Interactions of the RNA Polymerase with the Viral Genome at the 5’- and 3’-Ends Contribute to 20S RNA Narnavirus Persistence in Yeast*

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20S RNA narnavirus is a positive strand RNA virus found in the yeast Saccharomyces cerevisiae. The viral genome (2514 nucleotides) only encodes a single protein (p91), the RNA-dependent RNA polymerase and does not have capsid proteins to form intracellular virions. The genomic RNA has no 3’ poly(A) tail and perhaps no cap structure at the 5’-end; thus resembling an intermediate of mRNA degradation. The virus, however, escapes the host surveillance and replicates in the yeast cytoplasm persistently. The viral genome is not naked but exists in the form of a ribonucleoprotein complex with p91 in a 1:1 stoichiometry. Here we investigated interactions between p91 and the viral genome. Our results indicate that p91 directly or indirectly interacts with the RNA at the 5’- and 3’-end regions and to a lesser extent at a central part. The 3’-end site is identical to or overlaps with the 3’ cis signal for replication identified previously. The 5’-site is at the second stem loop structure from the 5’-end (nucleotides 72–104), and this structure also contains a cis signal for replication. Analysis of mutants in the structure revealed a tight correlation between replication and formation of complexes. These results highlight the importance of ribonucleoprotein complexes for the viral life cycle. We will discuss implications of these findings especially on how the virus escapes from mRNA degradation pathways and residues in the cytoplasm persistently despite the lack of a protective capsid.

A variety of viruses (DNA, RNA, and retroviruses) can establish persistent infections in the host (1). For long term infections, the virus must not only be invisible to immune surveillance but also replicate stably inside of the host cells. DNA viruses and retroviruses can integrate their DNA genomes into host chromosomes or keep their genomes in an episomal state and utilize the host machinery to replicate them in a cell cycle-dependent manner. RNA viruses, including hepatitis C virus, also can establish persistent infections. Although clinical aspects of their infection such as the host immune responses have extensively been studied, intracellular events leading to the maintenance and stable replication of the viral genome remain to be elucidated.

20S RNA narnavirus is a persistent positive strand RNA virus found in most laboratory strains of the yeast Saccharomyces cerevisiae (2, 3). The virus has no extracellular transmission pathway. The small and simple genome (2514 nucleotides (nt)²) that encodes a single 91-kDa protein (p91), the RNA-dependent RNA polymerase (4–6), makes this virus an attractive model system to investigate the mechanism of RNA virus persistence. Especially the absence of intracellular virions makes it easier to analyze the viral RNA inside of the cell. Replication proceeds from genomic to antigenomic to genomic strands. The amount of antigenomic strands counts less than a few percent compared with that of the genomic strands. W double-stranded RNA (7), a double-stranded form of the 20S RNA genome, is present in virus-harboring cells and is a byproduct of replication (8). Cells with vegetative growth contain a low amount of 20S RNA virus. Transferring them to nitrogen-starvation conditions, a common procedure to induce sporulation in diploid cells, increases the copy number of viral RNA to almost equivalent to those of rRNAs (9, 10). Increase in viral load may contribute to the efficient distribution of the virus to meiotic segregants.

The reading frame for p91 spans almost the entire length of the genome, with only 12 nt at both ends as non-coding regions. 20S RNA has 5-nt inverted repeats at both termini (5’-GGGGG…GCCCC-OH) (6). It lacks a poly(A) tail at the 3’-end and has perhaps no cap structure at the 5’-end, thus resembling an intermediate of the mRNA degradation pathway. 20S RNA is not encapsidated into proteinous or membranous structures and it migrates in sucrose gradients almost as naked RNA (11, 12). It thus appears that the viral genome would be vulnerable to the exonucleases involved in mRNA degradation. 20S RNA virus, however, resides and replicates stably in the cytoplasm (13). When antibodies against the RNA polymerase became available, it was found that p91 is associated with the viral genome (12). Further studies showed that most (if not all) of the genomic strands exist in the form of ribonucleoprotein complexes with p91 in a 1:1 stoichiometry (13), suggesting that formation of these complexes is a key factor for the stabilization of the viral genome inside of the cell. In this work we investigate the interactions of p91 with the viral RNA in ribonucleoprotein

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2 The abbreviation used is: nt, nucleotides.
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EXPERIMENTAL PROCEDURES

Immunoprecipitation of RNase-treated 20S RNA/p91 Complexes—Ribonucleoprotein complexes of 20S RNA virus were isolated from strain 37-4C (a kar1-1 leu1 20S RNA, 23S RNA, L-A-o, L-BC-o) (7) after converting the cells to spheroplasts and breaking them through a French pressure cell (5). Then complexes were purified by a differential centrifugation followed by a sucrose gradient, as described in Ref. 14. 100 μl of a sucrose gradient fraction (containing ~50 μg of protein) were diluted 10 times with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5 mM MgCl₂ and digested for 10 min at 25 °C with different concentrations of RNase A alone or along with RNase V1. 50 μg of yeast tRNA (Invitrogen) and 2 μl of anti-p91 antiserum (13) were added to the solution. The reaction mixture was kept at 4 °C for 30 min. 50 μl (wet volume) of protein A-conjugated Sepharose CL-4B (Amersham Biosciences) was added to the mixture, and it was incubated at 4 °C for another 30 min. The Sepharose was washed four times with 1 ml of TBST and suspended in 200 μl of 5 mM EDTA and 0.5% SDS. The RNA bound to Sepharose was extracted with phenol, phenol/chloroform and precipitated with ethanol. The pellet was dissolved in 100 μl of 50% dimethyl sulfoxide, 10% glyoxal, and 10 mM sodium phosphate, pH 7.0, and incubated at 50 °C for 1 h. The solution was diluted with 1 ml of 10× SSC and divided into aliquots. Each aliquot was blotted onto a Nytran N membrane (Schleicher and Schuell) using a Bio-Dot SF apparatus (Bio-Rad). RNA on the membranes was detected by hybridization using T7 or T3 RNA polymerase run-off transcripts as described in Ref. 15 or using deoxyligoligo probes (16). Quantification of the RNA recovered was performed with a phosphorimaguer.

Hydroxyl Radical Footprinting—Hydroxyl radical treatment was done as described in Ref. 17. We used 20S RNA/p91 complexes purified by a differential centrifugation instead of using a sucrose gradient fraction to avoid quenching of radicals by sucrose. The cleavage pattern was detected by reverse transcription (17) using the 5′-**32P**-labeled oligo primer NR2 (5′-GACGGCTCC-AACCGTAG-3′) complementary to nt 159–175 in 20S RNA (6).

Launching Assay—20S RNA virus was generated in vivo from a launching plasmid as described in Ref. 18. Formation of Ribonucleoprotein Complexes in Vivo—Pull-down assays of ribonucleoprotein complexes formed in vivo were performed as described in Ref. 19. Briefly, transformants of 20S RNA-negative strain 2928-4 (a ura3-1 trp1 his3, 20S RNA-o, 23S RNA-o, L-A-o) (18) with expression plasmids were grown and harvested in late logarithmic phase. Cells (3×10⁸) were added to the solution. The reaction mixture was kept at 4 °C for 30 min. 50 μl (wet volume) of protein A-conjugated Sepharose CL-4B. The RNA pulled-down was extracted and slot-blotted onto a Nytran N membrane, and 20S RNA transcripts on the membrane were detected by hybridization. Quantification of band intensities was done with a phosphorimaguer.

Complexes. Our results indicate that p91 interacts with the genomic strand at the 5′- and 3′-end regions and also weakly at the central part. Mutations at the 5′- and 3′-sites that impaired formation of complexes in vivo also abolished replication, indicating the importance of formation of ribonucleoprotein complexes in the viral life cycle. We will discuss possible roles of these complexes, especially on the stability of the viral genome during 20S RNA persistent infection.

FIGURE 1. p91 interacts with 20S RNA at three sites in a ribonucleoprotein complex. A, immunoprecipitation of intact 20S RNA molecules by anti-p91 antiserum. Sucrose gradient-purified 20S RNA/p91 complexes were immunoprecipitated with anti-p91 antisem (Anti-p91) or the preimmune serum (Preimmune). RNA was extracted from the precipitate, separated in an agarose gel, blotted, and hybridized with a 20S RNA genomic strand-specific probe. As control, 20S RNA directly extracted with phenol from the gradient fraction was analyzed in the same gel (Phenol). The mobility of some Lambda HindIII fragments is shown. B, 20S RNA fragments associated with p91 after RNase A treatment. 20S RNA/p91 complexes were digested with RNase A at the concentrations indicated and then immunoprecipitated with anti-p91 antiserum. RNA in the precipitates was extracted and blotted onto nylon membranes. We made nine sets of blots, and each membrane was hybridized with a **32P**-labeled probe that recognizes a different part of the 20S RNA molecule (1–9). The percentages of the RNA fragments recovered in the precipitates after treatment with 1 μg/ml of RNase A are shown below each panel. C, RNA probes (1–9) used for hybridization.
this work we used T3 run-off transcripts from SmaI-digested pAli-18 (18) as a 20S RNA-specific probe unless described otherwise. The probe contains the entire 20S RNA antigenomic strand sequence. RNA was also extracted directly from the lysates (Total RNA), slot-blotted, and hybridized with either the 20S RNA-specific probe or the 32P-labeled oligonucleotide RE368 (5’-CCCTCATAAAACTGATAGGGCTCTGCTATTCC-3’) complementary to nt 963–994 in 25S rRNA. The latter was used to detect 25S rRNA as a loading control.

In Vitro Mutagenesis—Site-directed in vitro mutagenesis was done as described (20). All mutations introduced were confirmed by DNA sequencing.

RESULTS

p91 Interacts with the 20S RNA Viral Genome at Three Sites—20S RNA forms ribonucleoprotein complexes with p91. These complexes, partially purified through a sucrose gradient, can be specifically immunoprecipitated with anti-p91 antiserum and intact 20S RNA molecules can be recovered in the precipitates (Fig. 1A). We wished to determine the p91 binding site(s) on the RNA. For this, complexes were first treated with various amounts of RNase A and then immunoprecipitated with anti-p91 antiserum. RNA fragments pulled down by the antibodies were analyzed by Northern blots using different RNA probes. We made nine sets of blots, and each membrane was hybridized with a 32P-labeled probe that recognized a different part of the 20S RNA molecule (Fig. 1C). After treatment with the highest concentration of RNase A, the 5’- and 3’-most fragments (detected by probes 1 and 9, respectively) were found in the precipitates at a level (70–80%) similar to the undigested control (Fig. 1B). Probes 3, 4, and 6–8, however, detected almost no RNA fragments. Probe 5 that recognized the central portion (nt 1253–1513) of 20S RNA also gave a lesser but significant signal (35% of the control). A secondary structure analysis with the MFOLD program (21) predicts that the 20S RNA genome has long distance interactions that connect the 5’-end, central, and 3’-end regions detected by probes 1, 5, and 9, respectively (Fig. 4A). Therefore, these data suggest that p91 interacts only with one or two of these sites and that the other sites are pulled down indirectly through RNA–RNA interactions. Alternatively, p91 may interact with all of three sites.

The 3’ cis Site Is Identical to or Overlaps with the 3’ Signal for Replication—The 20S RNA genome has 5-nt inverted repeats at both termini (5’-GGGGG . . . GCCCC-OH) and the 3’-terminal four C nt are attached to a stem loop structure (Fig. 2B). Previously we established an in vivo launching system of 20S RNA virus from a yeast expression vector (18). The vector contained the complete cDNA of the 20S RNA genome with a ribozyme sequence directly fused to its 3’-end. Using this system we found that the third and fourth C nt from the 3’-end are essential for virus generation. While the terminal and penultimate C nt were dispensable for in vivo generation, the launched virus recovered these terminal nucleotides. Furthermore, the fifth nucleotide from the 3’-end, which is a G, is dispensable but the nucleotide at this position needs to be hydrogen-bonded at the bottom of the stem for virus generation (18). The inactivation of the ribozyme by changing GGG 3’ to the cleavage site to AAA results in the failure of virus generation. The vector (the expression plasmid; Fig. 2A) still can transcribe the 20S RNA genome from the promoter and p91 can be translated from the transcript. This p91 forms complexes with the 20S RNA transcripts in vivo even though they have non-viral extensions at their 3’-ends (not shown). Thus these complexes can be pulled down with anti-p91 antiserum and 20S RNA transcripts in the precipitates can be detected by hybridization. Using this in vivo system we analyzed the effect of mutations at the 3’-end on complex formation. As shown in Fig. 2C, we found that, while changing the 3’-terminal or penultimate C to other nucleotides did not affect formation of complexes (Fig. 2, lanes 1 and 2), a modification at the third or fourth C impaired the activity by about 90% (Fig. 2, lanes 3 and 4). The replication-negative mutants still retain a basal level of complex forming activity (10%). As will be shown later, the cis site at the central part appears to be responsible for this basal activity. A mutation at the fifth nucleotide (G5A) that abolished replication also affected the formation of complexes (Fig. 2, lane 5).

FIGURE 2. The 3’ cis site is identical to or overlaps with the 3’ signal for replication. A, expression plasmid used for the in vivo assay of ribonucleoprotein complexes. The plasmid contains the entire 20S RNA genome (bold line) downstream of the constitutive PGK1 promoter (PGK1). The hepatitis delta virus antigenomic ribozyme (R) directly attached to the 3’-end of the 20S RNA genome has been modified (X) so that the plasmid does not generate the virus but can produce 20S RNA transcripts from the promoter, p91 and its conserved RNA-dependent RNA polymerase motifs (A–D) are shown. aa, amino acids. B, effects of 3’ mutations on replication activity. Individual RNA transcripts were pulled down by the antibodies or not and hybridized with a 20S RNA-specific probe or the 32P-labeled oligonucleotide. The results with the RNA probe indicate that the extracts contain fairly equal amounts of total RNA. The effects of these mutations on virus generation observed previously in launching experiments (18) are shown on the right of the panel (Launching). The relative amounts of 20S RNA transcripts pulled down with anti-p91 antiserum are also shown (Recovery).
ried out the experiments shown in Fig. 3. Consistent with the result with the G5A mutation, a G5C change also gave a basal level of complex forming activity (Fig. 3, lane 1). To restore base pairing at the bottom of the stem in the latter mutant, we introduced a compensatory mutation (C35G, numbered from the 3′-end) to the other side of the stem (Fig. 3, lane 2). Because this mutation also changed the amino acid sequence of p91 from Thr to Ser at codon 823 to form complexes and that the fifth nucleotide G from the 3′-end is dispensable for formation of complexes. The results further suggest that this fifth nucleotide from the 3′-end needs to be hydrogen-bonded for full activity. This was confirmed by the failure of transcripts having the C35G mutation alone in forming complexes even though they contained a wild-type G at the fifth position (Fig. 3, lane 2). There is a clear correlation between replication and formation of complexes among the mutants analyzed in Figs. 2 and 3. All the mutants that formed complexes at a wild type level generated 20S RNA virus when expressed from a launching plasmid, whereas the mutants that formed complexes at a basal level failed to generate the virus from the launching vector. These results demonstrate that the 3′ cis site for formation of ribonucleoprotein complexes is identical to or overlaps with the 3′ cis signal for replication identified previously.

**The 5′-Binding Site Is Located in the Second Stem Loop Structure from the 5′-End** — To locate the 5′-binding site more precisely, we took two different approaches. First, in an experiment analogous to the one shown in Fig. 1B, we used five small deoxyligo probes that covered the region recognized by RNA probe 1 (nt 1–326), to finely map the 5′-end site (Fig. 4B). After treatment with RNase A, the 5′-most stem loop structure (Stem-loop 1, nt 1–71) was not found in the precipitate, while the second stem loop structure (Stem-loop 2, nt 72–104) or second stem loop structure at the 3′-end (Stem-loop 2, nt 72–104) was maintained in the precipitate. Because this structure is critical for replication, we designate this region as the 5′-cis site for formation of ribonucleoprotein complexes, and we term this the ‘cis’ site for formation of ribonucleoprotein complexes.
5A). Non-cleaved RNA control produced a pattern of premature reverse transcription termination (Fig. 5A, lane 3), consistent with features of the two stem loop structures shown in Fig. 5B. When native complexes were treated with hydroxyl radicals, we found two wide areas (denoted as I and II) protected from cleavages (Fig. 5A, lane 2) compared with the pattern generated from phenol-extracted 20S RNA (Fig. 5A, lane 1). The protected nucleotides are located at both sides of the stem structure in Stem-loop 2, consistent with data from the pulled-down assay with deoxyoligo probes (Fig. 4). This result suggests that p91 physically interacts with the RNA at the 5’-site and eliminates the possibility that RNA fragments containing this site were pulled down indirectly by anti-p91 antibodies through RNA-RNA interactions.

**Stem-loop 2 at the 5’-End Contains cis Sites for Replication and for Formation of Complexes**—To confirm the above results, we introduced mutations into Stem-loop 2 and examined their effects on formation of complexes and also on virus generation. The long stem of this structure is divided into two parts by an asymmetric internal loop; a 9-bp upper stem (St-2a) and a 3-bp lower stem (St-2b) (Fig. 6A). To avoid changing the amino acid sequence of p91 we first introduced single mutations at the wobble positions in the structure. Although most of them did not affect virus generation and formation of complexes, C84G, C96G (Fig. 6), and G102C (not shown) reduced both virus generation and formation of complexes moderately. C84G and G102C also shorten St-2a and St-2b by one base pair, respectively. Then, to modify the Stem-loop 2 structure more extensively we changed the two Arg codons present in Stem-loop 2. When three bases of the Arg codon 97AGG99 were changed to another Arg codon CGC, thus opening St-2a at the lower edge by three base pairs, this mutant failed to generate 20S RNA virus from the launching plasmid and also formed complexes only at a basal level (10%) when transcribed from the expression plasmid (Fig. 6C, lane 1). Neither activity requires specific nucleotides at the Arg codon because single mutations at positions 97 and 99 (A97C and G99A, respectively) showed wild-type phenotypes for both activities (Fig. 6C, lanes 2 and 3). Furthermore, when a second mutation (U78G) was introduced into the 97CGC99 mutant, thus recovering two base pairs of hydrogen bonding, this compensatory mutation not only restored the ability to generate the virus but also brought complex formation back to a wild-type level (Fig. 6C, lane 4). When the other Arg codon 82CGC84 was changed to AGA, thus shortening the St-2a at the upper edge by three base pairs, this mutation resulted in the complete loss of virus generation and also reduced formation of complexes to a basal level (Fig. 6B, lane 3). Even the single mutation C84G, that shortens the stem by one base pair, reduced virus generation and formation of complexes moderately (Fig. 6B, lane 2). On the other hand, a double mutation in the loop (Fig. 6B, lane 1) or a mismatched pair at the upper part of St-2a (Fig. 6B, lane 4) affected none of the activities. When St-2a was disturbed in its middle part by the creation of a symmetric internal loop of four or six nucleotides by combining two single mutations (U78A and C96G, or U81A and C96G), the modified RNA failed to generate the virus and also formed complexes only at a basal level (Fig. 6D, lanes 2 and 3) and, as afore mentioned, C96G reduced virus generation and formation of complexes moderately (Fig. 6D, lane 1). These results indicate that Stem-loop 2 contains cis signals for
replication and also for formation of complexes and that the secondary structure is important for both activities.

The mutations in Stem-loop 2 described above can be divided into three groups based on the effects on replication and formation of complexes. 1) Those that did not affect virus generation from a launching vector produced complexes in amounts comparable to the wild-type control. 2) Those that failed to generate the virus formed complexes at a basal level (about 10% or less compared with the wild-type control) and 3) Those (C84G, C96G, and G102C) that generated virus at a reduced level formed complexes at an intermediate level. After curing the launching plasmids, the viruses generated from the last group of mutants were induced poorly under nitrogen starvation conditions (not shown), most likely because of the instability of ribonucleoprotein complexes. These data suggest that formation of stable complexes is a prerequisite for efficient replication. Furthermore, we observed that the amounts of 20S RNA transcripts in lysates (see Total RNA columns in Figs. 2, 3, 6, and 7) decreased by 30–60% compared with the wild-type control when mutants failed to form complexes at full activity.

FIGURE 6. A tight correlation between replication and formation of complexes at the 5′ Stem-loop 2. A, Stem-loop 2 with the nucleotide sequence. The stem can be divided into St-2a and St-2b by an asymmetric internal loop. Small bars and Arg indicate the coding frame of p91 and the two Arg codons present in the structure, respectively (nucleotides are numbered from the 5′-end). B, effects of mutations (lanes 1–4) at the upper part of St-2a on formation of complexes and virus replication. C, analysis of mutations in the lower part of St-2a. Mutations were introduced into the 97AGG99 Arg codon or the other side of the codon in the stem (lanes 1–4). Hydrogen bonding is indicated by horizontal bars. The MFOLD program predicts that the central C-G base pair in the 97C-G99 mutant (lane 1) is too unstable to be formed. D, analysis of mutations (lanes 1–5) in the middle of St-2a. Immunoprecipitation of 20S RNA transcripts and their detection were done as described in the legend to Fig. 2C, and the results are shown on the left panels. Lanes C, wild-type control, lanes –, cells without the expression plasmid. The effects of mutations on virus generation, assayed in a launching vector, are also shown in the right panels. The mutations introduced (indicated by filled circles) and their effects on the structures are shown in the diagrams posted above. The relative amounts of 20S RNA transcripts pulled-down with anti-p91 antiserum are shown under the mutant diagrams (Recovery). On the left panel in C, a mutation at the very end of the 5′-end (3′G to 3′U) is also analyzed (lane 5). This mutation caused a failure in virus generation when analyzed in the launching vector (18).
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when ribonucleoprotein complexes had been treated with phenol or when a p91 fragment truncated at Arg106 or Leu1274 was expressed from the vector (not shown). This indicates that the transcripts bearing the double mutation were still specifically associated with p91, thus suggesting the presence of another cis site for complex formation. These results, therefore, strongly suggest that the interaction at the central site is responsible for the basal level of complex formation exhibited by mutants at the 5’ and/or 3’ cis site.

DISCUSSION

mRNA degradation in eukaryotes usually begins with the shortening of the poly(A) tail at the 3’-end, followed by decapping at the 5’-end. Then the decapped RNA is degraded by the 5’ XRN1/SKI1 exonuclease. Alternatively, deadenylated RNA is digested by a 3’ exonuclease complex called the exosome. The exosome consists of multiple 3’ exonucleases, and to digest mRNA in the cytoplasm the exosome interacts with a heterotrimeric complex of Sk1p, Sk3p, and Sk8p through its cytoplasmic specific subunit Ski7p (22, 23). Because many RNA viruses lack the 5’-cap structure and the 3’ poly(A) tail in their genomes, the proteins involved in mRNA degradation pathways can play an important role in antiviral defense. Indeed, SKI genes were initially identified in yeast by their antiviral activities on the totivirus L-A and its satellite RNA (24). SKI2, SKI3, SKI7, and SKI8 genes also repress the copy number of 20S RNA virus3 (25). Because the 20S RNA genome is not sequestered into proteinous or membranous structures in the cytoplasm, it suggests that the 20S RNA genome is constantly challenged by the exosome.

In this work we have investigated the interactions of p91 with the 20S RNA genome in ribonucleoprotein complexes. From complexes predigested with RNase A, anti-p91 antiserum pulled down 20S RNA fragments containing the 5’- and 3’-end sequences and, to a lesser extent, a central region of the molecule as well. This suggests that p91 interacts with the RNA at three sites. The 3’-site is identical to or overlaps with the 3’ cis signal for replication identified previously. As shown in Fig. 2C, the modification of the third or fourth C from the 3’-end to A reduced formation of complexes to a basal level while changing the 3’-terminal or penultimate C to T did not affect complex formation. The fifth nucleotide G is located at the bottom of a stem adjacent to the 3’-end. Modifying it to A impaired both replication and formation of complexes. This nucleotide, however, is dispensable and can be changed to C without affecting either activity, provided that a compensatory mutation is introduced to other side of the stem to re-establish base pairing with the modified nucleotide (Fig. 3). This tight correlation between replication and formation of complexes is similar to the one observed in 23S RNA virus, another narnavirus present in yeast. Its genome contains a 3’ bipartite cis signal essential for generation of 23S RNA virus in vivo, consisting of the third and fourth C nt from the 3’-end and a mismatch pair of purines in the stem adjacent to the 3’-end (26). This signal is also required for formation of complexes between the 23S RNA genome and its RNA polymerase, p104 (19).

3 R. Esteban and T. Fujimura, unpublished results.
Hybridization with deoxyoligo probes and hydroxyl radical protection experiments located the 5′-site at Stem-loop 2. This stem loop structure also contains a cis signal for replication. Mutations in the structure analyzed in this work can be divided into three groups based on their effects on replication and formation of complexes. First, most of single mutations at the wobble positions did not impair replication or formation of complexes. Second, some of them, such as C84G, affected both activities moderately. Finally, those that caused extensive disturbance on the stem structure abolished replication and reduced complexes to a basal level. Here we again observed a good correlation between replication and formation of complexes. It suggests that formation of stable complexes is a prerequisite for efficient replication. A wide area of the stem structure was protected from hydroxyl radical in the complex (Fig. 5). It suggests that p91 interacts with the structure along the long stem. It is conceivable that single mutations in the second group partially disturbed the 5′ cis signal. This may also explain why an extensive disturbance in the stem structure was necessary to abolish replication completely. Fine mapping of the 5′ cis signal, however, awaits the development of a system in which p91 and modified RNA can be expressed separately.

Secondary structure analysis in silico predicts that the 5′-end, central, and 3′-end regions are brought together into proximity by intramolecular long distance interactions (Fig. 4A). This raises the possibility that anti-p91 antibodies pulled down some fragments indirectly through RNA-RNA interactions. We consider it unlikely based on the following evidence. Protection experiments from hydroxyl radicals suggest that p91 interacts with the structure along the long stem. It is conceivable that single mutations in the second group partially disturbed the 5′ cis signal. This may also explain why an extensive disturbance in the stem structure was necessary to abolish replication completely. Fine mapping of the 5′ cis signal, however, awaits the development of a system in which p91 and modified RNA can be expressed separately.

Protection experiments from hydroxyl radicals suggest that p91 and modified RNA can be expressed separately. It is conceivable that single mutations in the second group partially disturbed the 5′ cis signal. This may also explain why an extensive disturbance in the stem structure was necessary to abolish replication completely. Fine mapping of the 5′ cis signal, however, awaits the development of a system in which p91 and modified RNA can be expressed separately.

To determine whether the viral RNA is vulnerable to the exosome in the extracellular environment, the virus still needs to infect yeast cells persistently without killing them. The formation of ribonucleoprotein complexes with its RNA polymerase represents a prominent feature in the exclusively intracellular form of this virus; this structure is not only essential for replication but perhaps it also reflects the necessity of this virus to dodge or cope with host exonucleases surveillance. Although an infectious virus can protect its RNA genome by forming virions in the extracellular environment, the virus still needs to replicate inside of the cell, and thus has to deal with exonucleases. In poliovirus, a cloverleaf structure at the 5′-terminus of the genome forms a ternary ribonucleoprotein complex with the poliovirus 3CD protein and a cellular protein, poly(rC)-binding protein. It has been proposed that the viral genome is circularized by a protein bridge between the ternary complex and poly(A)-binding protein associated with the

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poly(A) tail at the 3’-end of the genome (30, 31); a circularization model analogous to the one proposed for stabilization of mRNA (32). Influenza virus has a segmented negative strand RNA genome, and each RNA segment is encapsidated into a ribonucleoprotein particle by the nucleoprotein NP. These particles serve as the template for transcription and replication. The polymerase of influenza virus is a heterotrimer and binds to both ends of viral RNA in the particle (33, 34). It is conceivable that the association of the polymerase may stabilize the RNA and protect it from exonucleases. Because most RNA viruses have linear RNA genomes, a mechanism(s) to maintain the integrity of both ends is vital for their survival. This could be a potential target to develop a new type of antiviral drugs against RNA virus infection.

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