Bipartite 3′-Cis-acting Signal for Replication in Yeast 23 S RNA Virus and Its Repair*

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23 S RNA narnavirus is a persistent positive strand RNA virus found in Saccharomyces cerevisiae. The viral genome is small (2.9 kb) and only encodes its RNA-dependent RNA polymerase. Recently, we have succeeded in generating 23 S RNA virus from an expression vector containing the entire viral cDNA sequence. Using this in vivo launching system, we analyzed the 3′-cis-acting signals for replication. The 3′-non-coding region of 23 S RNA contains two cis-elements. One is a stretch of 4 Cs at the 3′ end, and the other is a mismatched pair in a stem-loop structure that partially overlaps the terminal 4 Cs. In the latter element, the loop or stem sequence is not important but the stem structure with the mismatch pair is essential. The mismatched bases should be purines. Any combination of purines at the mismatch pair bestowed capability of replication on the RNA, whereas converting it to a single bulge at either side of the stem abolished the activity. The terminal and penultimate Cs at the 3′ end could be eliminated or modified to other nucleotides in the launching plasmid without affecting virus generation. However, the viruses generated regained or restored these Cs at the 3′ terminus. Considering the importance of the viral 3′ ends in RNA replication, these results suggest that this 3′ end repair may contribute to the persistence of 23 S RNA virus in yeast by maintaining the genomic RNA termini intact. We discuss possible mechanisms for this 3′ end repair in vivo.

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The abbreviations used are: RdRp, RNA-dependent RNA polymerase; RACE, rapid amplification of cDNA ends; dsRNA, double-stranded RNA; nt, nucleotides; Cs, cytidines.

Positive strand RNA viruses replicate their linear RNA genomes using RNA-dependent RNA polymerases (RdRps) in conjunction with host or other viral proteins. The viral genomes contain cis-acting elements at the 3′ ends, which direct the polymerase machinery (holoenzyme) to synthesize the viral negative strands in a milieu dominated by host RNAs. In contrast to DNA replication in eukaryotic cells, the polymerase machinery can start RNA synthesis without shortening the viral genome at the 3′ ends. However, during the replication cycle, their RNA termini may be vulnerable to host exonucleases that are usually involved in cellular RNA metabolism (1, 2). The viruses may have developed strategies to cope with this problem, e.g. by sequestering the viral RNA into membranous structures during replication (3) or by covalently attaching 5′-cap structures and 3′-poly(A) tails or peptide fragments to the RNA ends. Possessing efficient 3′ end repair mechanisms may also be beneficial. Various repair mechanisms have been proposed but are poorly understood (4–8). One of these studies suggests that the viral 3′ ends are not static but undergo rapid turnover in vivo (5). Particularly, persistent viruses such as the yeast 23 S RNA narnavirus may require efficient repair mechanisms to keep their genomic termini intact throughout their long term infection.

Most laboratory strains of Saccharomyces cerevisiae harbor 20 S RNA virus (ScNV-20S), and fewer strains carry 23 S RNA virus (ScNV-23S). These viruses were originally discovered as RNA species induced under nitrogen starvation conditions (9–12). They are positive strand RNA viruses belonging to the genus Narnavirus of the Narnaviridae family (13). They share many features in common. Their RNA genomes are small (2891 and 2514 nt in 23 S RNA and 20 S RNA, respectively) and have the same 5 nt-inverted repeats at their termini (5′-GGGGC . . . UCCCG-3′) (14). The double-stranded forms of 20 S and 23 S RNAs are called W and T, respectively (14, 15). Each genome encodes a single protein, a 104-kDa protein (p104) by 23 S RNA (12, 14) and a 91-kDa protein (p91) by 20 S RNA (11, 16). Both proteins contain four amino acid motifs well conserved among RdRps. Because the viral genomes do not encode coat proteins, they are not encapsidated into viral particles (17–19). Instead, the RNA genomes form ribonucleoprotein complexes with their cognate RdRps in a 1:1 stoichiometry and reside in the host cytoplasm (18–20). 20 S and 23 S RNA viruses are compatible in the same host and are maintained stably without excluding each other. They are transmitted vertically to daughter cells during mitosis or horizontally during mating. There is no known extracellular transmission pathway. Typical of fungal viruses, they do not kill nor render phenotypic changes to the host, which makes their genetic manipulation difficult.

Recently, we established an efficient launching system for 23 S RNA virus in yeast from an expression vector containing the entire 23 S RNA virus cDNA (21). In this study, we used this launching system to analyze the 3′-cis-acting signal for replication and found two cis-elements at the 3′ end of positive strands. We also found an efficient repair system of the 3′-terminal nucleotides in vivo. Because the viral genome is not encapsidated into viral particles but exists as a ribonucleoprotein complex with p104 in the yeast cytoplasm, the repair mechanism(s) may contribute to the 23 S RNA virus persistence by keeping the genomic termini intact for long term infection.

EXPERIMENTAL PROCEDURES

Strains and Media—A L-A-o derivative (2928 L-A-o) of strain 2928 (a ura3 trp1 his3, 20 S RNA, 23 S RNA-o) (22) was used throughout this
work with the exception of one launching experiment (indicated in Table 1) in which 23 S RNA-cured 2928 L-A-o was used. We have observed the curing of endogenous 20 S RNA virus at a low frequency from yeast cells when low uracil-containing media were used during launching. Cells were grown in either rich YPAD (1% yeast extract, 2% peptone, 0.04% adenine, and 2% glucose) or tryptophan-omitted synthetic (H-Trp) medium (23). Both media were supplemented with uracil at a concentration of 100 μg/ml.

**Launching Plasmid**—The standard launching plasmid pRE637 was described previously (21). Mutations were introduced into 23 S RNA cDNA sequence by oligonucleotide site-directed mutagenesis (24) and were verified by DNA sequencing.

**Launching Assay**—Yeast cells were transformed with launching plasmids according to the lithium acetate method (25). We used two independently isolated plasmids for each experiment. Three to six transformants were isolated from each plasmid and grown in H-Trp liquid medium for 2 days. Cells then were transferred to 1% potassium acetate and incubated for 16 h at 28 °C to induce 23 S RNA. The induced cells were broken with glass beads as described previously (21), and total RNA isolated was separated on a 1.3% agarose gel. 23 S RNA was visualized by ethidium bromide staining of the gel and also by Northern blots (26). The generation of 23 S RNA virus was confirmed by curing the launching plasmids from 23 S RNA-positive cells by growing them on rich YPAD medium. All of the mutations analyzed in this work showed all-or-none phenotype in virus launching. For the sake of simplicity, only a representative of each experiment is shown in the figures.

**Sucrose Gradients**—Sucrose gradient centrifugation was done as described previously (18). 

**Primer Extension**—Purification of T double-stranded RNA (dsRNA) and primer extension analysis of the negative strands of T dsRNA was performed as described previously (14).

**3′-RACE**—Total RNA isolated from launching plasmid-cured cells was poly(A)-tailed using poly(A) polymerase (Invitrogen). The poly(A)-tailed RNA was denatured with hydroxymethyl mercuric hydroxide as described previously (27), and cDNA was synthesized using oligonucleotide primer RE156 (5′-GACTCGAGTCGAGGATCC-3′) and NR23 (5′-TTCAATGGGCTCTGCCGC-3′). After cDNA synthesis, RNA was digested with RNase A and the unincorporated primer was eliminated by a G-50 minispin column (Worthington Biochemical Corp.). cDNA containing the 3′ ends of 23 S RNA-positive strands was PCR-amplified for 30 cycles using Taq polymerase (Promega) and primers RE157 (5′-GAGTCCACGTCGTAACGC-3′) and NR22 (5′-GAGTCCACGTCGTAACGC-3′). For amplification of the negative strand 3′ ends, we used oligonucleotides RE157 and NR22 (5′-GAGTCCACGTCGTAACGC-3′). The amplified products from positive and negative strand 3′ ends were digested with SpeI and BamHI and ligated into the Bluescript-KS+ vector (Stratagene). In the case of negative 3′ ends, PCR products were digested with Sall and BamHI and ligated into the Bluescript-KS+ vector.

**RESULTS**

**Launching Plasmid**—The standard launching plasmid pRE637 contains the entire 23 S RNA cDNA sequence (2991 bp) downstream of the PGK1 promoter (Fig. 1A). The plasmid has the TRP1 gene as a selectable marker. Transcripts from the promoter have the positive strand polarity of the viral genome. The hepatitis delta virus antigenomic ribozyme is the promoter for the positive strand polarity of the viral genome. There are three computer-predicted stem-loop structures in this region (Fig. 1B). When the nucleotides of each loop (L-III) were modified separately in the vector, none of them affected launching of 23 S RNA virus, even though the modification of loop III also changed the C-terminal two amino acids in p104 (Fig. 1C). The stem-loop structure proximal to the 3′ end contains a mismatched pair (A< >G) in the stem. Eliminating one of the mismatched nucleotides (G) impaired the generation of 23 S RNA virus (Fig. 1C). The importance of the mismatched bases and the stem structure encompassing them in viral replication was investigated in detail (see below).

When the nucleotide sequence of one side of the lower stem was replaced with that of the other or vice versa, thus destroying the lower stem structure, none of vectors generated the virus (Fig. 2A). The two base pairs at the bottom of the stem were left unmodified because they are part of the 3′-terminal 5 nucleotides that are complementary to those at the 5′ end of the viral genome (5′-GGGCG. . . . GCCCC-OH). When sequences of both sides in the lower stem were exchanged simultaneously, thus restoring the stem structure, the ability to generate the virus was recovered (Fig. 2A).

The viruses generated from this lower stem-exchanged plasmid and from the original standard launching plasmid were indistinguishable from endogenous 23 S RNA virus in sucrose gradients (Fig. 3). p104 and the 23 S RNA genomes co-migrated in the gradients as observed previously (18). With regard to the upper stem structure, we obtained the same results (Fig. 2B). When the upper stem was destroyed by replacing the 3 nt sequence of either side of the stem with that of the other side, the activity was lost. However, when both sequences were exchanged simultaneously, virus-generating activity was recovered. Therefore, these results indicate that the stem structure (perhaps functioning as a platform to present the mismatched pair), but not the stem sequences per se, is important for virus replication.

**A Mismatched Pair of Purines Is Essential**—When the 3′-G of the mismatched pair was eliminated or replaced with U (Fig. 4A), the modified vectors did not generate the virus, suggesting that the bulged non-base-paired nucleotide at the 3′ side is necessary. Additionally, A, but not C, could substitute this G without losing the activity, suggesting that the bulged nucleotide should be a purine. Similarly, when the 5′-A of the mismatched pair was eliminated or replaced with C or U, none of the modified vectors generated the virus (Fig. 4B), indicating that the bulged non-base-paired nucleotide at the 5′ side of the stem is also necessary. The ability of G to replace this A without affecting virus launching again highlights the importance of purines at this position. To confirm that purines at the mismatched pair are essential for replication, we generated three concerted mutations at these positions (Fig. 4C). When the pair (A< >G) was changed to (G< >A), the launching activity was unaffected. Therefore, any combination of purines at the mismatched pair is capable of replication. On the other hand, the mismatched pair (C< >A) inhibited virus launching, whereas the (A< >A) mismatch maintained virus-launching activity. These results suggest that the 3′ and 5′ bases of the mismatched pair should be purines. Finally, this was confirmed by the inability of the third concerted mutation (U< >U) to generate the virus. Because changes of the mismatched pair (A< >G) to (A-U) and (U-G) resulted in failure to produce the virus, these results together indicate that the mismatched pair is essential for virus replication and that both bases at the pair should be purines.

**3′-Terminal 4 Cs**—The 5′- and 3′-terminal 5 nucleotides of the 23 S RNA viral genome are complimentary. Previously, we have shown that changing the 3′ endpoints . . . . CCC-OH to . . . . AAAC-OH resulted in the failure to generate the virus (21). Thus, this region contains a cis-element for replication. In this study, we modified the 3′-terminal five nucleotides individually (Fig. 5A) and found that the terminal or penultimate C

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could be changed to A without affecting virus launching. However, the activity was lost when the third or fourth C (from the 3’ end) was changed to A or U. Because the fourth C is part of the stem that contains the essential mismatched pair, we chose U to replace it to minimize the effects on the stem structure. The modification of the fifth G to C or the addition of an extra A to the 3’ end did not affect virus generation (Fig. 5A). Consistently, up to two Cs but not three could be eliminated from the 3’ terminus without affecting virus launching (Fig. 5B). Therefore, the third and the fourth Cs from the 3’ end were essential for virus replication, whereas the last two Cs as well as the fifth G appeared to be dispensable. Accordingly, the 3’-terminal C could be changed to any other nucleotide without affecting virus launching (Fig. 6). It should be noted that changing the C to A or G breaks its complementarity with the 5’-terminal G. When this 5’-terminal G was changed to A or C, the generation of 23 S RNA virus was not affected. Furthermore, when their 3’-terminal C was changed to U or G, thus restoring complementarity between the 5’- and 3’-terminal nucleotides, again these double mutations did not affect virus launching. These results suggest that the terminal nucleotides at the 5’ and 3’ ends of the viral genomes are dispensable for virus replication. Alternatively, the virus may have somehow corrected these modifications, enabling only RNAs with proper viral sequences to be replicated. To examine these possibilities, we took two different approaches. First, we examined the viral 3’ end by primer extension (Fig. 7). 23 S RNA virus was launched from various plasmids. After curing the launching plasmids, T dsRNA was purified from virus-launched cells and the 5’ ends of the T-negative strands were reverse-transcribed using a 32P-labeled primer. The reverse transcripts from viral RNAs generated from cDNAs whose 3’-terminal C had been changed to U or G had the same length as those from the authentic endogenous viral RNA. These results do not distinguish the two possibilities mentioned above, however, they indicate that the 3’-terminal nucleotide either modified or corrected has been maintained. When we analyzed the 3’-terminal region of viral RNA generated from a cDNA that lacked the 3’-terminal C, we found that the transcripts had the same length as those from the authentic endogenous virus, thus indicating that the launched virus had acquired one nucleotide at the 3’ end absent in the cDNA sequence.

3’-RACE—Because primer extension does not provide information on the base composition of the sequences at the 3’ end, we used a second approach (3’-RACE) to address this point. As control, the 3’-terminal regions of endogenous virus and also of virus generated from the standard launching plasmid (pRE637) were cloned and sequenced. >80% of the clones in the
controls possessed the correct full-length 5' and 3' termini in the positive strands (Table I). When the 3'-terminal C was changed to G in the vector (pRE681), the majority of clones (6 of 7) from the launched virus had the authentic positive strand 3'-termini (... GCCCC-OH) and none of them maintained the terminal G present in the cDNA. The primer extension experiment shown in Fig. 7 gave the same result on the length of the viral 3'-termini generated from the plasmid but failed to provide information on the correction of the terminal nucleotide.

At the 5' ends, 7 of 8 clones possessed the full-length wild type sequence. Therefore, these results indicate that the launched virus recovered the terminal C at the 3' end of the viral genome, thus suggesting its indispensability in virus replication. To confirm this and also to examine any possible role played by the complementary base at the 5' end on the 3'-terminal repair, we analyzed viral RNAs generated from two cDNAs having concerted mutations at both termini. The 5'- and 3'-terminal bases G and C were changed to A and U, respectively, in one case (pRE718) and to C and G in the other (pRE719). In both cases, the generated viruses recovered the terminal C at the 3' end (8 of 8 clones in the former case and 6 of 8 in the latter) and also the terminal G at the 5' end (6 of 7 in the former case and 9 of 9 in the latter). None of the clones retained the mutations originally introduced to the cDNA. Therefore, the 3'- and 5'-terminal nucleotides were corrected to the wild type irrespective of the mutations introduced at those positions. This indicates that the complementarity of the terminal bases in the inverted repeats is not involved in the repairing processes.

Although the elimination of the terminal C (pRE698) or terminal and penultimate Cs (pRE739) from the vector did not affect virus launching, the virus generated regained these Cs at their 3' termini. In the latter case (pRE739), we analyzed two independently launched viruses. Therefore, these results clearly indicate that not only the third and fourth Cs but also the terminal and penultimate Cs at the 3' end are essential for replication. In the former experiment (pRE698), we used a yeast strain that had no endogenous 20 S RNA virus. This indicates that the acquisition of the 3'-terminal C does not require the presence of 20 S RNA virus.

In contrast to these terminal Cs, we obtained quite different results when the fifth base (G) from the 3' terminus was...
changed to C. To avoid any complication derived from the shortening of the lower stem structure adjacent to the 3' end, we used a plasmid (pRE748) that also harbors the compensatory mutation at the 5' side of the lower stem (C34G numbered from the 3' end). These compensatory double mutations did not affect virus launching and generated 23S RNA virus as efficiently as the single mutation (G5C numbered from the 3' end) (Fig. 5A and data not shown). When two independently launched viruses were examined, all of the clones derived from them retained this C (Table 1) as well as the compensatory mutation at the 5' side of the stem (data not shown), indicating that the fifth G at the 3' end is dispensable for replication. Furthermore, the generated viruses also retained the C at position 5 at the 5' end of viral genome. Thus, the base complementarity at the innermost positions of the inverted repeats is not essential for replication. Interestingly, among the viruses generated from this plasmid, we observed at a high proportion the viral RNAs having the 3' termini one nucleotide shorter, 3 of 9 in one case and 4 of 9 in the other. Because the shorter RNA still has a cluster of 4 Cs at the 3' end, this finding suggests that these 4 Cs endowed the shorter RNA genome with a substantial activity for replication. Finally, when viruses were generated from a plasmid (pRE755) in which the wild type mismatched pair (A< >G) was changed to (G< >A), all of the clones derived from two independently generated viruses retained the modified mismatched pair (Table 2). Viruses generated from cDNAs containing the wild type mismatched pair retained the same wild type mismatch, thus confirming our previous conclusion that any combination of purines at the mismatched pair has cis-acting activity for replication.
Fig. 7. Launched viruses have full-length 3' termini. A, 23 S RNA virus was generated from a plasmid containing 23 S RNA cDNA whose 3'-terminal C was deleted (lane 1) or changed to G (lane 2) or T (lane 3). After curing the plasmids, T-dsRNA was isolated from these cells. As controls, T-dsRNA from cells containing 23 S RNA virus generated from wild type cDNA (lane 4) or endogenous 23 S RNA (lane Endogenous) was also isolated. After denaturation, T-negative strands were reverse-transcribed using a 32P-labeled primer and the products were analyzed on a 8% acrylamide sequencing gel. An autoradiogram of the gel is shown. A sequence ladder was generated from a plasmid containing wild type cDNA. In lane C + Endogenous, an aliquot of sequence ladder C and the primer-extended products shown in lane Endogenous were mixed and analyzed. Premature termination of reverse transcription was seen at the position corresponding to the 3' side of the mismatched pair. B, the 3' terminus of endogenous 23 S RNA virus and the corresponding region in the primary transcripts from plasmids are shown. The modification and elimination of the terminal C are indicated by filled circles and A, respectively. Ribozyme cleavage sites are indicated by vertical arrows.

### Table I
3'-RACE

| Launching plasmid       | Clones examined | 3' Ends          | Clones examined | 5' Ends      |
|-------------------------|----------------|------------------|----------------|-------------|
| Endogenous              | 9              | 8...GCCC-OH      | 8              | 7.5'-GGGGGC..|
| 5'-GGGGC..GCC..OH       | 8              | 6...GCCC-OH      | 7              | 7.5'-GGGGGC..|
| pRE637 (wild type)      | 8              | 2...GCCC-OH      | 8              | 7.5'-GGGGGC..|
| 5'-GGGGC..GCC..OH       | 8              | 1...GCCC-OH      | 7              | 7.5'-GGGGGC..|
| pRE719                  | 8              | 6...GCCC-OH      | 9              | 7.5'-GGGGGC..|
| 5'-GGGGC..GCC..OH       | 8              | 2...GCCC-OH      | 7              | 6.5'-GGGGGC..|
| pRE718                  | 8              | 8...GCCC-OH      | 6              | 6.5'-GGGGGC..|
| 5'-GGGGC..GCC..OH       | 9              | 1...GCCC-OH      | 8              | 6.5'-GGGGGC..|
| pRE739-1                | 7              | 6...GCCC-OH      | 15             | 15'-GGGGGC..|
| 5'-GGGGC..GCC..OH       | 7              | 1...GCCC-OH      | 15             | 15'-GGGGGC..|
| pRE739-4                | 7              | 6...GCCC-OH      | ND             |             |
| pRE748-1                | 9              | 5...GCCC-OH      | 6              | 5.5'-GGGGGC..|
| 5'-GGGGC..GCC..OH       | 3              | 3...GCCC-OH      | 1.5'-Δ6        |             |
| pRE748-2                | 9              | 4...GCCC-OH      | 6              | 5.5'-GGGGGC..|
|                         |                | 1...GCCC-OH      |                |             |

A 20 S RNA-o strain was used for 23 S RNA virus launching.

a 3′-terminal sequences of negative strands are presented as 5′-terminal sequences of positive strands.

ND, not determined.

### DISCUSSION

**Bipartite 3′-cis-Signal**—We have used a recently established in vivo launching system to analyze the 3′-cis-acting signals for replication in yeast 23 S RNA virus. There are two cis-elements in the 3′-non-coding region of 23 S RNA virus. One is a mismatched pair present in a stem-loop structure adjacent to the 3′ terminus. Although the loop and stem sequences (with the exception of the fourth C from the 3′ terminus) are not impor-
tant, the mismatched pair with purine bases is essential for replication. Changing the pair to a single bulge at either side of the stem or converting it to a matched pair resulted in the loss of activity. Any combination of purines at the mismatched pair is vital for viral replication. This was confirmed by analyzing the virus generated from a cDNA in which the wild type mismatch (A<->G) was changed to (G<->A). All of the viral genomes retained the modified mismatched pair. The second cis-element is a cluster of 4 Cs at the 3' end. The viral genome bears 5 nt-inverted repeats at both termini (5'-GGGGC...GGGGC-3'). When each of the conserved 5 bases at the 3' end was modified, we found that the innermost G could be changed to C without affecting the replication. The viruses generated maintained the modified C without recovering the original G, thus confirming that the G at position 5 from the 3' end is dispensable for replication.

Because the generated virus also maintained the C at the corresponding position in the 5' end counterpart, the complementarity between the innermost bases of the terminal repeats is not necessary for 23 S RNA replication. The third and fourth Cs at the 3' end are essential for virus launching. Although the terminal and penultimate Cs can be eliminated or changed to other bases without affecting virus generation, the generated viruses have recovered these Cs at their 3' termini. The terminal 4 Cs may constitute the initiation site for negative strand synthesis, whereas the purine mismatched pair may provide template specificity for binding to replication machinery as proposed in the case of brome mosaic virus (28, 29). Our recent experiments indicate, however, that the mismatched pair and also the C at position 4 from the 3' end (so far examined) are essential to form a complex in vivo with p104.3 It is likely that these cis-elements are implicated not only in replication but also in the formation of a stable complex between the genomic RNA and its RdRp.

Terminal Repair Mechanisms—The 23 S RNA genome has no poly(A) tail at the 3' end and perhaps no 5'-cap structure, thus resembling intermediates of mRNA degradation. The formation of a complex may stabilize or protect the RNA genome from degradation. However, because p104 has only one-tenth of the molecular mass of the genome, it cannot protect the entire RNA molecule. The 5' end of 23 S RNA has to be accessible to the translational machinery to be decoded to produce p104. Additionally, during replication, the 3' end of the viral genome may interact with the replicase machinery to synthesize the negative strands. Thus, it is likely that the termini of the 23 S RNA genome are accessible to cytoplasmic 5'--3'-exonucleases and continually nibbled by these enzymes, which are usually involved in mRNA metabolism. In this context, to have a terminal repair mechanism(s) would be a requirement for 23 S RNA virus that resides and replicates persistently in the host cytoplasm without encapsidation.

It has been reported that satellite RNA C of turnip crinkle carmovirus can repair deletions of up to 6 nt from the 3' end by utilizing 4–8 nt oligonucleotides as primers, produced by abortive synthesis by viral polymerase from the 3' end of the helper

| Launching plasmid | Clones | Sequence |
|-------------------|--------|---------|
| pRE637            | 8b     | 8. C/A CGGUAUAAU ACCG/G/G. |
| _CGGUAUAAU ACCG/G/G._ |        | |
| pRE755-1          | 4      | 4. C/G CGGUAUAAU ACCG/G/G. |
| _CGGUAUAAU ACCG/G/G._ |        | |
| pRE755-2          | 4      | 4. C/G CGGUAUAAU ACCG/G/G. |

* Nucleotides in boldface and parenthesis are the mismatched purine pair. The loop I sequence is underlined.
* All of viral clones derived from launching plasmids listed in Table I also maintained the wild type mismatched pair of purines.

Fig. 8. Resemblance of the 3′-terminal secondary structures in the positive and negative strands of 23 S RNA virus to the top half-domain of tRNA. The top half-domain of eukaryotic tRNA34 was derived from Sprinzl et al. (39) The non-templated A residues at the viral 3′ termini (see “Discussion”) are indicated by parenthesis. Y, R, and N stand for pyrimidine, purine, and any base, respectively.

virus genome (6). Because both 20 S RNA and 23 S RNA genomes share the same 5 nt sequence at their 5′ termini and most launching experiments described in this work were performed by using a strain, which also harbors 20 S RNA virus, it raises the possibility that 23 S RNA virus utilizes abortive oligonucleotides produced by 20 S RNA virus as a primer to correct defects at the 3′ end. However, elimination of 20 S RNA virus from the strain did not affect 23 S RNA virus as a primer to correct defects at the 3′ end. However, elimination of 20 S RNA virus from the strain did not affect 23 S RNA virus utility, whether the generated virus also maintained the C at the corresponding position in the 5′ end counterpart, the complementarity between the innermost bases of the terminal repeats is not necessary for 23 S RNA replication. The third and fourth Cs at the 3′ end are essential for virus launching. Although the terminal and penultimate Cs can be eliminated or changed to other bases without affecting virus generation, the generated viruses have recovered these Cs at their 3′ termini. The terminal 4 Cs may constitute the initiation site for negative strand synthesis, whereas the purine mismatched pair may provide template specificity for binding to replication machinery as proposed in the case of brome mosaic virus (28, 29). Our recent experiments indicate, however, that the mismatched pair and also the C at position 4 from the 3′ end (so far examined) are essential to form a complex in vivo with p104.3 It is likely that these cis-elements are implicated not only in replication but also in the formation of a stable complex between the genomic RNA and its RdRp.

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4 R. Esteban and T. Fujimura, unpublished results.
plate-independent fashion and use these oligonucleotides to initiate negative strand synthesis at the 3′ end of truncated templates as reported in the RdRp of turnip crinkle carmovirus (8). In the mechanism proposed for the initiation of transcription in the bacteriophage Φ6 (30), the opened template strand of Φ6 dsRNA initially overshoots passing through the active site and looking into a specificity pocket in the carboxyl-terminal domain of the RdRp. The interaction between the penultimate C and GTP causes the template to ratchet back by one nucleotide so that the 3′-terminal and penultimate Cs can interact now with two cognate GTPs to start polymerization. Because the third and fourth Cs at the 3′ ends are essential for 23 S RNA virus launching, these bases may constitute the initiation site for negative strand synthesis. After synthesis of a dinucleotide G3, the template at the active site of p104 may ratchet back by one nucleotide (or two) until a tetranucleotide G4 is formed. Chain elongation starts using the G4 as a primer. In this case, the terminal and penultimate Cs may be considered as a protective tail. The 23 S RNA genome forms a stable resting complex with p104. If an activated p104 also interacts with the template RNA at the 3′-terminal two cis-sites during initiation of negative strand synthesis, the realignment of the primer postulated above could be driven by a conformational change in p104 in which one domain of the protein, the active site, polymerizing the oligonucleotide primer at the terminal Cs is pushed away along the RNA from a second domain anchored at the mismatched pair. Because the majority of progeny generated from cDNAs having deletions or modifications at the terminal Cs recovered the wild type species, these RdRp-mediated repair mechanisms infer that RNAs with modified 3′ termini were poor templates for RNA synthesis and that RNA with the wild type terminus, once generated, replicates autonomously and dominates the population in the absence of a selective pressure.

It is also possible that host proteins are involved in the 3′ end repair. A number of RNA viruses from plant origin such as brome mosaic virus possess tRNA-like structures at their 3′ termini (31). Many of these viral genomes can be aminocoylated and also function as a substrate for tRNA nucleotidyltransferase (CCA-adding enzyme). Because brome mosaic virus RNAs bearing mutations in the 3′-CCA-OH termini were corrected efficiently to the wild type sequences in vivo and the CCA-adding enzyme appears to be involved in this process (5), it raises the possibility that this enzyme is also implicated in the repair of 23 S RNA viral termini during launching. There are two lines of evidence that support this possibility. When the 3′ termini of viral RNA were examined by the nearest-neighbor analysis after radioactive pCP labeling, both the positive and negative strands of 23 S RNA genome have a non-templated A at 20–30% of their 3′ termini (14, 32, 33). The other line of evidence comes from structural features at the 3′ termini of viral RNA. As shown in Fig. 8, both the positive and negative strands of 23 S RNA genome have stem-loop structures with stems of 13 and 12 bp long, respectively, to which CCC(A)-OH tails are attached. These structures resemble the top half-domain of tRNA. The acceptor stem and T stem of tRNA stack with the wild type terminus, once generated, replicates autonomously and dominates the population in the absence of a selective pressure.

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