Differential Regulation of Multiple Glucose Transporter Genes in *Leishmania mexicana*.

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We have studied the structure and expression of glucose transporter genes in the parasitic protozoan *Leishmania mexicana*. Three distinct glucose transporter isoforms, LmGT1, LmGT2, and LmGT3, are encoded by single copy genes that are clustered together at a single locus. Quantitation of Northern blots reveals that LmGT2 mRNA is present at ~15-fold higher levels in promastigotes, the insect stage of the parasite life cycle, compared with amastigotes, the intracellular stage of the life cycle that lives within the mammalian host. In contrast, LmGT1 and LmGT3 mRNAs are expressed at similar levels in both life cycle stages. Transcription of the LmGT genes in promastigotes and axenically cultured amastigotes occurs at similar levels, as measured by nuclear run-on transcription. Consequently, the ~15-fold up-regulation of LmGT2 mRNA levels in promastigotes compared with amastigotes must be controlled at the post-transcriptional level. Measurement of LmGT2 RNA decay in promastigotes and axenic amastigotes treated with actinomycin D suggests that differential mRNA stability may play a role in regulating glucose transporter mRNA levels in the two life cycle stages.

*Leishmania* are important human pathogens (1) whose life cycle involves transmission of an extracellular flagellate (promastigote) from the alimentary tract of a sandfly vector to a mammalian host, where the parasite multiplies intracellularly in macrophages as an aflagellate (amastigote) (2). The pronounced biological changes that occur during the life cycle underscore the importance of developmental regulation of gene expression to parasite survival in these contrasting and hostile environments. Yet, the mechanisms responsible for regulation of gene expression in *Leishmania* and related kinetoplastid protozoa have not been extensively studied. To probe the mechanisms controlling gene expression during the parasite life cycle, we are studying a family of glucose transporters that contains both developmentally regulated and constitutively expressed members.

Metabolism of glucose is of considerable interest in these parasites since it provides a major source of energy to the promastigote (3), and it involves some novel biochemical features such as specialized membrane-bound organelles called glycosomes (4) that contain many of the glycolytic enzymes. In *Leishmania* species, the permeases for glucose are structurally related to the 12 transmembrane-spanning mammalian facilitative glucose transporters (5, 6). Previous studies with *Leishmania enriettii* (5) have shown that the mRNAs encoding these glucose transporters are present at relatively abundant levels in promastigotes but are dramatically down-regulated in amastigotes. Down-regulation of glucose transporter expression in the amastigote presumably reflects the lower glucose concentration that the parasite encounters in the macrophage. Indeed, *Leishmania* amastigotes transport much less glucose than promastigotes (7) and derive metabolic energy primarily from fatty acid oxidation (8). Thus, the glucose transporter genes encode proteins that are likely to play an important role in parasite life cycle adaptation, and they present an attractive model in which to address the molecular mechanisms of gene regulation during the parasite life cycle.

However, the study of developmentally regulated gene expression in *Leishmania* has been hampered by the inherent difficulty in obtaining substantial numbers of viable, pure amastigotes. Furthermore, experiments involving expression of wild-type or altered genes from extrachromosomal expression vectors (9) are impractical in intracellular amastigotes, since transfected parasites would have to be cultured in the presence of selective drugs, such as neomycin or hygromycin, which are toxic to the host macrophages. One way to overcome the technical difficulties of studying gene expression in amastigotes is to use axenically cultured amastigotes as a model system (10–12). *Leishmania mexicana* is one species that can be grown in axenic culture and exhibits many of the morphological and biochemical properties of intracellular amastigotes (13). To study the developmentally regulated expression of glucose transporter genes in this axenic model system, we have cloned glucose transporter genes from *L. mexicana*. Mapping and sequencing of genomic clones encoding these transporters (referred to hereafter as *LmGT* genes, indicating their identity as *L. mexicana* glucose transporters) demonstrate that there are three genes that encode three distinct isoforms of this permease, LmGT1, LmGT2, and LmGT3. LmGT2 mRNA is strongly regulated during the parasite life cycle, since it is present at a ~15-fold higher level in promastigotes compared with amastigotes. In contrast, the less abundant LmGT1 and LmGT3 mRNAs are present at similar levels in both life cycle stages. Transcription of the *LmGT* genes is

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‡ The abbreviations used are: LmGT1, LmGT2, and LmGT3, *L. mexicana* glucose transporter proteins 1, 2, and 3; LmGT1, LmGT2, and LmGT3, glucose transporter genes 1, 2, and 3 from *L. mexicana*; UTR, untranslated region; PCR, polymerase chain reaction; kb, kilobase pairs; GST, glutathione-S-transferase.

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Growth of Parasites and Isolation of Nucleic Acids—L. mexicana promastigotes (WHO reference strain M379) were grown at 26°C in Dulbecco’s modified Eagle’s medium-L (14) containing 10% heat-inactivated fetal calf serum. Axenic amastigotes were grown in a simplified version of JH-30 medium (15), developed by Dr. David Russell (Washington University, St. Louis) and designated JH-30A, consisting of M199 (11 g liter\(^{-1}\), glucose (2.5 g liter\(^{-1}\)), tryptica (5 g liter\(^{-1}\)), L-glutamine (0.75 g liter\(^{-1}\)), HEPES (5.96 g liter\(^{-1}\)), NaHCO\(_3\) (2.2 g liter\(^{-1}\)), and hemin (20 mg liter\(^{-1}\)). The pH was adjusted to 6.0 with HCl. Axenic amastigotes were transformed from mid-log phase promastigotes and axenically cultured spores by dilution into an equal volume of JH-30A medium, kept aerated for 12–24 h, and then unincorporated parasites were washed twice with ice-cold phosphate-buffered saline, pH 7.3 and detached by scraping. J774.G8 cells were lysed by repeated passage through a 30-gauge needle, and the amastigotes were purified by Percoll density gradient centrifugation (16).

Isolation of genomic DNA and preparation of Southern and Northern blots were performed as described (17). Total RNA was prepared as reported (18). The phage library of EcoRI-digested L. mexicana genomic DNA in the Lambda DASH II vector (Stratagene) was prepared according to protocols supplied by the manufacturer, and screening, isolation, and subcloning were performed by standard protocols (19).

DNA Sequencing and Analysis—DNA sequences were obtained from the two KpnI fragments and the two KpnI/EcoRI fragments, which together spanned the LmGT3 locus (Fig. 1B) using an ABI 373 automated sequencer (Perkin Elmer). Protein-coding regions and 5’-UTRs were sequenced completely on both strands, whereas only partial sequence was obtained for 3’-UTRs and intergenic regions. DNA sequences cloned PCR products and from other genomic clones derived from the LmGT locus were obtained using the Sequencher EXCEL DNA sequencing kit (Epicentre Technologies Inc.).

Comparisons of the LmGT-predicted protein sequences to protein sequences in the GenBank data base were performed using the FASTA program (20). Predicted membrane-spanning domains were determined using the Eisenberg algorithm (21).

Use of the Polymerase Chain Reaction to Map the 5’ Ends of LmGT Transcripts—Reverse transcription of total promastigote RNA and PCR amplification of resulting cDNA was performed as described previously (6). The forward primer was the 39-nucleotide sequence of the spliced leader from Leishmania donovani (22). Gene-specific reverse primers were the reverse complement of the nucleotide sequences encoding divergent regions within the NH\(_2\) terminus of the putative protein-coding regions for LmGT1, LmGT2, and LmGT3. Thus, the reverse primers were 5’-ATAGCTCTTTATGCGGATATAAGCTCC-3’ (LmGT1), 5’-GCTGAGGTTCCGAATTTGCGGATACGCTG-3’ (LmGT2), and 5’-GCTGAGGTTCCGAATTTGCGGATACGCTG-3’ (LmGT3), where the underlined nucleotides represent a BamHI site introduced to facilitate subcloning of the PCR products. Amplification products were either digested with BamHI and subcloned into the BamHI site of the plasmid vector pBluescript (Stratagene) or they were subcloned directly into the pSGEM-T vector (Stratagene), and the nucleotide sequence was determined to reveal the site for spliced leader addition.

Use of a Nested Polymerase Chain Reaction to Clone the 3’ End of the LmGT3 Transcript—Since the sequence encoding the COOH-terminal region of LmGT3 isorform was not contained within the 2.5-kb EcoRI fragment obtained from the genomic library (Fig. 1B), this region of the LmGT3 gene was obtained by PCR amplification of cDNA. Reverse transcription of total promastigote RNA was performed as described previously (6), using the following oligonucleotide designed to prime from the poly(A) tail: 5’-GGCCCCGTAGCCAATTTTGTTTTTTATT-3’. Two consecutive PCRs were performed, both using the above oligonucleotide as a forward primer. The primer in this primer provided an “anchor” that facilitated performing the PCR at a higher annealing temperature than is possible using oligo(dT) alone. The forward primer for the first reaction, primed with cDNA, was 21 nucleotides from the sequence that encodes the predicted hydrophilic loop between transmembrane segments 10 and 11, identical in all three LmGT genes (primer 1, Fig. 1B: 5’-CTGACTGAGCAAGGAGAGCCCG-3’). The forward primer for the second reaction, templated with a small aliquot of the product from the first reaction, was the 21 nucleotides upstream of the 3’ end of the EcoRI site near the predicted COOH terminus of LmGT3 (primer 2, Fig. 1B: 5’-AGGAGACGTGAGGCGGGA-ATTCC-3’). This primer is specific for LmGT3. The product of this PCR, called PCR1, was sequenced directly to obtain the sequence of the 3’-UTR (the last six amino acids) and part of the 3’-UTR of the LmGT3 gene. The observation that PCR1 is only 1.8 kb, whereas LmGT3 mRNA is 7.5 kb long and hence contains a 3’-UTR of almost 6 kb, indicates that the oligo(dT)-containing primer above described above was used to template cDNA synthesis did not actually prime from the poly(A) tail but initiated synthesis at an internal site within the 3’-UTR.

To confirm that this PCR product represented a sequence that was contiguous with the rest of the LmGT3 gene, PCR was exploited to amplify from cDNA the sequences flanking the EcoRI site within the predicted COOH terminus of LmGT3. The forward primer was the oligonucleotide used in the first reaction above (primer 1) and contained within the body of the LmGT3 coding region, whereas the reverse primer was the reverse complement of the nucleotide sequence −200 to −220 nucleotides downstream of the putative translation stop for LmGT3 (primer 3, Fig. 1B: 5’-AAAGGGCAAGACGCAACCGAGGT-3’), obtained from the sequence of PCR1. Sequence analysis of this second PCR product, PCR2, confirmed that the body of the LmGT3 protein-coding region was linked to the COOH-terminal coding region originally obtained by sequencing PCR1.

Blots Used for Detection of LmGT1, LmGT2, and LmGT3 RNAs—The LmGT3 coding region probe used for detection of RNAs on Northern blots (Fig. 4A) was an antisense RNA of −1.2 kb, derived from cloned DNA between the BglII site upstream of the LmGT3 open reading frame and the SstI site within the LmGT2 open reading frame (Fig. 1B). The LmGT1 probe (Fig. 4B) was a DNA probe of −1.2 kb, encompassing sequence from a SmaI site −1 kb downstream of the LmGT1 open reading frame (Fig. 1B) to the SstI site at the 3’ end of the open reading frame (Fig. 1B). The LmGT2 probe (Fig. 4C) was an antisense RNA of −1.4 kb, derived from cloned DNA between the SstI site at the downstream end of the LmGT2 open reading frame and a SmaI site −200 base pairs downstream of the KpnI site in the intergenic region between LmGT2 and LmGT3 (Fig. 1B). The LmGT3 probe (Fig. 4D) was an antisense RNA of −1.8 kb, derived from the cloned product of PCR1 (see above).
appropriate, α-amanitin was added to nuclei 10 min prior to other reaction components. Samples were extracted with 1 volume of phenol: chloroform (1:1), and the aqueous phase was extracted with 1 volume of chloroform. Nucleic acids were precipitated with 2 mM ammonium acetate (pH 7) and 3 volumes of ethanol and centrifuged at full speed for 20 min in a microcentrifuge, with 10 μg of yeast tRNA as a carrier, rinsed twice with 70% ethanol, and resuspended in 200 μl of water. Incorporation of [α-32P]UTP was determined by counting a 2-μl aliquot.

Labeled transcripts were hybridized to slot blots of linearized, alkali-denatured plasmid DNA (~5 μg per slot) (23). Slot blots were prehybridized for at least 2 h at 42 °C in a sealed plastic bag containing 5 ml of 50% formamide, 5 × SSC, 50 mM NaPO4 (pH 7.0), 1% SDS, 200 μg/ml yeast tRNA, 5 × Denhardt’s solution, 5 ml EDTA (19). Run-on transcripts were denatured by heating to 95 °C for 5 min and then added to the slot blot in prehybridization solution. Hybridization was allowed to proceed with gentle agitation for ~48 h. Blots were washed at 55 °C, three times for 30 min each in 0.1 × SSC, 0.1% SDS, 5 ml EDTA, dried, and exposed to film or phosphorimage screen, as described above.

Analysis of mRNA Decay—We first determined that 9 μg/ml−1 was the IC50 for actinomycin D (Boehringer Mannheim) inhibition of [3H]uridine incorporation in L. mexicana. Promastigote and amastigote cultures, at approximately 2 × 106 cells/ml−1, were incubated with 10 μg/ml−1 actinomycin D under normal culture conditions. Aliquots were removed at various time points, and nucleic acids were extracted and analyzed by Northern blot and exposure to a phosphorimager screen, as described above.

Transport Assays in Xenopus Oocytes—A BgII-ClaI restriction fragment containing the protein-coding region of the LmGT2 gene (labeled BC in Fig. 1B) was subcloned into the Bluescript SK+ plasmid (Stratagene), linearized with ClaI, and transcribed in vitro with T3 RNA polymerase (Life Technologies, Inc.) as described (24). Stage V-VI Xenopus oocytes were injected with 40 nl containing ~3 ng of RNA, incubated for 3 days at 18 °C, and assayed for uptake of 50 μM 2-deoxy-D-[3H]glucose as described previously (24).

Immuno blot Analysis—Lanes of promastigotes and axenic amastigotes were prepared by dissolving the cell pellet in Laemmli sample buffer (19) at a concentration of 2.5–5 × 106 cells/ml−1 and heating immediately to 65 °C for 5 min. Aliquots of the lysate were assayed in triplicate for protein content by the Bradford method (25). Samples (10 μl) were separated on a 10% SDS-polyacrylamide gel and electrophoresed onto nitrocellulose, and the blot was incubated overnight in TBST buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween 20, 3% (w/v) powdered milk) containing the affinity-purified P1L antibody used to raise the P1L antibody. Blots were developed with a horseradish peroxidase-conjugated secondary goat anti-rabbit (1:40,000) IgG and the Supersignal chemiluminescence kit (Pierce) using the manufacturer’s instructions and then exposed to XAR-5 film (Eastman Kodak Co.).

RESULTS

Cloning and Structure of LmGT Glucose Transporter Genes from L. mexicana—To study expression of glucose transporter genes using the L. mexicana axenic amastigote system, we first cloned these genes from L. mexicana. Southern blots of restriction-digested L. mexicana genomic DNA, probed with a protein-coding region of the L. enrietti Pro-1 glucose transporter gene, revealed the presence of single hybridizing bands in EcoRI, HindIII, EcoRV, and BamHI digests, suggesting that most or all of the L. mexicana glucose transporter locus was contained within the ~14-kb EcoRI fragment (Fig. 1A). Other Southern blots revealed that there were no other smaller bands that hybridized to the Pro-1 probe in genomic DNA digested with these four enzymes (data not shown). Therefore, a phage library of EcoRI-digested L. mexicana genomic DNA was screened with the L. enrietti Pro-1 probe, and 11 positive clones were isolated, all with an insert of ~14 kb. Restriction mapping of one of these inserts revealed three restriction fragments of 5.2 kb (EcoRI/KpnI), 3.8 kb (KpnI), and 2.7 kb (KpnI/EcoRI), which hybridized with the Pro-1 probe (Fig. 1B). Each of these restriction fragments was subcloned, partially sequenced, and found to contain a single copy of an L. mexicana Pro-1 homologue. The results indicate that this locus contains three closely related glucose transporter genes, with significant identity to L. enrietti Pro-1. The deduced amino acid sequences (Fig. 2) for these three genes indicates that they differ from each other mostly within their predicted NH2-terminal hydrophilic domains (thought to be located on the cytoplasmic side of the membrane (27)). LmGT1 has an NH2 terminus that is 45 amino acids longer than that of either LmGT2 or LmGT3, which have NH2 termini of similar length but divergent sequence. Downstream of the predicted NH2-terminal domains, all three sequences are more than 90% identical, and all have the same predicted membrane-spanning topology. However, there are 24 additional amino acid positions that are divergent among the three isoforms. Thus, LmGT1 and LmGT2 have identical COOH termini, whereas LmGT3 differs in 8 of the last 9 amino acids, and LmGT1 has a divergent cluster of 7 amino acids toward the cytoplasmic end of the predicted transmembrane domain IV. In addition, they appear to be either non-identical amino acids distributed throughout the primary amino acid sequence. Among the three isoforms, LmGT2 is the most closely related to L. enrietti Pro-1 (LmGT1, LmGT2, and LmGT3 have 78.0, 85.2, and 83.1% identity, respectively, to L. enrietti Pro-1 isoform 2).

5′- and 3′-UTRs of LmGT Genes—To define the 5′ end of the mRNA from LmGT1, LmGT2, and LmGT3, we amplified, sub-
cloned, and sequenced cDNAs representing the 5' ends of each mRNA (6). cDNA synthesized using a random oligonucleotide primer was amplified in the PCR using a reverse primer complementary to the unique NH2-terminus coding region of each mRNA and a forward primer representing the 3'-nucleotide spliced leader (22) present on all *L. donovani* mRNAs (see "Experimental Procedures"). The sequences of the cDNA subclones revealed that the spliced leaders were trans-spliced onto the 5'-ends of the *LmGT2* and *LmGT3* mRNAs at identical positions, 170 base pairs upstream of the first ATG codon. Comparison of the 5'-UTRs of the *LmGT2* and *LmGT3* mRNAs revealed that they were identical. In contrast, the 5'-UTR of *LmGT1* showed no sequence homology with that of *LmGT2* and *LmGT3*, and the site of spliced leader addition was 386 base pairs upstream of the first ATG codon. For each predicted transporter protein, the indicated initiation codon is the first in frame methionine codon in the RNA. The limited sequence we have obtained for the 3'-UTRs of each mRNA indicates that all three are divergent.

Functional Expression of LmGT2 Gene—To confirm that the *LmGT* genes encode bona fide glucose transporters, we expressed the *LmGT2* gene in *Xenopus* oocytes and assayed for uptake of the glucose analog 2-deoxy-D-[3H]glucose (24). Oocytes injected with *LmGT2* RNA exhibited robust linear uptake of the radiolabeled analog for at least 3 h, whereas oocytes injected with water did not take up this compound (Fig. 3).

Regulation of *LmGT* Gene Expression in Promastigotes and Axenic Amastigotes of *L. mexicana*—Biological criteria suggest that axenically cultured amastigotes of *L. mexicana* present a reasonable model for *in vivo* amastigotes (12, 13). However, if these axenic amastigotes are to be used as a model for investigating the regulation of *LmGT* gene expression, it is essential to establish that *LmGT* gene expression is correctly regulated in these cells. Consequently, we have used Northern blots to compare the expression of RNAs from several genes in promastigotes, axenically cultured amastigotes, and amastigotes derived from cultures of macrophage-like cells (Fig. 4). In these experiments, equal amounts of total RNA from promastigotes, macrophage-derived amastigotes, and axenic amastigotes were separated by gel electrophoresis. Following blotting and hybridization, the signals for each mRNA were quantitated by phosphorimaging and normalized to those for rRNA (28), allowing quantitative comparison of mRNA levels in each cell type. For all of the genes investigated, expression by axenically cultured amastigotes was very similar to expression by intracellular amastigotes, supporting the validity of these axenic amastigotes as a model for intracellular amastigotes.

Northern blots reveal that *LmGT1*, *LmGT2*, and *LmGT3* are encoded by three different mRNAs of 8.0, 3.5, and 7.5 kb, respectively (Fig. 4B), a 3.5-kb transcript for *LmGT2* (Fig. 4C), and a 7.5-kb

![FIG. 2. Alignment of the predicted amino acid sequences of the *L. mexicana* glucose transporters *LmGT1*, *LmGT2*, and *LmGT3.* Amino acids that are non-identical in all three proteins are indicated by a white background, whereas those that are identical in two out of three sequences are indicated by a gray background. The numbers at the left indicate the amino acid positions in each sequence, and the numbers at the end of each sequence indicate the total number of constituent amino acids. Spaces introduced to optimize the alignment are indicated by a period. Predicted transmembrane domains are indicated by numbered bars above the aligned sequences.](image-url)
transcript for \textit{LmGT3} (Fig. 4D). Hybridization with the \textit{LmGT2} protein-coding region alone detects each of these (Fig. 4A) and an additional \textasciitilde 6.0-kb transcript (labeled “X”) of unknown origin. Expression of \textit{LmGT2} RNA is down-regulated \textasciitilde 15-fold in both axenically cultured amastigotes and in intracellular amastigotes, compared with promastigotes, as determined by phosphorimaging of the 3.5-kb signal (Fig. 4A), indicating that the \textit{LmGT2} transcript is strongly developmentally regulated. In contrast, the larger \textit{LmGT1} and \textit{LmGT3} transcripts are present at similar levels in promastigotes, axenic amastigotes, and intracellular amastigotes, indicating that these mRNAs are constitutively expressed.

Expression of Other mRNAs in Promastigotes and Axenic Amastigotes—To further investigate axenic amastigotes as a model for studying gene regulation, we have examined the relative levels of expression of mRNAs from several other genes (Fig. 4, E–H). The mRNA encoding another developmentally regulated protein, the paraflagellar rod protein PFR-1 (29), was also down-regulated in both types of amastigotes (Fig. 4D). In contrast, the mRNA for the constitutively expressed \textit{\alpha}-tubulin (30) was expressed at similar levels in all three \textit{L. mexicana} cell types studied here (Fig. 4E). The RNA for the developmentally regulated cysteine protease gene \textit{Lpccys2} (31) was quantitatively up-regulated in both macrophage-derived and axenic amastigotes compared with promastigotes (Fig. 4F). Finally, a 2.4-kb \textit{\beta}-tubulin transcript decreased in abundance in both macrophage-derived and axenic amastigotes compared with promastigotes, and a 2.8-kb transcript increased in abundance in both types of amastigotes (Fig. 4H). A \textit{\beta}-tubulin transcript of \textasciitilde 5 kb (marked “Y” in Fig. 4H) increased in abundance in intracellular amastigotes but not in axenic amastigotes. A similar pattern of differentially regulated \textit{\beta}-tubulin transcript sizes has been reported for \textit{Leishmania amazonensis} (30). In summary, three constitutively expressed RNAs (\textit{\alpha}-tubulin, \textit{LmGT1}, \textit{LmGT3}), three RNAs that are down-regulated in amastigotes (\textit{LmGT2}, PFR-1, \textit{\beta}-tubulin 2.4-kb transcript), and two RNAs that are up-regulated in amastigotes (cysteine protease-2 and \textit{\beta}-tubulin 2.8-kb transcript) are correctly regulated in axenic amastigotes. Although a quantitative difference in the expression of the \textasciitilde 5-kb \textit{\beta}-tubulin transcript was observed between intracellular and axenic amastigotes, this latter transcript is larger in size than the mature \textit{\beta}-tubulin mRNAs and may represent unprocessed mRNA precursors. However, mature mRNAs are expressed at very similar levels in macrophage-derived and axenic amastigotes, confirming the utility of this \textit{in vitro} system for analysis of gene expression.

Transcription of \textit{LmGT} Genes in Promastigotes and Axenic Amastigotes—To determine whether the \textit{LmGT} genes are regulated at the transcriptional or post-transcriptional levels, we have quantitated the relative rates of transcription of several genes in promastigotes and axenically cultured amastigotes by nuclear run-on transcription. Radiolabeled run-on transcripts from promastigote or amastigote nuclei were hybridized with plasmid DNA immobilized on nylon filters, and the amount of labeled transcript in each hybrid was quantitated by phosphorimaging. The rate of transcription of each gene was determined relative to the rate of rRNA transcription by normalizing each hybridization signal to that obtained for the rRNA hybrid in each run-on transcription. Hence, the run-on transcriptions have been normalized to the same standard used to normalize the relative levels of mRNA abundance, as determined by quantitation of hybridization signals on Northern blots (Fig. 4). Probes for genes whose relative rates of transcription were measured in these experiments include the \textit{LmGT} genes (3.6-kb \textit{PstI} fragment labeled \textit{P} in Fig. 1B), \textit{L. enriettii} \textit{\alpha}-tubulin, \textit{L. mexicana} PFR-1, and \textit{L. enriettii} rRNA. The results (Fig. 5, A and C) show that transcription of all genes occurred at approximately the same rates, relative to rRNA transcription, in both promastigotes and axenic amastigotes. No hybridization was detected to \textit{P}Bluescript, a plasmid that contains no DNA of leishmanial origin. The results of three independent run-on transcription experiments were quantitated and are reported in Table I. The hybridization signal to the heterologous \textit{L. enriettii} rRNA clone was arbitrarily chosen as 1.0, and the other signals were reported relative to this value. Although there is considerable scatter for each normalized hybridization signal from one experiment to another, it is clear that the relative rates of transcription for all genes examined do not vary significantly between promastigotes and axenic amastigotes. Since all the genes investigated here are transcribed at similar rates in both life cycle stages, the developmentally regulated \textit{LmGT2} and PFR-1 genes must be post-transcriptionally controlled. The absolute rate of transcription per nucleus was approximately 10-fold lower in axenic amastigotes than in promastigotes, as determined by quantitation of radioactivity incorporated into total RNA during the run-on transcriptions (3.6 \textasciitilde 1 \times 10^6 cpm per 10^9 promastigote nuclei \textit{n} = 3) compared with 3.9 \textasciitilde 0.8 \times 10^6 cpm per 10^9 axenic amastigote nuclei \textit{n} = 3). Transcription of the \textit{LmGT}, PFR-1, and \textit{\alpha}-tubulin genes, but not the rRNA genes, was inhibited by 50 \textmu gml^-1 \textit{\alpha}-amanitin (Fig. 5B), a level of drug similar to that which inhibited transcription of other mRNA-encoding genes in \textit{Leishmania tarentolae} (32).

In the preceding experiments, we hybridized the nascent transcripts to a restriction fragment that contains both glucose transporter protein-coding regions and the intergenic region between the \textit{LmGT2} and \textit{LmGT3} protein-coding regions. This probe will hybridize most strongly to the \textit{LmGT2} transcript, since the probe contains the entire unique 3'-UTR of this mRNA, but it will also cross-hybridize to the \textit{LmGT1} and \textit{LmGT3} transcripts due to the high sequence identity of all three transcripts in the protein-coding regions. This cloned fragment was chosen, rather than a shorter target representing the unique 3'-UTR of \textit{LmGT2}, to ensure that a detectable level of signal for \textit{LmGT2} transcription could be obtained in the run-on experiments. Partial cross-hybridization of the probe to the unregulated \textit{LmGT1} and \textit{LmGT3} transcripts would not alter the interpretation of the results, since these RNA levels are not regulated during the life cycle. It is thus clear that the 15-fold regulation of \textit{LmGT2} mRNA steady-state levels (Fig. 4A) cannot be attributed to regulation of \textit{LmGT2} transcription.
rapidly, producing a biphasic decay curve in this life cycle. The first 2 h following drug addition and then decayed more slowly. This mRNA was quite stable in promastigotes for the first 2 h following drug addition and then decayed more rapidly, producing a biphasic decay curve in this life cycle stage. Although it is not possible to assign a unique half-life to LmGT2 mRNA in drug-treated promastigotes, the substantial difference in mRNA decay rates between promastigotes and amastigotes, especially during the initial 2 h of drug treatment, suggests that LmGT2 mRNA levels may be regulated during the life cycle, at least in part, by differential mRNA stability. In contrast, the decay of β-tubulin mRNAs (sum of the hybridization signals of the 2.4- and 2.8-kb transcripts) was essentially the same in promastigotes and axenic amastigotes (Fig. 6B). When the 2.4- and 2.8-kb β-tubulin transcripts were analyzed individually, the 2.4-kb transcript was found to be more stable in promastigotes, and the 2.8-kb transcript was more stable in axenic amastigotes (data not shown). Thus, differential message stability may further modulate expression of various β-tubulin mRNAs.

Expression of LmGT Proteins in Promastigotes and Axenic Amastigotes—To determine whether LmGT proteins are down-regulated upon the transformation of promastigotes to axenic amastigotes, we performed immunoblots on lysates containing similar amounts of protein from promastigotes and axenic amastigotes. These blots were probed (Fig. 7) with the P1L antibody (26) directed against the peptide TT76-Y143 (5) from the large extracellular loop of the L. enriettii Pro-1 glucose transporters, which is highly related in sequence (48 of 68 amino acids are identical) to the corresponding regions of the LmGT proteins. As in the case of L. enriettii, the L. mexicana lysates revealed a major band (Fig. 7, solid arrow) of 50–55 kDa that was not competed by GST (Fig. 7, lanes 1 and 2) but was competed by the GST-fusion protein used to generate the antiserum (Fig. 7, lanes 3 and 4), confirming that this band represents LmGT glucose transporters. This band presumably corresponds to both LmGT2 and LmGT3, since both proteins are of the same predicted molecular weight and contain the same sequence within the region recognized by the P1L antiserum (Fig. 2A). The intensity of this band was considerably greater in lysates from promastigotes (Fig. 7, lane 1) compared with axenic amastigotes (Fig. 7, lane 2). Furthermore, a background band that was not competed by either GST or the GST-fusion protein (Fig. 7, open arrow) was of similar intensity in the promastigote and axenic amastigote lanes, confirming that similar amounts of cellular protein had been loaded onto each lane. This result establishes that expression of the LmGT2 protein and the LmGT2 mRNA is similarly regulated during the parasite life cycle.

Decay of LmGT2 mRNA in Promastigotes and Axenic Amastigotes—To determine whether decreased RNA stability could contribute to the down-regulation of LmGT2 mRNA in amastigotes, we measured LmGT2 mRNA decay in promastigotes and axenic amastigotes that had been treated with 10 μg/ml1 2-aminopterin D, a drug concentration that inhibits RNA synthesis by greater than 90% (data not shown). Total RNA was isolated from cultures from 0 to 5 h following addition of drug, and the levels of LmGT2 and β-tubulin mRNAs were measured in each sample by quantitation of Northern blots with a phosphorimager. The results of three independent experiments revealed that LmGT2 mRNA was more stable in promastigotes than in amastigotes (Fig. 6A). Although LmGT2 mRNA decayed rapidly, with a half-life of approximately 1 h in amastigotes, this mRNA was quite stable in promastigotes for the first 2 h following drug addition and then decayed more rapidly, producing a biphasic decay curve in this life cycle stage. Although it is not possible to assign a unique half-life to LmGT2 mRNA in drug-treated promastigotes, the substantial difference in mRNA decay rates between promastigotes and amastigotes, especially during the initial 2 h of drug treatment, suggests that LmGT2 mRNA levels may be regulated during the life cycle, at least in part, by differential mRNA stability. In contrast, the decay of β-tubulin mRNAs (sum of the hybridization signals of the 2.4- and 2.8-kb transcripts) was essentially the same in promastigotes and axenic amastigotes (Fig. 6B). When the 2.4- and 2.8-kb β-tubulin transcripts were analyzed individually, the 2.4-kb transcript was found to be more stable in promastigotes, and the 2.8-kb transcript was more stable in axenic amastigotes (data not shown). Thus, differential message stability may further modulate expression of various β-tubulin mRNAs.

Expression of LmGT Proteins in Promastigotes and Axenic Amastigotes—To determine whether LmGT proteins are down-regulated upon the transformation of promastigotes to axenic amastigotes, we performed immunoblots on lysates containing similar amounts of protein from promastigotes and axenic amastigotes. These blots were probed (Fig. 7) with the P1L antibody (26) directed against the peptide TT76-Y143 (5) from the large extracellular loop of the L. enriettii Pro-1 glucose transporters, which is highly related in sequence (48 of 68 amino acids are identical) to the corresponding regions of the LmGT proteins. As in the case of L. enriettii, the L. mexicana lysates revealed a major band (Fig. 7, solid arrow) of 50–55 kDa that was not competed by GST (Fig. 7, lanes 1 and 2) but was competed by the GST-fusion protein used to generate the antiserum (Fig. 7, lanes 3 and 4), confirming that this band represents LmGT glucose transporters. This band presumably corresponds to both LmGT2 and LmGT3, since both proteins are of the same predicted molecular weight and contain the same sequence within the region recognized by the P1L antiserum (Fig. 2A). The intensity of this band was considerably greater in lysates from promastigotes (Fig. 7, lane 1) compared with axenic amastigotes (Fig. 7, lane 2). Furthermore, a background band that was not competed by either GST or the GST-fusion protein (Fig. 7, open arrow) was of similar intensity in the promastigote and axenic amastigote lanes, confirming that similar amounts of cellular protein had been loaded onto each lane. This result establishes that expression of the LmGT2 protein and the LmGT2 mRNA is similarly regulated during the parasite life cycle.

DISCUSSION

Coordinate regulation of gene expression in kinetoplastids is central to the complex life cycle switches that are characteristic of these organisms. Although higher eukaryotes control much of their gene expression at the level of transcription, current data suggest that in the Kinetoplastida, transcriptional regulation may not play a major role in expression of many genes.
Preincubated with GST, and for phages. Nuclear run-on transcriptions would be cumbersome to difficult to perform with amastigotes isolated from macrophages. Differences in levels of specific mRNAs in amastigotes compared to promastigotes or axenic amastigotes (lanes 2 and 4) were separated by SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose filters, and incubated with the P1L antibody (26) directed against the Pro-1 glucose transporter from L. enriettii. For lanes 1 and 2, the P1L antibody was preincubated with GST, and for lanes 3 and 4, the P1L antibody was preincubated with the GST-fusion protein used to generate the P1L antibody. The solid arrow indicates the band that is competed by GST-fusion protein but not by GST and hence represents the LmGT transporters. The open arrow indicates a background band whose intensity does not change significantly between promastigotes and axenic amastigotes. The numbers at the left indicate the mobility and relative molecular weights (kDa) of protein molecular weight standards.

(33). Though current examples are limited, expression of several genes in the related organism Trypanosoma brucei seems to be regulated primarily at a post-transcriptional level (34–38). In L. amazonensis, temperature-dependent induction of the developmentally regulated hsp83 is post-transcriptionally controlled and involves a change in the rate of mRNA turnover (39, 40). Developmentally regulated changes in mRNA stability have also been implicated in regulation of expression of the surface glycoprotein gp63 in Leishmania chagasi (41). Since the LmGT2 glucose transporter gene is among a limited number of strongly developmentally regulated genes that have been identified in Leishmania parasites, it provides a valuable model for probing the molecular mechanisms that control gene expression during the parasite life cycle.

An important reason for the current paucity of information regarding leishmanial gene regulation is the technical difficulty of working with amastigotes grown in host cells. Although it is possible to obtain sufficient numbers of amastigotes from infected macrophages to isolate nucleic acids and measure the differences in levels of specific mRNAs in amastigotes compared with promastigotes, more sophisticated experiments that probe the mechanism for regulating gene expression are very difficult to perform with amastigotes isolated from macrophages. Nuclear run-on transcriptions would be cumbersome to perform with amastigotes isolated from macrophages, as it would be hard to obtain enough parasites to generate the levels of labeled transcripts required for hybridizations. Furthermore, the purification of amastigotes from macrophages requires several hours, and the transcription levels of developmentally regulated genes could be significantly altered once the amastigotes are released from the environment of the parasitophorous vacuole. For similar reasons, measurements of mRNA stability in intracellular amastigotes are also problematic. In principle, these technical problems can be overcome using axenically cultured amastigotes, so that nuclear transcription and mRNA decay experiments can be performed in the same way they are done with cultured promastigotes.

However, to establish the validity of axenic amastigotes as a model for studying regulation of gene expression, it was first essential to demonstrate that axenic amastigotes regulate the expression of genes, especially the LmGT genes, in essentially the same way that intracellular amastigotes regulate their expression. We have determined the pattern of expression of a range of genes in promastigotes and purified macrophage-derived amastigotes and have shown that axenic amastigotes regulate expression all of these genes in a pattern that is very similar to intracellular amastigotes. This is true both for transcripts that are up-regulated or down-regulated in amastigotes. Thus, axenically cultured amastigotes represent an excellent model for studies of amastigate gene regulation and provide a compelling reason to undertake these experiments in L. mexicana.

**Structure and Multiplicity of Glucose Transporter Genes in L. mexicana**—To begin studies on glucose transporter gene expression in L. mexicana, we cloned the LmGT genes from this parasite and determined their sequence and genomic arrangement. Three LmGT genes are present at a single locus and encode closely related isoforms that differ from one another mainly at their predicted NH2 and COOH termini. The existence of three related but distinct glucose transporter isoforms in L. mexicana suggests that each of these three permeases may subserve a unique or specialized physiological function. However, the potentially distinct biological roles of these transporters remain to be determined.

The pronounced sequence similarity between the LmGT genes from L. mexicana and the Pro-1 genes from L. enriettii confirms that these are homologous genes from these two species of Leishmania parasite. Nonetheless, there are notable differences between the glucose transporter isoforms of L. mexicana and L. enriettii (42). Isoform 1 and isoform 2 of L. enriettii Pro-1 are targeted to different locations at the cell surface (43), and the structural information that directs this
targeting is contained within the divergent NH₂ termini (26). The rest of the coding region of the two L. enriettii isoforms, from the first transmembrane segment to the COOH terminus, is identical. Each of the three LmGT isoforms has unique NH₂-terminal hydrophilic domains, but, in contrast to the L. enriettii Pro-1 isoforms, LmGT3 has a divergent COOH terminus compared with LmGT1 and LmGT2. LmGT1 has a divergent cluster of amino acids at the cytosolic end of predicted transmembrane domain IV compared with LmGT2 and LmGT3. There is also a number of single amino acid differences that distinguish these isoforms from one another. It is possible that the distinct NH₂ termini of the three LmGT isoforms are involved in differential subcellular targeting, as in L. enriettii, and that the other polymorphic amino acids might confer some additional biochemical or kinetic differences between the two isoforms. Alternatively, it is possible that divergent sequences in both the NH₂ terminus and elsewhere are involved in differential subcellular targeting of the LmGT isoforms. Understanding the functional differences between the three isoforms of LmGT transporters will require further experimentation, including functional expression of both isoforms in a heterologous system as well as subcellular targeting studies similar to those performed on isoforms 1 and 2 of the L. enriettii Pro-1 transporters (43). Despite the divergence of the NH₂ and COOH termini, LmGT2 is nonetheless more closely related to the L. enriettii isoform 2 within both these domains than is either LmGT1 or LmGT3. This observation suggests the possibility that LmGT2 might be the functional homolog of isoform 2.

It is noteworthy that the LmGT2 and LmGT3 transcripts have identical 5'-UTRs, even though there are significant differences within the protein-coding regions. It is currently not clear why these two mRNAs are identical in this region, whereas the 5'-UTR of LmGT1 is divergent. In contrast, the limited sequence we have obtained indicates that the 3'-UTRs of all three LmGT mRNAs are divergent. It is possible that the different 3'-UTRs are involved in the differential regulation of these transcripts during the life cycle.

Regulation of Glucose Transporter Genes in L. mexicana—
The LmGT2 transcripts in L. mexicana are down-regulated ~15-fold in both isolated in vivo amastigotes and in axenically cultured amastigotes. Like LmGT2, both Pro-1 transcripts in L. enriettii (5) and the D2 hexose transporter transcript in L. donovani (6) are down-regulated in amastigotes compared with promastigotes. In contradistinction, the LmGT1 and LmGT3 transcripts are expressed at similar levels in both life cycle stages and thus represent a unique example of unregulated glucose transporter genes. A detailed kinetic analysis of transport by all three isoforms might suggest a physiological rationale for the differential regulation of the three genes.

Analysis of the regulation of LmGT2 gene expression was greatly facilitated by the availability of an axenic culture system for amastigotes. The nuclear run-on transcription experiments clearly show that the level of transcription of LmGTs (relative to constitutively transcribed ribosomal RNA) is very similar in both life cycle stages. Since the cloned α-tubulin and ribosomal RNA genes are from another Leishmania species, it is not possible to quantitate absolute rates of transcription in these experiments. However, it is clear that the ~15-fold down-regulation of steady-state LmGT2 mRNA in amastigotes cannot be explained by transcriptional regulation. Thus, LmGT2 expression is regulated primarily at the post-transcriptional level.

Down-regulation of mature mRNA might be effected at the level of post-transcriptional processing or via modulation of message stability. We utilized a transcription inhibitor, actinomycin D, to investigate a potential role for differential message stability in LmGT2 regulation. Since actinomycin D shuts off all RNA synthesis and can also affect other metabolic processes, such as translation, these experiments are potentially subject to artifacts, and the rates of mRNA decay measured in the presence of drug could differ from those operating in the absence of drug. The level of actinomycin D used in these experiments is not in excess of that required to efficiently inhibit transcription, and cells did not seem to be affected morphologically nor was their motility reduced during the 5-h incubation in drug. Nonetheless, we have attempted to measure mRNA decay more directly by in vivo pulse-chase experiments involving radioactive labeling of RNA with [3H]uridine (44), but we have not been able to achieve sufficient labeling of the moderately abundant LmGT2 transcript to perform quantitative decay experiments.

Although the biphasic shape of the decay curves (Fig. 6) precludes the determination of a quantitative half-life characteristic of simple exponential decay, the data point to a clear qualitative difference in LmGT2 mRNA stability between these two life cycle stages, at least in the presence of drug. This complexity in the decay curves makes it difficult to definitively determine the relative role of mRNA half-life in LmGT2 gene regulation during the parasite life cycle. Thus, if the true half lives are represented by the 0–2-h region of the decay curves, there is a dramatic difference in stability of the LmGT2 mRNA between promastigotes and amastigotes. Nonetheless, even if the difference in mRNA half lives is not as pronounced as suggested by the early region of the decay curves, the more modest difference suggested by following mRNA decay over the entire 5-h time course could still effect a very large difference in steady-state mRNA levels. Thus, Ross (45) has mathematically demonstrated that 2–4-fold differences in mRNA half lives can result in differences of at least two orders of magnitude in mRNA steady-state levels.

In contrast, the stability of total β-tubulin mRNA seems to be similar in both promastigotes and amastigotes, a finding that is consistent with our observation and a previous report (30) that total β-tubulin mRNA is constitutively expressed in L. mexicana. It is interesting, however, that the most abundant β-tubulin transcript shifts from 2.4 to 2.8 kb when promastigotes are compared with amastigotes and that these major transcripts are also more stable in promastigotes and axenic amastigotes, respectively.

This life cycle-dependent difference in transcript stability may be explained by either stabilization of LmGT mRNA in promastigotes or destabilization in amastigotes and predicts a role for cis-acting sequences in the control of mRNA stability. Cis-acting sequences in both the 5'- and 3'-UTRs have been implicated in differential decay of hasp83 mRNA during heat shock in L. amazonensis (39, 40), and sequences within the 3'-UTR have been demonstrated to play a role in the post-transcriptional regulation of the A2 amastigote-specific gene from L. donovani (46), establishing a precedent for this type of regulation among Leishmania species in response to changing environmental conditions. It should now be possible to define specific elements involved in the regulation of the LmGT2 gene during the parasite life cycle by systematic deletion and modification of discrete regions of sequence.

Expression of LmGT Proteins during the Parasite Life Cycle—Immunoblots of lysates from the two life-cycle stages confirm that the total LmGT protein is significantly less abundant in amastigotes compared with promastigotes (Fig. 7). Although the antiserum used in these experiments recognizes all glucose transporter isoforms, the band intensity in the promastigote lane probably represents primarily the LmGT2 isoform and
confirms that the level of this protein is down-regulated concomitantly with the down-regulation of the LmGT1 mRNA. Although we cannot directly determine whether or not the LmGT1 and LmGT3 polypeptides, encoded by the significantly less abundant mRNAs, are regulated during the life cycle, the constitutive expression of these RNAs during the life cycle suggests that the corresponding permeases are likely to be expressed at similar levels in both life cycle stages. This hypothesis is consistent with the presence of an LmGT1 band in the amastigote lane in exposures of the immunoblot that are longer than that shown in Fig. 7 (data not shown).

Additional Advantages of Studying Glucose Transporters in L. mexicana—The cloning and structural analysis of glucose transporter genes in L. mexicana offers several advantages for functional studies on these transporters. L. mexicana parasites can establish infections in laboratory-reared sandflies (47) and in J774.G8 tissue culture macrophages (16), unlike some other species of Leishmania such as L. enriettii. Hence, generation of glucose transporter null mutant (48, 49) strains in L. mexicana will allow phenotypic testing of these mutants within the insect vector and within the macrophage and will greatly enhance our ability to assess the biological functions of glucose transporters in intact Leishmania parasites.

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