LRV1 Viral Particles in *Leishmania guyanensis* Contain Double-Stranded or Single-Stranded RNA

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The 32-nm-diameter spherical viral particles found in the cytoplasm of *Leishmania guyanensis* CUMC1-1A sediment at 130S and have a buoyant density of ~1.4 g/ml in cesium chloride gradients. These particles contain a 5.3-kb double-stranded RNA, while single-stranded RNA that corresponds to the viral positive strand is associated with less-dense particles. These results suggest a conservative and sequential mode of LRV1 viral RNA replication that is exemplified by the ScV L-A virus of yeast.

Few viruses have been described in parasitic protozoa (26). Several double-stranded (ds) DNA viruses have been found in *Entamoeba histolytica* (5, 6), and viruslike particles have been observed by electron microscopy in kinetoplastid flagellates (3, 14, 15, 19). dsRNA viruses have been discovered in *Trichomonas vaginalis* (25), *Giardia lamblia* (4, 24), *Babesia bovis* (12), and *Eimeria* spp. (7, 16). The RNA virus LRV1 was originally found in *Leishmania guyanensis* and called LR1 (20, 23), and related viruses variously called LR2 (22, 23), LBV (28), or LRV1 related (10) have been identified in different isolates of *L. guyanensis* and *Leishmania braziliensis*. The LRV1 viral particle appears spherical or polyhedral by electron microscopy, has a sedimentation coefficient of 130S in sucrose density gradients, and contains a 5.3-kb RNA (23). RNA-dependent RNA polymerase activity has been found in *Leishmania* isolates that contain the viral particles but not in those lacking the virus (29), and the polymerase activity has been shown to copurify with the viral particles (30).

We report here the presence of two classes of LRV1 particles that can be separated on sucrose or cesium chloride (CsCl) density gradients. The denser particles contain dsRNA, while the lighter particles contain single-stranded (ss) RNA. The ssRNA corresponds to the viral positive strand, and this strand is in slight excess in total RNA. The virus could not be transmitted to a drug-resistant *Leishmania* strain by incubation with viral particles. The characteristics of noninfectivity, unsegmented RNA genome, and different particles containing positive-strand or dsRNA are shared by the yeast L-A virus, suggesting a similar mode of viral replication (27).

MATERIALS AND METHODS

Organisms and subcellular fractionation. *L. guyanensis* CUMC1-1A (23) promastigotes were grown at 24°C in Schneider's complete medium supplemented with 15% heat-inactivated fetal calf serum (18). Exponential cells were kept at a density of 5×10^6 to 5×10^7 cells per ml. Stationary-phase cells were grown to a density of 10^8 cells per ml.

Cytoplasm and nuclei were prepared after Triton X-100 treatment as previously described (23).

CsCl density gradient centrifugation. Mid- to late-exponential-phase promastigotes were washed in phosphate-buffered saline; resuspended at 10^{10} cells per ml in TKM buffer (25 mM Tris-HCl, pH 7.5, 150 mM KCl, 5 mM MgCl₂) containing 0.25 M sucrose, 78 U of RNasin (Promega) per ml, 3.5 mM dithiothreitol, 0.1 mg of leupeptin per ml, 10 µg of soybean trypsin inhibitor per ml, and 0.75 mM phenylmethylsulfonyl fluoride; and disrupted by sonication at 0°C (to 90% cell lysis). Cell debris and nuclei were removed by centrifugation once at $2,000 \times g$ for 15 min and twice at $12,000 \times g$ for 20 min. The sonicate (0.45 ml) was mixed with 4.3 ml of 1.41-g/ml CsCl in TKM buffer containing leupeptin and soybean trypsin inhibitor and spun at 35,000 rpm for 48 h in an SW55 rotor (Beckman), and 0.25-ml fractions were collected. Aliquots were treated with 1% (wt/vol) *N*-lauryl sarcosine and 40 µg of proteinase K per ml for 30 min at 65°C and analyzed on a 0.6% agarose-Tris-borate-EDTA gel as described previously (23).

Isolation and analysis of RNA. Total RNA from *L. guyanensis* promastigotes was prepared by the acid guanidinium thiocyanate-phenol-chloroform method (1). RNA was denatured by incubation in 4 M urea–5 mM EDTA–3% glycerol at 100°C for 3 min or in 2.0 M formaldehyde–11.5 M formamide–9 mM sodium phosphate, pH 7.4, at 65°C for 10 min. Denatured or nondenatured RNAs were separated by electrophoresis on 0.6% agarose-Tris-borate-EDTA gels, transferred to Nytran membranes as previously described (23), and UV cross-linked (2).

Hybridization. Riboprobes were prepared from a 388-bp LRV1 cDNA, LP5-30, after *Sma*I or *Sal*I digestion, using T3 or T7 RNA polymerase, respectively, in the presence of [³²P]UTP according to the instructions of the manufacturer (Stratagene). Northern blots of LRV1 RNA were hybridized for 12 to 18 h at 65°C with riboprobes (10^6 dpm/ml) in a mixture containing 50 to 100 µl of 50% (vol/vol) formamide per cm², 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 0.1 mM EDTA, pH 7.0), 1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 150 µg of denatured sheared salmon testis DNA per ml, 200 µg of heparin per ml, and 1% (wt/vol) sodium dodecyl sulfate (SDS). The filters were washed twice for 30 min in 2× SSPE–0.1% SDS and 0.1× SSPE–0.1% SDS at 65°C.

Infectivity trials. Cytoplasm from 3×10^9 *L. guyanensis* CUMC1-1A promastigotes (containing LRV1 particles) was

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added to 10-ml cultures containing 3×10^8 promastigotes from formamycin B-resistant *Leishmania donovani* FBD5 (17), which do not contain LRV1. The cells were cultured in DMEL medium (11) for 4 days, then formamycin B was added to 5 μ M, and the culture was continued for 9 days. RNA was extracted from aliquots (containing 1×10^8 to 2×10^8 parasites) of the cultures at 3-day intervals throughout this period and analyzed for the presence of LRV1 RNA by electrophoresis and hybridization, as described above.

RESULTS

We have previously shown that LRV1 RNA and particles containing this RNA are restricted to the cytoplasm of *L. guyanensis* CUMC1-1A (23). Northern blot analysis using riboprobes representing both strands of a 388-bp LRV1 cDNA clone (LP5-30) showed hybridization of both ss probes with the 5.3-kb LRV1 RNA in the cytoplasm of exponentially growing *L. guyanensis* CUMC1-1A (Fig. 1, lanes 4). Hybridization to the 5.3-kb RNA was reduced or undetectable unless the RNA was fully denatured before transfer to nylon filters (data not shown). Neither probe hybridized with RNA from *Trypanosoma brucei* or *L. braziliensis* M2903, a strain that does not contain LRV1 (data not shown). These results show that both strands of LRV1 RNA are present, since sequence analysis of the cDNA probe indicates no inverted repeat sequences. Neither strand was detected in the nuclear fraction (lanes 1), confirming the cytoplasmic location of the RNA. The absence of the 5.3-kb band in exponentially growing cells (but detection of signal in the well) when the proteinase K-SDS treatment is omitted (lanes 5) suggests that the RNA is present within a viral particle. The particle was disrupted by heating in the presence of SDS or SDS and proteinase K (lanes 3 and 4). It was also disrupted by heating to 65°C for 1 h in the absence of SDS and proteinase K, even in the presence of proteinase inhibitors (lanes 2; the small difference in the mobility of LRV1 RNA in lanes 2 is due to the absence of SDS). This suggests that LRV1 RNA is not covalently bound to protein within the particle. Stationary-phase cells contained more LRV1 RNA than exponential-phase cells (compare lanes 4 and 9). However, the increased RNA content appears to be due to a greater amount of LRV1 RNA not in stable particles in stationary cells, since the 5.3-kb RNA is seen even in the absence of heating and proteinase K-SDS treatment (compare lanes 5 and 10; the difference in mobility is due to the absence of SDS).

While both riboprobes (which are specific for different strands of the cDNA clone) detected the 5.3-kb RNA, the riboprobe prepared using the T3 promoter also detected a smear of hybridization with an apparent size of 1.5 to 3.0 kb (Fig. 1A, lanes 4, 8, and 9). The riboprobe from the other strand of the cDNA clone (prepared with the T7 promoter) did not detect any RNA other than the 5.3-kb species (Fig. 1B), indicating that the faster-migrating RNA species was ss. The faster migration and apparent heterogeneity in size of this RNA run under nondenaturing conditions reflect its ss nature. The absence of the ssRNA in untreated cytoplasm (Fig. 1A, lanes 5 and 10) suggests its presence in viral particles, but the particles containing the ssRNA may be more resistant to disruption than those containing the 5.3-kb dsRNA, since the ssRNA appeared not to be released by heating alone (Fig. 1A, lanes 2 and 7). However, this could also reflect the greater sensitivity of the ssRNA to nuclease activity, especially in the absence of SDS.

Analysis of the RNA under denaturing conditions resolved

FIG. 1. Subcellular location of LRV1 RNAs. Nuclei and cytoplasm from *L. guyanensis* CUMC1-1A were treated as indicated below, the RNAs electrophoresed on a 0.6% nondenaturing agarose gel and hybridized with the T3 (A) or T7 (B) riboprobe of LRV1 cDNA clone LP5-30. Lanes 1, exponential-phase nuclei (4×10^7) treated with 1% SDS and 20 μ g of proteinase K per ml for 1 h at 65°C; lanes 2, exponential-phase cell cytoplasm (20 μ l) heated to 65°C for 1 h in the presence of proteinase inhibitors (10 μ g of soybean trypsin inhibitor per ml, 100 μ g of leupeptin per ml, and 2 mM phenylmethanesulfonyl fluoride); lanes 3, exponential-phase cell cytoplasm treated with 1% SDS for 1 h at 65°C in the presence of proteinase inhibitors; lanes 4, exponential-phase cell cytoplasm treated with 1% SDS and 20 μ g of proteinase K per ml for 1 h at 65°C; lanes 5, exponential-phase cell cytoplasm kept at 0°C in the presence of proteinase inhibitors; lanes 6, stationary-phase nuclei (4×10^7) treated as in lane 1; lanes 7 to 10, stationary-phase cell cytoplasm (20 μ l) treated as in lanes 2 to 5. Marker sizes were derived from *Hind*III-digested λ DNA and are given in kilobases.

FIG. 2. Denaturation and strand separation of the LRV1 RNA. Total cellular RNA (5 μ g) from *L. guyanensis* CUMC1-1A was analyzed by electrophoresis on a nondenaturing 0.6% agarose gel following various denaturing treatments and hybridized to the T3 (A) or T7 (B) riboprobe of clone LP5-30. Lanes 1, stored on ice; lanes 2, incubated with urea-EDTA at room temperature; lanes 3, incubated with urea-EDTA at 100°C for 3 min; lanes 4, incubated with formamide-formaldehyde at room temperature; lanes 5, incubated with formamide-formaldehyde at 65°C for 10 min. High-molecular-weight RNA ladders from Bethesda Research Laboratories were run in urea-EDTA (A) or formamide-formaldehyde (B) to obtain markers; sizes are shown in kilobases.

the ssRNA with apparent size heterogeneity into a single band. Mild denaturation by treatment with urea at room temperature (Fig. 2, lanes 2) or with formamide-formaldehyde at room temperature (lanes 4) followed by electrophoresis on nondenaturing gels revealed a band detected with riboprobe prepared from the T3 (panel A) but not the T7 (panel B) promoter on clone LP5-30. Thus, this band corresponds to the ssRNA described above (Fig. 1A, lanes 4, 8, and 9). The more intense band with slower mobility that is detected with both strand-specific probes corresponds to the band seen after ethidium bromide staining and thus represents the dsRNA. The ssRNA has an apparent size of 5.3 kb in formamide-formaldehyde (relative to ssRNA markers examined under the same conditions). The ssRNA has a smaller apparent size (4.4 kb) relative to RNA markers in urea, reflecting base composition differences between LRV1 and marker RNAs, since urea changes the mass-to-charge ratio of RNA depending on G+C content (13). The slower migration of LRV1 RNA in formamide-formaldehyde (lanes 4) compared with that in urea (lanes 2) is due to chemical modification of the RNA by formaldehyde (13).

Denaturation of the dsRNA by heating to 100°C in urea (lanes 3) resulted in a single intense band with the same mobility as the ssRNA. The minor difference in mobility of the two strands of urea-denatured LRV1 RNA (compare lanes 3 in panels A and B) is due to differences in base composition rather than size (13). Heating the RNA in formamide-formaldehyde at 65°C resulted in a smear of hybridization (lanes 5) and its destruction at 100°C (data not shown), reflecting partial denaturation and chemical reaction of formaldehyde with RNA (especially at elevated tempera-

FIG. 3. CsCl density gradient analysis of cytoplasm from exponential-phase *L. guyanensis*. Sonicated cytoplasm (0.5 ml) was centrifuged on isopycnic CsCl gradients, and 8- μ l aliquots of each 0.25-ml fraction (numbering from the bottom of the gradient) were treated with 1% *N*-lauryl sarcosine and 40 μ g of proteinase K per ml for 30 min at 65°C, electrophoresed on a 0.6% agarose-Tris-borate-EDTA gel, and hybridized with the T3 (A) or T7 (B) riboprobe of clone LP5-30. Marker sizes, derived from *Hind*III-digested λ DNA, are given in kilobases.

tures) leading to degradation and cross-linking (13). Thus, these results show that the bulk of the LRV1 RNA is ds but a significant amount of ssRNA of one strand is present. They also show that within the limits of resolution of the gel, the sizes of the two strands present in the dsRNA are the same as that of the ssRNA (5.3 kb).

Cytoplasm from *L. guyanensis* CUMC1-1A was fractionated on CsCl density gradients, and RNA from each fraction hybridized with strand-specific riboprobes from LP5-30 (Fig. 3). Both riboprobes detected the 5.3-kb dsRNA, while the T3 riboprobe alone also detected the ssRNA described above. If the detergent-proteinase K treatment of the fractions was omitted, only a smear of hybridization in the 23-kb region of the gel was seen with both probes (data not shown), suggesting that both the 5.3-kb dsRNA and the T3-specific ssRNAs are present within viral particles. The gradient pellet contained both dsRNA and the T3-specific ssRNA (data not shown), probably representing free LRV1 RNA present in

the cytoplasm, although it may be due to lability of the LRV1 particles.

The peak signal for the LRV1 dsRNA detected by both probes was found in fractions 9 to 10 of the gradient, corresponding to a density of ~ 1.4 g/ml, while the T3-specific ssRNA was found in fractions 12 to 14, which have a lower density. The relative positions of the particles containing dsRNA and ssRNA in a CsCl density gradient differ slightly depending on the method used for cell disruption (data not shown). These results suggest that the particles containing the ssRNA are less dense than those containing the dsRNA.

Cytoplasm containing LRV1 viral particles from *L. guyanensis* CUMC1-1A promastigotes was added to cultures of formamycin B-resistant *L. donovani* FBD5 (17), which does not contain LRV1. After 13 days of growth, examination of RNA from these cultures failed to reveal the presence of LRV1 RNA by either ethidium bromide staining or hybridization with LP5-30 riboprobes (data not shown). Thus, LRV1 viral particles are not infective when added extracellularly.

DISCUSSION

The human pathogen *L. guyanensis* CUMC1-1A contains LRV1 viral particles that are 32 nm in diameter, sediment at 130S, are restricted to the cytoplasm, and are associated with 5.3-kb RNA (23). Although the stock of *L. guyanensis* which contains LRV1 was isolated from a human cutaneous lesion, there is as yet no evidence for a relationship between the presence of the virus and any disease characteristic, host range, or growth characteristic of the parasite. Both dsRNA and ssRNA, representing only one strand of the viral genome, are found in the cytoplasm of *L. guyanensis* CUMC1-1A. The ds nature of the majority of LRV1 RNA is evident from intense ethidium bromide staining, the shift in electrophoretic mobility upon denaturation (Fig. 2), hybridization to both strand-specific probes, and the lack of hybridization to the ethidium bromide staining band unless the RNA is denatured. Previous reports that LRV1 (or LR2 or LBV) RNA is ss (23, 28) were the result of methods used to isolate the RNA (i.e., preferential isolation of ssRNA from CsCl gradients or denaturation of the RNA during isolation with guanidinium thiocyanate).

The ssRNA is less abundant than the dsRNA, and its ss nature is evident from its hybridization to only one of two complementary strand-specific probes and its electrophoretic behavior in the presence and absence of denaturants (Fig. 2). Sequence analysis of LRV1 cDNA clones indicates that the ssRNA corresponds to the positive strand, since it encodes an open reading frame with homology to the RNA-dependent RNA polymerases from other viruses (21). Both ssRNA and dsRNA are more abundant in stationary-phase cells than in exponential-phase cells, suggesting a greater rate of LRV1 RNA synthesis relative to the slowed host cell synthesis in the former stage. The ability to resolve dsRNA without treatment to disrupt particles (Fig. 2, lanes 10) suggests that assembly of dsRNA containing viral particles may be altered or incomplete in the stationary phase. The apparent absence of ssRNA that is resolved from untreated particles may reflect assembly into altered or incomplete particles or nuclease degradation.

In exponentially growing parasites, most LRV1 dsRNA is associated with 130S particles which appear quite labile, since they can be dissociated by heat (65°C) alone, as well as by SDS or SDS and proteinase K treatment (Fig. 1). These

particles have a density of ~ 1.4 g/ml in CsCl gradients (Fig. 3). In addition, positive-strand ssRNA is associated with particles with lower density in CsCl gradients (Fig. 3). They may also be less labile than the particles containing dsRNA (Fig. 1).

Although morphologically similar to the dsRNA viruses of other protozoa (26), LRV1 virus is distinct by several criteria. LRV1 is not membrane bound like the *T. vaginalis* virus (25), nor is it found in the nucleus as is the *G. lamblia* virus (24). LRV1 does, however, show similarity to the yeast killer virus, L-A (27). Both viruses have unsegmented dsRNA genomes that are similar in size. Both appear not to be infective by extracellular transmission. The L-A viral particle is assembled from the positive-strand RNA, coat protein, and coat protein-RNA-dependent RNA polymerase fusion protein. The negative strand is synthesized within the particle by using the RNA-dependent RNA polymerase, resulting in a particle containing dsRNA. This particle in turn synthesizes the positive-strand ssRNA, which is extruded from the particle and is able to be translated and/or packaged (8, 9). The density of the L-A viral particles that contain ssRNA is less than that of those containing dsRNA (8).

Thus, both the LRV1 and L-A viruses are restricted to the cytoplasm, have unsegmented genomes, are packaged as ssRNA or dsRNA in particles with different densities, have associated RNA-dependent RNA polymerase activity (29), and appear not to produce particles that infect by extracellular transmission. In addition, nucleotide sequence analysis reveals that the conserved domains of RNA-dependent RNA polymerase of LRV1 have the greatest homology to that of the L-A virus when compared with sequences in the data base (21). These data suggest that LRV1 has a replication cycle similar to that of the L-A virus and that it may be transmitted by cell contact. These results also indicate that LRV1 is unlikely to have been recently acquired from the insect vector but appears to be an RNA virus of the type found in lower eukaryotes. The isolation of 12 independent isolates, all of which have homology to the isolate from CUMC1-1A but also substantial sequence divergence, indicates the existence of related RNA viruses in *Leishmania* spp. (10). Several other dsRNAs in yeast strains, some of which are deletion mutants of L-A virus, are packaged into L-A viral particles, and the viral sequences responsible for this packaging are known (27). Elucidation of similar sequences in LRV1 may open the way for development of novel transformation vectors for trypanosomatid parasites.

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