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Single-site cleavage in the 5′-untranslated region of *Leishmaniavirus* RNA is mediated by the viral capsid protein

(*Totiviridae*/double-stranded RNA virus/endoribonuclease/RNA processing/*Leishmania*)

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ABSTRACT ***Leishmaniavirus* (LRV) is a double-stranded RNA virus that persistently infects the protozoan parasite *Leishmania*. LRV produces a short RNA transcript, corresponding to the 5′ end of positive-sense viral RNA, both *in vivo* and in *in vitro* polymerase assays. The short transcript is generated by a single site-specific cleavage event in the 5′ untranslated region of the 5.3-kb genome. This cleavage event can be reproduced *in vitro* with purified viral particles and a substrate RNA transcript possessing the viral cleavage site. A region of nucleotides required for cleavage was identified by analyzing the cleavage sites yielding the short transcripts of various LRV isolates. A 6-nt deletion at this cleavage site completely abolished RNA processing. In an *in vitro* cleavage assay, baculovirus-expressed capsid protein possessed an endonuclease activity identical to that of native virions, showing that the viral capsid protein is the RNA endonuclease. Identification of the LRV capsid protein as an RNA endonuclease is unprecedented among known viral capsid proteins.**

The initial search for viruses in the protozoan parasite *Leishmania* was undertaken for the potential power these organisms might provide as molecular tools in probing the host-cell biology and as model viral systems (1). *Leishmaniaviruses* (LRVs) have now been identified in twelve strains of *Leishmania braziliensis* or *Leishmania guyanensis* (2) and one strain of *Leishmania major* (3). Much interest has developed over the role that this virus may play in the virulence and pathogenesis of leishmaniasis. There is a precedent in simple eukaryotes for double-stranded RNA (dsRNA) viruses playing a role in pathogenesis. The dsRNA virus that infects the Chestnut Blight fungus has been shown to confer hypovirulence to this fungus (4). Although the effect that LRV may have on the growth or virulence of *Leishmania* remains to be elucidated, much has been learned about the virus itself.

LRV is a dsRNA virus that persistently infects some strains of the protozoan parasite *Leishmania* (5). The complete sequences and genome organization for two LRV isolates from the New World protozoan strain *L. guyanensis* have been reported (6, 7). The virus genome is ≈5280 nt in length, 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. The protein reacted to antiserum generated against purified virus, demonstrating that ORF2 encoded the 82-kDa capsid protein (8). ORF3 is believed to encode the viral polymerase, as the predicted protein product of ORF3 possesses motifs characteristic of viral RNA-dependent RNA polymerases (9). In an *in vitro* polymerase assay, both double- and single-stranded RNAs are synthesized by purified virions, indicating that the viral polymerase possesses both replicase and transcriptase activities (10).

In addition to genome-length RNA transcripts, a short transcript corresponding to the 5′ end of viral positive-sense RNA is generated in polymerase assays of purified virions (11). This short transcript is also detected in infected cells by Northern blot analysis. We have recently shown that the short transcript of LRV1-4 is generated by cleavage within the 5′ untranslated region of viral RNA transcripts (12). The endonuclease activity responsible for the cleavage event was proteinaceous in nature and was only associated with intact viral particles. In this report, we identify the viral capsid protein as the responsible endonuclease by demonstrating that recombinant-expressed viral capsid protein possesses the endoribonuclease activity. Furthermore, a region of nucleotides at the cleavage site is required for RNA cleavage, and the endonucleolytic activity is species-specific.

MATERIALS AND METHODS

Parasite Strains and Cell Culture. *L. major* stock MHOM/SU/73/5ASKH and *L. guyanensis* stocks MHOM/BR/80/MCUMC1-1A and MHOM/BR/75/M4147, infected with viral species LRV2-1, LRV1-1, and LRV1-4, respectively, were grown at 23°C in M199 semidefined medium (GIBCO) supplemented with 5% fresh, filter-sterilized human urine (13).

Virus and Virus-Like Particle Purification. Native virions were purified as described (11). Briefly, *Leishmania* promastigotes (≈10^{10} cells) were harvested in early stationary phase, washed, and lysed in 1% Triton X-100. Cell lysates were fractionated on 10–40% sucrose gradients, and the fractions containing the peak of viral dsRNA were used in polymerase and cleavage assays.

Virus-like particles were generated essentially as described (8) by infecting 100 ml of spinner cultures of ≈10^6 *Spodoptera frugiperda* cells with recombinant baculovirus expressing LRV1-4 ORF2, encoding the viral capsid protein. The infected cells were harvested 3 days after infection and lysed in 1 ml of lysis solution [2% Nonidet P-40/10 mM Tris (pH 7.5)/5 mM $MgCl_2$/100 mM NaCl] for 3–4 hr on ice. Cell extracts were fractionated on 10–40% sucrose gradients, and the peak fraction of capsid protein was determined by SDS/PAGE and Coomassie brilliant blue staining. A negative control, nonrecombinant baculovirus-infected insect cell extract, was also fractionated on a sucrose gradient. The fraction of identical density to that in which virus-like particles migrate was used in the cleavage assay (see Fig. 3), although all fractions were tested.

Polymerase Assay. Polymerase activity was assayed in 20 μl of reaction mixtures containing 100 mM NaCl; 20 mM Tris base (pH 7.5); 10 mM $MgCl_2$; 8 mM dithiothreitol; 2 mM (each) ATP, CTP, UTP; 40 μM GTP; 7.5–15 nM [α-^{32}P]GTP (3000 Ci/mmol; 1 Ci = 37 GBq) (NEN); 20 units of recombinant RNase inhibitor, RNasin (Promega); and 5 μl of gradient fractions possessing viral

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Abbreviations: LRV, *Leishmaniavirus(es)*; ORF, open reading frame.

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particles. Incubation was for 1 hr at 37°C. Polymerase products were resolved on denaturing 4% polyacrylamide/7.5 M urea gels and visualized by autoradiography.

RNA Transcript Synthesis. The cloning of the LRV1-4 genome 5′ terminus upstream of the bacterial chloramphenicol acetyltransferase gene, generating a construct designated WT, has been described (12). Substrate RNA transcript, S-RNA, possessing the 5′ 447 nt of LRV1-4, was generated by *in vitro* transcription of the WT construct in the presence of [α-^{32}P]UTP as described (12). A cleavage-site deletion mutant of construct WT was generated by restriction endonuclease digestion of WT at a unique *Bam*HI site within the putative cleavage consensus site, treatment with S1 nuclease (Boehringer Mannheim), and self-ligation with T4 DNA ligase (New England Biolabs) according to manufacturers' instructions. The deletion was confirmed by DNA sequencing (United States Biochemical). Deletion mutant RNA, Δ6-RNA, was generated by *in vitro* transcription of this WT-derived construct that had been linearized at a *Pvu* II site, similarly to S-RNA. Plasmid pRSET-rev was linearized at a unique *Eco*RI site, and Rev RNA of ≈750 nt was generated by *in vitro* transcription. Full-length radiolabeled RNAs were gel purified from denaturing 4% polyacrylamide/7.5 M urea gels (14) and used in cleavage assays.

Cleavage Assay. Cleavage assays were done as described (12). Briefly, cleavage activity was assayed in 10-μl reactions containing ≈35 nM (2000 cpm) of substrate RNA, viral particle-containing sucrose-gradient fractions at 50% vol, and 20 units of the recombinant RNase inhibitor RNasin (Promega). Incubation was at 37°C for 20–30 min. Reaction products were either precipitated (see Fig. 3) or taken directly from the reactions (see Figs. 2 and 4), mixed with formamide loading dye, heat denatured, resolved on denaturing 4% polyacrylamide/7.5 M urea gels and visualized by autoradiography.

RESULTS

RNA Cleavage at Different Sites in Different LRV Isolates. Three viral isolates, LRV1-4, LRV1-1, and LRV2-1, were used in *in vitro* polymerase assays to compare the site of RNA cleavage in each viral genome. The cleavage site for LRV1-4 was previously mapped to nt 320 of the genome by using exogenously added substrate RNA transcripts in an *in vitro* cleavage assay (12). All of the sequence determinants necessary for accurate RNA cleavage were present in the 5′-terminal 447 nt of LRV1-4-positive-sense single-stranded RNA.

Because the 5′-terminal 450-nt sequence of isolate LRV1-1 is highly homologous to that of LRV1-4 (90% identical), cleavage of LRV1-1 RNA was predicted to occur at exactly the same site as in LRV1-4 RNA. The cleavage site and surrounding sequence of LRV1-4 RNA is shown in Fig. 1*A*. An identical sequence is found in LRV1-1 RNA, but this sequence is shifted such that it occurs 2 nt closer to the 5′ end of the genome. If cleavage occurs in a sequence-specific manner at the site mapped for LRV1-4 RNA (5′-GAUC˅CGAA-3′), the short transcript of LRV1-1 is predicted to be 2 nt shorter than the short transcript of LRV1-4. The short transcripts generated in *in vitro* polymerase assays of sucrose-purified LRV1-1 and LRV1-4 particles were resolved on a denaturing polyacrylamide gel to compare their sizes (Fig. 1*B*, lanes 1 and 2), and transcripts were accurately sized against a sequencing ladder (K.J.M. and J.L.P., unpublished data). The short transcript of LRV1-1 was smaller than that of LRV1-4 by exactly 2 nt, suggesting that cleavage was occurring at the identical sequence as in LRV1-4.

An LRV isolate identified in an Old World parasite has recently been cloned and sequenced (S. Scheffter, Y.-T. Ro, I.-K. Chung, and J.L.P., unpublished data). This Old World virus, LRV2-1, has a similar genome organization to that of the New World viruses, yet its sequence is significantly divergent (<50% homology). Polymerase assays on sucrose-purified LRV2-1 particles yielded a short transcript of ≈280 nt in length (Fig. 1*B*, lane 3). Because the short transcript originates from the 5′ end of the genome, its length maps the cleavage site. When the sequence of LRV2-1 was examined, the sequence 5′-GCUCCGUA-3′ was present in the vicinity predicted for the 3′ end of the short transcript. Comparing the cleavage sites of these three viruses suggests that cleavage occurs specifically at a putative consensus sequence 5′-GNUC˅CGNA-3′ (Fig. 1*A*).

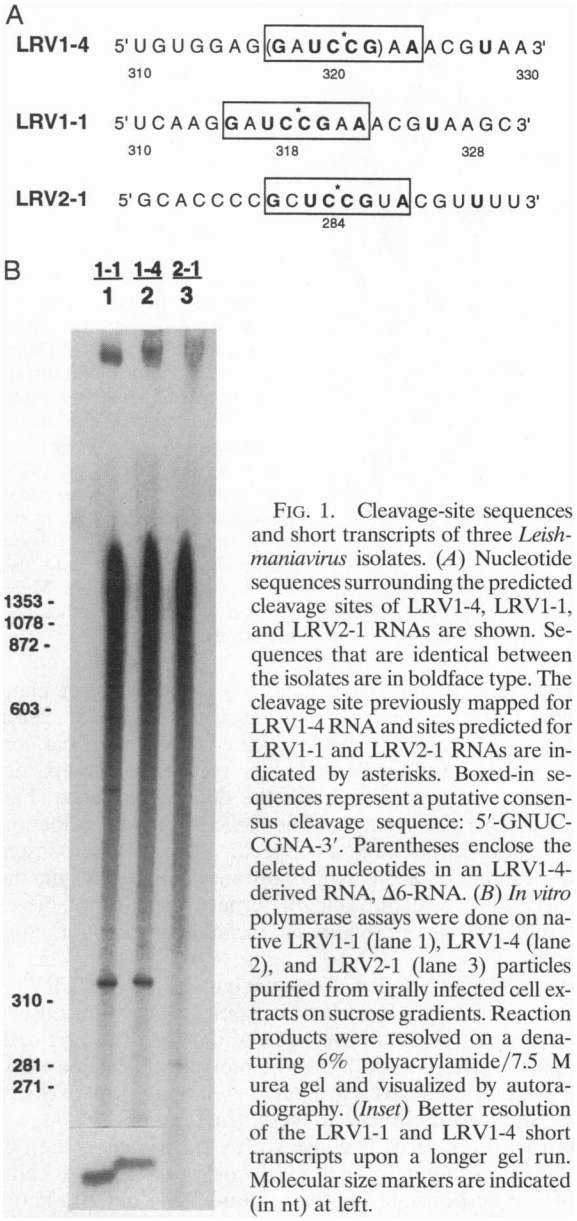

FIG. 1. Cleavage-site sequences and short transcripts of three *Leishmaniavirus* isolates. (*A*) Nucleotide sequences surrounding the predicted cleavage sites of LRV1-4, LRV1-1, and LRV2-1 RNAs are shown. Sequences that are identical between the isolates are in boldface type. The cleavage site previously mapped for LRV1-4 RNA and sites predicted for LRV1-1 and LRV2-1 RNAs are indicated by asterisks. Boxed-in sequences represent a putative consensus cleavage sequence: 5′-GNUC-CGNA-3′. Parentheses enclose the deleted nucleotides in an LRV1-4-derived RNA, Δ6-RNA. (*B*) *In vitro* polymerase assays were done on native LRV1-1 (lane 1), LRV1-4 (lane 2), and LRV2-1 (lane 3) particles purified from virally infected cell extracts on sucrose gradients. Reaction products were resolved on a denaturing 6% polyacrylamide/7.5 M urea gel and visualized by autoradiography. (*Inset*) Better resolution of the LRV1-1 and LRV1-4 short transcripts upon a longer gel run. Molecular size markers are indicated (in nt) at left.

Deletion of the Putative Consensus Sequence Abolishes RNA Cleavage. To determine whether the putative consensus sequence was required for specific RNA cleavage, we generated a deletion of this sequence in a substrate RNA and assayed for cleavage by viral particles. The substrate RNA, S-RNA, is a 644-nt RNA generated by *in vitro* transcription of a DNA construct possessing the 5′ 447 nt of the LRV1-4 genome. A 6-nt deletion of the putative cleavage site consensus sequence removed nt 317–322 of LRV1-4 sequence, yielding Δ6-RNA (Fig. 1*A*). S-RNA and Δ6-RNA were tested for their sensitivities to cleavage in an *in vitro* cleavage assay (Fig. 2). As previously shown (12), the S-RNA substrate was specifically cleaved when incubated in the presence, but not in the absence, of native LRV1-4 particles (Fig. 2, lanes 1 and 2). Cleavage at viral nt 320 in the S-RNA yielded

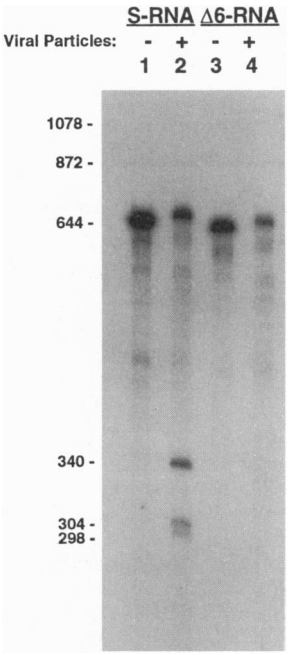

FIG. 2. Cleavage assays of native LRV1-4 particles on S-RNA and Δ6-RNA transcripts. Substrate RNA transcript S-RNA (lanes 1 and 2) and deletion-mutant RNA transcript Δ6-RNA (lanes 3 and 4) were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of purified native LRV1-4 particles in *in vitro* cleavage assays. Reaction products were resolved on a denaturing 4% polyacrylamide/7.5 M urea gel and visualized by autoradiography. Molecular size markers are indicated (in nt) at left.

cleavage products of 340 nt and 304 nt. The 298-nt band is believed to be a degradation product of the 340-nt cleavage product. When Δ6-RNA was incubated under identical conditions, this RNA was not cleaved in the presence of viral particles, indicating that cleavage requires the deleted sequence (Fig. 2, lanes 3 and 4). This result does not preclude the participation of other signals. In addition to a specific cleavage site consensus sequence, other cis-acting signals, distance limitations from the 5′ end, secondary structure and/or tertiary structure of the viral RNA molecule may be required to facilitate efficient targeting of cleavage.

Endonuclease Activity of Baculovirus-Expressed LRV Capsid Protein. The endonuclease responsible for the observed cleavage activity could have been of host or viral origin. The viral particles used in both the polymerase and cleavage assays were purified on sucrose gradients over which cell extracts of virally infected *Leishmania* were fractionated. Though the peak fraction of virus is dense with viral particles, the possibility that a cosedimenting or virally incorporated host cellular factor was responsible for the endonuclease activity had not been ruled out.

To determine whether the endonuclease activity was of host or viral origin, recombinant-expressed viral capsid protein was tested in the cleavage assay (Fig. 3). Virus-like particles were produced in *Spodoptera frugiperda* insect cells infected with recombinant baculovirus expressing the LRV1-4 capsid protein and purified on a sucrose gradient. When S-RNA was incubated in the presence of the baculovirus-expressed capsid particles, the RNA transcripts were specifically cleaved. The RNA cleavage products were identical to those produced upon incubation of S-RNA with native viral particles (Fig. 3, lanes 2 and 3), indicating that the capsid protein alone was the responsible endonuclease. S-RNA was not cleaved by sucrose gradient fractions from a wild-type baculovirus-infected cell extract (Fig. 3, lane 4). Therefore, other factors in the baculovirus-infected cells were not mediating the cleavage event. A negative control RNA, the human immunodeficiency virus type 1 Rev transcript, was not cleaved in any instance, showing the specificity of the cleavage reaction for the LRV RNA sequence. The possibility remains that an endoribonuclease conserved between *Leishmania* and *Spodoptera* is cosedimenting with the virus-like particles on sucrose gradients. However, given the extraordinary divergence between a protozoan parasite and an insect cell, this explanation is unlikely.

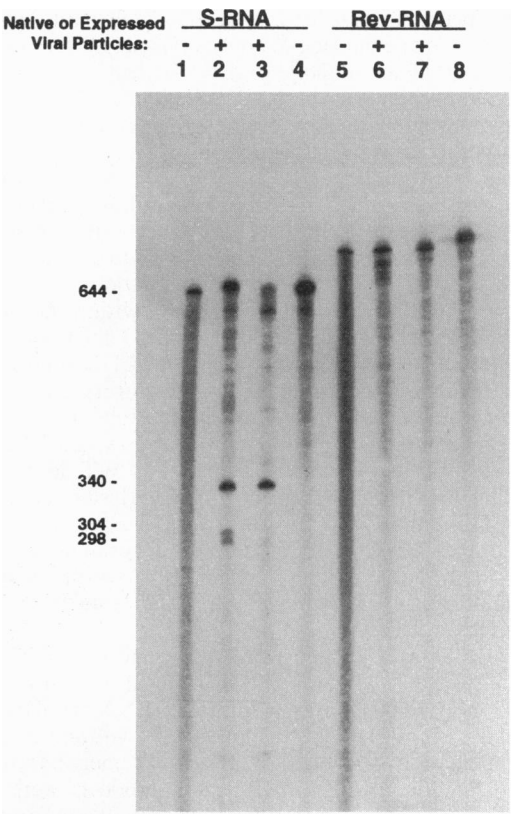

FIG. 3. Cleavage assays of native and baculovirus-expressed LRV1-4 viral particles. Substrate RNA transcript S-RNA (lanes 1–4) and a negative-control RNA transcript Rev RNA (lanes 5–8) were incubated alone (lanes 1 and 5) or in the presence of sucrose-gradient fractions containing native LRV1-4 viral particles (lanes 2 and 6), recombinant baculovirus-expressed LRV1-4 virus-like particles (lanes 3 and 7), or wild-type baculovirus-infected cell extract (lanes 4 and 8) in *in vitro* cleavage assays. Reaction products were resolved on a denaturing 4% polyacrylamide/7.5 M urea gel and visualized by autoradiography. Molecular size markers are indicated (in nt) at left.

Species-Specificity of LRV Capsid Endonuclease Activities. Native LRV1-4, LRV1-1, and LRV2-1 particles were tested in a cleavage assay using the LRV1-4-derived RNA substrate, S-RNA (Fig. 4). As before, the S-RNA was cleaved at a specific site by LRV1-4 particles (Fig. 4, lane 2). LRV1-1 particles cleaved the S-RNA into identical cleavage products to that generated by LRV1-4 particles (Fig. 4, lane 3), demonstrating that LRV1-1 can recognize and cleave the identical RNA sequence as the LRV1-4 particles. In contrast, LRV2-1 particles did not cleave the S-RNA (Fig. 4, lane 4), suggesting that this divergent virus has an alternate substrate-specificity for cleavage.

DISCUSSION

Because many RNA endonuclease activities have been reported in *Leishmania*, we were surprised to find that a viral protein was responsible for cleavage of LRV RNA. RNA editing is a feature of RNA processing in trypanosomatids, including *Leishmania*, and a mitochondrial endoribonuclease activity that may be involved in a cleavage mechanism for RNA editing has been identified (15). Trans-splicing of the spliced leader sequence onto the 5′ end of trypanosomatid mRNAs also requires RNA cleavage, although the mechanism for trans-splicing is unknown (16). Other nonspecific ribonuclease activities have also been identified in *Leishmania* (17, 18).

The identification of the LRV capsid protein as an RNA endonuclease is unprecedented among known viral capsid proteins. Capsid proteins serve a variety of functions to ensure

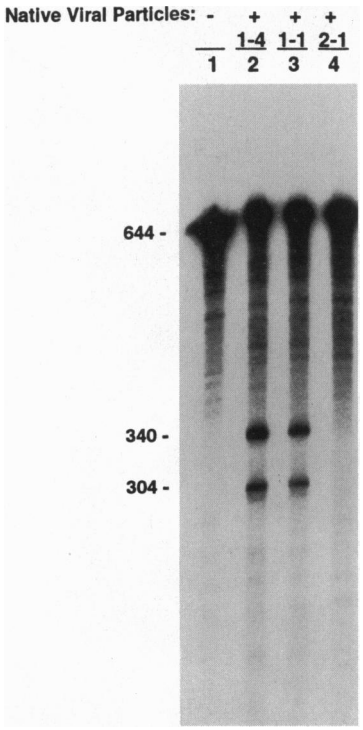

FIG. 4. Cleavage assays of three *Leishmaniavirus* isolates on an LRV1-4 derived RNA substrate. Substrate RNA transcript S-RNA was incubated alone (lane 1) or in the presence of purified native LRV1-4 (lane 2), LRV1-1 (lane 3), or LRV2-1 (lane 4) particles in *in vitro* cleavage assays. Reaction products were resolved on a denaturing 4% polyacrylamide/7.5 M urea gel and visualized by autoradiography. Molecular size markers are indicated (in nt) at left.

the successful propagation of viral genomes. In addition to protecting viral genomes from nucleases in the intracellular and extracellular environment, capsid proteins can package genomes, recognize receptors, and serve as proteases. The capsid protein of LRV is a single unit protomer of 82 kDa. Mutational analysis of the capsid protein of LRV1-4 revealed that carboxyl-terminal deletions of up to 115 amino acids did not affect the ability of the protein to assemble into virus-like particles (19). These deletion mutations do not disrupt the endonuclease activity of the capsid protein (K.J.M. and J.L.P., unpublished data). Consistent with these findings, the carboxyl-terminus of the capsid protein is believed to be located internally to the capsid (19), whereas the endonuclease activity is believed to be externally located due to its sensitivity to mild proteinase K treatment (12).

The exact functional role in viral replication for the LRV capsid endonuclease activity remains to be determined. Infection of *Leishmania* cells by LRV is believed to be persistent, as no extracellular virus has been observed (5). One model proposes that cleaving the 5′ end from positive-sense, genome-length viral transcripts could serve to create a subset of viral RNAs that are translation and replication incompetent. We have previously shown that the cleavage activity of viral particles can be specifically abolished by treatment with EGTA or by CsCl gradient purification (12). These conditions are known to disrupt assembled LRV capsids, and therefore an assembled capsid structure may be important for the endonuclease activity. Obviously a virus cannot destroy all of its functional mRNA and continue to replicate. Our model predicts that at low virus densities (i.e., during logarithm-phase growth of the *Leishmania*) a portion of genome-length transcripts will avoid cleavage, be translated, and subsequently be encapsidated. As the density of virus in the cell accumulates (i.e., during stationary-phase of the *Leishmania*), all viral transcripts will encounter a fully assembled capsid and be cleaved. Such a suicide mechanism would serve to tightly regulate the number of virions per cell at a low level, thereby maintaining a persistent infection.

Alternative roles for the endonuclease activity exist. Cleavage of viral transcripts could serve to generate functional mRNAs by removing potential translation-attenuating domains present at the 5′ end of full-length RNAs. Also, the cytoplasmic subcellular localization of LRV (8, 20) suggests that both viral and cellular RNAs present in the cytoplasm could be accessible to the capsid endonuclease activity. Differences in phenotypes between uninfected and virally infected *Leishmania* could reflect differences in expression of cleavage-susceptible mRNAs.

Although sequence-specific RNA cleavage can be catalyzed by ribozymes, no endoribonucleases are known to be sequence-specific. If the capsid endoribonuclease proves to be sequence-specific, the potential for developing an RNA restriction enzyme is an intriguing possibility. The fact that the LRV2-1 endonuclease does not function on the LRV1-4 sequence suggests that multiple endonucleases with various specificities may exist within the *Leishmaniavirus* genus. Protozoan parasites other than *Leishmania* are also infected by dsRNA viruses. These parasites include *Entamoeba histolytica, Giardia lamblia*, and *Trichomonas vaginalis* (21–23). It remains to be determined whether other viruses in this family possess capsid-encoded endonuclease activities.

Finally, advances in gene therapy and successes with ribozyme molecules as antivirals lead to the potential for developing a sequence-specific RNA endonuclease as a therapeutic agent against pathogens.

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