

IDENTIFICATION OF *LEISHMANIA* GENES ENCODING PROTEINS CONTAINING TANDEMELY REPEATING PEPTIDES

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Leishmania are protozoan parasites of mammals that have two discrete life stages. They exist as extracellular promastigotes within the gut of the sandfly vector and are introduced into the mammalian host when an infected insect takes a blood meal. Subsequently, promastigotes are ingested by mononuclear phagocytic cells, such as macrophages, and then convert into their intracellular amastigote form (1). Considerable attention has been focused on understanding the molecular basis of parasite–host cell interactions. Current evidence indicates that the uptake of *Leishmania* by macrophages is mediated by specific ligand–receptor interactions involving molecules on the surface of both the parasite and the host macrophage. Cell surface glycoproteins and/or glycoconjugates of promastigotes have been implicated in the binding to macrophage receptors. These include the major surface glycoprotein of apparent mol wt 63,000 (2–4) and a lipid-containing surface glycoconjugate (5). Specific macrophage receptors that have been shown to interact with ligands on the promastigote surface include the receptor for the complement component C3bi (CR3) (6–8), the manose/fucose receptor (6, 9), and the receptor for advanced glycoprotein end-products (10).

Immunity to *Leishmania* in man or in experimental animals may be induced during recovery from infection with virulent organisms or by immunization with various attenuated parasite preparations (1, 11). This suggests that protective immunization may be possible and, therefore, there is considerable interest in determining the molecular basis of acquired resistance. To identify *Leishmania* membrane proteins that may mediate interactions with host macrophages or may induce specific immunity, a *Leishmania major* DNA expression library has been screened with rabbit antibodies against *Leishmania* membranes. Several clones were identified and the two described in this report are of particular interest as they encode two *Leishmania* proteins containing different regions of tandemly repetitive amino acid sequences of 14 and 10 residues, respectively. The repetitive nature of these *Leishmania* proteins is strikingly similar to several malaria (12, 13) and trypanosome molecules (14, 15), where in malaria the repetitive sequences have been implicated in evasion of host immunity (12, 13).

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Materials and Methods

Strains. *L. major* promastigotes, NIH S strain (16), were provided by Dr. N. Reiner (University of British Columbia, Vancouver) and were maintained in tissue culture medium 199 (Gibco Laboratories, Grand Island NY) supplemented with 10% FCS (HyClone Laboratories, Logan, UT). Promastigotes of *Leishmania donovani* 1-S, Cl_{2-D} (17) were generously provided by Dr. D. Dwyer (National Institutes of Health, Bethesda, MD) and were grown in chemically defined RE-IX medium (Gibco Laboratories). Genotypes of both strains were confirmed by comparison of genomic DNA fragment hybridization patterns with known standard *Leishmania* strains (kindly carried out by Dr. T. Spithill, Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia).

Construction and Screening of DNA Libraries. The protocols of Huynh et al. (18) and Young et al. (19) with modifications as described (20) were used to construct and screen a λ gt11 genomic expression library. *L. major* promastigote genomic DNA was partially digested with the restriction enzymes Alu I or Hae III. The digests were combined, size-separated, and ligated to λ gt11 arms and packaged as described (Stratagene Cloning Systems, San Diego, CA). This procedure resulted in 2×10^7 phage and the library was not amplified.

For screening, phage were plated on an *Escherichia coli* Y1090(r⁻) (Stratagene) and screened as described previously (20) with a rabbit antiserum prepared by immunization with a purified membrane fraction prepared from *L. major* promastigotes (generously provided by Dr. T. W. Pearson, University of Victoria, British Columbia). Bound antibodies were detected using biotin-labeled anti-rabbit antibodies and avidin-biotin-peroxidase complexes (ABC Vectastain reagents; Vector Laboratories, Inc., Burlingame, CA). Peroxidase complexes were developed using 4-chloro-1-naphthol.

A λ EMBL3 library was constructed using *L. major* genomic DNA that had been partially digested with Sau IIIA following the procedures of Frischauf et al. (21). This library was screened as described (22) using DNA probes that had been nick-translated (23) with [³²P]dATP.

Characterization of DNA Clones. DNA clones were analyzed by restriction mapping using a variety of restriction enzymes. DNA fragments were subcloned from the original λ gt11 clones into the plasmid, pUC19, or the phage, M13mp19 (24), using standard procedures. DNA sequence analysis was carried out using the dideoxy chain termination technique (25) as described previously (22). In addition, exonuclease III deletion clones were prepared and sequenced as described (26).

Southern Blot Analysis. Genomic DNA isolated from *Leishmania* promastigotes was digested with a variety of restriction enzymes and transferred to nylon membranes (Hybond-N; Amersham Corp., Oakville, Ontario). Blots were prehybridized, hybridized, and washed following the manufacturer's protocol. DNA probes were labeled with [³²P]-dATP by nick translation.

Northern Blot Analysis. Total RNA was isolated from *Leishmania* promastigote as described by Chirgwin et al. (27) using cesium chloride gradients. Total RNA was size-separated on agarose formaldehyde gels as described (28). RNA was transferred to nylon membranes and hybridized as described above for Southern blot analysis.

Preparation of Antifusion Protein 20 Antibodies. Phage lysates were prepared from clone 20 as described by Young et al. (19), except that after lysis and centrifugation the pellet contained the majority of fusion protein and was, therefore, solubilized by boiling in 5% SDS. Phage extracts were reduced and alkylated by the addition of dithiothreitol to 50 mM and heating for 10 min at 90°C followed by the addition of iodoacetamide to 200 mM and incubation at room temperature for a further 40 min. Excess dithiothreitol and iodoacetamide were removed by dialysis against 500 times volume of 0.1% SDS.

Rabbits were immunized with 3 biweekly injections of ~500 μ g clone 20 extract in CFA and bled 2 wk after the last immunization.

Affinity Purification of Antifusion Protein 20 Antibodies. Antibodies were affinity-purified by binding and elution from strips of nitrocellulose onto which fusion protein 20 had been blotted. Proteins from clone 20 phage lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham Corp.) using a transblot apparatus

(Hoeffer Scientific Instruments, San Francisco, CA). The regions of the membrane that contained fusion protein 20 were then excised and these strips were used to affinity-purify the antifusion protein antibody. Rabbit serum was diluted 1:100 in 170 mM NaCl, 10 mM Tris, pH 7.5 (TBS),¹ 1% BSA and bound to the membrane strips, which had been blocked with 3% gelatin in TBS. After 60 min, unbound antibody was removed and the membrane strips were sequentially washed with TBS, TBS + 0.05% Triton-X 100, 50 mM Tris (pH 7.5), and TBS. Antibody was eluted from the membrane strips by incubation with 0.15 M NaCl, 50 mM diethylamine, 10 mM NaN₃ (pH 11.2), 1% BSA for 10 min with constant agitation. The eluted antibody was equilibrated to pH 7.0 with 1 M Tris-HCl. Affinity-purified antibody was further depleted of nonspecific phage lysate reactivity by the addition of wild-type *λgt11* bacterial lysate.

Western Blot Analysis of Leishmania Proteins and Phage Lysates. Total *Leishmania* proteins, prepared by boiling 10⁸ promastigotes per milliliter in 5% SDS, were reduced and alkylated as described above. Samples were mixed with an equal volume of SDS-sample buffer, boiled for 3 min, and separated by SDS-PAGE. Proteins were then transferred to a synthetic membrane (Immobilon; Millipore, Bedford, MA) using a trans blot apparatus. The membranes were handled according to the manufacturer's directions during the blotting procedure and in the subsequent antibody-screening steps. Antibody staining was carried out as described above.

Results

Identification of Leishmania DNA Encoding Proteins Containing Tandemly Repeating Peptides. Rabbit antibodies against purified *Leishmania* membranes were used to screen a *L. major* *λgt11* expression library. From a screen of 10⁶ plaques, 40 positives were isolated and 2 strongly staining plaques were selected for further study. These 2 clones, clones 20 and 39, contained *Leishmania* DNA inserts of 3.6 kbp and 2.0 kbp, respectively. The orientation of the DNA inserts in *λgt11* were determined and the insert fragments were subcloned into pUC19 and then into M13mp19 for DNA sequence determination. The DNA sequences of a portion of the inserts of clones 20 and 39 were determined and are shown in Fig. 1. The predicted amino acid sequences are striking, as each clone encodes a different protein consisting of tandemly repeating peptides of a specific sequence and length. The two sequences are not related, clone 20 encodes a repeat unit of 14 residues, while clone 39 encodes a repeat unit of 10 residues.

The restriction map of clone 20 is shown in Fig. 1*a*. There were unique sites within the clone for the restriction enzymes Xho I, Cla I, Bgl II, and Pvu II. Each repeat unit has a unique site for the restriction enzyme Pst I, which was useful in determining the extent of the repeats within the clone. There are a minimum of 20 repeat units in clone 20 DNA insert. DNA sequence analysis and restriction mapping of clone 20 showed that the 5' end of clone 20 insert is located within the repeats and that the repetitive sequence continues in the 3' direction for 1.0 kbp. The sequence of the repeats is highly conserved at both the nucleotide and the amino acid level; changes that occur in the DNA sequence do not result in amino acid substitutions. Following the repeat sequence were 177 bp of coding sequence that terminated in a TAG stop codon. Clone 20, therefore, contains 1.2 kbp of coding sequence, which corresponds to the 3' end of the gene, and 1.0 kbp of this sequence codes for a tandemly repeated peptide. There is a potential glycosylation site, Asn-Val-Thr, 22 amino acids from the COOH-terminus and the penultimate amino acid is a cysteine residue. The

¹ Abbreviation used in this paper: TBS, Tris-buffered saline.

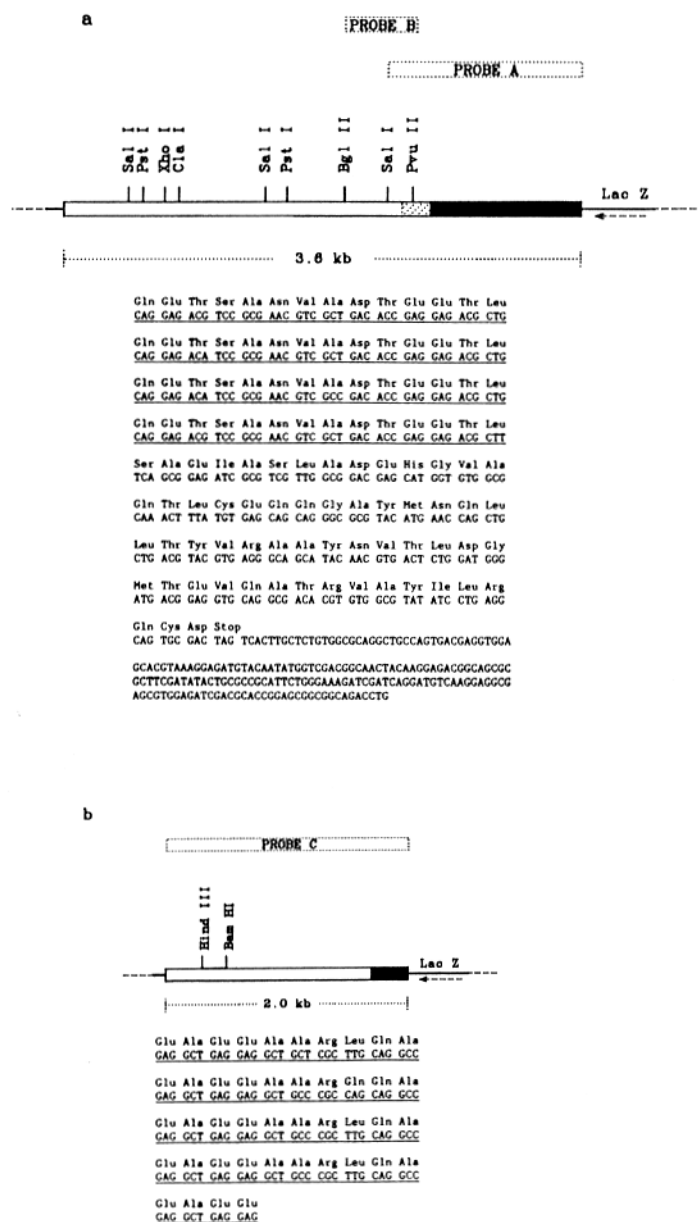


FIGURE 1. Restriction map, DNA sequence analysis and predicted protein sequence of λ gt11 inserts. (a) Clone 20, (b) clone 39. The shaded areas represent repetitive amino acid units. The boxed regions represent DNA fragments that were used as probes in Southern and Northern blots.

presence of a cysteine residue close to the COOH-terminus is of interest since certain membrane proteins are anchored to the surface membrane by an unusual lipid linkage possibly involving a COOH-terminus cysteine residue (29).

The restriction map for clone 39 (Fig. 1 b) is different from that of clone 20.

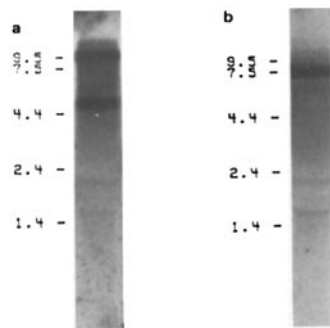


FIGURE 2. Analysis of *L. major* promastigote RNA using repetitive DNA probes labeled with [32 P]dATP. (a) Hybridized with clone 20, probe A. (b) Hybridized with clone 39, probe C. DNA fragments that were used as probes are shown in Fig. 1. The size markers used were an RNA ladder (Bethesda Research Laboratories, Burlington, Ontario) and are indicated as kilobases.

There are unique sites for the restriction enzymes Hind III and Bgl II. Sequence analysis of the repeat units of clone 39 showed that they are unrelated to those of clone 20. There was a high degree of sequence conservation among clone 39 repeat units, although there was one amino acid substitution.

No crosshybridization was observed between the inserts of clone 20 and clone 39; however, it was possible that the two inserts corresponded to different regions of the same gene. To determine whether clones 20 and 39 encode different proteins, DNA fragments from each clone (probes A and C, see Fig. 1) were used as hybridization probes in Northern blot analysis of total promastigote RNA to determine the size of their corresponding RNA transcripts. The results in Figure 2 show that clone 20 hybridized to two RNA species of 9.5 and 5.2 kb, while clone 39 hybridized to a RNA of 7.5 kb. Thus, clones 20 and 39 appear to correspond to different *Leishmania* genes. To examine whether the two clones corresponded to species-specific transcripts, RNA analysis was also carried out using promastigote RNA of *L. donovani*, the causative agent of visceral leishmaniasis. *L. donovani* expressed RNA transcripts that hybridized to probes prepared from clones 20 and 39 and the transcripts were of the same size as those expressed by *L. major* (data not shown). Thus, *Leishmania* species that cause different disease symptoms express both genes 20 and 39.

Genomic DNA from *L. major* was used in Southern blot analysis and hybridized with a fragment of clone 20 (probe A in Fig. 1) that contained repeat units (Fig. 3a). For most restriction enzyme digests, there were two genomic fragments that hybridized to this probe, even though this probe did not contain sites for the restriction enzymes used. Two possible explanations of this result are that either there is another group of repeat units upstream in the clone 20 gene or that there are two copies of clone 20 gene in the *Leishmania* genome. To differentiate between these two possibilities, a second probe from clone 20 was used which overlapped the 3' end of the original probe extending into the 3' untranslated region and lacked the repeat region (Probe B). In a Southern blot hybridization, this 3' probe hybridized to genomic DNA fragments of the same size as those that hybridized to probe A (Fig. 3b). Thus, the most likely explanation for the presence of two fragments in the Southern blot is the presence of two genes in the *Leishmania* genome. These data, together with the Northern blot results (Fig. 2) where clone 20 probe A hybridized to two RNA transcripts, support the contention that there are two genes corresponding to clone 20 in the *Leishmania* genome. The intensities of the bands on the Southern were not equal when

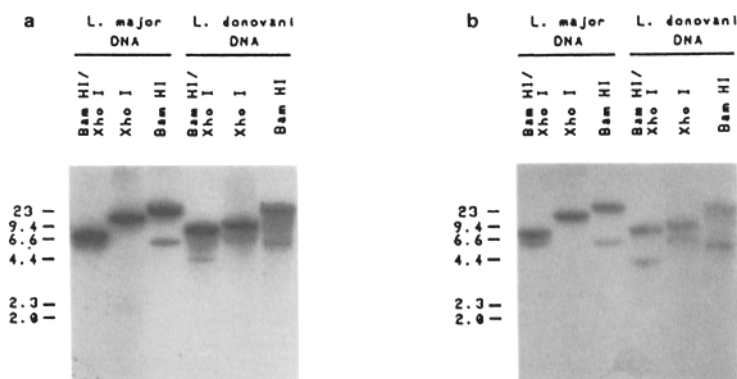


FIGURE 3. Genomic DNA analysis using both repetitive and nonrepetitive probes. Genomic DNA from *L. major* promastigotes was digested with the indicated restriction enzymes. DNA was transferred to a nylon membrane and hybridized with DNA probes labeled with [32 P]-dATP. (a) Hybridized with clone 20, probe A. (b) Hybridized with clone 20, probe B. DNA fragments that were used as probes are shown in Fig. 1. The size markers used were a Hind III digest of phage λ DNA and are indicated as kilobase pairs.

probe A, which contains repeat sequence, was used. One interpretation of this finding is that the two clone 20 genes contain different numbers of repeat units. On Northern analysis this difference in intensity was not as apparent, perhaps due to differential levels of RNA transcription. Further screening of the λ gt11 library and an EMBL3 genomic library with clone 20 probes resulted in the identification of three clones related to clone 20. Restriction mapping of these clones indicated that they corresponded to the gene represented by the less intense fragments, which hybridize to probe A on a genomic blot and contain a fewer number of repeat units than the λ gt11 clone 20. Thus, clone 20 corresponds to the gene that gives rise to the more intense hybridizing fragments on Southern analysis.

Southern blots hybridized with the insert from clone 39 indicated that there is a single copy of this gene, or possibly two identical copies, in the *Leishmania* genome (Fig. 4). Genomic blots carried out with DNA digested with the restriction enzymes Pvu II, Pst I, Sal I, and Bam HI all resulted in high molecular weight fragments (>9 kbp) when hybridized with the DNA insert from clone 39 (Fig. 4).

Identification of *Leishmania* Proteins Containing Repeat Peptides. The antibody used to screen the library was a polyclonal serum directed against *Leishmania* membranes and was not useful for identification of the corresponding *Leishmania* proteins. Rabbit antibodies, therefore, were prepared by immunizing with partially purified β -galactosidase fusion protein synthesized in *E. coli* lysogens. The fusion protein encoded by clone 20 had an apparent mol wt of 210,000 and required complete reduction, alkylation, and boiling in 5% SDS for solubilization. Rabbit antibodies to fusion protein 20 were prepared by immunizing with the insoluble fraction from clone 20 lysate, after reduction, alkylation and boiling in 5% SDS. No fusion protein could be detected in lysogenic cultures of clone 39, probably due to the instability fusion protein 39 (30).

Antibodies against clone 20 fusion protein were then used to identify the

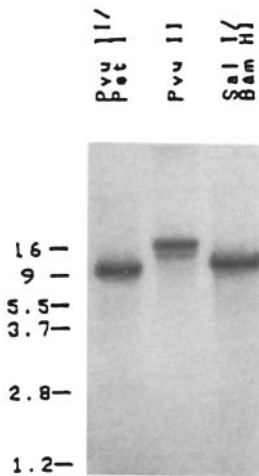


FIGURE 4. Genomic DNA analysis using clone 39 repetitive DNA. Genomic DNA from *L. major* promastigotes was digested with the indicated restriction enzymes. DNA was transferred to a nylon membrane and hybridized with [³²P]dATP-labeled DNA probe clone 39, probe C. DNA fragments that were used as probes are shown in Fig. 1. The size markers used were an Eco RI/Bam HI digest of phage λ DNA and are indicated as kilobase pairs.

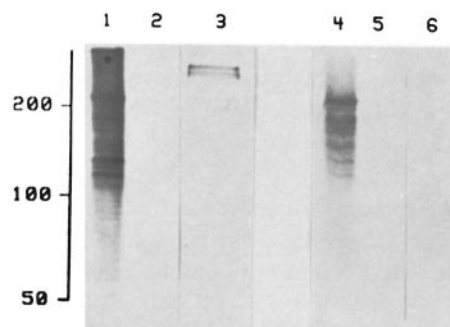


FIGURE 5. Identification of repetitive proteins. Recombinant bacterial lysates and total proteins from *L. major* promastigotes were subjected to SDS-PAGE analysis, transferred to synthetic membranes, and developed with anti-clone 20 fusion protein antibodies absorbed with wild type λ gt11 lysate as described in Materials and Methods. (1-3) developed with anti-clone 20 fusion protein antibody absorbed further with wild-type λ gt11 lysate; (4-6) developed with anti-clone 20 fusion protein antibody absorbed further with clone 20 recombinant lysate. (1 and 4) Recombinant clone 20 bacterial lysate; (2 and 5) wild-type λ gt11 bacterial lysate; (3 and 6) total *L. major* promastigote proteins.

corresponding *Leishmania* protein. The results of Western blot analysis of total *L. major* proteins, which were reduced and alkylated, are shown in Fig. 5. The anti-20 fusion protein antibody recognized a series of protein bands of apparent mol wt 250,000. The prior addition of clone 20 total lysate completely blocked this reaction against *Leishmania* proteins and greatly reduced the reaction with clone 20 lysate while the prior addition of wild-type λ gt11 lysate had no blocking effect (Fig. 5). The recognition of these proteins was greatly enhanced by the reduction and alkylation of the proteins before electrophoresis and transfer. This may be due to the fact that the antibodies were raised against reduced and alkylated fusion protein and may only recognize epitopes on denatured *Leishmania* proteins. The large size of the proteins reacting with antibodies against clone 20 fusion protein correlates with the high molecular weight of the RNA transcripts that hybridize with clone 20 DNA. The multiple bands detected on the Western blot may represent slight differences in glycosylation or some other form of posttranslational modification.

The anti-20 fusion protein antibody was used to surface-label promastigotes; however, no significant binding could be detected, presumably because this antibody reacts only with protein that is completely denatured. Although the antibodies used to identify clone 20 were raised against *Leishmania* membranes,

this does not rule out the possibility that the proteins are located intracellularly or even secreted. Antipeptide antibodies to repeat units of both clone 20 and 39 are currently being produced to investigate further the localization of these two *Leishmania* proteins.

Discussion

The host immune response to *Leishmania* is complex; however, it has been clearly shown (1) that resolution of an established infection and acquired resistance is dependent on cell-mediated rather than humoral immunity. Susceptibility and resistance to *Leishmania* has been extensively studied in inbred strains of mice. These studies have demonstrated that discrete genetic loci control both innate and acquired resistance to *L. donovani* (1, 31). Innate resistance to *L. donovani* appears to involve an inherent difference in the ability of macrophages to support parasite growth (31–34). Susceptibility to *L. major* in the mouse has also been shown to be genetically determined (35, 36).

Studies in both animals and man have revealed a variety of means by which *Leishmania* may evade host resistance mechanisms. For example, both *L. donovani* and *L. major* have been shown to infect macrophages without inducing the production of IL-1 (37, 38). In addition, once infected with *L. donovani*, murine macrophages do not express MHC class II molecules in response to IFN- γ (39). Furthermore, mouse strains susceptible to either *L. donovani* or *L. major* have been reported to develop *Leishmania*-specific T suppressor cells (1, 40).

An additional potential mechanism that may contribute to defective host resistance to *Leishmania* is evasion of an effective humoral response. A commonly reported feature of chronic *Leishmania* infections is polyclonal B cell activation resulting in high levels of serum Ig containing parasite-specific antibody. For reasons that are not clear, this B cell response appears to play no role in host protection (1). Recently, it has been proposed that regions of repetitive peptides, which have been found in many malarial antigens, may play a role in immune evasion and may contribute to chronic infection (12, 13). Thus, tandemly repeated sequences may provide a complex network of crossreacting epitopes that overstimulate B cells and interfere with the normal maturation of an effective antibody response. This may result in the failure to select or stimulate B cell clones synthesizing high-affinity antibody (12). The observation that *Leishmania*, a distinct protozoan species that causes chronic infection, also express proteins containing regions of tandemly repeating amino acid sequence suggests that these molecules may function in a similar manner and thereby confer a selective advantage to these parasites.

Summary

A genomic *Leishmania major* DNA expression library was screened using antibodies raised against *L. major* membranes. Two different clones were identified that encoded proteins containing regions of tandemly repeated peptides. Clone 20 encodes a repetitive peptide of 14 amino acids, while clone 39 encodes a repetitive peptide of 10 amino acids. DNA from clone 20 hybridized with two RNA species of 9,500 and 5,200 nucleotides in length, while DNA from clone

39 hybridized to a single RNA species of 7,500 nucleotides. Antibodies against clone 20 fusion protein recognized a series of *L. major* proteins of apparent mol wt 250,000.

Regions of repetitive peptides is a characteristic shared by many malarial protein antigens and this feature has been implicated in immune evasion. Intracellular parasites such as *Leishmania* and *Plasmodia*, therefore, may have evolved similar mechanisms consisting of the expression of proteins containing tandemly repeating peptides that are involved in immune evasion.

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