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Extrachromosomal genetic complementation of surface metalloproteinase (gp63)-deficient *Leishmania* increases their binding to macrophages

(transfection/tunicamycin resistance/Zn-proteinase/trypanosomatid protozoa/macrophage)

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ABSTRACT **A major surface glycoprotein of 63 kDa (gp63) has been previously identified biochemically and genetically as a zinc proteinase conserved in pathogenic *Leishmania* spp. The functional significance of this proteinase was analyzed by genetic approaches. A 15-kilobase DNA with a tunicamycin-resistance gene from *Leishmania amazonensis* was ligated in two different orientations into pBluescript containing a gp63 gene from *Leishmania major*. These plasmid constructs were used to transfect a variant of *L. amazonensis* deficient in gp63 expression. Both constructs were found to confer tunicamycin resistance with equal efficiency and remained structurally unchanged in the transfectants. RNA and immunoblot analyses showed over-expression of gp63 in the transfectants with one of the two plasmids constructed. The over-produced products were enzymatically active and expressed on the cell surface. Significantly, the transfectants with over-expressed gp63 increased by 2-fold over controls in their binding to macrophages. Evidence presented thus indicates that the gp63 gene constructed in the plasmid as described and introduced exogenously expresses in the gp63-deficient variants and that the expressed products are functionally active.**

Leishmania are a group of trypanosomatid protozoa, of which many species are pathogenic to humans (1). These organisms infect exclusively macrophages of the mammalian hosts and subsequently live in the phagolysosomes of these cells. Molecular determinants, which help *Leishmania* achieve such intracellular parasitism, contribute to their virulence. Multiple factors are presumably involved, and many are surface glycoconjugates, including a number of ectoenzymes (2).

Most prominent among the *Leishmania* ectoenzymes known is the major surface glycoprotein of 63 kDa (3–5). It constitutes up to 2% of the total cellular proteins and is conserved among all pathogenic species. It is a proteinase (6)—an unusual zinc enzyme (7, 8) subjected to posttranslational modifications—i.e., pre- and propeptide cleavages at the N-terminal end (9–11), glypiation (12) with glycosylphosphatidylinositol anchor (13) at the C-terminal end, and N-glycosylation (4) with high mannose-type asparagine-linked oligosaccharides (14). Previously, gp63 has been proposed to function as a surface ligand of *Leishmania* for their receptor-mediated endocytosis by macrophages (4, 5, 15) and for protection against lytic factors in their subsequent intralysosomal survival (7).

Recently, it has become possible to transfect *Leishmania* by electroporation with plasmids for the expression of foreign genes; the vectors used consist of regulatory sequences of *Leishmania* genes in combination with drug-selective markers (16, 17). Another avenue of molecular genetic approach is thus available for functionally analyzing *Leishmania* virulence determinants, such as gp63.

We report here the construction of a plasmid containing the gp63 gene together with a tunicamycin (TM)-resistance gene as a selective marker. Transfection of a gp63-deficient variant with this plasmid substantially increases the surface metalloproteinase of these cells and their binding to macrophages. The results not only provide genetic evidence for the functional importance of gp63 in leishmanial infection of macrophages but also allow further molecular dissection of the functional domains of this protein.

MATERIALS AND METHODS

Cells. Promastigotes of *Leishmania amazonensis* (LV78) and the murine macrophage line J774G8 were cultured as described (18). Briefly, promastigotes were grown to the stationary phase at 25°C in Hepes-buffered medium 199, pH 7.4/10% heat-inactivated fetal bovine serum. Macrophages were grown in Hepes-buffered RPMI 1640 medium, pH 7.4/20% heat-inactivated fetal bovine serum.

The gp63-deficient variant used in the present study was obtained by growing a stock of *L. amazonensis in vitro* as promastigotes for >3 yr. The virulence of these cells is known to be substantially reduced *in vitro* and *in vivo* (19). The level of gp63 protein and its proteolytic activity are both several-fold less in these cells than in the virulent variants, as found previously with different stocks of these cells (19).

Plasmid Constructs. The plasmid pBS10Rb.1, consisting of *Leishmania major* gp63 gene cloned in pBluescript, was from W. R. McMaster (University of British Columbia, Vancouver) (9). The insert includes ≈200-base-pair (bp) and 160-bp-flanking sequences at the 5′-end and 3′-end of the gp63-coding region, respectively. The plasmid pTR15 contains a 15-kilobase (kb) *Bam*HI fragment cloned into pBR322 (Fig. 1) from the 63-kb amplified circular DNA of TM-resistant *L. amazonensis* variants (20). The 15-kb DNA was found to contain an *N*-acetylglucosamine-1-phosphate transferase (NAGT)-encoding gene—i.e., TM-resistance gene (unpublished data). The 15-kb fragment derived from pTR15 was cloned into pBS10Rb.1 at the *Bam*HI site in two different orientations, designated as pBSgp63-TMR I and II, respectively (Fig. 1).

Transfection of *Leishmania*. Supercoiled plasmid DNAs were introduced by electroporation into cloned or uncloned cells of the gp63-deficient variant. Transfection of these cells by electroporation and their selection with TM were done as described (refs. 16 and 22, and unpublished data).

Nucleic Acid Techniques. DNAs were isolated by standard procedures (23). Total cellular RNAs were isolated by hot phenol extraction (24) and LiCl precipitation (23). RNAs

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Abbreviations: BSA, bovine serum albumin; NAGT, *N*-acetylglucosamine-1-phosphate transferase; TM, tunicamycin.

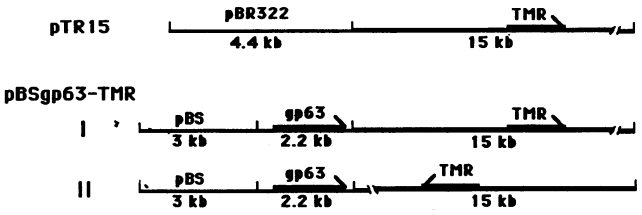

FIG. 1. Linear maps of circular plasmids constructed for expression of *Leishmania* gp63 gene. pTR15, 15-kb *Bam*HI fragment containing a 1.4-kb TM-resistance gene (TMR) cloned at *Bam*HI site into pBR322; pBSgp63-TMR, 15-kb fragment from pTR15 cloned into plasmid pBS10Rb.1, consisting of pBluescript (pBS) and a gp63 gene (gp63). Constructs I and II differ only in orientation of the 15-kb fragment with respect to the gp63 gene; arrows indicate orientation of genes.

were electrophoresed in 1% agarose gel with formaldehyde and Mops (23). For orthogonal field alternating gel electrophoresis, agarose blocks, each containing 1–2 × 10^7 cells, were prepared as before (25). Orthogonal field alternating gel electrophoresis was done in 0.7% agarose at 14°C in 0.5× Tris/borate/saline (TBE) (1× TBE is 89 mM Tris/89 mM boric acid/1 mM EDTA, pH 8.0) at 300 V for 20 hr with an interval of 40 sec.

All probes for Southern hybridization were labeled with [^{32}P]dCTP by using random hexanucleotide primers (United States Biochemical). Northern (RNA) and Southern blot analyses were done as described (25). Filters were washed four times in 0.1× standard saline citrate/0.1% SDS at 65°C.

Immunoblot Analyses. Cells were lysed by boiling in sample buffer with 2.5% SDS and 0.65 M 2-mercaptoethanol. Other cell samples were lysed in less denaturing conditions—i.e., 2% SDS and 0.5% Nonidet-P40 without boiling under nonreducing conditions. Samples were subjected to SDS/PAGE and transferred to nitrocellulose paper as recommended by the manufacturer (Schleicher & Schuell). Monoclonal antibody 6H12 (26) was used for detecting gp63 specific to the promastigotes of *L. amazonensis* under less denaturing conditions. Samples were also probed with polyclonal antibodies in antisera raised in rabbit against purified gp63, which are known to react with this antigen from all species (ref. 7; unpublished data).

Assay of Surface Proteinase Activity. Cells were fixed in 0.5% glutaraldehyde for 2 hr at 4°C. The fixed cells were extensively washed three times in ice-cold phosphate-buffered saline, pH 7.4. The surface proteinase activities were assayed by using either radioiodinated native bovine serum albumin (BSA) (27) or azocasein (6) as the substrates.

Albuminolytic activity was assayed in 50 mM acetate buffer, pH 4. Native BSA was radioiodinated by chloramine T as described (27). The specific activity of the ^{125}I-labeled BSA obtained was 170 cpm/ng of protein. Aliquots of 5 × 10^6 cells were each incubated with 7.5 μg of ^{125}I-labeled BSA with or without 1 mM *o*-phenanthroline at 37°C for 30 min. Undigested substrates were precipitated with 10% trichloroacetic acid. The radioactivity of trichloroacetic acid-soluble fractions was counted in a γ counter and converted to ng of protein digested for calculating specific enzyme activity.

Caseinolytic activity was assayed in phosphate-buffered saline, pH 7.4. The azocasein stock solution (20 mg/ml) was dialyzed extensively before use. Aliquots of 5 × 10^7 or 10^8 fixed cells were each incubated with 5 mg of azocasein at 37°C for 1 hr. The OD_{366} of trichloroacetic acid-soluble fractions was determined spectrophotometrically and converted to μg of azocasein digested per ml according to a standard curve.

Macrophage Binding Assay. This assay was done essentially as described (4). Briefly, cells were labeled with 4,5-[^{3}H]leucine (specific activity = 60 Ci/mmol; 1 Ci = 37 GBq) at 5 μCi/ml overnight and chased in complete medium for 3 hr. Labeled cells were washed three times and resuspended in Hanks' balanced salt solution. Aliquots of 10^7 labeled promastigotes were each added to 10^6 macrophages plated per coverslip. After incubation at 35°C for 5, 10, and 15 min, the coverslips in triplicate were rinsed extensively with Hanks' balanced salt solution to remove unbound promastigotes. Samples were then digested with Protosol and subjected to scintillation counting. Radioactivity was converted into cell numbers based on the labeling efficiency of individual cell samples separately determined (4).

RESULTS

Transfectants Selected for TM Resistance Contain Extrachromosomal Plasmids of the Original Constructs. The gp63-deficient variants were transfected with various plasmids (Fig. 1) by electroporation and selected for TM resistance. Cloned and uncloned cells served equally well as the recipients. TM-resistant transfectants were obtained from cells with pTR15 or pBSgp63-TMR I or pBSgp63-TMR II but not with pBS10Rb.1. Transfection efficiency was comparable among the three effective plasmids. Transfectants in all three cases emerged in ≈10–14 days of selection in medium containing 10 μg of TM per ml; the transfectants subsequently grew continually under such conditions as well as the parental cells in drug-free medium. This result is consistent with our observations of transfectants with other plasmids with the TM-resistance gene (unpublished data). Thus, the presence of pBluescript plus a gp63 gene does not impair the expression of NAGT gene in the 15-kb DNA to serve as a selective marker (see Fig. 1).

In the transfectants, the plasmids existed extrachromosomally and were structurally unaltered. This result was indicated initially by Southern blot analyses of the total DNAs isolated from various transfectants and their parental cells. When probed with the gp63 gene, the transfectant DNAs showed a hybridization pattern exactly as the combination of samples from the parental cells and plasmids (data not shown). The absence of additional bands indicated that there were neither apparent structural alterations of the plasmids nor recombination between the leishmanial DNA sequences in the plasmids and homologous chromosomal regions of the transfectants. The existence of the electroporated plasmids as extrachromosomal supercoiled DNA was also indicated by their characteristic migration in orthogonal field alternating gel electrophoresis gels (Fig. 2*A*). Southern hybridization of these gels with the gp63 gene revealed that the plasmids (pBSgp63-TMR) migrated as supercoiled DNAs off the course of the linear chromosomes (Fig. 2*B*, lane 5, star). The chromosomal signals for gp63 gene were of equal intensity between the transfectants and their parental cells (Fig. 2*B*, lane 5 vs. lane 1), suggesting no apparent chromosomal integration of the electroporated DNA. The intensive hybridization signals seen in the well of the transfectant DNAs (Fig. 2*B*, lane 5) were due to the presence of nicked plasmids as relaxed open circles. It has been shown (28) that such circular DNAs are often trapped in the agarose and, thus, fail to migrate into the gel. Several control samples were included in the same blot to show specificity of the hybridization: signals were absent from λ ladder (lane 4), very weak for the total DNAs from *Leishmania enriettii* (lane 3), and associated with chromosome bands of different mobility from other stocks of *L. amazonensis* (lanes 2 and 6).

Transfectants with Plasmid Construct I Over-Express gp63 Transcripts. Northern blot analysis showed that two broad transcripts of 3–4 kb and 4.5–6 kb were over-expressed in these transfectants (Fig. 3*A*, lane P1), whereas transcripts of the parental cells and the transfectants with pTR15 were slightly smaller in size and much less abundant (Fig. 3*A*, lanes P and w). Reprobing the same blot with β-tubulin gene

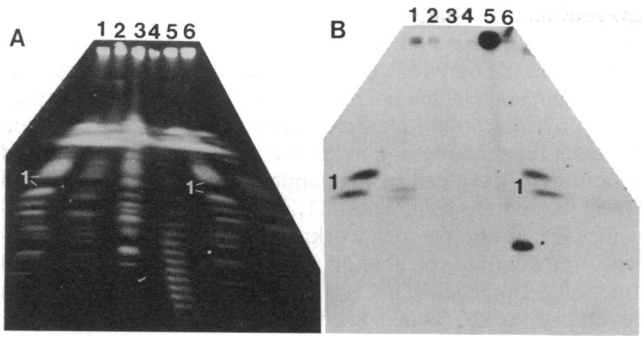

FIG. 2. Orthogonal field alternating gel electrophoresis of *Leishmania* wild-type, variant, and transfectant cells. (*A*) Ethidium bromide-stained gel. (*B*) Blot of the gel probed with the gp63 gene from pBS10Rb.1. Lanes: 1, parental variants for transfectants (cf. lane 5); 4, λ ladders as M_r marker; 5, transfectants with pTR15-gp63 I (cf. lane 1); 2, 3, and 6, different wild-type stocks. The electroporated plasmids as extrachromosomal circular DNAs (⋆) migrate along a path straighter than that of the linear chromosomal DNAs. The gp63 gene hybridizes to two endogenous chromosomal bands (labeled as 1) and to the pTR15-gp63 plasmids (star) present only in the transfectant cells. Note that the chromosomal hybridization intensity and pattern from the transfectant (lane 5) is similar to those of the parental cells (lane 1). Other wild-type stocks show lower hybridization intensity (lanes 2, 3, and 6).

revealed comparable signals among the three samples (Fig. 3*B*). The transcripts over-expressed in the transfectants thus appear to originate from the electroporated gene, as they differ in size from the endogenous mRNAs.

The Transfectants with Over-Expressed Transcripts Also Over-Produce gp63 Proteins. To differentiate the over-expressed products from the endogenous gp63, different antibodies were used for immunoblot analyses. The monoclonal antibody from the hybridoma, 6H12 (26), only recognizes gp63 from *L. amazonensis* promastigotes under nonreducing and less denaturing conditions. However, polyclonal antisera raised in rabbit against gp63 purified from this species recognizes this antigen from all species examined so far. The polyclonal antibodies in the antisera revealed 2- to 3-fold more gp63 in the transfectants with the pBSgp63-TMR I (Fig. 4*A*, lane P1) than controls—i.e., parental cells (lanes W), *L. major* promastigotes (lane w), transfectants with pTR15 (lane P), and those with the plasmid construct II (lane P2). The difference observed was not due to unequal loading of proteins, as indicated by Coomassie blue staining of these samples (Fig. 4*B*). The monoclonal antibody, however, revealed no significant differences in the banding intensity of this antigen between parental cells and all their transfectants

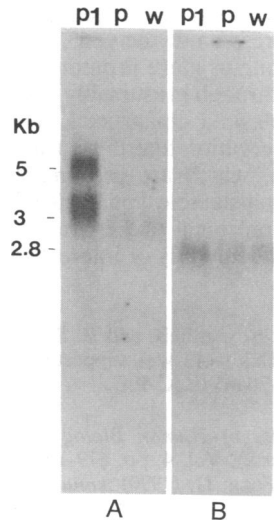

FIG. 3. Northern blot analysis of transfectants showing over-expression of gp63 mRNA. Ten micrograms of total cellular RNAs from cells transfected with pBSgp63-TMR I (P1), pTR15 (P) and parental cells (w) was electrophoresed in 1% agarose gel and blotted onto nitrocellulose paper. (*A*) Blot probed with gp63 gene. (*B*) Same blot reprobed with β-tubulin gene.

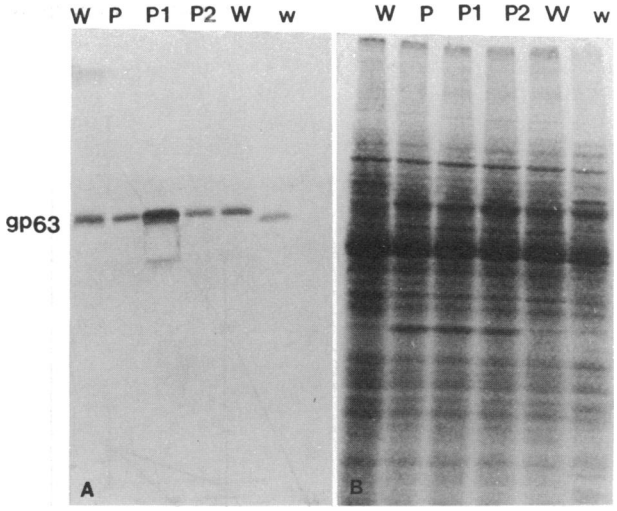

FIG. 4. Immunoblot analysis of transfectants showing over-expression of gp63 protein. (*A*) Immunoblot probed with anti-gp63 polyclonal antibody. (*B*) Same set of samples stained with Coomassie blue. Each sample contains proteins equivalent to 2×10^7 promastigotes. W, variants of *L. amazonensis* used for transfection; w, wild-type *L. major*; P, variants transfected with vector (pTR15) alone; P1 and P2, variants transfected with pBSgp63-TMR I and II, respectively.

(data not shown). This result suggests that the over-expressed gp63 does not contain the epitope (or complete epitope) specific to the monoclonal antibody used. The results, thus, further indicate that the over-produced gp63 is derived from the gene in the electroporated plasmids instead of the endogenous chromosomal copies of the recipient cells.

Over-Produced gp63 Molecules Are Enzymatically Active and Express on the Surface of Transfectants. Glutaraldehyde-fixed cells were assayed for proteolytic activities to demonstrate their surface localization (27). Both ^{125}I-labeled BSA and azocasein were used as substrates because a previous report indicated that they were susceptible to cleavages by gp63 optimally at acidic pH and neutral pH, respectively (29). The transfectants with plasmid construct I showed both albuminolytic and caseinolytic activities, which were ≈2- to 3-fold and 10-fold higher than those of the parental cells and other transfectants, respectively (Table 1). The substrate-dependent variation in the increase of specific proteolytic activities seen in the transfectants is not unexpected in view of the different assay conditions used. The enzyme activities observed fell within the range of those reported before for the same *Leishmania* species against identical substrates—i.e., BSA (27) and azocasein (29). The sensitivity of all samples to *o*-phenanthroline was assessed by using BSA as the assay substrate. The results showed complete inhibition of the proteolytic activity by this zinc chelator (data not shown), as has been observed (7).

Transfectants with Over-Expression of Surface gp63 Increase in Their Binding to Macrophages. Macrophage-binding

Table 1. Proteolytic activities of transfected cells

Transfection	Albumino-lytic*	% Control	Caseino-lytic†	% Control
None	67.7 ± 2.85	100	4.2 ± 1.8	100
pTR15	86.3 ± 0.7	120	ND	
pBSgp63-TMR I	185.3 ± 3.4	272	49.5 ± 4.05	1178
pBSgp63-TMR II	ND		4.2 ± 0.35	100

Glutaraldehyde-fixed promastigotes were used for all assays. ND, not determined.
*^{125}I-labeled BSA degraded per μg per 10^{10} cells per hr, pH 4.
†Azocasein degraded per μg per 10^8 cells per hr, pH 7.4.

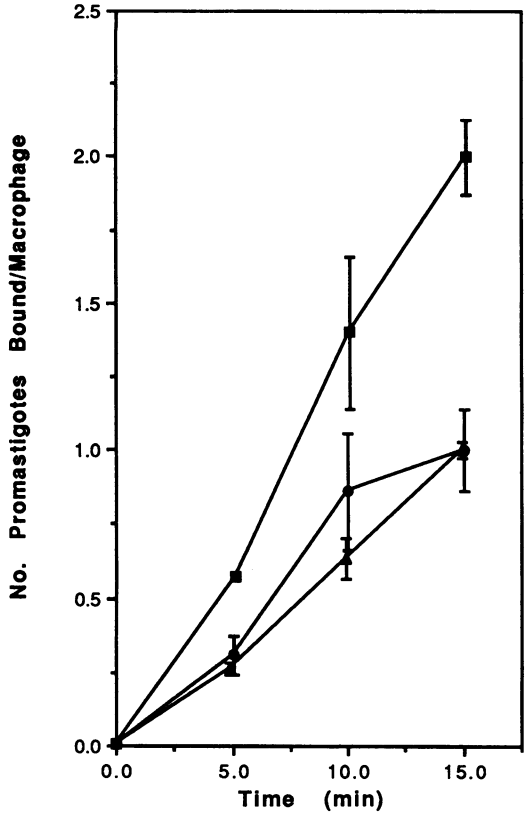

FIG. 5. Increase in binding to macrophages of transfectants with over-expressed gp63. Promastigotes were labeled with [^{3}H]leucine and chased in fresh medium. Aliquots of 10^7 promastigotes were each added to 10^6 macrophages grown as a monolayer on a coverslip. Triplate samples were removed at different time points as indicated and washed extensively to remove unbound promastigotes. Promastigotes bound were determined by scintillation counting. ■, Cells transfected with pBSgp63-TMR I; ●, cells transfected with pBSgp63-TMR II; ▲, parental cells.

activity was assessed by a radioassay using metabolically labeled promastigotes and the J774G8 line of macrophages described in *Materials and Methods*. As shown (30), the radioactivity of labeled cells was linear in relation to the cell number. Also, the assay was specific for macrophage binding, as the experiments were completed in 15 min when endocytosis of promastigotes by macrophages was minimal. By this assay, binding of the transfectants with pBSgp63-TMR I to macrophages was shown to be 2-fold higher than that of the transfectants with pBSgp63-TMR II or their parental cells (Fig. 5).

DISCUSSION

We demonstrate the use of a NAGT gene as the selective marker for the construction of a *Leishmania* expression vector. The ease and specificity of this gene in the selection of transfectants for TM resistance will be presented and discussed in detail elsewhere (unpublished data). Here, the 15-kb *L. amazonensis* DNA with this marker was placed randomly at a convenient restriction site of the pBluescript containing a *L. major* gp63 gene. Expression of this gene in the constructed plasmid is initially suggested by the finding of abundant gp63 transcripts and protein in the transfectants. This expression is further indicated by the antigenic specificity of the over-expressed protein and the size of their transcripts, both of which differ from the endogenous species. The over-expressed gp63 in the transfectants is apparently processed appropriately not only for the expression of enzyme activity but also for targeting to the cell surface. More importantly, such transfectants were found more competent than the parental cells in binding to macrophages. The role of gp63 in leishmanial infection of macrophages is, thus, shown by molecular genetic evidence. A similar conclusion has been reached previously on the basis of competition and inhibition types of cell biology approaches with purified gp63 (4) or its peptide fragments (31, 32) and gp63-specific antibodies (5). In the present work, we make available a prototype transfection system for molecular dissection of gp63 functional domains. This system differs from the vectors developed so far, which used regulatory sequences of the dihydrofolate reductase gene for expressing *Leishmania* gp46 and β-galactosidase (33).

Our results provide some insight into the possible defects of the variants in gp63 expression. Clearly these cells are not deletion mutants of the gp63 genes (see Fig. 2 and unpublished data). As a multigene family of tandemly repeated and dispersed chromosomal copies (34), their deletion is unlikely to occur spontaneously. The variants contained a limited amount of endogenous gp63 but apparently could process appropriately the products of exogenously introduced genes into functionally active molecules. The variants used in these studies thus appear to differ from those that have been characterized previously as deficient in posttranslational modifications to account for the lack of gp63 accumulation (35). Recently, *Leishmania donovani* has been found to contain three species of gp63 mRNAs with 3′ untranslated regions different in length and in sequence (21, 36). One species is produced constitutively, whereas the remaining two are transcribed growth phase-specifically, correlating with the abundance of gp63 found in cells grown to the stationary phase. If these events also occur in *L. amazonensis*, the transcription of one or more species of gp63 mRNAs may be somehow deficient in the variant cells, resulting possibly from the failure of transcription initiation or degradation of inappropriately processed mRNAs (cf. ref. 36). Whatever the mechanisms, it is clear, however, in our case that the gp63 gene from a heterogeneous *Leishmania* species exogenously introduced is not susceptible to such inhibition and expresses appropriately in these cells.

Worthy of further investigation are the possible contributions of the sequences in the 15-kb DNA and the flanking sequences of the gp63 gene in regulating its expression. Interestingly, both plasmids constructed are equally efficient in conferring TM resistance, but only the transfectants with pBSgp63-TMR I over-express gp63. The only difference between the two constructs lies in the orientation of the 15-kb NAGT gene-containing *Leishmania* DNA with respect to the gp63 gene (see Fig. 1). This fact suggests strongly that a certain sequence(s) in the 15-kb DNA not only is required but must be oriented correctly for expression of the gp63 gene. One possibility is the presence of one or more promoters in the 15-kb region, from which read-through presumably over-expresses gp63 transcripts from plasmid construct I. This proposal is consistent with the observation that these transfectants contain larger transcripts, which are presumably processed subsequently to form translatable gp63 mRNA. Transcriptional regulation of chromosomal and extrachromosomal gp63 genes in these transfectants is of interest for further investigation.

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