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RNA Polymerase Activity Is Associated with Viral Particles Isolated from *Leishmania braziliensis* subsp. *guyanensis*

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Viral particles purified from species of the protozoan parasite *Leishmania braziliensis* subsp. *guyanensis* by centrifugation in CsCl gradients were examined for the presence of viral polymerase. We demonstrated that RNA-dependent RNA polymerase is associated with viral particles. Viral transcription was studied in vitro with pulse-chase experiments and by assaying the RNase sensitivity of the viral transcripts. Viral polymerase synthesized full-length transcripts within 1 h. Double-stranded, genome-length, and single-stranded RNAs were produced in this system. The nature of the RNA extracted from virions was also tested by RNase protection assays; both single-stranded and double-stranded RNAs were found.

Viruses of protozoan parasites are a relatively recent discovery. Their presence has been documented in several genera pathogenic for humans, including *Giardia*, *Trichomonas*, and *Leishmania*. A small number of studies have focused on the molecular biology of these viruses (4–10). In *Giardia* and *Trichomonas* species, viruses with double-stranded RNA (dsRNA) genomes have been identified (7, 8). RNA-dependent RNA polymerase activity has been detected in *Giardia lamblia* virus-infected cells and culture supernatant (9). The existence of infectious particles has been proven only for *G. lamblia* virus (5). The mode of transmission of the other protozoan viruses is unknown.

As the pathways of gene expression in protozoa are still poorly understood, there is considerable interest in identifying and understanding viral systems in these organisms. Recently, Tarr et al. (6) and Widmer et al. (10) reported on similar RNA viruses which infect some stocks of *Leishmania braziliensis*, the causative agent of cutaneous leishmaniasis. These viruses, designated LR1 and LBV, respectively, were detected in the cytoplasmic fraction of lysates of the promastigote stage by several techniques. First, electrophoretic analysis of total RNA of some *L. braziliensis* subsp. *guyanensis* stocks revealed an RNA species of approximately 6 kilobases (kb) (6, 10) which was subsequently shown to be the viral genome. Second, RNA polymerase activity was detected in infected promastigote lysates (10). The transcripts synthesized during an in vitro viral polymerase reaction were found to be complementary, by Northern (RNA) blot analysis, to the viral genome. Third, capsidlike particles 30 nm in diameter were detected by electron microscopy in infected promastigotes (6, 10). To date nothing is known about the mode of replication or transmission of these newly described protozoan viruses.

To gain insight into the replication of LBV as well as to obtain pure viral transcripts, we examined isolated virions for virus-specific transcription. In this study we demonstrated that viral particles purified in CsCl gradients contain RNA-dependent RNA polymerase activity. In an attempt to unravel the replicative cycle of LBV, we have analyzed the products of this polymerase reaction as well as RNA from virions.

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MATERIALS AND METHODS

Virus purification. Promastigotes (3×10^8) in the late exponential phase of *L. braziliensis* subsp. *guyanensis* MHOM/BR/75/M4147 stock (1) grown at 28°C in Schneider's drosophila medium (GIBCO Laboratories) supplemented with 10% fetal bovine serum and 0.01% gentamicin sulfate were lysed by sonication in TNM (10 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 5 mM $MgCl_2$)–0.5 mM phenylmethylsulfonyl fluoride, and the lysate was cleared by centrifugation (4,000 × *g*, 15 min), loaded on a 30 to 38% (wt/wt) CsCl gradient in TNM, and centrifuged for 16 to 20 h at 200,000 × *g* in an SW41 or SW50.1 rotor. Ten to 14 fractions were diluted in TNM, and the virions were pelleted at 200,000 × *g* and resuspended in TNM. Portions of each fraction were extracted with phenol-chloroform, and the nucleic acid was precipitated in ethanol and analyzed on a native agarose gel. For routine virion purification, the visible virus band was collected from CsCl gradients with a syringe by puncture of the centrifuge tube.

Polymerase assay. Polymerase activity was assayed in 30-μl reaction mixtures containing 10 mM Tris hydrochloride (pH 7.5); 150 mM NaCl; 3 mM $MgCl_2$; 1,000 U of RNasin per ml; 400 μM dithiothreitol; 50 μM each ATP, CTP, and UTP; 5 μM GTP; 5 to 20 μCi of [α-^{32}P]GTP; and 15 μl of virion suspension. Incubation was for 1 h at room temperature. Pulse-labeling was carried out under the same reaction conditions for 10 min, and 1 mM unlabeled GTP was used for the chase. Reaction products were phenol-chloroform extracted and ethanol precipitated.

RNase protection assay. Viral polymerase products or LBV RNA extracted from gradient-purified virions were denatured in 5 mM Tris hydrochloride (pH 7.5)–5 mM EDTA at 85°C, slowly cooled to 30°C, brought to 10 mM Tris hydrochloride (pH 7.5)–300 mM NaCl (high salt concentration) or 10 mM Tris hydrochloride (pH 7.5)–10 mM NaCl (low salt concentration), and incubated with 40 μg of RNase A and 200 U of RNase T_1 per ml for 30 min at room temperature. The reaction was stopped with proteinase K-sodium dodecyl sulfate, and the products were phenol-chloroform extracted and ethanol precipitated.

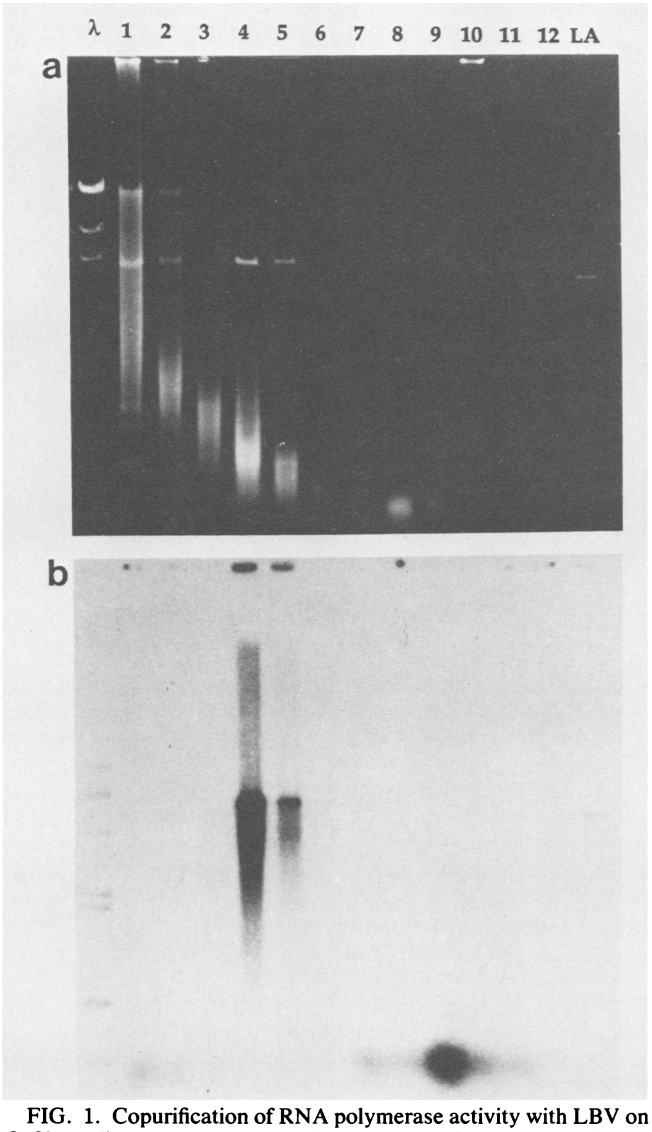

FIG. 1. Copurification of RNA polymerase activity with LBV on CsCl gradients. (a) A promastigote extract from *L. braziliensis* subsp. *guyanensis* was fractionated on CsCl, and the nucleic acid extracted from fractions (lanes 1 to 12) was analyzed on agarose. The viral genome was predominantly present in fractions 4 and 5. Sedimentation was from right to left; size markers were *Hin*dIII-digested λ DNA (lane λ) and yeast virus LA dsRNA (4.6 kb) (lane LA). (b) A portion of each fraction shown in panel a was assayed for RNA polymerase activity, and the reaction products were visualized on agarose. Size markers are as in panel a.

RESULTS

Viral particles were purified by isopycnic centrifugation in preformed CsCl gradients. Figure 1a shows the nucleic acid obtained by phenol-chloroform extraction of 12 density gradient fractions. The viral genome was mainly found in fractions 4 and 5 and migrated in native agarose at about 6 kb, as compared with DNA size markers. Yeast (*Saccharomyces cerevisiae*) virus LA dsRNA (4.6 kb) (2) had a slightly faster mobility, suggesting that the actual size of the LBV genome was about 4.7 kb. Relatively abundant viral RNA was present at the bottom of the gradient (lane 1), probably originating from degraded particles and/or unencapsidated viral transcripts. Host RNA was also present in this fraction, and a small nucleic acid of unknown origin was visible in the lower half of the gradient. An alternative centrifugation method was attempted with a vertical rotor (VTi 80 [Beckman Instruments, Inc.]; 220,000 × *g*; 24 h; initial CsCl density, 1.33 g/cm^3) but resulted in a less focused distribution of virions (data not shown).

The fractions from the CsCl gradient shown in Fig. 1a were assayed for RNA polymerase activity under conditions similar to those previously described for promastigote lysates (10). Portions of each of the 10 fractions were incubated in the presence of [α-^{32}P]GTP, and the radiolabeled glyoxalated products were resolved on an agarose gel (Fig. 1b). The polymerase activity was only detected in fractions 4 and 5, correlating with the distribution of viral RNA in the gradient. The small nucleic acid which cosedimented with the virions was not required for polymerase activity, as micrococcal nuclease pretreatment of virions did not affect polymerase activity (data not shown). The majority of the transcripts appeared to be of a length similar to that of genomic RNA. No polymerase activity was detected in fraction 1, confirming that the genomic RNA present in this fraction was not associated with intact virions. The viral origin of the transcripts was confirmed by hybridizing the products of the polymerase reaction to total nucleic acids of infected and uninfected *L. braziliensis* subsp. *guyanensis* stocks on a Northern blot. Consistent with our previous results (10), the transcripts only annealed to the viral genome (data not shown). The viral origin of the RNA and polymerase activity was further confirmed by electron microscopy. Viral particles, as observed previously (10), were consistently present in the same fractions as were genomic RNA and polymerase activity.

The nature of the polymerase reaction products and that of the viral genome were analyzed by assaying for RNase sensitivity in high and low salt concentrations (Fig. 2 and 3, respectively). The polymerase transcripts used for the experiment, obtained from a gradient other than that shown in Fig. 1, segregated into two size classes when electrophoresed on an agarose-formaldehyde gel (Fig. 2a, lane 1); a majority of transcripts ranged in apparent size from 1.4 to 2.3 kb, as compared with reovirus genomic segments, and a minority were of genome length. The large transcripts were completely resistant to RNase digestion in high salt concentrations (Fig. 2a, lane 3) but sensitive in low salt concentrations (lane 2), indicative of dsRNA. Genomic RNA from reovirus, which was used as a double-stranded control (Fig. 2b, lane 1), showed the expected RNase sensitivity (lane 2) and resistance (lane 3) in low and high salt concentrations, respectively. On the other hand, the fast-migrating transcripts were degraded by RNase regardless of the salt concentration (Fig. 2a, lanes 2 and 3), consistent with a single-stranded structure. As expected, rRNA present in the sample (Fig. 2b, lane 4) was also degraded under these conditions (lanes 5 and 6). Viral RNA from purified virions (Fig. 3, lane 5), when digested with RNases under the same conditions, was completely digested in low salt concentrations (lane 7) but only partially degraded in high salt concentrations (lane 6). The proportion of RNase-resistant RNA was variable, depending on the viral preparation. LA dsRNA (Fig. 3, lane 2), used as a control, showed the expected resistance (lane 3) and sensitivity (lane 4) in high and low salt concentrations, respectively.

Viral transcription was further investigated in a pulse-chase experiment. A polymerase reaction mixture was incubated under standard conditions in the presence of [α-^{32}P]GTP for 10 min. A portion of the reaction mixture was

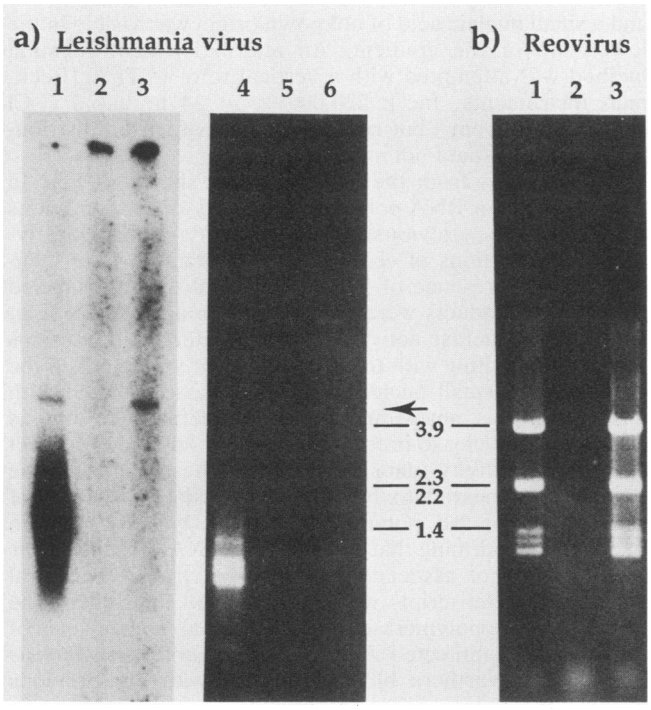

FIG. 2. Synthesis of ssRNA and dsRNA by viral polymerase in vitro. (a) Unmodified viral polymerase products (lanes 1 and 4) were compared with polymerase products digested with RNases in low (lanes 2 and 5) and high (lanes 3 and 6) salt concentrations. Lanes 1 to 3, Autoradiograph; lanes 4 to 6, ethidium bromide stain of the same gel. The arrow indicates the viral genome (the slow-migrating band in lanes 4 to 6 is contaminating *L. braziliensis* subsp. *guyanensis* DNA). The resistance of full-length RNA and the sensitivity of fast-migrating RNA to RNases in high salt concentrations are characteristic of dsRNA and ssRNA, respectively. (b) Reovirus dsRNA subjected, as a control, to the same treatment as the polymerase reaction products and electrophoresed on the same gel as that shown in panel a. Segment sizes are given in kilobases.

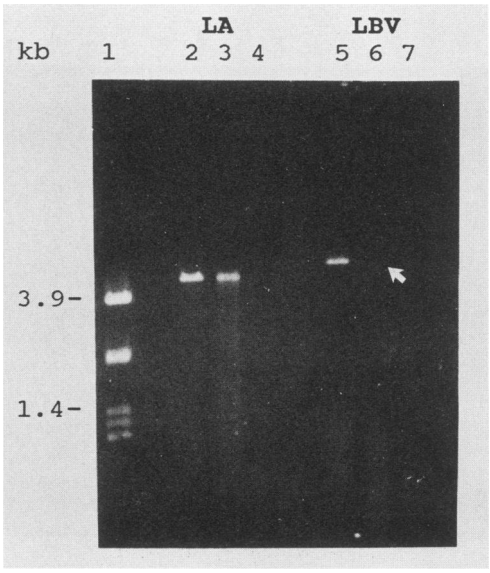

FIG. 3. Intermediate RNase sensitivity of virion-associated LBV RNA in high salt concentrations. Shown is an RNase protection assay with LBV RNA extracted from purified virions (lanes 5 to 7) and yeast virus LA dsRNA as a control (lanes 2 to 4). Samples were RNase digested in 10 mM NaCl (lanes 4 and 7) or 300 mM NaCl (lanes 3 and 6). Some RNase-resistant LBV RNA is present in lane 6 (arrow). Reovirus genomic segments were used as size markers (lane 1).

removed at this point, and the products were electrophoresed (Fig. 4a and b, lanes 1, −). Unlabeled GTP was added to a concentration of 1 mM, and the reaction was continued for 50 min. Portions of the reaction products synthesized after 30 min and after 60 min of total reaction time are shown in lanes 2, −, and lanes 3, −, respectively. The viral polymerase generated full-length transcripts within 1 h. A control reaction (60 min without chase) was run in the leftmost lanes. The lanes labeled + show the reaction products after digestion with RNases A and T_1 in 300 mM NaCl. No RNase-resistant transcripts were detected, indicating that all the products of this reaction were single stranded.

The electrophoretic mobility of the transcripts and the absence of dsRNA in the pulse-chase experiment are in contrast to the data shown in Fig. 2. To rule out the possibility that these discrepancies were due to the different electrophoretic conditions, we electrophoresed the same samples as those shown in Fig. 4a in a native gel (Fig. 4b) and in an agarose-formaldehyde gel (data not shown). Most of the transcripts comigrated with the 2.3-kb DNA marker but otherwise showed profiles similar to those of the denatured products (Fig. 4b).

DISCUSSION

The present study demonstrates that viral polymerase is associated with purified virions. The heterogeneity of the viral transcripts from some virion preparations, as revealed by differential sensitivity to RNase and electrophoretic mobility, indicates that the polymerase accomplishes a dual function, which we tentatively designate as replication and transcription. This assumption is based on a striking similarity of our data with those for yeast viruses, in which replicase and transcriptase activities synthesize double-stranded and single-stranded products, respectively, and copurify with virions banded in CsCl gradients (2).

A comparison between viral transcripts from different polymerase assays shows significant differences in their electrophoretic profiles. First, a large proportion of subgenomic RNA is synthesized by some polymerase preparations. Second, dsRNA is not consistently present (Fig. 2 and 4). We verified that the absence of fast-migrating RNA in some experiments was not due to the different electrophoretic conditions. It is unknown whether the variability in the synthesis of subgenomic RNA is caused by an experimental artifact or determined by a regulatory process. If LBV establishes a persistent infection, as is the case with double-stranded yeast viruses (3), viral replication and transcription might be a function of promastigote growth rate and could result in different compositions of polymerase reaction products. This argument might also explain the different proportions of RNase-resistant virion RNAs in different preparations. According to the yeast virus model, the viral replicative cycle alternates between single-stranded RNA (ssRNA)- and dsRNA-containing particles. Virions purified from parasite cultures in different phases of growth could consist of different ratios of single-stranded and double-stranded particles, which would result in various amounts of RNase-resistant RNA. This model is supported by observations on another protozoan virus, *G. lamblia* virus, in which the ratio of viral dsRNA to viral ssRNA was found to increase with the age of the parasite culture (4).

Observations on the RNase sensitivity of the LBV genome

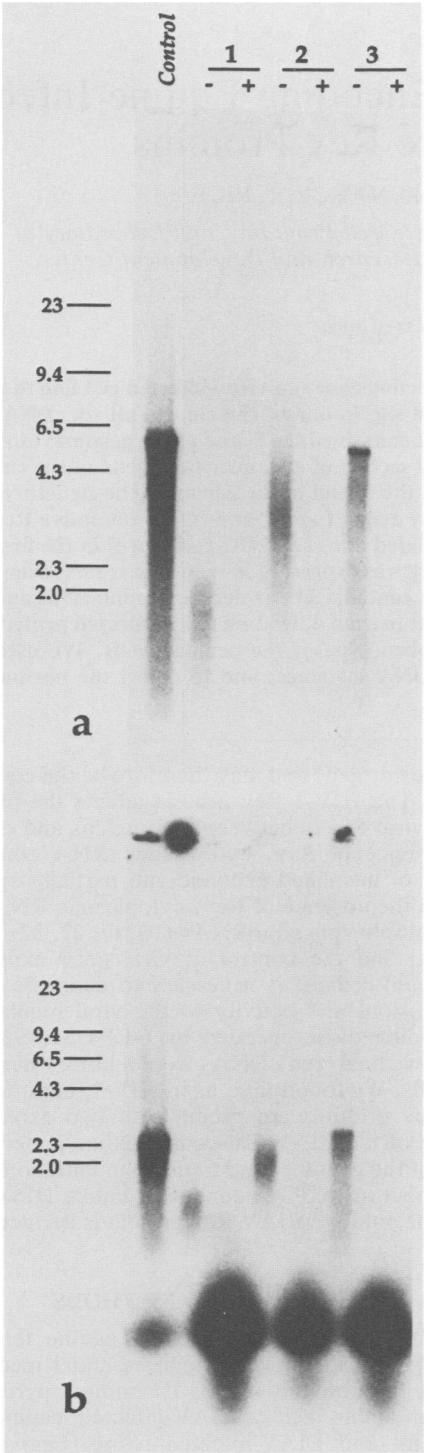

FIG. 4. Pulse-chase labeling of viral transcripts. A polymerase reaction was run for 10 min in the presence of [α-^{32}P]GTP (lanes 1) and chased with unlabeled GTP for 20 min (lanes 2) and 50 min (lanes 3). (a) Glyoxalated RNA. (b) Native RNA. −, Total transcripts; +, transcripts digested with RNases A and T_1 in 300 mM NaCl. Numbers at left are in kilobases.

based on the analysis of total cellular RNA (6, 10) suggested a single-stranded genomic structure. The present data show that genome-length RNA also exists as a double strand under certain conditions. It is likely that this structure is also present in vivo but that, because of the predominance of single strands, it remains undetected in analyses of total cellular RNA. The presence of polymerase activity associated with the virions, as demonstrated here, is compatible with a dsRNA or negative-stranded RNA virus. The assignment of LBV to either of these groups will require the analysis of extracellular particles, if such particles exist. Again, according to the yeast virus model, virions alternate between single-stranded and double-stranded stages during the replicative cycle and, in the absence of infectious particles, the definition of ssRNA or dsRNA virus is ambiguous.

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