Inducible Expression of Suicide Genes in *Leishmania donovani* Amastigotes*

(Received for publication, March 11, 1998, and in revised form, June 11, 1998)

Elodie Ghedin‡§, Hugues Charest¶, Wen-Wei Zhang§, Alain Debrabant**, Dennis Dwyer**, and Greg Matlashewski‡ ††

*From the ‡Institute of Parasitology, McGill University, Macdonald Campus, Ste-Anne-de-Bellevue, Quebec H9X 3V9, Canada and the Immunobiology Section and the **Cell Biology Section, Laboratory of Parasitic Diseases, NIAID, National Institutes of Health, Bethesda, Maryland 20892-0425*

This study tests the feasibility of using the A2 gene regulatory system to create a *Leishmania* cell line in which attenuation is developmentally regulated when the parasite differentiates from promastigotes to amastigotes. The *Leishmania donovani*-inducible A2 gene regulatory system was used to differentially express in amastigotes two potential suicide genes: a truncated version of the *L. donovani* 3'-nucleotidase/nuclease expressed in the cytoplasm and the herpes simplex virus thymidine kinase gene. These genes were inserted between A2 noncoding regulatory sequences for up-regulation of expression in amastigotes. The accumulation of toxic products affected cell replication and viability both *in vitro* and *in vivo*. The inducible expression of toxic gene products represents a valuable tool for the development of safe and effective vaccines.

The *Leishmania* species are parasitic protozoa responsible for various clinical forms of leishmaniasis ranging from self-curing skin ulcers to an often fatal visceral disease. The *Leishmania* parasite oscillates between two distinct host-specific developmental stages in the course of their life cycle. Flagellated promastigotes are found in the gut lumen of the sandfly vector, while nonmotile amastigotes exist intracellularly in the vertebrate host macrophages (1).

The *Leishmania donovani* A2 gene family of proteins was shown to be amastigote stage-specific (2) and essential for survival of this parasitic protozoan in its mammalian host (3). Previously, it was demonstrated that the A2 gene regulatory system could be used as a model to study the regulation of gene expression in *Leishmania* cells (4). The developmental expression of A2 gene transcripts and protein products can be induced *in vitro* in cultured promastigotes by a combination of pH and temperature shifts, conditions associated with the passage of the parasite from the insect vector to the phagolysosomal environment within the mammalian macrophage (2, 5). A2 gene expression is post-transcriptionally regulated, and selectable marker transcripts carrying the A2 mRNA 3'-untranslated region display amastigote-specific expression in transfected *Leishmania* cells (4).

In this study, we tested whether the A2 regulatory sequences could be used to developmentally express suicide genes toward developing an attenuated *L. donovani* strain. The suicide genes tested were the herpes simplex virus thymidine kinase gene (*hsv-tk*) previously used by LeBowitz et al. (6) as a negative selectable marker in *Leishmania major* and a truncated version of the *L. donovani* 3'-nucleotidase/nuclease (3'NT/Nu). The native *L. donovani* 3'NT/Nu is an externally oriented surface membrane enzyme capable of hydrolyzing 3'-nucleotides and nucleic acids (7–9). Developmental expression from both episomal and integrated A2-toxic gene chimeric constructs was tested *in vitro* in differentiated amastigotes. We demonstrate that the pattern of expression of these toxic genes under the control of A2 gene sequences was differential, although not completely amastigote-specific. The truncated 3'NT/Nu expression affected growth of amastigotes, and TK expression induced sensitivity of amastigotes to the anti-herpes drug, ganciclovir.

**EXPERIMENTAL PROCEDURES**

*Leishmania Strain and Culture Media*

Promastigotes and amastigotes of a cloned line of *L. donovani* (1S, clone 2p, from the 1S strain (10, 11), World Health Organization designation, MHOM/SD/82/1S-c12p) were cultured as follows. Promastigotes were grown at 28 °C in M199 medium (Life Technologies, Inc.) supplemented with 10% defined heat-inactivated fetal bovine serum (HyClone Laboratories Inc., Logan, UT) and 25 mM HEPES (ICN Biomedicals Inc., Aurora, OH), pH 6.8. Axenic amastigotes were grown at 37 °C in RPMI 1640 medium supplemented with 20% fetal bovine serum and 20 mM MES (Sigma), pH 5.5 (12). Cell growth assays of TK transfectants were performed in media supplemented with ganciclovir sodium (0.1 μM stock solution in deionized water; Cytovene; Hoffman-La Roche).

Axenic amastigotes used in Western and Northern blot experiments were obtained by transferring late log-phase promastigotes to medium at 37 °C, pH 5.5. Cells were used 12–18 h after the transfer.

*Nucleic Acids Preparations and Analyses*

Total RNA was extracted from promastigotes and axenic amastigotes by the phenol-chloroform-guanidium isothiocyanate method using TRizol (Life Technologies, Inc.). DNA samples were prepared from promastigotes using the “mini-prep” procedure described by Medina-Acosta and Cross (13). Southern and Northern blot hybridizations were carried out in 1 x NaCl, 1% SDS, and 10% dextran sulfate for 18 h at 65 °C. DNA probes were prepared from agarose gel-purified fragments.

* The abbreviations used are: TK, thymidine kinase; kb, kilobase pairs; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; MES, 2-(N-morpholino)ethanesulfonic acid.
Suicide Genes in L. donovani

**Fig. 1.** Schematic representation of the constructs used for up-regulation of foreign gene expression in amastigotes. **Upper panel,** schematic outline of the A2/A2rel genomic organization on a 850-kb chromosome. Arrows delineate the A2 and A2rel mature mRNA encoding regions. The open box represents the repeated region of the A2 open reading frame. This genomic map also shows the upstream (PRO) and downstream (TAIL) untranslated sequences from the A2 gene. **Panel A,** schematic representation of the constructs used in this study. **Panel B,** schematic representation of the constructs used in this study. **Schematic representation of the constructs used in this study.**

**Plasmid Constructs**

Schematic representations of plasmids used in this study are depicted in Fig. 1. **NEOPT and pKSneo—**The NEOPT plasmid was designed to allow the differential expression of the neo gene in amastigotes (4). The construct carries the Pro (P) element, which represents the A2/A2rel intergenic region and the 5’ end of the A2rel coding sequence, a synthetic 92-base pair trans-splicing element (pyt), the neomycin resistance gene, and the Tail (T) element, which represents the 3’ untranslated region of A2. The construction of the pKSneo plasmid was described in Zhang et al. (5). In distinction with the NEOPT plasmid, the neo gene in pKSneo is inserted upstream from the Pro element. The Pro element provides a polyadenylation site for the neo gene, whereas trans-splicing of neo at the 5’ end is controlled by a pyt element. Thus, in pKSneo, the neo gene mRNA is constitutively expressed in both promastigotes and amastigotes.

**pKSneo-3’NT—**A truncated version of the 3’-nucleotidease/nuclease gene (3’nt/nu) was amplified by a polymerase chain reaction (PCR) from plasmid CI-2 (14), which contains a 4.5-kb fragment carrying the 3’nt/nu coding sequence (1.4 kb). The signal peptide and the terminal anchor sequences were not included in the PCR product. Hence, the sense oligonucleotide (5’-gactcatgg atg aacctcttcatgctgca-3’) created an initiation codon, whereas the antisense oligonucleotide (5’-gacttgatca caagggcacaactgctgca-3’) created a stop codon. The PCR product (1.1 kb) was first inserted into the pCRII cloning vector (TA cloning system, Invitrogen). Subsequently, the SpeI and XbaI restriction sites from the pCRII vector restriction cassette were used to subclone it into the SpeI site of the pKSneo vector.

**Constructs for Targeting tk into the A2 Locus**

The neo gene was amplified from the pALTneo vector along with an α-tubulin intergenic region from L. enriettii, which provides a trans-splicing acceptor site and a polyadenylation acceptor site (15). The following primers were used: sense, 5’-gg atgact ctcttgctttcgac-3’ (bases in bold represent a BglII site); antisense, 5’-tacgactcactagggc-3’. The neo intergenic region (Neo-IG) product (1.8 kb) was subcloned BglII/BamHI in the BamHI site of vector pGEM-11zf (Promega). The Pro and Tail elements were inserted on either side of the neo-intergenic or neo-intergenic-th sequences to permit targeting by homologous recombination (Fig. 1B). For the pGEMneo construct, the Tail was subcloned directionally at the XbaI and HindIII restriction sites. For the pGEMneo-TK plasmid, the tk gene was first inserted upstream from the Tail element on a KS pBluescript (Stratagene, LaJolla, CA) (KSTail plasmid). The 1.3-kb tk coding region was amplified from the pFG5 vector which contains the tk gene (16). The following primers were used: sense, 5’-gg tctaga cctgaaccagcaca-3’; antisense, 5’-gg tctaga ggtctgggctgatc-3’ (bases in bold represent a XbaI site). The PCR product (1.3 kb) was first inserted in the PCRII cloning vector and then subcloned with the XbaI restriction sites from the PCRII vector in front of the Tail element. The plasmid-created KS/Tk-Tail was digested BglII/HindIII (the BglII site is located upstream from the tk initiation codon) and the TK-Tail element was subcloned BamHI/HindIII at the 3’ end of the pGEM construct.

**Transfections**

Transfections and selection of clones were performed as described previously (4). Uncloned populations of recombinant promastigotes were used for experiments with episomal copies of the truncated 3’nt/nu and neo genes. Recombinant promastigotes carrying neo and tk genes targeted into the chromosomal A2 gene cluster were selected with minimal doses of G418 (10 μg/ml), and clonal populations were selected by limiting dilutions.

**Protein Analyses**

Promastigotes and amastigotes were harvested by centrifugation and washed three times at 4 °C in 10 mM Tris, 150 mM NaCl, pH 7.5 buffer. Pellets were resuspended at a concentration of 5 × 10^6 cells/ml in 2 × SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, 100 mM dithiothreitol, 0.005% bromphenol blue), denatured by boiling for 10 min, and separated on a 10% SDS-PAGE (17). The 3’-nucle-cysteine protease activity of the native 3’NT/Nu and the truncated 3’NT/Nu were visualized in the SDS-PAGE bands by in situ staining using 3’-AMP as a substrate and malachite green as the staining agent (18). The 3’NT/Nu proteins were detected in Western blot analyses using a rabbit anti-3’NT/Nu peptide serum and a goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) (as described in Ref. 14). Bound secondary antibodies were detected using the ECL Western blotting detection system (Amersham Pharmacia Biotech). In Fig. 4B, the secondary antibody used was a biotinated goat anti-rabbit IgG, and bound antibodies were detected with streptavidin-conjugated horseradish peroxidase (Amersham Pharmacia Biotech) and 3,3’-diaminobenzidine (Sigma) in a 0.03% H₂O₂ solution.

**Infection of Macrophages in Culture**

Mouse bone marrow cell preparations and infections with L. donovani cells were performed as described previously (19). Late log-phase promastigotes were used to infect cultures of macrophages at a ratio of 20 parasites/macrophase for 24 h at 37 °C, after which nonphagocyted parasites were washed out by low speed centrifugation. Infected cells were incubated at 37 °C in RPMI 1640 medium supplemented with 10% fetal bovine serum, and intracellular infection levels were monitored daily by Giemsa staining.

**Infections in Mice**

Infections were performed as described in Zhang and Matlashewski (3). In brief, female BALB/c mice (Charles River, Montreal, Canada) 6–8 weeks old were infected via the tail vein with 1.5 × 10^6 wild-type or transfected promastigotes. Four weeks post-infection, mice were examined for L. donovani parasite burden by counting the number of amastigotes in imprints of the liver stained with Giemsa. Liver parasite...
burdens are expressed as Leishman-Donovan units (number of amastigotes/1000 cell nuclei × liver weight (g)) (20).

RESULTS

Neomycin (G418) Resistance of neo Transfectants—As a first step toward assessing the A2 gene regulatory sequences for the developmental expression of suicide genes in L. donovani, the effect of gene copy number versus basal protein expression levels was examined in promastigotes. Promastigotes were transfected with the neo gene, used both as a selectable marker and as a reporter gene, present in single copy (targeted) or in a multicopy plasmid. The effect of the differential expression of NEO was monitored indirectly by determining the growth of promastigotes cultured in the presence of various concentrations of the drug G418. The resistance to G418 of L. donovani promastigotes transfected with the NEOPT plasmid (episomal) and of a targeted clone (R2) carrying the neo gene into the A2 genomic locus are shown in Fig. 2. R2 and NEOPT are described in Charest et al. (4). Wild-type 1S2D promastigotes were very sensitive to G418 (Fig. 2, lower panel); the EC$\text{}_{50}$ was estimated to be 4 µg/ml.

R2 promastigotes multiplied slowly in medium containing up to 100 µg/ml G418; however, growth was inhibited at ≥150 µg/ml. In contrast, promastigotes carrying the neo gene in episomal vectors readily multiplied in culture medium containing up to 500 µg/ml. Thus, episomally transfected promastigotes, which contain plasmid constructs in multiple concatenated copies (21), were more resistant than targeted promastigotes. Nevertheless, an increase in drug concentration in the medium correlated with a decrease in growth for both targeted and plasmid-carrying promastigotes.

Because drug pressure was shown to increase the plasmid copy number in transfected Leishmania cells (21, 22), the concentration of G418 in the culture medium was used to modulate the plasmid copy number, and therefore the expression of episomal toxic genes, for the experiments described below.

Differential Expression of the Truncated 3'NT/Nu from an Episomal Construct—The A2 gene regulatory sequences were tested for the expression of toxic gene products from an episomal construct (refer to Fig. 1 for plasmid constructs). As a potential toxic gene, a truncated version of the L. donovani 3'NT/Nu was used in which the N-terminal signal and the C-terminal anchor sequences were deleted from the 3'NT/Nu sequence (pKSneo-3'NT). The assumption was that the lack of signal sequence would prevent the targeting of the protein to the endoplasmic reticulum, which is required for its expression to the surface of the cell. 3'NT/Nu activity would then be expressed in the cytoplasm and potentially be toxic to Leishmania.

Expression of the truncated 3'NT/Nu RNA in pKSneo-3'NT transfected cells grown in various G418 concentrations (ranging from 0 to 400 µg/ml) is shown in Fig. 3A. The size of the 3'NT/Nu transcript (2.5 kb) derived from the pKSneo-3'NT plasmid was consistent with RNA processing at an artificial trans-splicing acceptor site located at the 3' end of the Tail element (pyt). As expected for episomal copies, the truncated 3'NT/Nu mRNA was differentially expressed in axenic amastigotes and was increased in proportion to the G418 concentration. Denatometric analyses revealed that truncated 3'NT/Nu transcripts accumulated to an 8-fold higher level in axenic amastigotes grown in 400 µg/ml than in 25 µg/ml G418. Further, when grown at identical concentrations of G418, axenic amastigote expression of truncated 3'NT/Nu mRNA was on average 5-fold higher than in promastigotes.

Protein accumulation paralleled transcript expression in the transfected cells (data not shown). The endogenous 3'NT/Nu has an apparent molecular mass of 43 kDa whereas that of the truncated 3'NT/Nu is 38 kDa. In both promastigotes and axenic amastigotes there was a dose-dependent expression of the truncated 38-kDa protein associated with concentrations of G418 in the culture medium. Also in agreement with the Northern blot results of above, more truncated 38-kDa 3'NT/Nu was detected in axenic amastigotes than in promastigotes.

Activity of the truncated 3'NT/Nu was determined in promastigotes and axenic amastigotes by staining renatured proteins for 3'-nucleotidase activity using 3'-AMP as substrate. The levels of endogenous and truncated 3'NT/Nu proteins expressed in promastigotes and axenic amastigotes are shown in the left panel of Fig. 3B, whereas the right panel shows the
above, were subjected to SDS-PAGE, Western blot analysis (Fig. 4A, upper panel). In preliminary experiments, pKSneo transfected cells showed no diminution of growth in G418 concentrations ranging from 25 to 400 µg/ml. Further, there was no significant difference in cell growth between control pKSneo promastigotes cultured at 25 µg/ml G418 and pKSneo-3′NT promastigotes grown at G418 concentrations ranging from 0 to 400 µg/ml (Fig. 4A). These results strongly suggested that the accumulation of the truncated 3′NT/Nu, despite its apparent low specific activity, was toxic to *Leishmania* axenic amastigotes.

We examined in parallel the effect of the truncated 3′NT/Nu on amastigote survival in macrophages. Mouse bone marrow macrophages were infected for 24 h with transfected promastigotes previously cultured in G418 concentrations ranging from 0 to 400 µg/ml. Proliferation of the resulting amastigote population within macrophages was monitored over a 5-day period. There was no significant difference in the ability of pKSneo- and pKSneo-3′NT transfected promastigotes to infect macrophages (72% of macrophages were infected versus 65–82%, respectively). However, over the 5-day incubation period, there was a significant difference in growth among these transfected amastigotes (Fig. 4B). The growth of truncated 3′NT/Nu expressing amastigotes was significantly reduced when promastigotes were previously cultured at G418 concentrations ≥25 µg/ml, compared with the drug-free control and pKSneo transfected amastigotes. These results are in agreement with the *in vitro* cell growth studies (Fig. 4A), which showed that cell growth was hindered by increasing levels of the truncated 3′NT/Nu expression in axenic amastigotes. Further, macrophage infection levels were also reduced for amastigotes expressing the truncated 3′NT/Nu. At day 5, 48% of macrophages were infected with pKSneo-3′NT amastigotes previously grown as promastigotes in 25 µg/ml G418, whereas virtually all macrophages were infected with pKSneo amastigotes and pKSneo-3′NT amastigotes from promastigotes grown in G418-free medium.

The effect of pKSneo-3′NT transfection on the ability of *Leishmania* cells to establish an *in vivo* infection in mice was also determined. Promastigotes used to initiate the mouse infections were cultured in G418 concentrations ranging from 0 to 400 µg/ml. Four weeks post-infection, mice were sacrificed, and parasite burdens were determined by counting the number of amastigotes in liver impressions. Values are expressed as Leishman-Donovan units in Fig. 4C. No significant difference in the parasite burden was observed among mice infected with wild-type promastigotes, promastigotes transfected with the pKSneo control plasmid, or promastigotes transfected with the pKSneo-3′NT plasmid and cultured without G418. However, significant differences in amastigote burdens were observed in mice infected with transfected promastigotes previously cultured in 100, 200, and 400 µg/ml G418. These results are consistent with those obtained in the *in vitro* macrophage infection experiments above showing that expression of plasmid-derived 3′NT/Nu decreased either the growth or viability of amastigotes.

Targeting of a Suicide Gene into the A2 Coding Locus—In subsequent experiments, the efficacy of the developmental expression of a gene with toxic potential was tested from a construct targeted into the A2 locus. Because the truncated 3′NT/Nu did not appear to affect the viability of transfected cells carrying low episomal gene copy number, presumably because of its low specific intracellular activity, the herpes

---

**Fig. 3.** A, Northern blot analyses showing differential expression of the truncated 3′NT/Nu and increased accumulation through drug pressure. pKSneo-3′NT transfected promastigotes were cultured in media at different concentrations of G418. Total RNA was extracted from log-phase promastigotes. Amastigote RNA was obtained by switching log-phase promastigotes to amastigote culture conditions and extracting RNA 12 h later. Membranes were hybridized with a labeled 1.1-kb sequence from the 3′nt/nu gene. Equal loading was verified by staining the denatured RNA with ethidium bromide in the agarose gel prior to Northern blot (shown on the bottom panel). B, truncated 3′NT/Nu protein expression and activity. 2.5 µg of total protein from promastigotes (Pro) grown in 400 µg/ml G418 and amastigotes (Ama), obtained as above, were subjected to SDS-PAGE, Western blot analysis (left panel), and an enzyme activity assay (right panel). 3′NT activities were visualized in these gels by *in situ* staining using 3′-AMP as a substrate. The endogenous 3′NT/Nu is present at 43 kDa while the truncated 3′NT/Nu migrates at 38 kDa. Note: truncated 3′-nucleotidase activity was only observed in amastigotes.

activity stain for 3′-nucleotidase activity. The Western blot clearly demonstrated the overexpression of the truncated 3′NT/Nu in the amastigote stage compared with the promastigote stage. Enzyme activity of the truncated 3′NT/Nu was detected in the transfected axenic amastigotes but was not detectable in promastigotes. Clearly, the specific activity of the truncated 3′NT/Nu was remarkably low compared with that of the endogenous enzyme, probably because it was not targeted to the endoplasmic reticulum where its normal processing occurs (i.e., glycosylation).

**Induction of the Truncated 3′NT/Nu Leads to Reduced Amastigote Viability**—To test whether differential expression of the truncated 3′NT/Nu was toxic to the *Leishmania* cells, pKSneo-3′NT plasmid transfected promastigotes and axenic amastigotes were grown in their media supplemented with various concentrations of G418. Axenic amastigote growth was inhibited in proportion to the increasing drug concentrations in the medium (Fig. 4A, lower panel). This corresponded to the up-regulation of expression of the truncated enzyme in these cells. At 400 µg/ml, axenic amastigote growth was completely inhibited. In contrast, promastigotes transfected with the pKSneo-3′NT plasmid were not affected by the truncated 3′NT/Nu enzyme (Fig. 4A, upper panel).
simplex virus thymidine kinase gene (tk) was used for gene targeting experiments. The expression of tk per se does not affect Leishmania cell growth (6) but renders such transfec-
tants sensitive to nucleoside analogues (e.g. anti-herpes drug ganciclovir).

The strategy used to target both the neo and tk genes into the A2 locus by homologous recombination is outlined in Fig. 5A. Results of the Southern blot analyses confirmed the correct insertion of the neo and tk genes into the A2 locus (Fig. 5B) and the localization of the neo, intergenic, and tk sequences up-stream from the 3′-untranslated region in the genome.

Northern blots of the TK-targeted clone (R.tk) and of the control clone (R.ctl), which only contains the neo gene, demonstrated that induction of TK transcripts was up-regulated in the axenic amastigotes (Fig. 5C). The TK transcript length (2.8 kb) was consistent with trans-splicing occurring at the 3′-end of the A2 Tail sequence, and the α-tubulin intergenic region provided a polyadenylation site. As expected, the NEO transcript is not differentially regulated (cf. Fig. 1B). Its size (1.4 kb) corresponds to processing occurring in the Pro and the α-tubulin intergenic regions.

The cell growth of promastigotes and axenic amastigotes cultured in media containing different concentrations of ganciclovir were analyzed (Fig. 6A). L. donovani lines were inocu-
lated into media containing the indicated amounts of ganciclovir and allowed to proliferate until control cultures lacking ganciclovir had reached late log phase (1 \times 10^7 cells/ml) at which time the EC_50 (defined as the concentration of ganci-
clovir that reduced the cell numbers by 50%) was calculated (23). R.tk promastigotes and axenic amastigotes were differentially sensitive to ganciclovir, with estimated EC_50 of 25 μM and 4.5 μM, respectively. In contrast, the growth of control (R.ctl) promastigotes and axenic amastigotes was unaffected by ganciclovir (Fig. 6A). In vitro infection studies using bone marrow macrophages were performed with the R.tk clones (Fig. 6B).
Infected macrophages were incubated in medium at seven different ganciclovir concentrations ranging from 0 to 1 mM. Infection of macrophages and growth of amastigotes within macrophages were monitored over a 5-day period. At day 0, there was on average for all ganciclovir concentrations a ratio of 7 amastigotes to 1 infected macrophage. At day 5 this ratio was 8:1 for \( R.\) \( tk \) in drug-free medium and in 1 mM ganciclovir. At 10 mM ganciclovir and higher concentrations, amastigote growth was dramatically affected with infection levels at a ratio of 2:1 (Fig. 6B).

These data show that promastigotes that carried the \( tk \) gene in the A2 genomic locus were relatively unaffected in culture by the presence of ganciclovir until high concentrations of the drug were reached. In contrast, axenically cultured amastigotes that carried the \( tk \) gene in the A2 genomic locus were relatively unaffected in culture by the presence of ganciclovir until high concentrations of the drug were reached. In contrast, axenically cultured amastig-
ones or amastigotes within macrophages displayed a dose-dependent sensitivity to the drug. These results demonstrated the feasibility of targeting negative selectable markers into the A2 locus for their developmental up-regulation in amastigotes.

**DISCUSSION**

In this study, we have demonstrated the feasibility of using the A2 gene regulatory system to generate *L. donovani* recombinant lines in which attenuation is developmentally regulated during the promastigote to amastigote cytodifferentiation. The pattern of expression of toxic genes under the control of A2 gene sequences was differential, although not completely amastigote-specific. Whereas promastigotes were generally unaffected by low expression of the suicide genes, the growth of axenic amastigotes was hampered. Similarly, amastigote growth within host macrophages could be completely inhibited. We also showed by gene replacement that the A2 locus can control the expression of negative selection genes.

Two different suicide gene systems carried on episomal and targeting constructs were tested. Both suicide genes were placed under the control of the A2 untranslated regions in recombinant *L. donovani* cells. In a first series of experiments, a truncated version of the 3'-nucleotidase/nuclease, an endogenous *L. donovani* enzyme, was inserted between two A2 non-coding sequences to be developmentally expressed in amastigotes. The 3'-nucleotidase/nuclease is an externally oriented surface membrane glycoprotein responsible for the hydrolysis of 3' nucleotides and single strand nucleic acids (24). The truncated 3'NT/Nu used in our constructs, which lacked both the signal peptide and the membrane anchoring sequences, was expressed in the cytosol (data not shown), which led to reduced amastigote cell viability. The truncated 3'NT/Nu was not post-translationally modified by glycosylation because it was not targeted to the endoplasmic reticulum, where its normal processing occurs. Campbell et al. (25) have demonstrated that the glycosylation of the native enzyme plays an important role in regulating its specific activity. This is consistent with the observation that the 3'NT/Nu expressed in *Escherichia coli* had a 50-fold lower activity than the native enzyme of *Leishmania*.

To modulate the expression of the truncated 3'nt/nu gene in amastigotes as well as in promastigotes, we took advantage of the increase in plasmid copy number resulting from the G418 selective pressure. Despite its low activity, high levels of truncated 3'NT/Nu expression resulted in a reduced amastigote viability. Decreased cell growth of axenic amastigotes correlated with a decreased replication of amastigotes in macrophages and mice. In the absence of G418, pressure-transfected DNA is maintained in *Leishmania* cells for a relatively long period of time (26). However, in *vivo*, transfecants would be expected to eventually begin to lose their plasmids. Thus, a generation of stable attenuated cell lines requires the chromosomal integration of the suicide gene. Clearly, the low specific activity of the truncated 3'NT/Nu that was observed, even at high copy number, was not suitable for the generation of attenuated lines.

To directly assess the feasibility of differentially inducing in amastigotes the expression of suicide genes when targeted into the chromosomal A2 locus, we used the th/ganciclovir suicide gene system. LeBowitz et al. (6) and Muyombwe et al. (27, 28) describe the expression of the *hsu-tk* gene on episomal vectors in *L. major* for the creation of ganciclovir-sensitive strains. We showed that the *hsu-tk* gene under the control of A2 regulatory sequences is differentially expressed in amastigotes. As expected, the expression of the herpes virus *tk* gene in transfected promastigotes results in sensitivity to nucleoside analogues, such as the anti-herpes drug ganciclovir. However, the EC50 calculated for axenic amastigotes was 5 times lower than for promastigotes, correlating with an up-regulation of the *tk* gene expression in amastigotes.

The major advantage of the A2 gene regulatory system to establish attenuated cell lines is that attenuation can be developmentally regulated. Such attenuated *L. donovani* cell lines should prove useful in examining certain aspects of the immune response during infection, under conditions where the infection is not lethal. Further, it may hold potential for the development of an attenuated vaccine.

**Acknowledgments**—We thank Dr. Thomas Christie, Laboratory of Parasitic Diseases (NII), for the pPG5 plasmid and Dr. Dyann Wirth, Harvard School of Public Health, for the pAE7Tneo plasmid.

**REFERENCES**

1. Molyneux, D. H., and Killick-Kendrick, R. (1987) in *Leishmaniasis in Biology and Medicine* (Peters, W., and Killick-Kendrick, R., eds) Vol.1, pp. 121–176, Academic Press, New York
2. Charest, H., and Matlashewski, G. (1994) *Mol. Cell. Biol.* 14, 2975–2984
3. Zhang, W. W., and Matlashewski, G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 8807–8811
4. Charest, H., Zhang, W. W., and Matlashewski, G. (1996) *J. Biol. Chem.* 271, 17081–17089
5. Zhang, W. W., Charest, H., Ghedin E., and Matlashewski, G. (1996) *Mol. Biochem. Parasitol.* 78, 79–90
6. LeBowitz, J. H., Cruz, A., and Beverley, S. M. (1992) *Mol. Biochem. Parasitol.* 51, 321–332
7. Gottlieb, M., Gardiner, P. R., and Dwyer, D. M. (1988) *Comp. Biochem. Physiol.* 83B, 63–69
8. Dwyer, D. M., and Gottlieb, M. (1984) *Mol. Biochem. Parasitol.* 10, 139–150
9. Gottlieb, M., and Dwyer, D. M. (1983) *Mol. Biochem. Parasitol.* 7, 303–317
10. Dwyer, D. M. (1977) *Exp. Parasitol.* 41, 341–358
11. Stauber, L. A. (1966) *Exp. Parasitol.* 18, 1–11
12. Joshi, M., Dwyer, D. M., and Nakhasi, H. L. (1993) *Mol. Biochem. Parasitol.* 58, 345–354
13. Medina-Acosta, E., and Cross, G. A. M. (1993) *Mol. Biochem. Parasitol.* 59, 327–330
14. Debrabant, A., Gottlieb, M., and Dwyer, D. M. (1995) *Mol. Biochem. Parasitol.* 71, 51–63
15. Laban, A., Tobin, J. F., Curotto de Laflaive, M. A., and Wirth, D. F. (1990) *Nature* 345, 572–574
16. Colbere-Garapin, F., Chousterman, S. L., Horodniceanu, F., Kourilsky, P., and Garapin, A. C. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 3755–3759
17. Laemmli, U. K. (1970) *Nature* 227, 680–685
18. Zlotnick, G. W., Mackow, M. C., and Gottlieb, M. (1987) *Comp. Biochem. Physiol.* 87B, 629–635
19. Moor, R. J., Labrecque, S., and Matlashewski, G. (1993) *J. Immunol.* 150, 4457–4465
20. Murray, H. W., Oca, M., Granger, A., and Schreiber, R. (1989) *J. Clin. Invest.* 83, 1253–1259
21. Papadopoulos, B., Roy, G., and Ouellette, M. (1994) *Mol. Biochem. Parasitol.* 63, 39–49
22. Zhang, W. W., Charest, H., and Matlashewski, G. (1995) *Nucleic Acids Res.* 23, 4073–4080
23. Ellenberger, T. E., and Beverley, S. M. (1989) *J. Biol. Chem.* 264, 15094–15103
24. Sacci, J. B., Campbell, T. A., and Gottlieb, M. (1990) *Exp. Parasitol.* 71, 158–168
25. Campbell, T. A., Zlotnick, G. W., Neubert, T. A., Sacci, J. B., Jr., and Gottlieb, M. (1991) *Mol. Biochem. Parasitol.* 47, 109–111
26. Coburn, C. M., Otteman, K. M., McNeely, T., Turco, S. J., and Beverley, S. M. (1991) *Mol. Biochem. Parasitol.* 46, 169–180
27. Muyombwe, A., Olivier, M., Ouellette, M., and Papadopoulos, B. (1997) *Exp. Parasitol.* 85, 35–42
28. Muyombwe, A., Olivier, M., Harvie, P., Bergeron, M. G., Ouellette, M., and Papadopoulos, B. (1998) *J. Infect. Dis.* 177, 188–195

A. Debrabant and D. Dwyer, unpublished data.