Glycoprotein 46 mRNA Abundance Is Post-transcriptionally Regulated during Development of *Leishmania chagasi* Promastigotes to an Infectious Form*

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GP46 is an abundant glycoprotein of 46 kDa on the surface of the promastigote form of most *Leishmania* species. We show that the steady state level of GP46 mRNA increases >30-fold as *Leishmania chagasi* promastigotes develop in vitro from a less infectious form during logarithmic growth to a highly infectious form in the stationary phase of cultivation. Nuclear run-on experiments demonstrate that this increase in GP46 mRNA abundance is regulated post-transcriptionally. Plasmids containing the 3′-untranslated regions (UTRs) and downstream intergenic regions (IRs) of two different GP46 genes fused immediately downstream of the β-galactosidase coding region were transfected into *L. chagasi*, and β-galactosidase activity and mRNA levels were examined. The presence of the 3′-UTR + IR of one GP46 gene (gp46A) resulted in a steady increase in β-galactosidase activity and mRNA level as the transfected promastigotes developed from logarithmic to stationary phase. This differential effect parallels that of the 3′-UTRs + IRs of a family of genes for an unrelated *Leishmania* surface glycoprotein, GP63. Thus, post-transcriptional regulation of the genes for two different surface glycoproteins of *Leishmania* occurs via a similar mechanism.

Protozoan parasites of the genus *Leishmania* cause a diverse group of diseases collectively called leishmaniasis, which range in severity from spontaneously healing cutaneous ulcers to potentially fatal visceral disease. These parasites have a digenetic life cycle, passing from the infected sandfly vector to the mammalian host as the female fly takes a blood meal. In the fly, *Leishmania* exist as extracellular flagellated promastigotes within the alimentary canal, and in mammals, they exist as intracellular flagellate amastigotes within phagolysosomes of macrophages.

While multiplying in the sandfly gut, promastigotes progress through a series of morphologically distinct developmental stages culminating in the highly infectious metacyclic stage (1). Some aspects of this development are mirrored *in vitro* during the growth of promastigotes from logarithmic (less infectious) phase to stationary (highly infectious) phase in liquid culture medium (2). For example, in several *Leishmania* species, the glycosyl side chain of the abundant lipophosphoglycan on the surface of promastigotes elongates as parasites grow to their infectious metacyclic form in stationary phase (3, 4). This increase in metacyclic lipophosphoglycan size has been correlated with enhanced virulence of the parasite (5, 6). Another major surface constituent of promastigotes is a well characterized 63-kDa glycoprotein called GP63, a zinc protease with broad substrate specificity and a wide pH optimum (7, 8). In *Leishmania chagasi*, the cause of South American visceral leishmaniasis, the amount of GP63 increases 11-fold as the promastigotes grow from logarithmic to stationary phase (9). Biological functions ascribed to GP63 include evasion of complement-mediated lysis, attachment to macrophages, cleavage of various substrates including C3b, and elicitation of both humoral and cellular immune responses that are protective in several mouse models (10–14).

In earlier studies of GP63 expression, we found that *L. chagasi* has >18 tandemly arrayed GP63 genes divisible into three classes on the basis of their developmental expression (9, 12). Genes of the class encoding 2.7-kb mRNAs occurring predominantly in logarithmic phase promastigotes are called *mspL*, where *msp* and *L* refer to the genes encoding major surface protease (GP63) and logarithmic phase, respectively. Genes of the class encoding 3.0-kb mRNAs found predominantly in stationary phase promastigotes are called *mspS*. Promastigotes at an intermediate growth phase possess both RNAs. Class *mspC* is comprised of a single gene that is constitutively expressed at a low level throughout promastigote growth as 2.6- and 3.1-kb mRNAs. The 3′-UTR and downstream intergenic region (IR) of the *mspS* genes play an important role in the stationary phase expression of these genes, whereas the corresponding 3′-UTR + IR sequence of the *mspL* genes does not seem to be responsible for their differential RNA expression (15).

Another abundant protein present on the surface of promastigotes is a 46-kDa glycoprotein called GP46 or promastigote surface antigen 2 (16, 17). Genes encoding GP46 have been detected in *Crithidia fasciculata* and in all *Leishmania* sp. examined except for members of the *Leishmania braziliensis* complex (18, 19). The organization of this gene family has not been fully characterized in any *Leishmania* species, but in

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1 The abbreviations used are: GP63, glycoprotein of 63 kDa; GP46, glycoprotein of 46 kDa; βGAL, β-galactosidase; gp46A and gp46B, GP46 genes; UTR, untranslated region; IR, intergenic region; kb, kilobase(s); bp, base pair(s); msp, major surface protease; mspC, constitutive GP63 genes; mspL, logarithmic GP63 genes; mspS, stationary GP63 genes; PCR, polymerase chain reaction; UTR, untranslated region.
those that have been investigated, multiple nonidentical copies of GP46 genes are arranged in clusters (18). Although the biological function(s) of GP46 are not known, immunization with GP46 partially protects experimental mice against challenge with *Leishmania amazonensis* (20, 21). Recent experiments have demonstrated that *Leishmania major* amastigotes and promastigotes express different GP46 mRNAs and proteins (22), indicating that these genes undergo developmental regulation.

Since GP63 genes are differentially expressed during *L. chagasi* promastigote growth, we questioned whether GP46 gene expression also varies during promastigote growth. We found that the abundance of GP46 RNA in *L. chagasi* promastigotes increases dramatically as they grow from logarithmic to stationary phase promastigotes have approximately equivalent steady state levels of GP46 and stationary phase promastigotes have been transfected with plasmids and plated onto solid medium containing 25 or 40 µg/ml G418 for selection and isolation of clonal transfectants as described (29). Clonal isolates were grown in liquid culture, and aliquots of 1.5 × 10⁶ promastigotes were removed daily, washed three times (centrifuged for 5 min at 4000 × g) and resuspended in 1.4 ml phosphate-buffered saline, and then stored at −70 °C. For βGAL assays, aliquots were thawed quickly with the addition of 2 volumes (200 µl) of lysis buffer (100 mM KH₂PO₄, pH 7.8, 0.33% Triton X-100), lysed by three freeze-thaw cycles in dry ice/ethanol and a 37 °C water bath, and centrifuged (5 min at 10,000 × g). The supernatant was assayed for protein concentration (bichinchonic acid reagent and assay, Pierce) and for βGAL activity using Galacton-Star™ chemiluminescent substrate in a fluorometric assay, CLONTECH, Palo Alto, CA). Fluorescence was measured in a Monolight™ 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA).

**General Methods**—Standard techniques of molecular biology were used (25). DNA was sequenced using dye terminator cycle sequencing chemistry and analyzed on a 373A stretch fluorochrome automated sequence analyzer (Perkin-Elmer). The radioactivity of the DNA was quantified using InstantImager™ electronic autoradiography (Packard Instrument Co.). DNA and protein sequences were compared using the University of Wisconsin Genetic Computer Group program version 7.0.

**RESULTS**

Isolation of a cDNA for GP46 of *L. chagasi*—The cDNA sequences encoding GP46 have been reported for *L. major* and *L. amazonensis* (30, 31) but not for *L. chagasi*. Attempts to PCR-amplify a partial GP46 cDNA using degenerate and non-degenerate primers designed from the *L. amazonensis* sequence yielded a 1.3-kb product with *L. mexicana* genomic DNA template but no product with *L. chagasi* genomic DNA. This 1.3-kb fragment was shown by DNA sequencing to encode GP46 and was used to screen 2.7 × 10⁹ phage in an *L. chagasi* cDNA library. Fourteen phage clones hybridized to the probe (0.05%), and 10 were plaque-purified for further characterization. The complete 2.4-kb sequence of the largest of these 10 cDNA inserts was determined and found to be a partial-length cDNA lacking the 39-nucleotide-spliced leader found on the 5' end of all *Leishmania* mRNAs (32).

To isolate the missing 5' segment, a reverse transcriptase-PCR was conducted on total *L. chagasi* promastigote RNA using a forward primer containing part of the spliced leader

**REFERENCES**

1. Post-transcriptional Regulation of GP46 mRNA Abundance 17361

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**EXPERIMENTAL PROCEDURES**

**Parasites**—An isolate of *L. chagasi* derived from a Brazilian patient with visceral leishmaniasis was maintained in hamsters. Promastigotes were cultured in *vitro* at 26 °C as described (9) in a modified minimal essential medium (HOMEM) supplemented with 10% fetal bovine serum and 5 µg/ml hemin (23). Logarithmic and stationary phase promastigotes were defined by morphology and concentration criteria (24). Medium for stably transfected promastigotes contained 40 µg/ml G418 (Life Technologies, Inc.).

**Genomic and cDNA Libraries**—A *L. chagasi* genomic DNA library and a *L. chagasi* cDNA library made from RNA isolated from stationary phase promastigotes and a forward primer containing a partially spliced leader sequence (5'-GGGGACGAGCGACTTCAC-3') and reverse primer, 5'-CAGGCGCACAGACCACGAGA-3', were designed from the coding sequence for *L. amazonensis* (accession number U09612). A series of primers designed from the coding sequence for *L. chagasi* and *Leishmania mexicana* genomic DNA. The reverse primer for this reversed transcriptase-PCR, 5'-9CTGGGACGAGCGACTTCAC-3', and reverse primer, 5'-9CAGGCGCACAGACCACGAGA-3', successfully amplified a fragment from *L. mexicana* DNA that was subsequently used to screen the *L. chagasi* cDNA library.

To obtain a cDNA sequence corresponding to the 5' end of GP46 mRNA, reverse transcriptase-PCR was conducted using RNA isolated from stationary phase *L. chagasi* promastigotes and a forward primer containing a partially spliced leader sequence (5'-AAGCTAACCTAATAAGTGACATT-3', and reverse primer, 5'-CGAGGTTGGTGTACGTGCA-3', was designed from the sequence of a partial length (2.4 kb) GP46 cDNA isolated from the *L. chagasi* cDNA library.

**PCR Amplification of Partial GP46 cDNA Clones**—A series of primers designed from the coding sequence for *L. amazonensis* (GenBank accession number M38368) were used in attempts to PCR-amplify a partial-length GP46 coding region from *L. chagasi* and *Leishmania mexicana* genomic DNA. The forward primer, 5'-GCGAGAIGCCAGGCCTAC-3', reverse primer, 5'-CAGGCGCACAGACCACGAGA-3', successfully amplified a fragment from *L. mexicana* DNA that was subsequently used to screen the *L. chagasi* cDNA library.

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**Southern and Northern Blot Analysis**—Total *L. chagasi* DNA was isolated using DNAzol™ (Life Technologies), and Southern blots were conducted as described (25). For Northern blots, the *L. chagasi* RNA was isolated using a guanidinium-based method (26). RNA was separated by electrophoresis on a 1.2% agarose gel (6% formaldehyde-buff-}

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**DNA Constructions**—The starting plasmids were pBluescript™ SK (Stratagene) and pX-βgal 2 (a generous gift from S. Beverley). A fragment containing the 3'-UTR and IR of gp46A was isolated after NorI digestion of recombinant plasmid DNA containing this genomic region of *L. chagasi* DNA and then ligated into the NorI site of pX-βgal 2. NorI cleaves 76 bases upstream (5') of the stop codon in gp46A; therefore, this clone contains a small piece of the coding region. A partial 3'-UTR and IR of gp46B was similarly isolated from recombinant plasmid DNA by XhoI digestion, blunt ended, and ligated into pX-βgal 2 that had previously been digested with NorI, blunt ended, and dephosphorylated. The orientation of the inserts was determined by sequence across the insert boundaries. The pX-βgal 2 plasmid constructs containing the 3'-UTRs and IRs of the GP63 genes have been described (15).

**Nuclear Run-on Assays**—Promastigotes were harvested in logarithmic or stationary phase, pelleted by centrifugation for 5 min at 3000 × g, and washed twice in Hanks’ balanced salt solution. Disruption of cells and labeling of nuclei were as described previously (28).

**DNA Transfections and βGAL Assays**—*L. chagasi* promastigotes were transfected with plasmids and plated onto solid medium containing 25 or 40 µg/ml G418 for selection and isolation of clonal transfectants as described (29). Clonal isolates were grown in liquid culture, and aliquots of 1.5 × 10⁶ promastigotes were removed daily, washed three times (centrifuged for 5 min at 4000 × g) and resuspended in 1.4 ml phosphate-buffered saline, and then stored at −70 °C. For βGAL assays, aliquots were thawed quickly with the addition of 2 volumes (200 µl) of lysis buffer (100 mM KH₂PO₄, pH 7.8, 0.33% Triton X-100), lysed by three freeze-thaw cycles in dry ice/ethanol and a 37 °C water bath, and centrifuged (5 min at 10,000 × g). The supernatant was assayed for protein concentration (bichinchonic acid reagent and assay, Pierce) and for βGAL activity using Galacton-Star™ chemiluminescent substrate in a fluorometric assay, CLONTECH, Palo Alto, CA). Fluorescence was measured in a Monolight™ 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA).

**General Methods**—Standard techniques of molecular biology were used (25). DNA was sequenced using dye terminator cycle sequencing chemistry and analyzed on a 373A stretch fluorochrome automated sequence analyzer (Perkin-Elmer). The radioactivity of the blasts was quantified using InstantImager™ electronic autoradiography (Packard Instrument Co.). DNA and protein sequences were compared using the University of Wisconsin Genetic Computer Group program version 7.0.
sequence and a reverse primer based on the cDNA sequence. The resulting 500-bp PCR product showed complete sequence identity in a 194-bp overlap with the cDNA. The combined GP46 cDNA sequence of 2754 bp (Fig. 1A) is consistent with the 2.8-kb size of the major GP46 mRNA species observed on a Northern blot (Fig. 2A). The deduced nascent protein sequence of 44 kDa (Fig. 1B) is similar to GP46 proteins of other Leishmania species.

The Steady State Level of GP46 mRNA Is Developmentally Regulated by Post-transcriptional Events—Total RNA was isolated from promastigotes at various times during growth in culture and probed in Northern blots with the GP46 cDNA (Fig. 2). Promastigotes in these cultures entered logarithmic phase of growth within 1–2 days after passage and reached stationary phase by day 7, according to morphology and concentration as previously defined (24).

Panel A of Fig. 2 shows that two GP46 RNA species that hybridize to the cDNA probe exist in stationary phase \textit{L. chagasi} promastigotes, a major species of 2.8 kb and a minor one of 4.8 kb. Both species steadily increase in abundance as the cells grow from logarithmic to stationary phase. InstantImager analysis of the blot indicated that the steady state level of the 2.8-kb GP46 RNA is 30-fold higher at day 7 than at days 3–4. The 4.8-kb RNA increases a similar amount. On the basis of size similarity, the cDNA shown in Fig. 1A is likely derived from the 2.8-kb GP46 RNA.

Panel B shows the same RNAs probed with the GP63 coding region. The hybridization pattern is very similar to the pattern reported previously (15). A GP63 RNA of 2.7 kb occurs in logarithmic phase promastigotes (days 3–4), and a GP63 RNA of 3.0 kb occurs in stationary phase promastigotes (days 6–7). Promastigotes at an intermediate phase of growth (day 5) have both GP63 RNA species. A comparison of panels A and B demonstrates that the abundance of GP46 RNA closely parallels that of the stationary 3.0-kb GP63 RNA. Both RNAs are very rare in days 3–4, begin to appear in day 5, and increase in abundance during days 6–7.

Panels C and D show a hybridization with a tubulin probe and an ethidium bromide stain, respectively, to detect variations in RNA loadings to the gel lanes. Both panels indicate that in this particular experiment more RNA was added to lane 5 (day 5) and less to lane 7 (day 7) than to the other lanes. When these differences are taken into account, the increases in GP46 and stationary GP63 RNA in stationary phase promastigotes (day 7) is even more dramatic than those indicated by the relative band intensities in panels A and B. As shown in panel C, the control hybridization with the \textit{T. brucei} a- and b-tubulin probe resulted in the typical one a- and three b-tubulin band pattern seen in prior studies (28). To eliminate this pattern of

**FIG. 1.** \textbf{cDNA and deduced protein sequence of GP46.} \textbf{A,} the GP46 cDNA of 2754 bp includes a partial 5' spliced leader (overline), the ATG start and TGA stop codons of the open translation reading frame (boxes), and a 3' poly(A) tail. \textbf{B,} the 417-residue protein sequence deduced from the cDNA sequence includes a putative hydrophobic signal peptide (amino acids 1–24), seven leucine-rich repeats of 24–25 residues (107–276), regions rich in Thr and Ser (305–346) or in acidic residues (390–418), and a hydrophobic tail preceded by an Asp (389) that may serve as the site of attachment to a glycosylphosphatidylinositol membrane anchor. *, stop.

**FIG. 2.** \textbf{Northern blots showing GP46 mRNA abundance during promastigote growth in culture.} Total RNA was isolated from cultured promastigotes in logarithmic phase (days 3–4), late logarithmic/early stationary phase (days 5–6), and stationary phase (day 7). Panels A–D, the RNAs (5 μg per lane) were loaded onto agarose gel, separated by electrophoresis, and transferred to a nylon membrane. One-half of the membrane was probed with the coding region of the GP46 cDNA (panel A), and the other half was probed with the GP63 coding region (panel B). The membranes were stripped and reprobed with a \textit{Tryptosoma brucei} tubulin gene (panel C). An ethidium bromide stain of one-half of the gel is shown in panel D. The experiment shown represents one of four independent time courses of promastigote growth from which RNAs were isolated for subsequent Northern blots. Panel E, RNAs on a separate blot were probed with the 1.3-kb region within the 3'-UTR of gene \textit{gp46B} that is not in the cDNA or \textit{gp46A} (see Fig. 4).
The relative rates of transcription of the GP46 genes in logarithmic and stationary phase promastigotes. Plasmids pBlue- 
script (Control) or pBlueScript containing cDNA inserts encoding GP46, GP63, ATPase, or α-tubulin of L. chagasi were applied in duplicate to 
nylon membranes via slot blots (2 µg/slot). The spots were probed with 
nuclear run-on [32P]RNA from nuclei isolated from logarithmic (L) or 
stationary (S) phase promastigotes. The radioactivity hybridizing to 
the DNA in each slot was determined by InstantImager™ analysis and 
normalized to that hybridizing to the tubulin DNA in the respective L 
or S group. The ratio S/L is the ratio of the normalized values; for 
example, the ratio of the normalized values for GP46 was calculated to 
be 0.44/0.87 or 0.51. By definition, the ratio of normalized S/L α-tubulin 
values was 1.0/1.0, or 1.0. nd, not determined.

The similar sizes of the GP46 and GP63 RNAs (2.8 kb and 3.0 
kb) precluded a quantification of their relative abundance in a 
single Northern blot analysis. Thus, Northern blots hybridized 
separately with each probe were incubated in the hybridization 
solution with a second filter containing serial dilutions in slot 
blots of DNA fragments possessing the GP46 and GP63 coding 
regions. The relative amount of GP46 and GP63 RNA was 
estimated by comparing the intensities of their signals on the 
Northern blot filter to the signals of the serially diluted DNAs 
on the slot blot filter. Using this approach, the abundance of 
GP46 2.8-kb RNA was found to be about the same as that of 
GP63 RNA in stationary phase day 7 cells (data not shown).

To determine whether the increase in the steady state level of 
GP46 mRNA that occurs as promastigotes grow from loga-
rithmic to stationary phase is due to enhanced transcription 
initiation or to post-transcriptional events, nuclear run-on ex-
periments were conducted using nuclei isolated from logarithmic 
and stationary phase promastigotes (Fig. 3). The radioactive 
RNA isolated from logarithmic nuclei consistently had a 
higher specific activity than that obtained from stationary nuclei, 
probably indicating that logarithmic phase cells are transcrip-
tionally more active. Thus, the amount of radioactive RNA 
hybridizing to each of the test DNAs in Fig. 3 was normalized 
to the amount hybridizing to DNA encoding α-tubulin. The 
normalized ratio of GP46 run-on RNA in stationary versus 
logarithmic nuclei was found to be about 0.51, approximating 
the corresponding ratio of GP63 run-on RNA. This result indi-
cates that there is no difference in the transcription rate of the 
GP46 genes in logarithmic and stationary phase promastigotes 
relative to α-tubulin gene transcription despite the 30-fold 
increase in the steady state level of GP46 mRNA in stationary 
phase cells. Thus, this increase in GP46 mRNA must be regu-
lated primarily by post-transcriptional events.

Expression of a βGal Reporter Gene—To examine which se-
quences of the GP46 genes might contribute to the increased 
level of their mRNAs in stationary promastigotes, we first 
screened a bacteriophage λ library of L. chagasi genomic DNA 
for genomic DNA clones that contain GP46 genes. Two such 
clones were isolated and characterized by restriction mapping, 
and several of their restriction fragments were subcloned for 
DNA sequencing. The genomic DNA segments in these two 
clones were found to overlap in a region of the genome contain-
ing two GP46 genes (Fig. 1A). A 3.2-kb NotI fragment containing the 3′-UTR and downstream IR of 
GP46A was cloned downstream of the βGal gene in plasmid pX-βgal 2 as indicated. A 4.2-kb XhoI fragment containing part of the 3′-UTR + 
IR of gp46B was also cloned at the same site.

Post-transcriptional Regulation of GP46 mRNA Abundance

Fig. 3. Relative rates of transcription of the GP46 genes in logarithmic and stationary phase promastigotes. Plasmids pBlue-
script (Control) or pBlueScript containing cDNA inserts encoding GP46, GP63, ATPase, or α-tubulin of L. chagasi were applied in duplicate to 
nylon membranes via slot blots (2 µg/slot). The spots were probed with 
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example, the ratio of the normalized values for GP46 was calculated to 
be 0.44/0.87 or 0.51. By definition, the ratio of normalized S/L α-tubulin 
values was 1.0/1.0, or 1.0. nd, not determined.

Fig. 4. Diagrams showing a genomic DNA region containing 
two GP46 genes and the pX-βgal 2 expression vector. The map of 
the genomic DNA region was deduced from a characterization of two 
overlapping genomic DNA clones isolated from a genomic DNA library of L. chagasi. Restriction sites are shown for AccI (A), NotI (N), and 
XhoI (X). Large solid line open rectangles indicate the coding regions of 
two GP46 genes, gp46A and gp46B. Since one of the two genomic DNA 
clones ends within the gp46A coding sequence, the dashed rectangle at the 5′ region of gp46A indicates the sequence predicted from the cDNA sequence (see "Results"). Thin rectangles denote 3′-UTRs of the GP46 genes. The shaded region in the 3′-UTR of gp46B indicates a 1.3-kb sequence that does not occur in the 3′-UTR of gp46A. Fragments contain-
ing the two genes were subcloned and sequenced. The percentages 
shown below the map indicate the nucleotide identity of the indicated 
regions with the corresponding regions of the GP46 cDNA (Fig. 1A). A 
3.2-kb NotI fragment containing the 3′-UTR and downstream IR of 
gp46A was cloned downstream of the βGal gene in plasmid pX-βgal 2 as indicated. A 4.2-kb XhoI fragment containing part of the 3′-UTR + 
IR of gp46B was also cloned at the same site.
growth in the presence of the drug G418. In earlier studies, we had already constructed derivatives of pX-gal2 in which the corresponding 3′-UTRs and IR regions of the three GP63 gene classes, i.e. mspS (stationary), mspL (logarithmic), and mspC (constitutive), were cloned at the same site downstream of the βGAL gene (15). These five plasmids containing the various Leishmania 3′-UTRs + IRs and the parent plasmid pX-gal2 were introduced into promastigotes, and clones of stable transfectants were selected (listed in Table I).

Fig. 5 shows the βGAL enzymatic activities that were determined during growth of the six transfectants from logarithmic to stationary phase and corresponds to experiment 1 of Table I. No change in βGAL activity with time occurred in the transfectants containing the parent plasmid and the plasmids with the 3′-UTRs + IRs of mspL and mspC, as demonstrated previously (15). Likewise, the presence of the partial 3′-UTR + IR of gp46B did not change the βGAL activity from that of the parent plasmid, possibly due to the lack of a full-length 3′-UTR. However, the 3′-UTR + IR of gp46A stimulated a dramatic increase in βGAL activity with time in culture, similar to that of the 3′-UTR + IR of mspS. In both cases, the βGAL activity in logarithmic phase promastigotes was lower on day 3 than in the other transfectants, increased to the level observed in the other transfectants by day 4, and subsequently increased steadily until by day 8 it was about 20–30-fold higher than on day 3. In two other experiments similar to that shown in Fig. 5, the ratio of βGAL activity on day 8 versus day 3 was ≥ 12 in transfectants containing the 3′-UTRs + IRs of gp46A and mspS, whereas no substantive difference was observed in transfectants bearing the other plasmids (Table I). Thus, the gp46A and mspS 3′-UTRs + IRs exert parallel effects on βGAL activity when placed downstream of the βGAL gene.

The βGAL Activity in the Transfectants Reflects the Steady State Level of βGAL mRNA—To ensure that the increase in βGAL enzymatic activity observed in the transfectants bearing plasmids with the 3′-UTRs + IRs of gp46A and mspS was due to increased βGAL mRNA, Northern blots were conducted on RNAs extracted from the transfectants. Fig. 6A shows that in transfectants containing the 3′-UTRs + IRs of gp46A and mspS, the steady state level of βGAL mRNA is much higher in stationary phase (S, day 8) than in logarithmic phase (L, day 3). In addition, the βGAL mRNA containing the 3′-UTR of gp46A (1.3 kb) is slightly larger than the βGAL mRNA with the 3′-UTR of mspS (1.1 kb), as expected from the known sizes of these 3′-UTRs. In contrast, in the transformant bearing only the parent plasmid (called β-gal in Fig. 6), the level of βGAL RNA does not vary as much with respect to the phase of growth. In addition, the major βGAL RNA species in this transformant is about 7.0 kb, suggesting that its primary polyadenylation site occurs about 4 kb downstream of the 3′-kb βGAL coding region. The membrane shown in Fig. 6A was stripped and reprobed with the α-tubulin gene to determine the relative amounts of RNA added to each lane (Fig. 6B). The relative increase in βGAL mRNA in stationary versus logarithmic phase transfectants was 13-fold for the clone containing gp46A sequence, 12-fold for the clone containing mspS sequence, and <2-fold for the parent plasmid. These increases in steady state mRNA levels are consistent with the increases observed in the βGAL enzymatic activity.

To verify that the plasmid copy number does not change

| Transfectant        | Stationary/logarithmic |
|---------------------|------------------------|
| gp46A 3′-UTR + IR   | 21                     |
| gp46B 3′-UTR + IR   | 0.92                   |
| mspS 3′-UTR + IR    | 31                     |
| mspL 3′-UTR + IR    | 0.37                   |
| mspC 3′-UTR + IR    | 0.94                   |
| βgal                | 0.66                   |

| Activity (x 10^6 units/μg protein) | DAYS IN CULTURE |
|-----------------------------------|-----------------|
| 2                                 | 3               |
| 4                                 | 5               |
| 6                                 | 7               |
| 8                                 | 9               |

**Fig. 6.** Northern blots showing the steady state levels of βGAL mRNA in promastigotes stably transfected with derivatives of pX-βgal 2. The transfected promastigotes possessed pX-βgal 2 containing the 3′-UTRs + IRs of gp46A (●), gp46B (○), mspS (■), mspC (▲), mspL (logarithmic (♦)) and no insert (▲). The βGAL enzymatic activities shown are the results of one experiment in which extracts of the various transfected cells collected at the indicated times were assayed in triplicate for activity. The coefficients of variation for all relative fluorescent units are <10. The results shown for the gp46A 3′-UTR + IR transfectant are representative of three independent experiments (summarized in Table I).
during growth from logarithmic to stationary phase, DNA was extracted from the transfectants at days 3 and 8 and probed with the βGAL coding sequence (Fig. 7A). The same blot was stripped and reprobed with GP46 coding sequence to adjust for DNA loading (Fig. 7B). The relative amount of the plasmid in each transformant was found to vary by <10% during growth of the transfectants.

In these Southern blots, the βGAL probe hybridizes only to a single 3.6-kb AccI fragment of the plasmid, since the probe is contained within this fragment. Conversely, because the plasmid constructs do not contain GP46 coding sequence, the GP46 coding region probe hybridizes only to GP46 genes in the L. chagasi genome. Thus, in panel B, all of the fragments are derived from the genome. The 2.0-kb fragment and one of the ~7.5-kb doublet fragments correspond to expected fragments based on the locations of AccI sites in the genomic and cDNA sequences shown in Fig. 3. The additional 1.6, 2.7, and ~7.5-kb fragments to which the GP46 probe hybridizes with varying intensities indicate the existence of other genes besides gp46A and gp46B. These results are consistent with similar studies of GP46 gene organization in other Leishmania species that showed that GP46 is encoded by a family of nonidentical genes (18). The weak signals of fragments whose sizes are not indicated in Fig. 7B may suggest either the presence of additional sequences in the genome that have partial identity to the GP46 coding region probe or incomplete digestion of genomic DNA.

DISCUSSION

Previous work with in vitro cultured L. chagasi has documented differential expression of RNAs from distinct msp genes encoding the surface glycoprotein GP63 (9). The current study was based on the hypothesis that the genes coding for other surface proteins might be similarly regulated. We tested this hypothesis with another Leishmania gene family encoding GP46, which, like GP63, is a glycoprotein that is expressed on the parasite surface membrane.

The deduced amino acid sequence of L. chagasi GP46 shown in Fig. 1B displays 61% identity to GP46 of L. major (30) and 65% identity to GP46 of L. amazonensis (31). All of these GP46 sequences contain hydrophobic amino and carboxyl termini that are probably post-translationally cleaved during the translocation of the protein across the endoplasmic reticulum and its linkage to membrane-anchored glycosylphosphatidylinositol, respectively. Additionally, all contain 3 (L. major) to 7 (L. chagasi) leucine-rich repeats of 24 residues. Leucine-rich repeats of 24 residues are components of many other proteins and are thought to be sites of specific protein-to-protein interactions (33, 34), suggesting a similar role for these repeats in GP46. Protozoan parasites of the genus Giardia also have a surface glycoprotein that contains leucine-rich repeats of 24 residues (35), raising the possibility that the surface glycoproteins of these two distantly related genera may have analogous function.

Based upon our work on GP46 gene transcription and mRNA stability, we would predict that GP46 levels vary during promastigote development, as has been shown for GP63. Recent analysis in L. major showed that different GP46 RNAs are expressed in amastigotes and promastigotes, and that the glycosylphosphatidylinositol-anchored GP46 in amastigotes, but not promastigotes, is resistant to hydrolysis by phosphatidylinositol-specific phospholipase C (22). The work presented here establishes the need for a similar study of GP46 expression throughout promastigote development.

Northern blot and nuclear run-on experiments showed that the varied abundance of GP46 mRNA in promastigotes is due to post-transcriptional events (Fig. 2 and 3). The steady state level of a number of RNAs in the order Kinetoplastidae are also regulated post-transcriptionally (36–38). Such regulation may be linked to the observation that in this order, many highly expressed proteins are encoded by tandemly repeated genes present in a linked cluster. Some, if not all, of these gene clusters are transcribed as polycistronic precursor RNAs that subsequently undergo processing by the addition of a 39-nucleotide-spliced leader at the 5′ end and polyadenylation at the 3′ end of each mRNA. 5′ processing of a downstream gene is coupled to polyadenylation of the upstream gene product (39). Thus, post-transcriptional regulation would provide the means for controlling levels of specific mRNAs derived from a polycistronic transcript.

The parallel expression profiles of GP46 and mspS RNA in promastigotes as well as the equivalent effects of the 3′-UTRs + IRs of gp46A and mspS on βGAL activity and mRNA levels suggest that both genes are post-transcriptionally regulated by a similar mechanism. From this observation, one would hypothesize that homologous regions within the 3′-UTRs of these two genes may be responsible for the increased abundance of these mRNAs during stationary phase growth. Comparison of the 3′-UTR + IR sequences of gp46A and mspS with BESTFIT
Fig. 8. Alignment of a region of sequence similarity in the 3'-UTRs of gp46A and mspS. The 34-nucleotide overlined segment has 79% identity, and the entire 92-nucleotide region has 66% identity. These segments are located 416 bp (gp46A) and 213 bp (mspS) upstream of the polyadenylation site. Numbers refer to GP46 (Fig. 1) and mspS (9) cDNAs. Vertical lines indicate identity, and periods indicate gaps that were introduced to optimize the alignment.

indicates several regions of similarity within their 3'-UTRs, the highest scoring of which is depicted in Fig. 8. In contrast, there is little identity within their IRs. A test of whether these homologous DNA segments in the 3'-UTRs are responsible for the stationary-specific expression pattern will require further transfection experiments with these putative regulatory regions either deleted or inserted downstream of a reporter gene. The 3'-UTR of gp46B also contains this region of similarity, leading us to hypothesize that the reason for the lack of growth-regulated expression of the Bgal construct containing the partial length gp46B 3'-UTR + IR is that all sequences essential for its growth-regulated expression (Fig. 2E) were not contained in this construct.

The actual molecular mechanism by which the GP46 and mspS genes are regulated remains unclear. Our prior work documented differences in the mechanisms regulating expression of the mspL and mspS genes within the same cluster (15, 28). In experiments comparable to those reported here, the 3'-UTR + IR of mspS, but not of mspL, was responsible for augmenting both the steady state RNA and protein expression levels of an upstream reporter gene during growth of L. chagasi from logarithmetic to stationary phase (15). In other experiments, the levels and half-lives of mspL RNAs, but not of mspS RNAs, were dramatically up-regulated by the addition of protein synthesis inhibitors that did not influence the rate of transcription, implying the involvement of a negative regulatory protein factor in targeting mspL RNAs for rapid degradation (28). Similar studies with the GP46 genes hold the promise of revealing further intricacies about gene regulation in this class of protozoan parasites.

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