and 23 S RNA molecules form complexes with their cognate RNA polymerases, p91 and p104, respectively. A and B, a crude cell lysate from strain 37-4C (20 S RNA) was applied directly onto a native agarose gel (lane 1); after pretreatment with the anti-p91 antiserum (lane 4), its preimmune serum (lane 2), or the anti-p104 antiserum (lane 3); or after being protein-striped by phenol extraction (lane 5). Two sets of experiments were run in parallel on the same gel. One set was electroblotted onto a nitrocellulose membrane (A), and the other onto a positively charged nylon membrane (B). 20 S RNA on the membranes was detected by hybridization using a 20 S RNA-specific probe. The mobilities of 20 S RNA, 20 S RNA-p91 complexes, and the supercomplex are indicated. C and D, a crude lysate from the same strain was applied directly onto a native agarose gel (lane 1); after pretreatment with the anti-p104 antiserum (lane 4), its preimmune serum (lane 2), or the anti-p91 antiserum (lane 3); or after being protein-striped by phenol (lane 5). Two sets of experiments were done as described above, and 23 S RNA on the nitrocellulose (C) or positively charged nylon (D) membrane was detected using a 23 S RNA-specific probe. The mobilities of some λ HindIII markers are shown to the right of the panels. kb, kilobases.

Persistent Fungal RNA Viruses—A variety of DNA- and RNA-containing viruses can establish long-term infections in their hosts. To be persistent viruses, they have to fulfill three general requirements (29). The viral infection should avoid cytopathic effects on the host. Second, the virus must have means to maintain or replicate its genome for a long-term infection. Finally, the virus has to be invisible to immunological or other surveillance of the host.

Fungal RNA viruses are noninfectious and do not kill the host. They are transmitted vertically or horizontally through gomer, let us say a dimer for simplicity, of p91 (and p91-GFP) binds to 20 S RNA, and if the dimer consists of a random mixture of p91 and p91-GFP, it can be calculated that the ratio of p91 and p91-GFP precipitated with the anti-GFP antibodies should be 7:10. Since p91 was not detectable in the immuno precipitates with the anti-GFP antibodies (Fig. 6B), this again does not fit with the experimental data. Furthermore, if the GFP part of p91-GFP accidentally inhibits the formation of the dimer, then the data imply that dimer formation is not essential for binding to 20 S RNA. Therefore, these results strongly suggest that a single molecule of p91 binds to 20 S RNA in the ribonucleoprotein complex.

Virtually All 20 S and 23 S RNAs Exist in Vivo as Ribonucleoprotein Complexes with Their Cognate RNA Polymerases—Here we directly addressed the following question: how much 20 S RNA in the induced cells actually forms complexes with its cognate RNA polymerase (p91)? To answer this question, we tried to measure the proportion of 20 S RNA that can form supercomplexes with the anti-p91 antibodies.

A crude cell lysate was prepared from nitrogen-starved cells that harbored both 20 S and 23 S RNA viruses. The lysate was subjected to native agarose gel electrophoresis, and the RNA was electrolotted onto a nitrocellulose or positively charged nylon membrane. Under the blotting conditions used, 20 S RNA, when protein-striped by phenol extraction, did not bind to the nitrocellulose membrane because of the repulsion of negative charges between them (Fig. 7A, lane 5). In contrast, when the native cell lysate was used, 20 S RNA was retained on the membrane (lane 1), and its mobility on the gel was specifically slowed down by preincubation with the anti-p91 antiserum (lane 4). The preimmune serum or the anti-p104 antiserum did not affect the mobility (lanes 2 and 3). These results therefore indicate that all the 20 S RNA molecules bound to the nitrocellulose membrane existed as part of the ribonucleoprotein complex with p91 and that they formed the supercomplex with the anti-p91 antibodies. When the RNA was electroblotted onto a positively charged nylon membrane (Fig. 7B), 20 S RNA, whether native or protein-striped, bound equally to the membrane (lanes 1 and 5). The native 20 S RNA moved slightly slower than the protein-striped 20 S RNA, but the same as the 20 S RNA/p91 complex detected on the nitrocellulose membrane. When the lysate was preincubated with the anti-p91 antiserum, the band corresponding to the native or protein-striped 20 S RNA completely disappeared, and a slower moving band appeared (lane 4). Because the preimmune serum and the anti-p104 antiserum did not produce this new band (lanes 2 and 3), it is evident that the appearance of the slower band was the result of a supercomplex formation between the anti-p91 antibodies and the 20 S RNA/p91 complex. Furthermore, the complete disappearance of the band corresponding to the protein-striped 20 S RNA by immunocomplex formation indicates that all of the 20 S RNA molecules in the lysate formed complexes with p91.

The same sets of experiments were done to examine the proportion of 23 S RNA/p104 complexes in the total 23 S RNA population (Fig. 7, C and D). The results were the same as the ones obtained for 20 S RNA/p91 complexes and are summarized as follows. 1) Protein-striped 23 S RNA moved slightly faster than native 23 S RNA/p104 complexes on the gel, but only the latter was retained on the nitrocellulose membrane, which was verified by supercomplex formation with anti-p104 antibodies. 2) All of the 23 S RNA molecules in the crude extract formed the supercomplexes by preincubation with the anti-p104 antiserum, and there was no free 23 S RNA unbound to p104, as verified by Northern blotting onto the positively charged nylon membrane.

These ribonucleoprotein complexes are not formed artifactually during the preparation of cell extracts. We used several different methods including those such as breaking cells gently after converting them into spheroplasts or harshly using a French pressure cell. We got the same results. These RNA-protein interactions are quite stable; and so far, we have been unable to dissociate them into separate components under native conditions or to reconstruct the complexes by adding an excess amount of purified 20 S or 23 S RNA to cell extracts. Moreover, those isolated complexes have an in vitro RNA polymerase activity, indicating their functional integrity (18). The fluorescent observations of p91 and p104 under induced or uninduced conditions also indirectly support that those ribonucleoprotein complexes are genuine and present in vivo in the cell cytoplasm (Figs. 3 and 4). Since almost all of the 20 S and 23 S RNAs and their cognate RNA polymerases in the induced cells are recovered in the crude extract fraction (Ref. 18 and data not shown), we therefore concluded that virtually all of the 20 S and 23 S RNA molecules exist in vivo as parts of ribonucleoprotein complexes with their cognate RNA polymerases.

DISCUSSION
mating or cytoplasmic mixing, which appears to occur frequently in nature (30). Their genomes encode their own RNA-dependent RNA polymerases to replicate them so that the viral genomes are not diluted out during the host cell divisions. Although it is not clear whether fungi have developed active intracellular surveillance against viral infections, most of the fungal viruses appear to protect their genomes by sequestering them into capsids (Totiviridae, Partiviridae, and Barnaviridae) or membranous structures (Hypoviridae). In the case of the yeast totivirus L-A, the double-stranded RNA genome is encapsidated into viral capsids. To produce progenies, however, the (+)-strand should be translated into capsid proteins in the cytoplasm and encapsidated into new particles (31). Since the L-A (+)-strands have no poly(A) tails and apparently lack the 5'-end CAP structure, they appear to be vulnerable to cytoplasmic exoribonucleases. Several host mutants (ski) have been isolated that increase the copy number of the viral RNA (32, 33). Interestingly, many of these gene products are involved in the turnover of the host mRNA as components of the exoribonucleases or modulators thereof (34–36). Thus, the study of how fungal RNA viruses survive in their host may provide rich information on how the host cells control their own RNAs and also distinguish them from non-self-RNAs.

Ribonucleoprotein Complexes as Viral Entities in Vivo—In this study, we show that yeast 20 S and 23 S RNAs and their RNA polymerases do not reside in mitochondria. Instead, p91, p104, and their GFP-conjugated derivatives were observed in the cytoplasm of induced cells by fluorescence microscopy. Given that most of p91 and p104 associate with their cognate RNAs in induced cells (9, 10) and that all of the 20 S and 23 S RNA molecules form complexes in vivo with p91 and p104, respectively (Fig. 7), these data indicate that 20 S and 23 S RNAs are also present in the cytoplasm. This is consistent with the following evidence. Genetic and cell fractionation data show their non-nuclear localization. Furthermore, 20 S RNA-p91 and 23 S RNA-p104 complexes are not associated with the plasma or endoplasmic reticulum membranes in sucrose gradients, but move at the sedimentation coefficients of 20 S and 23 S, respectively (9, 10). Therefore, they do not associate with membranous structures or organelles. Interestingly, the mobilities of phenol-treated 20 S and 23 S RNAs are almost indistinguishable in sucrose gradients from those of native 20 S RNA-p91 and 23 S RNA-p104 complexes, respectively (9, 10). These observations suggest that the molecular masses of the complexes are not significantly different from those of the naked RNA molecules. Consistently, the stoichiometry of the viral RNA and its cognate RNA polymerase in the complex is 1:1 as shown in immunoprecipitation experiments (Fig. 6B), and the molecular masses of the polymerases count for only 10% of those of the respective viral RNAs. We do not know how many host proteins are involved in the complex formation, but the evidence above suggests that there are not many. This may be because the majority of the complexes in induced cells are not replicating, but are in a resting status.

Our observations from fluorescence microscopy experiments suggest that p91 and p104 form aggregates in vivo when the cognate viral RNA is absent or insufficient for complex formation (Figs. 3 and 4). Consistently, a large part of p91-GFP, when expressed in cells without endogenous 20 S RNA viruses, was insoluble and recovered in the pellet even at low speed centrifugation. When the supernatant was further subjected to sucrose gradients, the fusion protein moved much faster and more broadly than the authentic 20 S RNA-p91 complexes (Fig. 5B). On the contrary, p91-GFP expressed in the presence of endogenous 20 S RNA viruses comigrated with 20 S RNA in the gradient (Fig. 5A). This is not an artifact related to the conjugation of GFP to the polymerase or the expression of the fusion protein from a vector since we have made similar observations on endogenous p91. Log-phase cells contain <1% of 20 S RNA compared with nitrogen-starved cells, but both contain similar amounts of p91 (Fig. 3) (10). Again, a large part of p91 from log-phase cells, but not from nitrogen-starved cells, was insoluble as judged by low speed centrifugation. Therefore, these observations suggest that p91 and p104 are stabilized by the formation of complexes with the cognate viral RNAs, but form aggregates in their absence. It should be pointed out that these RNA-protein interactions are specific since p91 does not form complexes with 23 S RNA, nor p104 with 20 S RNA (Fig. 7) (10).

A Possible Mechanism to Escape the Host Surveillance—Yeasts have two major pathways of mRNA decay. One pathway occurs by shortening the poly(A) tail followed by a decapping reaction. The decapped mRNA is then degraded by the 5' to 3' exoribonuclease encoded by the XRNI/SKI1 gene (34, 37). The second pathway occurs by the 3' to 5' degradation of mRNA carried out by an exoribonuclease complex termed the exosome (35, 36). The exosome consists of the five proteins: Rrp4p, Rrp4p/Ski6p, Rrp42p, Rrp43p, and Rrp44p. The Rrp4p/Ski6p protein has homology to the E. coli 3' to 5' exoribonuclease RNase PH. The composition of the exosome appears to be modulated by the SKI2, SKI3, and SKI8 genes. 20 S and 23 S RNA molecules have no poly(A) tails at their 3'-ends and perhaps no 5'-CAP structures (4). Since they reside in the cytoplasm, they resemble intermediates of mRNA decay. In fact, when we examined ski2-1, ski6-1, and ski8-1 mutants, all of them had elevated amounts of 20 S RNA compared with wild-type cells (38). This thus suggests that p91 and p104 protect 20 S and 23 S RNAs, respectively, from exoribonuclease cleavage perhaps by sequestering their ends in the complexes. Our preliminary RNAi protection experiments indicate that p91 interacts with 20 S RNA at both the 5'- and 3'-ends. This also explains why all of the 20 S and 23 S RNA molecules need to form complexes with their cognate RNA polymerases. If the ribonucleoprotein complexes were necessary only for replication purposes, the involvement of a small fraction of 20 S and 23 S RNA molecules in complex formation may suffice. In this regard, these complexes resemble the small circular plant pathogenic RNAs, viroids, because of their apparent lack of the RNA ends for RNA degradation. Circular RNAs are often found to be more stable compared with their linear counterparts in vivo (39, 40). Therefore, we suggest that one of the major host surveillances that these fungal RNA viruses have to evade is the cytoplasmic exoribonucleases that are usually involved in the turnover or processing of the host RNA.

Replacement of Viral Capsid by RNA Polymerase—Although similar in their genomic organization and also in the RNA polymerase consensus sequences, the yeast narnaviruses differ from the mitoviruses in their cellular localization. 20 S and 23 S RNA viruses reside in mitochondria, whereas the mitoviruses reside in the cytoplasm. Their idiosyncratic distribution from the mitoviruses in their cellular localization. 20 S and 23 S RNA molecules form complexes in vivo with their cognate RNA polymerases. If the ribonucleoprotein complexes were necessary only for replication purposes, the involvement of a small fraction of 20 S and 23 S RNA molecules in complex formation may suffice. In this regard, these complexes resemble the small circular plant pathogenic RNAs, viroids, because of their apparent lack of the RNA ends for RNA degradation. Circular RNAs are often found to be more stable compared with their linear counterparts in vivo (39, 40). Therefore, we suggest that one of the major host surveillances that these fungal RNA viruses have to evade is the cytoplasmic exoribonucleases that are usually involved in the turnover or processing of the host RNA.

**Replacement of Viral Capsid by RNA Polymerase**—Although similar in their genomic organization and also in the RNA polymerase consensus sequences, the yeast narnaviruses differ from the mitoviruses in their cellular localization. 20 S and 23 S RNA viruses reside in the cytoplasm, whereas the mitoviruses reside in mitochondria. Their idiosyncratic distribution from the mitoviruses in their cellular localization (41) might be attributed to the adaptability of RNA viruses because of their high mutational rates, if we assume a common ancestor.

Fungal RNA viruses have no extracellular transmission pathway. This probably eliminated tremendous pressure to evolve new strategies to evade host defenses.
keep the genes necessary for exit from and re-entry to the new host. This resulted in their simpler genomic organizations and virion structures compared with the infectious counterparts found in other kingdoms. For example, the yeast totovirus L-A has one double-stranded RNA genome that encodes only two proteins (42). L-A virions contain the RNA polymerase, but lack the outer capsid. Thus, they correspond to the inner cores of reoviruses in higher eukaryotes. In this context, it is noteworthy to mention that the consensus sequences present in the narnavirus and mitovirus RNA polymerases are most closely related to those of the replicases of RNA coliphages such as Qβ (43, 44). In addition, 20 S and 23 S RNAs have 3′-end sequences and secondary structures similar to those found in RNA coliphages (4). Thus, if we eliminate the genes necessary for extracellular transmission from the coliphages, these hypothetical viruses now resemble 20 S and 23 S RNA viruses in their genomic structure and organization, i.e. the genome contains only one gene that encodes the RNA polymerase. However, the capsid not only provides exit and re-entry functions, but also protects the encapsidated RNA genome. 20 S and 23 S RNA viruses (and mitoviruses?) perhaps have evolved in such a way that their RNA polymerases substitute the protein capsid and protect their genomic RNAs by binding to them. In turn, this binding may also stabilize the RNA polymerases themselves since they tend to form aggregates in the absence of the cognate viral RNAs. Therefore, 20 S and 23 S RNA viruses survive in the host as persistent viruses in the form of ribonucleoprotein complexes. Since they have no extracellular transmissible structures, we can consider these complexes as their true viral entities.

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