Genomic Structure and RNA Polymerase Activity in *Leishmania* Virus

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Viral particles infecting some stocks of the protozoan parasite *Leishmania braziliensis* subsp. *guyanensis* contain a double-stranded RNA genome of ca. 5 kbp and are associated with an RNA-dependent RNA polymerase which synthesizes in vitro double-stranded and single-stranded, genome-length transcripts. The majority of viral transcripts are single-stranded and templated from one genomic strand. The putative replicase generates double-stranded RNA by synthesizing the opposite strand on a preexisting RNA template. These data are compatible with a replicative cycle proposed for the yeast viruses. Purification of the *Leishmania* virus on CsCl yields virus without double-strand synthesis activity, while this activity is consistently present in unpurified virus and in particles from sucrose gradients. The deficiency in double-strand synthesis in CsCl-derived virions correlates with the accessibility of the viral polymerase and genomic RNA to exogenously added enzymes, indicative of a structural modification of the viral capsid.

Some stocks of the protozoan parasite *Leishmania braziliensis* subsp. *guyanensis* are infected with RNA viruses designated LR1 (7) and LBV (for *L. braziliensis* virus) (11). There is considerable interest in these and other protozoan viruses for several reasons. First, parasitic protozoa have until recently been refractory to traditional gene transfer techniques, and these viruses could potentially be used as vectors. A significant step in this direction was the infection of *Giardia lamblia* with *G. lamblia* virus by electroporation of *G. lamblia* virus single-stranded RNA (ssRNA) into uninfected cells (4). Second, since viruses are dependent for their replication on host cell molecular pathways, they have been successfully used as experimental systems with which to study and understand the molecular biology of eukaryotes. In view of the many unconventional gene expression pathways in protozoa, such as *trans*-splicing and RNA editing, LBV could give new insights into the mechanism and significance of these processes in *Leishmania* spp. It is presently unclear whether these viruses have any relevance to the host-parasite interaction.

Some similarities between the *Leishmania* viruses and those infecting other protozoa (8, 9) and *Saccharomyces cerevisiae* (10) have become apparent. These are the relatively small double-stranded RNA (dsRNA) genome and the capsidlike viral particles. Like particles of other viruses with genomic negative-stranded RNA or dsRNA, CsCl-purified LBV particles were shown to be associated with RNA-dependent RNA polymerase activity. The in vitro products of this activity were identified as mainly ssRNA and some dsRNA transcripts. The activity generating the latter products was variable in these preparations (12).

In this article, we demonstrate that LBV particles contain two complementary RNA strands and that the products of the putative viral transcriptase and replicase are of opposite polarities. Significant differences between CsCl- and sucrose-purified virions were found, which are consistent with a disruption of the viral capsid during purification on CsCl.

MATERIALS AND METHODS

Sucrose gradient purification. Between 3×10^9 and 7×10^9 *L. braziliensis* subsp. *guyanensis* promastigotes (stock MHOM/BR/75/M4147 [1]) grown as described before (12) were lysed by sonication. The lysate was cleared by two low-speed centrifugations of 10 min each at 4°C and loaded on a 10 to 40% (wt/vol) sucrose gradient in 10 mM Tris-HCl (pH 7.5)–150 mM NaCl. Centrifugation was for 2.5 h at $120,000 \times g$ and 10°C.

From 14 to 16 fractions were recovered. One-twentieth of each fraction was phenol-chloroform extracted and ethanol precipitated, and the nucleic acid was visualized on a 0.7% agarose gel in TAE (40 mM Tris-acetate, 1 mM EDTA).

Strand separation. Viral RNA was phenol-chloroform extracted from sucrose-purified particles, precipitated in ethanol, and resuspended in loading buffer (3% glycerol, 0.05% each bromophenol blue and xylene cyanol, 5 mM EDTA, 3.5 M urea, sodium dodecyl sulfate 0.1% [SDS] [6]). Radiolabeled dsRNA from an in vitro polymerase reaction was gel-purified on 1% low-melting-point agarose, phenol extracted, ethanol precipitated, and dissolved in the same loading buffer. Samples were denatured for 3 min at 100°C, chilled on ice, and immediately loaded on a 1.2% agarose gel in TAE. Electrophoresis was done initially for 3 min at 90 mA and then for 3 to 4 h at 40 to 50 mA.

Northern (RNA blot) analysis. RNA was transferred to Immobilon N hybridization membranes (Millipore) in 5 mM NaOH and hybridized at 68°C as recommended by the manufacturer. Posthybridization washes were done once in $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate [pH 7]) at room temperature, once in $2 \times$ SSC–0.1% SDS at 60°C, and twice in $0.2 \times$ SSC–0.1% SDS at 60°C, each for 15 min.

Other methods. The polymerase assay on particles from CsCl was done by the method of Widmer et al. (12). Polymerase activity in sucrose gradients was assayed by incubating 8 to 10 μ l of fraction in 20 μ l of the same reaction mixture. Activity in cell extracts was assayed according to Widmer et al. (11). RNA was 3' end labeled with 5'-[³²P]pCp and RNA ligase as described by England et al. (2). Nucleic acid samples were glyoxalated at 50°C for 40 to 60 min in 8% glyoxal–67% dimethyl sulfoxide–13 mM NaH₂PO₄ (pH 7).

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FIG. 1. Denaturation of viral RNA yields two ssRNA species. LBV RNA, phenol-chloroform extracted from sucrose-purified particles, was heat denatured in the presence of 3.5 M urea and 0.1% SDS (lane 3) and cofractionated in 1.2% agarose-TAE with native RNA obtained in the same way (lane 2). The mobilities of the native and denatured RNA are indicative of a dsRNA and ssRNA structure, respectively. Lane 1, lambda *Hind*III-digested DNA as size markers; the molecular sizes of the fragments are 23, 9.4, 6.5, 4.4, 2.3, 2.0, and 0.6 kb.

RESULTS

Previous analyses of the genomic structure of LBV obtained from either cell lysates or CsCl-purified virions by RNA protection assays gave inconsistent results (11, 12). RNase protection analyses showed that the proportions of RNase-resistant and -sensitive RNAs were variable. An alternative approach was attempted here with LBV purified on sucrose gradients. RNA extracted from purified particles was heat denatured, and the products were separated on agarose gels. These experiments (Fig. 1) clearly showed that genomic RNA (lane 2) can be dissociated into two strands of about equal intensity (lane 3). The shift in electrophoretic mobility occurring after denaturation is characteristic of a change from dsRNA to ssRNA structure. This difference in mobility is also observed between ssRNA and dsRNA viral transcripts (see Fig. 5 and 7). The structure of these transcripts was previously determined by RNase protection (12).

The same denaturation protocol was used with 3'-end-labeled LBV RNA (Fig. 2). As a control, LA dsRNA from yeast virus, labeled in the same way, was fractionated in native and denatured condition (lanes 2 and 3, respectively). Genomic RNA extracted from LBV showed properties similar to those of the LA dsRNA; in lane 5, the denatured

FIG. 2. RNA ligase labels both strands of the genomic RNA. LBV RNA obtained as described in the legend to Fig. 1 was 3' end labeled with [32 P]pCp (lanes 4 and 5) and electrophoresed in parallel with yeast virus LA dsRNA (lanes 2 and 3) and sucrose-purified LBV particles (lanes 6 and 7) labeled in the same way. Strand-separated LBV RNA (lane 5) yields a doublet with an additional band of unknown origin. Only trace amounts of label are visible with RNA from native particles. Even-numbered lanes, native samples; odd-numbered lanes, denatured samples; lane 1, lambda *Hind*III-digested DNA as size markers.

RNA appeared as a doublet with an additional band of lower intensity, confirming the observation with unlabeled RNA (Fig. 1) and showing that both genomic strands terminate in 3' hydroxyls. The origin of the third band of slightly faster mobility is unknown. As it does not appear in the LA control, it is unlikely to be an experimental artifact. Lanes 6 and 7 show the absence of labeled LBV RNA when native virions were used as the substrate.

Earlier experiments demonstrated that LBV genomic RNA is the template for the virion-associated RNA-dependent RNA polymerase and that a majority of the transcripts generated in vitro by viral polymerase are single-stranded (11, 12). Using the strand-separation protocol in conjunction with Northern blot analysis, we determined which strand was the template for this activity. Figure 3 shows an RNA blot probed with internally labeled transcripts generated in vitro with purified virus. In lanes 2 and 3, unlabeled, native and heat-denatured LBV RNAs, respectively, were fractionated in parallel with denatured 3'-end-labeled LBV RNA as size markers (lane 4). The probe in lane 3 hybridized only to the slow-migrating strand, identifying the transcriptional template. This experiment also shows that the fainter, fast-migrating species sometimes visible in denatured LBV RNA (see also Fig. 2, lane 5) does not hybridize to the probe, indicating that it is either a host RNA or a viral subgenomic transcript.

The origin of the dsRNA synthesized by the viral poly-

FIG. 3. Northern blot analysis of LBV RNA. Unlabeled LBV RNA from purified virions was fractionated in native (lane 2) and denatured (lane 3) conditions in parallel with denatured, pCp-labeled LBV RNA (lane 4), blotted to a nylon membrane, and hybridized with viral transcripts. The transcripts hybridized in lane 3 to the slower band only, the template strand for transcription. Lane 1, size markers (see Fig. 1 legend).

merase was investigated by the same strand-separation protocol with gel-purified dsRNA from a polymerase reaction. This approach revealed that during the reaction, only the slow-migrating strand incorporated radioactivity (Fig. 4, lane 2), suggesting that the polymerase synthesized a full-length strand on an unlabeled, preexisting RNA template. In conjunction with the data in Fig. 3, this observations showed that the labeled products of ssRNA and dsRNA synthesis were of opposite polarity.

As an alternative to the purification of LBV on CsCl gradients, which yields particles with unstable or missing dsRNA synthesis activity, a protocol with a sucrose gradient was developed with the aim of obtaining virus preparations with polymerase activity similar to that observed in unpurified virions, which consistently express dsRNA and ssRNA synthesis activity. Sucrose gradient fractions obtained as described in Materials and Methods were assayed for polymerase activity. The products of the reactions, visualized on a native agarose gel (Fig. 5), contained some dsRNA but predominantly ssRNA, as seen with cell extracts. The absence of dsRNA synthesis in CsCl-purified samples was tentatively attributed to the requirement for an exogenous protein or RNA factor. This hypothesis was not supported by experiments in which polymerase reactions with CsCl-purified virus supplemented with protein, RNA, or cell extract failed to synthesize dsRNA (not shown).

It is conceivable that dsRNA synthesis remained undetec-

FIG. 4. dsRNA synthesis activity copies a new RNA strand on preexisting RNA. Gel-purified, radiolabeled dsRNA generated in an *in vitro* polymerase reaction was analyzed by strand separation (lane 2). A comparison with pCp-labeled genomic strands (lane 3) reveals that only the slower band incorporated radioactivity. Lane 1, DNA markers from a lambda *Hind*III digest; fragment sizes are shown in kilobases.

ted in particles from CsCl gradients owing to particle disruption by the high ionic strength of the gradient. This possibility was suggested by the observation that CsCl-purified virions appeared to be empty by electron microscopy (11). We tested the possibility that virions might have been modified in CsCl by two approaches. First, particles purified on CsCl and sucrose were incubated in 3'-end-labeling reaction mixes with RNA ligase and 5'-[³²P]pCp with the aim of testing whether the viral RNA in CsCl particles was exposed. The results of this experiment, shown in Fig. 2 and 6, support this view. RNA from sucrose-purified virions was not a substrate for the labeling reaction (Fig. 2, lanes 6 and 7), while labeling of CsCl-purified virions yielded a band of the expected mobility, compared with the DNA markers. We verified that the absence of label in sucrose-purified virions (Fig. 2, lanes 6 and 7) was not due to an inhibition of

FIG. 5. Polymerase reaction in sucrose-purified virions reveals ssRNA and dsRNA synthesis. A promastigote extract was fractionated on a sucrose gradient, and 16 fractions were recovered. Polymerase activity was assayed in fractions 3 to 9 and visualized on a 0.7% agarose gel in TAE. Sedimentation was from right to left. Leftmost lane, size markers (see Fig. 1 legend).

FIG. 6. RNA from CsCl-purified particles is a substrate for RNA ligase. CsCl-purified LBV particles were 3' end labeled in parallel with dsRNA standards from reovirus (reo) and yeast virus (LA). All RNA samples and DNA markers were glyoxalated and separated on a 0.7% agarose gel. The largest genomic segment of reovirus is 3.9 kb; that of LA RNA is 4.6 kb.

the RNA ligase by supplementing an identical reaction mix with reovirus dsRNA. In this control reaction, reovirus RNA was labeled, demonstrating normal RNA ligase activity under these conditions (not shown). In Fig. 6, two dsRNA samples of known molecular size, yeast virus LA dsRNA (4.6 kb) and reovirus genomic dsRNA segments (3.9 to 1.2 kb), were glyoxalated and cofractionated with LBV RNA denatured in the same way. From this comparison, the size of the LBV genome was estimated to be ca. 5 kb.

Second, based on the same rationale as for the end-labeling experiment, the resistance of viral polymerase to degradation by proteinase K in CsCl- and sucrose-purified virus preparations was probed by using unpurified particles in a cell extract as a positive control (Fig. 7). Viral polymerase activity in the cell extract (lane 7) and particles from sucrose gradients (lane 5) was not affected by the protease treatment. However, in particles from CsCl gradients, the activity disappeared following protease digestion (lane 3). Control polymerase reactions with undigested samples from CsCl, sucrose, or cell extract generated the expected pattern (lanes 2, 4, and 6, respectively). The extent of the proteinase K reaction was monitored by electrophoresing a portion of treated cell extract on an SDS-polyacrylamide gel. Except for the protease band, no proteins were visible after digestion (not shown). This observation is in agreement with the end-labeling experiment and suggests that in CsCl-purified

FIG. 7. Proteinase K resistance of viral polymerase activity. LBV particles from a CsCl gradient, a sucrose gradient, and a cell extract were digested in 100 μ g of proteinase K per ml (+ lanes) for 15 min at room temperature, and the samples were assayed for polymerase activity. Viral polymerase was protected from proteinase K in virions in cell extracts (lane 7) or purified on a sucrose gradient (lane 5). Polymerase from virions banded in CsCl was susceptible to the protease (lanes 3). Lanes 2, 4, and 6 are undigested controls for lanes 3, 5, and 7, respectively.

particles, both the viral RNA and the polymerase are accessible to exogenous enzymatic activities.

DISCUSSION

This study demonstrates that each strand of the dsRNA genome of LBV is the template for a distinct polymerase activity, transcription of ssRNA and synthesis of dsRNA. By analogy with the model of yeast virus replication (3), these RNA polymerase activities are tentatively designated transcriptase and replicase, respectively, defining the polarity of their products as positive and negative, respectively. This model also has some similarity to the replication of the multisegmented reovirus and trisegmented dsRNA phage $\phi 6$ (5). The abundance of ssRNA transcripts in the polymerase products is in agreement with their function as mRNAs postulated by this model, but the polarity of the polymerase products has not yet been demonstrated experimentally.

The strand separation analyses of unlabeled and 32 P-labeled genomic RNA illustrated in Fig. 1 and 2, respectively, are consistent with a double-stranded genomic structure. This conclusion is supported by a previous RNase protection experiment with in vitro polymerase products but is in contradiction to the apparent partial RNase sensitivity of genomic RNA from virions (12). A possible explanation for this discrepancy is an experimental error leading to loss or degradation of the RNase-protected sample in our earlier analysis (12).

The products of the RNA polymerase activities in cell extracts and in sucrose-purified particles were indistinguishable, consisting of ssRNA and dsRNA. This indicates that virions obtained through this purification procedure more closely resemble the native particles found in the cell than

virions from CsCl gradients, in which dsRNA synthesis is often undetectable. Our interpretation of the difference between sucrose- and CsCl-purified virions assumes that a structural modification induced by the high ionic strength of the gradient is affecting dsRNA synthesis. Both the end-labeling and protease sensitivity assays support the view that in these preparations, capsids are modified in a way that exposes the polymerase and RNA. This modification might be the cause of the lack of stable dsRNA synthesis. It is conceivable that damaged capsids release a factor required for this activity. The observation that protease treatment does not affect dsRNA synthesis indicates that this putative factor is protected from protease digestion.

The data on the replicative cycle of LBV obtained so far conform to the model proposed for other dsRNA viruses, in particular for the yeast viruses. A major gap in our understanding of LBV replication is the process of encapsidation, and we are currently approaching this problem by using in vitro systems. This work will lead into the study of regulatory processes linking the parasite growth cycle and virus replication.

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