The Short Transcript of *Leishmania* RNA Virus Is Generated by RNA Cleavage

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*Leishmania* RNA virus 1 (LRV1) is a double-stranded RNA (dsRNA) virus of the family Totiviridae that infects the New World protozoan parasites *Leishmania braziliensis* and *Leishmania guyanensis*. The complete sequences and molecular organizations of two LRV1 isolates, LRV1-4 and LRV1-1, have been reported (12, 13). The genome size is approximately 5,280 nucleotides (nt), and two large open reading frames (ORFs) are present in both isolates. When ORF2 was expressed in a recombinant baculovirus expression system, the expressed protein self-assembled into virus-like particles, demonstrating that ORF2 encodes the capsid protein (2). ORF3 is believed to encode the viral polymerase, as the predicted protein product of ORF3 possesses motifs characteristic of viral RNA-dependent RNA polymerases (RDRP) (1). Together, ORFs 2 and 3 constitute 90% of the viral genome. Small ORFs can be identified in the 5′-terminal 450 nt of different LRV1 isolates, and yet these ORFs have not been shown to directly encode gene products and they are not conserved among isolates. However, the nucleotide sequence at the 5′ end of LRV1 isolates is highly conserved (12). Strong conservation of nucleotide sequence at the genomes’ 5′ ends suggests that this region serves an essential viral function.

Purified LRV1 particles contain genome-length dsRNA and single-stranded RNAs (ssRNAs) (17). The presence of both dsRNA and ssRNAs in purified virions suggests that the viral polymerase accomplishes a dual function of replication and transcription, on the basis of analogy with a yeast virus system (5). For the yeast L-A virus, the polymerase serves as a transcriptase, generating positive-sense ssRNA transcripts, and as a replicase, synthesizing negative-sense ssRNAs which yield dsRNA (16). Both dsRNAs and ssRNAs are also synthesized in an in vitro polymerase assay with either virally infected cell extracts or virions purified on a sucrose gradient as the source of RDRP and RNA template (14, 18). Through analysis of in vitro polymerase assay products from purified viral particles, the replication cycle of LRV1 is believed to proceed via the replication of full-length encapsidated positive-sense ssRNA transcripts in a conservative manner, yielding dsRNA which is transcribed to synthesize more positive-sense ssRNA transcripts, which are extruded from the particle and translated and/or encapsidated, thus completing the cycle (14, 18).

A short viral transcript corresponding to the 5′ end of viral positive-sense ssRNA was recently identified in vitro polymerase assays with sucrose-purified virus (3). This short transcript was also found in vivo by Northern (RNA) analysis of total RNA from LRV1-4-infected cells. Generation of the short transcript was originally hypothesized to be the result of premature transcription termination. Evidence presented here shows that generation of the short transcript is mediated by a site-specific cleavage event within the 5′ untranslated region of viral message.

**MATERIALS AND METHODS**

**Parasite strain and cell culture.** *L. guyanensis* stock MHOM/BR/75/M4147 (M4147) was grown at 23°C in M199 semisynthetic medium (4) ( Gibco Laboratories) supplemented with 5% fresh, filter-sterilized human urine.

**Virus purification and virion treatment.** Virus isolated from M4147 cells, designated LRV1-4, was isolated as previously described, with minor differences (3). Briefly, promastigotes (~10^7 cells) were harvested in early stationary phase, washed, and lysed in 1% Triton X-100 for 1.5 h on ice. Cell lysates were fractionated on 10 to 40% (wt/vol) sucrose gradients or 30 to 40% (wt/wt) CaCl

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 gradients, and the fractions containing the peak of viral dsRNA were used as the source of enzyme and template RNA for viral polymerase reactions and the source of enzyme in cleavage assays.

Proteinase K-treated sucrose-purified particles were prepared by incubation...
with protease K (100 ng/μl) (United States Biochemical Corp.) for 20 min at 23°C. Ethylene glycol-bis-(β-aminoethly ether) N,N,N′,N′-tetraacetic acid (EGTA)-treated sucrose-purified particles were prepared by addition of EGTA to a final concentration of 90 mM. Sucrose-purified particles were heat inactivated by incubation at 90°C for 2 min.

Polymerase assay. Polymerase activity was assayed in 20-μl reaction mixtures containing 100 mM NaCl, 20 mM Tris-base (pH 7.5), 10 mM MgCl₂; 8 mM dithiothreitol; 2 mM (each) ATP, CTP, and UTP; 40 μM GTP; 7.5 to 15 mM [α-32P]-ATP (3,000 Ci/mmol) (New England Nuclear); 20 U of recombinant RNAsin inhibitor (Promega); and 5 μl of viral particles from gradient fractions. Incubation was for 1 h at 37°C. Polymerase products were resolved on denaturing 4% polyacrylamide–7.5 M urea gels and visualized by autoradiography.

Primer extension. Primer extension was performed according to manufacturer’s directions with Superscript II reverse transcriptase (Gibco BRL) on 20 μg of RNA produced in a polymerase assay of sucrose-purified particles with 2 μM unlabeled GTP. The synthetic oligonucleotide primer used was LRV-ORF2 FRAME TEST (5'-ATCATAACACACGTTCGTCG-3'), an antisense primer to viral protein at nucleotide 554 to 572. This primer was end labeled with [γ-32P]ATP (6,000 Ci/mmol) (New England Nuclear) and T4 polynucleotide kinase (New England Biolabs). Reaction products were resolved on a 4% denaturing polyacrylamide–7.5 M urea gel and visualized by autoradiography.

cDNA cloning and RNA transcript synthesis. Total nucleic acid isolated from ~2 × 108 M1417 promyelocytes was used as a template for reverse transcription with retrovirus avian leukemia virus reverse transcriptase (Boehringer Mannheim) and PCR amplification with Taq DNA polymerase (Boehringer Mannheim) and the synthetic oligonucleotide primers LRV3-AUG (5'-CTGACTGGATGCCAATATGGAATACATCACAGGAGCTCGCGAAT-TG-3') and LRV3-5' (5'-CCGAGCCTCCGAAATTCGAGTATACTGGTAGTCGAG-3'), according to the manufacturer’s directions. The PCR product, corresponding to the viral 5'-terminal 447 nt, was ligated into a TA Cloning vector (Invitrogen Corp.) according to the manufacturer’s instructions. This construct was designated number 2. The viral sequence was subcloned from number 2 by excision with SacI and NcoI, gel purification of the desired fragment, blunting of the ends with deoxyadenosine triphosphates and Klenow (Boehringer Mannheim), and ligation into pBRAMS CAT1 (7) that had been digested with SpeI and SacI and blunted with Klenow fragment polymerase. This construct was designated WT and possesses the viral 5'-terminal 447 nt juxtaposed upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene.

RNA transcripts were generated in vitro in transcription reaction mixtures containing 40 mM Tris-base (pH 7.5); 20 mM MgCl₂; 1 mM spermidine; 30 mM dithiothreitol; 0.5 mM (each) ATP, CTP, and GTP; 50 μM UTP; 1.25 μM [α-32P]UTP (800 Ci/mmol) (NEN); 20 U of RNAsin; 10 mM NaCl; 20 U of T3 RNA polymerase (Promega); and 20 mM DNA template generated by digestion of WT with Smal or PvuII. Incubation was at 37°C for 1 h. Template DNA was removed by addition of 1 U of RQ1-DNase (Promega) and incubation for 15 min. Labeled RNAs were phenol-chloroform extracted and ethanol precipitated to remove unincorporated [α-32P]UTP.

Cleavage assay. Cleavage activity was assayed in 10- to 16.5-μl reaction mixtures containing substrate RNA, viral particles at 20 to 50% volume, and 20 U of RNAsin. Incubation was at 37°C for 30 to 60 min. The amount of viral particles added to each reaction was equilibrated to account for concentration alterations resulting from proteinase K treatment. Following the reaction, portion of each reaction mixture was mixed with formamide loading dye and heat denatured at 90°C for 2 min. Reaction products were resolved on denaturing 3.5 to 4% polyacrylamide–7.5 M urea gels and visualized by autoradiography.

RESULTS

The ability to generate short transcript in in vitro RDRP reactions can be specifically abolished. In an attempt to understand the mechanism of short-transcript synthesis, we began by biochemically investigating the source of short-transcript generation. Since sucrose-purified viral particles produce the short transcript in a polymerase assay, while CsCl-purified viral particles do not, the nature of the short-transcript-generating activity could be analyzed by using sucrose-purified particles in in vitro polymerase assays.

The ability of sucrose-purified particles to generate the short transcript in a polymerase assay was tested following treatment of viral particles with proteinase K, heat, or EGTA. Prior to addition of the polymerase assay reaction components, viral particles were preincubated in the presence or absence of proteinase K. In agreement with previous results (3), untreated sucrose-purified particles produced the short transcript, while untreated CsCl-purified particles did not (Fig. 1A, lanes 1 and 2). Mild proteinase K digestion of CsCl-purified particles resulted in the complete loss of polymerase activity, as no transcripts were generated (Fig. 1A, lane 4). When sucrose-purified particles were digested with proteinase K in an identical manner, synthesis of genome-length transcripts was unaffected, but no short transcript was observed (Fig. 1A, lane 3). All polymerase activity could be destroyed by treatment of sucrose-purified particles with higher concentrations of proteinase K (data not shown). Heat treatment of sucrose-purified particles also abolished all polymerase activity (data not shown). The ability to specifically abolish the short-transcript-generating activity of sucrose-purified particles with proteinase K indicates that a proteinase factor is involved in short-transcript production.

Polymerase assay products generated by sucrose-purified particles that were treated with increasing concentrations of EGTA, a calcium chelator, were examined next. Sucrose-purified particles specifically lost the ability to generate the short transcript in the presence of high EGTA concentrations (Fig. 1B). Polymerase activity was unaffected in the presence of 0 to 10 mM EGTA. At a 90 mM EGTA concentration, production of the short transcript was specifically abolished, while genome-length transcript synthesis appeared unaffected. Polymerase reaction products from untreated and EGTA-treated sucrose-purified viral particles were also resolved on a nondenaturing agarose gel in order to distinguish dsRNA and ssRNA reaction products (Fig. 2). EGTA-treated sucrose-purified viral particles retained both dsRNA and ssRNA synthesis activities, indicating that the polymerase’s replicase and transcriptase functions were intact. This is evidenced by the presence of a 5-kbp band and a smear in the 2-kbp range, representing viral dsRNA and ssRNAs, respectively (see reference 18 for comparison). The ability to specifically abolish the short-transcript-generating activity of sucrose-purified particles with proteinase K and EGTA indicates that a proteinase factor and divalent cations are required components of this activity. Furthermore, the short-transcript-generating activity is independent of the polymerase’s transcriptase and replicase activities.

Primer extension maps a position corresponding to the putative cleavage site. The putative cleavage site was determined by measurement of the size of the short transcript generated in polymerase assays. The size of the short transcript was measured at 318 nt by comparison of its electrophoretic mobility with that of denatured DNA sequencing markers (data not shown). Since the short transcript was previously shown to be generated from the viral genome’s 5’ terminus, the length of the short transcript should map the cleavage site.

If the short transcript is generated by cleavage of genome-length transcripts, two cleavage products should be produced. Unfortunately, resolving the cleavage product downstream of the cleavage site is difficult, as it is expected to be 4,965 nt, similar in size to the genome-length transcript of 5,283 nt. In an effort to identify the 5’ end of the predicted downstream cleavage product, primer extension analysis was performed on the RNA products from a polymerase assay of sucrose-purified particles. With a primer which bound downstream of the predicted 5’ end, synthesis of genome-length transcriptswas specifically abolished, primer extension to the 5’ end of the predicted downstream cleavage product would yield a 572-nt product and primer extension to the 5’ end of the predicted downstream cleavage product would yield a 254-nt product. Several products were observed in such a primer extension reaction (Fig. 3, lane 1). A single major band was produced in the 500- to 600-nt range. This product is likely to represent primer extension to the 5’ end of genome-length transcripts. A distinct band of 252 nt (accurately sized against a sequencing ladder) was also produced. Primer extension to this position maps within 2 bases of...
the 3’ end of the short transcript, suggesting that the 5’ end of the downstream cleavage product may have been identified. Primer extension results must be interpreted cautiously, since RNA secondary structure can also terminate progression of reverse transcriptase. The prominent band of 109 nt is believed to result from such a pause site. The origin of bands representing extremely long extension products is unknown and is assumed to be a result of mispriming at sites far downstream in the viral genome.

**Exogenous RNA transcripts possessing the viral 5’ 447 nt are cleaved in vitro.** To more definitively determine whether or not the short transcript is generated by a cleavage event of longer RNA transcripts, we asked whether or not exogenous RNAs containing viral sequences would be cleaved by viral particles. To test for the processing of substrate RNA transcripts, in vitro-synthesized RNAs that possessed the 5’ 447 nt of LRV1-4 positive-sense ssRNAs near their 5’ ends were incubated with sucrose-purified particles and cleavage was assessed by resolution of reaction products on a denaturing polyacrylamide gel. Two substrate transcripts were used (Fig. 4). T3 RNA polymerase-mediated transcription of plasmid WT digested with Smal produced an RNA of approximately 1,700 nt; this RNA was designated L-RNA. T3 transcription of plasmid WT digested with PvuII produced an RNA of 644 nt; this RNA was designated S-RNA. When RNA substrates were incubated with sucrose-purified particles, both L-RNA and S-RNA substrates yielded prominent, discrete cleavage products that were smaller than the input transcript (Fig. 5A, lanes 2 and 4). Incubation of RNA substrates alone resulted in no specific degradation (Fig. 5A, lanes 1 and 3). The appearance

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**FIG. 1.** Effect of proteinase K and EGTA on the polymerase activity of LRV1-4 particles. (A) LRV1-4 particles purified on either sucrose (lanes 1 and 3) or CsCl (lanes 2 and 4) gradients were used in in vitro polymerase assays after preincubation in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of proteinase K. (B) Sucrose-purified LRV1-4 particles were assayed for polymerase activity in the presence of final EGTA concentrations of 0 mM (lane 1), 1 mM (lane 2), 10 mM (lane 3) and 90 mM (lane 4). Polymerase products were resolved on 3.5 to 4% denaturing polyacrylamide gels and visualized by autoradiography. Full-length and short transcripts are indicated.

**FIG. 2.** dsRNA and ssRNA synthesis by EGTA-treated LRV1-4 particles. Polymerase assay reaction products shown in Fig. 1B, lanes 1 and 4 (lanes 2 and 3), were resolved on a 1.0% agarose gel and visualized by autoradiography. Lane 1, λ DNA digested with HindIII. Molecular size markers are indicated (in base pairs) on the left. dsRNA and ssRNAs are indicated on the right.
of cleavage products when RNA substrates were incubated in the presence, but not in the absence, of viral particles shows that a factor(s) present in the viral particle preparation is mediating endonucleolytic cleavage of the RNAs. A control RNA that did not possess any viral sequences was also tested in the cleavage assay (Fig. 5B). This nonviral RNA transcript, CAT-RNA, was not cleaved in the presence of sucrose-purified particles, indicating that cleavage is specific to the viral sequences present in the L- and S-RNA substrates.

S-RNA cleavage products were measured at 340 and 304 nt in length by comparison of their mobilities with that of a sequencing ladder (data not shown). The combined size of these two cleavage products exactly equals that of the input transcript (644 nt). Furthermore, the 340-nt band comigrates with a cleavage product from the L-RNA transcript. The predicted 1,360-nt cleavage product from L-RNA transcripts is not easily distinguished from full-length transcripts as a result of their close migration on the gel system in use. The larger cleavage product has been visualized on 1.5% agarose gels (data not shown). A 298-nt cleavage product is also present in both L- and S-RNA cleavage reactions of Fig. 5. This cleavage product was inconsistently produced and is believed to be derived from the 340-nt cleavage product.

When the 20 additional nucleotides present at the 5' ends of the substrate RNAs (because of incorporation from the plasmid polylinker) are taken into account, the 3' end of the 340-nt cleavage product maps to nt 320 of the viral genome. nt 320 is exactly the same position that was mapped by primer extension to the 5' end of the predicted downstream cleavage product counterpart to the short transcript. This site is within 2 nt of the 3' end of the short transcript, as determined by sizing of the short transcript generated in polymerase assays. Therefore, cleavage is occurring specifically in the region mapped by the end of the short transcript, suggesting that the short transcript may be generated through a site-specific cleavage event.

**Cleavage activity and short-transcript production in polymerase assays are associated.** We next tested the ability of altered sucrose-purified particles, which were unable to synthesize the short transcript in polymerase reactions, to cleave substrate RNA transcripts. This was done to determine whether cleavage activity and the ability of viral particles to generate the short transcript were associated. S-RNA was incubated with sucrose-purified particles treated with proteinase K, heat, or EGTA, as was done in the polymerase assays. As previously seen, the RNA substrate was cleaved in the presence, but not in the absence, of sucrose-purified particles (Fig. 5C, lanes 2 and 1). Incubation of RNA substrate with only the sucrose buffer used for gradient purification of the virus did not gen-
erate cleavage products (Fig. 5C, lane 7). Sucrose-purified particles treated with heat or EGTA were inactive in the cleavage assay (Fig. 5C, lanes 3 and 4), while particles treated with proteinase K showed a dramatic deficiency in the amount of cleavage products generated (Fig. 5C, lane 5). Furthermore, CsCl-purified particles, which do not generate the short transcript in polymerase assays, did not cleave the RNA substrate (Fig. 5C, lane 6). Identical results were obtained when L-RNA was used (data not shown). In summary, viral particles which were incapable of short-transcript synthesis in polymerase assays were also defective in the cleavage assay.

**DISCUSSION**

We have shown that the short transcript identified in LRV1-4-infected cells and produced in in vitro polymerase assays is generated by a specific cleavage within the 5' untranslated region of viral transcripts. Exogenously added substrate RNA transcripts that possessed the 5' 447 nt of LRV1-4 positive-sense ssRNAs were cleaved upon incubation with sucrose-
purified viral particles. The cleavage event was site specific, occurring after nt 320 of the viral genome.

Results from the polymerase assays indicate that the ability of sucrose-purified particles to generate the short transcript can be specifically abolished. Previous experiments had shown that all polymerase activity of CsCl-purified particles was sensitive to mild proteinase K digestion, while the polymerase activity of sucrose-purified particles was resistant (18). In those experiments, however, short-transcript production was not assessed. This paper shows that mild proteinase K digestion of sucrose-purified particles completely abolishes generation of the short transcript without otherwise affecting polymerase activity. The proteinaceous factor involved in the generation of the short transcript could be the polymerase protein itself or an additional protein factor.

EGTA-treated sucrose-purified particles also specifically lost the ability to generate the short transcript in polymerase assays, although the transcriptase and replicate activities of the polymerase were intact. The effect of EDTA on the polymerase activity of sucrose-purified particles was also tested (data not shown). EDTA concentrations as low as 1 mM completely abolished all polymerase activity, including full-length transcript synthesis. Since EDTA chelates both Ca$^{2+}$ and Mg$^{2+}$, and Mg$^{2+}$ is a required component for many polymerases’ activities, we believe that EDTA’s effect occurs because of Mg$^{2+}$ chelation. This leads us to believe that EGTA’s effect is specific to Ca$^{2+}$ chelation. On the assumption that EGTA is acting by chelating Ca$^{2+}$ ions present in the reaction, these results suggest that Ca$^{2+}$ is required for generation of the short transcript. Loss of the ability to generate the short transcript could be due to disassembly of the viral capsid when EGTA is present, as many capsid proteins require Ca$^{2+}$ for stable assembly. Electron micrograph observation of EGTA-treated virions showed that the capsid proteins were indeed disassembled (9a). Disassembly of the viral capsid could result in either a conformational change in the responsible protein which alters its activity or the dissociation of a factor which is required for short-transcript production. Alternatively, the responsible activity may have an inherent requirement for Ca$^{2+}$.

We have previously reported that CsCl purification disrupts viral particles’ integrity and replicate activity (18). Since CsCl-purified particles and EGTA-treated particles behave similarly in polymerase assays, it appears that particle disruption is associated with an inability to generate the short transcript. If the viral polymerase itself is the protein responsible for short-transcript production, the ability to separate genome-length-transcript synthesis from short-transcript synthesis suggests that these two activities are distinct.

A cleavage site responsible for generation of the short transcript was defined by three independent criteria. Primer extension analysis was used to identify the 5’ end of the predicted downstream cleavage product that would result from cleavage of viral transcripts to produce the short transcript. A distinct primer extension product of 252 nt maps to nt 320 of the viral genome. The short transcript itself was estimated at 318 nt. It is not known whether or not the short transcript is primed from the precise 5’ terminus of the virus. If this assumption is made, the length of the short transcript would map the cleavage site to nt 318. From these data, it appeared that the region of nt 318 to 320 in viral positive-sense transcripts serves as a cleavage site. Conclusive evidence that this region contains a cleavage site was obtained by assaying for cleavage by viral particles when this region was attached to a heterologous RNA.

For the cleavage assay, two substrate RNAs, L-RNA and S-RNA, that possessed viral sequences containing the putative cleavage site near their 5’ ends, were created. When incubated with sucrose-purified particles, both L-RNA and S-RNA were specifically cleaved. S-RNA was cleaved into two distinct products whose combined lengths equaled that of the input transcript, suggesting that cleavage had occurred and that no further processing of the cleavage products had ensued. Cleavage of L-RNA generated a cleavage product identical in size to the larger of the two S-RNA cleavage products. Since the 5’ ends of L- and S-RNAs are identical, while their 3’ ends differ by more than 1 kb in length, the 340-nt cleavage product in common between the two substrate RNAs must have originated from their 5’ ends. Another band of 298 nt is sometimes present in both L- and S-RNA cleavage reactions, suggesting that it also originates from the 5’ end of the transcripts. Since no corresponding partner cleavage product is observed with S-RNA cleavage (predicted to be 340 nt), we conclude that it is derived from the 5’ 340-nt cleavage product. When the 20 nonviral nucleotides present at the 5’ ends of the substrate RNAs are taken into account, the cleavage site of in vitro-processed RNAs maps to viral nt 320.

All sequence determinants required for accurate RNA cleavage are present in the 447 viral nucleotides used in construction of the substrate RNAs. The cleavage reaction is endonucleolytic, since both upstream and downstream cleavage products can be identified, and does not appear to be autocatalyzed, since the reaction did not occur in the absence of protein. Cleavage of the substrate RNAs shows that the viral sequence does not need to be at the exact 5’ terminus of transcripts to be cleaved, since 20 nt of plasmid polylinker sequence is present in these RNAs. Furthermore, there appear to be no restrictions on what sequences may be present 3’ of the viral sequence.

The cleavage activity we have identified is directly associated with the ability of viral particles to generate the short transcript in vitro polymerase assays. Only viral particle preparations which were capable of producing the short transcript in polymerase assays were also capable of cleaving exogenously added substrate RNAs in the cleavage assay, suggesting that the generation of the short transcript is mediated by a cleavage event. Cleavage of viral transcripts appears to be a feature common to members of the genus *Leishmania*, as short transcripts are generated in polymerase assays of other LRV isolates, including LRV1-1 and the Old World isolate LRV2-1 (9b).

It remains to be determined whether the endonuclease activity is of viral or host origin. If cleavage is found to be mediated by the viral polymerase, this is not without precedent. The first step in transcription initiation of influenza virus and bunyavirus is the endonucleolytic cleavage of capped cellular RNAs mediated by a polymerase-associated endonuclease activity (10, 11). Also, a number of different RNA polymerases in ternary complexes have been shown to possess a chain-retracting activity that proceeds via hydrolytic cleavage of the growing end of nascent transcripts (reviewed in reference 8). In addition to eukaryotic RNA polymerase II, similar RNA hydrolytic activities are associated the vaccinia virus RNA polymerase (6) and yeast RNA polymerase III (15).

Infection of *Leishmania* cells by LRV1-4 is believed to be persistent, since no extracellular virus has been observed. The ability to exist as a persistent infection must require a regulatory mechanism that maintains the number of virions per cell within a range compatible with cellular function. Measurements of the number of viral genomes present per cell indicate that LRV1-4 infection is maintained at relatively low levels. The viral genome is present at about 100 copies per cell, as measured by competitive reverse transcription-PCRs (2a).

Regulation of the amount of viral genome competent for replication could be achieved by transcriptional and/or posttran-
scriptional mechanisms. Cleaving the 5' end off of positive-sense, genome-length viral transcripts could serve to remove those cleaved RNAs from the replication cycle and therefore reduce the viral load on a cell. Such a cleavage mechanism may be used to maintain a persistent infection. A model for the replication cycle of LRV is diagrammed in Fig. 6.

Cleavage of viral transcripts could also serve to generate a functional mRNA. Computer-assisted modeling of potential secondary structure at the 5' ends of LRV1-1 and LRV1-4 transcripts predicts five conserved stem-loops and two conserved AUGs (12). The presence of extensive secondary structure, as well as upstream ORFs, is known to attenuate translation of downstream ORFs (9). Removal of this putative translation-attenuating region of viral transcripts through cleavage could result in the generation of mRNAs which as a result are translated much more efficiently. Genetic analysis of the functional role for cleavage of viral transcripts awaits the development of an infectious clone for LRV.

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REFERENCES


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