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Hygromycin B Resistance Mediates Elimination of *Leishmania* Virus from Persistently Infected Parasites

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A series of pX63-HYG derivatives encoding *Leishmania* RNA virus 1-4 (LRV1-4) sequences were electroporated into cells of *Leishmania* strain M4147, a virus-infected strain of *L. guyanensis*. After 6 weeks of drug selection (hygromycin B), transfected parasites lacked detectable quantities of viral genomic double-stranded RNA, viral capsid protein, and RNA-dependent RNA polymerase (RDRP) activity. Evidence of viral infection was not recovered upon removal of the drug. While viral RNA transcripts were produced from electroporated expression vectors, as determined by reverse transcription-PCR, viral antigens were not detected, suggesting that the antiviral effects of hygromycin B are mediated through translation inhibition. A short-term selection study suggests that the LRV1-4 elimination may not only be a function of hygromycin B as a protein synthesis inhibitor but also possibly related to the mechanism of hygromycin B resistance in *Leishmania* strains.

The impetus for seeking viruses in protozoan parasites of the genus *Leishmania* is the possibility that such viruses will provide vehicles for understanding the molecular biology of the host cell as well as representing a potentially novel viral system (43, 44). Since the first double-stranded RNA (dsRNA) virus was discovered in *Leishmania* sp. in 1988 (40), similar viruses have been found in at least 13 strains of *Leishmania braziliensis*, *L. guyanensis*, or *L. major* (6, 33, 37, 39). Much of the initial work on *Leishmania* RNA virus (LRV) focused on characterization of the viral RNA genome (36, 37, 39) and identification of viral genes and proteins, specifically the capsid and the RNA-dependent RNA polymerase (RDRP) (5, 6, 35).

Translation of LRV RNA yields a capsid-RDRP fusion protein in vitro (26), similar to those described in other known totiviruses such as yeast L-A virus and *Giardia* virus (11, 16, 41). However, definitive evidence of an LRV fusion product in virus-infected parasites is lacking. Recently, we used an in vitro cleavage assay to suggest that a cellular cysteine-like protease may cleave the predicted capsid-polymerase fusion protein in vivo (35).

To investigate the possibility of in vivo proteolytic cleavage of an LRV polyprotein by a cellular protease (35), we transfected cultured parasites with derivatives of a recombinant expression vector, pX63-HYG (9), encoding the relevant LRV1-4 gene sequences. These constructs, which also encode a hygromycin phosphotransferase (*hph*) gene (20), were electroporated into the LRV1-4-infected *Leishmania* strain designated M4147. Drug-resistant parasites were selected by growth in medium supplemented with hygromycin B (50 μg/ml). After 6 weeks of selection, cells were examined for expression of viral dsRNA and proteins. Surprisingly, the transfected cells lacked detectable quantities of genomic viral RNA and virus capsid and of RDRP activity.

Hygromycin B is known as an aminocyclitol antibiotic produced by *Streptomyces hygroscopicus* (34) which inhibits protein synthesis in both prokaryotes and eukaryotes through interference with ribosomal translocation as well as with aminoacyl-tRNA recognition (4, 12, 19, 38). In this study, we report that LRV was eliminated from persistently infected parasites during the hygromycin B selection. We also discuss possible mechanisms of elimination of LRV by hygromycin B as a protein synthesis inhibitor.

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MATERIALS AND METHODS

Parasite strain and cell culture. *L. guyanensis* M4147 (MHOM/BR/75/M4147) and *L. braziliensis* M6244 (MTAM/BR/80/M6244) served as virus (LRV1-4)-infected and uninfected strains, respectively. Cells were grown at room temperature in semidefined medium M199 (GIBCO-BRL) supplemented with 5% fetal bovine serum (HyClone) and 1% fresh, filter-sterilized human urine (21).

Construction of pX63-HYG derivatives. LRV1-4 genes were assembled in the *Leishmania* expression vector pX63-HYG (9). Plasmid pX63-HYG encodes a selectable *hph* gene, conferring resistance to hygromycin B (20). A 5.3-kb fragment from *Xba*I/*Xho*I-cut pBSK-FULL14 or pBSK-INFRAME (35) was end filled with Klenow fragment (Promega) and subsequently purified from a low-melting-point agarose gel (NuSieve GTG agarose; FMC Bioproducts). The purified fragment was ligated into pX63-HYG previously cut with *Sma*I and dephosphorylated by alkaline phosphatase (calf intestine; New England Biolabs). The resulting plasmids are referred to as pHYG-FULL14 or pHYG-INFRAME, respectively.

Electroporation and hygromycin B selection. *Leishmania* cells were harvested from mid-log-phase cultures, washed twice in electroporation buffer (21 mM HEPES [pH 7.5], 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose) (3), and resuspended at a density of 10^8 cells/ml in electroporation buffer. Aliquots (0.4 ml) of the cell suspension were decanted into an electroporation cuvette (0.2 cm; Bio-Rad), mixed with 10 μg of plasmid DNA, and exposed to a 0.45-kV pulse (500-μF capacitance; average time constant, 3.0 ms; Bio-Rad gene pulser). After electroporation, cells were incubated on ice for 10 min, transferred into 10 ml of culture medium M199, and grown overnight without drug. After 24 h, hygromycin B (Sigma) was added to the culture medium (25 μg/ml, final concentration) and incubation continued as before. After 10 days growth, the cells were diluted to 1:20 (vol/vol) in fresh medium M199 and passaged subsequently every week for 6 weeks. The hygromycin B concentration was increased to 50 μg/ml at 2 weeks posttransfection.

To distinguish whether drug selection completely abolished viral infection or temporarily reduced virus copy number to an undetectable level, resistant parasites were transferred to tissue culture medium M199 lacking hygromycin B. Cells were passaged by dilution into fresh medium every week for 5 weeks. Control cultures were maintained in medium supplemented with hygromycin B (50 μg/ml) as previously described. Equal numbers of cells were collected at the end of weeks 2 and 5 posttransfection, washed once with TMN buffer (10 mM Tris [pH 7.5], 5 mM $MgCl_2$, 150 mM NaCl), and stored at −80°C for further study.

Preparation of nucleic acid extracts and cell lysates. *Leishmania* cells were resuspended in nuclease-free TMN buffer, and the suspension was extracted twice with phenol-chloroform-isoamyl alcohol (Sigma). Total RNA templates for reverse transcription (RT)-PCR were prepared from whole cells with Tri Reagent-LS (Molecular Research Center) according to the manufacturer's recom-

TABLE 1. Oligonucleotide primers used

Name	Sequence[a] (5′→3′)
R3	CCACAGTTATTGCACACAG
RF	TGTTACTTACCCTACGACTC
F3	TGTGTAAGAAGTCAACT
Tail-11	GTGAACACACAAGCCACG
L3-400	GCGGCCGCATCATTAGGATTGGATAGCTC
Tail-14	GGATTGAAGTCGCTCC
E6-rev	GCACTCTTGCCATATTCC
H1	TGCAAGAAGCGTGCCTGC
H2	CCACCTCTGACTTGAGCG

[a] Viral sequences are underlined.

mendations. Nucleic acids were examined by electrophoresis on a 1% agarose (Sigma) gel followed by staining with ethidium bromide (EtBr) solution (Sigma). For Western blot analysis, cells were lysed in TMN buffer supplemented with 1% (vol/vol) Triton X-100 (Sigma) for 3 h at 4°C, and a clarified supernatant was prepared by centrifugation at 12,000 × *g* for 10 min.

RT-PCR and Western blot analysis. PCR was accomplished with *Taq* DNA polymerase (Boehringer Mannheim) for 30 cycles according to the manufacturer's recommendations. RT reactions were performed with Moloney murine leukemia virus (MMLV) reverse transcriptase (GIBCO-BRL) at 37°C for 1 h. The oligonucleotide primers are listed in Table 1. The reaction strategy and predicted products are described in Fig. 1. Primers H1 and H2 are complementary to pX63-HYG vector sequences and were used to demonstrate presence of the plasmid in drug-resistant parasites.

Western blot analysis was performed as described previously (6), with minor modifications. Briefly, an equal volume of cell lysates prepared from an identical number of cells was resolved on a 10% polyacrylamide gel with sodium dodecyl sulfate (SDS) and electroblotted to a polyvinylidene difluoride membrane (Millipore Immobilon-P), using a Semi-Phor electrotransfer unit (Hoesfer). LRV1-4 capsid-specific antiserum (5) was diluted to 1:7,500 (vol/vol) in phosphate-buffered saline supplemented with 0.05% (vol/vol) Tween 20 (TPBS) for Western blot analysis. Secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim).

RDRP assay. Viral polymerase activity in cell extracts was measured as described by Widmer et al. (45) except that [α-^{32}P]UTP (NEN) was used instead of [α-^{32}P]GTP. Cell lysates were prepared as described above, and 10 μl of lysate was used in a 20-μl final reaction volume.

Effects of hygromycin B on *Leishmania* growth. To determine the effects of hygromycin B on *Leishmania* growth and on viral protein synthesis, wild-type (nontransfected) *Leishmania* cells were harvested at late log phase and resuspended to 2.2×10^7 cells/ml in medium M199 supplemented with hygromycin B (25 μg/ml) as previously described. Control cultures were grown in medium lacking hygromycin B. Identical numbers of cells were harvested daily for 4 days and assayed for evidence of LRV by agarose gel electrophoresis and Western blotting. Nucleic acid extracts were prepared as previously described, resolved on a 1% agarose gel, and visualized by EtBr. An equal number of whole cells was by mixed with SDS-sample buffer (24), heated at 100°C for 4 min, and resolved by electrophoresis on a SDS-polyacrylamide gel with 10% polyacrylamide (PAGE). Viral capsid protein was detected by Western blotting with LRV1-4 capsid-specific antisera (5) as described before.

RESULTS

Transfection studies of *Leishmania*. Two constructs (pHYG-FULL14 and pHYG-INFRAME) were assembled in the *Leishmania* expression plasmid pX63-HYG and electroporated into M4147 cells as described in Materials and Methods. One construct encodes a complete viral genome, and the other has one nucleotide deleted to yield a capsid-RDRP gene fusion. After 6 weeks of selection, drug-resistant parasites exhibited growth kinetics similar to those of wild-type cultures. PCR analysis using primers specific for the pX63-HYG vector or viral cDNA insert confirmed the presence of recombinant plasmid in drug-resistant parasites (data not shown). To our surprise, however, these same parasites now lacked sufficient quantities of the 5.3-kb LRV genomic dsRNA (43, 44) to detect by EtBr staining. Furthermore, Western blot analysis failed to detect the virus capsid protein. These original observations, suggesting that the transfection-drug selection protocol had eliminated LRV from persistently infected M4147 parasites, prompted us to perform a more rigorous analysis of the transfected parasites.

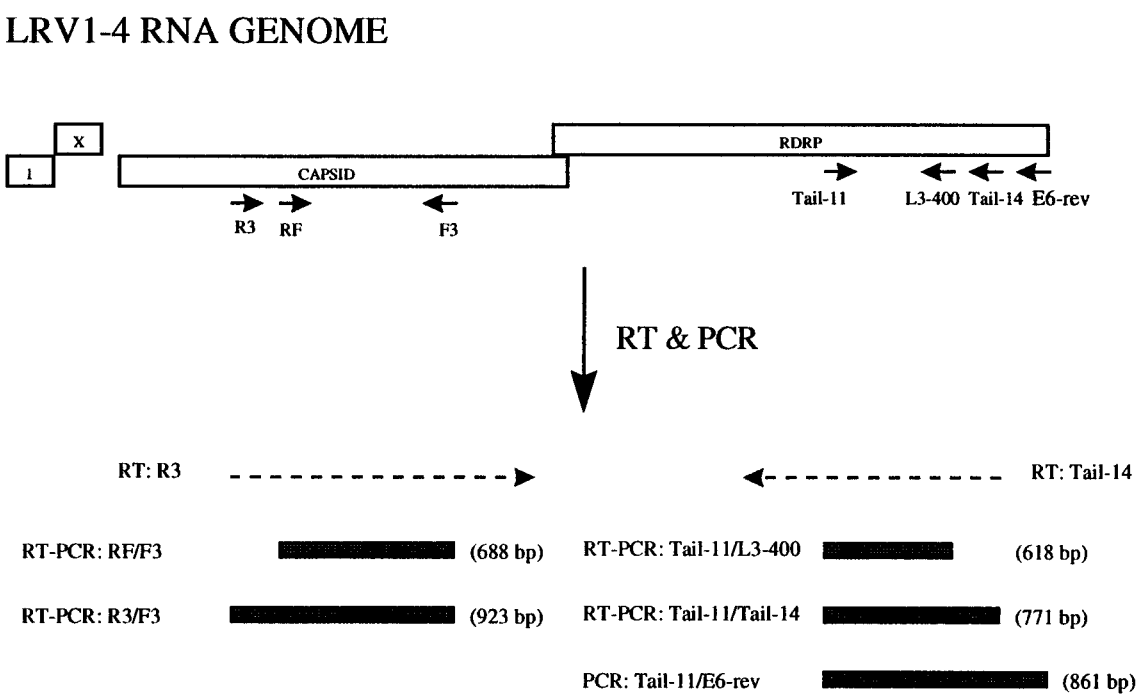

FIG. 1. Genomic organization of LRV1-4 RNA and schematic representation of the expected RT-PCR products. RT was accomplished by using MMLV reverse transcriptase (GIBCO-BRL) and the indicated primer pairs as described in Materials and Methods. Primer sequences are shown in Table 1. Arrows indicate the polarity of each primer. The expected sizes of PCR products are shown in parentheses.

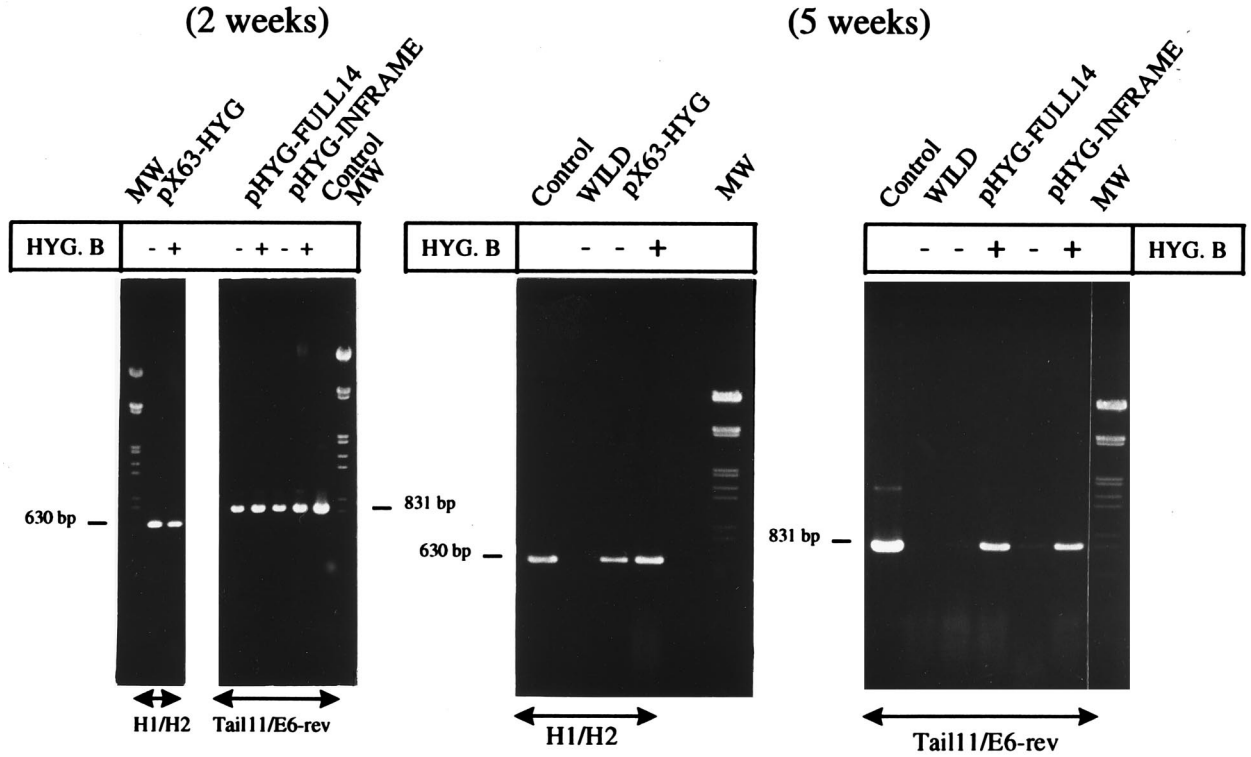

FIG. 2. PCR-based detection of DNA vectors in hygromycin B-resistant cells. Total nucleic acids were prepared from drug-resistant cells grown in the absence (−) or presence (+) of hygromycin B (HYG. B; 50 μg/ml) as indicated. Parasites were harvested after 2 or 5 weeks of growth as described in Materials and Methods. PCR-based detection of vector sequences in cell lysates was accomplished by using H1 and H2 as primers. Viral sequence-specific primers Tail-11 and E6-rev were used to amplify viral cDNA sequences encoded in expression plasmids. Molecular size markers are *Eco*RI/*Hin*dIII-digested λ DNA (MW). The transfection plasmids pX63-HYG and pHYG-FULL14 served as a positive PCR control (Control). Lanes contain PCR products obtained from DNA templates isolated from wild-type *Leishmania* strain M4147 cells (WILD) or drug-resistant M4147 cells transfected with pX63-HYG, pHYG-FULL14, or pHYG-INFRAME.

Loss of virus during growth in medium containing hygromycin B. To distinguish whether drug selection cures viral infection or simply reduces the virus copy number, parasite cultures were transferred into fresh medium M199 lacking hygromycin B and passaged weekly for 5 weeks. Equal numbers of cells were harvested from the second and fifth passages and processed for analysis by PCR and Western blotting with LRV1-4 capsid-specific antisera. PCR analysis yielded the expected-size DNA fragments from primers directed against either the pX63-HYG vector (H1/H2) or the LRV1-4 RDRP gene (Tail-11/E6-rev) (Fig. 2). The EtBr-stained PCR products from cells grown in the absence or presence of hygromycin B are roughly equivalent after 2 weeks. However, after 5 weeks of consecutive passage, the relative amount of plasmid appeared reduced in parasites grown in medium lacking hygromycin B. Thus, drug selection is necessary for maintenance of the resistance plasmid. Total nucleic acid preparations obtained from both parasite cultures lacked the 5.3-kb viral dsRNA genome (Fig. 3). As expected, the wild-type M4147 parasites grown in medium M199 contained easily detectable quantities of an RQ1 DNase-resistant 5.3-kb nucleic acid molecule (Fig. 3, wild lane 1).

In another analysis, parasite lysates were examined by Western blotting with LRV1-4 capsid-specific antiserum (Fig. 4). The antiserum recognizes the 82-kDa capsid protein as well as a second 42-kDa antigen present in lysates prepared from wild-type *Leishmania*. The latter antigen is also present in uninfected *L. braziliensis* M6244 stock and is unrelated to viral infection (data not shown). Lysates prepared from drug-resistant parasite cultures grown continuously in the presence of hygromycin B or passaged in hygromycin-free medium for as long as 5 weeks lacked detectable quantities of the 82-kDa viral capsid protein. Thus, absence of detectable quantities of viral dsRNA correlates with an absence of viral capsid antigen in transfected cells. The findings further indicate that transfected parasites were cured of viral infection during selection with hygromycin B.

Transfected parasites lack viral RNA by RT-PCR. Total cellular RNAs purified from the parasite cultures either maintained in or released from drug selection were examined for viral RNA by RT-PCR. Total RNA was incubated with MMLV reverse transcriptase and a plus-sense primer specific for the viral minus-strand (R3). Amplification of minus-strand cDNA generated from wild-type M4147 parasites, using RF and F3 as primers (see Fig. 1 for schematic strategy), yielded the expected 688-bp fragment and a 923-bp product which is presumably amplified by excess RT primer (R3) in combination with oligonucleotide F3 (Fig. 5). These products were not detected when reverse transcriptase was excluded from the RT reaction mixture, supporting the conclusion that the amplified products originated from an RNA template. RT-PCR of RNA isolated from parasites electroporated with pX63-HYG failed to yield any product, suggesting the cells lack viral minus-strand sequences. Cells transfected with vectors encoding LRV1-4 cDNA sequences were also tested for presence of virus by RT-PCR. The result showed that the predicted RT-PCR products are detected from hygromycin B-resistant parasites, though the quantity of viral RNA sequences, presum-

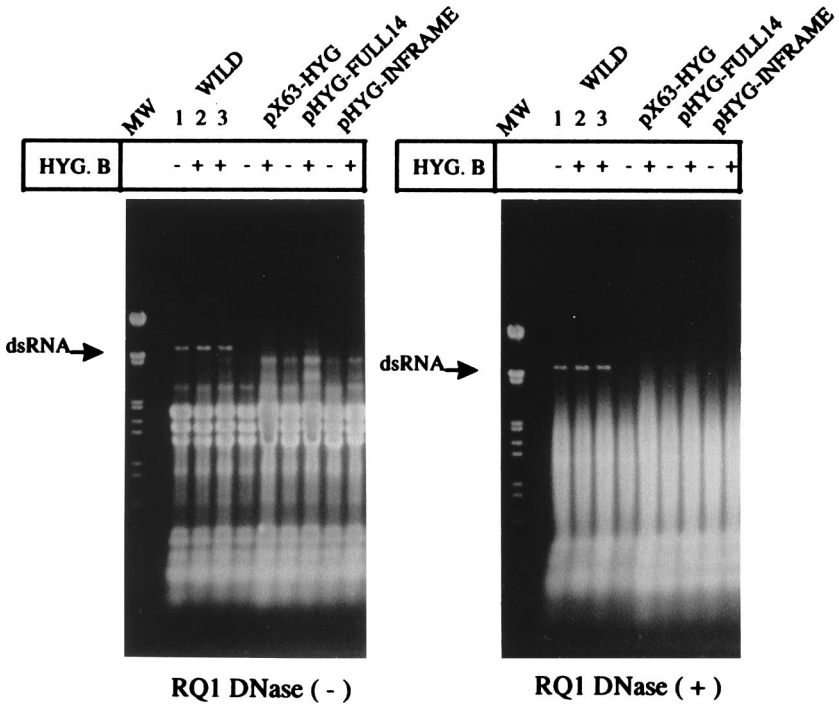

FIG. 3. Viral dsRNA is absent from drug-selected parasites. Total nucleic acids prepared from cultured *Leishmania* cells by phenol-chloroform extraction were incubated without or with 2 U of RQ1 DNase (Promega) for 3 h at 37°C prior to examination by agarose gel electrophoresis and EtBr staining. Lanes 1 to 3 contain RNA extracted from wild-type M4147 parasites (WILD) grown in the absence (−) or presence (+) of hygromycin B (HYG. B; 10 or 25 μg/ml [lane 2 or 3, respectively]) for 4 days. Remaining lanes contain nucleic acids from drug-resistant parasites either released from drug selection (−) or maintained continuously in medium containing hygromycin B (+) for 5 weeks. Parasites were transfected with plasmid pX63-HYG, pHYG-FULL14, or pHYG-INFRAME, all of which encode resistance to hygromycin B, as indicated. Arrows indicate the position of LRV dsRNA. Molecular size markers are *Eco*RI/*Hin*dIII-digested λ DNA (MW).

ably transcribed from the plasmid copy, was greatly reduced relative to that from wild-type M4147 cells, as judged by the intensity of EtBr staining. However, the quantity of viral minus-sense RNA judged by RT-PCR products was much different between drug-selected parasites either grown continuously in hygromycin B or released in medium M199 lacking the antibiotic. The result may reflect increased contaminating plasmid DNA in cultures maintained under constant selective pressure. The notion is consistent with the observations of a similar PCR product in the absence of RT and of no PCR product from parasites transfected with pX63-HYG, which has no viral sequences.

Transfected parasites lack detectable viral RDRP activity. Further evidence of viral infection was sought by assaying cells for viral RDRP activity in an in vitro assay. To accomplish this, cell extracts were prepared from electroporated (drug-resistant) M4147 cells either maintained in medium supplemented with hygromycin B or released from selective medium for an additional 8 weeks prior to harvest. In a parallel experiment, cell extracts were prepared from an uninfected *Leishmania* strain, designated M6244, that had been electroporated with the same pHYG-FULL14 and selected by growth in medium containing hygromycin B for six consecutive weekly passages. Positive control RDRP assays detected synthesis of full-length viral transcripts from sucrose gradient-purified LRV1-4 particles and lysates from wild-type M4147 cells (Fig. 6). Additionally, the assay produced the short RNA molecule generated by capsid-dependent cleavage of the full-length transcript (27, 28). Consistent with previous data showing that transfected cells lacked detectable quantities of viral RNA (Fig. 3 and 5) and viral capsid protein (Fig. 4), drug-resistant M4147 (previously infected) or M6244 (uninfected) parasites did not yield detectable quantities of viral polymerase products in the in vitro assay, irrespective of the presence of hygromycin B.

Hygromycin B does not interfere with transcription of viral genes. To investigate the possibility that elimination of persistent infection is caused by hygromycin B-dependent inhibition of RNA transcription, an RT-PCR experiment was performed with a minus-sense primer to detect the viral plus-sense RNA transcripts from the parasites electroporated with plasmids encoding viral sequences (see Fig. 1 for the schematic strategy). RNA samples were prepared from drug-resistant cells grown for 8 weeks in medium M199 alone or supplemented with hygromycin B, as previously described. RT-PCR of nucleic acids isolated from wild-type M4147 parasites produced an expected 618-bp DNA product, generated from oligonucleotide primers Tail-11 and L3-400, and a 771-bp fragment from Tail-11 in combination with excess RT primer (Tail-14) (Fig. 7). Similar-size RT-PCR products were generated in reactions primed with total cellular RNA isolated from parasites electroporated with either pHYG-FULL14 or pHYG-INFRAME. Because the prepared RNA samples lack viral genomic dsRNA or plasmid DNA, and no products were generated without RT (data not shown), the 618-bp PCR product is amplified exclusively from the viral plus-sense RNA transcribed from the electroporated expression vector. Moreover, when an identical experiment was performed with the RNA prepared from drug-resistant M6244 cells used in RDRP activity assay (Fig. 6), an expected 618-bp DNA fragment absent from wild-type M6244 cells was amplified in RT-PCR of RNA prepared from transfected (uninfected) parasites (Fig. 7), showing that the product is the viral plus-sense RNA transcript

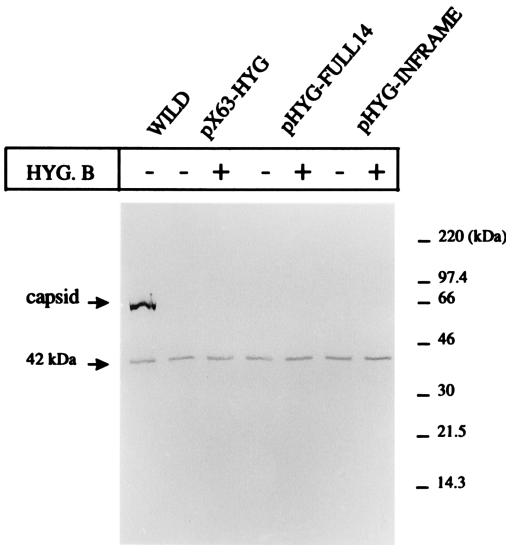

FIG. 4. Drug-resistant parasites lack detectable quantities of LRV capsid protein. Lysates were prepared from *Leishmania* cells grown in either medium M199 (−) or medium M199 supplemented with 50 μg of hygromycin B (HYG. B) per ml (+). Sample preparation, SDS-PAGE, and Western blotting with antisera raised against the virus capsid protein were performed as described in Materials and Methods. Lanes contain protein lysates prepared from an identical number of wild-type *Leishmania* strain M4147 cells (WILD) or M4147 cells transfected with plasmid pX63-HYG, pHYG-FULL14, or pHYG-INFRAME. Positions of molecular size markers are indicated on the right.

from the transfected plasmid. Taken together, the results clearly demonstrate the existence of RNA transcripts encoding viral proteins in transfected parasites. Thus, drug selection does not interfere with transcription of viral genes from the plasmid DNA vector. It would appear, however, that the vector-encoded transcripts are not efficiently translated (Fig. 4 and 6).

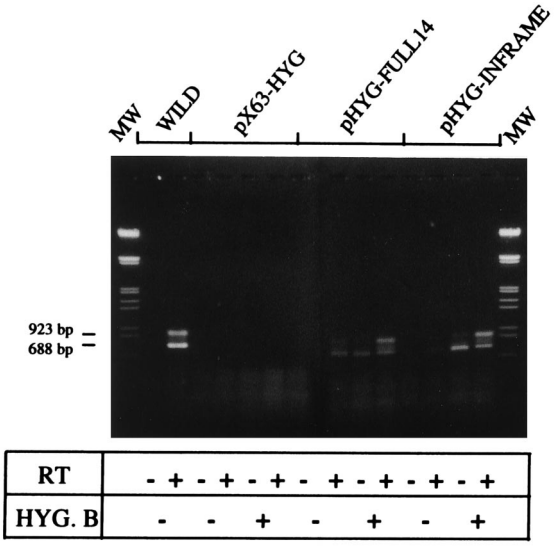

FIG. 5. Drug-resistant parasites lack detectable quantities of minus-sense LRV1-4 RNA. Total cellular RNA was extracted from either wild-type *Leishmania* strain M4147 cells (WILD) or hygromycin B (HYG. B)-resistant parasites transfected with the indicated plasmid as previously described. RT and PCR reactions were accomplished exactly as described for Fig. 1. Control reactions were performed in the absence of reverse transcriptase (RT; −). Alternating lanes contain PCR or RT-PCR products obtained with RNA template isolated from parasites grown in either the absence (−) or presence (+) of hygromycin B at a final concentration of 50 μg/ml. The molecular size markers are *Eco*RI/*Hin*dIII-cut λ DNA (MW).

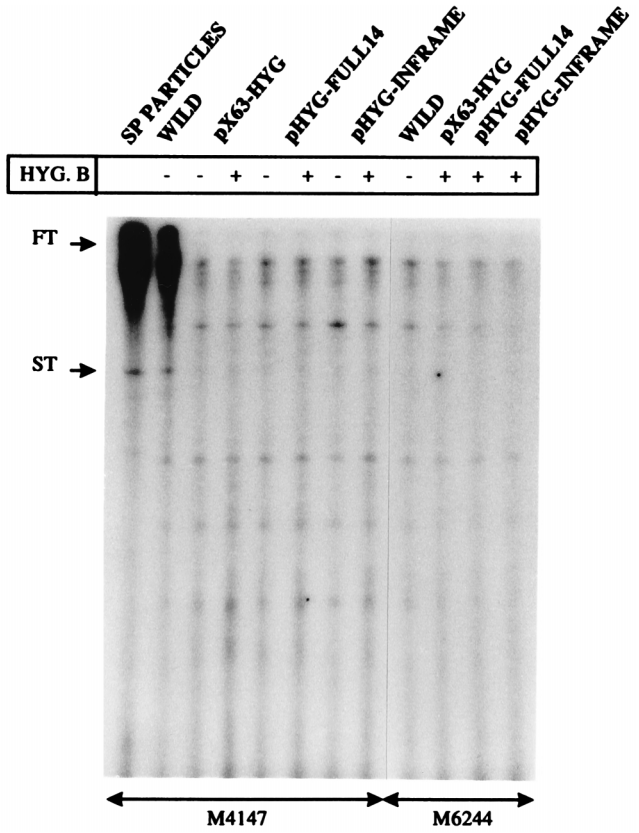

FIG. 6. Viral polymerase activity is not detected in lysates prepared from hygromycin B-resistant cells. Lysates were prepared as previously described from parasites transfected with the indicated recombinant DNA vector (top). Drug-selected parasites were grown in either the absence (−) or the presence (+) of hygromycin B (HYG. B; 50 μg/ml). Viral polymerase activity was assayed in vitro as described in Materials and Methods. Sucrose gradient-purified particles (SP PARTICLES) were prepared from *Leishmania* strain M4147 cells as described by Cadd and Patterson (5) and used for a positive control along with lysates from wild-type M4147 cells (WILD). FT, full-length transcript; ST, short transcript.

Effects of hygromycin B on *Leishmania* growth and viral protein synthesis. To examine the effects of hygromycin B on *Leishmania* growth and viral protein synthesis, wild-type *Leishmania* M4147 cells were grown in medium supplemented with increasing concentrations of hygromycin B. The lowest hygromycin B concentration (5 μg/ml) used in this study had shown a mild effect on parasite morphology and growth rate (data not shown). At 25 μg of hygromycin B per ml, however, cell division rates and parasite survival were severely reduced. Nucleic acids prepared from the same number of cells grown in medium containing either 10 or 25 μg of hygromycin B per ml were examined for the presence of viral dsRNA by EtBr staining (Fig. 3, wild lanes 2 and 3, respectively). Even though cell growth was inhibited at a higher concentration of hygromycin B (25 μg/ml), the intensity of EtBr-stained viral dsRNA was nearly identical to that observed in cells grown in medium lacking the drug. Because wild-type *Leishmania* cells do not survive long-term growth in medium containing hygromycin B at 25 μg/ml (the concentration used for selection of resistant cells in earlier experiments), we examined the effects of the drug selection protocol in short-term parasite growth studies.

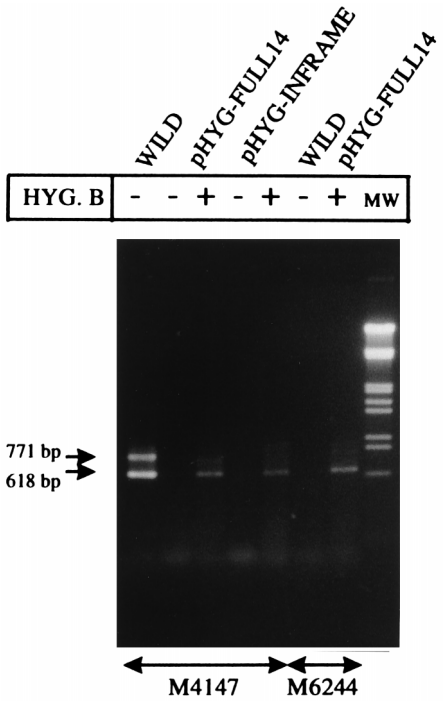

FIG. 7. Expression constructs direct the synthesis of plus-sense viral mRNA in vivo. Whole-cell RNA isolated from identical numbers of M4147 and M6244 cells transfected with the indicated expression vector (top) were reverse transcribed by using Tail-14 as a primer and amplified by a PCR using primers Tail-11 and L3-400. Cells were grown in the absence (−) or presence (+) of hygromycin B (HYG. B; 50 μg/ml) as indicated. Wild-type M4147 and M6244 cells (WILD) were used as controls. Molecular size markers are *Eco*RI/*Hin*dIII-cut λ DNA (MW).

To accomplish this, late-log-phase cultures of *Leishmania* were harvested and resuspended in medium M199 with or without the addition of hygromycin B (25 μg/ml). Equal numbers of cells were harvested every day up to 4 days and processed for total nucleic acid and cell proteins. Hygromycin B (25 μg/ml) dramatically retarded the growth of wild-type parasites (Fig. 8A). EtBr staining of nucleic acids isolated from cultured parasites visualized similar quantities of viral dsRNA regardless of whether parasites were grown in the presence or absence of hygromycin B (Fig. 8B). Likewise, the amounts of immunologically detectable capsid antigen are similar in cells grown in the presence and absence of hygromycin B (Fig. 8C). We could not perform a similar experiment with greater concentrations of hygromycin B (50 μg/ml), such as those used in the drug selection regimens described in earlier experiments, because the parasites do not survive in the absence of a resistance gene (data not shown).

DISCUSSION

There is presently no evidence for the existence of extracellular LRV particles. Weeks et al. (42) have hypothesized that LRV persistently infects its host in the absence of an infectious cycle. Persistent viral infection is well known to modulate gene expression in animal cells (17). We do not yet know whether LRV modulates gene expression in *Leishmania.*

In an effort to examine for possible effects of LRV on parasite phenotype via virus transfer studies, we had prepared a series of cDNA constructs in which viral sequences were assembled in the *Leishmania* expression vector pX63-HYG (9, 10, 14), encoding the selectable gene marker *hph*. Previously, Armstrong et al. (1) showed that LRV particles can be successfully transferred into both uninfected and virus-infected parasites via electroporation, though persistent infection was not established in the study. In this study, we electroporated a selectable plasmid which encodes a full-length cDNA copy of the LRV1-4 genome, including a silent PCR tag within the virus capsid gene sequence, for the purpose of overexpressing LRV1-4 proteins in their natural host, *L. guyanensis* M4147. The experiment is based on the assumption that M4147 cells encode all of the machinery necessary for synthesis of viral protein as well as maintenance of a persistent infection. One of our expectations was that overexpression of viral RNA from a cDNA plasmid template may identify a predicted LRV capsid-polymerase fusion protein and/or provide additional evidence of a polyprotein cleavage event in vivo (35). Much to our surprise, however, the experimental protocol not only eliminated viral infection but also prevented viral protein synthesis from the plasmid vector, presumably due to the actions of hygromycin B.

Hygromycin B is an aminocyclitol antibiotic produced by *S. hygroscopicus* (34) which inhibits protein synthesis in both prokaryotes and eukaryotes through interference with ribosomal translocation as well as with aminoacyl-tRNA recognition (4, 12, 19, 38). Due to the low permeability of hygromycin B in most normal cells, previous studies on the drug as an antiviral agent have concentrated mainly on picornavirus-infected cells (7, 22, 23) and other animal DNA or RNA virus-infected cells (2, 18). In this study, we show that hygromycin B inhibits *Leishmania* growth dramatically at a lower drug concentration (25 μg/ml). In the absence of a resistance gene, cells did not survive at higher drug concentrations (50 μg/ml). However, when a plasmid encoding the *hph* gene was introduced into *Leishmania* cells by electroporation and hygromycin B-resistant cells were selected for several passages, the resistant cells could grow as rapidly as the normal cells even at greater drug concentrations (50 μg/ml), consistent with its extensive use as a selectable marker.

After electroporation and drug selection, the plasmid marker was detected by PCR, confirming that the vector replicates and propagates successfully in *Leishmania* cells. However, drug-resistant parasites harboring selectable vectors encoding LRV1-4 RNA sequences not only failed to establish or maintain virus infection but also failed to express viral proteins. Loss of virus infection could be due to a selective effect of hygromycin B on the translation of viral RNA transcripts. Through RT-PCR, we showed that the synthesis of vector-derived RNA transcripts in *Leishmania* was not prevented by hygromycin B (Fig. 7). However, assays for viral polymerase and capsid proteins were negative, suggesting that inhibition occurs at the level of translation. Several reports show that hygromycin B can inhibit the synthesis of viral proteins, consequently blocking viral replication via its effects on expression of the viral polymerase (18, 23, 29, 31).

Studies with mouse hepatitis virus (MHV) show marked reductions in the level of virus-specific RNA in the presence of hygromycin B (0.5 mM) (30, 32). In other experiments, hygromycin B (0.5 mM) cured (after 108 h) mouse fibroblast LM-K cells of persistent infection by MHV (30). However, in the short-term inhibition experiments presented here (25 μg of hygromycin B per ml; 96 h), the levels of viral dsRNA and capsid protein appeared unaffected despite the severe growth inhibition and the appearance of morphological effects in the parasite host. The working concentration of hygromycin B used in this study was 50 μM, approximately 10-fold lower than that used in MHV-infected mouse L cells. Therefore, the cur-

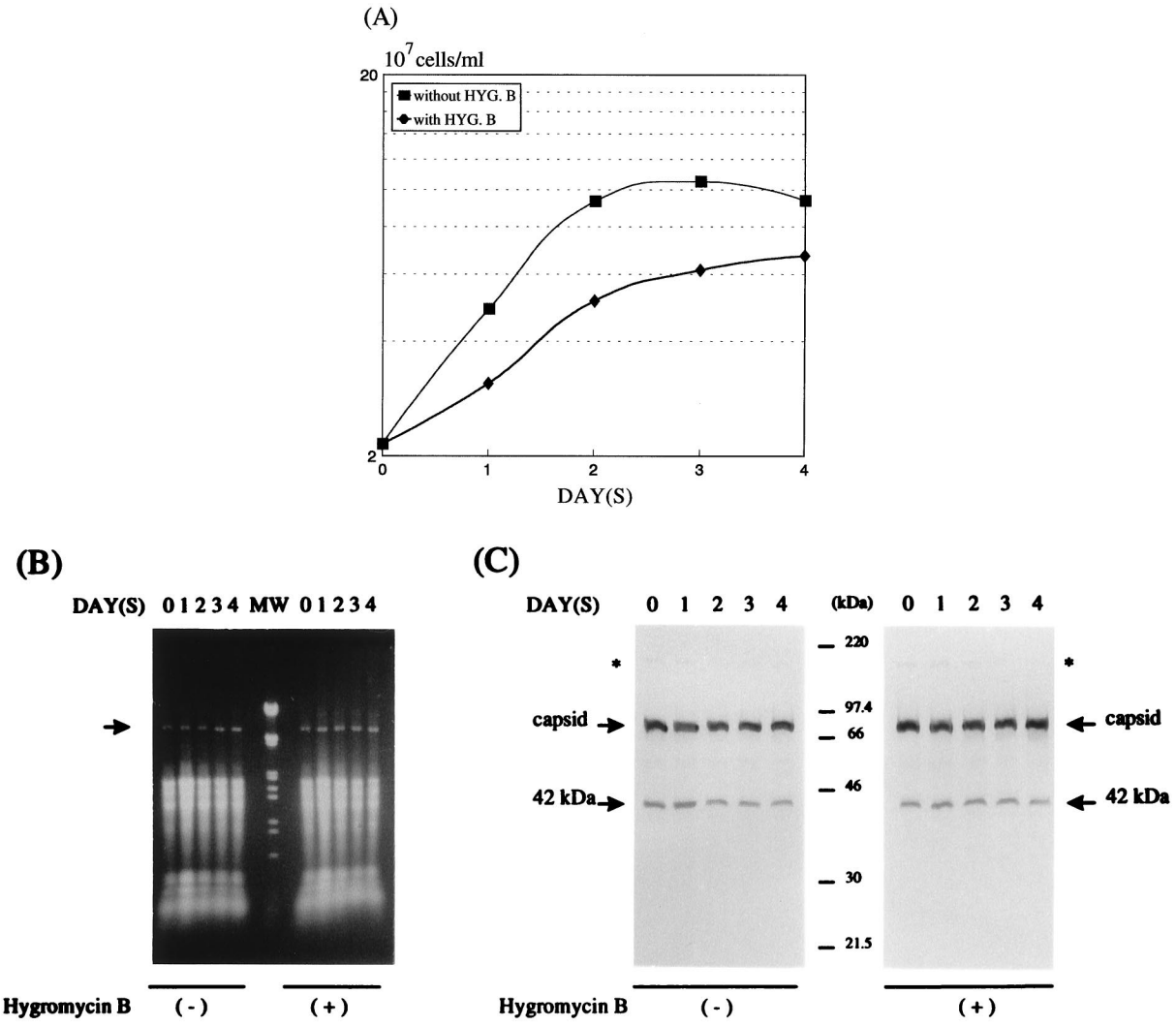

FIG. 8. Effects of hygromycin B on parasite growth, and the synthesis of viral dsRNA and capsid protein. (A) Growth curve of *Leishmania* cultured in the absence or presence of hygromycin B (HYG. B; 25 μg/ml). Cultures were assayed every 24 h over a 4-day period. Equal numbers of cells were harvested daily, and the washed cell pellets were stored at −80°C prior to analysis. (B) Total nucleic acids were prepared by phenol-chloroform extraction, resolved on a 1% agarose gel, and stained by EtBr. The position of viral dsRNA is indicated by an arrow. (C) Equal volumes of frozen whole cells were subjected to SDS-PAGE (10% polyacrylamide) and Western blot analysis with LRV1-4 capsid-specific antiserum as described in Materials and Methods. Size markers (MW) are as in Fig. 7.

ing of *Leishmania* in these experiments may be caused not by an acute response to hygromycin B but rather by a long-term effect wherein the drug preferentially reduces internal translation initiation on viral RNA transcripts. However, hygromycin B is known to bind RNA directly (31) and may exert the antiviral effects reported here through direct binding of viral transcripts, preventing their interaction with cellular translation machinery. Also, because wild-type parasite cultures passaged continuously (6 weeks) in medium containing low concentrations of hygromycin B (10 μg/ml) show no evidence of a reduction in viral protein or viral genomic RNA (data not shown), elimination of LRV from persistently infected parasites could involve the hygromycin B resistance gene product (*hph*) in *Leishmania.*

A *Leishmania* expression vector, pX63-HYG (a modified version of the expression vector pX [9, 25]), was used in this study for the purpose of overexpressing LRV1-4 proteins in their natural parasite host. However, the results of this study indicate that hygromycin B selection is inconsistent with expression of LRV genes and ultimately led to elimination of the persistent viral infection. In the case of *Saccharomyces cerevisiae*, low levels of cycloheximide, a protein synthesis inhibitor, result in the selective curing of M_1 satellite virus in yeast cells containing both L-A and M_1 viruses (13), and cycloheximide is known to act on L29, another 60S subunit protein in yeast cells (15). It has been hypothesized that the curing mechanism in yeast results from a deficiency in 60S subunits that leads to a preferential decrease in translation of the viral mRNA transcripts (8).

Finally, these studies demonstrate that hygromycin B, a protein synthesis inhibitor, can be used to eliminate LRV from persistently infected parasites. While the precise mechanism is not yet known, clearly the cured parasites will provide a valuable tool in studies aimed at delineating the possible effects of LRV on parasite phenotype.

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