The Bipartite 3′-cis-Acting Signal for Replication Is Required for Formation of a Ribonucleoprotein Complex in Vivo between the Viral Genome and Its RNA Polymerase in Yeast 23 S RNA Virus*

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23 S RNA narnavirus is a persistent positive strand RNA virus found in Saccharomyces cerevisiae. The viral genome (2.9 kb) encodes only its RNA-dependent RNA polymerase, p104, and forms a ribonucleoprotein complex with p104 in vivo. Previously we succeeded in generating 23 S RNA virus in yeast from an expression vector containing the entire viral cDNA sequence. Using this system, we have recently identified a bipartite 3′ cis-acting signal for replication. The signal consists of a stretch of four cytidines (Cs) at the 3′ end and a mismatched pair of purines in a stem-loop structure that partially overlaps the terminal four Cs. Although the 3′ terminal and penultimate Cs are not essential for virus launching, the generated viruses efficiently recovered these terminal nucleotides. In this work, we expressed RNA transcripts containing the entire 23 S RNA genome but incapable of generating the virus because of the presence of non-viral extra sequences at the 3′ ends. These transcripts could form complexes with p104 in vivo, and a detailed analysis indicated that the mismatched pair of purines as well as the third and fourth Cs from the viral 3′ end was essential for this complex-forming activity. Given that 23 S RNA virus does not have genes for capsid proteins, the binding of p104 to the viral 3′ end, in addition to the efficient 3′ terminal repair, may play a crucial role in virus persistence by protecting and maintaining the correct viral 3′ end in vivo.

A wide variety of viruses can establish persistent infections in the host. For a long-term infection, the viruses must evade host immune responses and limit their cytolytic effects but also need to replicate their genomes to be maintained stably in the cells. It is well known that retroviral DNA is integrated into the host genome after its synthesis by the reverse transcriptase. Viral DNA can also be maintained as an extrachromosomal element (1–3), and its synthesis is linked to the replication of the host chromosome. Although persistent infection also occurs in RNA viruses, such as the clinically important hepatitis A and C viruses, the basis for their persistence, especially how they replicate and maintain their genomes within the host cells, remains to be elucidated.

20 S and 23 S RNA narnaviruses are persistent positive strand RNA viruses found in the yeast Saccharomyces cerevisiae (4). These viruses were discovered originally as RNA species induced under nitrogen starvation conditions (5, 6). As is typical of fungal viruses, they do not kill the host or render phenotypic changes to the host. The viruses share many features. Their RNA genomes are small (2514 and 2891 nt1 in 20 S and 23 S RNAs, respectively) and each genome encodes a single protein, a 91-kDa protein (p91) by 20 S RNA and a 104 kDa protein (p104) by 23 S RNA (7–10). Both proteins contain four amino acid motifs well conserved among RNA-dependent RNA polymerases (RdRps) and these motifs are related most closely to those of RdRps of RNA coliphages (11). The double-stranded forms of 20 S and 23 S RNAs are called W and T, respectively (10, 12). Because 20 S and 23 S RNA viruses do not encode capsid proteins, their RNA genomes are not encapsidated into viral particles (13–15). Instead, these RNAs form ribonucleoprotein complexes with their cognate RdRps in a 1:1 stoichiometry and reside in the host cytoplasm (14). 20 S and 23 S RNA genomes lack 3′-poly(A) tails and have perhaps no cap structures at the 5′ ends, thus resembling intermediates of mRNA decay. Therefore, one of the interesting questions concerning these viruses is how they can reside and persist in the host cytoplasm without their genomes being degraded by the exonucleases involved in mRNA degradation pathways.

We succeeded recently in generating 23 S RNA virus in yeast from an expression vector containing the entire 23 S RNA cDNA sequence (15). The hepatitis δ virus antigenomic ribozyme was directly fused to the 3′ terminus of the viral genome. An active ribozyme as well as an active p104 were essential for virus generation. Using this launching system, we have identified a bipartite cis-acting signal for replication in the 3′ non-coding region of the 23 S RNA genome (16) (Fig. 1C). The signal consists of a cluster of 4 Cs at the 3′ terminus and a mismatched pair of purines in a stem-loop structure that partially overlaps this cluster of 4 Cs. Any combination of purines at the mismatched pair enabled the RNA to generate 23 S RNA virus; however, eliminating the mismatched pair or substituting the purines by pyrimidines abolished the activity.

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1 The abbreviations used are: nt, nucleotides; RdRp, RNA-dependent RNA polymerase; Cs, cytidines.
four Cs at the 3' end are essential for virus replication and that 23 S RNA virus has an effective 3' terminal repair mechanism(s) in vivo.

In this work, we analyzed the formation of 23 S RNA/p104 ribonucleoprotein complexes in vivo and found that the 3' bi-partite cis-signal for replication (the mismatched pair of purines as well as the third and fourth Cs at the 3' end) is essential for the binding of p104, thus suggesting physical interactions between them. The formation of complexes, along with the efficient 3' end repair in vivo, may play an essential role in protecting and maintaining the proper viral 3' end for 23 S RNA virus persistence.

EXPERIMENTAL PROCEDURES

Strains and Media—An L-A-o derivative (2928 L-A-o) of strain 2928 (a ura3 trp1 his3, 20 S RNA, 23 S RNA-o) (17) was used throughout this work. Cells were grown in either rich YPAD (1% yeast extract, 2% peptone, 0.04% adenine, and 2% glucose) or cryptophan-osmotic synthetic (H-Trp) medium (18). Both media were supplemented with uracil at a concentration of 100 µg/ml.

Plasmids—All of the 23 S RNA expression plasmids used in this work were derivatives of the 23 S RNA launching plasmids used in the previous study (16). The original launching plasmids were modified either by deleting the antigenomic hepatitis delta virus ribozyme sequence fused to the 3' end of the viral sequence, or by substituting the GGG sequence at the ribozyme cleavage site with AAA by site-directed mutagenesis (19). Because the generation of the viral 3' end by ribozyme cleavage was critical to launch 23 S RNA virus, these derivatives were unable to generate 23 S RNA virus in vivo. All of modifications were confirmed by DNA-sequencing.

Immunoprecipitation—Yeast transformants were grown in H-Trp medium and harvested at the logarithmic growing phase. These cells had been grown ~30 generations after receiving a 23 S RNA expression plasmid to obtain enough quantity. Cells (3 × 10^8) were washed with lysis buffer (20 mM Tris, pH 8.0, 100 mM NaCl), re-suspended in the same buffer (100 µl) and broken with glass beads by vortex mixing (15 s, 10 times). After adding 350 µl of fresh buffer, the cell suspension was centrifuged at 13,000 rpm for 1 min to remove cell debris and unbroken cells. The lysate was then used 1) to analyze p104 by Western blotting, 2) to extract total 23 S RNA transcripts with phenol for Northern blot analysis, and 3) to immunoprecipitate 23 S RNA transcripts with anti-p104 antibodies. Immunoprecipitation of 23 S RNA transcripts was carried out as follows. To 150 µl of the lysate prepared as described above, 1 ml of Triton-buffered saline-Tween 20 (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20), 1 mM dithiothreitol, 40 units of RNasin (Promega), 2 µg of yeast tRNA (Invitrogen) and 2 µl of anti-p104 antiserum or its preimmune serum were added and incubated at 4 °C for 30 min. 25 µl of 50% dimethyl sulfoxide, 1 mM glyoxal, and 10 mM sodium phosphate, pH 7.0, and incubated at 50 °C for 1 h. The solution was diluted with 150 µl of 10× SSC and blotted onto a Nytran N membrane (Schleicher and Schüll) using a BioDot SF apparatus (Bio-Rad). RNA on the membrane was detected by Northern hybridization as described previously (20). Treatment of lysates with benzotene was done as follows: to 50 µl of the cell lysate described above, 2.5 µl of benzotene (3%/w/v) was added. After a brief incubation at 4 °C, the lysate was centrifuged at 13,000 rpm for 1 min to separate benzotene. p104 in the supernatant was analyzed by Western blotting.

Northern Probes—A 23 S RNA positive strand-specific probe was made from SmaI-digested pALI-38 by run-off transcription with T3 RNA polymerase. The probe contained the entire nucleotide sequence of the 23 S RNA genome and also with probes recognizing different parts of the 23 S RNA genome (Fig. 3B). As control, we prepared a lystate from 23 S RNA virus-generated cells from which the launching plasmid (pRE637) had been cured. The three probes specific to different parts of 23 S RNA genome hybridized with RNA downstream of the 23 S RNA genome present in the transcripts immunoprecipitated with anti-p104 antiserum. Antiserum—Anti-p104 antiserum used in this work was described previously (21). It was raised against an N-terminal fragment (amino acids 11 to 265) of p104.

Sucrose Gradients—Sucrose gradients (10–40%, w/v) were done as described in Ref. 22 using a SW40 rotor run at 36,000 rpm for 4 h 30 min at 4 °C.

RESULTS

23 S RNA Expression Plasmids—23 S RNA virus exists in vivo in the form of a ribonucleoprotein complex between the 23 S RNA genome and its RdRp p104. To analyze cis-acting signals for the formation of these complexes we expressed 23 S RNA transcripts from a vector bearing various mutations at the 3' non-coding region in the viral genome and examined their ability to form complexes in vivo with p104 by an immunoprecipitation assay using anti-p104 antibodies. To avoid any complications derived from 23 S RNA virus generation, we used launching plasmids constructed previously (16) but modified them as follows (Fig. 1A). The standard launching plasmid (pRE637) contains the complete 23 S RNA cDNA sequence (2891 base pairs) downstream of the PGK1 promoter. Transcripts from the promoter have the positive polarity of the viral genome. The hepatitis delta virus antigenomic ribozyme is directly fused to the 3' terminus of the viral genome. The transcription termination site for the FLP gene (23) of the 2 µm plasmid is located ~0.7 kb downstream of the 23 S RNA genome. Because the ribozyme sequence is flanked by two unique restriction sites (SmaI and EcoRI), we digested the plasmid with these enzymes to eliminate the ribozyme. The larger fragment containing the 23 S RNA sequence was Klenow-treated and ligated to produce plasmid pTF662 (Fig. 1A). We have shown previously that an active ribozyme fused to the viral 3' end was critical for virus generation. Consistently, transcripts from pTF662 failed to generate 23 S RNA virus in vivo even after 100 generations, as judged by the absence of T double-stranded RNA, an indicator of negative strand synthesis, as well as the absence of 23 S RNA after curing the plasmid (data not shown). All the 23 S RNA expression plasmids used in this work except those shown in Fig. 9 were constructed in this way; they are thus incapable of generating 23 S RNA virus and contained the same flanking sequence to the viral 3' end.

p104 Can Bind to 23 S RNA with Extra Non-viral Sequence at the 3' End—Yeast cells having no endogenous 23 S RNA virus were transformed with pTF662 or the standard launching plasmid (pRE637). Lysates prepared from growing transformants contained p104 when analyzed by Western blots using anti-p104 antiserum (Fig. 2A). The amount of p104 expressed from the standard launching plasmid was several fold higher than the one from pTF662. This reflects that a significant proportion of cells (20–50%) transformed with the launching plasmid had already generated 23 S RNA virus (15). When p104 in the lysates was immunoprecipitated with anti-p104 antiserum, the precipitates from both transformants were found positive with a 23 S RNA-specific probe in Northern blots (Fig. 2B). Control experiments with the preimmune serum or without serum gave no signals. Because pTF662 does not generate 23 S RNA virus, the results suggest that transcripts from this plasmid can bind to p104 even though they contain extra non-viral sequences at the 3' ends. To confirm this, we analyzed the transcripts in the immunoprecipitates with a probe specific to the vector sequence downstream of the 23 S RNA genome and also with probes recognizing different parts of the 23 S RNA genome (Fig. 3B). As control, we prepared a lystate from 23 S RNA virus-generated cells from which the launching plasmid (pRE637) had been cured. The three probes specific to different parts of 23 S RNA genome hybridized with RNA downstream of the 23 S RNA genome present in the transcripts immunoprecipitated with anti-p104 antiserum. Antiserum—Anti-p104 antiserum used in this work was described previously (21). It was raised against an N-terminal fragment (amino acids 11 to 265) of p104.

Sucrose Gradients—Sucrose gradients (10–40%, w/v) were done as described in Ref. 22 using a SW40 rotor run at 36,000 rpm for 4 h 30 min at 4 °C.
Fig. 1. Diagrams of 23 S RNA launching plasmid and 23 S RNA expression plasmid (A) and the bipartite 3′ cis signal for replication (B). A, the standard launching plasmid pRE637 contains the complete 23 S RNA cDNA sequence (bold line) downstream of the PGK1 promoter (PGK1). The antigenomic hepatitis delta virus ribozyme (R), its cleavage site (vertical arrow), two unique restriction sites for SmaI (S) and EcoRI (E) flanking the ribozyme sequence, p104, and its conserved RdRp motifs (A–D) are shown. The ribozyme sequence was eliminated from pRE637 by digesting the plasmid with SmaI and EcoRI and the larger fragment was Klenow-treated and re-ligated, resulting in the 23 S RNA expression plasmid pTF662. Because of the lack of the ribozyme sequence, pTF662 cannot generate 23 S RNA virus in vivo. B, the bipartite 3′ cis signal for replication consists of a stretch of 4 Cs at the 3′ end (boxed) and a mismatch pair of purines (circled) at the stem adjacent to the 3′ end.

Bipartite 3′ cis signal

extracted from both immunoprecipitates and the relative intensities of the signals between them are quite similar irrespective of whether the probe recognized the 5′, middle, or 3′ portions of the 23 S RNA genome (Fig. 3A). These results suggest that the pTF662 transcripts immunoprecipitated with anti-p104 antiserum contained intact 23 S RNA viral genome. Consistently, the probe specific to the vector sequence flanking the viral 3′ end recognized the transcripts from pTF662 in the immunoprecipitates but not the viral RNA in the control (Fig. 3A). Therefore, these results clearly indicate that p104 could form complexes with transcripts from pTF662 even though they contained extra non-viral sequences at the 3′ ends.

The Stem Structure at the Viral 3′ End Is Essential for the Formation of Complexes with p104—The 23 S RNA genome contains a 59-nt untranslated region at the 3′ end. This region can form three stem-loop structures (Fig. 4B). When the nucleotide sequences of each loop (I–III) were modified separately in the vector, none of the modifications significantly affected the formation of complexes between the modified transcripts and p104 (Fig. 4A), although the modification in the loop III sequence also changed the last two amino acids in p104. As shown previously (16), these modifications did not affect replication of 23 S RNA virus. In contrast, the change of the fourth C from the 3′ end to a U, an essential nucleotide for replication, abolished the formation of complexes. This suggests an involvement of the bipartite cis signal for replication in the formation of 23 S RNA-p104 complexes. The importance of the stem I structure containing the mismatched pair of purines to form complexes is shown in Fig. 5. When the nucleotide sequence of one side of the lower stem was changed to that of the other side of the stem, or vice versa, thus destroying the lower stem structure, the modified transcripts no longer formed complexes with p104 (lanes 4 and 5). However, exchanging them simultaneously, thus re-establishing the lower stem structure but with nucleotide sequences different from the original ones, restored the activity to form complexes with p104 (lane 6). We obtained the same results concerning the upper stem structure (lanes 1–3). These results, therefore, indicate the importance of the lower and upper stem structures surrounding the mismatched pair of purines in the formation of 23 S RNA/p104 complexes.

p104 Expression—Because the formation of ribonucleoprotein complexes requires 23 S RNA transcripts as well as p104 decoded from them, we examined the expression of p104 in transformants analyzed in Figs. 4 and 5. As shown in Fig. 6, A and C, all of the transformants examined expressed p104. There is, however, variation in the amounts of p104 expressed among them. We noticed that cells expressing 23 S RNA transcripts capable of forming complexes contain higher amounts of p104 compared with cells producing transcripts defective in forming complexes. This may be a mere coincidence, and slight modifications at the 3′ non-coding region might have affected the expression of p104 as observed. On the other hand, because we were detecting the steady state levels of p104, this can be explained in terms of a stabilizing effect on the protein caused by the formation of a ribonucleoprotein complex. Consistent with the latter explanation, we also noticed that the steady state levels of 23 S RNA transcripts were higher when they could form complexes with p104 (Figs. 4 and 5).

In the course of experiments, we found that p104, depending
Transcripts containing the intact 23 S RNA sequence can form complexes in vivo with p104. A, p104 expressed from the standard launching plasmid pRE637 and from the expression plasmid pTF662. A lysate prepared from log-phase cells transformed with pRE637 or pTF662 or without plasmid (—) was subjected to SDS-PAGE followed by Western blot analysis using anti-p104 antiserum. The position of p104 is indicated by the arrow. B, lysates as described in A were incubated with anti-p104 antiserum (lane 1), its preimmune serum (lane 2), or without serum (lane 3), and the immunocomplexes were separated with protein A-conjugated Sepharose. After phenol extraction, RNA in the complexes was blotted onto a nylon membrane. As control, the lysates were extracted directly with phenol and blotted onto the membrane (lane 4). The relative amounts of pTF662 transcripts to launched virus RNA in the immunoprecipitates detected by the 23 S RNA-specific probe and visualized by autoradiography.

Fig. 2. Transcripts containing the intact 23 S RNA sequence can form complexes in vivo with p104. A, p104 expressed from the standard launching plasmid pRE637 and from the expression plasmid pTF662. A lysate prepared from log-phase cells transformed with pRE637 or pTF662 or without plasmid (—) was subjected to SDS-PAGE followed by Western blot analysis using anti-p104 antiserum. The position of p104 is indicated by the arrow. B, lysates as described in A were incubated with anti-p104 antiserum (lane 1), its preimmune serum (lane 2), or without serum (lane 3), and the immunocomplexes were separated with protein A-conjugated Sepharose. After phenol extraction, RNA in the complexes was blotted onto a nylon membrane. As control, the lysates were extracted directly with phenol and blotted onto the membrane (lane 4). The relative amounts of pTF662 transcripts to launched virus RNA in the immunoprecipitates detected by the 23 S RNA-specific probe and visualized by autoradiography.

The Mismatched Pair of Purines Is Essential for Formation of Complexes—The stem-exchange experiments (Fig. 5) indicate that the stem structure of the stem-loop I is important for the formation of complexes. Because the loop sequence was not involved in this complex formation (Fig. 4), these results suggest that the stem structure is necessary to form a platform to mount the mismatched pair of purines that is essential for the formation of 23 S RNA/p104 complexes. To test this possibility, we modified nucleotides at the mismatched pair and examined their effects on the formation of complexes (Fig. 8). When the 5'-A of the mismatched pair was eliminated (lane 1) or replaced with C (lane 3) or U (data not shown), the modified transcripts no longer formed complexes with p104. However, when this A was changed to G, the modified transcripts with the G< >G mismatch could form complexes with p104 with a slightly diminished activity (lane 2). Likewise, changing the 3'-G of the
mismatched pair to C (lane 6) of eliminating this nucleotide (lane 4) abolished the complex-forming activity in the modified transcripts. Again, an A could substitute for this G but with a slightly reduced activity (lane 5). These results suggest that the bulged non-base-paired nucleotides at the stem are essential for the formation of complexes and that their bases should be purines. To confirm this, we introduced concerted mutations at the mismatched pair and analyzed their effects on the formation of complexes. Because changing the 5′-A at the mismatched pair to C (lane 3) or U resulted in the elimination of the mismatch from the stem by a C-G or U-G pairing, we changed the wild-type mismatched pair to the pair (C<->A) and found that transcripts bearing these mutations failed to form complexes with p104 (lane 7). This suggests that the 5′ base of the mismatched pair should also be a purine. Consistently, transcripts bearing a A<->G (lane 5) or G<->A (lane 8) mismatched pair formed complexes with p104. Furthermore, transcripts with a U<->G mismatched pair failed to form complexes (data not shown). These results clearly indicate that the bases of the mismatched pair should be purines and that any combination of purines bestowed the activity of forming complexes with p104 on 23 S RNA transcripts. Therefore, 23 S RNA virus requires the same spectrum of nucleotide bases at the mismatched pair, not only for replication but also for the formation of ribonucleoprotein complexes with p104.

The Third and Fourth Cs from the Viral 3′ End Are Essential for the Formation of Complexes—The 23 S RNA viral genome has 5-nt terminal inverted repeats (GGGGC...GCCCC-OH). As shown previously (16), the fifth G from the 3′ end could be changed to C, along with the compensatory mutation C34G (numbered from the 3′ end) at the other side of stem I, in the launching plasmid without losing virus-generating activity, and the generated viruses retained the modified nucleotides. Likewise, transcripts having these G5C and the compensatory C34G mutations retained a complex-forming activity with p104, although less compared with the control (Fig. 8, lane 9). As shown in Fig. 4B, when the fourth C from the 3′ end was changed to U, the modified transcripts were unable to form complexes with p104. We chose a U to minimize the effects on the stem I structure. We also tried to analyze the last three Cs at the viral 3′ end. Because these Cs are part of the unique SmaI site used to eliminate the ribozyme sequence from launching plasmids, we modified each of these terminal Cs in the standard launching plasmid by site-directed mutagenesis. To avoid virus generation, we also changed the GGG sequence at the cleavage site to AAA. This modification destroys or modifies the substrate-bearing P1 helix as well as a G-U wobble at the cleavage site in the ribozyme core structure (23–25) and, as shown previously, eliminated virus-generating activity from the launching plasmid (15). The control plasmids with the complete 23 S RNA virus sequence, constructed either in this way or by deleting the ribozyme sequence, expressed comparable amounts of transcripts and both transcripts had similar activities to form complexes with p104. When the terminal C at the viral 3′ end was changed to A, the modified transcripts showed a slightly reduced activity to form complexes (Fig. 9, lane 1). Changing the penultimate C to A significantly reduced the activity (lane 2). Modifying the third C to A (lane 3) or the fourth C to A or U (lanes 4 and 5) completely eliminated the ability to form complexes from the transcripts. Therefore, these nucleotides at the third and fourth positions constitute an essential part of the 3′ cis signal to form complexes with p104. These results together indicate that the bipartite 3′ cis signal for replication determined by virus generation from a vector is also involved in the formation of ribonucleoprotein complexes in vivo with the viral RdRp, p104.

**DISCUSSION**

Yeast viruses, like other fungal viruses, have no extracellular transmission pathway. They are transmitted through mat-
ing or, vertically, to daughter cells (26). Because mating or hyphal fusion is part of the host life cycle, it is, perhaps, more economic for the viruses to rely on the host’s ability to find partners to be transmitted rather than to have their own extracellular infectious cycle. Therefore, two characteristics are evident among these viruses. First, they are persistent viruses. Because there is no extracellular phase that allows the virus to escape, they cannot kill the host. They have to replicate within

Fig. 5. The 3' terminal stem structure is essential for the formation of complexes with p104. A, the upper and lower stem sequences of the 3' terminal stem-loop I in the 23 S RNA expression plasmid were modified as indicated in B. Yeast cells were transformed with the control (lane C) of modified plasmids (lanes 1–6) and lysates were prepared from the transformed cells. 23 S RNA immunoprecipitated with anti-p104 antiserum (Anti-p104) or its preimmune serum (Preimmune) and total RNA extracted from the lysates (Total RNA) were analyzed as described in the legend to Fig. 2B. The effects of the modifications on virus launching observed in the previous work (16) are indicated on the right. B, wild-type (lane C) and modified (lanes 1–6) nucleotide sequences at the 3' terminal region of 23 S RNA analyzed in A. Modified nucleotides at the upper (lanes 1–3) or lower (lanes 4–6) stem are indicated by filled circles. The wild-type stem-loop I and restored stem structures by simultaneous exchange of the upper (lane 3) or lower (lane 6) stem sequences are shown on the right with the modified sequences boxed.
out hurting the cells and have to evade the intracellular surveillance of the host. Second, because there is no infectious cycle, the viruses do not need elaborate machinery for exit and reentry to a new host. This makes its structures and genomes much simpler than those of infectious counterparts found in other organisms. L-A double-stranded RNA virus and Ty retroelements have no envelopes, and their genomes only contain gag and pol genes (27, 28). L-A particles resemble, structurally and functionally, the inner core of reoviruses in higher eukaryotes. 20 S and 23 S RNA narnaviruses even have no coat proteins, and their genomes only encode their RdRps. Their simpler genomes, or a small number of proteins encoded by them might have helped these viruses to establish persistent infections. It is well known that persistent infection, for viruses that are normally lytic, generally requires restriction of viral gene expression or the generation of deletion mutants lacking one or more components of the wild-type genome. Considering the availability of versatile genetics and well-developed molecular biology techniques in yeast; therefore, these viruses are ideal to study not only their replication mechanisms but also intracellular virus-host interactions involved in viral persistence.

Recently, we have established a launching system to generate 23 S RNA virus in vivo from a vector containing the entire viral cDNA. Using this system, we have begun reverse genetics to study the replication mechanism of 23 S RNA virus. We found that the 23 S RNA genome contains a bipartite cis signal for replication in the 3’ non-coding region. The signal consists of a row of four Cs at the 3’ end and a mismatched pair of purines in a stem-loop structure adjacent to the 3’ end. Although the terminal and penultimate Cs at the 3’ end are not essential for virus generation, the generated viruses recovered these terminal Cs. This suggests that 23 S RNA virus has an efficient 3’ terminal repair mechanism(s). Because these viruses have no coat proteins, their genomes are not encapsidated into intracellular particles. Instead, 20 S and 23 S RNAs form ribonucleoprotein complexes with their cognate RdRps, p91 and p104, respectively, in a 1:1 stoichiometry. As shown in this work, RNA transcripts containing the entire 23 S RNA genome could form complexes with the polymerase p104, even though they had non-viral extra sequences at the 3’ ends. Our results indicate that the bipartite cis-signal for replication is also essential for forming these complexes with p104.

The 23 S RNA genome contains three stem-loop structures
There are two implications of this finding relevant to 23 S RNA virus replication. First, the formation of ribonucleoprotein complexes brings the viral RNA and its RdRp in close contact. This may be important because 23 S RNA virus resides and replicates in the host cytoplasm that is filled with a great variety of host RNAs. Therefore, the formation of complexes between them will not only ensure an efficient replication of the viral genome but also increase its fidelity by reducing synthesis of non-viral RNA. Furthermore, the involvement of the bipartite 3’ cis signal in the formation of complexes indicates that p104 can bind only to replication-competent 23 S RNA genomes having intact viral 3’ ends. Second, the formation of complexes may protect and stabilize the viral genome. 23 S RNA genome has no poly(A) tail at the 3’ end and perhaps no 5’-cap structure, thus resembling degradation intermediates of host mRNAs. Shortening the poly(A) tail triggers decapping of mRNA and then the decapped mRNA is quickly degraded by the potent XRN1/SKI1 5’-3’ exonuclease as well as by a 3’-5’ exonuclease complex called exosome (29, 30). The copy numbers of 20 S and 23 S RNA viruses are increased by host mutations in SKI2, SKI6, and SKI8 genes (31). These gene products are reported to be a component of the exosome or modulators of the exosome (32–34). It has also been proposed that these gene products suppress the expression of RNAs lacking the poly(A) tails, a common feature seen in yeast viral RNAs (35–37). We do not know whether p104 interacts directly with the bipartite 3’ cis signal to form complexes or a host factor(s) might also be involved in this recognition process. At any rate, because the third and fourth Cs from the viral 3’ end are essential for the complex-forming activity, we expect that such protein/RNA interactions will protect the viral 3’ end from

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(I–III) at the 3’ non-coding region, and none of these loop sequences is important for the formation of complexes. However, the stem I structure surrounding the mismatched pair of purines was essential for this complex-forming activity. This suggests the importance of the mismatched pair of purines for this activity. Consistently, eliminating one of the mismatched pair of purines or changing it to C or U abolished the activity to form complexes with p104. Any combination of purines at the mismatched pair bestowed the activity to form complexes on the RNA. Therefore, the spectrum of nucleotides at the mismatched pair necessary to form complexes with p104 is identical to the one required for 23 S RNA virus replication. Furthermore, the third and fourth Cs from the viral 3’ end were also essential to form complexes with p104. The same bipartite 3’ cis signal identified by a virus-launching assay is also required for the formation of complexes with p104 in vitro, which indicates the importance of complex forming in 23 S RNA virus replication.

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*Esteban and T. Fujimura, unpublished results.
exonuclease cleavage. The steady-state levels of 23 S RNA transcripts unable to form complexes with p104 were consistently lower than those of transcripts able to bind to the protein.

It is interesting that a modification of the 3′ terminal C to A slightly affected the complex-forming activity, and changing the penultimate C to A substantially reduced the activity. We observed previously that modification or elimination of any of these last two Cs was efficiently repaired to wild-type nucleotides during virus maturation. It is tempting, therefore, to speculate that these alterations destabilize the complex so that the modified 3′ end is now allowed to interact more easily with the repair machinery. Because the stem-loop structure at the 3′ end of 23 S RNA that contains the bipartite cis signal resemble the top half domain of tRNA, the yeast CCA-adding enzyme (tRNA nucleotidyl transferase) may be involved in this repair process (16).

We do not know whether the bipartite 3′ cis signal is sufficient for forming complexes with p104 or other cis sites are also required. If this 3′ signal is important to protect the viral 3′ end from degradation, then, the 5′ end of 23 S RNA genome must also be protected from 5′-3′ exonucleases by binding to p104 or by other means. In the case of the closely related 20 S RNA, our data indicate that its RdRp, p91, interacts with these structures to protect the genome physically and chemically during the extracellular stage. RNA viruses in eukaryotes may also have developed these intracellular supramolecular structures to achieve dual tasks: to protect the transcription and replication machinery in the hostile intracellular environment and, at the same time, to fulfill viruses’ reliance on the host metabolism.

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