IDENTIFICATION OF *Leishmania mexicana* AMASTIGOTE-SPECIFIC GENES AND PROTEINS

by

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ABSTRACT

The leishmaniases are parasitic human diseases that constitute a public health problem in many parts of the world. The diseases are caused by the protozoan *Leishmania* that as amastigotes are intracellular parasites of the host's macrophages. The efforts to control these diseases will be greatly aided by a better understanding of the *Leishmania* amastigote and its complex relationship with its mammalian host. *Leishmania* genes that are preferentially expressed in amastigotes encode proteins that are likely involved in amastigote-specific functions. The first approach used in this thesis was the identification and isolation of amastigote surface proteins. The surface proteins of the amastigote are likely to have a significant role in the host-parasite relationship as they stand in the interphase between the two organisms. This approach was pursued by the generation of monoclonal antibodies directed against the surface of amastigotes. Major surface proteins of *L. mexicana* axenic-culture amastigotes were found to be glycoprotein 63 and a novel protein complex, consisting of three polypeptides of 110, 86 and 70 kDa. This protein complex appeared to be amastigote-specific as it was not detected on the surface of *L. mexicana* promastigotes and appeared on the surface of cultured *Leishmania* when they differentiated from promastigotes to amastigotes. The second approach of the thesis was the identification and characterization of amastigote-specific genes by subtractive hybridization. Two amastigote-specific genes were identified and sequenced: A600 and A850. A600 was abundantly expressed in the amastigotes and found to code for a novel small polypeptide of 93 amino acids, the first 42 of which were a predicted signal peptide, implying that the A600 polypeptide may be secreted by the amastigotes. The other amastigote-specific gene identified, A850, encoded a β-tubulin isogene. The A850 mRNA had a unique 3' UTR that hybridized with two copies out of the multiple β-tubulin genes. The amino acid sequence of the A850 gene was compared to that of the three other reported *Leishmania* β-tubulin genes and the four genes were found to be highly conserved with variable amino acids at only a few defined positions.

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LIST OF ABBREVIATIONS

BCIP	5-bromo-4-chloro-3-indolylphosphate-p-toludine salt
BSA	bovine serum albumin
CDNA	complementary deoxyribonucleic acid
CKII	casein kinase II
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAse I	deoxyribonuclease I
DTT	dithiothreitol
E64	trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane
EndoF	endoglycosidase F
FBS	fetal bovine serum
FITC	fluorecein isothiocyanate
GP63	major surface glycoprotein of *Leishmania* promastigotes: metallo-proteinase
GPI	glycosylphosphatidylinositol
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
γ-IFN	gamma-interferon
IL	interleukin
kDa	kilodalton
LmA600p	predicted polypeptide product of the A600 gene
LmA600sp	predicted secreted polypeptide product of the A600 gene
Lmcpa	*L. mexicana* cysteine proteinase a
Lmcpb	*L. mexicana* cysteine proteinase b
LPG	lipophosphoglycan
mAb	monoclonal antibody
mRNA	messenger ribonucleic acid

NBT	nitroblue tetrazolium chloride
PAGE	polyacrylamide gel electrophoresis
PBMNC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
PV	parasitophorous vacuole
rA600sp	proposed recombinant polypeptide based on the A600 gene product
RNA	ribonucleic acid
RT-PCR	reverse transcription – polymerase chain reaction
SDS	sodium dodecylsulfate
SL	spliced leader
TBS	tris buffered saline

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IDENTIFICATION OF *Leishmania mexicana* AMASTIGOTE-SPECIFIC GENES AND PROTEINS

1.- INTRODUCTION

1.1.- *Leishmania* AND LEISHMANIASIS

Leishmania are protozoan parasites that cause disease in mammals including humans. Different species cause a spectrum of clinical disease consisting of cutaneous and mucocutaneous and visceral leishmaniasis. Cutaneous leishmaniasis is the most prevalent, producing skin ulcers that may take more than a year to heal. After cure, the host will have developed protective immunity against subsequent infection. Ocassionally, the cutaneous lesions disseminate covering a large area of the skin in what is called diffuse cutaneous leishmaniasis. This is probably due to a deficient cellular immune response of the host. Cutaneous leishmaniasis is caused by *L. mexicana*, *L. amazonensis*, *L. pifanoi*, *L. braziliensis*, *L. peruviana*, *L. guyanensis* and *L. panamiensis* in the New World and by *L. major*, *L. tropica* and *L. aethiopica* in the Old World. Mucocutaneous leishmaniasis is caused by *L. braziliensis* in the Amazonian basin. The disease starts as a cutaneous lesion which may heal but in approximately 10% of the cases will reappear as a secondary lesion in the mucous tissue of the nose and mouth. This reactivation and metastasis of the parasite may occur years after the primary lesion heals. Left untreated it will disseminate to contiguous mucous tissue and can cause hideous disfigurement and extensive destruction of the lips, palate, nose and pharynx. Visceral leishmaniasis is caused by *L. donovani* and *L. infantum* in the Old World and by *L. chagasi* in South America. It is a very severe systemic disease associated with hepatomegaly, splenomegaly and severe anemia and is nearly always fatal if left untreated.

These diseases constitute a public health problem in many parts of the world. Leishmaniasis, one of the six major parasitic diseases recognized by the World Health Organization, accounts for an estimated 3 million cases worldwide, with 1.5 million new cases reported each year, of which 500,000 are visceral leishmaniasis (Modabber 1993).

The genus *Leishmania* is in the order Kinetoplastida and the family Trypanosomatidae. *Leishmania* is an asexual diploid unicellular organism whose genome contains 36

chromosomes (Wincker *et al.* 1996). *Leishmania* has a digenic life cycle that takes place in two hosts: an insect vector, female sandfies of the generas *Lutzomya* and *Plebotomus*, and a mammalian host. Leishmaniasis is considered a Zoonosis: humans are not normally part of the life cycle of *Leishmania* but get infected when they are bitten by blood-feeding sandflies in endemic areas, although some anthroponotic leishmaniasis may occur (Modabber 1993) such as with cutaneous leishmaniasis caused by *L. tropica.* The normal mammalian hosts or reservoirs for *Leishmania* are wild rodents. Dogs are apparently accidental hosts but may serve as reservoirs for transmission to humans. That leishmaniases are zoonotic diseases implies that the parasite's mechanisms to avoid the host immune system have not evolved in humans but in other mammals with similar but not identical immune systems. This fact might lead to pathogenesis instead of to a stable subclinical parasitosis when the parasite uses its adaptive genetic repertoire to survive in the human host. For reviews on the biology of *Leishmania* and on clinical Leishmaniasis see Chang *et al.* 1985 and Jeronimo & Pearson 1992.

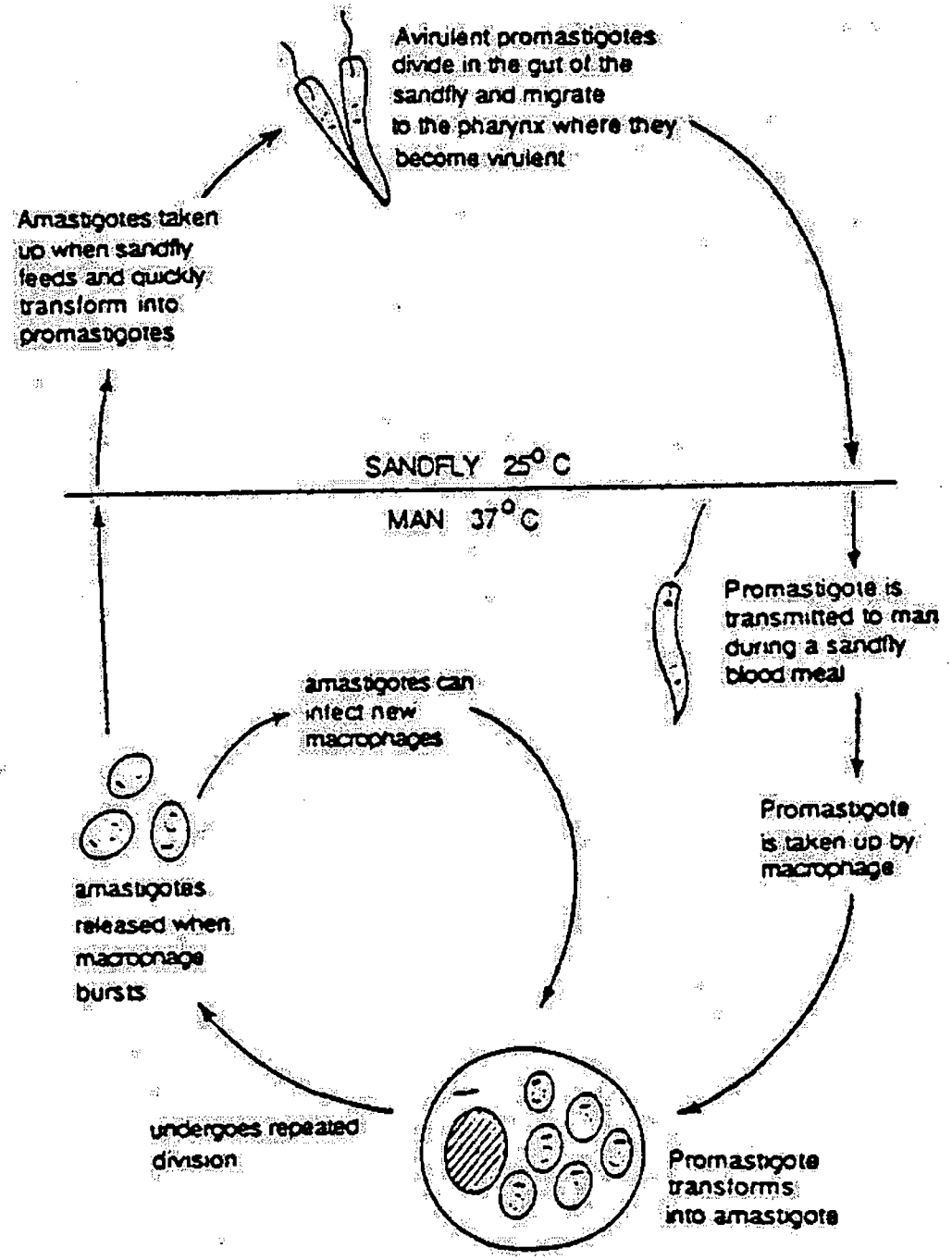
1.2.- THE LIFE CYCLE OF *Leishmania*

The life cycle of Leishmania is presented in figure 1. When a sandfly acquires a bloodmeal from an infected host, it acquires either free *Leishmania* amastigotes or amastigote-infected mononuclear cells. In the gut of the fly the amastigotes transform into the dividing flagellated procyclic promastigotes that become attached to the midgut epithelium with their flagella inserted between microvilli. From day 5 after the bloodmeal onward, increasing numbers of slender, non-replicating, rapidly moving promastigotes can be observed in the lumen of the anterior midgut and foregut of the fly. They constitute the highly infectious metacyclic form of the promastigotes that is delivered to the vertebrate host. During a bloodmeal, the sandfly regurgitates infective metacyclic promastigotes that are quickly taken up by the vertebrate host's tissue macrophages and by monocytes.

Within the acid environment of the parasitophorous vacuole or phagolysosomes of the macrophage, the parasites differentiate into rounded, aflagellated non-motile amastigotes. The amastigotes are capable of surviving and proliferating within the macrophages and of disseminating by destruction of their host cells and infection of neighboring macrophages.

FIGURE 1

LIFE CYCLE OF LEISHMANIA



Amastigotes are ingested by sandflies during a bloodmeal, thus completing the life cycle (Mosser and Brittingham 1997).

Promastigotes from all species of *Leishmania* can be easily grown in culture, in what constitutes a model for their growth as procyclic promastigotes in the insect vector. These cultured promastigotes have provided abundant material for the biochemical characterisation of this stage of the parasite's life cycle. The surface of the promastigotes has been well studied. The two most abundant molecules on the surface of promastigotes are a lipophosphoglycan (LPG) and a metalloproteinase membrane glycoprotein of an apparent molecular mass of 63,000 Daltons, (glycoprotein 63; GP63). Both surface molecules are attached to the surface membrane by a glycosyl-phosphatidylinositol (GPI) linkage. Promastigotes of *L. mexicana*, *L. major* and *L. donovani* have $1\text{-}3x10^6$ LPG molecules per parasite (Bahr *et al.* 1993). In promastigotes there are approximately $5x10^5$ molecules of GP63 per parasite, representing approximately 0.5 to 1% of the total parasite protein (Bordier 1987; Bahr *et al.* 1993).

The structure of LPG is composed of four domains: the GPI anchor, a glycan core, a repeating saccharide-phosphate region and an oligosaccharide cap, the last two components varying between *Leishmania* species and between procyclic and metacyclic promastigotes.

Differentiation into the non-dividing and infective metacyclic promastigotes in the midgut of sandfly vectors or in the stationary phase of axenic culture is accompanied by modifications of the surface LPG. There is a two to three fold increase in LPG size due to the increase in the number of phosphorylated saccharide units and a change in the composition of the terminal sugars. In *L. major*, the majority of the terminal sugars of procyclic promastigotes are galactose while in metacyclic promastigote they are an arabinopyranose (McConville et al. 1992). In *L. donovani* the terminal sugars are masked in the metacyclic promastigotes (Sacks et al. 1995). The down-regulation of terminally exposed galactose residues on metacyclic LPG appears to permit the selective release of infective promastigotes from adhesion to midgut epithelial cells (Pimenta *et al.* 1992). The parasites can then migrate to the proboscis and pharynx of the insect to be transmitted to the vertebrate host by the insect's bite.

The reduced presentation of LPG terminal sugars in metacyclic promastigotes resulted in the loss of binding to the lectin peanut agglutinin (PNA) and this property was used to purify them. The PNA(-) metacyclic promastigotes were found to accumulate in the stationary phase of axenic culture of *Leishmania* promastigotes (Da Silva and Sacks 1987; Turco and Descoteaux 1992).

The metalloproteinase glycoprotein GP63 is the major surface protein of the promastigotes of all species of *Leishmania* (Etges 1992). GP63 is abundant on the surface of the procyclic promastigotes residing inside the gut of the sandfly and on the surface of metacyclic promastigotes. While GP63 seems to play a role in protecting the metacyclic promastigotes from complement-mediated lysis, its possible role inside the insect vector is not clear. The role of *L. major* gp63 genes 1-6 in parasite development within the sandfly vector *P. argentipes* has been analyzed. Targeted gene expression was used to delete *gp63* genes 1-6 encoding the highly expressed promastigote (genes 1-5) and constitutively expressed GP63 (gene 6). The complete developmental pathway from lesion amastigotes to metacyclic promastigotes was observed for wild-type promastigotes, GP63 1-6 null mutants and mutants transfected with *gp63* gene 1. Therefore GP63 does not appear to play a significant role in the development of *L. major* in the insect vector (Joshi *et al.* 1998).

1.3.- IMMUNITY IN LEISHMANIASIS

Leishmania parasites evade and exploit the host immune system being the intracellular parasites of macrophages, the very cells that the immune system uses for killing Leishmania when the host organism is able to mount an effective curative response.

Murine experimental leishmaniasis has been instrumental in establishing the relevance of the Th1/Th2 paradigm for the immune response to infectious diseases *in vivo* (Lohoff et al. 1998; Reiner & Locksley 1995). A few inbred strains of mice such as BALB/c present uncontrolled parasitism with *L. major*, associated with the expansion of parasite reactive Th2 CD4 lymphocytes. The majority of inbred strains of mice present a restricted parasitism with the development of a polarized parasite-specific Th2 CD4 response (Launois *et al.* 1997)

In human leishmaniasis, there appears to be a mixed cytokine profile associated with active cutaneous or mucosal disease and a dominant Th1-type response associated with healing.

The peripheral blood mononuclear cells (PBMNC) of patients suffering from American cutaneous leishmaniasis were studied before therapy (active lesion) and after cure. The PBMNC were stimulated *in vitro* by *Leishmania* antigens. During active disease, the predominant stimulated T cells were CD4+ with mixed Th1 and Th2-type cytokine production (γ-IFN, IL-2 and IL-4). After healing, similar proportions of CD4+ and CD8+ T cells were stimulated with a Th1-type cytokine production (γ-IFN, IL-2 and very low IL-4) (Coutinho *et al.* 1996).

Neither B cells nor Leishmania-specific antibodies are significantly protective in murine or human leishmaniasis.

Multiple evasion mechanisms have been proposed for Leishmania based on *in vitro* studies or on artificially-infected inbred strains of mice. Mechanisms that could permit the initial as well as long term survival of the parasites in the host organism are the passive protection of the parasite against anti-leishmanial products and the retreat into "safe target cells", the active suppression of the synthesis of reactive oxygen or nitrogen intermediaries, the modulation of the host cytokine response, the inhibition of antigen presentation and T cell stimulation and the induction and expansion of counterproductive T helper cells (Bogdan & Rollinghff 1998). Some of these mechanisms may be significant *in vivo*, where the parasite is transmitted by the bite of a sandfly. Some of these mechanisms may be significant in leishmaniasis in our own species, while others will not be relevant to infection in a heterogeneous human population.

Important events of a curative immune response against *L. major* in experimental murine leishmaniasis are the efficient activation of NK cells, the presentation of protective antigens, induction and expansion of MHC class II restricted CD4+ Th1 cells and the activation of macrophages via γ-IFN followed by nitric oxide (NO)-dependent killing of the parasites (Bogdan *et al.* 1996). In humans on the other hand, a role for the (NO)-dependent killing of the parasites has not been demonstrated.

Some of the evasion mechanisms may be specific to a particular species of *Leishmania.* Different species cause different pathology and may have evolved particular survival strategies. For example, differences are likely between *L. donovani*, that causes visceral leishmaniasis and *L.major* or *L. mexicana* that cause cutaneous leishmaniasis. One obvious

difference is their temperature restriction *in vivo*, that is reflected in the temperature at which they are converted into amastigotes *in vitro*, 37°C for *L. donovani* and 32°C for *L. mexicana*. *L. donovani* selectively parasitizes resident macrophages in the liver, spleen and bone marrow. In experimental visceral leishmaniasis, in innately susceptible BALB/c mice, visceral infection initially progresses, but acquired resistance develops and the infection is controlled (Murray 1994). In human visceral leishmaniasis, the disease is progressive and does not cure without therapy. In experimental cutaneous leishmaniasis by infection of BALB/c mice with *L. major*, the disease is progressive. In contrast, *L. major* in humans causes cutaneous leishmaniasis that self-heals.

1.3.1.- Surface components of the metacyclic promastigotes are virulence factors:

Multiple macrophage receptors, parasite ligands and host opsonins have been implicated in the binding of promastigotes to macrophages. As the promastigotes encounter serum immediately upon infection of the host, it is likely that they encounter macrophages in a serum-opsonized state. The metacyclic promastigotes avoid the lytic effects of complement and resist fixation of the terminal complement components. At the same time they exploit the complement system by fixation of opsonic complement to invade host mononuclear phagocytes efficiently (Mosser and Brittingham 1997).

LPG (DaSilva et al. 1989) and GP63 (Russell 1987; Brittingham *et al.* 1995) have been reported to be major acceptors for activated fragments of the third component of complement (C3b, iC3b) that serve to opsonize the parasites for enhanced phagocytosis by host macrophages (Mosser and Brittingham 1997).

The two complement receptors CR3, the receptor for iC3b, and CR1, the receptor for C3b, can cooperate to mediate the initial complement-dependent adhesion of *L. major* metacyclic promastigotes to human monocyte-derived macrophages. CR3 is the predominant complement receptor responsible for the phagocytosis of complement-opsonized *L. major* metacyclic promastigotes (Rosenthal *et al.* 1996).

Leishmania complement fixation, in addition to increasing parasite phagocytosis, may increase their intracellular survival. The uptake of complement-coated parasites triggered a smaller respiratory burst in the macrophage than the uptake of uncoated parasites (Mosser and Edelson 1987; Wright and Silverstein 1983).

When an infected vector bites a mammal, the metacyclic promastigotes are transmitted in their saliva. Biochemical changes on the surface of the parasites during metacyclogenesis seem to constitute a pre-adaptation for infection and survival in the mammalian host. Infective or metacyclic *L. donovani* or *L. major* promastigotes have a thickened glycocalix due to the elongation of the surface LPG. This glycocalix protects metacyclics from complement-mediated lysis by hindering access of the membrane attack complex (lytic C5b-9) to the cell membrane (Sacks 1992). LPG additionally is one of the molecules mediating attachment and entry of promastigotes and amastigotes of *L. major* into macrophages (Bogdan et al. 1996). The elongation of LPG on metacyclics promotes complement activation and C3 deposition in a non-lethal manner, opsonizing the promastigotes for attachment to macrophage complement receptors and uptake by phagocytosis (Sacks 1992).

LPG may enhance the survival of *Leishmania* after uptake by the macrophage during its differentiation from metacyclic promastigotes to amastigotes, the form most adapted to survival in the infected macrophage. LPG may delay the biogenesis of phagolysosomal vacuoles by inhibiting fusion of the parasitophorus vacuoles with endosomes and lysosomes. Limited fusion has been reported after phagocytosis of *L. donovani* in contrast with extensive fusion after phagocytosis with mutants lacking surface LPG. Genetic complementation as well as opsonization with purified LPG restored the LPG-defective mutant's ability to inhibit phagosome-endosome fusion to a degree similar to that of wild-type promastigotes (Desjardins and Descoteaux 1997). Eventually, fusion must occur as the amastigotes proliferate in acidic phagolysosomal vacuoles, but a delay in fusion may assist the *L. donovani* metacyclic promastigotes in establishing intracellular residence. LPG may increase the viability of the host cell, thus enhancing parasite survival. Bone marrow-derived macrophage infection by *L. donovani* promastigotes or treatment of the macrophages with LPG inhibited the apoptosis in the macrophages induced by the removal of exogenous growth factor (Moore and Matlashewski 1994).

LPG continues to be detectable for at least 48 hours after entry to the macrophage and the coat of LPG may protect the parasite's surface from the digestive enzymes in the phagolysosome. LPG may be shed into the parasitophorous vacuole and counteract macrophage killing activities. LPG has been shown to inhibit lysosomal enzymes *in vitro*

(Turco and Descoteaux 1992) and both LPG and glycoinositolphospholipids (GIPLs) are inhibitors of nitric oxide synthesis in murine macrophages (Liew *et al.* 1997).

Another change observed during metacyclogenesis was the increase in the number of GP63 molecules on the surface of *L. braziliensis*, *L. panamiensis*, *L.guyanensis, L. peruviana*, *L. mexicana*, *L. amazonensis* and *L. chagasi* (Kweider et al. 1987 and 1989; Ramamoorthy 1992). GP63 appears to play a role similar to that of LPG in metacyclic promastigotes: resistance to complement degradation and opsonization for complement receptor mediated uptake by the macrophage.

The role of GP63 in resistance to complement-mediated lysis in *L. major* was probed by the deletion of *gp63* genes 1-6, encoding the highly expressed promastigote and constitutively expressed GP63, by targeted gene replacement (*gp63* 1-6 null mutants). The procyclic and metacyclic promastigotes of *gp63* 1-6 null mutants showed increased sensitivity to complement-mediated lysis. The level of resistance of the wild type parasites was restored to the *gp63* 1-6 null mutants by transfection with *gp63* gene 1 (Joshi *et al.* 1998). In another study, *L. amazonensis* promastigotes that were deficient in the level of expression of GP63 were shown to be more sensitive to complement lysis than wild-type *L. amazonensis*. Resistance to complement was restored by the expression of GP63 introduced by transfection with the cloned *L. major gp63* gene 1 but not with expression of a proteolytically-inactive active site mutant of GP63. This showed that the proteolytic activity of the metalloproteinase GP63 was required for resistance to complement-mediated lysis. The parasites expressing wild-type GP63 on their surface fixed only small amounts of the terminal complement components and more rapidly converted C3b to iC3b, interacting avidly with cells expressing Mac-1 (CR3), the receptor for iC3b (Brittingham *et al.* 1995).

GP63 may have a role in intracellular protection of *Leishmania.* GP63 purified from *L. amazonensis* was shown to be capable of protecting liposome-encapsulated proteins from phagolysosomal degradation by macrophages. This protection was provided by native GP63 but not by heat denatured GP63 that had lost its enzymatic activity (Chaudhuri *et al.* 1989). Attenuated *L. amazonensis* variants were isolated after prolonged cultivation in axenic culture and found to contain 20 to 50 fold less surface GP63. Coating of the attenuated parasites with proteolytically active GP63 protected them from degradation inside of macrophage phagolysosomes (Seay *et al.* 1996). *In situ* inhibition of GP63 proteinase

activity inside *Leishmania*-infected macrophage phagolysosomes with targeted delivery of the inhibitor 1,10-phenanthroline selectively eliminated intracellular *L. amazonensis* amastigotes (Seay et al. 1996). This result strongly suggests that GP63 is required for intracellular survival of *L. amazonensis*, but it is not clear if the GP63 involved is on the surface of the amastigote. *L. amazonensis* is closely related to *L. mexicana*, where the majority of the amastigote GP63 is not on its surface but present as a hydrophilic enzyme in its megasomes (Bahr *et al.* 1993). It is probable that *L. amazonensis* amastigotes present a similar distribution of GP63. Another megasomal proteinase, cysteine proteinase b, of *L. mexicana* has been shown to be important for the intracellular survival of their amastigotes (Mottram *et al.* 1996; Alexander *et al.* 1998).

While there is some evidence for a role of GP63 in intracellular survival in *L. amazonensis*, that does not appear to be the case for the products of *L. major gp63* genes 1 to 6. *L. major gp63* 1-6 null mutants, generated by gene replacement, were capable of infecting mouse macrophages in culture and of differentiating into amastigotes. The mutants were also capable of generating lesions on BALB/c mice and thus the *gp63* genes 1-6 do not play an essential role in the survival of *L. major* within mouse macrophages (Joshi *et al.* 1998). The role of *L. major gp63* gene 7, that is expressed in metacyclic promastigotes and amastigotes (Voth et al. 1998), remains to be determined.

In conclusion, both LPG and GP63 in the metacyclic promastigotes are considered *Leishmania* virulence factors. They exploit the opsonic properties of complement while avoiding its lytic effects. LPG and GP63 may also protect the parasites within the parasitophorous vacuoles of the host macrophage during the critical period of their differentiation into intracellular amastigotes.

1.3.2.- *Leishmania* and the parasitophorous vacuole of the infected macrophage:

Growth of the intracellular amastigotes occurs within organelles of the macrophages known as parasitophorous vacuoles (PV). The metacyclic promastigotes are taken up by phagocytosis. The phagosomes fuse with endocytic organelles, resulting in PV formation. In this period of time, the metacyclic promastigotes differentiate into amastigotes, a process that takes several days to complete. In *L. mexicana* infected macrophages, typical megasomes were first identified by day five after infection, being more prevalent by day seven. Cysteine

proteinase activity was first detected on day three and increased thereafter (Galvao-Quintao *et al.* 1990). Only the parasites belonging to the mexicana group (*L. mexicana*, *L. amazonensis* and *L. pifanoi*) posses the enlarged lysosomal compartment or megasomes in their amastigote stage.

The PV possesses membrane proteins characteristic of a lysosome and MHC class II molecules. The PV of *L. amazonensis*-infected rat bone marrow-derived macrophages were shown to be acidic with an approximate pH of 5, similar to that of the lysosomes (Antoine et al. 1990). As the vacuole matures, it gains mannose 6-phosphate receptors and becomes more accessible to endocytosed ligand, which suggests maturation from a lysosomal to a late endosomal compartment. The amastigote acquires material endocytosed by the macrophage that is later observed in the flagellar pocket and inside the parasite (Russell *et al.* 1992). Leishmania amastigotes live in acidic modified late endosomes, which can be considered persistent phagolysosomes. The amastigote within the PV must resist degradation by lysosomal hydrolases, exploit the host cell as a source of nutrients and avoid the macrophage's antigen-presenting capabilities (Russell et al. 1992).

The glycocalix coat of promastigotes is composed of LPG, GPI anchored proteins like GP63 and a family of low molecular weight GIPLs. This coat is absent from the amastigote surface as the expression of LPG and GPI-anchored proteins is massively down-regulated. Instead the plasma membrane of amastigotes is coated by a densely packed layer of parasite-derived GIPLs and host-derived glycosphingolipids (Winter *et al.* 1994). This down-regulation of promastigote surface macromolecules and acquisition of host glycolipids by amastigotes may be a strategy to avoid detection by the host's immune system (McConville and Ralton 1997).

The PV has been shown to contain the following enzymes synthesized by the macrophages: acid phosphatase, trimetaphosphatase, arysulphatases A and B, β-glucoronidase and the proteinases dipeptidylpeptidases I and II and cathepsins B, D, H and L (Antoine *et al.* 1998, Prina and Antoine 1990, Russell *et al.* 1992; Lang *et al.* 1994b). The amastigote must be resistant to all these macrophage hydrolases. The PV also contains the following macromolecules synthesized and secreted by the amastigotes: acid phosphatase by *L. donovani* amastigotes and proteophosphoglycan (aPPG) by *L. mexicana* amastigotes. The polyanionic PPG is a high molecular weight structure composed of serine-rich polypeptide

chains and phosphooligosaccarides capped by mannooligosaccharides. The aPPG is secreted in large amounts by amastigotes via their flagellar pockets into the PV of the host macrophage (Ilg et al. 1995). *L. mexicana* amastigotes grow in huge PV and their secreted aPPG may be responsible for vacuole enlargement given that purified PPG caused vacuolization of peritoneal macrophages *in vitro* (Peters *et al.* 1997a). Some of the glycans in aPPG were shown to be identical to oligosaccharides from *L. mexicana* promastigote LPG and secreted acid phosphatase. The majority of the aPPG glycans were novel amastigote stage-specific structures, which suggested the presence of developmentally regulated amastigote glycosyl-transferases (Ilg *et al.* 1998). An amastigote-specific secreted aPPG must have an amastigote-specific function, interfering with the macrophage. One function that has been proposed for the aPPG is that of activating complement in the lesion. The purified aPPG has been shown to efficiently activate complement. Cutaneous lesions induced in mice by *L. mexicana* have been found to contain abundant amounts of aPPG, released into the tissue, together with free amastigotes, upon the rupture of infected macrophages. It has been proposed that aPPG may cause complement depletion at the lesion site with the generation of anaphylactic peptides C3a, C4a and possibly C5a. These anaphylatoxins would attract infectable monocytes to the site of infection (Peters *et al.* 1997b).

The amastigotes within the PV have easy access to metabolites such as proteins, lipids, nucleic acids and polysaccharides that are degraded in the PV (Schaible et al. 1999). *Leishmania* amastigotes appear to subvert the endocytic traffic of the infected macrophage. The traffic of human transferrin (HTf)-gold conjugates was investigated in infected and uninfected macrophages. In uninfected macrophages Htf segregated to a different compartment than bovine serum albumin (BSA). In *Leishmania*-infected macrophages both Htf and BSA colocalized in the PV. Htf was delivered to the PV, formed patches on the amastigote surface and was endocytosed via the flagellar pocket. Within the amastigotes, Htf was found in the cysteine-proteinase-rich megasomes, where it was presumably degraded providing iron to the parasite (Borges *et al.* 1998). Conversely, *Leishmania* macromolecules may gain access to the macrophage's cytoplasm as was shown by the fact that a surface leishmanial antigen GP46/M-2 was presented to CD8 + T cells after being processed in the cytoplasm of the infected macrophage via the classical pathway of MHC class I presentation

(Kima *et al.* 1997). Amastigotes of *L. amazonensis*, *L. mexicana*, *L. pifanoi* and *L. donovani* are not free in the PV but are tightly bound to its membrane via their posterior pole and may thus interact directly with PV membrane components.

The macrophages are the host cells for *Leishmania*, but also part of the immune system being the effector cells responsible for killing the parasites. The *Leishmania* amastigotes appear to deactivate effector activities of the macrophages. For example, the presence of intracellular parasites of *L. major*, *L. mexicana* or *L. donovani* decreased hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) production in human monocytes treated with γ-IFN (Passwell *et al.* 1994). Another example was the impairment of γ-IFN signaling in human mononuclear phagocytes (phorbol ester-differentiated U-937 cell line and peripheral blood phagocytes) infected with *L. donovani*. The infection led to inhibition of γ-IFN-mediated tyrosine phosphorylation and selective effects on the Jak-Stat 1 pathway (Nandan and Reiner 1995).

1.3.3.- *Leishmania* interference with the host's immune response:

Leishmania may interfere with host immune responses by limiting or inhibiting antigen presentation and T cell stimulation or by promoting the development of a counter-protective Th2-type response.

a) ANTIGEN PRESENTATION

Leishmania-specific CD4+ T cells have been shown to play a fundamental role in the immune responses of the mammalian host. In order for the infected macrophage to function efficiently as an antigen presenting cell for *Leishmania*-specific CD4+ T cells, it requires MHC class II and co-stimulatory molecules and the availability of parasite-derived peptides for loading on the MHC molecules. Following infection by *L. mexicana*, *L. amazonensis* or *L. donovani*, a significant proportion of the MHC class II molecules but not of the MHC class I molecules associates with the PV (Lang *et al.* 1994a; Lang *et al.* 1994b; Antoine *et al.* 1998). In mouse bone-marrow derived macrophages infected with *L. mexicana* amastigotes and activated with γ-IFN, MHC class II molecules and amastigotes co-localized in the PV, with the MHC class II molecules concentrating at the attachment zone of the amastigotes to the PV membrane. This interaction of amastigote and MHC class II molecules appeared to

be specific as other PV membrane components were homogeneously distributed (Lang *et al.* 1994a).

Intracellular *L. amazonensis* amastigotes have been shown to internalize and degrade MHC class II molecules from infected mouse bone marrow-derived macrophages. Study of infected macrophage sections by immuno-electron microscopy showed internalized MHC class II molecules in the megasomes of the amastigote, where they accumulated when the megasomal cysteine proteinases were inhibited. The internalization of MHC class II moleculas appeared to be selective as other PV membrane components where not detected in the amastigote megasome after protease inhibition (De Souza Leao *et al.* 1995). MHC molecules H-2M showed the same distribution as MHC class II molecules in *Leishmania*-infected macrophages: they were detected in the PV, polarized towards amastigote-binding sites in the PV membrane and internalized and degraded by the amastigotes (Antoine *et al.* 1998).

To test if parasite antigen-class II molecule complexes could be formed in the PV, reach the cell surface and be recognized by *Leishmania*-specific CD4+ T cells, macrophages where infected with *L. mexicana* that overexpressed a model antigen. A membrane-bound acid phosphatase (MAP) normally expressed in low amounts within *Leishmania*, was overexpressed either on the surface of the parasites or in a soluble form that would be secreted into the PV. It was shown that macrophages containing live amastigotes with surface or secreted MAP efficiently presented it to MAP-specific T cell lines. The intracellular MAP of wild-type cells or the abundant megasomal cysteine proteinases were not, or only inefficiently, presented. After killing of the amastigotes, abundant antigens such as cysteine proteinases can stimulate T cells. It was concluded that intracellular proteins of intact amastigotes were not available for antigen presentation (Wolfram et al. 1996). A similar result was obtained with CD4+ cell lines against the proteins P8 and GP46 that are exposed on the surface of amastigotes. Macrophages infected with amastigotes presented little parasite antigen. Stationary phase promastigote-infected macrophages presented endogenous parasite molecules to CD4 + T cells, but only for a limited time with minimal presentation by 72 hours after the infection. This suggested that that amastigote antigens were sequestered from the MHC class II pathway of antigen presentation (Kima et al. 1996). A similar experiment was performed with a T cell clone against the antigen LACK

(*Leishmania* homologue of receptors for Activated C Kinase) a protein that is expressed by promastigotes and amastigotes of *Leishmania*. γ-IFN-treated murine macrophages infected with live promastigotes were able to activate LACK-reactive T cells during the early infection period but lost that capacity at later times, even if live intracellular parasites persisted. Antigen presentation appeared to correlate with partial killing of intracellular promastigotes. Macrophages infected with amastigotes did not present LACK antigen (Prina et al. 1996). In a later study, it was shown that if the population of promastigotes was selected for pure metacyclic promastigotes, the stimulation of LACK-reactive T cells was barely detectable. Furthermore, it was shown that the killing of intracellular *L. amazonensis* amastigotes did not lead to LACK antigen presentation. Both virulent stages of *Leishmania*, metacyclic promastigotes and amastigotes, appeared to avoid or minimize their recognition by CD4+ T cells (Courret et al. 1999).

MHC class I molecules are not detectable in the PV but some *Leishmania* antigens find their way into the macrophage cytoplasm as an endogenous leishmanial antigen processed via the MHC class I pathway was shown to be presented by *L. amazonensis*-infected macrophages to CD8+ T cells (Kima *et al.* 1997).

b) IL-12 SYNTHESIS

Interleukin 12 (IL-12) is a critical cytokine involved in the differentiation and expansion of Th1 cells. IL-12 enhances the γ-IFN production by Th1 cells that is crucial for cure of leishmaniasis. The macrophages, the host cells of *Leishmania* are a main source of IL-12 *in vivo*. It has been reported that infection of murine macrophages *in vitro* with *L. major* metacyclic promastigotes did not cause induction of IL-12 synthesis. In contrast, infection of macrophages *in vitro* with lesion-derived amastigotes did induce IL-12. Infection of mice *in vivo* with *L. major* metacyclic promastigotes also did not induce IL-12 synthesis. The appearance of IL-12 transcripts ocurred only after 7 to 10 days after infection *in vivo*, a period by which amastigotes had spread in the lesion (Reiner *et al.* 1994). LPG, a major component of the metacyclic promastigote surface that is shed after phagocytosis by the macrophage may mediate the inhibition of IL-12 synthesis. It has been reported that phosphoglycan, a component of LPG, was able to inhibit IL-12 release in a dose-dependent manner (Piedrafita et al. 1999). The delay in induction of IL-12 after infection with the

infective metacyclic promastigotes may be a mechanism of parasite evasion of the immune response. CD4+ differentiation would start in the absence of IL-12 with the possible outcome of a counter-protective Th2-type response (Reiner and Locksley 1995).

Recent studies have reported that *Leishmania* amastigotes did not induce the *in vitro* or *in vivo* infected macrophage to produce IL-12, in contrast with the report of Reiner *et al.*, 1994. Infection of quiescent murine macrophages with *L. mexicana* or *L. major* amastigotes did not induce IL-12 production. Infection with the amastigotes suppressed IL-12 secretion by macrophages activated by LPS, by CD40 cross-linking or cognate interaction with Th1 cells. Surprisingly, phagocytosis of latex beads produced a degree of suppression of IL-12 secretion. It was therefore suggested that *Leishmania* amastigotes might be viewed as a kind of inert particle (Weinberger *et al.* 1998). Another study reported that there was a selective impairment of IL-12 induction in mouse inflammatory macrophages by *in vitro* infection with *L. major* metacyclic promastigotes. This was demonstrated at the single cell level by two-color flow cytometry and intracellular staining for parasites and cytokines. IL-12 was not produced in response to infection itself and virtually every infected cell had lost its ability to produce IL-12 in response to γ-IFN /LPS. Low multiplicity infection of inflammatory macrophages *in vivo* using either metacyclic promastigotes or amastigotes also resulted in the complete and selective inhibition of IL-12 responses of infected cells (Belkaid et al. 1998).

While macrophages may not produce IL-12 when infected by *Leishmania* amastigotes, other cells might, like epidermal Langerhans cells, immature dendritic cells present at the site of infection on the skin. *L. major* amastigotes but not promastigotes entered murine Langerhans cell-like dendritic cells *in vitro*. Amastigote internalization was associated with an increase in surface MHC class I and II antigens and co-stimulatory molecules and by the release of IL-12 (von Stebut et al. 1998). In other study, an *in situ* analysis of IL-12 producing cells in the spleen early after *L. donovani* infection suggested that dendritic cells, but not macrophages, produced IL-12 (Gorak *et al.* 1998). These results may explain the temporal association seen between the appearance of amastigotes and IL-12 during experimental murine leishmaniasis *in vivo* (Reiner *et al.* 1994).

1.3.4.- The search for an effective vaccine for leishmaniasis:

A main focus of research in leishmaniasis is the development of vaccines against human cutaneous leishmaniasis, paving the way for a vaccine against the visceral disease. Vaccination presents the best long-term hope for controlling the leishmaniases (Modabber 1993). The metacyclic promastigotes are in contact with the host for only a short period so vaccine development efforts should concentrate on the amastigote forms of the parasite. Vaccines against human cutaneous leishmaniasis should be feasible as the lesion usually self-heals after a period of time and protective immunity ensues. Nevertheless, the multiple mechanisms that the parasite may employ to evade the host's immune system present a formidable obstacle to the development of an effective human vaccine. The knowledge being gained on the mechanisms of *Leishmania* amastigote evasion of the host immune system will aid the rational design of vaccines.

Susceptible BALB/c mice vaccinated with unfractionated preparations of *Leishmania* membrane proteins were partially protected from *Leishmania* challenge (Murray *et al.* 1989). In humans, clinical trial are being carried out with first generation vaccines, killed *Leishmania* with or without BCG, with only modest success so far (Sarifi *et al.* 1998; Engers *et al.* 1996).

Since in leishmaniasis some antigens may elicit protective T cell responses, while others may accelerate disease progression (Scott *et al.* 1988), it is expected that a mixture of "protective" purified antigens will be a better vaccine than unfractionated protein preparations. Nevertheless, both Th1 and Th2 effector cells can be derived from the same antigen depending on the conditions during priming (Reiner and Locksley 1995).

Purified or recombinant *Leishmania* proteins have mostly been screened for their potential as immuno-prophylactics by one of two approaches: either their capacity to protect mice against experimental leishmaniasis or their capacity to stimulate human peripheral blood mononuclear cells (PBMNC) from leishmaniasis patients or from individuals that have been cured. In the latter case, the capacity of the stimulated PBMNC to express a Th1-type cytokine profile (γ-IFN, IL-12) was considered potentially protective, while the expression of a Th2-type cytokine profile (IL-4, IL-10) was considered counter-productive. Both approaches have limitations as a model for human leishmaniasis, where both the pathogen

and the host are heterogeneous, but taken together they provide a suitable screen for vaccine candidate antigens.

Examples of the use of the murine system are the following: Immunization of mice with *Salmonella typhimurium* and bacille Calmette-Guerin (BCG) containing the *L. major gp63* gene produced partial protection against challenge with *Leishmania* (Yang *et al.* 1990; *Connell et al.* 1993). Similarly, recombinant vaccinia viruses expressing GP46/M-2, a promastigote surface glycoprotein, partially protected mice against infection with *L. amazonensis* (McMahon-Pratt *et al.* 1993). The LACK antigen successfully protected BALB/c mice against *L. major* infection but only when given to mice immuno-modulated so as to favour a Th1 response. LACK protected mice when given with neutralizing antibody to IL-4 or with recombinant IL-12 (Wakil et al. 1996).

Examples of the use of patient's PBMNC are the following: Recombinant *L. major* GP63, the major promastigote surface protein, cloned and produced in *E. coli* (Button *et al.* 1991), was recognised by T cells from *Leishmania*-infected humans (Russo *et al.* 1991). Other *Leishmania* antigens that have shown immuno-protective promise by this approach are the P-4, P-8 and A2 proteins from *L. pifanoi* amastigotes (Coutinho *et al.* 1996, Silveira *et al.* 1998) and a protein fraction of *L. major* consisting mainly of a cysteine proteinase (Rafati *et al.* 1997).

An example of the use of both approaches is rLeIF, a recombinant *L. braziliensis* homologue of the eukaryotic ribosomal protein eIF4. The parasite's lysate stimulated the patient's PBMNC to produce a mixed Th1/Th2-type cytokine response while the rLeIF stimulated the production of γ-IFN, IL-2 and TNF-α but not IL-4 or IL-10. The rLeIF4 stimulated the production of IL-12 in cultured PBMNC from both leishmaniasis patients and uninfected individuals (Skeiky *et al.* 1995). BALB/c mice immunized with rLeIF4 were partially protected against *L. major*. It was suggested that rLeIF4 may serve as a Th1-type adjuvant and as a vaccine when used with other *Leishmania* antigens (Skeiky *et al.* 1998).

An alternative vaccine approach is that of DNA immunization. The DNA encoding the LACK parasite antigen was used to immunize BALB/c mice, which were later challenged with *L. major* promastigotes. The protection induced by LACK DNA was similar to that produced by immunization with LACK protein plus IL-12 and superior to that produced by

only LACK protein. CD8+ T cells appeared to have a role in the protective response induced by the LACK DNA vaccine (Gurunathan *et al.* 1997).

Another approach is that of attenuated live vaccines such as the cysteine proteinase null mutants of *L. mexicana*. *L. mexicana* cysteine proteinases are predominantly and abundantly expressed in the amastigotes and are located in the amastigote-specific megasomes. The most abundant cysteine proteinases-b (Lmcpb) are the product of a 19 tandem gene array while cysteine proteinase-a (Lmcpa) is produced by a single gene. Null mutants of Lmcpb (Δcpb), of Lmcpa (Δcpa) and double null mutants lacking Lmcpb and Lmcpa (Δcpa/cpb) have been produced by targeted gene disruption (Mottram *et al.* 1996). The mutants were tested for their ability to infect BALB/c mice. The wild-type *L. mexicana* produced a progressive disease with a predominantly Th2-type immune response. Infections with the Δcpb mutant produced very slowly growing small lesions and with the double mutant Δcpa/cpb did not induce lesion growth and were associated with a Th1-type immune response. Vaccination of mice with the Δcpa/cpb mutants provided a degree of protection against infection with wild-type parasites (Alexander *et al.* 1998).

While the Δcpa/cpb mutant is a promising candidate for an attenuated vaccine, it could be improved if it was made to express a non-virulent Lmcpb, as Lmcpb is likely to be a good potential immuno-protective antigen. An *L. major* amastigote cysteine proteinase-enriched fraction strongly stimulated PBMNC of individuals that had recovered from cutaneous leishmaniasis with production of γ-IFN but not of IL-4 (Rafati *et al.* 1997). Similarly, a *L. amazonensis* amastigote cysteine proteinase used to immunize BALB/c mice produced a degree of protection against challenge with *L. amazonensis* amastigotes, that was associated with a Th1 T cell response (Beyrodt *et al.* 1997). It is likely that if the active site of Lmcpb was mutated by site-directed mutagenesis, the proteolytically inactive mutant Lmcpb(-) protein would lose its virulence activity but not its immunogenicity. The ideal attenuated vaccine would be a mutant where the wild type *Lmcpb* genes were replaced with an array of proteolytically inactive *Lmcpb(-)* mutant genes. These genes would conserve their flanking sequences so that the Lmcpb(-) would be abundantly produced only in the amastigotes and secreted into their megasomes. There would be no phenotypic change in the metacyclic promastigotes, so the parasites would be infective to the macrophages but not virulent.

When the avirulent mutant Δcpa/cpb-cpb(-) amastigotes were killed by the macrophages, the abundant Lmcpb(-) antigen would be presented to CD4+ T cells. It has been shown for macrophages infected with *L. mexicana* that efficient stimulation of T cells specific for *Lmcpb*-encoded cysteine proteinases required the killing of the amastigotes (Wolfram *et al.* 1995 and 1996). The infected macrophage would act as an efficient antigen presenting cell and the immune response could be boosted by immunization with recombinant Lmcpb(-) protein.

1.4.- DEVELOPMENTAL STAGE-SPECIFIC GENE EXPRESSION IN *Leishmania*:

During its life cycle, Leishmania parasites have to adapt to different environments by undergoing a profound morphological and physiological transformation, including variation in the composition of proteins and carbohydrates on their cell surfaces. This differentiation must be due in great part to regulation of gene expression. Understanding these changes will give insight into the pathogenesis of leishmaniasis.

1.4.1.- Examples of *Leishmania* stage-specific genes:

An example of amastigote-specific gene expression is the A2 gene in *L. donovani*. The amastigote-specific A2 mRNA is encoded by a locus of at least seven A2 genes. The predicted protein contains a putative signal peptide, so the A2 proteins are probably secreted. Most of the predicted A2 protein is composed of a repetitive sequence composed of a stretch of 10 amino acids repeated 19 times (Charest and Matlashewski 1994). Antibodies prepared against a recombinant A2 protein, revealed that *L. donovani* amastigotes produced a family of A2 proteins ranging from 45 kDa to about 100 kDa, varying in the number of repeat amino acid units. No A2 proteins were detected in *L. donovani* promastigotes (Zhang *et al.* 1996). The A2 genes are present in *L. donovani* and *L. mexicana* and the recombinant A2 protein was recognized by sera of human patients infected only with those species of *Leishmania* (Ghedin *et al.* 1997).

Another example of stage-specific gene expression is that of the cathepsin L-like cysteine proteinases-b in *L. mexicana* (Lmcpb). While Lmcpb is expressed in amastigotes and metacyclic promastigotes, it is considered a marker for the amastigotes (those derived from lesions as well as axenic amastigotes) because of its abundance in that developmental stage.

The abundant Lmcpb is located in the lumen of the extended lysosomes or megasomes of the amastigotes.

As to its function, Lmcpb appears to degrade MHC class II molecules internalized into the amastigote megasome from the PV of the infected macrophage (De Souza Leao *et al.* 1995). Lmcpb are megasomal proteins but can be seen in the PV and extracellularly in the lesion tissue presumably as a result of macrophage rupture (Ilg *et al.* 1994). *L. mexicana* amastigote cysteine proteinases are virulence factors as shown by a Lmcpb null mutant (Δcpb) produced by targeted gene disruption of the *Lmcpb* gene array. The infectivity of the mutant was reduced by 80%. The Δcpb mutant was efficient in invading macrophages but survived in only a small proportion of infected cells. The product of a single *lmcpb* gene re-expressed in the Δcpb mutant was enzymatically active and restored infectivity of macrophages to the level of wild-type *L. mexicana* (Mottram *et al.* 1996). The Δcpb mutant, the null mutant of cysteine proteinase-a (Δcpa), an enzyme product of a single gene that is expressed throughout the life cycle of *L. mexicana* but more abundantly in amastigotes (Mottram *et al.* 1992) and null mutants for both Lmcpa and Lmcpb (Δcpa/cpb) were tested for their ability to infect BALB/c mice. The Δcpa mutant produced a disease similar to that produced by wild-type *L. mexicana*, the Δcpb mutant appeared to be attenuated, producing very slowly growing small lesions and the double mutant Δcpa/cpb did not induce lesion growth (Alexander *et al.* 1998).

The Lmcpb enzymes are encoded in a nineteen gene tandem array. The first two genes, *cpb1* and *cpb2* are expressed predominantly in metacyclic promastigotes. The sixteen genes *cpb3* to *cpb18* are predominantly expressed in amastigotes while *cpb19* is a pseudogene. Transfection of the Δcpb null mutant with different *cpb* genes showed that individual cpb isoenzymes differed in their substrate preferences and in their ability to restore virulence to the null mutant (Mottram *et al.* 1997).

Examples of promastigote-specific genes are those that code for components of the paraflagellar rod (PFR). These proteins are not required by the aflagellated amastigotes but are required for motility in the promastigotes of *L. mexicana* (Santrich *et al.* 1997). Three tandemly repeated genes code for the PFR-2 protein and their mRNA was fifteen-fold more abundant in promastigotes than amastigotes of *L. mexicana* (Moore *et al.* 1996).

1.4.2.- Examples of gene families whose expression varies during the life cycle of *Leishmania*:

A well characterised example of a gene family whose expression varies during the life cycle of *Leishmania* is GP63.

In *L. mexicana*, the GP63 is encoded by three distinct tandemly-repeated gene families. The C1 and C2 *gp63* gene clusters contain four to five copies each while the C3 gene may be single copy. The promastigotes contain mRNA from all three classes while the amastigotes only present *gp63* C1 gene mRNA. The sequence of the C1 genes predicted a unique carboxyl terminus that would not be substituted with a GPI anchor (Medina-Acosta *et al.* 1993). In another study, most of the amastigote GP63, in a form that lacked a GPI anchor, was found in the flagellar pocket (Medina-Acosta *et al.* 1989). This GP63 is presumably the product of the *gp63* C1 genes. A small fraction of GP63 could be iodinated on the amastigote cell surface. Despite the downregulation of surface GP63 during the differentiation to amastigotes, it still was the most abundant protein on the surface of the amastigotes (Medina-Acosta *et al.* 1989). In other studies, it was reported that a water-soluble form of GP63 in the amastigotes was mainly located in the megasome of *L. mexicana* amastigotes (Ilg *et al.* 1993; Bahr *et al.* 1993). While in *L. mexicana* promastigotes the surface GP63 is amphiphilic and comprises about 1% of cellular proteins, in amastigotes it is predominantly hydrophilic. The amastigote GP63 is localised mainly in the lumen of their megasomes and corresponds to only about 0.1% of cellular proteins (Bahr *et al.* 1993).

In *L. major*, there are five homologous tandemly repeated *gp63* genes, followed by two more *gp63* genes downstream. The genes 1-5 were highly expressed in promastigotes while gene 6 was constitutively expressed at a lower level throughout the parasite's life cycle. Gene 7 was expressed predominantly in stationary phase promastigotes and amastigotes (Voth *et al.* 1998). GP63 has been detected on the surface of *L. major* amastigotes (Pimenta *et al.* 1991). The structure of *L. major* amastigote GP63 was shown to be different from that of promastigote GP63 when analysed by western blot using two different monoclonal antibodies raised against recombinant promastigote GP63 (the product of the cloned *L. major gp63* gene 1). When analysed after SDS-PAGE under non-reducing conditions, the amastigote GP63 was present as a large molecular weight complex, composed of GP63 monomers bound by disulfide bonds. It was calculated that it formed a tetramer. In contrast,

the promastigote GP63 was present as a monomer, containing internal disulfide bonds, as is the case with all previously reported *Leishmania* GP63 (Bellatin, J and W.R. McMaster, unpublished observations). A different GP63 structure in the amastigotes, as described for both *L. major* and *L. mexicana*, may reflect the need for a different and amastigote-specific function for GP63.

During growth in culture *L. chagasi* promastigotes differentiate into a highly virulent form (metacyclic promastigotes) as they progress to the stationary phase of culture. The increase in virulence is accompanied by an 11- fold increase in the amount of GP63 per cell (Ramamoorthy *et al.* 1992). The major surface protease GP63 in *L. chagasi* is encoded by 18 or more tandem *gp63* genes belonging to three classes differing in their unique 3' UTR and in their differential expression. During the logarithmic phase of growth in culture (a model for procyclic promastigotes) the mRNA from the *mspL* genes predominates. The mRNA from the *mspS* genes are present mainly in stationary phase (a model for metacyclic promastigotes) and mRNA from the *mspC* genes are present throughout growth in culture. All three classes of *gp63* genes are constitutively transcribed by promastigotes during their growth in culture, so their expression is post-transcriptionally regulated (Ramamoorthy et al. 1995).

In *L. mexicana*, there are three distinct glucose transporters: LmGT1, LmGT2 and LmGT3. The level of total *LmGT* mRNA was higher in the promastigotes, reflecting the fact that the promastigote relies on glucose more than the amastigote, that obtains its metabolic energy primarily from fatty acid oxidation. The *LmGT1* and *LmGT3* genes were expressed constitutively while the *LmGT2* mRNA was present at 15 fold higher level in promastigotes than in amastigotes, being the most highly expressed *LmGT* gene. Transcription of the three *LmGT* genes ocurred at similar levels in promastigotes and axenically cultured amastigotes, as measured by nuclear run-on transcription, so the higher levels of *LmGT2* mRNA in promastigotes were due to a post-transcriptional control. The decay of *LmGT2* mRNA was measured in the presence of the transcriptional inhibitor actinomycin D and *LmGT2* mRNA was found to be more stable in promastigotes than in amastigotes. A similar regulation by differential mRNA stability was found for the stage-specific β-tubulin mRNA in *L. mexicana*, with a predominant 2.4 kb species in promastigotes and a predominant 2.8 species

in amastigotes. The 2.4 kb mRNA was found to be more stable in promastigotes and the 2.8 kb mRNA was more stable in axenic amastigotes (Burchmore and Landfear 1998).

1.4.3.- Gene expression in *Leishmania*:

No promoter for RNA polymerase II has been found in *Leishmania*. *Leishmania* mRNA is transcribed as a polycistronic message that is processed at the 5' end by *trans*-splicing and at the 3' end by polyadenylation. *Trans*-splicing introduces a 35 to 39 nucleotide mini-exon or spliced leader (SL) to all *Leishmania* mRNA that serves for 5' end capping. The SL RNA is a short, non-polyadenylated, capped transcript of 140 nucleotides encoded by tandem array of genes. The 35 to 39 nucleotide SL, at the 5' terminus of the SL RNA is transferred to an internal 3' acceptor site on the mRNA precursor. The *trans*-splicing reaction requires the presence of a polypyrimidine tract and an AG splice acceptor site in the nascent mRNA precursor. The polyadenylation of an upstream gene is functionally coupled to *trans*-splicing of a downstream gene (Wong 1995). Poly A site selection is specified by the position of the downstream *trans*-splice acceptor site and sequence elements recognized by the splicing machinery are also required for polyadenylation. Although the mechanism of *trans*-plicing is similar to *cis*-splicing, the protein coding genes in *Leishmania* do not contain the usual eukaryotic cis intervening sequences. Gene regulation occurs through post-transcriptional mechanisms (Nilsen 1994 & 1995).

In *Leishmania* a post-transcriptional mechanism of stage-specific gene expression by differential stability of mRNA involved the 3' UTR of mRNA. Reporter genes fused with the 3' UTR plus its intergenic region (IR) of some *Leishmania* stage-specific genes were transfected into *Leishmania* and the level of reporter gene mRNA was measured. The 3' UTR of the amastigote-specific *L. donovani A2* gene (Charest *et al.* 1996 & Ghedin *et al.* 1998); the stationary phase promastigote-specific *L. chagasi gp63* and *gp46* genes (Ramamoorthy *et al.* 1995; Beetham *et al.* 1997) and the promastigote-specific *L. major gp63-1* gene (Kelly, B. and McMaster, W.R. personal communication) determined the stage-specific expression of reporter genes. The intergenic region (IR) after the 3' UTR was probably required to provide a suitable *trans*-splicing signal and splice acceptor site, signals required for the maturation of the reporter gene transcript at both the 5' and 3' ends of the mature mRNA as splicing is linked to polyadenylation. In the study of the *L. donovani* A2

3'UTR, the IR was not used but a 92 bp synthetic element (pyt) that consisted of a polypyrimidine tract and an AG *trans*-splicing acceptor site. Both the A2 3' UTR and the pyt element were required for differential gene expression of the reporter gene (Charest *et al.* 1996).

Unique sequence motifs or secondary structure features in the 3' UTR of the mRNA may be recognised by stage-specific proteins to either stabilise or target for degradation the regulated mRNA. Such a model, while fitting the evidence, begs the question of how are the genes that code for the putative stage-specific 3' UTR-recognition proteins regulated.

There is evidence for two different mechanisms of regulation of the expression of the *gp63* (*msp*) genes of *L. chagasi*. Plasmids containing the three different *msp* gene 3'UTR and their downstream IR fused downstream to the reporter gene *β-gal* have been tested for differential expression in *L. chagasi*. When the plasmid containing the 3' UTR of *mspS* plus its IR was transfected into *L. chagasi*, an increase of about 20 fold in β-galactosidase activity and mRNA was observed in stationary phase relative to logarithmic phase cells. The 3' UTR of *mspL* plus its IR had no effect and that of *mspC* had little effect on *β-gal* expression (Ramamoorthy *et al.* 1995). In contrast, the use of protein synthesis inhibitors like cycloheximide affected the expression of mspL and mspS genes differently. *L. chagasi* promastigotes switch from the predominant expression of a 2.7 kb log *gp63* mRNA (the product of *mspL* genes) in logarithmic growth to the predominant expression of a 3.0 kb stationary *gp63* mRNA (the product of *mspS* genes) during stationary phase of growth in culture. The addition of the protein synthesis inhibitor cycloheximide four hours prior to extraction of RNA increased the level of 2.7 kb *mspL* mRNA 16.5 fold in log phase promastigotes and more interestingly, led to the appearance of the 2.7 kb gp63 mRNA in stationary phase promastigotes. Cycloheximide treatment had no significant effect on the level of *mspS* or *mspC* mRNA or on the level of α- and β-tubulin mRNA. Nuclear run-on assays showed that the effect of cycloheximide was not due to an increased rate of transcription but to an increase in the half-life of *mspL* mRNA. These results suggested that cycloheximide specifically stabilised log phase *gp63* (*mspL*) mRNA and that a highly labile negative regulatory protein, such as an RNAase, may specifically target log *gp63* mRNA for degradation (Wilson et al. 1993). The specific target sequence would not appear to be on the

3' UTR of the *mspL* mRNA (Ramamoorthy *et al.* 1995). This negative regulatory protein was present in the logarithmic phase of growth and more relevantly, in the stationary phase where in the absence of the cycloheximide treatment no 2.7 kb *mspL* mRNA could be detected. The more dramatic effect on stationary phase promastigotes may be due to increased concentration of the negative regulatory protein or to a different and more efficient one. In contrast, the level of the 3.0 kb *mspS* mRNA was not affected in stationary phase promastigotes nor did it appear in log phase promastigotes as a consequence of cycloheximide treatment. In consequence, the stage-specific elements controlling *mspS* gene expression, through recognition of its 3' UTR, are either not proteins or are less labile proteins, so that four hours of cycloheximide treatment would not dramatically affect their concentration. In conclusion, the amounts of *mspL* and *mspS* mRNA are each post-transcriptionally regulated by different molecular mechanisms.

1.5.- AXENIC CULTURE AMASTIGOTE MODELS

Most of the information available about *Leishmania* has been obtained from studying axenic culture promastigotes, a model for the parasite's procyclic promastigotes, the developmental stage present in the insect vector. Although amastigotes can be obtained from experimentally infected animals or from infected, cultured macrophages (Glaser *et al.* 1990; Saraiva *et al.* 1983), the study of their biochemistry, surface proteins or mRNA has been hindered by the presence of host tissue contaminants. An alternative model is that of amastigote-like *Leishmania* in axenic culture. *Leishmania* strains from six species: *L. pifanoi*, *L. amazonensis, L. braziliensis*, *L. panamiensis*, *L. donovani* and *L. mexicana* have been cultured as axenic amastigotes by modifications in temperature, in pH or in both culture conditions with the intent of mimicking the conditions prevailing in the PV of the infected macrophage (Rainey *et al.* 1991; Hodgkinson *et al.* 1996; Eperon & McMahon-Pratt 1989; Doyle *et al.* 1991; Saar *et al.* 1998; Bates *et al.* 1992). These cultured amastigotes have been shown to resemble amastigotes from infected macrophages or murine lesions by morphological, biological, immunological and biochemical criteria (Bates 1993; Pan *et al.* 1993; Saar *et al.* 1998), thus validating their use as a model. Given that the amastigotes are not likely to find such conditions (32°C and pH 5.4 for *L. mexicana*) in nature outside of their host cells, they should still be considered obligate intracellular parasites.

For the present study *L. mexicana* was used because it was very well characterized and has been shown to constitute a reliable model for amastigotes recovered from lesions. They showed the same ultrastructural features as amastigotes recovered from murine lesions such as the presence of megasomes (large lysosomal compartment characteristic of the amastigotes of the mexicana group: *L. mexicana*, *L. amazonensis* and *L. pifanoi*) and non-emergent flagellum lacking a paraxial rod. They also resembled lesion amastigotes and differed from promastigotes in the presence of the megasomal amastigote-specific cysteine proteinase b (Bates *et al.* 1992; Pral *et al.* 1993). The relative levels of expression of four developmentally regulated genes were compared between axenic culture promastigotes and amastigotes and macrophage-derived amastigotes of *L. mexicana* by Northern blot analysis. The regulated genes were the promastigote-specific paraflagellar rod protein PFR-1, the amastigote-specific cysteine proteinase genes, the promastigote-specific glucose transporter gene-2 *LmGT2* and the differentially expressed β-tubulin mRNA species. In all those cases, the pattern of gene expression of the axenic amastigotes resembled that of the macrophage-derived amastigotes (Burchmore and Landfear 1998). Furthermore, the change in size of the GP63 bands between promastigotes and amastigotes observed in a western blot (See figure 5, lanes 7 and 9) was the same as that reported for *L. mexicana* amastigotes purified from mouse lesions (Frommel *et al.* 1990).

Axenic-culture *in vitro* models exist for the entire *L. mexicana* life cycle (Bates 1994): cultured pure promastigotes and amastigotes (Bates et al. 1992) and enriched populations of infective metacyclic promastigotes (Bates and Tetley 1993; Mallinson and Coombs 1989; Zakai *et al.* 1998) can be produced by simple changes in culture conditions.

1.6.- THE PRESENT WORK

Leishmaniasis is a parasitic human disease that constitutes a public health problem in many parts of the world. The efforts to control the disease, by developing much needed new therapies and vaccines, will be greatly aided by a better understanding of the *Leishmania* amastigote and its complex relationship with its mammalian host. This work was based on the explicit assumption that genes preferentially expressed in amastigotes encode proteins that are involved in amastigote-specific functions. Those amastigote-specific functions are the parasite's contribution to the host-parasite relationship. Identification, isolation,

sequencing and characterization of amastigote-specific genes should throw light on amastigote-specific functions. The long-term objective is to determine the biological role of *Leishmania* amastigote-specific genes and proteins in the relationship between the parasite and its host. This information would provide a significant improvement in our understanding of the pathogenesis of the disease. In the present work this approach was pursued by the use of axenic culture *L. mexicana* amastigotes and by the identification of amastigote-specific cDNA by subtractive hybridization. Two amastigote-specific genes were identified and sequenced: A600 that codes for a novel predicted secreted polypeptide and A850, a β-tubulin isogene.

The isolation and sequencing of genes preferentially expressed in the amastigote will also produce important indirect benefits. The availability of more developmentally regulated genes, and of their 3' UTR sequences, should aid in defining the mechanisms of stage-specific gene regulation in *Leishmania*. Furthermore, the identification of amastigote-specific genes enables the production of recombinant amastigote proteins that could provide new candidate molecules to test for immuno-prophylaxis. The desired characteristics for a vaccine candidate are abundance in the amastigote stage and immunogenicity. It is expected that A600, the most abundant amastigote protein identified and isolated in this work, will be tested in the near future for its immunogenicity and potentially added to the arsenal of antigenic components for vaccine development.

A second approach used in this thesis was the identification and isolation of amastigote surface proteins. The surface proteins of the amastigote are likely to have a significant role in the host-parasite relationship as they stand in the interphase between the two organisms. However, relatively little is known about these surface proteins and their specific functions. One major hypothesis of this thesis was that the surface proteins of the amastigote stage of *Leishmania* interact with molecules and cells of the mammalian host. Another hypothesis is that such interactions are important for the continuous infection of host macrophages and for the survival of the parasites within them. This approach was pursued by the generation of monoclonal antibodies directed against the surface of the amastigotes. The immunodominant surface proteins of *L. mexicana* axenic amastigotes were found to be GP63 and a novel amastigote-specific protein complex.

2.- MATERIALS AND METHODS

Leishmania CULTURE:

Leishmania mexicana (WHO designation MNYC/B2/M379) promastigotes were cultured at 26°C in M199 medium (Gibco BRL) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah), 40 mM N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid] (HEPES) and the antibiotics penicillin (50 units) and streptomycin (50 μg per ml). Cultures were maintained at densities ranging from 1 x 10^5 to 5 x 10^7 cells per ml.

The axenic cultures of amastigotes were started by transferring amastigotes obtained from murine lesions to medium UM54 pH 5.5 (medium M199 plus 0.25% glucose, 0.5% trypticase, 25 mM HEPES, 5.14 mM glutamine and 0.035% $NaHCO_3$) – 20 % FBS or by gradually transforming cultured promastigotes. Promastigotes growing in medium M199 – 10% FBS were transferred to 32°C and grown overnight. The cultures were diluted one in one with UM54 medium pH 5.5 – 20% FBS, thus gradually reducing their pH. Dilutions were made daily or as required to maintain a culture density of between 5 x 10^5 to 3 x 10^7 parasites per ml of culture.

BIOTIN LABELING OF *Leishmania* SURFACE PROTEINS:

In order to label surface proteins, the reagent Biotin N-hydroxysuccinimide ester containing a six atom spacer between the biotin and the target ligand was used (Biotin-X-NH_2) (Calbiochem, San Diego, California). This reagent reacts with primary amines. Cultured promastigotes or amastigotes were counted, and washed 5 times by centrifugation with excess phosphate buffered saline (PBS) pH 7.0. The cells were resuspended in PBS pH 8.0 at a concentration of 5 x 10^8 cells/ml and a 5% volume of freshly prepared 5mM NH_2-X-Biotin in DMSO was added and incubated for 1hr at 4°C. The reaction was stopped by adding a 5% volume of 1M Tris-HCl pH 6.8, the cells were washed twice in excess tris buffered saline (TBS) pH 7.0 and solubilized by resuspending cells at 4°C in TBS pH 7.0 - 2% Zwittergen 3-14 - 1mM PMSF - 10 ug/ml Leupeptin - 5 μM E64 - 25 mM 1,10 Phenonthalein - 5 mM Iodoacetamide. After incubation for 5 min at 4°C, the solubilized cells were centrifuged at maximum speed in a microfuge at 4°C for 15 min. The supernatant

was stored at -20°C. The extract was used for immunoprecipitation or SDS-PAGE. Surface labeled polypeptides were visualized in Western Blots with Streptoavidin-Alkaline Phosphatase (GIBCO-BRL) at a 1/12,000 dilution and developed with NBT-BCIP (Immuno-Select; GIBCO-BRL).

PREPARATION OF MONOCLONAL ANTIBODIES AGAINST SURFACE PROTEINS:

BALB/c mice were immunized subcutaneously with 2 x 10^8 axenic culture amastigotes emulsified with complete Freunds adjuvant. A second subcutaneous immunization in incomplete Freunds adjuvant was performed three weeks later. Ten days after the second immunization, the sera of the mice were analyzed by flow cytometry and found to bind to the surface of both *L. mexicana* axenic culture amastigotes and promastigotes. Two mice were given an intravenous boost with axenic culture amastigotes in saline four days prior to the fusion. The spenocytes of the two mice were pooled. The fusion of the mouse spenocytes with myeloma X63.Ag8.653 cells and the selection and cloning of the hybridomas was done with the ClonaCell HY^{TM} method following the instructions of the technical manual of the manufacturers (StemCell Technologies Inc., Vancouver, B.C. and Vancouver Island Antibodies Ltd., Victoria, B.C.). The supernatants of the cloned hybridomas were assayed for binding to the surface of axenic culture amastigotes or promastigotes by flow cytometry.

FLUORESCENCE FLOW CYTOMETRY

L. mexicana axenic culture promastigotes and amastigotes were labeled for indirect immuno-fluorescence. The cells (5 x 10^6 parasites) were washed with binding buffer, TBS containing 0.5% bovine serum albumin (BSA) and incubated for 1 hour at 4°C with 100 µl of mAb supernatant. The cells were then centrifugated and washed twice with 1 ml of binding buffer and incubated for 40 minutes at 4°C with 2 µl of FITC-conjugated secondary antibody (FITC-GAM) (Goat F(ab')2-anti-mouse IgG-FITC, Human adsorbed, Southern Biotechnology Associates Inc., Birmingham, Alabama) diluted in 50 µl of binding buffer. The cells were washed with PBS, fixed with 0.4% formaldehyde in PBS and resuspended in PBS for flow cytometry using a Becton Dickinson FACScan analyser. Negative controls were incubation in binding buffer only or with the supernatant of mAb 96, that was raised

against GP63 of *L. major* but does not bind to *L. mexicana*, followd by incubation with FITC-GAM.

ISOLATION OF AMASTIGOTE-SPECIFIC cDNA FRAGMENTS BY SUBTRACTIVE HYBRIDIZATION

The procedure used was as described by CLONTECH SMART PCR cDNA Synthesis Kit User Manual Protocol # PT3041-1, Version # PR75803.CLONTECH, Sections VII A to E and CLONTECH PCR-Select™ cDNA Subtraction Protocol # PT1117-1, Version # PR7X314, Sections IV F to I (CLONTECH, Palo Alto, California). The procedure is outlined and explained in section 4.1.1 of this thesis.

OLIGONUCLEOTIDES USED (CLONTECH's primers):

SMART II oligonucleotide:

5' - AAGCAGTGGTAACAACGCAGAGTACGCGGG - 3'

cDNA synthesis (CDS) primer:

5' - AAGCAGTGGTAACAACGCAGAGTAC$T_{(30)}$N$_{-1}$N - 3'

PCR primer (Second strand synthesis primer- DScDNA primer):

5' - AAGCAGTGGTAACAACGCAGAGT - 3'

Adaptor 1:

5' - CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT - 3'

PCR primer 1:

5' - CTAATACGACTCACTATAGGGC - 3'

Nested PCR primer 1:

5' - TCGAGCGGCCGCCCGGGCAGGT - 3'

Adaptor 2R:

5' - CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT - 3'

Nested PCR primer 2R:

5' - AGCGTGGTCGCGGCCGAGGT - 3'

B-SL primer: In bold characters 30 mer sequence corresponding to the spliced leader (SL) sequence present at the 5' end of all *Leishmania* mRNAs. The SL sequence was identical in *L. major*, *L. amazonensis* and *L. enriettii*.

5' - ATCAGGATCC**TATATAAGTATCAGTTTCTGTACTTTATTG** - 3'

FIRST STRAND cDNA SYNTHESIS

The procedure is illustrated in figure 2. For each sample (amastigote RNA and promastigote RNA) the following reagents were combined in a sterile 0.65 ml microfuge tube: 1-3 µl total RNA sample (1 µg of *L. mexicana* promastigote (driver) or amastigote RNA (tester)), cDNA synthesis (CDS) primer (10 µM), SMART II oligonucleotide (10 µM), Deionized water to complete 5 µl of reaction volume. The reactions were incubated at 70°C for 2 minutes, cooled on ice for 2 minutes and centrifuged briefly at room temperature.

The following was added to each tube: 2 µl of 5X first-strand buffer, 1 µl of DTT (20 mM), 1 µl of dNTP (10 mM), 1 µl of Superscript II Reverse Transcriptase (200 units/µl). The reagents were mixed gently by pipeting and centrifuging the tubes briefly. The reactions were incubated at 42°C for 1 hour, after which 40 µl of TE Buffer (10 mM Tris [pH 7.6], 1 mM EDTA) were added and the reactions heated at 72°C for 7 minutes. One µl from each reaction was used for second strand synthesis and the rest of the first strand cDNA preparation was aliquoted and stored at -20°C.

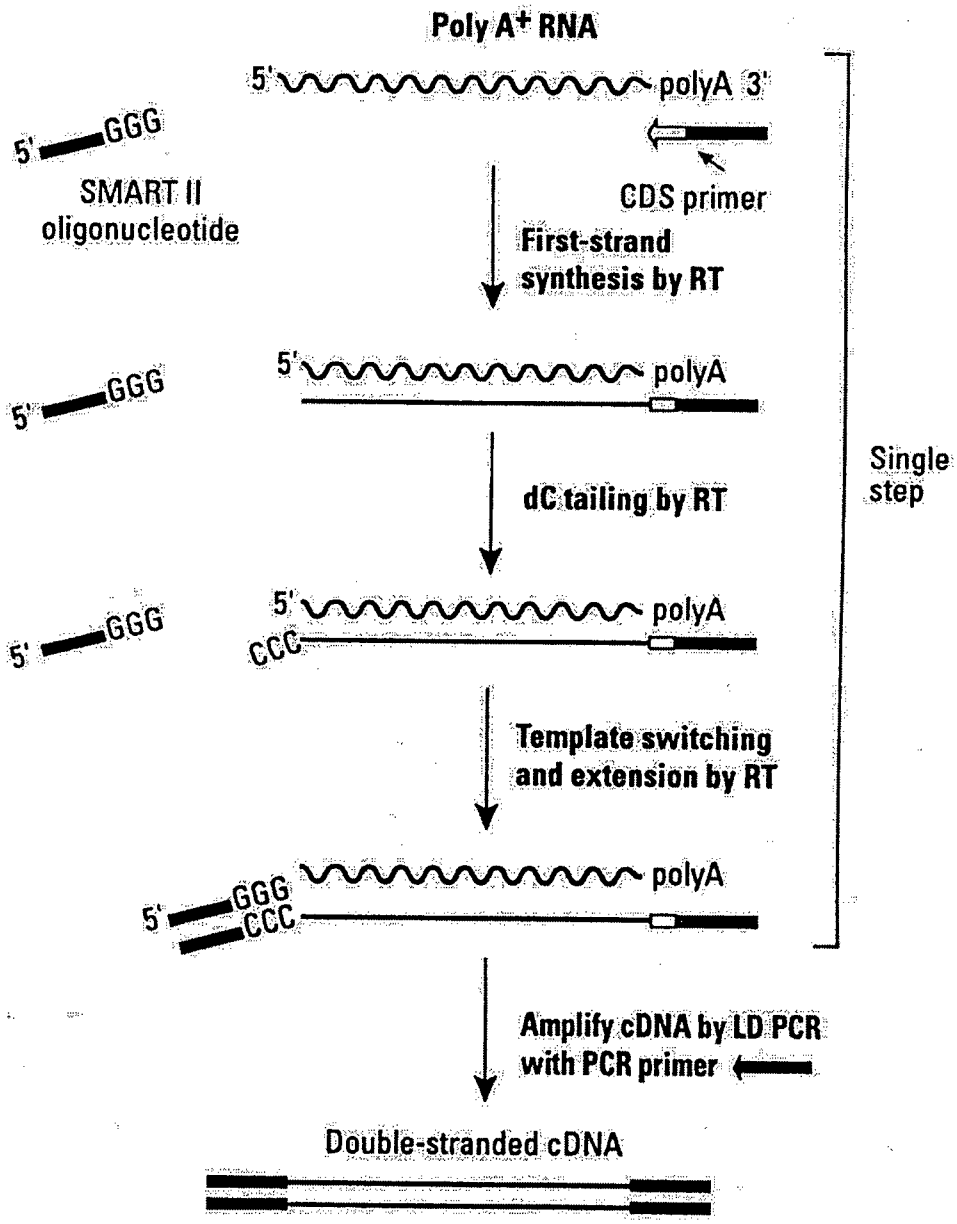
SECOND STRAND cDNA SYNTHESIS

The number of cycles and amount of single stranded template had to be determined experimentally so as not to over amplify. The number of cycles required varies according to the template preparation. The ds cDNA population should be representative of the relative amounts of mRNA in the sample and therefore a Southern Blot of the ds-cDNA would constitute a "Virtual Northern Blot".

PCR MIX (for 1 reaction): 81.8 µl of MilliQ H_2O; 10 µl of 10x Klen Taq Buffer Mix; 2 µl of 10 mM 4 dNTP mix; 3.2 µl of 6.4 µM DScDNA primer (or 2 µl of 10 µM) (CLONTECH's PCR primer) and 2 µl of 50x Advantage Taq Pol Mix.

FIGURE 2

CLONTECH SMART™ PCR cDNA SYNTHESIS



NOTES: The SMART II oligonucleotide, CDS primer and PCR (DScDNA) primer all contain a stretch of identical sequence
This figure was copied from CLONTECH's Protocol # PT3041-1

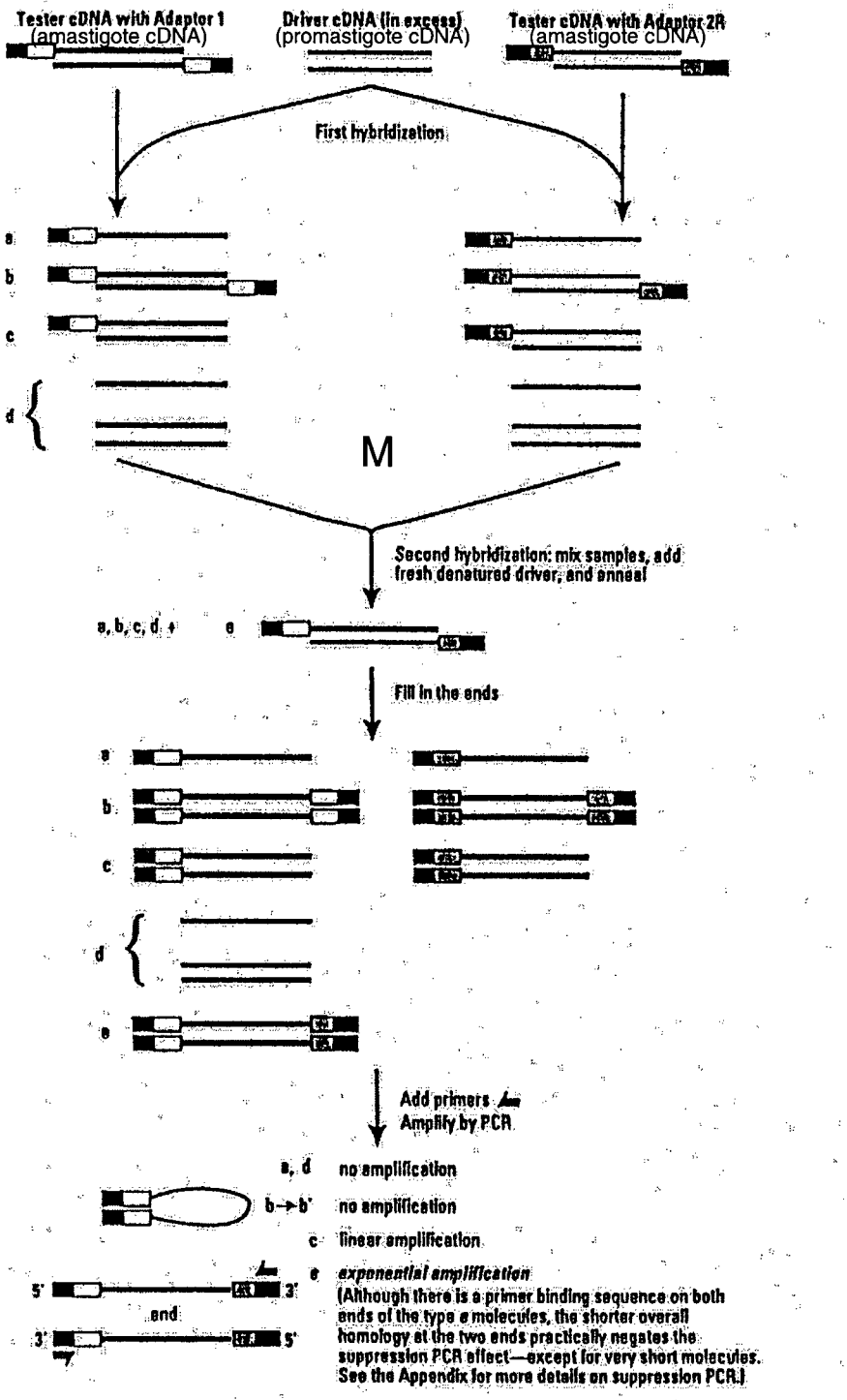
PCR PROTOCOL

The reactions were mixed cold in ice. To a thin walled PCR tube was added either 1 µl of *Leishmania mexicana* promastigote single stranded cDNA per reaction or 1 µl of amastigote single stranded cDNA per reaction. To each reaction tube were added 99 µl of ice-cold PCR mix. This was mixed by pipetting up and down a few times. The PCR protocol was initiated when the temperature reached 95°C and pause was pressed. The PCR tubes were transfered from the ice to 95°C at the thermocycler PCR machine for a hot start PCR. A hold of 1 min at 95°C was followed by 15 to 24 cycles of 15 sec at 95°C; 30 sec at 65°C; 6 min at 68°C. The yield of a 100 µl reaction has been measured as 3 µg of double stranded cDNA, for both promastigote and amastigote templates. It was measured by purifying the PCR products with QIAGEN's QIAquick PCR purification kit and measuring absorbance at 260 nm.

Three tubes of double stranded amastigote cDNA and three tubes of double stranded promastigote cDNA (ds-cDNA) were prepared in order to have sufficient material to start the subtraction protocol. After 15 cycles of PCR, two tubes each of the promastigote and amastigote cDNAs were removed to 4°C and the PCR reaction was continued for the third tubes, taking aliquots every 3 cycles. In this way the optimum number of cycles was determined, one cycle before the appearance of high molecular weight amplification products. For the preparations described, 20 cycles of PCR were found to be optimum. The double-stranded cDNA was purified using a Qiaquick PCR purification kit (QIAGEN) following the manufacturer's instruction manual. The ds-cDNA were digested with rhe restriction enzyme Hae III to produce smaller blunt ended cDNA fragments. The reduction in the average size of the cDNA was observed by agarose gel electrophoresis. The tester sample (amastigote cDNA) was divided into two portions, and ligated to either adaptor 1 or adaptor 2R following the procedure in the manual (CLONTECH PCR-select cDNA subtraction manual section IV-F). The adaptors were not ligated to the driver cDNA. The two hybridization and two PCR amplification procedures were performed as in the manual and are outlined in figure 3

FIGURE 3

CLONTECH PCR-SELECT™ cDNA SUBTRACTION PROTOCOL



NOTES: M shows the point were additional steps are introduced in the modified protocol outlined in Figure 4
Figure copied from CLONTECH's Protocol # PT1117-1

Modifications to the procedure were performed in order to obtain more amastigote-specific bands. The first modification consisted if diluting the tester cDNA 10- and 100-fold before the first hybridization, resulting in an increase in the driver cDNA to tester cDNA ratio.

A second modification of the procedure is outlined in figure 4. After the first hybridization, the samples were removed and the 4 µl hybridization mixes were diluted at room temperature by adding 46µl of a mix containing 39 µl of distilled water, 5 µl of 10x Klenow Polymerase Buffer, 1 µl of 10 mM dNTP and 1µl of Klenow Polymerase fragment (exo-)(5 units). The samples were incubated for 30 minutes at room temperature to fill in the single stranded adaptors of type **b** and **c** molecules. The samples were diluted by adding 150 µl of a mix consisting of 131 µl of distilled water, 15 µl of NEB 10x Buffer 3, 2 µl of Tris-HCl pH 8.0, 1 µl 5 M NaCl, 0.25 µl of $MgCl_2$ and 1 µl of the restriction enzyme Eag I. The samples were digested for 2 hours at 37°C and the enzyme was deactivated by incubation at 65°C for 20 minutes. The samples were purified by use of the QIAquick PCR purification kit and eluted with 30 µl of 5 mM Tris-HCl pH 8.5. The Eag-digested tester 1 and 2R samples were subjected to the second hybridization procedure after adding excess driver cDNA and the remainder of the protocol is as described in the CLONTECH manual.

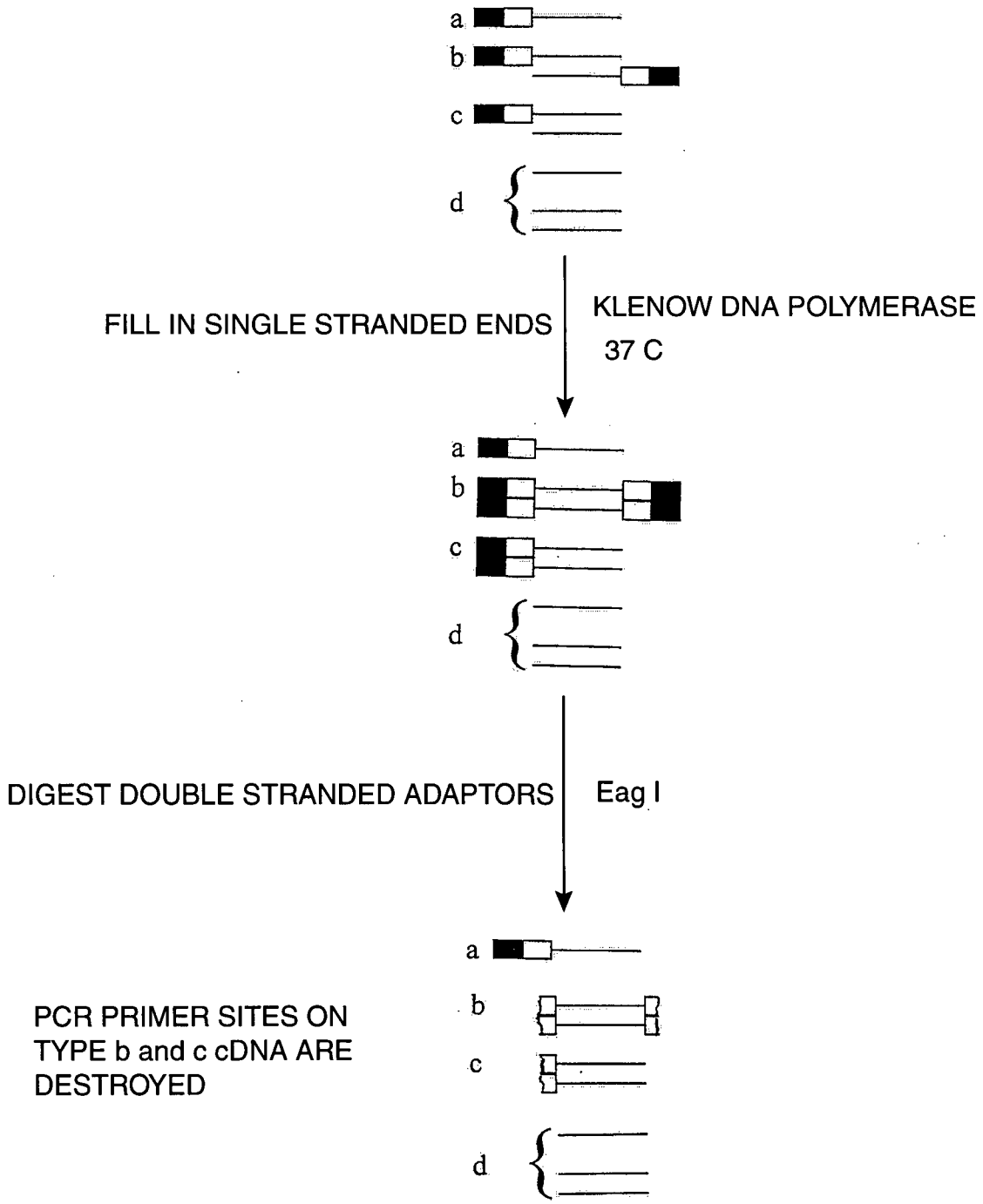
VIRTUAL NORTHERN BLOTS

Electrophoresis of 500 or 800 ng of promastigote and amastigote double stranded cDNA per slot was performed using a 1.5 % agarose gel. The two slots containing Amastigote and promastigote cDNA were flanked by empty slots or slots containing DNA markers in order to later cut slices of the blot. The gels were electrophoresed slowly at 30 to 50 Volts to ensure good resolution. After electrophoresis photographed the EtBr stained gel was photographed with a ruler to determine the position of the size markers. DNA was transfered to Hybond-N+ nylon membrane (Amersham Life Science Inc., Arlington Heights, Illinois) in a 0.4 N solution of NaOH using a Vacuum Blotter (Model 785: BIO-RAD, Richmond, California). The operating instructions as described in section 3 of the Vacuum Blotter Instruction Manual were followed with the following modifications: The gel was soaked in the 0.4 NaOH denaturing and transfer solution for 15 to 30 min. Depurination was not

FIGURE 4

MODIFICATION OF SUBTRACTION HYBRIDIZATION PROCEDURE

FOR EACH OF THE TWO TESTER cDNA PORTIONS, WITH ADAPTORS 1 OR 2R, AFTER FIRST HYBRIDIZATION OF NORMAL PROCEDURE:



PURIFY cDNA AND RESUME NORMAL PROCEDURE
WITH SECOND HYBRIDIZATION

necessary. The gel was transfered using the same solution. After setting up the gel, it was perforated with a pencil at the top and bottom of the empty slots that separated the future nylon slices. The pencil marks were visible in the nylon membrane under the gel. Transfer was performed for 90 min at 5 Hg of pressure. The membrane was washed with 2x SSC (20x stock solution of SSC: 3M NaCl, 0.3M Na_3citrate) for 5 minutes, dried and slices containing adjacent amastigote and promastigote cDNAs were cut and used for hybridization. The pre-hybridization solution was made up of 5x SSPE (20x SSPE stock: 3.6M NaCl; 0.2M Sodium phosphate and 0.02M EDTA pH 7.7); 5x Denhardt's solution (100x stock: 2% (w/v BSA; 2% (w/v) Ficoll and 2% (w/v) polyvinylpyrrolidone) and 0.5% (w/v) SDS. It also contained a 1/50 volume of denatured sheared salmon DNA. This DNA was denatured by boiling for 5 minutes and chilling on ice previous to adding to the pre-hybridization mix.

The cDNA probes were labeled by the N9 random priming method: 25 ng of a DNA template (cDNA fragment or insert from cloned cDNA removed by restriction enzymes digestion and purification from agarose gel slice using QIAEX II Gel Extraction Kit, QIAGEN Inc., Canada) in 28 µl of distilled water (dH_2O) was added to 10 µl of N9 primer (27 $O.D._{260nm}$/ml) and the mix was boiled for 5 minutes and collected by brief centrifugation. To the reaction tube at room temperature the following reagents were added: 5 µl of 10x concentrated Klenow DNA Polymerase (New England Biolabs); 1 µl of 1mM dGTP, dATP, dTTP mix; 5 µl of (α-^{32}P) dCTP and 1 µl of DNA Polymerase, Klenow fragment, (exo^-) (New England Biolabs). The sample was incubated at 37°C to 40°C for 10 minutes, and the reaction stopped by adding 2 µl of 0.5 M EDTA, pH 8.0. The probe was purified using a QIAquick Nucleotide Removal Kit following the instructions from the manufacturer (QIAGEN Inc., Canada). The labeled probe was boiled for 5 minutes and put on ice immediately before adding to the pre-hybridization mix.

The blots were hybridized for 16 to 24 hours at 65°C in a rotating tube. The blots were washed by twice incubating them in 2x SSC, 0.1% SDS at room temperature for 10 minutes; a wash with 1x SSC, 0.1% SDS at 65°C and two high-stringency washes of 0.2x SSC, 0.1% SDS at 65°C. The blots were exposed to autoradiographic X-ray film or to a phosphoimaging screen (BIORAD).

SOUTHERN BLOTS

Leishmania mexicana genomic DNA was prepared as described (Medina-Acosta and Cross, 1993), concentrated by ethanol precipitation and quantitated by measuring absorvance at 260 nm. The DNA was digested with various restriction enzymes and run slowly in a 0.8% Agarose gel.

The Southern blots were performed as described above for Virtual Northern Blots with the following differences: After agarose gel electrophoresis, the gel was placed in 0.25M HCl until the dyes change color and leaved for an additional 10 minutes. The gel was then rinsed in dH_2O and placed in denaturation buffer (1.5M NaCl; 0.5M NaOH) for 30 minutes with gentle rocking. The gel was rinsed in dH_2O and placed twice in neutralization buffer (1.5M NaCl; 0.5M Tris-HCl pH 7.2; 0.001M EDTA) for 15 minutes. The gel was transferred to the Hybond-N+ nylon membrane by capillary blot (Sambrock *et al.*, 1989) for 16 hours by alkali blotting with 0.4M NaOH and the membrane briefly washed with 2x SSC before hybridization.

3.- IDENTIFICATION OF AMASTIGOTE-SPECIFIC SURFACE PROTEINS

RATIONALE

Leishmania undergoes profound morphological and biochemical changes during its life cycle in order to survive and grow in very different environments. The most extreme differences are between the promastigote stage that exists naturally in the insect vector and the amastigote stage that exists naturally as an intracellular parasite of the macrophages in mammalian hosts, including humans. A better understanding of the amastigote stage of *Leishmania* and its relation with its host is needed to better control the human disease Leishmaniasis. The *Leishmania* amastigote must extensively interact with the macrophage on which it depends for nutrients. It must also counteract and perhaps interfere with the parasiticidal and parasititatic activities of the macrophage. Changes in protein expression within the parasite should be responsible for changed morphology and metabolism. Changes in molecules on the surface of the parasite and in molecules secreted by the amastigotes should play a major role in interacting with, and perhaps modifying, the hostile environment of the parasitophorous vacuole of the infected macrophage. Nevertheless, little is known of the proteins on the surface of the amastigotes, largely because it had been difficult to differentiate these from those of the macrophage. The availability of a suitable axenic culture model for the amastigote stage of *Leishmania* has facilitated its biochemical analysis. Monoclonal antibodies were raised against axenic culture *L. mexicana* amastigotes which allowed the identification of a novel surface protein complex.

3.1.-RESULTS

3.1.1.- Surface labeling of cultured *L. mexicana* amastigotes and promastigotes:

Axenic culture amastigotes from *L. mexicana* were obtained by shifting the culture temperature from 26 to 32 degrees centigrade and gradually decreasing the pH of the medium to 5.5. The first noticeable change was that the parasites lost motility and their flagellum. The process of differentiation took between five and seven days to complete. The cultured amastigotes appeared by light microscopy to be smaller and rounder than the elongated promastigotes and tended to bind in clusters. They would grow and proliferate in culture

with high viability for up to a month as shown by meeasuring their internal FDA esterase (Jackson & Papas 1985). The starting material for axenic culture amastigotes was amastigotes obtained from a lesion from an infected mouse or from culture promastigotes. The culture promastigotes used had been usually frozen shortly after passage through a mouse and had not been in culture for long periods of time.

To compare the pattern of surface proteins from cultured promastigotes and amastigotes, cells were washed and labeled with Biotin-X-NH_2. This reagent labels primary amine groups in the polypeptides exposed on the surface of cells. The pattern of surface label provided a good approximation to the relative abundance of the surface proteins, given the fairly homogeneous distribution of the lysines on proteins. The pattern of surface labeled proteins of log-phase promastigotes, stationary-phase promastigotes and amastigotes is shown in figure 5. It was clear that the surface proteins of amastigotes (lanes 3 and 6) differed from that of promastigotes (lanes 1 and 4) as judged by this method, with some of the most intense promastigote bands not present in the amastigote. Lanes 7 to 9 corresponded to the major surface proteinase GP63. It was detected with monoclonal antibody (mAb) 235 that was raised against *L. major* promastigote GP63 but that cross-reacted with *L. mexicana* GP63. The amastigote GP63 (lane 9) was less abundant and of a larger apparent molecular weight than the promastigote GP63 (lane 7). The change in size of the GP63 bands between promastigote and amastigote corresponded to that reported *for L. mexicana* amastigotes purified from mouse lesions (Frommel *et al.* 1990).

3.1.2.- Monoclonal antibodies against surface proteins of *L. mexicana* amastigotes:

Monoclonal antibodies were prepared to identify amastigote-specific surface proteins. BALB/c mice were immunized with axenic culture amastigotes in Freund's complete adjuvant. The animals developed antibodies against the surface of both promastigotes and amastigotes as tested by flow cytometry. A fusion was performed using spleen cells from two immunized animals and X63 Ag8-653 myeloma cells. The hybridoma cells were cloned and the supernatants of the clones assayed by flow cytometry on intact axenic amastigotes.

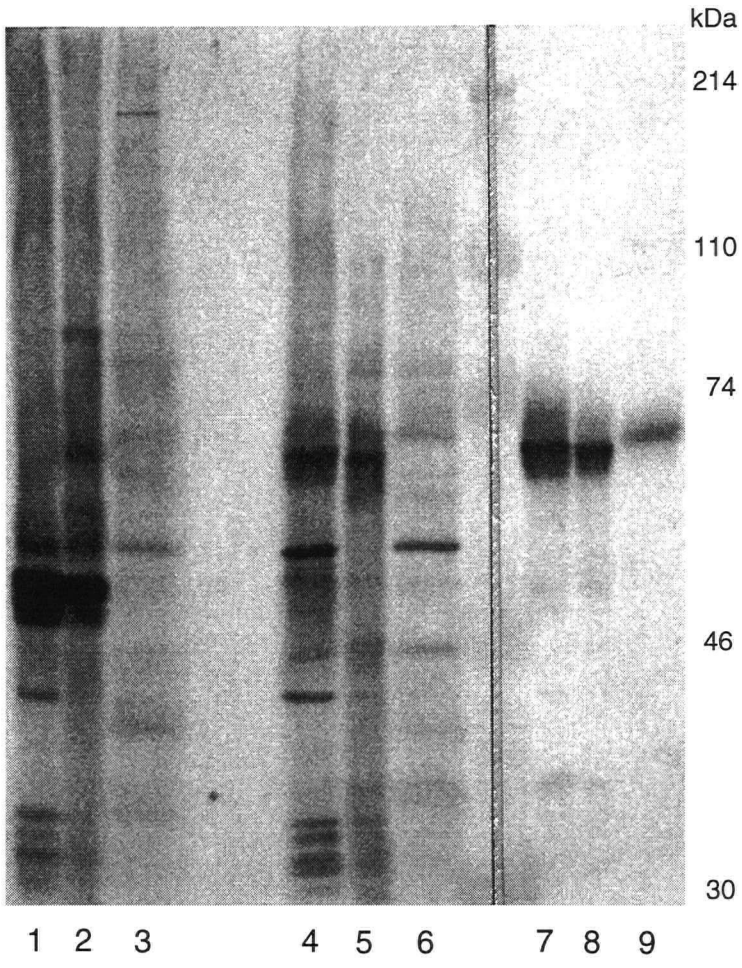


FIGURE 5

BIOTINYLATION OF PROTEINS ON THE SURFACE OF THE PROMASTIGOTE AND AMASTIGOTE STAGES OF *Leishmania*

Axenic culture promastigotes and amastigotes of Leishmania mexicana were surface labeled with Biotin-X-NH2. Detergent solubilized cell extracts were fractionated by SDS-PAGE and transferred to a membrane. Surface labeled proteins were visualized by a chromogenic reaction with enzyme-linked Streptoavidin (lanes 1-6). The major surface proteinase GP63 was detected by protein immunoblot with a monoclonal antibody (lanes 7-9). Lanes 1-3: non-reduced proteins. Lanes 4-9: proteins reduced with DTT. Lanes 1, 4 and 7: Log phase promastigotes. Lanes 2, 5 and 8: stationary phase promastigotes. Lanes 3, 6 and 9: amastigotes.

This screening procedure was designed to select mAbs that recognized surface antigens. More than four hundred hybridoma clones were tested and the thirty positives were re-tested versus amastigotes and promastigotes of both *L. mexicana* and *L. major* to assess if they were stage and species specific. The screening produced four mAbs: 13F2, 11F2, 16F2 and 13C10 that were specific for *L. mexicana* amastigotes. The flow cytometric analysis of the binding of one of these amastigote-specific mAb, 13F2 is shown in figure 6. The mAbs 11F2 and 16F2 showed identical binding patterns to that of 13F2 while the binding of mAb 13C10 was less intense. Two other mAbs, 12G6 and 14F11, bound to both *L. mexicana* amastigotes and promastigotes, as well as to *L. major* promastigotes. Mab 12G6 and 14F11 produced identical flow cytometric binding patterns.

The binding of mAb 13F2 to the surface of *L. mexicana* during the process of transformation from promastigotes to amastigotes in culture is shown in figure 7. The binding of mAb 13F2 increased as the morphology typical of the amastigotes was established, a process that took five to seven days to complete.

The supernatants of these mAb were incubated with amastigotes growing in culture in concentrations that saturated the cells for flow cytometry. None of them had any effect on cell viability or rate of division in culture. Presumably these mAb did not interfere with a necessary function of the amastigotes, such as nutrient uptake, at least under axenic culture conditions.

3.1.3.- Identification of the surface polypeptides bound by the monoclonal antibodies:

In order to identify the antigens recognized by the mAb, the amastigotes were surface biotinylated and a detergent extract prepared. The IgG of the mAb supernatant was bound by Protein-G sepharose beads. The biotinylated extract was incubated with the IgG-beads and washed extensively by centrifugation. The surface antigen absorbed to them was visualized by SDS-PAGE and blotting with streptavidin-alkaline phosphatase and substrate.

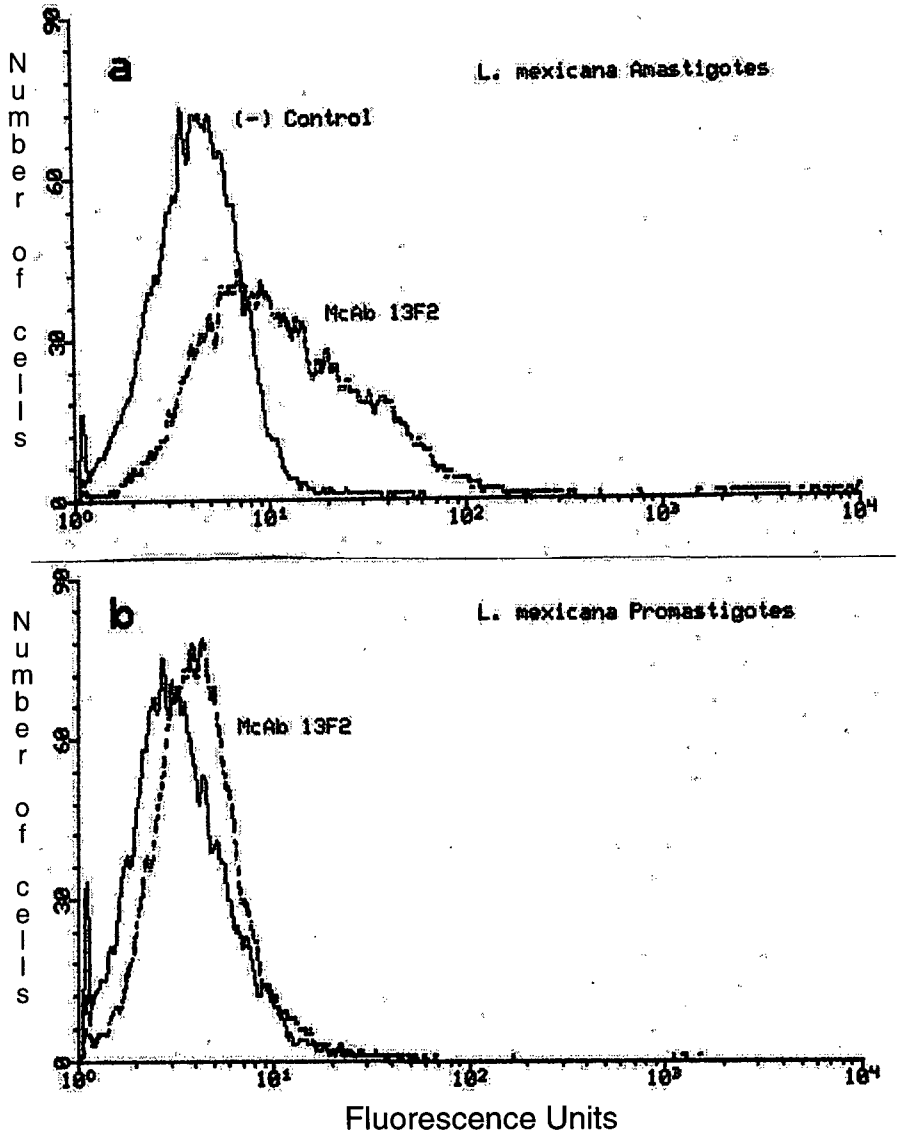


FIGURE 6

BINDING OF MONOCLONAL ANTIBODY 13F2 TO *Leishmania*

Flow cytometric analysis of the binding of monoclonal antibody 13F2 to the surface of *L. mexicana* culture amastigotes (a) or promastigotes (b).

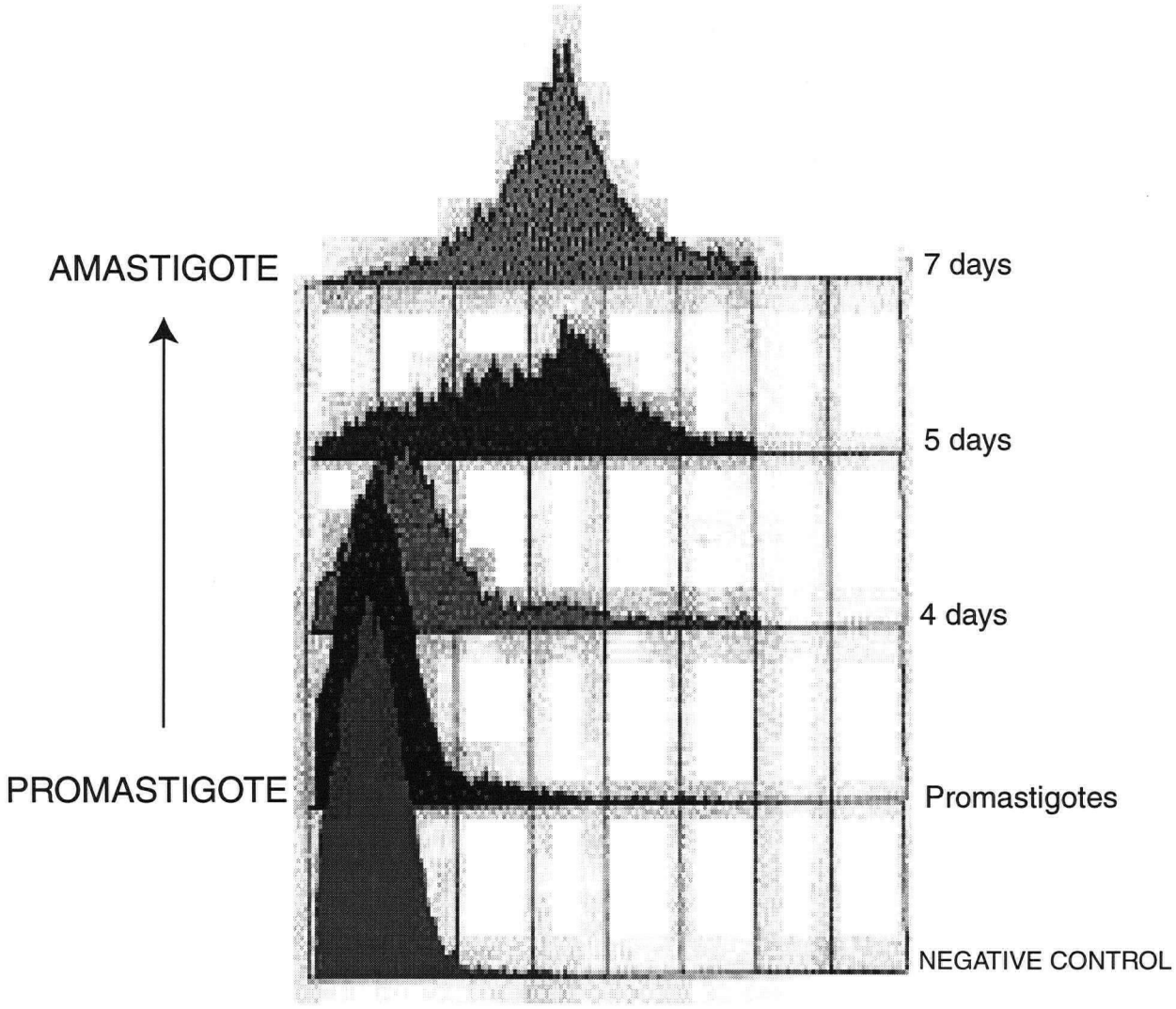


FIGURE 7

BINDING OF MONOCLONAL ANTIBODY 13F2 TO *Leishmania* DURING DIFFERENTIATION FROM PROMASTIGOTE TO AMASTIGOTE IN CULTURE

Flow cytometric analysis of the binding of monoclonal antibody 13F2 to the surface of *Leishmania mexicana* during differentiation from promastigotes to amastigotes in culture. Promastigotes differentiate to amastigotes after increasing culture temperature from 26 C to 32 C and by gradually decreasing the pH from 7.2 to 5.5, conditions that resemble those of the amastigote in the phagolysosome of the infected macrophage. The period in days after the shift in conditions is indicated in the figure. Negative control: No monoclonal antibody, just binding buffer.

The pattern of surface biotinylated polypeptides of amastigotes is shown in figure 8a, lane 1. In lane 2 the polypeptides absorbed to mAb 12G6 IgG, consisting mainly of an intense band of approximately 65 kDa, are shown. The same pattern was observed with mAb 14F11 (data not shown). The polypeptides absorbed by mAb 13F2 (shown in lane 3) corresponded to three bands, of approximately 110, 86 and 70 kDa, the most intense band being the 70 kDa one. The three polypeptides were tightly bound as they were co-precipitated, even after extensive washing of the beads. In one experiment, washing with 1 M NaCl produced the same pattern of three polypeptides bound. These same polypeptides were absorbed by the other three amastigote-specific mAb 16E2, 11F2 and 13C10. It was of interest that while mAb 13F2, 16E2 and 11F2 had identical flow cytometric profiles of binding to amastigotes, mAb 13C10 bound significantly less. One explanation would be that mAb 13C10 recognized a lower number of epitopes in the surface of the parasites than the other three mAb. It is possible that mAb 13F2, 16E2 and 11F2 bound to the same epitope or to different epitopes in the same polypeptide, while mAb 13C10 bound to a different polypeptide within the protein complex. Unfortunately, none of these mAb recognized their corresponding polypeptides in Western blots, probably because they were raised against native proteins, so a direct test of this hypothesis was not possible. An alternative explanation is that mAb 13C10 had a significantly lower affinity for the same epitope. This however seems unlikely in view of the immuno-absorption of the complex even after extensive washes.

In conclusion, two different surface polypeptide sets were absorbed, one set by mAb 12G6 and 14F11, with a predominant band of approximately 65 kDa (figure 8b, lane 1) and a different set of approximately 70, 86 and 110 kDa (figure 8b, lane 2) by mAb 13F2, 16E2, 11F2 and 13C10. Taken together, these 2 groups of mAb defined a large proportion of the Biotin-X-NH_2 surface labeled polypeptides of the axenic culture amastigotes (figure 8a, lane 1). Furthermore, these surface polypeptides appear to be immunodominant as no other mAbs were found that bound intact amastigotes after extensive screening. It is possible that other relatively abundant surface polypeptides are present as some polypeptides might not label with Biotin-X-NH_2, and could be revealed with other labeling procedures.

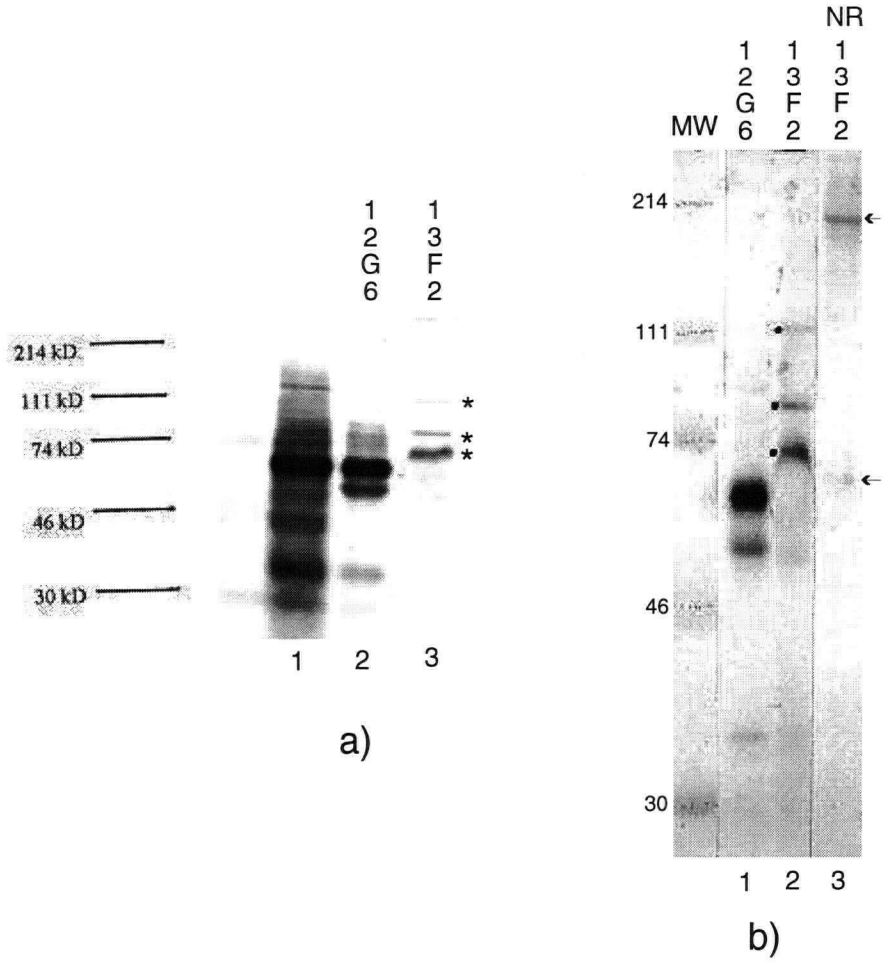


FIGURE 8

IMMUNOABSORPTION OF SURFACE LABELED POLYPEPTIDES FROM *L. mexicana* AMASTIGOTES

a) *L. mexicana* axenic culture amastigotes were labeled with Biotin-X-NH2 and solubilized with the detergent Zwittergent 3-14. The extract was incubated with protein G-agarose beads loaded wirh monoclonal antibody (mAb) 12G6 (lane 2) or mAb 13F2 (lane 3). After washing, the beads were boiled in the presence of SDS and DTT and separated by SDS-PAGE. The gel was transferred to a membrane and developed with Streptavidin-alkaline phosphatase and substrate to visualize surface labeled bands. In lane 1, the surface labeled extract (Biotin-amastigote) was loaded. b) Higher resolution SDS-PAGE. Lane 1: Biotinylated polypeptides absorbed by mAb 12G6. Lanes 2 and 3: Biotinylated polypeptides absorbed by mAb 13F2, lane 2 shows reduced polypeptides and lane 3 shows non-reduced ones (NR).

The polypeptides bound by mAb 13F2 were analyzed under non-reducing conditions (Figure 8b, lane 3). This pattern was different than that of the mAb 13F2 bound polypeptides analyzed under reducing conditions (lane 2). The non-reduced polypeptides presented two major bands at approximately 200 and 65 kDa (shown by arrows in figure 8b, lane 3), quite different from the major reduced band of approximately 70 kDa (figure 8b, lane 2). This result suggested the presence of disulfide bonds both between polypeptide chains as well as within the chains, possibly explaining the strong association between the three polypeptides that the mAb co-absorb.

3.1.4.- Search for potential disulfide bonds between the polypeptides bound by mAb 13F2:

To test the hypothesis that disulfide bonds between the polypeptides were responsible for the strong association of the three polypeptides co-absorbed by mAb 13F2, 16E2, 11F2 and 13C10, a two-dimensional gel electrophoresis analysis was performed.

The procedure consisted of first separating a mixture of polypeptides by SDS-PAGE under non-reducing conditions. Then a vertical strip of gel was cut, reduced by incubation in a solution containing DTT, laid horizontally on top of a second gel and electrophoresed in the second dimension. Polypeptides not containing disulfide bonds would be seen as spots in an imaginary 45 degree diagonal line. Polypeptides having bonds between chains would be seen in a vertical line below the diagonal, a single spot if identical monomers were linked (an example is mammalian transferrin receptor) and more than one spot if it was a hetero-complex (an example is mammalian insulin receptor). Polypeptides having internal bonds within the chain, as for example *Leishmania* GP63, will be seen above the diagonal.

The procedure was performed on the surface labeled polypeptides bound by mAb 13F2 IgG-beads as shown in figure 9. A strip of the first dimension SDS-PAGE gel containing the non-reduced polypeptides and identical to that shown in lane 1, was cut, incubated with the reducing agent DTT and electrophoresed in the second dimension with the result shown in figure 9b. It was observed that the major component of the 13F2 surface complex, the 70 kDa polypeptide was indeed linked by disulfide bonds between the chains, being present in a mix of monomers, dimers, trimers and tetramers, the most abundant being monomers and tetramers. These disulfide bonds were not generated after parasite lysis as it was performed

in the presence of the alkylating reagent Iodoacetamide, which should have modified all the available sulfhydryl groups in cysteine residues. A second observation was that the 70 kDa monomers themselves had internal disulfide bonds. Additional observations were that the 86 kDa polypeptide appeared to be a disulfide bound homo-dimer and that the 110 kDa polypeptide appeared to have internal disulfide bonds.

It was concluded that the other polypeptides of the surface complex, the 86 and 110 kDa polypeptides, were not bound to the 70 kDa polypeptide by disulfide bonds. The strong association between the three different components of the complex must be explained therefore by non-covalent interactions.

3.1.5.- Purification of the 13F2 bound polypeptides:

In order to purify the 13F2 antigen by affinity chromatography, purified mAb 13F2 IgG was bound to Sepharose 4B. Detergent extracts from 2.3 and 3.6 x 10^{10} axenic culture amastigotes were passed through the 13F3 IgG-Sepharose column. The eluted protein was concentrated, electrophoresed, transferred to a membrane and stained (figure 10a). Bands corresponding in size to the surface labeled polypeptides of 70, 86 and 110 kDa were observed and were indicated by arrowheads in figure 10. Other lower molecular weight bands did not correspond to surface labeled ones and could be degradation products. The 70 kDa bands were excised and subjected to N-terminal sequencing (Matsudaira 1987). Two independent sequencing reactions were performed and the consensus sequence of eighteen amino acids corresponding to the amino terminus of the 70 kDa polypeptide is presented in figure 11. This sequence should correspond to the amino terminus of the surface polypeptide after a signal peptide was removed.

FIGURE 9

TWO DIMENSIONAL GEL ANALYSIS OF DISULFIDE BRIDGES IN THE ANTIGEN RECOGNIZED BY mAb 13F2

ONE DIMENSIONAL SDS-PAGE

Surface-biotinylated *Leishmania mexicana* protein extracts were electrophoresed in an 8% polyacrylamide gel. A vertical strip of gel that was an identical duplicate of that shown in lane 1 was excised and used for the second dimension electrophoresis. The rest of the gel was electro-transferred to a membrane and surface labeled polypeptides were visualized by blotting with streptavidin-alkaline phosphatase and developing with BCIP/NBT. Lanes 1 to 4: Non reduced proteins. Lanes 6 to 9: Polypeptides reduced with DTT. Lanes 1 and 6: Biotin-labeled amastigote polypeptides absorbed with mAb 13F2: extract from 1 x 10^8 labeled amastigotes was absorbed. Lanes 2 and 7: Biotin-amastigote polypeptides absorbed with mAb 12G6, extract from 0.5 x 10^8 labeled amastigotes was absorbed. Lanes 3, 4, 8 and 9: Surface labeled Biotin-amastigote extract, the equivalent of 8.25 x 10^6 amastigotes was loaded per well.

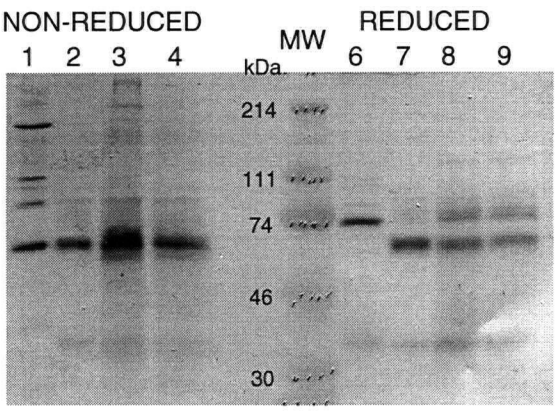
SECOND DIMENSION ELECTROPHORESIS

The vertical strip of first dimension polyacrylamide gel containing the electrophoresed mAb 13F2 absorbed non-reduced polypeptides, was incubated for 15 min by rolling in a solution containing 50 mM DTT, 2% SDS, 0.125 M Tris HCl pH 6.8, 20% Glycerol and BPB at room temperature. The reduced strip was then lowered horizontally on top of a SDS-PAGE stacking gel and covered with SDS running buffer. The separating gel was 8% polyacrylamide and of the same length as the first dimension gel. After electrophoresis, the gel was transferred to a membrane and developed with SA-AP. Photographs of the strips corresponding to lanes 1 and 6 of the one-dimensional gel blot were attached to the photograph of the corresponding portions of the second dimensional gel blot. In the bottom of the figure, a diagonal line was drawn to indicate the position of spots that did not contain disulfide bonds.

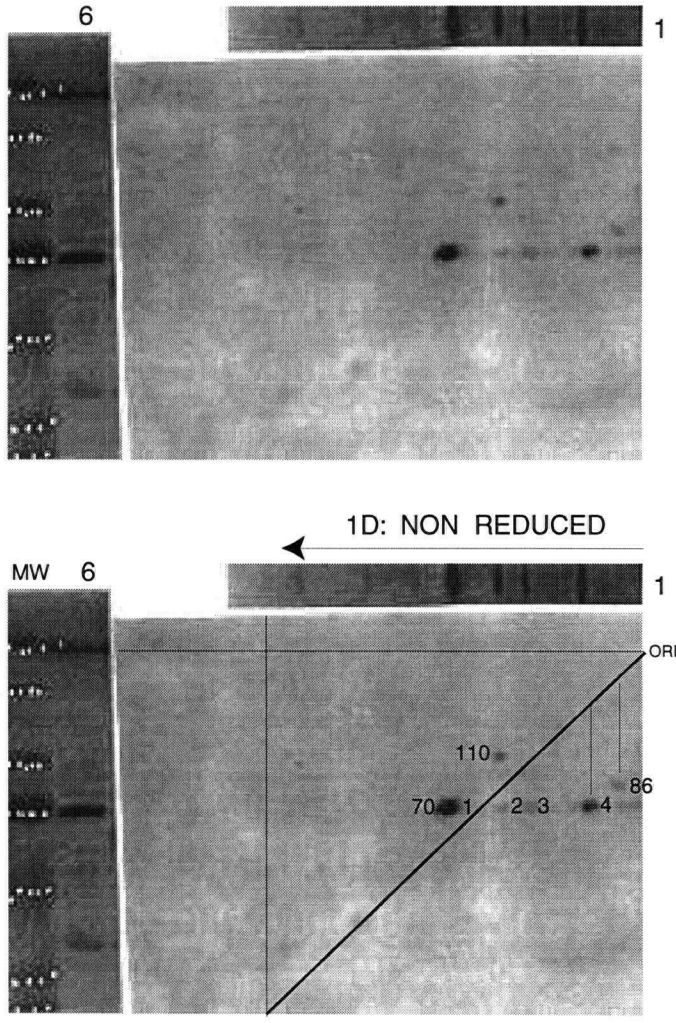
FIGURE 9

TWO DIMENSIONAL GEL ANALYSIS OF DISULFIDE BRIDGES IN 13F2 ANTIGEN

a)



b)



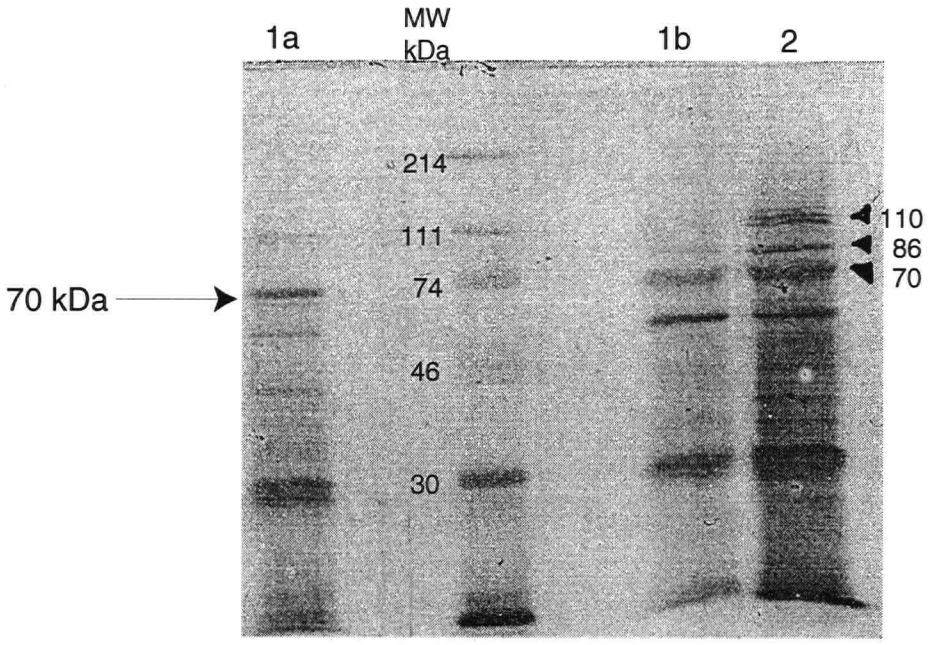


FIGURE 10

PURIFICATION OF THE 13F2 ANTIGEN BY AFFINITY CHROMATOGRAPHY

A 13F2 IgG-Sepharose column was used to purify its antigen from detergent extracts of Leishmania mexicana amastigotes. The eluted protein was separated by gel electrophoresis and transferred to the membrane shown for N-terminal protein sequencing of the major band of 70 kDa. Lanes 1a and 2 were from two independent preparations. Lane 1b was from a second loading of the affinity chromatography column with the passthrough of the same preparation shown in Lane 1a. The 70 kDa band, indicated by the arrow, was excised from the Coomassie blue stained membrane for sequencing. Two independent sequencing procedures were performed, one with the 70 kDa band from preparation 1a and the other from preparations 1b and 2. Arrowheads indicated the 110, 86 and 70 kDa bands that corresponded to the immunoabsorbed surface labeled ones. Other lower molecular weight bands did not correspond to surface labeled ones and could be cross-reacting proteins.

3.1.6.- Approach to the identification of the gene coding for the 70 kDa polypeptide:

A systematic effort was made to clone the gene coding for mAb 13F2-70 kDa antigen. The approach is illustrated in figure 11. *Leishmania* has a preference for G or C in the third bases of codons. Based on a table of *Leishmania* codon usage (Langford *et al.* 1992) the approximate DNA sequence coding the N-terminal amino acids of the 70 kDa polypeptide was predicted. The primer JB-1, corresponding to the predicted DNA sequence for amino acids eight to fourteen, was designed to be paired with the SL primer, based on the spliced leader present on the 5' end of every *Leishmania* mature mRNA, to perform the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The expectation was that a small PCR product comprising of about 100 to 200 bp of the gene's 5' UTR plus the sequence corresponding to the first seven N-terminal amino acids would be detected. This band was expected to be more abundant when the template was amastigote RNA than when it was promastigote RNA, reflecting the difference of abundance of its protein product in the surface of *Leishmania* of those stages, as seen by differential binding of mAb 13F2 (see figure 6). A third expectation was that the the PCR product would code for the first seven amino acids of the 70 kDa polypeptide, corresponding with the data of the N-terminal protein sequencing (figure 11), providing proof that the correct sequence had been amplified by the JB-1 and SL primers.

When the experiment was performed, a discrete set of bands was seen with both amastigote and promastigote RNA templates. There was no band that was apparently amastigote-specific or enriched. Nevertheless, all discrete bands were excised from the gel, purified, cloned and sequenced. None of the sequences corresponded to the expected protein sequence. The most abundant PCR product, a 400 bp fragment was found to be homologous to a published *Leishmania* gene sequence, *L. enriettii* H2B histone (Genske *et al.* 1990), and upon examination, a sequence homology was found for the 3' end of primer JB-1.

Variations were performed in the RT-PCR approach such as designing a new primer, JB-2 that overlapped the JB-1 primer to perform nested PCR. Additionally, the annealing temperature was increased. These variations produced new sets of PCR products but in no case was there a significant difference between the amastigote and the promastigote RNA generated sets. In total the clones from twenty three different PCR products were sequenced

FIGURE 11

GENE CLONING STRATEGY

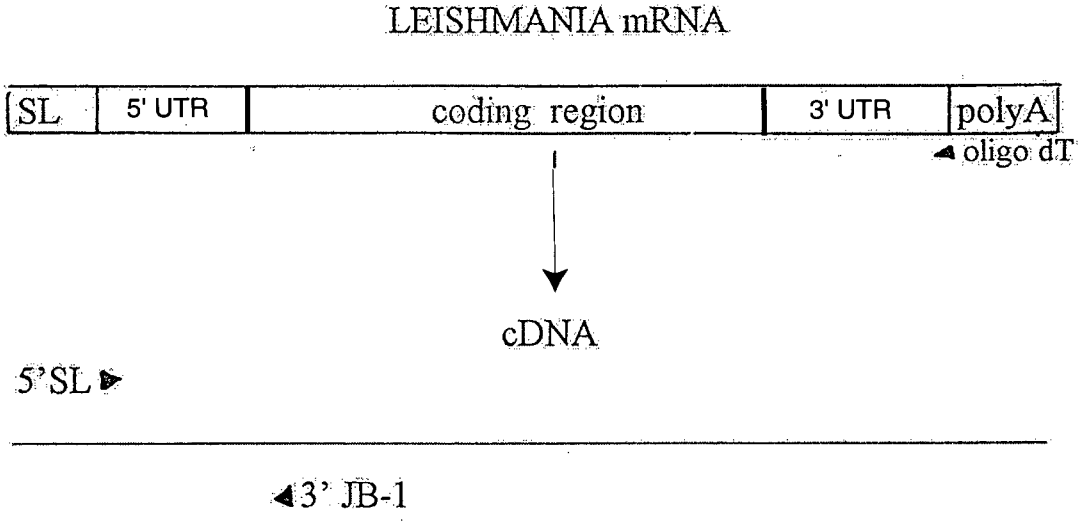
PCR amplification, cloning and sequencing of PCR products.

AMINO TERMINUS SEQUENCE OF 70,000 Da POLYPEPTIDE

Gln Pro Gln Pro Cys Tyr Asp Pro Ala Glu Arg Gln Gly Thr Cys Cys Lys Phe

CAG CCG CAG CCG TGC TAC GAC CCG GCG GAG CGC CAG GGC ACG TGC TGC AAG TTC

3' GGC CGC CTC GCG GTC CCG TG
JB-1 PRIMER



5' SL primer based on splice leader sequences from several Leishmania species.
3' JB-1 primer based on table of prefered codon usage in Leishmania.

without obtaining the expected sequence. Several PCR products generated by different conditions produced the same sequence and the approach was deemed to have reached the end of its usefulness for this particular sequence.

An alternative approach to cloning the gene coding the 70 kDa polypeptide was to use the 54 bp of the predicted sequence as a probe to screen a genomic DNA library of *L. mexicana*. Two positive colonies were obtained but upon sequencing, none was found to code for the known N-terminal protein sequence.

3.1.7.- Monoclonal antibodies 12G6 and 14F11 were directed against GP63:

The major polypeptide bound by mAb 12G6 and 14F11 corresponded to the major labeled band on the surface of amastigotes. That fact and its apparent molecular mass of approximately 65 kDa (see figure 8) led to the hypothesis that its antigen was the metalloproteinase GP63. This hypothesis was proven by showing that mAb 12G6 and 14F11 bound *L. major* GP63 (figure 12a, lanes 5, 6, 15 and 16). Furthermore, the antigen bound by 14F11 in *L. mexicana* promastigotes and amastigotes was shown to cross-react with a mAb raised against *L. major* GP63 (see figure 12a, lanes 7 and 8).

The antigen bound by 14F11 in *L. mexicana* amastigotes was a glycoprotein, whose apparent molecular weight decreased upon digestion with the enzymes Endoglycosidase F and N-glycosidase F as shown in figure 12b, in the same manner as observed in GP63 (Chang *et al.* 1986).

The metabolic labeling of culture amastigotes, followed by absorption to mAb 12G6 shown in figure 12c, proved that its antigen was indeed produced by *Leishmania*. The shift in apparent molecular weight upon reduction is characteristic of GP63 which has internal disulfide bonds.

3.1.8.- The surface GP63 differed from the intracellular GP63 of *L. mexicana* amastigotes:

A difference was revealed when comparing surface labeled *L. mexicana* amastigote GP63 with total cellular GP63. In the Western blot that labels all the GP63 both surface and internal, the shift in apparent molecular weight upon reduction was as expected with the non-reduced GP63 showing a lower apparent molecular weight (figure 12e, lanes 8 and 5). That

was not the case for the surface labeled GP63 with no apparent shift (lanes 1 and 4) and its molecular weight appears to be lower than that of the total GP63 (lanes 4 and 5). The surface GP63 may be either the product of a different *gp63* gene or may be differentially processed. Most of the amastigote GP63, as seen by Western blot, was of a size that did not correspond with the surface-labeled GP63.

FIGURE 12

MONOCLONAL ANTIBODIES 12G6 AND 14F11 ARE DIRECTED AGAINST GP63

a) Protein immunoblot with mAb against GP63

Monoclonal antibodies used for binding GP63 to protein-G Sepharose beads:
mAb 96, an antibody raised against native *L. major* GP63 that did not recognize *L. mexicana* GP63, mAb 12G6 and 14F11.
Monoclonal antibodies used for protein immunoblot (Western blot):
mAb 235 and 139 raised against recombinant *L. major* GP63-1. mAb 235 cross-reacted with *L. mexicana* GP63 while mAb 139 did not produce a significant cross-reaction in a previous experiment.

Detergent (Zwittergen 3-14) extracts of *L. major* A2 promastigotes and *L. mexicana* promastigotes and amastigotes were absorbed to mAb Ig bound to protein G beads. After washing, the beads were boiled in SDS sample buffer containing DTT. The samples were separated by SDS-PAGE and transferred to a membrane. Immunoblots were performed by incubating membranes with mAb 235 or 139, washing and incubating with goat anti-mouse Ig–alkaline phosphatase (GAM-AP). The blots were then developed with the chromogenic AP substrate BCIP-NBT. Lanes 1, 2, 3, 11, 12, 13 show protein extracts from *L. major* promastigotes (lanes 1 and 11), *L. mexicana* promastigotes (lanes 2 and 12) and *L. mexicana* amastigotes (lanes 3 and 13). Lanes 4 and 14: *L. major* promastigote extract absorbed by mAb 96. Lanes 5 and 15: *L. major* promastigote extract absorbed by mAb 14F11. Lanes 6 and 16: *L. major* promastigote extract absorbed by mAb 12G6. Lane 7: *L. mexicana* promastigote extract absorbed by mAb 14F11. Lane 8: *L. mexicana* amastigote extract absorbed by mAb 14F11. Lanes 9 and 10 show control protein-G beads containing mAb 14F11 and mAb 96 respectively, boiled in SDS/DTT sample buffer to show the position of the heavy chain IgG detected by GAM.

b) EndoF treatment

Axenic culture *L. mexicana* amastigotes were surface labeled with Biotin-X-NH_2 and absorbed to mAb 14F11 IgG bound to protein G beads. After washing, the sample was

boiled and then incubated with Endo F (Boehringer Mannheim's Endoglycosidase F/N-GlycosidaseF) for 26 hours at 37°C. The sample was then subjected to SDS-PAGE, transferred to a membrane and developed with SA-AP to visualize surface labeled polypeptides.

c) Metabolic label

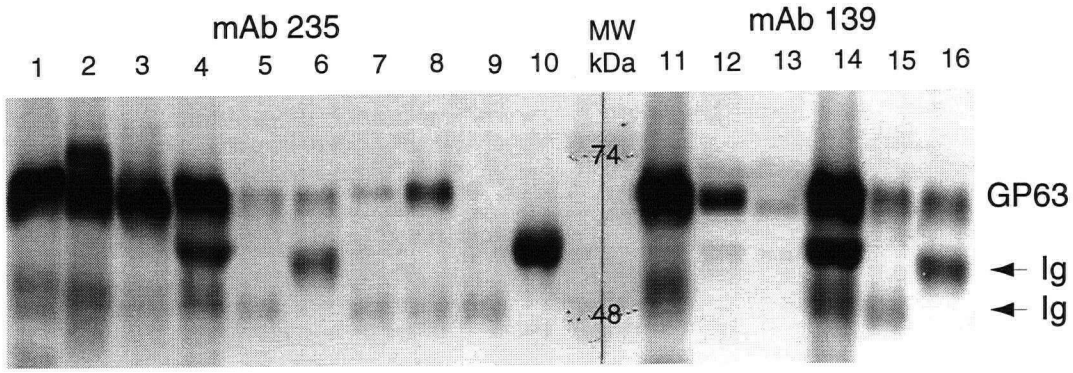
An axenic culture of *L. mexicana* amastigotes was grown overnight in a medium lacking methionine and cysteine and supplemented with dialysed serum and containing ^{35}S-methionine. The culture was then washed and lysed with a solution containing the detergent Swittergen 3-14 and protease inhibitors. The extract was absorbed to mAb 12G6 IgG-protein G-beads and separated by SDS-PAGE. The gel was dried and exposed for three weeks. The autoradiography is shown in figure 12c. NR was a non-reduced sample, R was reduced while C was a negative control: the labeled extract was absorbed by mAb 96, that does not bind *L. mexicana* GP63.

d) Surface label vs. Immunoblot

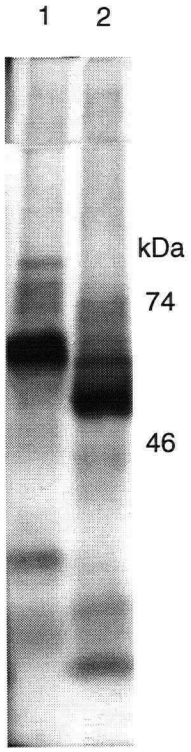
Lanes 1 to 4: blot developed with SA-AP for surface-labeled protein visualization. Lanes 5 to 8: immunoblot with mAb 235 and GAM-AP to reveal GP-63. Lanes 1 and 8: non-reduced biotinylated amastigote extract. Lane 2: non-reduced biotinylated amastigote extract absorbed by mAb 12G6 IgG-beads. Lanes 3 and 5: reduced biotinylated amastigote extract. Lanes 4 and 6: reduced biotinylated amastigote extract absorbed by mAb 12G6 IgG-beads. Lane 7: control, mAb 12G6 IgG-beads boiled with SDS and DTT to show the heavy chain IgG (indicated by * in the figure) labeled by GAM-AP.
G indicates GP63 in the figure.

FIGURE 12

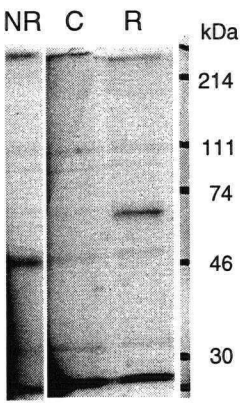
MONOCLONAL ANTIBODIES 12G6 AND 14F11 ARE DIRECTED AGAINST GP63



a) Protein Immunoblot with monoclonal antibodies against GP63

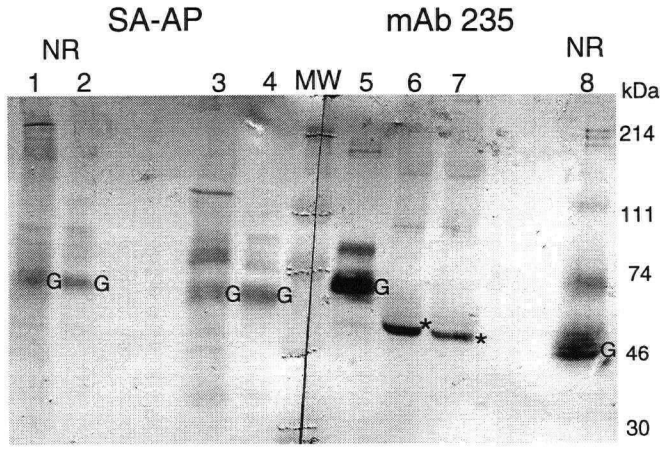


b) EndoF treatment



c) Metabolic label

C: Control
NR: Non Reduced
R: Reduced



d) Surface label vs Immunoblot

* Ig Heavy Chain G GP63

3.2.- DISCUSSION

In order to identify amastigote surface proteins, monoclonal antibodies were produced by immunising BALB/c mice with *L. mexicana* amastigotes grown in axenic culture (Bates, P.A. *et al* 1992). Hybridoma clones secreting monoclonal antibodies against surface antigens were selected by flow cytometry on intact amastigotes. Amastigote surface antigens were identified by immunoprecipitation of biotin surface-labeled proteins. Four monoclonal antibodies reacted with a novel surface protein complex that consisted of three polypeptides of apparent molecular masses of 110, 86 and 70 kDa (see figure 8). Although the three polypeptides co-immunoprecipitated, they were not covalently bound. The most abundant polypeptide in the protein complex was the 70 kDa one. It was present in the protein complex as monomers or as disulfide bound dimers, trimers and tetramers (see figure 9). The protein complex appeared to be amastigote-specific as it was not detected on the surface of *L. mexicana* promastigotes (see figure 6). The protein complex appeared in the surface of cultured *Leishmania* when they differentiated from promastigotes to amastigotes (see figure 7). These polypeptides were purified by affinity chromatography (see figure 10) and an eighteen amino acid N-terminal amino acid sequence of the major 70 kDa polypeptide was obtained (see figure 11). Comparison of that sequence with those in the current databases showed two potential related sequences. The first was with human granzyme A precursor, also known as cytotoxic T-lymphocyte proteinase 1 or Hanukah factor serine protease precursor, a protease found as disulfide-linked homodimers in cytoplasmic granules (Gershenfeld *et al.* 1988). The identity was 75% (9/12) and positives 83% (10/12):

13F2 p70	2 PQPCYDPAERQG	13
	P PCYDPA R+G	
Granzyme A	102 PYPCYDPATREG	113

The N-terminal sequence of the 70 kDa polypeptide should correspond to its amino terminus after removal of a signal peptide. In the granzyme A precursor, the first 28 amino acids correspond to the signal peptide and an activation peptide, the active enzyme starting at amino acid 29. The region of similarity between the two proteins therefore does not appear to correspond to similar domains.

The second similarity was with murine TNF receptor associated factor 1 (TRAF1), a signal transducer associated with the cytoplasmic domain of the tumor necrosis factor receptor. It

can be present as homodimers or heterodimers of TRAF1 and TRAF2. The heterocomplex can bind to the N-terminal of inhibitor of apoptosis proteins 1 and 2, to recruit them to the tumor necrosis factor receptor 2 (Rothe *et al.* 1994). The identity was 53% (8/15) and positives 66% (10/15):

13F2 p70	2 PQPCYDPAERQGTCC	16
	P PC DP+E + CC	
TRAF1	18 PAPCQDPSEPRVLCC	113

Given the small portion of the sequence of the 70 kDa polypeptide available, these similarities could be taken only as suggestive.

The unsuccessful attempts to clone the gene coding for the 70 kDa polypeptide might be due to low abundance of its mRNA. The internal primers, based on the protein sequence of the 70 kDa polypeptide, might partially cross-react with more abundant cDNA as was seen by amplification of a cDNA fragment corresponding to the *Leishmania* H2B histone. The gene cloning strategy outlined in figure 11 could probably succeed in the future if the amino acid sequence of a number of internal peptides from the polypeptide were obtained in order to design additional internal primers.

The other mAb generated against the surface of *L. mexicana* amastigotes, mAb 12G6 and 14F11, recognised the metallo-proteinase GP63 (see figure 12) in both amastigotes and promastigotes. GP63 constituted the most abundant surface protein of the amastigotes, as judged by surface biotin label (see figure 8a), although at a lower level than in the surface of promastigotes. Most of the amastigote GP63, as seen by Western blot, was of a size that did not correspond with that of the surface labeled GP63 (see figure 12d), leading to the conclusion that the surface GP63 was only a fraction of the total GP63. This finding correlated well with published reports on *L. mexicana* amastigote GP63. It had been reported that a water-soluble form of GP63 in the amastigotes was mainly located in the megasome (Bahr *et al.* 1993). In another study, most of the GP63, in a form that lacked a GPI anchor, was found in the flagellar pocket, although a small fraction of GP63 could be iodinated on the amastigote cell surface (Medina-Acosta *et al.* 1989).

The surface-labeled GP63 differed from the internal GP63 and all other reported *Leishmania* GP63, in not possessing internal disulfide bonds (see figure 12d). The mAb 12G6 and 14F11 recognized both conformations, that with disulfide bonds in the parasite's surface and that

with internal disulfide bonds in an internal compartment of the parasite. In *L. mexicana*, three *gp63* gene classes have been reported. Gene classes C2 and C3 were promastigote-specific while *gp63* gene class C1 was expressed constitutively. The *L. mexicana* C1 *gp63* gene encoded a predicted protein that had an extended carboxyl terminus and did not possess the GPI anchor addition sequence found in other *gp63* gene products (Medina-Acosta *et al.* 1993). It was not known if the particular surface GP63 of *L. mexicana* amastigotes identified in this study was the product of a particular *gp63* C1 gene or whether the surface bound fraction of the products of the C1 gp63 genes was differentially processed.

The mAb 12G6 and 14F11 bound both culture *L. mexicana* promastigotes and amastigotes with similar flow cytometric binding profiles (data not shown). As *L. mexicana* promastigotes have much more surface GP63 than the amastigotes (see figure 5) it was concluded that these mAb recognized only a fraction or subset of the promastigote surface GP63. One hypothesis was that mAb 12G6 and 14F11 recognized the products of the constitutive C1 *gp63* genes but not those of the promastigote-specific *gp63* C2 and C3 genes. This hypothesis can be tested experimentally by using these mAb to purify *L. mexicana* promastigote and amastigote GP63 by immuno-affinity chromatography. The immuno-absorbed GP63 molecules would then be partially sequenced to determine if they possess the carboxyl terminus characteristic of the C1 genes.

The mAb 12G6 and 14F11 also bound to the surface of *L. major* promastigotes. The flow cytometric analysis showed a positive binding of those mAb, although at a much lower level than the binding of mAb 96, that was generated against native *L. major* promastigote GP63 (data not shown). Furthermore, the heterologous mAb 14F11 and 12G6 immuno-absorbed *L. major* promastigote GP63 (see figure 12a, lanes 5 and 6), but only a fraction of the GP63 absorbed by the homologous mAb 96. It was concluded that these mAb recognized only a fraction or subset of the *L. major* promastigote surface GP63. The promastigotes of *L. major* express the abundant products of the promastigote-specific *gp63* genes 1 to 5 and the product of the constitutive *gp63* gene 6. The mRNA of *gp63* gene 6 is expressed at a lower level than that corresponding to genes 1 to 5 (Voth *et al.* 1998) and presumably the gene 6 product constitutes only a small part of the total GP63 in promastigotes. The sequence of the constitutive *gp63* gene 6 of *L. major* predicts an extended C-terminus, similar to the protein predicted by the constitutively expressed *L. mexicana* C1 and *L. chagasi* C genes (Voth *et al.*

1998; Medina-Acosta *et al.* 1993; Ramamoorthy *et al.* 1992). One hypothesis is that mAb 12G6 and 14F11 recognize the product of the constitutive *gp63* gene 6 but not those of the promastigote-specific gp63 genes 1 to 5. This hypothesis is analogous to that presented above for the *L. mexicana* promastigote C1 *gp63* gene and can be similarly tested experimentally.

The surface GP63 bound by mAb 12G6 and 14F11 was not stage specific. It is not known at present if its special feature of lacking internal disulfide bonds is an amastigote-specific characteristic. If it is determined experimentally that only the amastigotes differentially process its surface bound GP63, then an amastigote-specific function could be proposed. This can be achieved by comparing the biotin surface-labeled GP63 bound by 12G6 IgG-beads with total GP63 in the *L. mexicana* promastigotes, as was done for amastigotes (figure 12d).

The novel polypeptides identified by binding to mAb 13F2 plus the surface GP63 and its degradation product constituted most of the biotin surface-labeled polypeptides observable in the surface of amastigotes. The amastigote-specific protein complex bound by mAb 13F2 is likely to have an amastigote-specific function. Elucidating that function remains a challenge for the future. Possible functions are acquisition of nutrients, receptors for signal transduction, degradation or modification of macrophage molecules in the parasitophorous vacuole and ligands for invasion of other macrophages. During differentiation to the amastigote stage, *Leishmania* down-regulates its surface proteins, probably to minimize leishmanial antigen presentation by the macrophage. The presence of these proteins on the amastigote surface is likely to carry a cost in terms of parasite viability and is it therefore likely that the function of these surface proteins is of importance for the parasite.

4.- IDENTIFICATION OF GENES THAT ARE PREFERENTIALLY EXPRESSED IN AMASTIGOTES

RATIONALE

Leishmania parasites undergo profound morphological and biochemical changes during their life cycle in order to survive and grow in very different environments. The most extreme differences are between the promastigote stage that exists naturally in the insect vector and the amastigote stage that exists naturally as an intracellular parasite of the macrophages in mammalian hosts, including humans. A better understanding of the amastigote stage of *Leishmania* and its relation with its host is needed to better control the human disease Leishmaniasis. Many housekeeping genes should be expressed constitutively while the morphological and biochemical differences between the stages should in large part result from the stage-specific expression of a discrete number of regulated genes. The hypothesis underlying this work was that amastigote-specific proteins are responsible for amastigote-specific functions and furthermore, that amastigote-specific functions and the host responses to them should explain *Leishmania* pathogenesis. A subtraction hybridization procedure was used to identify, isolate and characterize amastigote-specific genes, opening promising avenues of research into their presumably amastigote-specific function.

4.1.- RESULTS

4.1.1.- Selection of amastigote-specific cDNA fragments by subtractive hybridization:

In order to identify genes that are preferentially transcribed in *Leishmania* amastigotes, henceforth referred to as amastigote-specific genes, a cDNA subtractive hybridization procedure was used (Diatchenko *et al.* 1996; Gurskaya *et al.* 1996; Clontech protocol # PT1117-1). The procedure was detailed in the Materials and Methods section and illustrated in figures 2 and 3. The starting material for cDNA synthesis was equal amounts of total RNA prepared from axenic cultures of *L. mexicana* amastigotes (tester cDNA) and promastigotes (driver cDNA). The amastigote and promastigote cDNAs were digested with either RSA I or Hae III, restriction enzymes that recognize a four basepair sequence and produce blunt ends. Both enzymes produced a reduction in the average size of the cDNA fragment population. The amastigote cDNA fragments were divided into two portions and

one half ligated to adaptor 1 and the other to adaptor 2R. The adaptors were single stranded oligonucleotide chains that lacked a terminal phosphate group so that they ligated only to the 5' ends of the amastigote cDNA fragments. The 5' end of both adaptors was identical and the binding site for PCR primer 1. Adaptors 1 and 2R differed in their 3' ends, the binding sites for nested PCR primers 1 and 2R respectively.

The subtractive hybridization procedure consisted of two hybridizations. In the first hybridization a 33-fold excess of digested promastigote cDNA (driver cDNA) with no adaptor was added to each of the two samples of tester cDNA, the amastigote cDNA fragments containing either adaptor 1 or adaptor 2R. Each of the two samples was heat denatured and left to anneal for eight hours at 68°C. The first hybridization led to the formation of type-**a**, -**b**, -**c** and -**d** molecules (see figure 3). The re-annealed double stranded amastigote cDNA type-**b** molecules should correspond mostly to highly expressed constitutive and amastigote-specific genes, as re-annealing should be faster for the more abundant molecules and the hybridization was stopped after 8 hours before re-annealing was completed. This equalization effect should have produced a single stranded type-**a** cDNA fragment population where low and high abundance sequences were similarly represented. The single stranded type-**a** molecules should also be enriched for amastigote-specific sequences, as cDNA from constitutively expressed genes should hybridize with the excess single stranded promastigote driver cDNA (type-**d** molecules) to form the double stranded type-**c** molecules.

After the first hybridization, the two samples, corresponding to adaptors 1 and 2R, were mixed together without denaturing, fresh denatured promastigote driver cDNA was added and the samples were returned to 68°C and left overnight for the second hybridization. This hybridization should have produced a new type of double stranded cDNA hybrid molecules or type-**e** molecules by the hybridization of type-**a** single stranded molecules containing adaptor1 with those containing adaptor 2R (see figure 3) in addition to type-**a**, -**b**, -**c** and -**d** molecules. The type-**e** molecules, that should consist of amastigote-specific equalized sequences, had binding sites for PCR primer-1 at both ends and different binding sites for the nested primers-1 and -2R on their 5' and 3' ends.

The single stranded ends of double stranded cDNA species, corresponding to the adaptors, were filled in by DNA polymerase and PCR was performed using PCR primer-1. The

promastigote type-**d** molecules lacked the primer annealing site and could not be amplified. Type-**a** and -**c** molecules had only one primer annealing site and could not be amplified exponentially. Most type-**b** molecules, which had the same adaptor at both ends, should have formed a pan-like structure that prevented their exponential amplification due to the suppression PCR effect (Siebert *et al.* 1995). Only the amastigote-specific type-**e** molecules, that had two different adaptors, should be amplified exponentially. A second PCR was performed using the nested PCR primers-1 and 2R to reduce background due to possible internal priming of cDNA sequences.

The result of a selection experiment is shown in figure 13. Lanes 2 and 3 show the amplified fragments of unsubtracted amastigote and promastigote cDNA respectively. The subtractive procedure was expected to produce a smear consisting of many amastigote-specific cDNAs but instead a discrete number of defined bands were obtained as seen in lane 4, most of which were of the same size as that of unsubtracted promastigote (lane 2) and amastigote cDNA (lane 3). These bands probably corresponded to fragments of abundant transcripts of constitutively expressed genes. There were a few bands in the subtracted cDNA (lane 4) that did not appear to be present in the unsubtracted samples (lanes 2 and 3), notably a band of about 800 bp and a less intense one of 600 bp, that were named S800 and S600. These unique bands were predicted to correspond to cDNA fragments from amastigote-specific genes.

4.1.2.- Modifications to the subtractive hybridization method:

The method was modified with the aim of obtaining more unique bands, that were assumed to be amastigote-specific. It was hypothesized that common bands predominated because of the abundance of constitutively expressed mRNA, leading to two potential problems: The type-**a** molecules after the first hybridization would probably not be sufficiently enriched for amastigote-specific sequences. Additionally, very high levels of type-**b** double strand cDNA from constitutive genes would probably be formed after the first hybridization, so that the suppression PCR mechanism would not be sufficiently efficient to completely prevent their amplification. This hypothesis led to modifications to the procedure aimed at further enriching the type-**a** population for amastigote-specific sequences and at reducing the concentration of type-**b** molecules after the first hybridization by increasing the concentration

of the hybrid type-**c** molecules. The first modification consisted in increasing the driver (promastigote cDNA) to tester (amastigote cDNA) ratio and the results are shown in figure 13, lanes 5 and 6. A subtractive hybridization was performed on which the concentration of tester cDNA (amastigote) was reduced 10 fold (lane 5) and 100 fold (lane 6) while keeping the concentration of driver cDNA (promastigote) unchanged. This produced an excess of the driver over tester cDNA concentration of 330 and 3300-fold instead of the normal 33-fold excess. The modification was successful for the 10-fold reduction in the tester to driver ratio (lane 5), where some new unique bands are visible, notably an 850 bp one named S850, thus validating the hypothesis. The 100-fold reduction in the tester cDNA appeared to have been excessive as very few bands were apparent and those only after an extra amplification by PCR (lane 6). It is probable that those few bands were the amplification products of the very few templates left and mostly came from common abundant species.

The results of a different modification are shown in lane 7, the purpose of which was again to reduce the concentration of type-**b** molecules. The modification, illustrated in figure 4, consisted of introducing some new steps between the first and the second hybridizations (see figure 3): After the first hybridization, the two cDNA samples were removed from their 68°C incubation and the single stranded ends of the double stranded type-**b** and -**c** molecules, corresponding to the adaptors, were filled by the Klenow DNA Polymerase, thus generating sites for the restriction endonuclease Eag I. Eag I could destroy the primer binding sites in the adaptors of type-**b** and -**c** molecules but could not affect the single stranded adaptors of the type-**a** molecules that contain the amastigote-specific species. The enzyme could also not make internal cuts within the cDNA sequences because it contained in its six base pair target sequence (5' CGGCCG 3') the four base pair target sequence (5' GGCC 3') of the enzyme Hae III, that was used to generate the small size amastigote cDNA fragments before adding the adaptors. After the Eag I digestion, the cDNA was purified without denaturation, fresh denatured driver cDNA was added and the normal procedure re-started at the second hybridization stage (see figures 3 and 4). This modification of the procedure also produced the desired result as is seen in lane 7: the common bands were much reduced both in number and concentration and the most abundant bands appeared to be unique. The two most abundant were S800 and a new band of 300 bp called S300, that appeared to be heterogeneous.

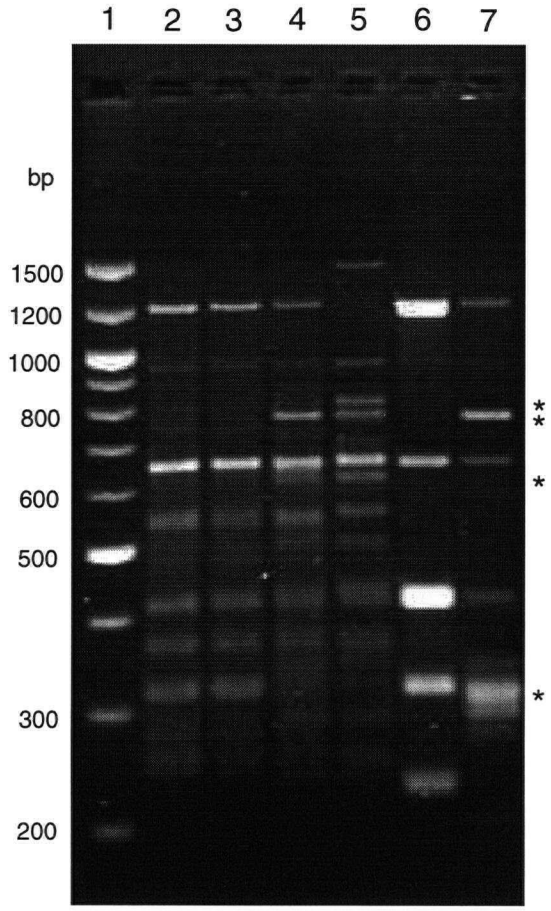


FIGURE 13

AMASTIGOTE SPECIFIC cDNA SELECTION BY SUBTRACTIVE HYBRIDIZATION

Lane 1: DNA molecular weight markers.
Lane 2: Unsubtracted promastigote cDNA.
Lane 3: Unsubtracted amastigote cDNA.
Lane 4: Subtracted cDNA.
Lane 5: Subtracted cDNA obtained by increasing the driver to target cDNA ratio by 10 fold.
Lane 6: Subtracted cDNA obtained by increasing the driver to target cDNA ratio by 100 fold.
Lane 7: Subtracted cDNA obtained by digestion of the adaptors with Eag I.

* Selected amastigote specific cDNA fragments:
From top: S850: present in lane 5 S800: present in lanes 4, 5, 6
S600: present in lanes 4, 5 and S300: present in lane 6

4.1.3.- Screening of clones from subtracted cDNA fragments by Virtual Northern Blots:

Every unique band produced by the subtractive hybridization procedures was cut from the agarose gel, purified and cloned. The inserts from selected clones were labeled with ^{32}P and used as probes in virtual Northern blots. These blots had as target electrophoresed double stranded cDNA, produced by RT- PCR synthesis (see figure 2). As a limited number of amplification cycles were used for the second strand synthesis, the cDNA population was expected to be representative of the mRNA population. The method provided a simple screen for stage-specific cDNA and was performed with equal amounts of amastigote and promastigote cDNA (figure 14a). As a positive control, a probe from the amastigote-specific gene cysteine proteinase-b from *L. mexicana*, Lmcpb (Souza *et al.* 1992; Mottram *et al.* 1997) was used (figure 14b). Fifteen clones, each corresponding to a unique band in a subtraction procedure, were tested by virtual Northern Blots. Eight were shown to be amastigote-specific (see figures 14 and 15). Three other clones gave no detectable signal while the other three showed no significant differences between amastigote and promastigote. The images obtained by phospho-imaging were quantified: the positive control Lmcpb gave an average difference of 20-fold higher concentration in amastigotes. S600 gave 46-fold, S800 an average of 16-fold, S850 gave 11-fold and S300 gave an average of 7-fold for each of its two bands, higher concentration in amastigotes. The clones shown in figure 15 gave smaller differences: R580 gave 1.7-fold, R380 gave 2.9-fold, R420 gave 2.3-fold and R600 gave 2-fold increases in amastigotes. It was decided to concentrate on characterizing the four cDNA species that gave the highest differences: S600, S800, S850 and S300.

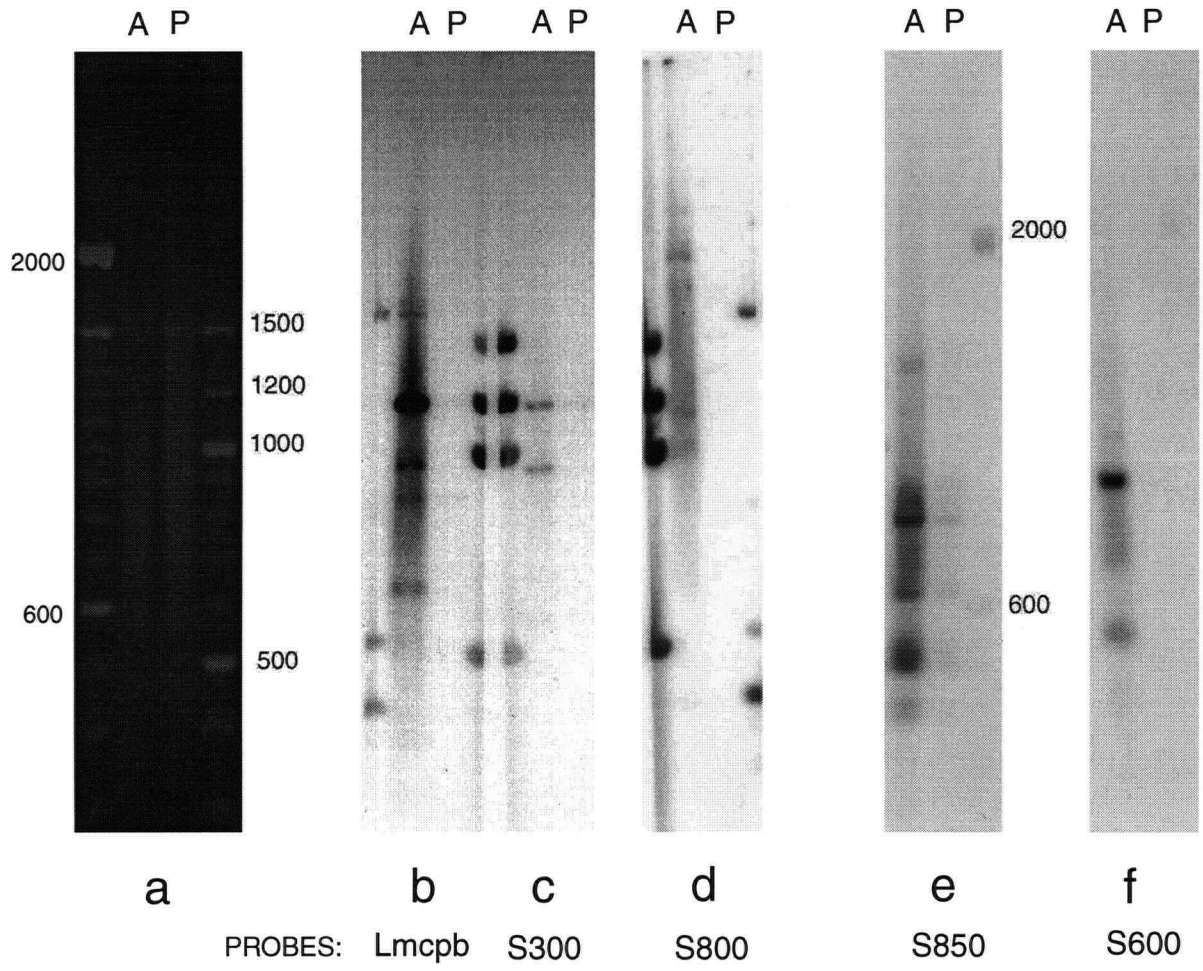


FIGURE 14

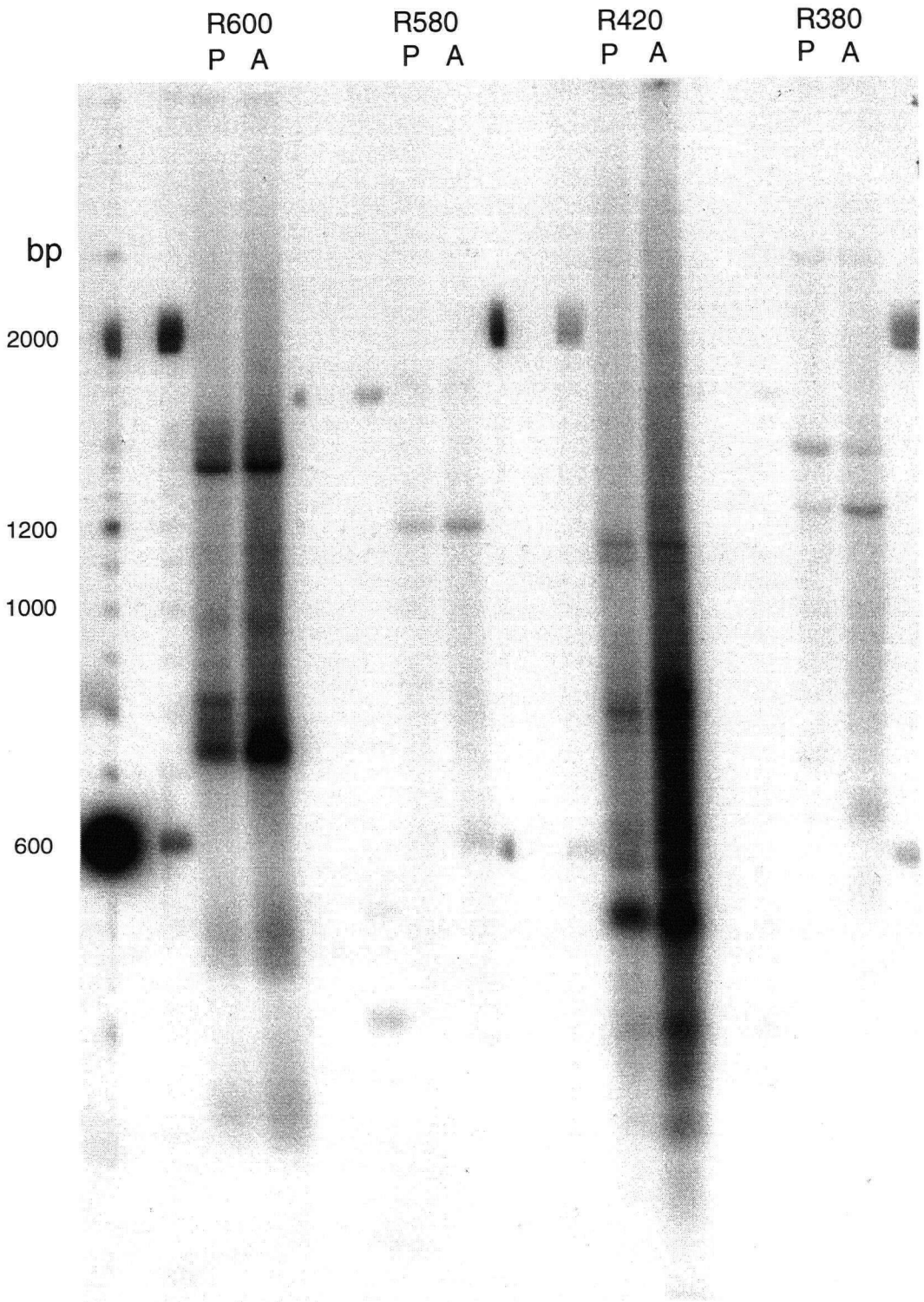
VIRTUAL NORTHERN BLOTS PROBED WITH SUBTRACTED cDNAs

Hybridization of blots containing equal amounts of *L. mexicana* amastigote (A) and promastigote (P) cDNA with radioactively labelled amastigote specific cDNA clones prepared from subtracted cDNA fragments. a) Ethidium Bromide stained agarose gel b) Positive control probe *L. mexicana* cysteine proteinase b (Lmcpb). c) S300 probe. d) S800 probe. e) S850 probe. f) S600 probe.

The labeled bands at each side of the strips correspond to non-specific binding of the probes to the very abundant molecular weight marker bands.

FIGURE 15

VIRTUAL NORTHERN BLOTS PROBED WITH SUBTRACTED cDNAs II



4.1.4.- Characterization of the amastigote-specific cDNA fragments:

In order to validate the results of the virtual Northern blot, Northern or RNA blots were performed and are shown in figure 16. Northern blots were probed with ^{32}P-labeled cloned inserts of S600 (figure 16a) and of a fragment of S850 (figure 16b). Both probes bound to amastigote RNA and not significantly to promastigote RNA. In both cases they produced a labeled band of approximately 3 kbp. When quantified in the phosphoimager, the S600 band was 12-fold and the S850 band 6.5-fold more abundant in amastigote RNA.

Clones of the amastigote-specific cDNA fragments S600, S800, S850 and S300 were sequenced and in all four cases the sequences appeared to be non-coding or untranslated regions (UTR), most probably 3' UTR.

4.1.5.- RT-PCR extension from the amastigote-specific cDNA fragments to the ends of its mRNA:

The sequence of the coding region corresponding to these subtracted UTR could give insight into the function of these amastigote-specific genes. The coding regions were obtained by PCR amplification from the amastigote-specific subtracted fragments to the spliced leader (SL) or mini exon present at the 5' end of all mature mRNA in *Leishmania*. The experimental design is explained in figure 17a. The amplification was successful for S800 (figure 17b and c), S850 (figure 17d) and S600 (figure 17e) and extension cDNA fragments of approximately 1800 bp, 1800 bp and 900 bp respectively were obtained. As expected, in all three cases the amplification product appeared to be more abundant with the amastigote than with the promastigote templates as judged by being detectable with fewer cycles of PCR. All these extension cDNA fragments were sequenced and found to be contiguous to the subtracted cDNA fragments.

The amplification towards the spliced leader failed for S300, even with the use of three different primers. It was successful for S300 in the other direction, towards the Poly A end of the message (see figure 18), where a 350 bp amastigote-enriched band was seen and upon cloning and sequencing, found to be contiguous to S300.

An amastigote enriched band of 800 bp for amplification to the Poly A end was found for S800 and one of 1100 bp for S850. This allowed the estimates of 3 kbp for the full mRNA

corresponding to S800 and of 2.8 kbp for that of S850, both correlating well to the approximate sizes of the single band seen for each in Northern blots (figure 16).

4.1.6.- The amastigote-specific A600 gene coded for a novel polypeptide:

Sequencing the extensions of S600 and S800 towards the spliced leader revealed that both amastigote-specific cDNA fragments corresponded to the 3' UTR of the same mRNA, being two non-contiguous fragments flanked by Hae III sites. The sequence of the mRNA of this amastigote-specific gene, henceforth called A600, is presented in figure 19. The sequence of S600 is underlined and that of S800 is doubly underlined. Min Zhao at our laboratory also sequenced independently most of this mRNA and her sequence confirmed that shown in figure 19.

Three potentially coding open reading frames (ORF) were observed, ORF 1 from base 105 to 386 was rather small but its start codon was located at the usual distance from the trans-splicing site in *Leishmania* genes. ORF 2 and 3 were slightly longer and mostly overlapped in two reading frames, only one of them could be a potential coding sequence. In order to determine which of these ORF was most likely to code for the gene product, they were compared by codon usage, using a table of *Leishmania* codon use (Appendix I). As seen in Table I, in ORF 1 the codons used correlated very well with those used generally in *Leishmania* coding sequences. That was not the case for ORF 2 and 3, that seemed to make random potential use of the codons and were most likely part of the 3'UTR of the A600 gene. It was concluded that ORF 1 was the only one that could code for a *Leishmania* gene.

The predicted polypeptide coded by the amastigote-specific A600 gene, shown in figures 19 and 20, was a 93 amino acid polypeptide corresponding to a molecular weight of 10.46 kDa. Its sequence was not significantly similar to any sequence in the current databases, thus constituting a novel gene.

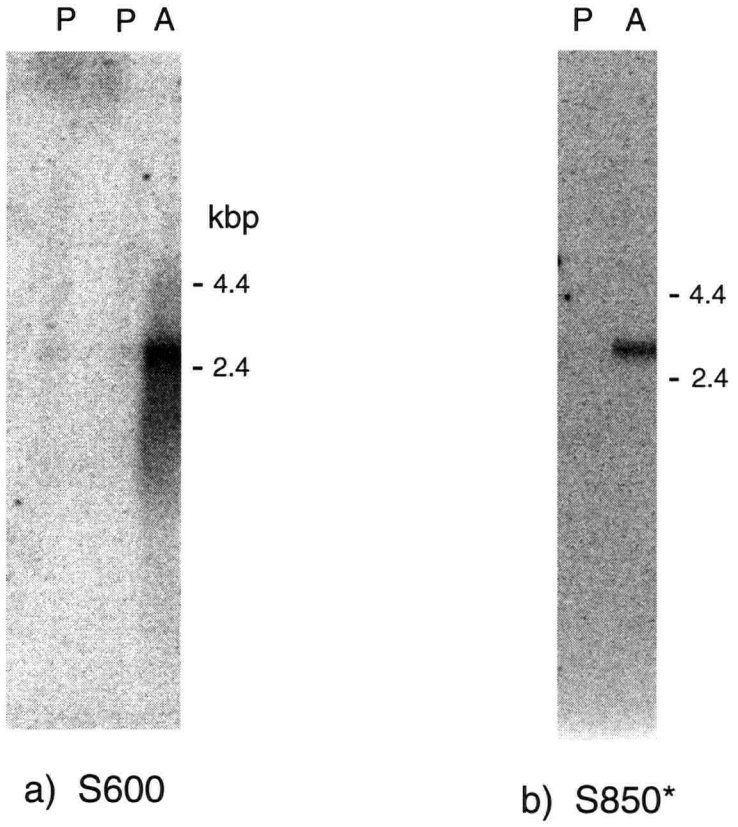


FIGURE 16

NORTHERN BLOTS PROBED WITH SUBTRACTED cDNAs

The same amount of total RNA from *Leishmania mexicana* promastigotes (P) and amastigotes (A) was analyzed by Northern Blot.
a) The probe was a clone of the amastigote specific cDNA fragment S600
b) The probe was a 620 bp segment of the amastigote specific cDNA fragment S850. This segment was obtained by digestion with Eco RV and corresponds to positions 1528 to 2148 in the A850 sequence shown in Figure 26.

FIGURE 17

AMPLIFICATION OF cDNA FROM SUBTRACTED cDNA FRAGMENT TO SPLICED LEADER

a) Experimental design

Single stranded cDNA was prepared from equal amounts of *Leishmania* amastigote (A) or promastigote (P) total RNA by reverse transcription using Clontech's CDS primer that binds Poly A. PCR was performed using B-SL, a primer designed using the sequence of the spliced leader from several species of Leishmania that is present in the 5' end of all Leishmania mature mRNAs and a primer contained in the sequence of the amastigote specific cDNA fragments obtained by subtractive hybridization. That fragments are indicated in the figure by two letters H, to represent the Hae III sites used to cut the cDNA prior to linking the adaptors and hybridization.

b) B-SL/800

The orientation of the amastigote specific cDNA fragments in relation to the spliced leader was unknown, so two primers were designed for each fragment based on their sequence. The primers were in complementary strands and in opposite directions. In order to amplify the correct template for the internal primers, five initial cycles of non-exponential PCR were performed with only the B-SL primer: 30 sec at 95°C , 30 sec at 55°C and 3 min at 68°C. The reaction was paused at 95°C, the second primer was added and a further 30 cycles of PCR were performed: 30 sec at 95°C , 30 sec at 55, 60 or 65°C of annealing temperature as indicated in the figure and 3 min at 68°C. The primers used were either 800-3 or 800-5, indicated as 3 and 5 in the figure. These primers were based on the sequence of the selected S800 cDNA fragment (see figure 13) and its position in the sequence was shown in figure 19. From this experiment it was concluded that the primer complementary to B-SL was 800-3 and that the higher annealing temperature, 65°C produced the most specific amplification. In the experiments shown in c, d and e and in those of figure 18, the annealing temperature used was 65°C and the Tm of the primers was designed accordingly.

c) PCR amplification using B-SL and 800-3 primers

Aliquots of the reaction were taken after 12, 15, 18, 21, 24, 27 and 30 cycles as indicated in the figure. This allowed for a comparison of RT PCR from amastigote (A) or promastigote (P) RNA. A band of approximately 1.8 kbp appeared to be more abundant in amastigote than in promastigotes, as judged from the fact that it could be detected after fewer cycles. The band was excised from the gel, cloned and sequenced and shown to be a contiguous sequence with that of S800.

d) PCR amplification using B-SL and 850-3 primers

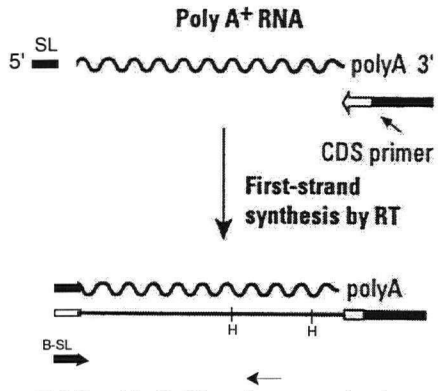
The correct internal primer for fragments S850 and S600 (see figure 13) was determined experimentally (data not shown). The position of the 850-3 primer was shown in figure 26. Amplification produced a 1.8 kbp fragment that was detected at fewer cycles with the amastigote template than with the promastigote one. The band was excised from the gel, purified, directly sequenced and shown to be a contiguous sequence with that of S850

e) PCR amplification using B-SL and 600-3 primers

An approximately 900 bp band was produced that is more abundant in amastigotes than in promastigotes. The band was excised from the gel, purified, directly sequenced and shown to be a contiguous sequence with that of S600. The position of the 600-3 primer was shown in figure 19.

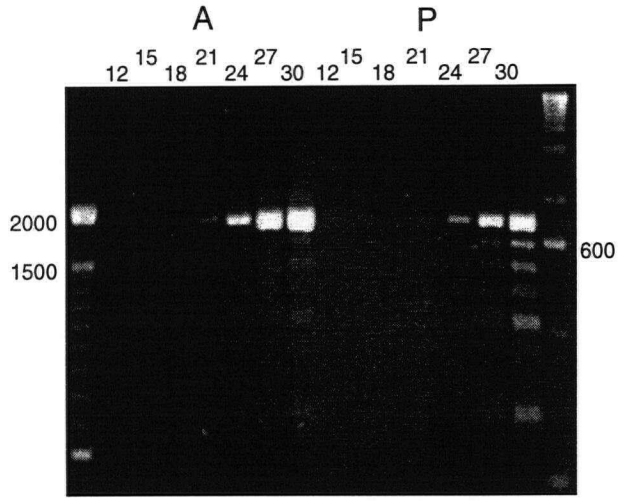
FIGURE 17

AMPLIFICATION FROM SUBTRACTED cDNA FRAGMENT TO SPLICED LEADER

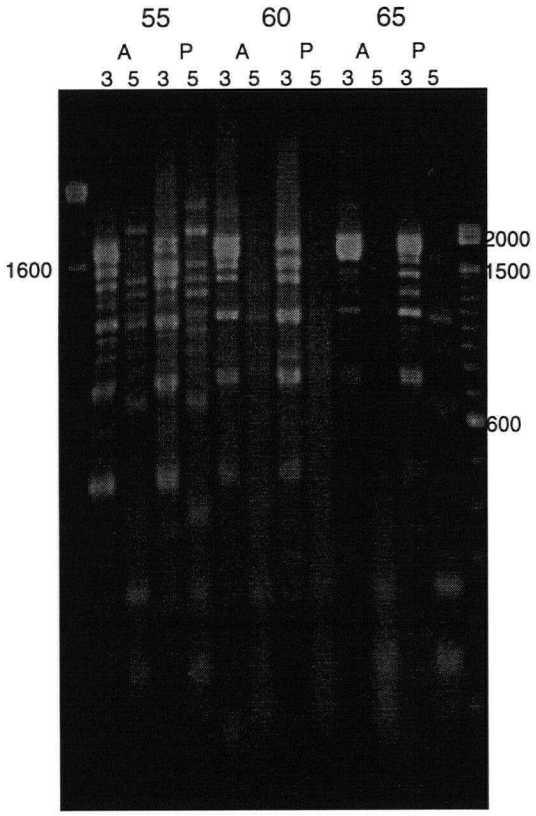


PCR with B-SL primer and primer from subtracted amastigote-specific cDNA fragment sequence (H-----H)

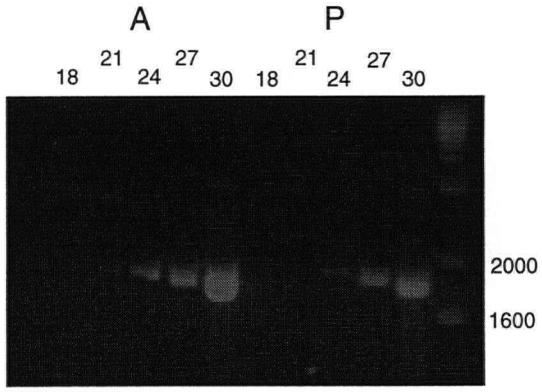
a) EXPERIMENTAL DESIGN



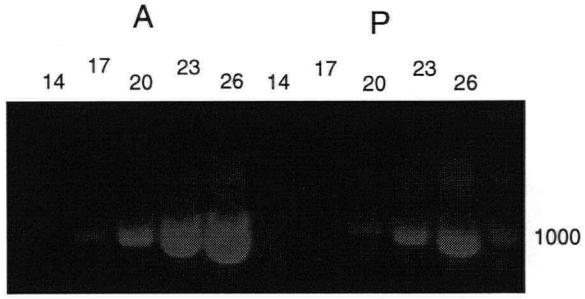
c) B-SL/800-3



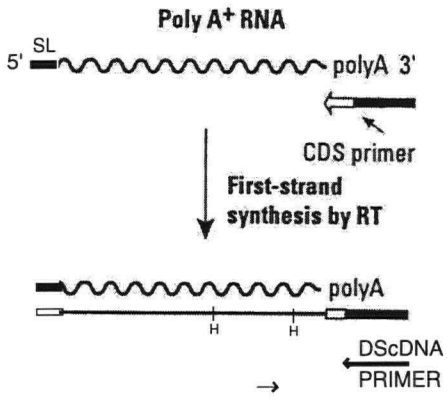
b) B-SL/800



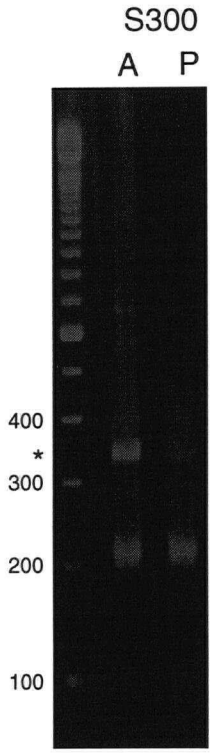
d) B-SL/850-3



e) B-SL/600-3



PCR with DScDNA primer and primer from subtracted amastigote-specific cDNA fragment sequence (H-----H)



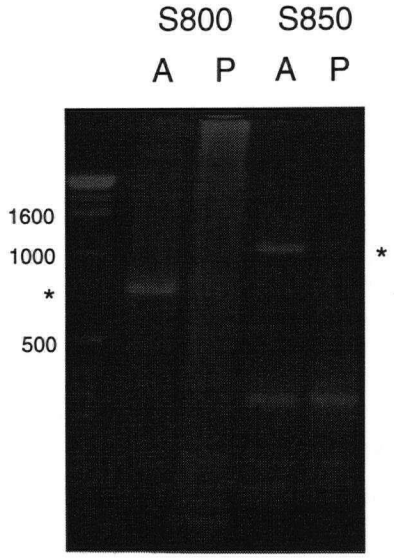


FIGURE 18

AMPLIFICATION FROM SUBTRACTED cDNA FRAGMENT TO POLY-A TAIL

Single stranded cDNA was prepared from equal amounts of *L. mexicana* amastigote and promastigote total RNA by reverse transcription using Clontech's CDS primer that binds PolyA. PCR was performed using DScDNA, a nested primer for CDS and a primer contained in the sequence of the amastigote specific cDNA fragments obtained by subtractive hybridization. These internal primers were in the opposite orientation and in the complementary strand as those use to amplify towards the splice leader, or 5' end of the mesenger RNA. Amastigote specific PCR products are indicated by * The primer pairs used were: 300-3/DScDNA that produced an amastigote specific product of aproximately 350 bp. DScDNA/800-5 that produced a 800 bp fragment and DScDNA/850-4 that produced a 1100 bp fragment.

TABLE I

COMPARISON OF THE A600 OPEN READING FRAMES BY CODON USE

	Order of codon use in Leishmania[1]	ORF 1 +3: 105-386	ORF 2 +2: 521-979	ORF 3 +3:528-1034
2 Codon AA:	1	14	29	30
	2	5	30	32
4 Codon AA:	1	20	9	11
	2	11	8	14
	3	1	12	6
	4	1	7	17
6 Codon AA:	1	7	9	2
	2	7	14	13
	3	6	7	10
	4	1	12	12
	5	2	4	8
	6	5	7	4

G + C CONTENT[2]:

ORF 1: 58.5 % G + C

ORF 2: 49.5 % G + C

ORF 3: 49.3 % G + C

[1] The order of codon use means that if in the ORF the codon used was the most commonly used in Leishmania coding sequences, the score given was 1, if the codon was the second most used, the score was 2 and so forth. *Leishmania* Codon Use Table in Appendix I

[2] *Leishmania* coding G + C is 64.48%

FIGURE 19

A600 SEQUENCE

```
SL→ 1 ACUUCUUCGC GCCUCCUCUU CCCAAAGCCA UCUCAACCCU CAUCUACGCA AGCCCUCAGA
                                                    M  P  S  M  L
  61 AUCACUCAAG CCGUUACCUC UUCUCUCACG CAUACUUGAU CACCAUGCCC UCUAUGCUCA
      N  L  V  P  A  V  E  T  T  M  T  R  T  P  M  Y  V  E  V  R
 121 ACCUUGUCCC GGCGGUGGAG ACGACGAUGA CCCGCACCCC GAUGUAUGUC GAGGUGAGGG
      V  N  A  V  P  L  M  M  V  F  G  V  S  L  V  L  A  L  V  Y
 181 UGAAUGCCGU GCCGUUGAUG AUGGUCUUUG GUGUCUCACU UGUGCUGGCG CUGGUGUACA
      T  L  W  K  L  L  P  R  I  R  S  G  E  L  S  S  S  N  T  E
 241 CUCUGUGGAA GCUUCUCCCG AGGAUCCGCA GUGGCGAGCU CUCGUCCUCG AAUACGGAGG
      A  N  F  R  A  G  L  L  N  R  K  L  K  R  E  K  V  R  S  E
 301 CCAACUUUCG UGCGGGGCUG CUGAACCGGA AGCUGAAGAG GGAGAAGGUG CGCUCGGAGG
      D  D  S  S  A  D  M  V
 361 AUGAUUCAUC UGCGGACAUG GUGUAAGGUG UUGACACUGA CGUCCUUCGU GACGGGAAGU
 421 CGCUCUCGCC UCUGAGGAUU CGACUUGCGA UCGCUGAAGU GUUCACGCAC UCGGAUGUGA
 481 GCUUGUCAGA GAGGCGUGGU UCGAAGUACC GAUAAGGAGC AUGGAGAAUG UUUCUCUGUU
 541 ACUCUUUUUA CCGGCAAAUA ACUUUUGCUU UCUGCAGUUU AGCGUGGAGU UUCAACAGGG
 601 ACUCUUACAA AACCGUCCUG UUUUCUCUCG GUGUGCAGCA UCACUGUACU CUUUGUUCUA
 661 UCUCGCCCAC GAGUGCGUGU GUGUGGGUAG GUGGAGAAGG AGGGGACGGU GGAGGUGCAA
 721 GUUGUGCAGC UGCGCACGGA AACGGAUCUG CUGUGCAGGU UGUCUUGCAU UUUUCUCUCC
 781 AAGCUGUCAC UCUUCAGUGC GAUCGGCCUC GACUCAGGCA UUUUUUUUAC UGUGCUUUCU
 841 GUUCUCCGGA UCUUUUCUCU AUUUUGAUUU CCUUUUUUUU UGCUUGUGUG UGCGCCAGUC
                                                          ←-----
 901 UCUCUGCCGA GUUCUCGAUG UCGGUGCGUG UGUACGGUGC UUUUCUUCUG CUCCACCGUC
      ---600-3----------
 961 UCCCCACGUC UCGGCGUGAC ACAUGUUUCC AUGAACAGUG GAGAAAACGG CGGAAACGAC
1021 AGACAAGAAG GUGAAGAGUG UAACUCACCU CACCGGCGAA AGAGAAACGA AAGAGCGAAA
1081 AAAAAAACGA AAAGAAAAGG CAGAAAAAGA AAAAAAGAAC GUUGAGAUGA GAUCACGCAC
1141 CCACACACGC ACGUACGAGG CGAAGGCUCA UGGUGCACGA UGGAGGCUGU GCGAGAGAGA
1201 AGUGAACCAA GCGAAAAAAA AAGUCCCUGA AACAUCGACG AAACGAACGA GUAGCGAACG
1261 UGUCAAACCC CCUUUUUUCC CCCAAGCGAA AAAUUGCUGC UUUGCUAGAA GGCGUGCUCG
1321 UACAUUUGUG UGCUCAUGCC ACUGAACAGU UGAUUGAGAG AUGUGUGUGG GUCUAGGGCC
1381 GCAGGCGUUA CGUUUCCUCG UCUCUUGCCU GUGUUUGGUU GCCGGUGUAU ACGCCUGACU
1441 CGAGUUUUUU CGCUCCACCC GCGCGCAUUU UUCCCCGUCC UCGCUCUCAU CUCUGGUAAC
1501 CUCAGCACUG UCGACCCCCG ACAUCUUUGU UUCUAGCUCC CUACUUCUCA ACCGCAGCUU
1561 CGCUUUUCGU UUUCCUUCUC CAGUGUGCUU UGCGUGUUCU UCGUCUGGGC UUGCGUUGAG
1621 GCCAGUCGAG UCCAUGCUUU CGCCCUGUUG ACGGCUUGUU CUUACGAGUG CGUAGGAGUU
1681 GUUCUCAUGA CCGUUUCUUC CGCCUAUAGU UGGUGUCUUU GAGAGCAAAG AGGGGCAAAG
                                                          ←-----
1741 UGCUCUCAGA CGCCGGUAGA UGGUAUGGAC AGAGAGAUGC GUGCACGCGA ACUUCUCUGU
      --800-3-----------
1801 ACAUGAUACA CACAUGAGCA CUCUCUUGGU GUCUUCUGUU GCGACUGGAU GACCAUGCUG
1861 CAAAACGAGC AGAAAGACGA CUGCGCUGAC UUUCACGGUC CCGUAACCUC UCGUGAUGAU
1921 GUCUGUCUCC CUCUUUCUUU UAGCGGUCGU UAACUGCGGC ACGGGAGACU GUCUUCCUCU
1981 UCUUCAUCCA UACAGCUCCG CUUUCUCCGU CUCUCCGUGA GGCUGCCCGA UCUGACAGAU
2041 GACCCGACCU CGCGCUCGCU CUUUCUUUUU CGUCGUUUCG UUUCGGGAUA GUCCUUUUUU
2101 CCUCCCACCU CUUCUUGCUC UCUCUCUCUA UCUCUCGCUC UUUCCUUCAU GGUGACGCGA
2161 GUGCUUCCCC UACUUGGUGG CGCUUCGAUC GUUGUGUGCU GCUGCAUUGU CGUGACAGUG
                          ---------800-5---------→
2221 UUACACGUAC UUAAGGGGUU UCGGCCCCAC UGCUGAUUUU UUUAUUUAUU UUUAUGUUUA
2281 UUUAUAUAUA UUUUUUUCUC CGUCCCUCUC ACCUCACCUC CGUUUUUUUC UGUUCUGUUU
2341 UACACGG..... ~ 600 bp ..... Poly A  3'
```

UNDERLINED: S600 cDNA fragment. DOUBLE UNDERLINED: S800 cDNA fragment

FIGURE 20

PREDICTED POLYPEPTIDE FROM OPEN READING FRAME 1

MPSMLNLVPAVETTMTRTPMYVEVR**VNAVPLMMVFGVSLVLA|LVYTLW**KLLPRI
RSGELSSSNTEANFRAGLLNRKLKREKVRSEDDSSADMV

VON HEIJNE'S METHOD FOR SIGNAL PEPTIDE RECOGNITION
(SIGNALP V1.1):
Seems to have a cleavable signal peptide (1 to 42 AA: 4.61 kd; after cleavage: 51 AA: 5.85 kd). Predicted site of cleavage marked by |

SOSUI ANALYSIS:
This amino acid sequence is of a membrane protein which have one transmembrane helix, indicated by **bold** case from aminoacids 26 to 48.

Double Underline: Casein kinase II phosphorylation sites.
Consensus pattern: [ST]-x(2)-[DE]
[S or T is the phosphorylation site]. Pinna L.A. Biochim. Biophys. Acta 1054:267-284 (1990).

4.1.7. Characteristics of the predicted polypeptide coded by the A600 gene:

As the predicted polypeptide did not have an anchoring sequence at the carboxyl terminus, the signal peptide would indicate that the polypeptide may be a secreted product of 51 amino acids and a molecular weight of 5.85 kDa. An alternative possibility, if the putative signal peptide was not actually cleaved under natural conditions, would be that the polypeptide was a type I membrane protein, with a transmembrane helix from amino acids 26 to 48 and the carboxyl-terminus half of the chain remaining in the cytoplasm. Three potential Casein Kinase II phosphorylation sites were predicted for the carboxyl terminus of the predicted A600 polypeptide.

Figure 21 shows the analysis of the predicted polypeptide for hydrophobicity (Kyle and Doolittle 1982), potential transmembrane regions (Engelman *et al.* 1986) and charge density. The amino-terminus half of the molecule contained the predicted signal peptide with a polar n-region and a hydrophobic h-region (positive peak in figure 21a). The hydrophilic carboxyl-terminus half (two negative peaks in figure 21a) was the predicted secreted polypeptide. The potential transmembrane region (figure 21b) corresponded to the hydrophobic peak (figure 21a). The predicted polypeptide had an isoelectric point of 9.72. The charge density, calculated at pH 7.0, showed two positively charged regions and two negatively charged regions above the thresholds indicated by the punctuated lines (see figure 21c). All the four charged regions were in the hydrophilic carboxyl-terminus corresponding to the predicted secreted polypeptide. The charged regions were intercalated: positive, negative, positive and negative.

Figure 22 shows the comparison between the sequence of the A600 predicted polypeptide and the amino terminus sequences of known *Leishmania* proteins that require signal peptides, such as the membrane protein *L. major* GP63-1 (Button et al. 1988), and proteins that are secreted to the lysosomal compartment: *L. mexicana* Cysteine Proteinase a (Mottram *et al.* 1992) and Cysteine Proteinase b 2.8 (Souza *et al.* 1992). These *Leishmania* proteins shared with A600 the characteristic of having a signal peptide as predicted by von Heijne's method (Nielsen *et al.* 1997) and like A600 showed a predicted transmembrane domain near the amino terminus as predicted by the Dense Alignment Surface method. This domain corresponded to the peaks over the cutoff line in figure 22 and would represent the hydrophobic h-region of the putative signal peptide.

FIGURE 21

ANALYSIS OF THE A600 PREDICTED POLYPEPTIDE I

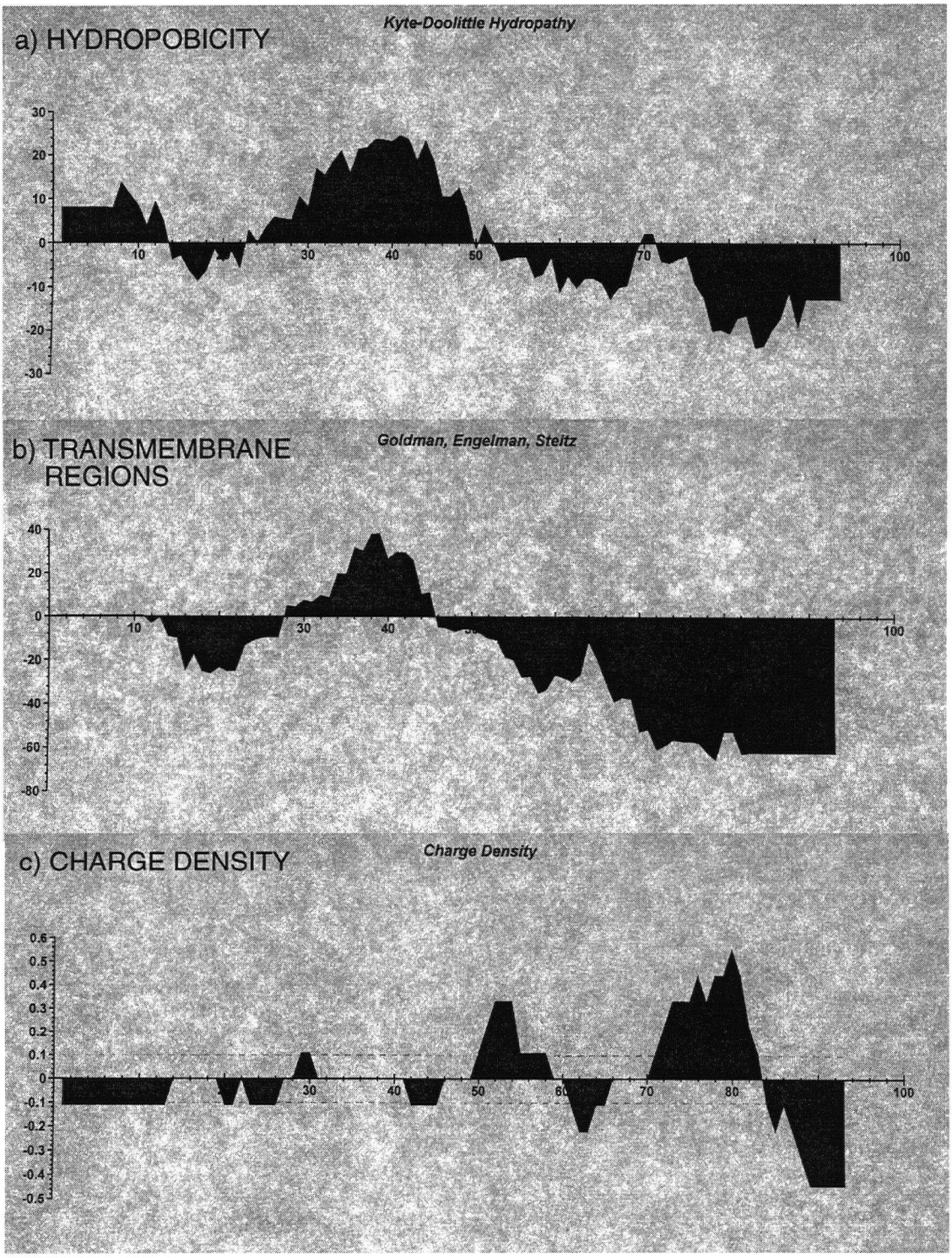


FIGURE 22

ANALYSIS OF THE A600 PREDICTED POLYPEPTIDE III

COMPARISON WITH THE N-TERMINAL SEQUENCE OF LEISHMANIA POLYPEPTIDES THAT REQUIRE A SIGNAL PEPTIDE

Predicted transmembrane domain by the Dense Alignment Surface (DAS) method (Cserzo, M., Wallin, E., Simon, I., von Heijne, G. & Elofsson, A. Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: Application of the Dense Alignment Surface method. http://www.biokemi.su.se/~server/DAS/abstract.html). The peaks over the cutoff line represent predicted transmembrane domains and would correspond to the hydrophilic region-h of an N-terminal signal peptide.

| Predicted cleavage site by the SIGNAL V1.1 signal peptide recognition program (Nielsen *et al.* 1997).

N-terminal sequences of selected Leishmania membrane and secreted proteins, that require signal peptides. The underlined sequences represented conserved aminoacids in similar proteins in other *Leishmania* species:

LmCPa MARRNPLLFAIVVTILFVVCYGSALIAQTPPVDNFVASAHYGSFKKR

LpCPa1 MARRNPLLFAIVVTILFVVCYGSALIAQTPPVDNFVASAHYGSFKKR

LdchCP1 MAR- NPFFFAIVVTILFVVCYGSALIAQTPLGVDDFIASAHYGRFKKR

LmCPa: N-terminal sequence of *L. mexicana* cysteine proteinase-a, expressed in all stages but more abundantly in amastigotes. The underlined sequence corresponded to conserved aminoacids with LpCPa1 and LdchCP1.

LpCPa1: N-terminal sequence of *L. pifanoi* cysteine proteinase a-1. This proteinase, was expressed 4 times more in amastigotes than in promastigotes (Traub-Cseko *et al.* 1993).

LdchCP1: N-terminal sequence of L. chagasi amastigote-specific cystein proteinase-1 (Omara-Opyene & Gedamu 1997).

LmCPb2.8 MATSRAALCAVAVVCVVLAAACAPARAIHVGTPAAALFEEFKRTY

LmCPb18 MATSRAALCAVAVVCVVLAAACAPARAIHVGTPAAALFEEFKRTY

LmCPb1 MATSRAALCAVAVVCVVLAAACAPARAIHVGTPAAALFEEFKRTY

LpCPa2 MATSRAALCAVAVVCVVLAAACAPARAIHVGTPAAALFEEFKRTY

LmajorCP MATSRAALCAVAVVCVVLAVACAPARAIYVGTPAAALFEEFKRTY

LdchCP2 MATSRAALCAVAVVCVVLAAAVAARAIY- VGTPAAALFEEFKRTY

LmCPb2.8: N-terminal sequence of *L. mexicana* cathepsin L-like cysteine proteinase. The underlined sequence corresponded to conserved aminoacids with LmCPb18, LmCPb1, LpCPa2, LmajorCP and LdchCP2. LmCPb18 corresponded to one of the 16 amastigote-specific genes and LmCPb1 corresponded to one of the two metacyclic promastigote-specific genes (Mottram *et al.* 1997).

LpCPa2: N-terminal sequence of *L. pifanoi* amastigote-specific cysteine proteinase a-2. This proteinase, expressed 15 times more in amastigotes than in promastigotes, was associated with the megasome, a unique lysosomal organelle found in amastigotes of the *L. mexicana* complex (Traub-Cseko *et al.* 1993).

LmajorCP: L. major cathepsin L-like cysteine protease (Sakanari *et al.* 1997).

LdchCP2: N-terminal sequence of L. chagasi constitutive cystein proteinase-2 (Omara-Opyene & Gedamu 1997).

LmaGP63-1: MSVDSSSTHRRRCVAARLVRLAAAGAAVTAVGTAAAWA|HA

N-terminal sequence of the metallo-proteinase GP63, the major promastigote surface protein, product of the *L. major gp63* gene 1 (Button & McMaster 1988). The underlined sequence corresponded to conserved aminoacids in twelve gp63 genes in five species of *Leishmania* (Voth, B. M.Sc. Thesis, University of British Columbia, 1994).

FIGURE 22

ANALYSIS OF THE A600 PREDICTED POLYPEPTIDE II

N-TERMINAL SEQUENCES OF SELECTED *LEISHMANIA* MEMBRANE AND SECRETED PROTEINS

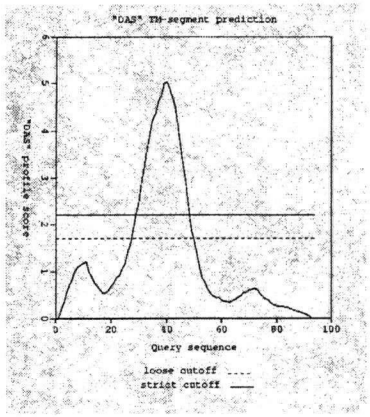
A600 MPSMLNLVPAVETTMTRTPMYVEVRVNAVPLMMVFGVSLVLA|LV
YTLWKLLPRIRSGELSSSNTEANFRAGLLNRKLKREKVRSEDDSSAD
MV

LmCPa MARRNPLLFAIVVTILFVVCYGSALIAQTPPVDNFVASAHYGSFKKR

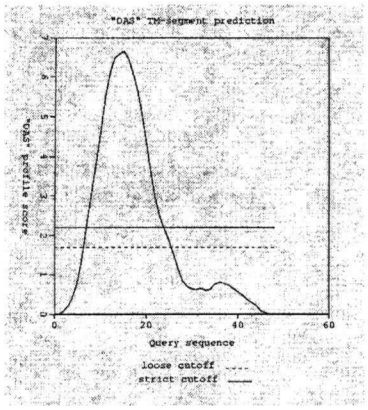
LmCPb2.8 MATSRAALCAVAVVCVVLAAACAPARA|IHVGTPAAALFEEFKRTY

LmaGP63-1 MSVDSSSTHRRRCVAARLVRLAAAGAAVTVAVGTAAAWA|HA

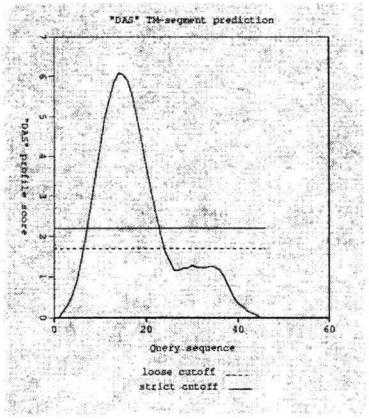
DENSE ALIGNMENT SURFACE (DAS) METHOD FOR TRANSMEMBRANE PREDICTION



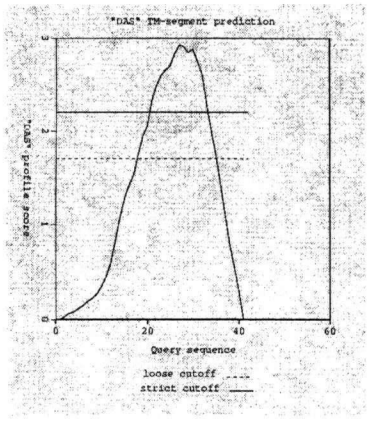
a) A600



b) LmaCPa



c) LmCPb2.8



d) LmaGP63-1

4.1.8.- A600 appeares to be a single copy gene abundantly expressed in amastigotes:

A Southern blot of *L. mexicana* genomic DNA digested with ten different restriction enzymes and probed with labeled S600, showed that A600 was a single copy gene (figure 23).

The relative abundance of the A600 mRNA was investigated by semi-quantitative RT-PCR as shown in figure 24. The mRNA for A600 was compared to that coding for β-tubulin, that is known to be one of the most abundant proteins in Leishmania, especially in the flagellated promastigotes. As expected, the A600 band was shown to be more abundant in amastigote than in promastigotes and the β-tubulin more abundant in promastigotes. In amastigotes, A600 appeared to be more abundant than β-tubulin mRNA. The mRNA for A600 in amastigotes was less abundant, but within the same order of magnitude, than the mRNA for β-tubulin in promastigotes, a product of multiple gene copies. This result suggested that the A600 mRNA was a highly abundant mRNA in the amastigotes.

4.1.9.- The amastigote-specific S300 cDNA fragment appeared to correspond to a single copy gene:

That A300 was apparently a single copy gene, two per diploid genome, was shown by its Southern blot (figure 25b). The sequence of A300, consisting of the amastigote-specific subtracted S300 cDNA fragment (see figures 13 and 14) and its extension to the 3' end of its mRNA (see figure 18) is shown in figure 25a. The sequence appeared to correspond to a 3' UTR. There was a fifty amino acid ORF but analysis of codon usage, such as that presented in Table I, indicated that it did not correspond to a *Leishmania* coding sequence. When the PCR product of the extension towards the Poly A end (see figure 18) was cloned, two distinct clones varying slightly in the size of the insert were obtained. In both cases the sequences were contiguous to S300 and for most of their length were identical between them. The only difference between the clones was the sequence immediately adjacent to the Poly A, where the sequence totally diverged, in one case having 48 bp and in the other 34 bp. This result lead to the conclusion that *L. mexicana* was heterozygous at the A300 locus, with the alleles differing at their 3' UTR. The size differences would be too small to be apparent in a Southern blot.

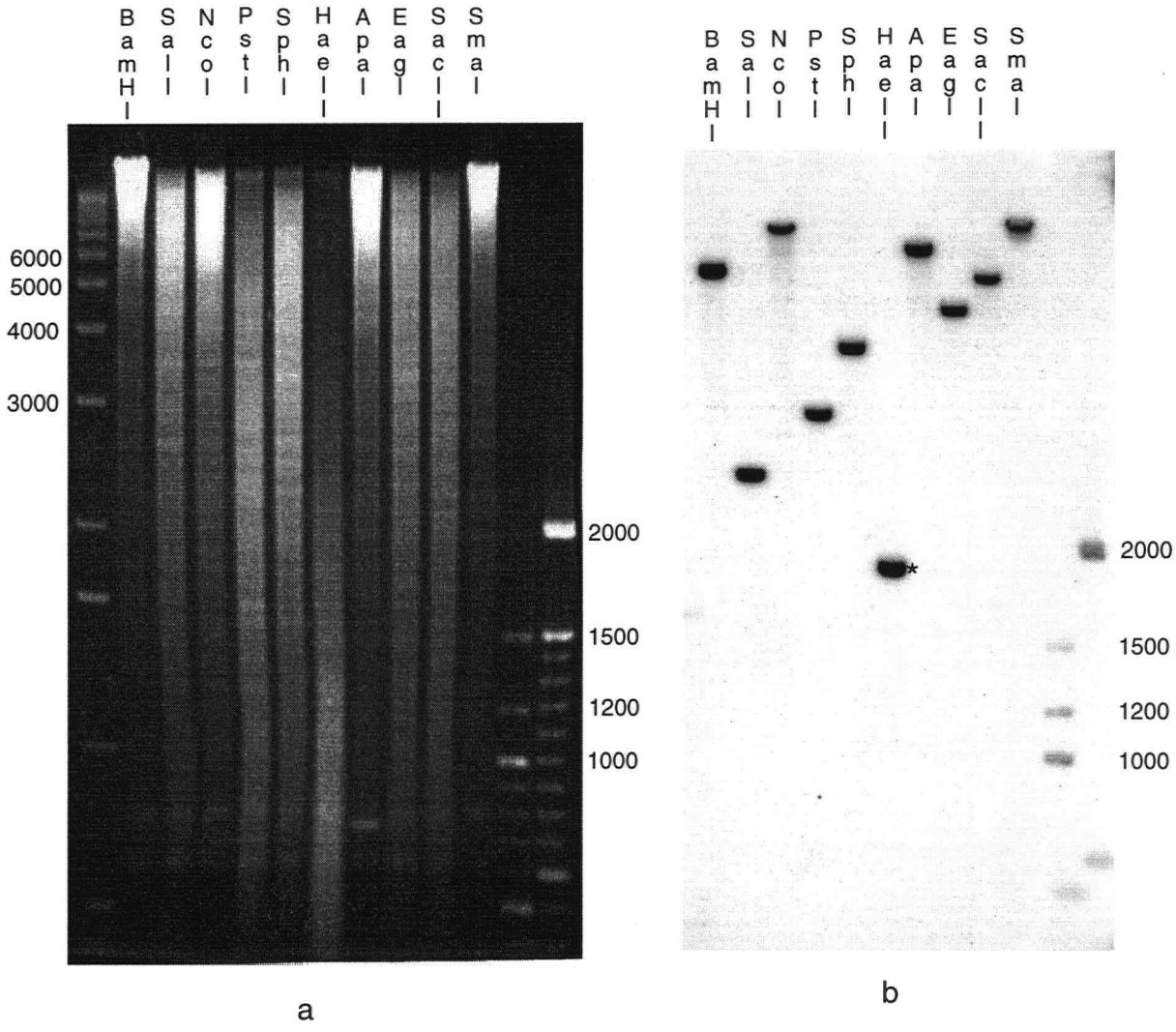


FIGURE 23

SOUTHERN BLOT OF A600

a) Ethidium Bromide staining of 0.8% agarose gel electrophoresis of Restriction Enzyme digested *L. mexicana* genomic DNA. Photo was taken before transfer to nylon membrane.
b) Autoradiography of membrane after Southern Blot with radioactively labeled insert from a clone of the amastigote specific S600 cDNA fragment.
* The labeled band corresponding to the HaeII digest is 1952 bp, according to the sequence presented in Figure 19.

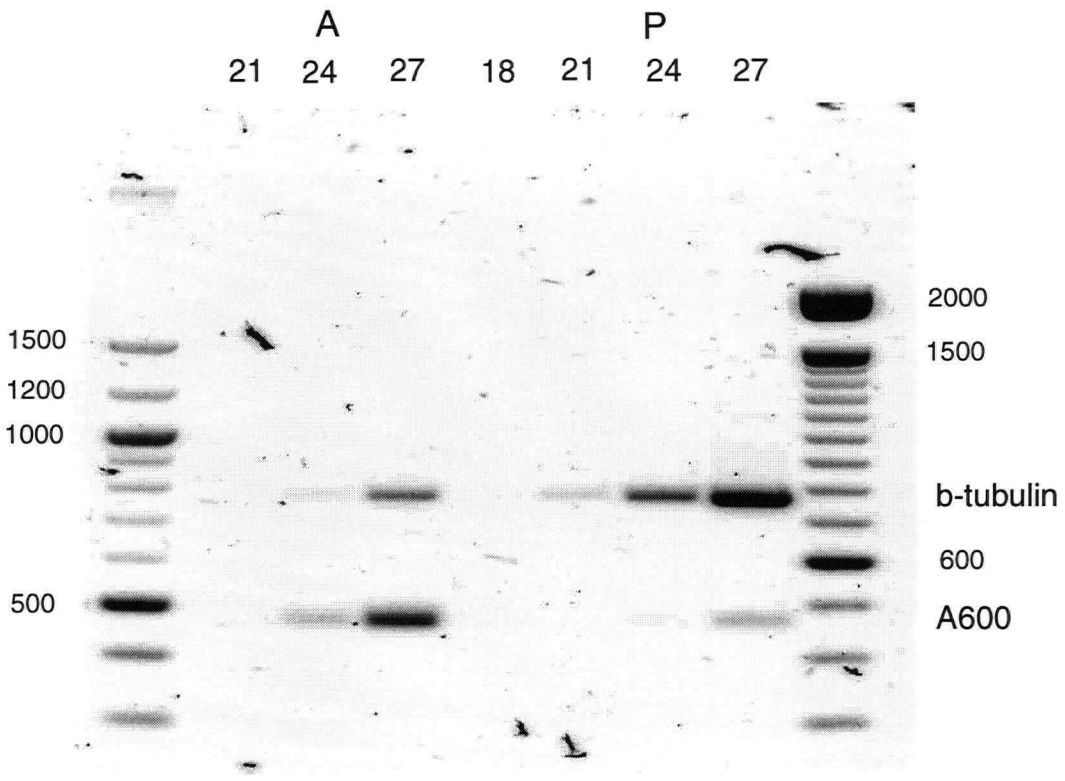


FIGURE 24

RT-PCR OF A600 AND BETA TUBULIN

The same amount of amastigote (A) or promastigote (P) RNA was used for single stranded cDNA synthesis by Reverse Transcriptase. This template was mixed with the PCR master mix minus the primers and aliquoted into separate tubes containing either primers 600-5 and 800-3 for A600 amplification (see Figure 19) or primers 850S and 850AS2 for amplification of the coding sequence of beta-tubulin (see Figure 26). Aliquots of each reaction were removed after 18, 21, 24 and 27 cycles and the products of A600 and beta-tubulin were mixed in the same amounts. A previous experiment had shown that there were no bands overlaping in size. The samples were electrophoresed, stained with Ethidium Bromide and visualized with a phosphoimager. A600 shows the position of its 463 bp product and b-tubulin identifies its 754 bp product.

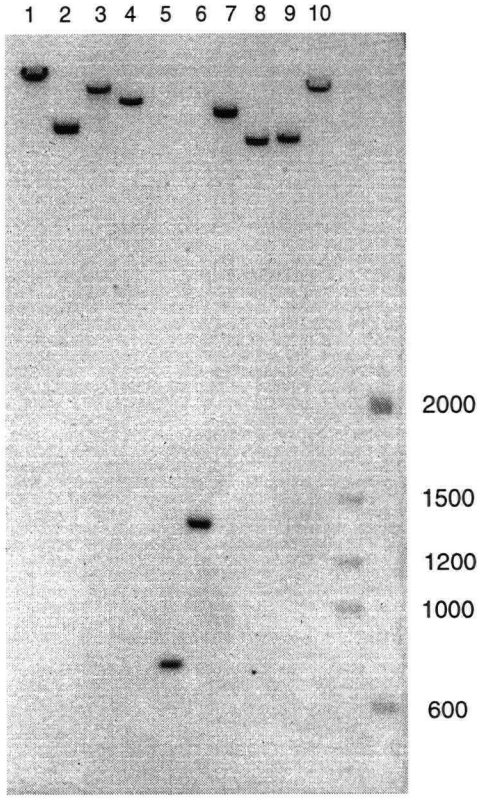
FIGURE 25

a) A300 SEQUENCE

```
  1 CCUUGCCGCC CACGCCACAC CCCUCUCCUU UUCUCUGACA UGUACACACU CUGCAACAUG
 61 UGCAAUCUGA GAUAAGGCGC GCUGCACUGC AUUCCUUCCC CGUCACCGCC UCUCUUUUCG
121 UUCACACAAG GGAGGGAGGA ACUCGCAGGG AACGCUAAAG UGAGGAAGUG CCAGUGGUGU
181 GAAAGCGGCG CAGACGAAGA AUACAACGUU CAACUGAGGC AUAAGUAUGC CGGACACUUC
        ------------300-3-------->
241 CUGCGUUGAU GCGUACUUUU UUCUGACAAU GUGUAUGGCG GGUUUCUUUG CGGGGCCUAA
301 AAUCUGCAAC GAUAAGGCAC GCCGCGAUUC AUCGUUCGUU CUGCCCUCUC UGUAUAUAUU
361 ACAGUACAGG UGUACCUAAG AAAGGUGAGU GAUCACGAAU CUCACUUCAC UUCUCCUCCC
421 CU.. 48 or 34 bp .. Poly A   3'
```

Underlined: S300 fragment

b) A300 SOUTHERN BLOT



The blot corresponds to the gel shown in figure 23. The membrane was stripped and rehybridized with the radioactively labeled insert of a clone of the amastigote specific cDNA fragment S300. *L. mexicana* genomic DNA digested with ten different restriction enzymes was shown in the following lanes: 1) Bam HI 2) Sal I 3) Nco I 4) Pst I 5) Sph I 6) Hae II 7) Apa I 8) Eag I 9) Sac II 10) Sma I

4.1.10.- The amastigote-specific A850 gene was a β-tubulin isogene:

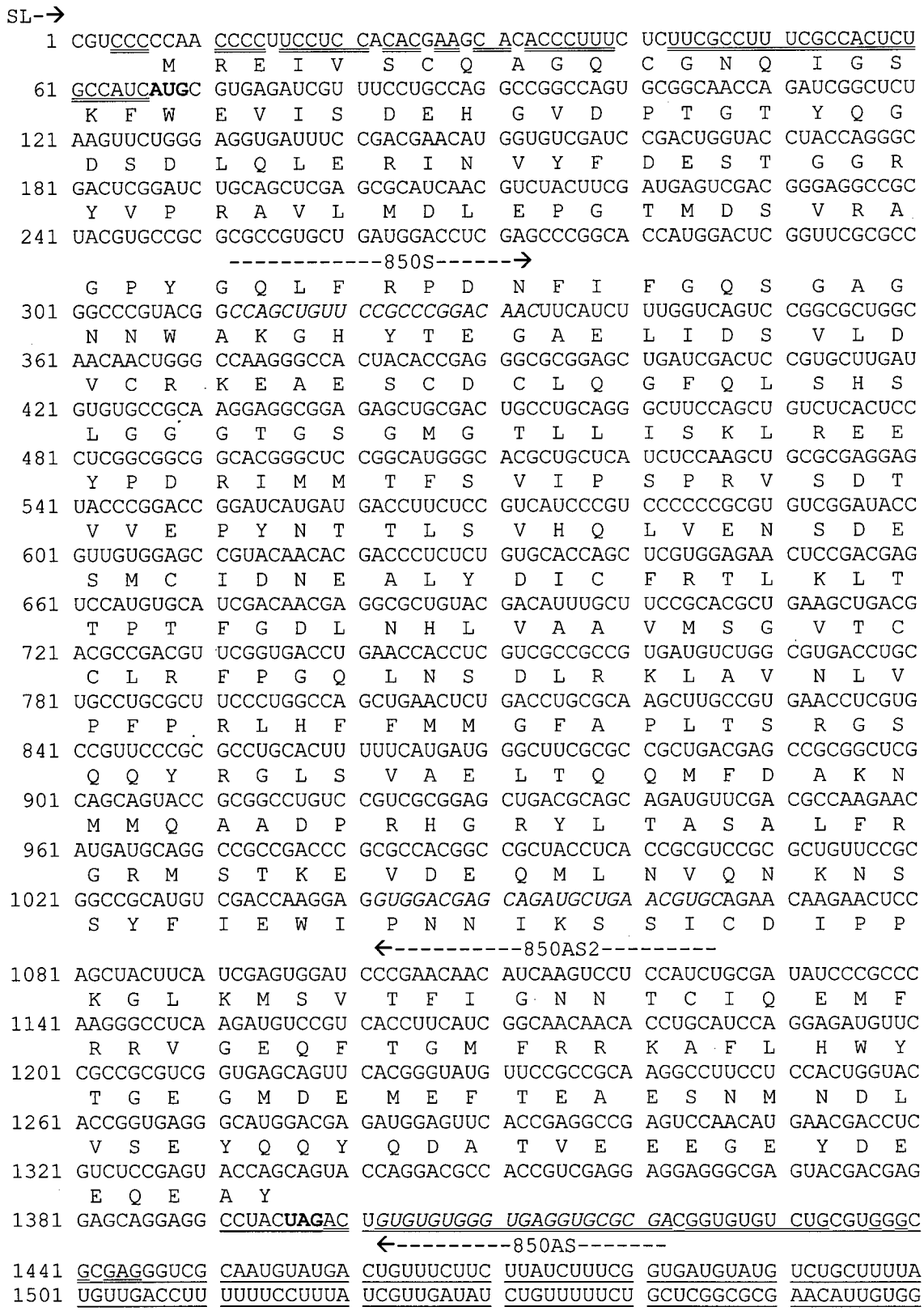
Sequencing the extension of S850 to the spliced leader revealed a β-tubulin gene whose sequence is presented in figure 26. The sequence of the amastigote-specific cDNA fragment S850 (see figures 13 and 14) corresponded to the last two amino acids of the coding sequence, the stop codon and the initial fragment of its 3' UTR (figure 26). The predicted size of the mRNA, calculated from extensions towards the 5' and 3' ends (figures 17d and 18) was 2.8 kbp. This predicted size fit well with the size of the mRNA band in the Northern blot (figure 16b). It also fit with the reported size of the most abundant *L. mexicana* amastigote β-tubulin mRNA, that was 2800 nucleotides, the most abundant promastigote mRNA being 2400 nucleotides (Burchmore & Landfear 1998). Comparison of the UTR of the A850 β-tubulin isogene with that of reported *Leishmania* β-tubulin genes revealed that they all shared a high degree of identity in the 5' UTR and in the 3' UTR immediately before the start codon and after the stop codon (see figure 27).

In figure 28, the protein sequence of the amastigote-specific *L. mexicana* A850 β-tubulin was compared with the published sequences of the amastigote-specific *L. major* 2.8 β-tubulin, the promastigote-specific *L. major* 2.2 β-tubulin (Coulson *et al.* 1996) and a *L. amazonensis* β-tubulin gene (Fong & Lee, 1988). While the proteins were highly conserved, differences in some positions were seen, differences which presumably generated differential function and that should eventually provide an explanation for stage-specific expression and the presence of isogenes. The *L. amazonensis* gene was very similar to the promastigote-specific *L. major* 2.2 gene. The *L. major* 2.2 promastigote gene differed from the amastigote gene 2.8 in a number of positions, the differences being most abundant in their carboxyl terminus (Coulson *et al.* 1996). It was noted that the A850 *L. mexicana* isogene resembled the *L. major* amastigote-specific gene 2.8 in its amino terminus but conversely resembled the *L. major* promastigote specific gene in its carboxyl terminus.

The only amino acid where A850 differed from the three other *Leishmania* β-tubulin genes was at position 440, almost at the carboxyl terminus where it had a glutamine instead of a glutamic acid, in the middle of a cluster of glutamic acid residues.

FIGURE 26

A850 SEQUENCE



```
1561 CCGUCUCCCC GUUUUUGCCG CUCUCUGUGA CAGGGCUUCU GAUACGGAAG GCGGAUUGAU
                                                 ------------
1621 GGAGAGUUGG UGCGCAGAAC UGGUGCGAGA GUAGUACAGU GAACGAACGU GAAGUCAACC
     --850-4----->
1681 ACAUACACCG GACUAGAAAC AUAUAGAAGA ACAAAGAGCA AAGGGUGACC CGUAGAAGCG
1741 CGCAAAUCGA UCCCUGUCAU CUUUCCCUAC CCUUCACCAU GUCAGGUCCU UCGAUGAGCA
                                     <-----------850-3------------
1801 UUGGUCUGGG ACUGGGGUGG GUGGGGAAGU CGAUAGCAGG AGAACAACGG AGAGAACCGC
1861 GACACUUCCU UCCGCCUAGA GAAAAAGGAA GAGGGGAGAA AGGAAAAAGC GCAGCCGACA
1921 GAUGUGGGCG AAAAGUGAUG CGCUUCAUGC GUUUCUUGUC GCUGAUUCCU UUUGCUCUCU
1981 UUCUUCUUUU CCUCGCACUG AUUUUUGUCU UUUUUUAUGA CUUUAAAGAA CGCUGUACUC
2041 CUUGAGUGGA GAGACGGAGA GAGUGUGUCU CUGUGCUUGC GUGAACACGA CCGCCAUACA
2101 CCCUCUCGCC CCCUUCUCUU CAAGACUCCU CUCUUUUCUU UUGUUUGG.....
     ..... ~ 570 bp .....Poly A   3'
```

Underlined: S850 fragment
Double Underline: Conserved Leishmania beta Tubulin 5′ and 3′ UTR
Italics: Sequence corresponding to primers
-----→ Top strand primer
←----- Complementary strand primer
SL: Spliced Leader

FIGURE 27

Conserved Leishmania beta Tubulin 5' UTR

51/63=81% Identity

L.m.mex.: 4 CCCCCAACCCCUUCCUCCACACGAAGCACACCCUUU

L.major 2.2: SL-UAACCGUCCCCAACACCCCCUCCUCCGCACAAAGCAUACCCUUU

L.major 2.8: 1 UAACCGUUCCCAACACCCCCUCCUCCACACAAAACAUACCCUUU

L.donovani: 41 UAACCGUCCCCCAG---CCCCCUCCUCCACACAAAGCAUACCCUUU

L.m.mex.: CUCUUCGCCUUUCGCCACUCUGCCAUC**AUG** 70

L.major 2.2: CGUUUCGCCUUUCGCCACUCUGCCAUC**AUG** 123

L.major 2.8: UGUUUCGCCUUUCGCCACUCUGCCAUC**AUG** 76

L.donovani: CUUUUCGCCUUUCGCCACUCUGCCAUC**AUG** 103

Conserved Leishmania beta Tubulin 3' UTR

33/47=83%; 33/35=94% Identity

L.m.mex: 1396 **UAG**AC*UGUGUGUG*GGUGAGGUGCGCGACGG*UGUG*UCUG

L.major 2.2: 1449 **UAG**ACAGUGUGUGGGUGAGGUGCGCGAAGGUGUGUCUG

L.m.amaz.: 1336 **UAG**ACGGUGUGUGGGUGAGGUGCGCGACGGUGUGUCUG

L.m.mex.: CGUGGGCGCGAG 1445

L.major 2.2: UCGGUGGGGGAG 1498

L.m.amaz.: UCUGUGGGGGAG 1385

Underlined: Identical sequence **Bold**: Start and stop codons *Italics*: UG repeat

FIGURE 28

COMPARISON OF *LEISHMANIA* BETA TUBULIN PROTEIN SEQUENCES

L. mexicana A850 and *L. major* 2.8 are Amastigote specific
L. major 2.2 is Promastigote specific
Aminoacids corresponding to the *L.major* 2.2 and 2.8 genes or to the *L. amazonensis* gene only shown where their sequence differs from that of the *L. mexicana* A850 gene.

```
                          5        10        15        20        25        30
L mex A850     1  M R E I V S C Q A G Q C G N Q I G S K F W E V I S D E H G V
L maj 2.8                                                      S
L maj 2.2                                                      A
L m amaz                                                       A

L mex A850    31  D P T G T Y Q G D S D L Q L E R I N V Y F D E S T G G R Y V
L maj 2.8             T                                        T
L maj 2.2             S                                        A
L m amaz              S                                        A

L mex A850    61  P R A V L M D L E P G T M D S V R A G P Y G Q L F R P D N F
L maj 2.8
L maj 2.2
L m amaz

L mex A850    91  I F G Q S G A G N N W A K G H Y T E G A E L I D S V L D V C
L maj 2.8
L maj 2.2
L m amaz

L mex A850   121  R K E A E S C D C L Q G F Q L S H S L G G G T G S G M G T L
L maj 2.8
L maj 2.2
L m amaz

L mex A850   151  L I S K L R E E Y P D R I M M T F S V I P S P R V S D T V V
L maj 2.8
L maj 2.2
L m amaz

L mex A850   181  E P Y N T T L S V H Q L V E N S D E S M C I D N E A L Y D I
L maj 2.8
L maj 2.2
L m amaz
```

```
L mex A850  211  C F R T L K L T T P T F G D L N H L V A A V M S G V T C C L
L maj 2.8
L maj 2.2
L m amaz

L mex A850  241  R F P G Q L N S D L R K L A V N L V P F P R L H F F M M G F
L maj 2.8                        K                 L
L maj 2.2                        K                 F
L m amaz                         NR                F

L mex A850  271  A P L T S R G S Q Q Y R G L S V A E L T Q Q M F D A K N M M
L maj 2.8                        Q                 E L
L maj 2.2                        E                 E L
L m amaz                         E    Q            D V

L mex A850  301  Q A A D P R H G R Y L T A S A L F R G R M S T K E V D E Q M
L maj 2.8                                                  R
L maj 2.2                                                  K
L m amaz                                                   K

L mex A850  331  L N V Q N K N S S Y F I E W I P N N I K S S I C D I P P K G
L maj 2.8                                  L         R
L maj 2.2                                  I         K
L m amaz                                   I         K

L mex A850  361  L K M S V T F I G N N T C I Q E M F R R V G E Q F T G M F R
L maj 2.8           A   S                                      R
L maj 2.2           V   F                                      L
L m amaz            V   F                                      F

L mex A850  391  R K A F L H W Y T G E G M D E M E F T E A E S N M N D L V S
L maj 2.8           A
L maj 2.2           A
L m amaz            R

L mex A850  421  E Y Q Q Y Q D A T V E E E G E Y D E E Q E A Y       443
L maj 2.8                                  Y       E               443
L maj 2.2                                  Y       E               443
L m amaz                                   F       E               445
```

Underlined: 2 extra aminoacids in *L. m. amazonensis* beta-Tubulin

4.1.11.- The A850 amastigote-specific β-tubulin mRNA is encoded by at least two genes:

If A850 is an isogene, then its amastigote-specific 3' UTR should not hybridize with all of the multiple genes for β-tubulin present in the genome of *L. mexicana*. This prediction was tested by comparing S850, the 3' UTR of the A850 gene, to the highly conserved coding sequence of the gene as probes in a Southern blot. The results are shown in figure 29. The β-tubulin coding sequence bound to at least 7 bands in all of ten different restriction-enzyme digests of *L. mexicana* genomic DNA (figure 29b). In contrast, the amastigote-specific S850 bound only two bands (figure 29a). The conclusion of this experiment was that A850 was the product of at least two copies of an isogene but definitely of only a minority of the β-tubulin genes in *L. mexicana*.

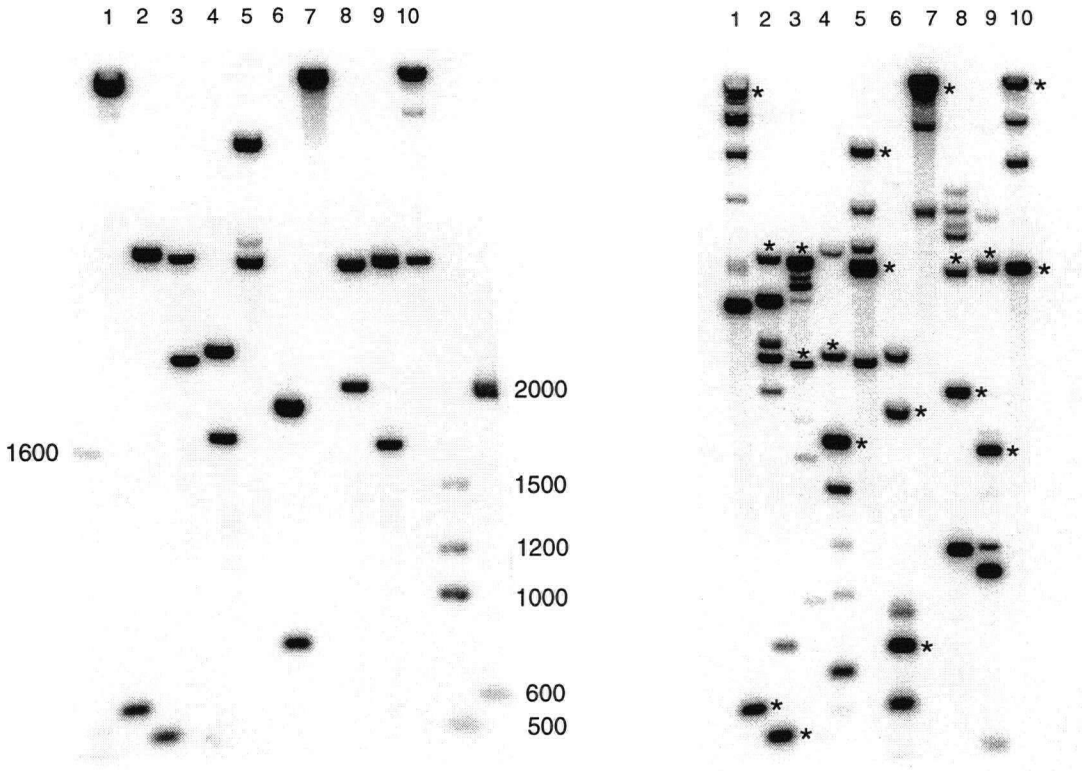
4.1.12.- Relative abundance of *L. mexicana* β-tubulin mRNA:

The relative abundance of the β-tubulin mRNA was explored by a semi-quantitative RT-PCR method as shown in figure 30. Three sets of primers were used, whose position is indicated in figure 26. All use the primer 850S, that bound within the coding sequence. The second primers were: 850AS2 from a highly conserved area of the coding sequence (it was the same as that used in figure 24); 850AS that bound to the conserved 3' UTR segment immediately after the stop codon (see figure 27) and 850-3 that bound to the amastigote-specific 3' UTR of gene A850.

As expected, the total β-tubulin mRNA, represented by the coding region (primers 850S/850AS2, b-tub 1 PCR fragment in figure 30), was more abundant in the flagellated promastigotes than in the amastigotes. Similar observations have been reported for *L. amazonensis* and *L. pifanoi* (Fong & Chang 1981; Fong *et al.* 1984; Landfear & Wirth 1984).

It was also concluded that most of the promastigote genes must present the conserved 3' UTR segment sequence (see figure 27), as the concentration of the product using the primer 850AS, that bound in that sequence (b-tub 2 in figure 30), was similar to that of the coding region product (b-tub 1). In the amastigotes, both PCR product bands showed also comparable intensity.

Based on this RT-PCR method, it appeared that only a fraction of the β-tubulin mRNA in amastigotes consisted of the amastigote-specific A850 β-tubulin isogene mRNA (represented by b-tub 3 in figure 30). This observation raised the probability that there are other *L. mexicana* amastigote-specific isogenes, which would presumably have differential function.



a) S850: b-tubulin 3' UTR

b) b-tubulin coding sequence

FIGURE 29

A850 SOUTHERN BLOT

The blot corresponded to the gel shown in figure 23. The membrane was stripped and rehybridized. *L. mexicana* genomic DNA digested with ten different restriction enzymes was shown in the following lanes: 1) Bam HI 2) Sal I 3) Nco I 4) Pst I 5) Sph I 6) Hae II 7) Apa I 8) Eag I 9) Sac II 10) Sma I

a) The probe was the insert of a clone of the amastigote specific cDNA fragment S850. This fragment consisted almost exclusively of the 3' UTR of an amastigote specific beta tubulin isogene and it was shown as the sequence underlined in Figure 26.

b) The probe is a 754 bp fragment obtained by PCR from the coding sequence of the A850 beta tubulin gene. It corresponds to positions 312 to 1056 bp in Figure 26.

* Bands common to both blots.

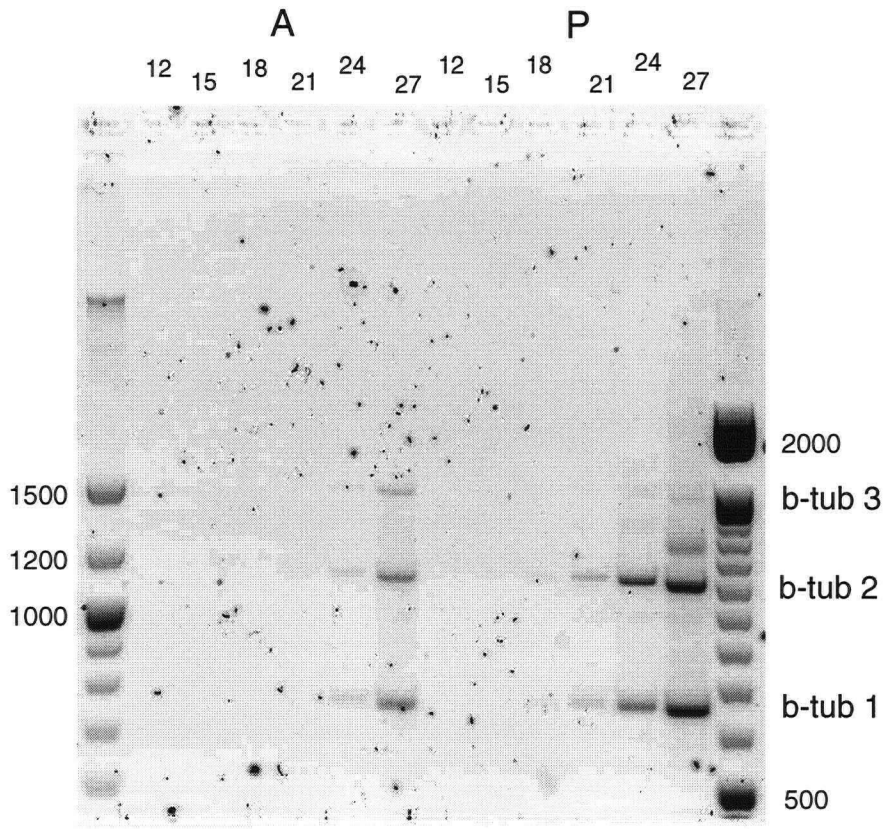


FIGURE 30

RT-PCR OF BETA TUBULIN

The same amount of amastigote (A) or promastigote (P) RNA was used for single stranded cDNA synthesis by Reverse Transcriptase. This template was mixed with the PCR master mix minus the primers and aliquoted into separate tubes all of which contain primer 850S that binds to a conserved coding sequence of *Leishmania* beta-tubulin (see Figure 26). The second primer was either 850AS2 that binds also in a conserved coding sequence producing a product of 754 bp indicated as b-tub 1; 850AS that binds in the conserved 3' UTR region immediatly after the stop codon, producing a 1100 bp fragment (b-tub 2) or 850-3 that binds to the amastigote specific 3' UTR of the A850 beta-tubulin isogene, producing the b-tub 3 fragment of 1486 bp. Aliquots of each reaction were removed after 12, 15, 18, 21, 24 and 27 cycles and the products of the three primer pairs were mixed in the same amounts. A previous experiment had shown that there were no bands overlaping in size. The samples were electrophoresed, stained with Ethidium Bromide and visualized with a phosphoimager.

4.2.- DISCUSSION

4.2.1.- Isolation of amastigote-specific cDNA by subtractive hybridization:

A cDNA subtraction hybridization protocol was used successfully to identify and isolate *L. mexicana* amastigote-specific cDNA fragments. The cDNA was produced from cultured amastigote and promastigote RNA. The gene for a cysteine proteinase, reported to be amastigote-specific (Mottram *et al.* 1997), was used as positive control. The procedure was modified and optimized and a total of fifteen potentially amastigote-specific defined bands were identified and isolated (see figure 13). These subtracted cDNA fragments were cloned and used to probe amastigote and promastigote cDNA in virtual northern blots (see figures 14 and 15). The cDNA corresponding to eight of these subtracted fragments were more abundant in amastigotes than promastigotes and four of those had between 6 and 46 fold excess.

Clones from the four amastigote-specific subtracted cDNA fragments S800, S600, S850 and S300 (see figure 14) were sequenced and all were found to correspond to 3'UTR. Three of these clones were successfully extended up to the spliced leader at the 5' end of their original mRNA (see figure 17). The two most abundant amastigote specific fragments, S600 and S800 were found to correspond to two fragments of the 3' UTR of the same transcript (see figure 19). The coding region of this gene, that was termed A600, was found to code for a novel small polypeptide of 93 amino acids, the first 42 of which were a predicted signal peptide, containing an hydrophobic domain (see figure 20). The predicted protein therefore was probably a secreted polypeptide. Alternatively, it may be anchored to the membrane, as a type I membrane protein with most of the polypeptide facing the inside of the cell. That A600 expression was amastigote-specific was confirmed by Northern blot (see figure 16) and a Southern blot established that it was present as a single gene in the *L. mexicana* genome (see figure 23). A notable feature of the A600 mRNA was its apparent abundance in the amastigotes, being more abundant than the β-tubulin mRNA, as determined by time course RT PCR (see figure 24).

A third amastigote-specific cDNA fragment, S850, upon extension to the spliced leader and sequencing was found to encode a β-tubulin gene. The mRNA coding for this amastigote-

specific β-tubulin had a unique 3' UTR that hybridised with what appeared to be two copies out of the multiple β-tubulin genes (see figure 29). The amastigote-specific A850 β-tubulin isogene is the first reported *L. mexicana* β-tubulin gene sequence (see figure 26). The amino acid sequence of the A850 gene was compared to that of the three other reported *Leishmania* β-tubulin genes (see figure 28) and the four *Leishmania* β-tubulin genes were found to be highly conserved with variable amino acids at only a few defined positions.

As expected, the cDNA subtraction hybridization protocol was used successfully to identify and isolate *L. mexicana* amastigote-specific genes, A600 and A850, opening avenues of research into what is expected to be their amastigote-specific function. Some of that proposed research approach is outlined in section IV-C-5 below.

The subtractive hybridisation procedure was expected to produce a smear, but instead produced a discrete set of bands (see figure 13, lane 4). Just a few of the subtracted defined bands were unique, different from the predominant bands common to the unsubtracted amastigote and promastigote cDNAs (see figure 13, lanes 2 and 3). This was probably due to the fact that the starting cDNA also presented a set of bands, instead of the expected smear (see figure 13, lanes 2 and 3). This characteristic was probably not a feature of *L. mexicana* cDNA as in later experiments the expected cDNA smear was obtained (see figure 14a). It was possible therefore that the discrete band pattern was not fully representative of the mRNA population. The band pattern of the subtracted cDNA was fortunate as unique cDNA fragments could be studied instead of screening cDNA libraries. It was presumed that unique discrete bands in the subtracted cDNA represented amastigote-specific cDNA fragments and this hypothesis was proven largely correct. Modifications were made to the procedure in order to obtain more unique bands and it was very simple to monitor their success by agarose gel electrophoresis (see figure 13). A relatively small number of bands could then be sliced out of the agarose gel, cloned and sequenced. As there are very few amastigote-specific genes known, this was a very profitable approach to the identification of some major stage-specific mRNAs.

Not all amastigote-specific genes were identified, for example the positive control amastigote-specific cysteine proteinases (Mottram *et al.* 1996; Mottram *et al.* 1997; Souza *et al.* 1992) were not selected. In the future it may be desired to produce more extensive

subtracted cDNA libraries with the goal of identifying all of the amastigote specific genes, including low abundance ones. The conclusions from the present work will be invaluable in such an undertaking. If the procedure started with a different cDNA population, it is possible that a subtracted cDNA smear consisting of many different species could be obtained. Performing also the modifications to the selective procedure described in figure 13 might be useful to obtain the largest possible number of amastigote-specific cDNA species. It is also likely that the subtracted cDNA library would not be totally amastigote-specific, requiring screening the clones for stage-specific gene expression.

One difficulty that is foreseen is that of detecting the low abundance mRNA species during the screening procedure. Some of the mRNAs that encode important amastigote-specific proteins, such as those involved in signal transduction or transcription activators, may be scarce. The subtractive protocol allows low abundance species to be identified, but because not all subtracted cDNA are amastigote-specific, those species have to be confirmed as amastigote-specific by screening.

Before attempting to identify the majority of the amastigote-specific genes, a more sensitive primary screen assay must be found. None of the screening methods used in this work would be adequate as a primary screen to compare the concentration of low abundance mRNA between promastigotes and amastigotes. The virtual Northern blot was more sensitive than the RNA blot but a number of clones from unique subtracted cDNA bands did not produce any detectable band with that method. RT-PCR can be more sensitive, but it requires sequencing the clones so as to be able to design the primers, which would not be practical as a first screen of a fairly large cDNA library. Additionally, semi-quantitative RT-PCR produced clear cut differences between the amastigote and promastigote templates for the abundant A600 transcript (see figures 17c, 17e and 24), but less clear differences with the less abundant A850 β-tubulin isogene mRNA (figures 17d and 30). It is unlikely to be a very useful screen for even less abundant transcripts.

Spotting the clones of a subtractive cDNA library and differentially screening them with labeled RNA or cDNA from promastigotes and amastigotes would also work well for abundant mRNA species but not for low abundance ones. This may be the reason why the 40,000 clones of a *L. donovani* amastigote cDNA library differentially screened with stage-specific cDNA probes produced only seven positive amastigote-specific clones, five of which

corresponded to the high abundance A2 mRNA (Charest & Matlashewski 1994). The rapidly evolving technology of DNA microarrays and two-color fluorescent probe hybridization (Schena *et al.* 1995, Shalon *et al.* 1996) has promise as a more sensitive assay for differentially expressed genes for the near future.

4.2.2.- Characteristics and possible function of the predicted A600 polypeptide: LmA600p

The sequence of the predicted polypeptide did not show any significant similarity with any sequence in the current databases. Nevertheless, some information can be gained by the analysis of the predicted protein sequence. The predicted characteristics of the LmA600p lead to a plausible hypothesis about its possible biological function. This hypothesis can be tested experimentally as proposed in section IV-C-5 below. LmA600p was predicted to have the following characteristics:

a) amastigote-specific:

The fact that the mRNA of the A600 gene was more abundant in the amastigote stage (see figures 14 and 16) suggested that the polypeptide it encoded would also be predominantly present in amastigotes and leads to the hypothesis that its function was related to its special environment. The rationale for this study had been that amastigote-specific proteins would be involved in amastigote-specific functions, in the survival of the parasite within its host. Such functions could be changes in parasite morphology and metabolism, acquisition of nutrients from the host, modification of the contents of the phagolysosome, inhibition or destruction of macrophage hydrolases or other effector molecules, interference with macrophage activation or antigen presentation and macrophage lysis and infection of new macrophages.

b) secretion:

Analysis of the predicted LmA600p by von Heijne's method for signal peptide recognition, SIGNALP V1.1 (Nielsen *et al.* 1997) showed a potentially cleavable signal peptide corresponding to amino acids 1 to 42. This potential signal peptide consisted of a polar n-region, followed by hydrophobic h-region from amino acids 26 to 48. The predicted cleavage site at position 42 follows the (-3, -1) rule that states that those residues (valine and

alanine) must be small and neutral. As the predicted polypeptide did not have an anchoring sequence at the carboxyl terminus, the signal peptide would indicate that the polypeptide may be a secreted product of 51 amino acids and with a molecular mass of 5.85 kDa.

The sequence of the predicted LmA600p was compared to the amino terminal sequences of *Leishmania* proteins that require signal peptides, such as the membrane protein *L. major* GP63-1, and proteins that are secreted to the lysosomal compartment: *L. mexicana* Cysteine Proteinase-a and Cysteine Proteinase-b 2.8, as seen in figure 22. The underlined sequences represented conserved amino acids in similar proteins in other *Leishmania* species, as detailed in the figure's legend. These *Leishmania* proteins shared with LmA600p the characteristic of having a signal peptide as predicted by von Heijne's method (Nielsen *et al.* 1997) and all also showed a predicted transmembrane domain near the amino terminus by the Dense Alignment Surface (DAS) method. This domain would correspond to the hydrophobic h-region of the putative signal peptide.

The amino terminus sequence of a larger group of *Leishmania* membrane or secreted proteins was analyzed and found to also share with LmA600p the characteristics of predicted signal peptide and hydrophobic domain by the SIGNAL V1.1 and DAS methods. This group of proteins consisted of the amastigote-specific *L. donovani* A2 protein (Charest & Matlashewski 1994); *L. chagasi* GP46 (Beetham *et al.* 1997); the integral membrane proteins in the endoplasmic reticulum: *L. amazonensis* N-acetylglucosamine-1-phosphate transferase (Lui & Chang 1992), *L. donovani* Galactofuranosyl glycosyl transferase (Ryan *et al.* 1993) and *L. donovani* glucose transporter D1 (Langford *et al.* 1992).

The N-terminus of a second group of *Leishmania* membrane proteins did not show the characteristics of predicted signal peptide and hydrophobic domain by the SIGNAL V1.1 and DAS methods and must therefore use a different type of signal. This second group consisted of: *L. donovani* glucose transporter D2 (Langford *et al.* 1992); the multidrug resistance proteins (P-glycoproteins) of *L. enriettii* (Chow *et al.* 1993) and *L. tarantolae* (Oullette *et al.* 1990); *L. major* promastigote surface antigen 2 precursor (Murray *et al.* 1989); *L. donovani* H^+ transporting ATPases 1A and 1B (Meade *et al.* 1987); *L. enriettii* membrane transport proteins (Stack *et al.* 1990; Cairns *et al.* 1989) and *L. infantum* integral membrane protein (Myler *et al.* 1994).

In conclusion, the amino terminus of LmA600 was similar to that of a large group of *Leishmania* membrane and secreted proteins known to have signal peptides. It was therefore likely that LmA600p possessed a signal peptide and was a secreted polypeptide.

A polypeptide secreted by *Leishmania* amastigotes into the parasitophorus vacuole would be likely involved in a host-parasite interaction. It could be involved in the acquisition of nutrients or in interfering with the macrophage response to the parasite. The polypeptide might be secreted constitutively or be stored in vesicles to be secreted in response to an environmental stimulus. A third possibility is that of being exported to the parasite's lysosomal compartment or megasomes. The LmA600p could then be released into the parasitophorus vacuole when some of the amastigotes were lysed. This had been observed for the amastigote-specific cysteine proteinases, that are megasomal proteins but can be seen in the parasitophorus vacuole and extracellularly in the lesion tissue presumably as a result of macrophage rupture (Ilg *et al.* 1994).

c) abundance:

The concentration of the mRNA of the A600 gene was compared to that of the mRNA from the multiple β-tubulin genes, β-tubulin being one of the most abundant proteins in promastigotes and to a lesser degree, in amastigotes. The comparison was done by RT-PCR by using the same amount of template and differential primers. Aliquots of the reactions were removed at various times of the PCR and analyzed in order to ensure that the amplifications were in the same logarithmic scale.

The RT-PCR analysis, presented in section IV-B-8 above, suggested that the mRNA produced by the single copy gene A600 was more abundant than β-tubulin mRNA in the amastigotes (see figure 24). The A600 mRNA was present in the amastigotes at a concentration in the same order of magnitude as the abundant mRNA produced by multiple β-tubulin genes in the promastigotes (see figure 24). It was much more abundant than the amastigote-specific A850 mRNA (data not shown), that is produced by at least two β-tubulin isogenes (see figure 29). These results suggested that the A600 gene's mRNA was highly abundant in amastigotes. Abundance of its mRNA suggested abundance of the polypeptide it encoded.

d) small polypeptide:

LmA600p was predicted to be translated as a 93 amino acid polypeptide and after removal of the putative signal peptide to had a size of 51 amino acids for a molecular weight of 5.85 kDa (LmA600sp). It was clear that it could not be an enzyme but it might be an enzyme inhibitor. The LmA600sp may be compared with aprotinin, a small polypeptide inhibitor of proteinases in bovine tissues. Aprotinin is translated as a 100 amino acid polypeptide and after removal of the signal peptide and a propeptide domain has an active proteinase inhibitor size of 65 amino acids for a molecular weight of 6.5 kDa (Anderson & Kingston, 1983; Creighton & Charles, 1987).

If these four predictions are confirmed experimentally, a likely hypothesis for the function of the amastigote-specific abundant small polypeptide LmA600sp secreted into the phagolysosomal compartment would be that of an inhibitor of a macrophage hydrolase. This hypothesis can be tested experimentally, as described in section 5b below.

An alternative hypothesis would be that the LmA600sp polypeptide interfered with macrophage activation or with antigen presentation, thus dampening the immune response of the host to the parasite. Such *Leishmania* effects on the macrophage have been reported (Reiner 1994; Boddan & Rollinghoff 1998) and one possible mechanism for them would be for the amastigote to secrete molecules that could either inhibit or misdirect macrophage signaling pathways. A clue to the possible involvement of LmA600sp in such a mechanism was the presence of three potential Casein kinase II (CKII) phosphorylation sites (Pinna, l.A. 1990; Meggio *et al.* 1994) in a thirty amino acid stretch corresponding to the two acid regions of the predicted secreted polypeptide (see figures 20 and 21c). It will be of interest to ascertain experimentally *in vitro* and *in vivo* if the LmA600sp is indeed phosphorylated and if so, if it is phosphorylated by a parasite or a macrophage kinase. It is possible that the LmA600sp is a substrate for the macrophage CKII and by being phosphorylated it may disrupt the normal macrophage signaling. An alternative hypothesis would be that the LmA600sp be an inhibitor of macrophage CKII. The CKII also called protein kinase CK2 is a ubiquitous serine/threonine-specific protein kinase required for viability and for cell cycle progression. The CKII is highly pleiotropic with more than 160 proteins known to be phosphorylated by it (Pinna & Meggio, 1997; Pinna, L.A. 1997; Allende & Allende, 1995). It has been shown that LPS can stimulate CKII activity in macrophages, thus inducing its

phosphorylation of the transcriptional regulatory factor PU.1 and enhancing the capacity of PU.1 to activate transcription in macrophages (Lodie, T.A. *et al.*).

The structure of the predicted secreted polypeptide, with two basic regions flanking the two acid regions that contain the putative CKII phosphorylation sites (see figure 21c) suggests the possibility of the LmA600sp binding strongly to CKII and inhibiting it. The CKII inhibitor hypothesis can be tested experimentally as proposed in section 5b below. The LmA600sp might interfere with macrophage signaling in the parasitophorus vacuole or in the macrophage's cytoplasm. That some *Leishmania* proteins may gain access to the cytoplasm of the macrophage was shown by the fact that *Leishmania* antigens were presented in the macrophage surface in association with MHC class I and must therefore have been processed in the cytoplasm (Kima *et al.* 1997).

4.2.3.- Comparison of the 3' UTR of *A600* to that of other amastigote-specific genes:

The 3' UTR of mRNA has been implicated in the regulation of gene expression through mRNA localization, translation and stability (Wickens *et al.* 1997). The 3' UTR of some *Leishmania* stage-specific genes had been shown to regulate the stage-specific expression of reporter genes transfected into Leishmania. That had been the case for the 3' UTR of the amastigote-specific *L. donovani A2* gene (Charest *et al.* 1996 & Ghedin *et al.* 1998); the stationary phase promastigote-specific *L. chagasi gp63* and *gp46* genes (Ramamoorthy *et al.* 1995; Beetham *et al.* 1997) and the promastigote-specific *L. major gp63-1* gene (Kelly, B. and McMaster, W.R. personal communication). In the kinetoplastid *Trypanosoma brucei*, the 3' UTR has been shown to participate in the stage-specific regulation of the insect stage surface coat procyclin genes and particular regulatory elements within the procyclins' 3' UTR have been identified (Hehl *et al.* 1994; Furger *et al.* 1997 and Schuch *et al.* 1997). Adenylate/uridylate elements were found in the 3' UTR of many mRNA that code for proto-oncogenes, nuclear transcription factors and cytokines in mammalian cells. These motifs determine mRNA stability (Chen & Shyu, 1995).

The RT-PCR analysis suggested that the mRNA produced by the single copy gene *A600* was highly abundant in amastigotes, at a concentration in the same order of magnitude as the abundant mRNA produced by multiple β-tubulin genes in promastigotes. It was much more abundant in the amastigotes than the amastigote-specific A850 mRNA product of at least two

β-tubulin isogenes (data not shown). *Leishmania* transcripts are thought to be polycistronic, with the mRNA produced post-transcriptionally by trans-splicing and poly-adenylation. For the *A600* gene, an abundant mRNA from a *Leishmania* single copy gene strongly suggested an enhanced stability of its mRNA. As the abundance was stage-specific, the suggestion was for a mechanism for differential mRNA stability of its mRNA, either by promoting stability in the amastigote stage or by promoting or targeting its degradation in the promastigote stage. The 3' UTR of the *A600* gene could be involved in this regulation of gene expression via the differential stability of its mRNA. It is here proposed as a working hypothesis that the 3' UTR sequence of the *A600* gene plays a role in the regulation of its amastigote stage specific gene expression. A proposed experimental exploration of the potential regulatory role for the *A600* gene 3' UTR is outlined in section IV-C-5f below.

If it is established that the 3' UTR of the *A600* gene controls the differential stability of the A600 mRNA, that 3' UTR could be used to express other genes specifically in the amastigotes of *L. mexicana*. A similar approach was followed for the 3' UTR of the amastigote-specific *A2* gene family in *L. donovani*. The 3' UTR of the *A2* genes plus a trans-splicing site was reported to increase the stability of reporter gene transcripts in culture amastigotes but not promastigotes (Charest *et al.* 1996). In a later study, suicide genes were inserted between *A2* non-coding regions to up-regulate their expression in amastigotes. The level of expression of the toxic genes was increased in amastigotes, although it was not completely amastigote-specific (Ghedin *et al.* 1998a). This is the only reported *Leishmania* amastigote-specific gene whose 3' UTR has been tested for a regulatory function. Furthermore, if the predictions about the A600 protein, being a secreted and abundant amastigote-specific polypeptide, are confirmed, a system would be available to use transfected *L. mexicana* for efficiently delivering large amounts of a desired polypeptide to the parasitophorous vacuole of *in vitro* or *in vivo* infected macrophages. Such a delivery system could form part of a vaccine design for leishmaniasis. The gene coding for an immuno-protective antigen fragment would be fused with the sequence coding for the A600 signal peptide and to the *A600* gene 3'UTR and inserted into the genome of *L. mexicana*. Ideally the parasite would be an attenuated strain, such as the *L. mexicana* Δcpa/cpb null mutants for cysteine proteinase (Mottram *et al.* 1996). Infections with the mutant Δcpa/cpb did not induce lesion growth in BALB/c mice and were associated with a Th1-type immune

response (Alexander *et al.* 1998). The attenuated Δcpa/cpb mutants would have two potential amastigote-specific delivery systems for abundant protective antigens: secretion into the megasomes using *Lmcpb* regulatory sequences and into the parasitophorous vesicle using *A600* regulatory sequences. Being infective as metacyclic promastigotes but not virulent as amastigotes, such parasites would transform the infected parasites into efficient antigen presenting cells. The immuno-protective *Leishmania* antigens to be delivered would be chosen by the characteristic of being abundantly expressed in the amastigotes of several species of *Leishmania* that cause human leishmaniasis. The candidate antigens should also not be virulence factors or interfere with the host immune system. It is at present not known if the A600 protein will fulfill such conditions. A promising candidate would be a mutant GP63 whose active site was rendered inactive by site directed mutagenesis (McMaster *et al.* 1994). Such protein should not be a virulence factor but should still be immunogenic. If an enzymatically inactive *Leishmania* cysteine proteinase could be similarly produced, it would be a good antigen candidate since cysteine proteinases appear to be abundant in the amastigotes of several species of *Leishmania* and are immunogenic (Rafiti *et al.* 1997; Beyrodt *et al.* 1997).

One output of this thesis work was the sequence of large fragments of the 3' UTR of two amastigote-specific genes: *A600*, 1961 bp out of an estimated 2600 bp 3' UTR and the *A850* β-tubulin isogene, 750 bp out of an estimated 1300 bp 3' UTR.

The sequence of the *A600* 3' UTR was compared to that of other reported amastigote-specific genes. Of particular interest was the comparison with the 2037 nt 3' UTR of the *L. donovani* amastigote stage-specific *A2* genes, where a regulatory role for its 3' UTR was established experimentally. The other amastigote-specific gene's 3' UTR used in the comparison with the *A600* 3' UTR were the *A850* β-tubulin (see figure 26); the *L. chagasi* cysteine proteinase 1 (Ldchcys1) with 815 nt of 3' UTR sequence (Omara-Opyene and Gedamu 1997) and the *L. pifanoi* cysteine proteinase 2 (Lpcp2) with 1161 nt of 3' UTR sequence (Boukai 1993).

Comparison between the *A600* 3' UTR and the *A2* 3' UTR by local alignment revealed the sequence CACCUCACC repeated twice in the *A600* 3' UTR at positions 660 and 1924 bp (see figure 19) and present once in the complete *A2* 3' UTR at position 1445 (Charest &

Matlashewski 1994). This sequence was however not present in the available 3' UTR sequences of the other amastigote-specific genes, so its significance remained doubtful.

One interesting feature revealed by the local alignment analysis was the presence of dinucleotide repeats in the 3' UTR, usually three or more tandem repeats of UG. They were not however related to amastigote-specific expression as they were also present in the 3' UTR of promastigote-specific genes and of constitutively expressed genes (see Appendix II). These repeats were too numerous in *Leishmania* 3' UTR to be random sequences so they probably had a function that was selected for.

It is expected that as more *Leishmania* 3' UTR sequences become available, especially those from regulated genes, their comparison will reveal conserved sequence motifs or RNA secondary structures that could be involved in the regulation of gene expression.

4.2.4.- Proposed future work on A600:

The questions that the A600 gene raises are the following: Is the A600 protein secreted into the phagolysosome of the infected macrophage? Is it an abundant protein in that compartment? What is the biological function of the A600 protein, is it an inhibitor of macrophage function? What is the role the A600 protein in the parasite's survival, its relation with the host and in pathogenesis? Is A600 a feature of other Leishmania species? Is the A600 protein a suitable candidate as antigen for vaccine development? Is the 3' UTR of the A600 mRNA responsible for the stage specific expression of the gene? Framing these questions suggests avenues for experimental exploration.

a) Localization and abundance of the A600 polypeptide:

The first priority is to identify the protein product of the A600 gene and to determine its abundance and localization. One approach would be to produce recombinant A600 protein in *E. coli* (Button *et al.*, 1991), both of the full 93 amino acid polypeptide (rA600p) and of the predicted 51 amino acid secreted polypeptide (rA600sp). The recombinant polypeptides would be purified and injected into laboratory animals to raise specific antibodies, preferentially monoclonal antibodies. Because of the small size of the A600 polypeptide, especially in its putative secreted form, and its apparent simplicity it is predicted that the recombinant rA600sp is likely to retain the biological function of the normal gene product

(LmA600p). Furthermore it is predicted that some monoclonal antibodies would recognize the native protein LmA600p by immuno-histology, while others would recognize the partially denatured polypeptide in Western blots. The monoclonal antibodies would be used to absorb potentially secreted A600 polypeptide from the supernatant of amastigotes grown in axenic culture. The secreted polypeptide would then be analyzed by N-terminal protein sequencing to determine the cleavage point under natural conditions. The mAb would be used for Western blot of both amastigotes and promastigotes to identify the natural product of the A600 gene, its size and its relative abundance as compared with a positive control, GP63 detected with the cross-reacting mAb 235.

The mAb would also be used to perform immuno-histological staining in macrophages infected with *L. mexicana* in *in vitro* culture and in biopsies of the lesions of experimentally infected BALB/c mice, the rationale being that a secreted protein may be secreted constitutively or in response to an environmental challenge. The amastigote-specific A2 proteins from *L. donovani* for example were predicted to be secreted (Charest & Matlashewski 1994). They were not detected in the supernatant of cultured amastigotes but were detected in the cytoplasm of the parasites by fluorescence microscopy (Zhang *et al.* 1996). It may be possible that they were present in secretory vesicles and that they were not secreted in axenic culture. The immuno-histological approach outlined for the A600 protein should give a more detailed picture. The immuno-histology would be both by light microscopy, either antibodies that were fluorescent or chromogenically labeled, and electron microscopy using immuno-gold complexes. The monoclonal antibodies against native *L. mexicana* GP63, mAb 12G6 and 14F11 produced in the present work, should provide a useful positive control both in terms of cellular localization and relative abundance. In *L. mexicana* amastigotes, GP63 has been reported as being mostly in the megasome (Bahr *et al.* 1993) or in the flagellar pocket, with a small proportion on the cell surface (Medina-Acosta *et al.* 1989).

b) Search for the function of the A600 polypeptide:

The hypothesis that the A600 predicted secreted polypeptide could be an inhibitor of an hydrolytic enzyme present in the phagolysosome of the macrophage could be tested experimentally. *In vitro* assays of enzymes that resemble those present in phagolysosome

(Antoine *et al.* 1998) would be performed in the presence or absence of recombinant rA600sp. It would be assumed that the recombinant rA600sp has similar biological activity as the normal parasite secreted polypeptide. Enzyme kinetic analysis of the results of the assays would show if rA600sp acts as an inhibitor.

The hypothesis that the A600 predicted secreted polypeptide could be an inhibitor of a macrophage Casein kinase II would be tested experimentally by adding rA600sp to an *in vitro* assay of the commercially available enzyme and an synthetic substrate (Marin *et al.* 1994). A possible effect of rA600sp on signal transduction on macrophages could also be tested more generally by measuring its effect on *in vitro* assays of protein phosphorylation by macrophage cell extracts.

c) Role of the A600 polypeptide in parasite viability and infectivity:

Being amastigote-specific and apparently abundant, it is probable that the A600 gene has an important function. It may be vital for the amastigote under natural conditions. A parallel could be drawn to the report that the inhibition of the expression of another amastigote-specific gene, A2 in *L. donovani* by anti-sense RNA severely reduced that parasite's virulence in mice. The only amastigotes that survived in mice had restored A2 protein expression (Zhang and Matlashewski 1997). Similarly, the deletion of the mainly amastigote-specific *lmcpb* gene array of *L. mexicana* demonstrated that the megasomal cysteine proteinases were virulence factors (Mottram *et al.* 1996).

To determine if the A600 gene is required for amastigote survival and infectivity, the gene would be removed from the genome by targeted gene deletion producing A600 null mutants. This work would be aided by the fact that A600 is a single copy gene, two per diploid *Leishmania* genome. A second advantage is that because it is an amastigote-specific gene, it is very likely that it can be deleted in the promastigotes without suffering any negative selective pressure. The mutant phenotype would only be made manifest when the parasites are converted to amastigotes by changing the temperature and pH of the culture. In that sense, the A600 null mutants would be the functional equivalent of conditional mutants.

Two constructs, each with an antibiotic resistance selective marker would be separately inserted between the DNA fragments that flank the A600 coding sequence. One construct would be used for each round of gene knockout, as described by Joshi *et al.* (1998). The 3'

flanking sequence could be the S600 or the S800 cDNA fragments, part of the 3' UTR of the A600 gene (see figure 19). The required 5' flanking sequence would be identified from restriction enzyme digested *L. mexicana* genomic DNA by Southern blot using as probe the cDNA corresponding to the 5' UTR of the A600 mRNA. This probe can be prepared by digesting an A600 cDNA clone with Hha I at bp11 and Bcl I at bp 97 (figure19). These enzymes cut after the B-SL primer and just before the start codon and a 89 bp A600 5' UTR fragment can be purified from an agarose gel. A suitably sized clone of the genomic DNA region upstream of the A600 coding sequence can then be selected from a size fractionated DNA library of *L. mexicana* by colony hybridization with the same probe.

The constructs would be transfected into *L. mexicana* promastigotes in two rounds of gene knockout and antibiotic resistant clones analyzed by Southern blot with an A600 coding region probe (Bcl I/ Hinc II digest of A600 cDNA clone) and with probes consisting of the antibiotic resistance gene sequences. The A600 gene coupled to a third selective antibiotic resistance gene could be reintegrated into its locus in order to assess later if any lost phenotype could be rescued.

The null mutants would be converted to amastigotes in culture. Failure to sustain growth as axenic culture amastigotes would signify that the A600 protein is essential for that stage. It is possible that the null mutants may grow as axenic amastigotes but not infect macrophages or survive in the hostile environment of their phagolysosomes. The wild type *L. mexicana* would be compared with the A600 null mutant for their infectivity of cultured macrophages and for their ability to produce lesions on BALB/c mice.

d) Possible presence of A600 in other species of *Leishmania*:

The possible presence of the A600 gene in other species of *Leishmania*, especially those of public health interest, would be determined by performing a Southern blot on their DNA with a probe consisting of the coding sequence of the *L. mexicana* A600 gene. This sequence is much more likely to be conserved across species lines than the 3' UTR. The probe would be prepared by digesting a clone of the A600 cDNA with the restriction enzymes Bcl I and Hinc II which cut just before the start codon and after the stop codon respectively of the cDNA for A600.

The next step would be to determine if the A600 gene is also amastigote-specific in other *Leishmania* species that are shown to possess a similar gene. An ELISA can be used to

determine if the recombinant rA600sp is recognized by the sera of patients with Leishmaniasis caused by various species. This would imply that it was expressed in their amastigotes *in vivo*. This approach was used for the amastigote-specific *L. donovani* A2 proteins, whose gene locus was present exclusively in parasites of the *L. donovani* and *L. mexicana* families and also recognized only by the sera of patients infected with those parasites (Ghedin, E. *et al.* 1998a and b).

Assessing stage specific expression would require growing axenic cultures of both promastigotes and amastigotes from the selected *Leishmania* species and measuring their A600 gene expression by either Western blot using mAb or Northern blot by using the *L. mexicana* A600 gene coding sequence as probe.

e) Evaluation of the A600 polypeptide as a candidate for immuno-prophylaxis:

The most important application of the study of *Leishmania* is without doubt the development of a useful human vaccine. In this quest it is of value to identify and purify potential candidates for protective antigens. These antigens should be abundant in the amastigote stage and different from host proteins, characteristics predicted for the putative secreted polypeptide product of the A600 gene.

To determine if the A600 polypeptide is a prominent antigen during human Leishmaniasis, the recombinant rA600p and rA600sp would be tested in an ELISA with sera from patients with Leishmaniasis, of those individuals that have recovered from the disease and of negative controls. The same recombinant polypeptides would be used to stimulate *in vitro* the T cells of individuals from those groups. For these experiments, peripheral blood mononuclear cells (PBMNC) would be isolated and stimulated with various concentrations of the recombinant proteins in microtiter plates. The stimulation would be measured by increased incorporation of ^{3}H-Thymidine. The supernatant of the stimulated PBMNC would be tested for the presence of the cytokines gamma interferon, interleukin 4 and interleukin 10 by ELISA. Alternatively, RNA would be prepared from the cells and RT-PCR for those cytokines performed. The stimulation of gamma interferon production would be an indication of a TH1 type response that would be desirable in a potential vaccine candidate for a parasitosis while the stimulation of interleukin 4 would be an indication of a TH2 type response.

The recombinant polypeptides rA600p and rA600sp would also be used to immunize mice to assess if they are protected from a later challenge with *Leishmania*.

In all these experiments, recombinant GP63 from *L. major* (Button *et al.* 1991) would be used as a positive control.

f) Analysis of the role of the A600 3' UTR in amastigote-specific gene expression:

The ability of the A600 3' UTR to regulate the expression of a reporter gene in a stage specific fashion would be tested. A genomic DNA fragment consisting of the 3' UTR of the A600 gene plus the trans-splicing site of the adjacent gene, a fragment of approximately 2.7 to 3 kbp, would be isolated from a DNA library. It would be then linked to a reporter gene in a plasmid suitable for *Leishmania* transfection (Joshi *et al.* 1995), that contained a constitutively expressed antibiotic resistance selection gene. *L. mexicana* promastigotes would then be transfected and stable transfected clones selected with the antibiotic. The level of expression of the reporter gene in both culture promastigotes and amastigotes would be measured by Northern blot using the reporter gene DNA as probe.

If a role for the A600 3' UTR in amastigote stage-specific mRNA concentration is proven, further studies would be performed to reveal the regulation mechanism. The concentration of the reporter gene's mRNA measured by Northern blot would be compared with its transcriptional level measured by nuclear run-on analysis to determine if the regulation is post-transcriptional. This comparison had led to the conclusion that the amastigote-specific *L. donovani* A2; the promastigote-specific *L. mexicana* glucose transporter 2 and the stationary phase promastigote-specific *L. chagasi* gp63 and gp46 genes were post-transcriptionally regulated, probably by differential mRNA stability (Charest *et al.*, 1996; Burchmore et. al., 1998; Ramamoorthy *et al.*, 1995, Beetham *et al.* 1997).

4.2.5.- Amastigote-specific β-tubulin isogene:

A850, an amastigote-specific β-tubulin isogene was identified and isolated by its unique 3' UTR (see figure 14). This gene produced a single mRNA as judged by Northern blot (see figure 16) and was the product of at least two of the multiple genes that code for β-tubulin (see figure 29).

In eukaryotes, families of tubulin genes give rise to multiple isoforms of tubulin. The synthesis of isotubulins can be spatially and temporally regulated producing different tubulins within an organism (McRae & Langdon 1989). Vertebrates have six classes of β-tubulin isotypes, each displaying a distinct pattern of expression (Haber *et al.* 1995). Examples are the variation of the expression of tubulin isogenes during mammalian neural development (Oblinger & Kost 1994) and during the cell cycle (Dumontet *et al.* 1996). The products of different tubulin isogenes are thought to produce different function and β-tubulin isoforms have been found not to be functionally equivalent (Hoyle & Raff 1990).

Changes in gene expression of β-tubulin during their life cycle had been reported for several species of *Leishmania.* β-tubulin was more abundant in promastigotes than amastigotes, probably reflecting the presence of the flagellum in promastigotes (Fong & Chang 1981; Landfear & Wirth 1984). In *L. mexicana*, the major β-tubulin mRNA of axenic culture amastigotes and amastigotes recovered from infected macrophages was a 2800 nucleotides (nt) species, while a 2400 nt species predominated in promastigotes (Burchmore *et al.* 1998). In *L. amazonensis*, the predominant promastigote mRNA species was 2800 nt, with other 3600 and 4400 nt species present and the amastigote had a single 3600 nt species (Fong *et al.* 1984).

In *L. major* the predominant mRNA was 2200 nt in log phase promastigotes, 3200 nt in stationary phase metacyclic promastigotes and 2800 nt in amastigotes. These mRNA are the products of different genes that had highly divergent 3' UTR. Both the promastigote 2200 nt mRNA and the amastigote 2800 nt mRNA were produced by tandem arrays of multiple β-tubulin genes located in different chromosomes in *L. major* (Coulson *et al.* 1996). The protein sequences for one clone of each of these two stage-specific mRNA sizes was available and compared with that of a clone of a β-tubulin gene in *L. amazoniensis* (Fong & Lee 1988) and with that of the A850 gene sequence (see figures 26 and 28).

The amino acid sequence of the *L. mexicana* amastigote-specific A850 β-tubulin isogene differed from that of either the reported *L. major* amastigote-specific 2.8 and promastigote-specific 2.2 β-tubulin genes in only eleven positions (see figure 28).

A850 differed from both *L. major* genes in two positions: 385 and 440. A850 differed from the promastigote-specific *L. major* gene 2.2 and presented the same amino acid as the amastigote-specific 2.8 gene in four positions, mostly in the amino terminus half of the molecules: 25, 35, 55 and 280. It differed from the amastigote-specific *L. major* gene 2.8 and presented the same amino acid as the promastigote-specific 2.2 gene in five positions, mostly in the carboxyl terminus half of the molecules: 260, 324, 345, 365, 367.

Five of those eleven differences were for similar amino acids: the polar amino acids threonine for serine at position 35; the hydrophobic amino acids phenylalanine for leucine at position 260, isoleucine for leucine at position 345 and valine for alanine at position 365 and the basic amino acids lysine for arginine at position 324. Those changes for similar amino acids were unlikely to produce changes in the protein's conformation and therefore unlikely to account for differential biological activity.

The six positions where there was a change to a different type of amino acid were the following: In position 440, near the carboxyl terminus, where A850 presented the neutral polar amino acid glutamine (Q) instead of the acidic amino acid glutamic acid (E) as found with the other isogenes. This change occurred in the middle of a cluster of four consecutive glutamic acids encoded in the other genes. A850 differed from both *L. major* proteins in position 385, where it presented the hydrophobic amino acid phenylalanine (F), similar to the hydrophobic amino acid leucine (L) for the promastigote-specific *L. major* 2.2 molecule but different from the basic amino acid arginine (R) for the amastigote-specific *L. major* 2.8 isoform. A850 also differed with the amastigote-specific *L. major* 2.8 molecule in position 367, where it presented the hydrophobic amino acid phenylalanine instead of the polar amino acid serine. A850 differed from the promastigote-specific *L. major* 2.2 protein in positions 25 and 55 presenting the polar aminaoacids serine (S) and threonine (T) instead of the hydrophobic amino acid alanine (A) and in position 280, where it presented the polar neutral amino acid glutamine (Q) instead of the acidic amino acid glutamic acid (E).

The *L. amazoniensis* β-tubulin gene was similar to the promastigote-specific *L. major* 2.8 gene, differing significantly from that gene, and from the amastigote-specific *L. mexicana* A850 and *L. major* 2.8 genes, in only two positions in the carboxyl terminus of the proteins

coded: arginine for alanine in position 393 and phenylalanine for tyrosine in position 436. It also encoded two additional amino acids (see figure 28).

In conclusion, the *L. mexicana* amastigote-specific A850 isogene differed significantly in its coded amino acid sequence from a *L. major* amastigote-specific 2.8 isoform in three positions: 367, 385 and 440. It differed significantly from a *L. major* promastigote-specific 2.2 isoform in four positions: 25, 55, 280 and 440. It differed significantly from a *L. amazonensis* protein in six positions: 25, 55, 280, 393, 436 and 440.

Based in this comparison, the A850 isogene did not appear to be the *L. mexicana* equivalent of either of those other three *Leishmania* isogenes. The results of the RT-PCR (see figure 30) suggested that the A850 was not the only amastigote-specific isogene in *L. mexicana.* It is possible that another *L. mexicana* amastigote-specific isogene exists that more closely resembles the sequence and the specific function of the *L. major* amastigote-specific isogene 2.8 clone. There could also be another 2800 nt mRNA species in *L. major* amastigotes whose coding sequence resembles that of the *L. mexicana* A850 isogene mRNA. It is hoped that when the sequences of more *Leishmania* stage-specific β-tubulin isogenes are known, it will be possible to assign them to functional groups based on amino acid changes at a few critical positions.

β-tubulin interacts with alpha tubulin to form heterodimers and with other heterodimers to form protofilaments and microtubules. It also interacts with microtubule-associated proteins (MAPs) and GTP. Some of the domains or sequences involved in these interactions have been identified by mutational analysis in various species (Savage *et al.* 1994; de la Vina et al. 1988; Farr & Sternlicht 1992). The major variable regions within the vertebrate β-tubulin isotypes were found in the carboxyl terminus beyond residue 430 (Sullivan 1988). The small carboxyl terminal region was required for the binding of MAPs (Cross *et al.* 1991) Comparing the reported functional sequences with the variable amino acid positions in *Leishmania* β-tubulin, they do not appear to correlate, with the exception of the A850 variant amino acid in position 440 (see figure 28), in what is the most variable region for β-tubulin isotypes.

The available 3' UTR sequence of the amastigote specific *L. mexicana* A850 β-tubulin isogene, 750 nt out of an estimated 1300 nt 3' UTR, was compared with that of the only other reported substantial 3' UTR sequence of a *Leishmania* β-tubulin gene, the 739 nt 3' UTR of the *L. major* promastigote-specific 2.2 gene (Coulson *et al.* 1996). The *L. major* gene 2.2 contained a motif named 'RNA zipcode' that localized β-actin mRNA within fibroblasts (Kislauskis *et al.* 1994). The "RNA zipcode' was not found in the available 3' UTR sequence of A850. The only similarity found by local alignment between the two 3' UTR was in the conserved 3' UTR region immediately after the stop codon (see figure 27).

There were conserved non-coding sequences just before the start codon and after the stop codon of A850 and other reported *Leishmania* β-tubulin genes (see figure 27). A much less significant degree of conservation was found for the 5' UTR sequence of a *L. enriettii* β-tubulin gene (Landfear et al. 1986) that had only a 40% identity over the 63 nt region that had a 81% identity between the *L. mexicana* A850, the *L. major* 2.2 and 2.8 and a *L. donovani* β-tubulin isogenes (see figure 27). While the conserved 3' UTR was sequenced in only A850, one of probably several β-tubulin isogenes in *L. mexicana*, it was suggested from an RT-PCR experiment (see figure 30) that most of its β-tubulin isogenes must present the conserved 3' UTR segment sequence. The concentration of the PCR product (b-tub 2 in figure 30) using a primer that binds in that 3' UTR sequence was very similar to that of the product (b-tub 1) using primers in the conserved coding region. This degree of conservation of non-coding sequences across species lines might imply that there was a function for them, but did not correlate with the expression of the genes in one particular stage of the parasite's life cycle (see figure 27). One possible function for the conserved sequences flanking the coding sequence of the multiple copy *Leishmania* β-tubulin genes was that they were used for gene duplication by recombination.

To identify and isolate the other *L. mexicana* β-tubulin isogenes, the following approach is proposed. The amastigote and promastigote single stranded cDNA would be used as templates for PCR. The PCR primers would be: DScDNA that binds in what corresponded to the Poly A tail of all the mRNAs (see figure 2) and the 850S primer that binds in a conserved region of the coding sequence of *Leishmania* β-tubulin (see figure 26). It would be expected that all the β-tubulin isogenes would be amplified, those promastigote-specific,

those amastigote-specific and those constitutive. It would also be expected that the sequence of 3' UTR of the various isogenes will be divergent. The PCR products would be visualized by Ethidium bromide staining after agarose gel electrophoresis and the bands excised from the gel, purified and cloned. Clones corresponding to each band would be partially sequenced in both directions. The partial sequence in one of the directions would determine if the clone corresponded to a β-tubulin gene. The partial sequence in the other direction would provide the sequence of the end of its 3' UTR. Unique 3' UTR partial sequences would be used to cluster the clones into the various isogene groups. Isogene specific probes would be constructed from unique 3' UTR sequences and used to probe Northern blots of amastigote and promastigote RNA to determine the stage at which each isogene is expressed and the relative abundance of each isogene's mRNA. One clone from each of the isogenes would be completely sequenced and the amino acid sequences compared as shown in figure 28, to define the variable amino acid positions. This would create the basis for a functional analysis of critical amino acids by site-directed mutagenesis studies. Similarly, the complete 3' UTR from one clone corresponding to each isogene group would be completely sequenced. The sequences of the 3' UTR of the various β-tubulin isogenes would be compared by local alignment. This comparison could reveal conserved sequence motifs or RNA secondary structures that could be involved in the regulation of gene expression.

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APPENDIX I

Leishmania major **Codon Usage Table**
126 CDS's (63481 codons)

	order of use		percentage of use
Phenylalanine (F)	2	UUU (6.9)	24%
(28.6)	1	UUC (21.7)	76%
Leucine (L)	6	UUA (0.8)	1%
(87.3)	3	UUG (8.0)	9%
	3	CUU (8.0)	9%
	2	CUC (25.7)	29%
	5	CUA (3.3)	4%
	1	CUG (41.5)	48%
Isoleucine (I)	2	AUU (6.3)	19%
(32.7)	1	AUC (24.4)	75%
	3	AUA (2.0)	6%
Methionine (M)		AUG (23.7)	100%
Valine (V)	3	GUU (7.0)	9%
(74.0)	2	GUC (23.5)	32%
	4	GUA (4.1)	6%
	1	GUG (39.4)	53%
Serine (S)	4	UCU (7.0)	9%
(80.6)	3	UCC (16.6)	21%
	6	UCA (4.8)	6%
	2	UCG (20.9)	26%
	5	AGU (5.4)	7%
	1	AGC (25.9)	32%
Proline (P)	4	CCU (6.5)	11%
(58.4)	2	CCC (12.4)	21%
	3	CCA (9.7)	17%
	1	CCG (29.8)	51%
Threonine (T)	4	ACU (4.5)	8%
(59.9)	2	ACC (18.4)	31%
	3	ACA (8.1)	14%
	1	ACG (28.9)	48%

Alanine (A)	4	GCU (14.2)	12%
(119.6)	2	GCC (39.5)	33%
	3	GCA (17.9)	15%
	1	GCG (48.0)	40%
Tyrosine (Y)	2	UAU (2.9)	12%
(24.1)	1	UAC (21.2)	88%
Stop		UAA (0.7), UAG (0.5), UGA (0.7)	
Histidine (H)	2	CAU (5.7)	20%
(28.1)	1	CAC (22.4)	80%
Glutamine (Q)	2	CAA (4.9)	13%
(38.6)	1	CAG (33.7)	87%
Asparagine (N)	2	AAU (3.9)	14%
(27.3)	1	AAC (23.4)	86%
Lysine (K)	2	AAA (3.3)	9%
(35.4)	1	AAG (32.1)	91%
Aspartic Acid (D)	2	GAU (12.3)	24%
(51.5)	1	GAC (39.2)	76%
Glutamic Acid (E)	2	GAA (8.1)	15%
(55.8)	1	GAG (47.7)	85%
Cysteine (C)	2	UGU (3.5)	18%
(19.9)	1	UGC (16.4)	82%
Tryptophan (W)		UGG (10.7)	100%
Arginine (R)	3	CGU (8.7)	12%
(70.1)	1	CGC (36.3)	52%
	4	CGA (5.7)	8%
	2	CGG (13.3)	19%
	6	AGA (1.7)	2%
	5	AGG (4.4)	6%
Glycine (G)	2	GGU (13.2)	19%
(71.3)	1	GGC (42.1)	59%
	4	GGA (5.2)	7%
	3	GGG (10.8)	15%

APPENDIX II

UGUGUG MOTIF IN THE 3' UTR OF LEISHMANIA MESSENGER RNA

AMASTIGOTE SPECIFIC MESSENGER RNA:

A600: 1961 nt 3' UTR:

291 299 501 506
.....**GUGUGUGUGG**.....**GUGUGUG**CGCCAGUCUCUCUGCCGAGUUCUCGAUGU
941 946
CGGUGCGUGUGUA.....**UGUGUG**CUCAUGCCACUGAACAGUUGAUUGAGAGA
976 983
UGUGUGUGGGUCUAG.....

Ld A2: 2036 nt 3' UTR:

165 172 547 552
.....GUGGGG**UGUGUGUGA**.....UGUGCC**UGUGUG**GGCUGAUGA.....UGC
1830 1836
GUGUGUGCGUUGA

Ldchcys1: 815 nt 3' UTR:

32 50
.....GUGUGGGUGGCGU**UGUGUGUGUGUGUGUGUGG**GUG.....GUGC
757 764
GUGUGUGUGCGUGCGUGUGCGUGA.....

Lpcp2: 1161 nt 3' UTR:

4 11 16 21 23 30 399 405
AGG**UGUGUGUG**CCCU**UGUGUGCUGUGUGUG**GGUGG.....**UGUGUG**CUGUGC.....

Lmabt1 Promastigote specific: 739 nt 3' UTR

5 10
ACAG**UGUGUG**GGUGAGGUGCGCGAAGGUGUGUCUGUCGGUGGGGGAGCUCGCGC
57 62 151 158
GG**UGUGUG**.....**UGUGUGUG**.....

Ldchgp63S: Stationary Phase Promastigote specific: 1038 nt 3' UTR

488 494 581 586 781 786
.....**GUGUGUGUG**GGUGUG.....**UGUGUG**UUGU.....GUUGUGU**UGUGUG**.....
822 826
.....UGUGCGCGUC**UGUGUG**CGGAGCUGUG.....