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Cleavage Site Mapping and Substrate-Specificity of *Leishmaniavirus* 2-1 Capsid Endoribonuclease Activity[1]

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The *Leishmaniavirus* capsid protein possesses an RNA endoribonuclease activity that cleaves viral positive-sense RNA at a specific, single site within the 5′ untranslated region. The site of cleavage in LRV1-4 RNA was previously mapped to nucleotide 320 of the LRV1-4 genome. Here we show that an LRV2-1-derived substrate RNA transcript is also cleaved at a single site in an *in vitro* cleavage assay with LRV2-1 virions. Precise RNA cleavage site mapping in this divergent Old World virus, LRV2-1, confirms that cleavage is occurring within a region of homology to the LRV1 isolates. Substrate RNA transcripts possessing viral sequences from LRV1-4 or LRV2-1 genomes were assayed for susceptibility to cleavage by the cognate and noncognate capsid endoribonucleases to determine the level of substrate specificity.

Key words: capsid protein, cleavage site mapping, *Leishmania* RNA virus, site-specific RNA endonuclease activity, substrate specificity.

Leishmaniaviruses (LRV) are members of the family *Totiviridae* that persistently infect some strains of the protozoan parasite *Leishmania* (*15*). LRV have been identified in 12 isolates of the New World species *L. guyanensis* and *L. braziliensis*, and one strain of the Old World species *L. major* (*3, 9*). Full-length cDNA clones have been generated for two New World isolates, LRV1-1 and LRV1-4, and one Old World isolate, LRV2-1 (*18-20*). The LRV genome is approximately 5.3 kbp of double-stranded RNA (dsRNA), and two large open reading frames (ORF2 and ORF3) are present in all isolates. Short ORFs are present at the 5′ terminus of all LRV genomes, but these ORFs are not believed to encode proteins, as they are not conserved among isolates. When ORF2 is expressed by recombinant baculovirus in *Spodoptera frugiperda* cells, the expressed protein self-assembles into virus-like particles of identical morphology to native virions, demonstrating that ORF2 encodes the major capsid protein (*4*). ORF3 possesses motifs characteristic of viral RNA-dependent RNA polymerases, and purified LRV virions possess both transcriptase and replicase activities that generate positive and negative-sense genome-length RNAs, respectively (*24, 25*).

In addition to full-length RNAs, a short viral RNA is also detected in an *in vitro* polymerase assay and *in vivo* (*6*). The short transcript is generated by endoribonucleolytic cleavage at a single site in the 5′ untranslated region of viral RNA (*11*). Recombinant-baculovirus-expressed viral capsid protein was shown to cleave substrate viral RNA in an *in vitro* cleavage assay, thereby identifying the viral capsid protein as the endoribonuclease that mediates this cleavage event (*12*). Short RNA transcripts are generated in polymerase assays of all LRV isolates tested, suggesting that the endoribonuclease activity is conserved in the entire LRV genus (*12*). The cleavage site in LRV1-4 RNA was previously mapped to nucleotide 320 of the viral genome by primer extension to the 5′ terminus of the downstream cleavage product (*11*). Although the sequence of Old World virus LRV2-1 is significantly divergent from the sequences of the New World LRV1 isolates (<50% homology), a sequence in the 5′UTR of LRV2-1 was found to be homologous to the cleavage site sequence in LRV1-4 RNA (*12, 19*). To determine if cleavage of LRV2-1 RNA occurs within the region of sequence homology to LRV1 isolates, we have accurately mapped the cleavage sites of LRV1-4 and LRV2-1 RNAs by sizing the 5′ cleavage products generated in an *in vitro* cleavage assay.

Previously, we demonstrated that an LRV1-4-derived RNA substrate was susceptible to cleavage by LRV1 capsid endoribonuclease, but it appeared resistant to cleavage by the divergent LRV2-1 capsid endoribonuclease (*12*). Here we present a more thorough analysis of the cleavage activity of both LRV1-4 and LRV2-1 capsid endoribonucleases on both cognate and noncognate RNA substrates, in order to quantitatively compare the cleavage efficiencies of each endoribonuclease on different substrates. A possible functional role for RNA cleavage in affecting the translational efficiency of LRV RNA is discussed in analogy to site-specific endoribonucleolytic cleavages in the 5′UTRs of other viral mRNAs.

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MATERIALS AND METHODS

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

Virus Purification—Native *Leishmaniavirus* virions were purified as previously described (*6*). Briefly, *Leishmania* promastigotes ($\sim 10^{10}$ cells) were harvested in early stationary phase, washed, and lysed in 1% Triton X-100. Cell lysates were fractionated on 10 to 40% sucrose gradients, and the fractions containing the peak of viral dsRNA were used in cleavage assays.

Virus-like particles were produced using recombinant-baculovirus expressing the LRV1-4 capsid protein, as previously described (*12*). LRV1-4 native virions and virus-like particles are interchangeable in the *in vitro* cleavage assay, as both particles generate identical cleavage products (*12*).

cDNA Cloning—The cloning of the LRV1-4 5′ untranslated region (UTR) cDNA into a transcription vector, designated WT, has been described (*11*). An LRV2-1 5′ UTR cDNA was generated by reverse transcription of total nucleic acid from late-log stage 5ASKH promastigotes, using Moloney murine leukemia virus reverse transcriptase (Boehringer Mannheim) and primer 2-24 (5′-CGAACCTGTAATCTCGACC-3′), according to the manufacturer's instructions. The LRV2-1 cDNA was amplified by PCR, using *Taq* DNA polymerase (Boehringer Mannheim) and the synthetic oligonucleotide primers 2-8b (5′-GTGTGCCCGGCTTACATGTGACG-3′) and 2-5′end (5′-CCGCGGAATTCAAACCCAATC-3′), according to the manufacturer's directions. The PCR product, corresponding to LRV2-1 nucleotides 1-547, was ligated into transcription vector pCRII (Invitrogen), according to the manufacturer's instructions, and designated 2-1:5′.

Substrate RNA Transcript Synthesis—WT DNA linearized with *Pvu*II [New England Biolabs (NEB)] and 2-1:5′ DNA linearized with *Hinc*II (NEB) were used as templates for *in vitro* transcription, using T3 RNA polymerase (Promega) and T7 RNA polymerase (*14*), respectively. RNA transcribed from the LRV1-4 cDNA is referred to as RNA-1, and RNA transcribed from the LRV2-1 cDNA is referred to as RNA-2. RNA-1 is a 643 nt RNA, possessing 447 nt of LRV1-4 sequence, 20 nonviral 5′ nt and 176 nonviral 3′ nt. RNA-2 is a 471 nt RNA, possessing 395 nt of LRV2-1 sequence and 76 nonviral 5′ nt.

Transcription reactions (1-2 ml) for unlabeled RNAs contained 40 mM Tris-base (pH 7.5), 20 mM $MgCl_2$, 1 mM spermidine, 30 mM dithiothreitol (DTT), 2 mM each NTP, 0.25 unit/μl RNasin (Promega), and 15-30 nM linearized DNA template. Incubation was at 37°C for 1-2 h. Template DNA was removed by RQ1-DNase (Promega) treatment for an additional 10 min. RNAs were phenol-chloroform extracted, ethanol precipitated, resuspended in water and 0.25 volume loading buffer [44% deionized formamide, 5.5 mM EDTA (pH 8), 5 mM urea, 0.03% xylene cyanol, 0.03% bromphenol blue], heat denatured at 90°C for 3 min, and resolved on 1.5 mm thick 6% polyacrylamide-8.3 M urea gels. Resolved RNAs were located by UV-shadowing (*8*). Bands were excised, crushed into elution buffer (2 M ammonium acetate, 1% sodium dodecyl sulfate) and incubated at 37°C 1 h, room temperature 10-14 h (on rotary shaker), and again at 37°C 1 h. Eluates were filtered through prewetted 0.45 μm filters (Millipore) and phenol-chloroform extracted. RNAs were ethanol precipitated, resuspended in water, and quantitated by absorption spectroscopy.

Transcription reactions for internally labeled RNA-1 were performed as above, except that 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 50 μM UTP, and 1.25 μM [α-^{32}P]-UTP (800 Ci/mmol) [New England Nuclear (NEN)] were used, and RNA was purified from 0.4 mm thick 5% polyacrylamide-8.3 M urea gels.

End-Labeling of RNAs—Gel-purified RNAs were treated with calf intestinal phosphatase (NEB) to remove 5′-terminal phosphates. Phosphatase reactions contained 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT (pH 7.9), and 1-13 μM RNA. Incubation was at 50°C for 1 h, followed by 30 min at room temperature. The enzyme was inactivated by incubation at 75°C for 10 min in the presence of 50 mM EDTA. Dephosphorylated RNAs were recovered by phenol-chloroform extraction and ethanol precipitation.

RNAs were 5′ end-labeled with T4 polynucleotide kinase (NEB) and [γ-^{32}P]ATP (NEN). Kinase reactions contained 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.6 μM [γ-^{32}P]ATP (6,000 Ci/mmol), and 1-15 μM RNA. Incubation was for 2 h at 37°C. Phenol-chloroform extracted RNAs were fractionated on 0.4 mm thick 5% polyacrylamide-8.3 M urea gels and purified, as described above for the unlabeled RNAs.

Cleavage Assays—For the cleavage kinetics and RNA-2 cleavage site mapping experiments, cleavage assays were performed in reactions containing 3.8 nM 1-4 or 2-1 substrate RNA (77 fmol and 4-19$\times 10^4$ cpm), 4 units/μl RNasin, and native LRV1-4 or LRV2-1 sucrose gradient-purified virions at 50% volume [contributes final concentration of 5 mM Tris-base (pH 7.5), 2.5 mM $MgCl_2$, 50 mM NaCl, 19% sucrose, and approximately 16 ng/μl total protein]. Incubation was at 37°C for 0-40 min. At 0, 2, 5, 10, 15, 30, and 40 min, an aliquot of each reaction was placed on ice to terminate the reaction. Reaction products were phenol-chloroform extracted, mixed with loading buffer, heat denatured at 90°C for 3 min, and equal cpm of each reaction were resolved on 4% polyacrylamide-8.3 M urea gels. Known amounts of each substrate RNA were loaded on all gels to serve as RNA standards for determining moles of product formed. Substrate and product bands were quantitated using a Molecular Dynamics phosphorimaging system. Product accumulation was quantitated by setting background counts to zero for the position of product migration at the zero min time point. Assays were performed in duplicate or triplicate.

For the RNA-1 cleavage site mapping and turnover experiment, recombinant-baculovirus-expressed LRV1-4 capsid protein was used in each cleavage assay at an approximate concentration of 10 nM. Optimal reaction conditions for the expressed capsid were 5 mM Tris-base (pH 7.5), 8 mM $MgCl_2$, 0.25 mg/ml yeast tRNA, and 1 unit/μl RNasin. For the turnover experiment, capsid endoribonuclease was preincubated in two separate tubes with 0 or 15 nM of unlabeled substrate RNA-1 at 37°C for

15 min. Following the preincubation, 40 nM of internally radiolabeled substrate RNA-1 was added to each tube and the reaction was allowed to proceed at 37°C for an additional 30 min. Aliquots of each reaction were taken, following the addition of radiolabeled RNA, at 0 min (immediately after addition), 15 and 30 min. Reaction products were resolved on a 4% polyacrylamide-8.3 M urea gel and visualized by autoradiography.

Base-Specific Endoribonuclease Digestions—RNA ladders were generated by incubating 5′-end-labeled RNAs in the presence of base-specific ribonucleases under partial digestion conditions. Ribonuclease T1 (G-specific cleavage), ribonuclease Phy M (A and U-specific cleavage), and ribonuclease *B. cereus* (U and C-specific cleavage) were obtained from Pharmacia Biotech and used according to the manufacturer's recommendations. Ribonuclease T1 and Phy M reactions contained 16 mM sodium citrate (pH 5.0), 0.8 mM EDTA, 0.02% xylene cyanol, 0.04% bromphenol blue, 0.7 mg/ml carrier tRNA, 3.5 M urea, and 1–10 pmol ($2\text{-}56\times10^5$) cpm 5′-end-labeled RNA. Ribonuclease T1 was used at 0.005 unit/μl and ribonuclease Phy M was used at 0.1 unit/μl. Incubation was at 50°C for 14 min. Ribonuclease *B. cereus* was used at 0.2 unit/μl and reactions were performed in the same buffer as for ribonucleases T1 and Phy M, except for the exclusion of dyes, which were added following the reaction. Incubation was at 55°C for 25 min. Reaction products were resolved on 10% polyacrylamide-8.3 M urea gels and visualized by autoradiography.

RESULTS

Endoribonuclease Activity of LRV2-1 Virions in a Cleavage Assay—The endoribonuclease activity of the LRV capsid protein has been assayed using an *in vitro* system with purified viral particles and *in vitro*-synthesized substrate RNA transcripts possessing the viral cleavage site (*11, 12*). In previous studies, the cleavage activity of New World isolate LRV1-4 was analyzed on substrate RNAs possessing LRV1-4 sequences. The complete genome sequence of a newly identified and divergent LRV isolate, LRV2-1, is now available (*19*). In order to determine if the endoribonuclease activity described for the LRV1-4 capsid/substrate system could be reproduced with an LRV2-1 capsid/substrate system, the *in vitro* cleavage assay was performed using native LRV2-1 virions and an LRV2-1-derived substrate RNA.

RNA-2 is a 471 nt RNA possessing the 5′ 395 nt of the LRV2-1 genome (Fig. 1A). 5′-End-labeled RNA-2 was incubated with purified native LRV2-1 virions and cleavage was assessed by resolving reaction products on a denaturing polyacrylamide gel (Fig. 1B). A prominent, single cleavage product of approximately 280 nt in length was generated only when substrate RNA-2 was incubated in the presence (Fig. 1B, lane 2) of viral particles, showing that LRV2-1 virions possess an endoribonuclease activity that is functional in the *in vitro* cleavage assay.

Cleavage Site Mapping in Two Divergent LRV Isolates—To accurately map the position of cleavage in LRV1-4 and LRV2-1 RNAs, cleavage products were sized by comparison to RNA ladders generated by base-specific ribonuclease digestion of the same RNAs. Substrate RNAs possessing the 5′ untranslated sequences of each viral RNA were 5′ end-labeled and cleaved *in vitro* with the cognate capsid endoribonuclease to generate cleavage products. RNA-1 is a 643 nt RNA possessing the 5′ 447 nt of the LRV1-4 genome (Fig. 1A), and RNA-2 was described above.

The RNA ladders generated by base-specific ribonuclease (RNase) cleavage of the substrate RNAs showed the expected pattern of nucleotide sequences, indicating that the proper substrates had been gel-purified. The 5′ cleavage product of RNA-1 migrated between RNAs generated by RNase Phy M and T1 cleavage of RNA-1 at nucleotides 319 and 322, respectively, and comigrated with an RNA generated by RNase *B. cereus* cleavage of RNA-1 at nucleotide 320 (Fig. 2A). Therefore, the cleavage site in RNA-1 maps to LRV1-4 viral nucleotide 320. Cleavage at this site is consistent with a previous mapping study that identified the same position using primer extension to determine the 5′ end of the downstream cleavage product (*11*).

The 5′ cleavage product of RNA-2 migrated between RNAs generated by RNase T1 cleavage of RNA-2 at nucleotides 281 and 286, and comigrated with an RNA generated by RNase Phy M cleavage of RNA-2 at nucleotide 283 (Fig. 2B). Therefore, the cleavage site in RNA-2 maps to LRV2-1 viral nucleotide 283. Cleavage at this site in LRV2-1 is within the region predicted on the basis of homology to LRV1 isolates, although the exact site of cleavage within the conserved sequence is shifted by one nucleotide (Fig. 2C) (*12*). Possible reasons for this one nucleotide discrepancy are considered in "DISCUSSION."

Endoribonuclease Turnover—The results of initial experiments to characterize the kinetics of the cleavage reaction suggested that the endoribonuclease was not rapidly turning over to process multiple substrates (data not shown). To test for rapid turnover, the capsid endoribonuclease was incubated in the absence or presence of

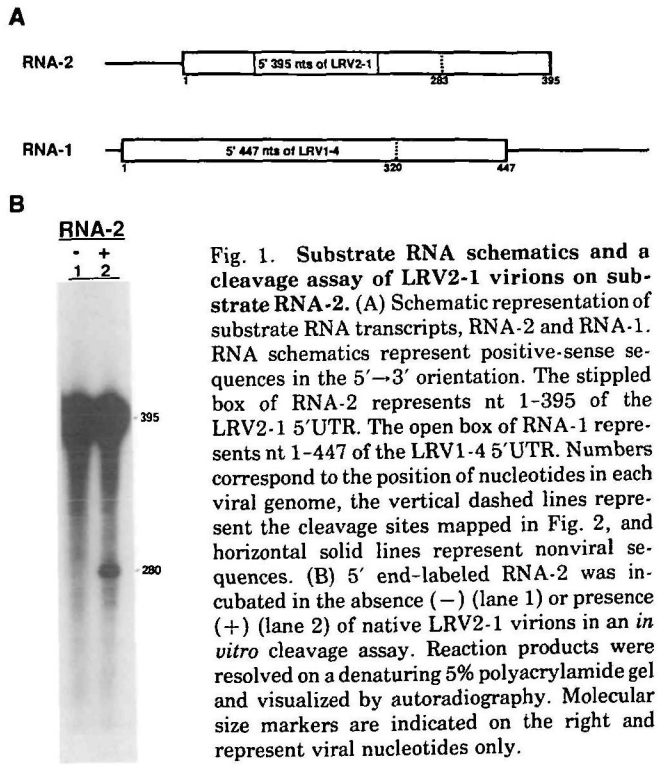

Fig. 1. **Substrate RNA schematics and a cleavage assay of LRV2-1 virions on substrate RNA-2.** (A) Schematic representation of substrate RNA transcripts, RNA-2 and RNA-1. RNA schematics represent positive-sense sequences in the 5′→3′ orientation. The stippled box of RNA-2 represents nt 1-395 of the LRV2-1 5′UTR. The open box of RNA-1 represents nt 1-447 of the LRV1-4 5′UTR. Numbers correspond to the position of nucleotides in each viral genome, the vertical dashed lines represent the cleavage sites mapped in Fig. 2, and horizontal solid lines represent nonviral sequences. (B) 5′ end-labeled RNA-2 was incubated in the absence (−) (lane 1) or presence (+) (lane 2) of native LRV2-1 virions in an *in vitro* cleavage assay. Reaction products were resolved on a denaturing 5% polyacrylamide gel and visualized by autoradiography. Molecular size markers are indicated on the right and represent viral nucleotides only.

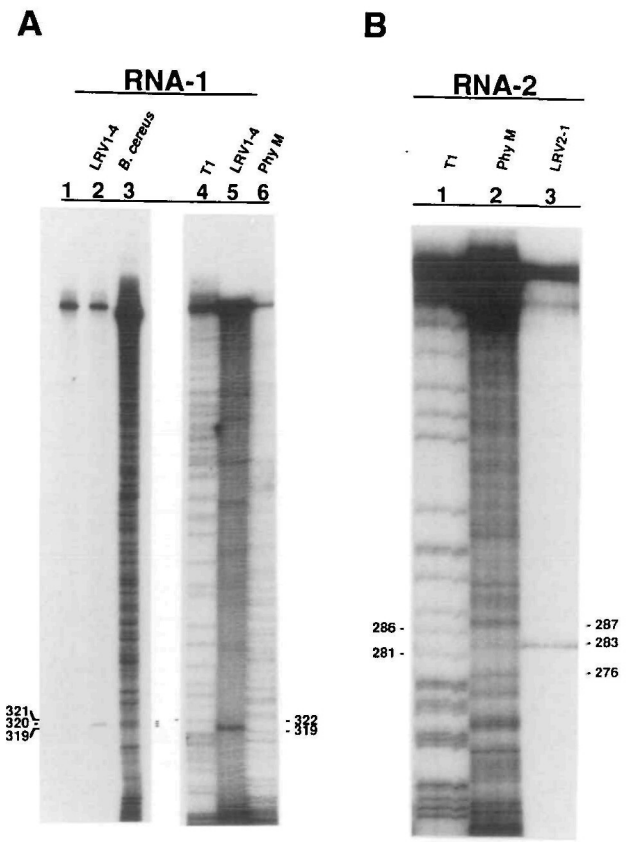

Fig. 2. **Sizing of RNA-1 and RNA-2 5′ cleavage products.** (A) 5′ end-labeled RNA-1 was incubated alone (lane 1) or in the presence of the LRV1-4 capsid endoribonuclease (lanes 2 and 5), ribonuclease *B. cereus* (lane 3), ribonuclease T1 (lane 4), or ribonuclease Phy M (lane 6). (B) 5′ end-labeled RNA-2 was incubated in the presence of ribonuclease T1 (lane 1), ribonuclease Phy M (lane 2), or the LRV2-1 capsid endoribonuclease (lane 3). Cleavage products were resolved on denaturing 10% polyacrylamide gels and visualized by autoradiography. Molecular sizes represent viral nucleotides only. Hatch marks in part A indicate the position of bands not adjacent to their size labels. (C) Nucleotide sequences surrounding the cleavage sites mapped for RNA-1 and RNA-2. Arrows mark the site of cleavage, 3′ to the indicated nucleotide. A region of sequence homology between LRV1-4 and LRV2-1 RNAs is boxed.

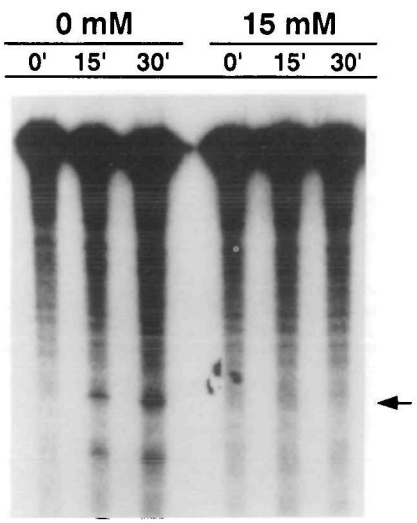

Fig. 3. **Lack of rapid enzyme turnover in the cleavage assay.** Following a 15 min preincubation of approximately 10 nM baculovirus-expressed LRV1-4 capsid protein alone (0 mM), or in the presence of 15 nM unlabeled RNA-1 (15 nM), 40 nM internally radiolabeled substrate RNA-1 was added and incubated for an additional 30 min. Reaction products taken at 0, 15, and 30 min incubation time were resolved on a denaturing 4% polyacrylamide gel and visualized by autoradiography. The position of the 5′ cleavage product is marked by an arrow.

unlabeled substrate RNA, prior to the addition of excess radiolabeled RNA (Fig. 3). Cleavage products were apparent after 15 and 30 min incubation only when the endoribonuclease was preincubated in the absence of unlabeled RNA, showing that the unlabeled RNA was preventing the radiolabeled RNA from being processed. Multiple enzyme turnovers would have been evident, since the radiolabeled RNA was added at greater than 2.5-fold excess of unlabeled RNA, and the reaction was allowed to proceed for twice the preincubation time. Therefore, endoribonuclease turnover is slow or nonexistent in the *in vitro* conditions used for these experiments. Lack of rapid turnover could arise due to a slow rate of catalysis and/or tight/irreversible product association with the enzyme.

Substrate-Specificity of the LRV2-1 Capsid Endoribonuclease Activity—The cleavage site mapping experiments revealed that RNA cleavage in each of the divergent viral isolates occurs within a short region of sequence homology between the LRV2 and LRV1 RNAs. To compare the ability of the LRV2 and LRV1 capsid endoribonucleases to recognize and cleave the RNA of the divergent viral isolates, cleavage assays were performed with LRV2-1 and LRV1-4 virions on equimolar amounts of both cognate RNAs, RNA-2 and RNA-1, respectively, and noncognate RNAs, RNA-1 and RNA-2, respectively. Steady-state kinetics do not apply to the cleavage assay conditions used in these experiments, due to the lack of rapid enzyme turnover, and therefore defined catalytic efficiencies were not determined. The efficiency of endoribonuclease cleavage on different substrates was compared as a function of cleavage product accumulation, without consideration to differences in K_{cat} and K_m values. A representative autoradiogram of the kinetics of cleavage product accumulation is shown in Fig. 4A. The band intensities of RNA-1 and RNA-2 in Fig. 4A are not representative of the actual molar

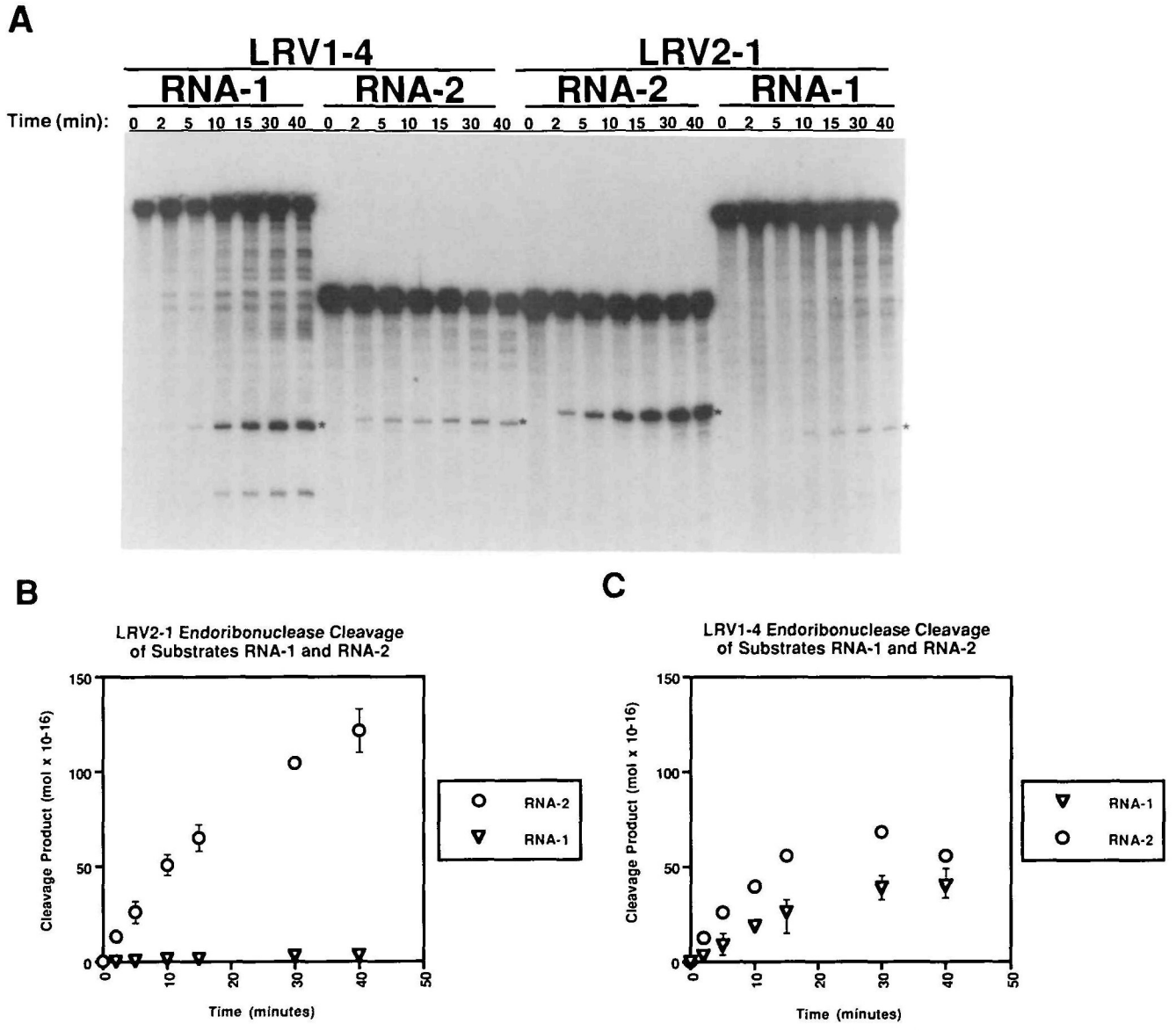

Fig. 4. **Kinetics of cleavage product accumulation using RNA-1 and RNA-2 substrates with LRV1-4 and LRV2-1 capsid endoribonucleases.** (A) A representative autoradiogram of cleavage product accumulation using cognate and noncognate enzyme/substrate pairs. Equimolar amounts of substrate RNA transcripts RNA-1 and RNA-2 were incubated in the presence of native LRV1-4 or LRV2-1 virions. Reaction products at times from 0-40 min were resolved on a denaturing 4% polyacrylamide gel, with equal cpm loaded in each lane for visualization purposes. The specific activity of RNA-1 was 1/5 the specific activity of RNA-2. Asterisks indicate the specific cleavage product quantitated for parts B and C. (B) Quantitation of cleavage product accumulation for LRV2-1 capsid endoribonuclease cleavage of substrates RNA-1 and RNA-2. The amount of cleavage product per reaction at 0, 2, 5, 10, 15, 30, and 40 min was assessed by phosphorimage quantitation. Average values for moles of product per reaction are plotted as a function of time, with the range of values from duplicate or triplicate experiments represented by error bars. (C) Quantitation of cleavage product accumulation for LRV1-4 capsid endoribonuclease cleavage of substrates RNA-1 and RNA-2. See part B for plot description.

amounts of each product, as a result of a 5-fold difference in the specific-activities of RNA-1 and RNA-2. The actual moles of product formation are shown in Fig. 4, B and C.

Cleavage product accumulation was quantitated for cleavage of RNA-2 and RNA-1 by both LRV2-1 and LRV1-4 endoribonucleases (Fig. 4, B and C). Although other bands accumulate to some extent, only the predominant cleavage product was quantitated. Many of the higher molecular weight bands are believed to result from nonspecific degradation because these bands continue to accumulate in the presence of $ZnCl_2$, a specific inhibitor of the capsid endoribonuclease activity (data not shown). In these experiments, the LRV2-1 capsid endoribonuclease converted 14% of cognate substrate RNA-2 to product after 30 min incubation, while only 0.4% of noncognate substrate RNA-1 was converted to product at this time (Fig. 4B). Therefore, the LRV2-1 capsid endoribonuclease preferentially cleaved the LRV2 RNA sequence with 35-fold better efficiency than the divergent LRV1 RNA sequence.

The RNA-1 cleavage product generated by the LRV2-1 endoribonuclease was identical to the cleavage product generated by the LRV1-4 endoribonuclease, indicating that

the LRV2-1 endoribonuclease cleaved the RNA-1 sequence at the wild type LRV1-4 cleavage site. When both RNA-1 and RNA-2 were simultaneously incubated with the LRV2-1 endoribonuclease, the RNA-2 cleavage product accumulated at a rate similar to the rate observed in the absence of noncognate RNA-1 (data not shown), arguing against the possibility that the noncognate RNA preparation contained inhibitory factors that could account for the diminished cleavage efficiency of the noncognate enzyme/substrate pair in isolation.

The LRV1-4 endoribonuclease converted 5% of cognate substrate RNA-1 to product after 30 min incubation, and 9% of noncognate substrate RNA-2 was converted to product at this time (Fig. 4C). Therefore, the LRV1-4 endoribonuclease displayed no significant substrate specificity, cleaving the LRV1 RNA sequence with approximately equal or lower efficiency to the divergent LRV2 RNA sequence. Interestingly, the RNA-2 cleavage product generated by the LRV1-4 endoribonuclease was approximately 10 nt smaller than the RNA-2 cleavage product generated by the LRV2-1 endoribonuclease, indicating that the LRV1-4 endoribonuclease cleaves RNA-2 at a site slightly 5′ to the wild type cleavage site utilized by the cognate LRV2-1 endoribonuclease. Therefore, although the efficiency of cleavage with the noncognate enzyme/substrate pair was similar to that of the cognate pair, the noncognate pair displayed a difference in cleavage site specificity. Cleavage efficiencies of the LRV1-4 and LRV2-1 endoribonucleases on their respective cognate RNAs cannot be directly compared as the amounts of capsid protein used were not equal.

DISCUSSION

A substrate RNA possessing the 5′ terminal 395 nt of LRV2-1 positive-sense RNA is cleaved at a single site *in vitro* by an endoribonuclease activity of purified native LRV2-1 virions. Therefore, as shown in the LRV1-4 system, all of the determinants that target the accurate processing of viral RNA are present in the 5′UTR of LRV2-1 RNA.

Although the sequences of LRV1 and LRV2 genomes are highly divergent, we had previously recognized a short region of homology around the predicted sites of endoribonucleolytic cleavage within the 5′UTR of each viral RNA. Sequence conservation in this region suggested that RNA cleavage may occur sequence specifically within a putative consensus sequence. Precise mapping of the cleavage sites in LRV2-1 and LRV1-4 RNAs has confirmed our prediction that cleavage occurs within the region of sequence homology between these divergent viral isolates. The cleavage site in LRV1-4 RNA is after viral nucleotide 320, between the two cytosine residues of the sequence 5′ GAUC*CGAA3′. The cleavage site in LRV2-1 RNA is after viral nucleotide 283, immediately 5′ of the two cytosine residues of the sequence 5′GCU*CCGUA3′. Although the cleavage sites in these two viral RNAs did not map to exactly the same position within the consensus sequence 5′ GxUCCGxA3′, the sites mapped to within one nucleotide of each other. This one nucleotide discrepancy may represent a true difference in the site of cleavage for each virus, or may be attributed to an experimental limitation. Cleavage at nucleotide 284 of LRV2-1 could be mistaken for cleavage at nucleotide 283 if a substrate transcript one nucleotide short at the 5′ end was labeled and purified, or if a subsequent cleavage of one additional 3′ nucleotide followed an initial cleavage at nucleotide 284. Furthermore, a one nucleotide discrepancy might be accounted for by subtle variations in the running of marker and experimental lanes on the gel. Regardless, it is clear that the cleavage site in these two viral RNAs is within the region of conserved sequence between LRV1 and LRV2 species. We have previously shown that endoribonucleolytic processing does not occur on an RNA with a six nucleotide deletion that removes this conserved region (*12*). The conservation in divergent viral isolates and functional requirement of the 5′ GxUCCGxA3′ sequence suggests that this element is an important component of the determinant for targeting the specific endoribonucleolytic cleavage in LRV RNAs.

Putative consensus sequences for RNA cleavage have been proposed for a number of endoribonucleases as a result of the identification of common sequences in multiple substrates of a particular enzyme (*10, 17*). *In vitro* cleavage analyses of various RNA substrates have shown that, in almost all cases, primary nucleotide sequence alone cannot suffice to target cleavage because many canonical sequences are not cleaved, while sequences other than the putative consensus are cleaved. As an example, the mutational analysis of a consensus sequence substrate for an *Escherichia coli* endoribonuclease, RNase III, revealed almost no critical residues for cleavage (*5*). Flexibility in cleavage site sequences suggests that the mechanism by which endoribonucleases cleave specific sites is likely to involve the recognition of complex higher order RNA structures formed through secondary and tertiary configurations of an RNA molecule that may be achieved from divergent RNA sequences. In one example, a 19 nucleotide RNA consensus sequence has been shown to confer cleavage susceptibility to a foreign RNA by an endoribonuclease activity of *Xenopus* oocyte extracts (*2*). The authors report that the small sequence element is unlikely to be structured, suggesting that perhaps primary sequence alone may be able to target some endoribonucleases.

Substrates RNA-1 and RNA-2 were cleaved by the capsid endoribonuclease activities of the noncognate viral species. For RNA-1, the same site of cleavage was utilized by both LRV1-4 and LRV2-1 capsid endoribonucleases, indicating that the sequence/structural determinants of this site were sufficient for the accurate processing by both endoribonucleases. In contrast, the LRV1-4 capsid endoribonuclease cleaved the noncognate substrate, RNA-2, at a site approximately 10 nt 5′ to the site utilized by the cognate enzyme/substrate pair. A subtle variation in the sequence/structural determinant for RNA-2 cleavage apparently permits recognition, albeit altered processing, by the noncognate enzyme. It is possible that differences in reaction requirements for the two capsid endoribonuclease preparations could alter the specificity of cleavage site selection. Previously we reported that the native LRV2-1 particels did not cleave an LRV1-4 derived substrate (RNA 1) (*12*). In this study, however, RNA 1 was cleaved by the native LRV2-1 particles. This discrepancy can be explained by the usage of a more sensitive assay system in the present study. Also it may be explained by the viral preparation from the different batch of cells. The cleavage activity by noncognate enzyme/substrate pair may need a sufficient

amount of expressed capsid endoribonuclease or a more stable enzyme.

The kinetic analyses of product accumulation, using the LRV capsid endoribonucleases on both cognate and noncognate substrates, showed a substrate-specificity for only the LRV2-1 capsid endoribonuclease. The LRV2-1 capsid endoribonuclease preferentially cleaved the substrate RNA possessing LRV2-1 sequence over the substrate RNA possessing LRV1-4 sequence, with 35-fold better efficiency. In contrast, the LRV1-4 capsid endoribonuclease was equally efficient at cleaving both substrates, although, as previously mentioned, a different site of cleavage was utilized in the noncognate substrate. The ability of the LRV1-4 capsid endoribonuclease to cleave the noncognate substrate as efficiently as its cognate substrate suggests that the major determinants of RNA sequence/structure are present in both substrates. The ability of the LRV2-1 capsid endoribonuclease to discriminate between these substrates suggests that the mechanism by which this enzyme either recognizes, or is able to process, its substrate has diverged from that of LRV1-4.

A recent phylogenetic comparison of LRV isolates and their *Leishmania* hosts showed co-evolution of viral and host sequences, suggesting that the ancestral *Leishmania* species was infected with LRV prior to divergence into *L. guyanensis* (LRV1-infected) and *L. major* (LRV2-infected) lineages (*23*). Genetic recombination of LRV is not believed to occur because no extracellular infectious cycle for the virus has been observed, and *Leishmania* reproduction is predominantly asexual in nature (*21, 22*). As a result of these two limitations to genetic transfer between viruses, clonal populations of host cells would independently evolve with clonal populations of viruses. Considering the significant divergence of LRV2 and LRV1 genomes, it is not surprising that differences exist between the endoribonuclease activities of the LRV2 and LRV1 capsids. In addition to weak sequence homology between LRV2 and LRV1 isolates, Western blot analysis using an LRV1-specific antiserum failed to detect any LRV2 proteins, including the predominant capsid antigen (*3*). Despite their divergence, limited regions of amino acid sequence homology do exist between the LRV1 and LRV2 capsid proteins. Mutational analysis of conserved regions may provide information as to the location of functional domains in these capsid proteins.

We have not determined the functional consequence of the cleavage event on the LRV life cycle. Site-specific endoribonucleolytic cleavages in the 5′UTR of viral RNAs have been described in other systems, including RNase III-mediated cleavage of specific early mRNAs expressed by bacteriophage λ and T7 (*7*) and RegB-mediated cleavage of T4-related bacteriophage early mRNAs (*16, 17*). Cleavage in the 5′UTRs of specific bacteriophage λ and T7 mRNAs serves to activate translation of downstream ORFs by removing translation attenuating secondary structures that inhibit ribosome binding at translation initiating sequences (*7*). In contrast, cleavage in the 5′UTR of specific bacteriophage T4 mRNAs serves to inactivate translation by removing sequences required for ribosome binding (*17*). RegB is a virally encoded endoribonuclease. Interestingly, a RegB-specific cleavage site is present in the 5′UTR of its own mRNA, and RegB expression has been shown to be autoregulated as a function of cleavage at this site (*17*).

Endoribonucleolytic cleavage within the 5′UTR of LRV RNA is also mediated by the endoribonuclease encoded by the downstream ORF of the viral message. In analogy to the RegB system, it is tempting to speculate that the persistent nature of LRV infection is autoregulated as a function of a capsid-mediated endoribonucleolytic cleavage, event that serves to affect the translational efficiency of viral RNA. A recent study suggests that the 5′UTR of LRV can function as an internal ribosome entry site for cap-independent translation in *Leishmania* (*13*). Furthermore, a 120 nt deletion, in the region that is removed by cleavage, was shown to reduce translation by 10-fold. In one model, at high capsid protein concentrations, all unencapsidated viral RNA would be functionally inactivated by cleavage. Such a mechanism could serve to tightly control the level of virus at a low level. The conservation of a capsid endoribonuclease activity in divergent LRV isolates, infecting different species of *Leishmania*, suggests that viral RNA cleavage is likely to serve an important role in maintenance of viral persistence.

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REFERENCES

1. Armstrong, T.C. and Patterson, J.L. (1994) Cultivation of *Leishmania braziliensis* in an economical serum-free medium containing human urine. *J. Parasitol.* **80**, 1030-1032
2. Brown, B.D., Zipkin, I.D., and Harland, R.M. (1993) Sequence-specific endonucleolytic cleavage and protection of mRNA in *Xenopus* and *Drosophila*. *Genes Dev.* **7**, 1620-1631
3. Cadd, T.L., Keenan, M.C., and Patterson, J.L. (1993) Detection of *Leishmania* RNA virus 1 proteins. *J. Virol.* **67**, 5647-5650
4. Cadd, T.L. and Patterson, J.L. (1994) Synthesis of viruslike particles by expression of the putative capsid protein of *Leishmania* RNA virus in a recombinant *Baculovirus* expression system. *J. Virol.* **68**, 358-365
5. Chelladurai, B.S., Li, H., and Nicholson, A.W. (1991) A conserved sequence element in ribonuclease III processing signals is not required for accurate *in vitro* enzymatic cleavage. *Nucleic Acids Res.* **19**, 1759-1766
6. Chung, I.K., Armstrong, T.C., and Patterson, J.L. (1994) Identification of a short viral transcript in *Leishmania* RNA virus-infected cells. *Virology* **198**, 552-556
7. Court, D. (1993) RNA processing and degradation by RNase III in *Control of Messenger RNA Stability* (Belasco, J. and Brawerman, G., eds.) pp. 71-116, Academic Press, San Diego
8. Ellington, A. (1992) Purification of oligonucleotides using denaturing polyacrylamide gel electrophoresis in *Short Protocols in Molecular Biology*, 2nd ed. (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds.) pp. 2-38, John Wiley & Sons, New York
9. Guilbride, L., Myler, P.J., and Stuart, K. (1992) Distribution and sequence divergence of LRV1 viruses among different *Leishmania* species. *Mol. Biochem. Parasitol.* **54**, 101-104
10. Krinke, L. and Wulff, D.L. (1990) The cleavage specificity of RNase III. *Nucleic Acids Res.* **18**, 4809-4815
11. MacBeth, K.J. and Patterson, J.L. (1995) The short transcript of *Leishmania* RNA virus is generated by RNA cleavage. *J. Virol.* **69**, 3458-3464
12. MacBeth, K.J. and Patterson, J.L. (1995) Single-site cleavage in the 5′-untranslated region of *Leishmaniavirus* RNA is mediated by the viral capsid protein. *Proc. Natl. Acad. Sci. USA* **92**, 8994-8998
13. Maga, J.A., Widmer, G., and Lebowitz, J.H. (1995) *Leishmania* RNA virus 1-mediated cap-independent translation. *Mol. Cell. Biol.* **15**, 4884-4889
14. McKinney, P.A., Scott, S.W., Swanson, M., Ge, X., and Gehrke, L. (1996) A plant viral coat protein RNA binding consensus

sequence contains a crucial arginine. *EMBO J.* **15**, 5077-5084
15. Patterson, J.L. (1993) The current status of *Leishmania* virus 1. *Parasitol. Today* **9**, 135-136
16. Sanson, B. and Uzan, M. (1993) Dual role of the sequence-specific bacteriophage T4 endoribonuclease RegB. *J. Mol. Biol.* **233**, 429-446
17. Sanson, B. and Uzan, M. (1995) Post-transcriptional controls in bacteriophage T4: Roles of the sequence-specific endoribonuclease RegB. *FEMS Microbiol. Rev.* **17**, 141-150
18. Scheffter, S., Widmer, G., and Patterson, J.L. (1994) Complete sequence of *Leishmania* RNA virus 1-4 and identification of conserved sequences. *Virology* **199**, 479-483
19. Scheffter, S., Ro, Y.T., Chung, I.K., and Patterson, J.L. (1995) The complete sequence of *Leishmania* RNA virus LRV2-1, a virus of an Old World parasite strain. *Virology* **212**, 84-90
20. Stuart, K.D., Weeks, R., Guilbride, L., and Myler, P.J. (1992) Molecular organization of *Leishmania* RNA virus 1. *Proc. Natl. Acad. Sci. USA* **89**, 8596-8600
21. Tibayrenc, M., Kjellberg, F., and Ayala, F.J. (1990) A clonal theory of parasitic protozoa: The population structures of *Entamoeba, Giardia, Leishmania, Naegleria, Plasmodium, Trichomonas,* and *Trypanosoma* and their medical and taxonomical consequences. *Proc. Natl. Acad. Sci. USA* **87**, 2414-2418
22. Tibayrenc, M., Kjellberg, F., Arnaud, J., Oury, B., Breniere, S.F., Darde, M.-L., and Ayala, F.J. (1991) Are eukaryotic microorganisms clonal or sexual? A population genetics vantage. *Proc. Natl. Acad. Sci. USA* **88**, 5129-5133
23. Widmer, G. and Dooley, S. (1995) Phylogenetic analysis of *Leishmania* RNA virus and *Leishmania* suggests ancient virus-parasite association. *Nucleic Acids Res.* **23**, 2300-2304
24. Widmer, G., Keenan, M.C., and Patterson, J.L. (1990) RNA polymerase activity is associated with viral particles isolated from *Leishmania braziliensis* subsp. *guyanensis. J. Virol.* **64**, 3712-3715
25. Widmer, G. and Patterson, J.L. (1991) Genomic structure and RNA polymerase activity in *Leishmania* virus. *J. Virol.* **65**, 4211-4215