

UBIQUITIN GENE EXPRESSION DURING  
DIFFERENTIATION OF LEISHMANIA MAJOR

by

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## ABSTRACT

Leishmania major (L. major) is an intra-macrophage protozoan parasite which differentiates from a promastigote to an amastigote upon transmission from its insect vector at 25°C to its mammalian host at 37°C. This temperature shift occurs in the same range as that used to elicit the heat shock response in prokaryotes and higher eukaryotes in which the induction of genes encoding heat shock proteins is seen. Ubiquitin is a heat inducible protein and one of the most conserved eukaryotic proteins known. Genomic libraries made from L. major DNA were initially screened with the ubiquitin gene from yeast. DNA sequence analyses of positive clones revealed at least 5 ubiquitin coding elements arranged head to tail without intervening sequences. The predicted protein sequence showed that ubiquitin in Leishmania differs from that of yeast and barley at 5 out of 76 amino acid positions and from that of human at only 2 positions. Further characterization revealed another ubiquitin encoding locus believed to carry only one ubiquitin encoding element. Comparisons of ubiquitin mRNA levels from L. major grown at 26°C, 37°C, and 42°C suggest that ubiquitin gene expression in these particular parasites is constitutive and that prolonged exposure at a non-lethal temperature results in a reduction of ubiquitin-specific mRNA. However, a direct correlation between parasite differentiation and ubiquitin gene expression was not defined as it could not be determined whether the described experimental conditions actually established differentiated states of L. major.

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**LIST OF ABBREVIATIONS**

ATP	riboadenosine 5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
CFU	Colony Forming Units
cpm	counts per minute
DEP	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dATP	deoxyriboadenosine 5'-triphosphate
ddATP	dideoxyriboadenosine 5'-triphosphate
dCTP	deoxyribocytidine 5'-triphosphate
ddCTP	dideoxyribocytidine 5'-triphosphate
dGTP	deoxyriboguanosine 5'-triphosphate
ddGTP	dideoxyriboguanosine 5'-triphosphate
dTTP	deoxyribothymidine 5'-triphosphate
ddTTP	dideoxyribothymidine 5'-triphosphate
dNTP	deoxyribonucleoside 5'-triphosphate
ddNTP	dideoxyribonucleoside 5'-triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
EtOH	ethanol
gm	gram(s)
GuHCl	guanidine hydrochloride
GuSCN	guanidine monothiocyanate
IPTG	isopropylthiogalactoside



kb	kilobase pair(s)
kd	kilodalton(s)
LiCl	lithium chloride
l	litre(s)
LB	Luria-Bertani
ug	microgram(s)
ul	microlitre(s)
um	micrometer(s)
mg	milligram(s)
ml	millilitre(s)
MOPS	3-(N-morpholino)propanesulfonic acid
ng	nanogram(s)
NH <sub>4</sub> Ac	ammonium acetate
NaAc	sodium acetate
PBS	phosphate buffered saline
PEG	polyethylene glycol
PFU	Plaque Forming Units
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
Tris	tris(hydroxymethyl)aminomethane
U	units
UT	untranslated
UV	ultraviolet
V	volts
W	watts
X-gal	5-dibromo- 4-chloro 3-indolylgalactoside

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## INTRODUCTION

### I. THE BIOLOGY OF LEISHMANIA

Leishmania, the etiologic agents of leishmaniasis, are obligate intra-macrophage protozoan parasites. They are members of the trypanosomatidae family and may have evolved from lower insect trypanosomes (McGhee and Cosgrove, 1980). Of the numerous species capable of infecting mammals, at least 14 can cause disease conditions in man (Wirth et al., 1986).

Leishmaniasis is largely a zoonotic disease, but it can be transmitted to humans from various rodent and canine reservoirs by the blood-sucking phlebotomine sandfly. Although accurate data on the world-wide incidence of leishmaniasis are unavailable, estimates suggest there may be as many as 12 million people infected (Walsh and Warren, 1979) and 400,000 new cases arising annually (Handman, 1986). The disease is pandemic in regions, particularly the tropics and sub-tropics, where the insect vectors breed successfully.

The clinical manifestation of leishmanial infection has long been recognized as complex and diverse. Since the disease outcome is largely dependent on the leishmania species as well as the immune status and competence of the host, the concept of a disease spectrum was introduced in the 1960's (Turk and Byrceson, 1971). This spectrum accommodates the varying degrees of immunoreactivity and histopathology among infected individuals as exemplified by the three main clinical forms of the disease: simple cutaneous leishmaniasis (SCL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL). SCL involves a skin lesion or ulceration that is usually self healing whereas MCL involves the dissemination of parasites to the nose, mouth and pharynx resulting in erosion of the cartilage and soft tissues in these regions. The latter often occurs as a relapse after a primary SCL infection. VL or kala-azar, however, involves slow parasite dissemination to the internal organs which is fatal if untreated. Table I lists several leishmania species and the disease they cause. In general, the clinical classification describes the site of leishmania-infected macrophages.

TABLE I. Major Leishmania species causing human disease.

Disease	Species	Geographical Location
<b>Cutaneous leishmaniasis</b>		
Simple	<u>L. mexicana mexicana</u>	Mexico, Central America
cutaneous	<u>L. mexicana amazonensis</u>	Brazil, Amazon region
leishmaniasis	<u>L. mexicana pifanoi</u>	Venezuela
	<u>L. major</u>	Southern U.S.S.R., Middle East
	<u>L. tropica</u>	Asia, Southern Europe, Northern and western Africa
	<u>L. braziliensis guyanensis</u>	North and South America
	<u>L. braziliensis panamensis</u>	Central America
	<u>L. braziliensis peruviana</u>	Peru
Diffuse	<u>L. mexicana amazonensis</u>	Brazil, Amazon region
cutaneous	<u>L. aethiopica</u>	Ethiopia and Kenya
disease		
Mucocutaneous	<u>L. braziliensis</u>	Western and northern
disease	<u>braziliensis</u>	South America
<b>Visceral leishmaniasis</b>		
	<u>L. donovani</u>	India, Africa
	<u>L. donovani infantum</u>	Mediterranean area
	<u>L. chagasi</u>	Northern, South America

Source of data: Wirth et al., (1986).

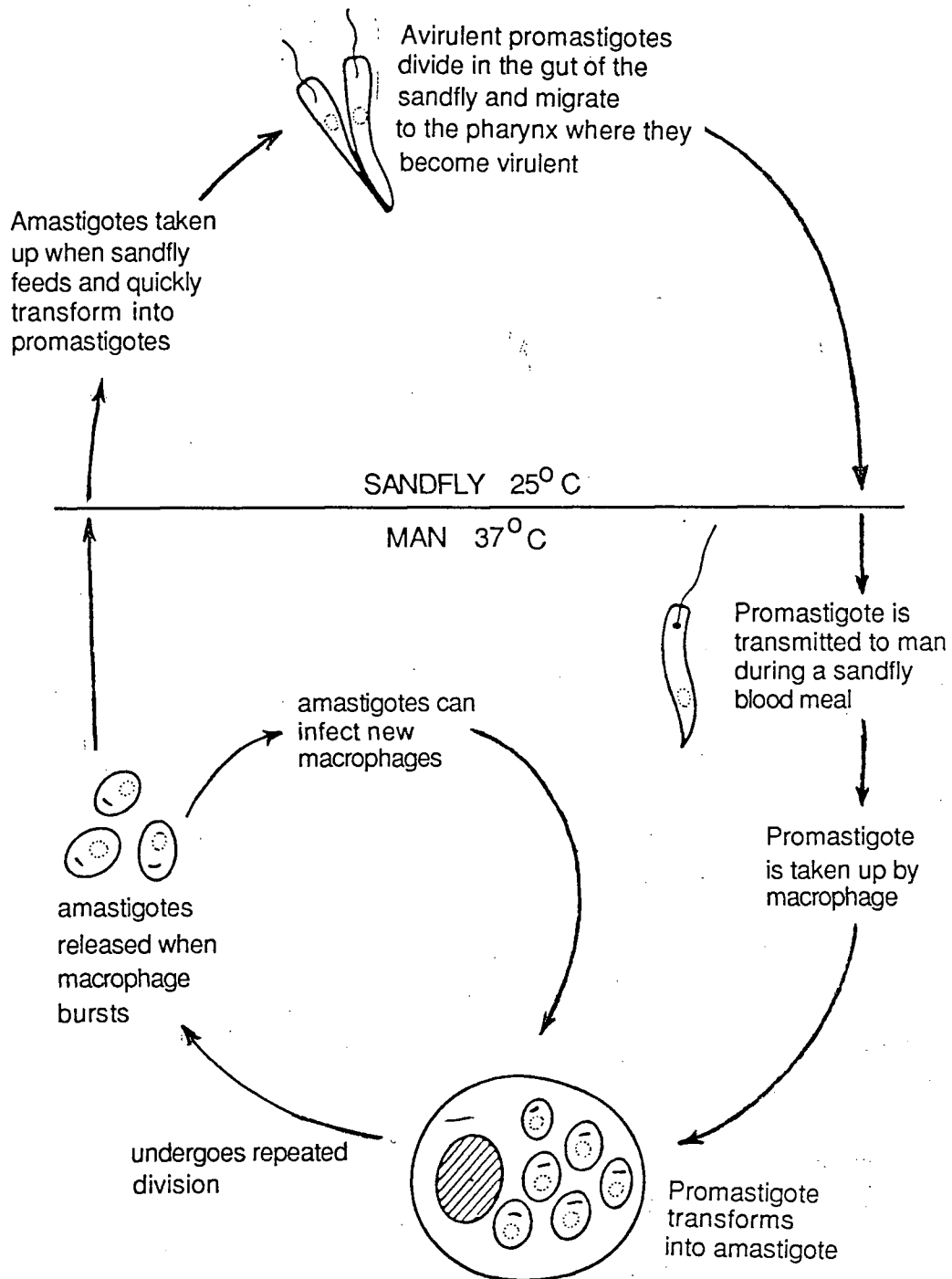
## A. THE LIFE CYCLE

Leishmania is a dimorphic parasite in which cytodifferentiation correlates with its cyclic transmission. Within the poikilothermic environment of the sandfly (22<sup>o</sup>-26<sup>o</sup>C), leishmania exists as a free-swimming or attached promastigote (2-20um) characterized by a slender cell body and anterior flagellum. After colonizing the gut upon ingestion, the parasites which are generally avirulent at this stage, will divide by binary fission. By day 6 or 7, the midgut becomes nutrient depleted causing the promastigotes to transform into an infective stage presumably in response to adverse growth conditions (Sacks and Perkins, 1984). Then, via attachment by their flagella to the microvilli of the gut epithelia, parasites migrate to the pharynx (Chang et al., 1985). Those that reach the proboscis are now virulent and non-dividing. The generation of the infective stage in the gut prior to movement to the mouthparts ensures that parasites which are to be transmitted are preadapted to survival in the vertebrate (Sacks and Perkins, 1984).

During the sandfly feeding process, virulent promastigotes are introduced into the skin of the host. Recent evidence suggests that the parasites then attach to macrophages via receptor-ligand interactions (Handman, 1986). Once inside the homeothermic host macrophage (34<sup>o</sup>-37<sup>o</sup>C), the parasites rapidly differentiate into amastigotes which are small (2-5um), round, and lacking the characteristic flagellum. After the amastigotes multiply repeatedly in the phagolysosome vacuole, the host cell bursts and releases amastigotes which can subsequently invade new macrophages. When parasitized macrophages from an infected individual or reservoir animal are taken up by the vector during a blood meal, only a small number of amastigotes are actually ingested (Chang et al., 1985). These forms quickly differentiate into promastigotes, thus completing the life cycle and cyclic transmission of the parasite.

One of the most intriguing aspects of the leishmania life cycle is the parasite's ability to survive and multiply within the macrophage, precisely the cell that is responsible for disposing of invading organisms. The mechanisms by which leishmania evades intracellular killing are not well understood, but studying various animal models implicates the involvement of several

Figure 1. The Life Cycle of Leishmania.



potential factors. For example, Mosser and Edelson (1984: 1987) have suggested that fixation of the third component of complement enhances parasite survival by significantly inhibiting the macrophage respiratory burst. As leishmania are susceptible to killing by oxygen metabolites, impairment of the host defense mechanism at its early stage is crucial for parasite survival (Buchmuller-Rouiller and Mauel, 1987). Others claim that an excreted factor protects the amastigote from digestion by lysosomal enzymes by binding to the intracellular  $\text{Ca}^{++}$  that is required for enzyme activity (Eilam et al., 1985). Alternatively, the excreted factor may simply provide a protective, negatively-charged shell around the parasite as suggested by Hernandez (1986). It should be noted, however, that the presence alone of leishmania in macrophages is not enough to cause a disease condition (Hill et al., 1983).

#### B. PROMASTIGOTE TO AMASTIGOTE DIFFERENTIATION

Promastigote to amastigote differentiation is a critical event in leishmanial parasitization of macrophages since only the amastigote can multiply within the adverse environment of the phagolysosome compartment (Chang and Fong, 1982). This process has become amenable to study in recent years through the use of tissue culture techniques. Large numbers of promastigotes and amastigotes of certain leishmania species can be generated easily and quickly in liquid media, thereby often providing enough material for diagnostic and preliminary research purposes. Parasites maintained in culture at 26°C and 37°C mimic naturally occurring promastigotes and amastigotes respectively.

In vitro studies have shown that in addition to the salient morphological differences between the two developmental stages of leishmania, there are other features that vary qualitatively or quantitatively during differentiation. Firstly, it has been demonstrated that there is a switch from oxidative phosphorylation in the sandfly to anaerobic respiration in the mammalian macrophage (Bowman and Flynn, 1976). Mukkada et al (1985) noted that the rate of glucose metabolism was considerably higher in L. donovani promastigotes than amastigotes. This observation correlates well with the observed levels of enzyme activities in the Embden-Meyerhof pathway (Meade et al., 1984). Also in agreement is the finding that the enzymes for

$\beta$ -oxidation of fatty acids are at a lower level in promastigotes than amastigotes (Hart et al., 1981; Hart and Coomb, 1982). Chang et. al (1985) suggest that the latter may reflect the in vivo situation in which there is a poor hexose supply in the sandfly gut versus an abundance of fatty acids in the phagolysosome vacuole of the host cell. Secondly, promastigote and amastigotes have been shown to express stage-specific and strain-specific antigens with the use of monoclonal antibodies (Handman and Hocking, 1982). Similarly, antigenic changes during the differentiation of L. mexicana in cultured macrophages has also been observed (Chang and Fong, 1982). Thirdly, Fong and Chang (1981) have demonstrated that the biosyntheses of  $\alpha$ - and  $\beta$ -tubulins are developmentally regulated. Both of these proteins are clearly more abundant in promastigotes than amastigotes which is not surprising since amastigotes lack flagella. Recent RNA studies by the same group (1984) have shown the presence of a single  $\beta$ -tubulin mRNA species in L. mexicana amastigotes as opposed to three different mRNA species in promastigotes and suggest that regulatory control occurs at the post-transcriptional level.

Interestingly, culturing leishmania under laboratory conditions has also unmasked the importance of temperature on leishmanial differentiation. Since naturally occurring parasites must undergo a temperature change during their transmission cycle, it has been postulated that a temperature shift from 25°C to 37°C is necessary and possibly directly responsible for triggering promastigote to amastigote transformation (Van der Ploeg et al., 1985; Lawrence and Robert-Gero, 1985). As suggested by Mardsen (1979) and Hill (1986), there may be a correlation between certain strains and their predilection for anatomical sites based on temperature tropism. Similarly, Sacks et al. (1983) have shown that New World strains of leishmania are significantly more sensitive to higher temperatures than Old World strains an observation which again supports the hypothesis that temperature plays a regulatory role during parasite development.

Although the influence of temperature on development has been observed in many organisms such as *Xenopus* (Bienz, 1982), yeast (Kurtz et al., 1986), and *Drosophila* (Mason et al., 1984; Zimmerman et al., 1983), the effects of temperature on parasite development have



only recently become apparent. For instance, it is now thought that trypanosomes (Van der Ploeg, 1985; Davis et al., 1987), plasmodia (Bianco et al., 1986) and schistosomes (Hedstrom et al., 1987) all require a shift in temperature in order to complete their life cycles. Although many investigators have already recognized the striking similarity between the temperature differences in the respective vector/host systems and the conditions characteristic of the heat shock response observed in prokaryotes and higher eukaryotes, the significance of such a parallel is not yet clear. However, identification and characterization of the molecules involved will contribute greatly to the expanding field of molecular parasitology in addition to providing insight to those investigating heat shock as a system for studying gene regulation.

## II. THE HEAT SHOCK RESPONSE

When cells are exposed to temperatures a few degrees celsius above their normal physiological temperature, they respond by rapidly altering their usual patterns of transcription and translation and by selectively synthesizing a group of highly conserved proteins called heat shock proteins (Hsps) (Schlesinger, 1982). This phenomenon is commonly referred to as the heat shock response but can be induced by other agents such as ethanol, hypoxia, amino acid analogues, arsenite, and glucose starvation. The main functions of this response are presumably to protect the cell from irreversible damage during the stress period and to aid in complete recovery of cellular activities once the stressful agent has been removed.

The heat shock response was first documented cytologically by Ritossa (1962) who observed substantial changes in the puffing pattern of salivary gland polytene chromosomes from *Drosophila* larvae that had been exposed to brief treatments of heat. Little attention was given to this documentation until a decade later when Tissieres et al (1974) showed that the production of heat-induced proteins accompanied the appearance of previously inactive puffs. These molecular findings made it clear that the puffs seen in the DNA of heat shocked fruit flies denoted regions of active RNA transcription of genes encoding heat shock proteins.

## A. THE HEAT SHOCK PROTEINS

The heat shock proteins themselves have been named according to their molecular weights on SDS polyacrylamide gels, but the precise weight varies somewhat among different organisms. In the eukaryotic cell there are three families of HSPs based upon their structural homologies. The first group is composed of a single member with molecular weight in the 80-90 kd region, usually designated Hsp83. The second group, called the 70K family, is comprised of one or two members in the 65-75 kd region. The four small and closely related hsp's, such as hsp22, hsp23, hsp26, and hsp27 in *Drosophila*, characteristically form the third group.

Although the *Drosophila* system remains the best characterized, the heat shock responses of numerous other organisms have also undergone rigorous investigation. Collectively, the data indicate remarkable conservation at the protein and gene structure levels throughout evolution with the larger proteins more highly conserved than the smaller ones (Schlesinger, 1986). This "universality" of HSPs clearly indicates that the heat shock system is a fundamental and vital cellular response for organismal survival, despite the variability of the response among different cell types.

## B. REGULATION OF HEAT SHOCK GENE EXPRESSION

Genes encoding heat shock proteins can be regulated at either the transcriptional or translational level although the former appears to be the primary mechanism. A heat shock gene has the potential for transcriptional control when it possesses in its 5' region at least one copy of the symmetrical sequence C--GAA--TTC--G known as the heat shock consensus element (HSE) (Pelham, 1982). This sequence serves as an inducible promoter for mRNA transcription even in heterologous systems (Pelham, 1982; Pelham and Bienz, 1982;) which suggests that all eukaryotic heat shock genes share a common regulatory mechanism.

Of equal interest is the trans-acting heat shock transcription factor (HSTF) (Parker and Topol, 1984) which must also be present in the cell if heat shock gene expression is to be

induced. Both in vivo and in vitro, this factor selectively binds to the HSE resulting in conformational changes of the DNA which may be important components in the inducible pathway of HSP gene expression (Shuey and Parker, 1986). Since this factor has been isolated in several organisms from cells grown at normal temperature as well as those under heat shock and its induction is extremely rapid under heat shock conditions (Kingston et al., 1987), it is believed that the HSTF is activated rather than synthesized de novo upon heat shock.

Unlike the mechanisms underlying transcriptional control, those involved in translational control are activated selectively by high temperatures and not other stress agents. The response in these instances is highly effective and also the only practical means by which the stressed cell can quickly produce a particular heat shock gene product. For example, the control mechanism of the Hsp70 gene in *Xenopus* oocytes is entirely at the translational level because it would be impossible for a cell having such a high ratio of cytoplasmic volume to nuclear material to synthesize enough of the gene product via activation of gene transcription (Bienz, 1982).

Regardless of whether a heat shock gene is under transcriptional or translational regulation, the mechanisms involved are complex ranging from increased synthesis and stability of heat shock induced mRNAs to the preferential translation of those mRNAs presumably due to recognition sequences in their 5' untranslated leader sequences (McGarry and Lindquist, 1985). The concept of regulation is further complicated by the fact that some heat shock genes, such as the ones encoding the small hsps, are expressed during normal development and differentiation (Zimmerman et al., 1983). Such observations suggest that heat shock gene expression can be uncoupled from heat shock, thus non-coordinate regulation also exists (Kurtz et al., 1986).

### C. HSP70

Of all the heat shock proteins described in the literature, excluding ubiquitin, Hsp70 is the most conserved and omnipresent (Bienz, 1985). Most organisms carry several copies of the gene which are found in complex clusters at one or more loci. There are other non-heat inducible genes similar to the Hsp70 gene, and their gene products are referred to as Hsp70

cognates. The expression of Hsp70 and related proteins depends on the organism as some may be induced substantially by heat whereas others are strictly developmentally regulated.

Although the gene for HSP70 has been well characterized, the function of its gene product remains unclear. One hypothesis is that Hsp70 repairs damaged pre-ribosomes upon heat shock since it has been found concentrated at the nucleolus bound to partially assembled ribosomes (Welch and Suhan, 1986). Another hypothesis is that Hsp70 binds to proteins that are denatured as a result of the elevated temperature thereby preventing the aggregation of the denatured proteins into insoluble masses which could harm the cell (Munro and Pelham, 1985). The Hsp70 cognates, however, appear to have multiple functions which include primary roles during sporulation and oogenesis (Kurtz et al., 1986) and ATPase activity during the uncoating of clathrin coated vesicles in vitro (Chappell et al., 1986).

### III. UBIQUITIN

Ubiquitin is a 76 amino acid polypeptide found in all eukaryotic cells examined to date. It exists in the cell either free or covalently attached to various nuclear, cytoplasmic, and cell surface proteins via its carboxy terminal glycine residue. Ubiquitin is a heat shock protein in chicken embryo fibroblasts (Bond and Schlesinger, 1985) and is likely to be the most conserved eukaryotic protein known. Because the roles of ubiquitin in protein modification and degradation have been well researched, it is the first heat shock protein described with known functions. Furthermore, it is apparent that the ubiquitin system is closely coupled to the heat shock response making the gene expression of ubiquitin particularly interesting to study.

#### A. PROTEIN STRUCTURE

Much of the early work on ubiquitin involved protein sequencing and functional assays since the polypeptide could be purified easily from bovine and porcine reticulocyte extracts. The complete structure, however, was elucidated only after several groups cloned the ubiquitin coding sequences from different eukaryotes. The data unequivocally revealed that the 76 amino acid protein is in fact largely synthesized by the proteolytic cleavage of a polyubiquitin

acid protein is in fact largely synthesized by the proteolytic cleavage of a polyubiquitin precursor containing ubiquitin coding elements organized in spacerless, tandem arrays. As indicated in Figure 2, cleavage occurs after each glycine residue at amino acid position 76 to generate the active monomer. This protein structure and processing is consistent among the plant, animal and fungal kingdoms (Gausing and Barkardottir, 1986; Sharp and Li, 1987).

Some organisms possess a single non-ubiquitin residue at the carboxy terminal of the precursor such as valine in human polyubiquitin (Wiborg et al., 1985) and asparagine in yeast polyubiquitin (Ozkaynak et al., 1984), but the significance of these residues is uncertain. One hypothesis is that it serves to protect polyubiquitin from participating in reactions involving the monomer (Ozkaynak et al., 1984). On the other hand, because the C-terminus of the precursor is identical to that of mature ubiquitin in Xenopus laevis, the extra residue may not be essential (Dworkin-Rastl et al., 1984).

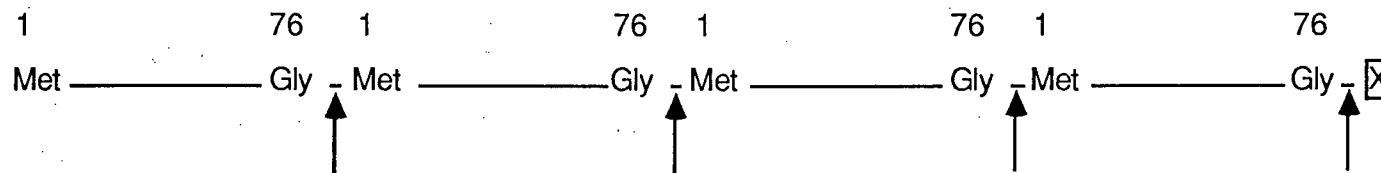
Unlike most polyproteins previously described, ubiquitin is neither a secretory nor viral gene product. Ubiquitin also does not require processing to remove spacer sequences and is internally repetitive to an extent matched only by the yeast  $\alpha$ -factor precursor (Kurjan and Herskowitz, 1982). Thus, the polyubiquitin precursor protein is novel in many respects.

## B. GENE STRUCTURE

The ubiquitin gene, as represented by the protein, is comprised of contiguous direct repeats of the 228 base pair ubiquitin coding element. While the number of repeats per gene varies from five in yeast (Ozkaynak et al., 1987) to greater than 12 in Xenopus laevis (Dworkin-Rastl et al., 1984), the gene structure is highly conserved. The number of loci can also vary.

Another important feature of the ubiquitin gene is that despite differences between repeat units at the nucleotide level, the amino acid sequences are identical within an organism (Ozkaynak et al., 1984; Wiborg et al., 1985; Bond and Schlesinger, 1985; Dworkin-Rastl et al., 1985; Gausing and Barkardottir, 1986). This observation suggests that the gene may have

**FIGURE 2.** Structural Organization of the Polyubiquitin Precursor Protein.



The polyubiquitin precursor consists of exact repeats of the 76-residue amino acid sequence of mature ubiquitin joined in tandem without intervening sequences. The only non-ubiquitin coding residue is shown in the box. The arrows indicate sites of proteolytic cleavage that generate the monomer.

undergone concerted evolution of tandem repeats involving both unequal crossover and gene conversion events (Sharp and Li, 1987). Furthermore, it is thought that the selective pressure which has maintained the amino acid identity of the repeat units has similarly acted on the primary structure throughout eukaryotic evolution (Ozkaynak et al., 1984).

The ubiquitin gene has also been shown to constitute a multigene family (Wiborg et al., 1985). Interestingly, many of its members are pseudogenes and natural gene fusions. Examples of the former have been identified in humans and they appear to have arisen by reverse transcription events (Baker and Board, 1987). However, the latter which has been characterized in yeast (Ozkaynak et al., 1987) encodes nascent hybrid proteins containing ubiquitin fused to an unrelated "tail" amino acid sequence. Remarkably, even this tail polypeptide is reasonably conserved among mouse (St. John et al., 1986), human (Lund et al., 1985), and yeast, and may function as a ubiquitin-free, nucleic acid binding protein.

### C. GENE EXPRESSION

Although any given organism can possess several loci encoding ubiquitin, not all the loci have proven to be heat inducible. For instance, in yeast, the gene encoding the polyubiquitin precursor and possessing the heat shock element is the only one induced by stress agents. The gene fusions do not show an increase in expression under the same conditions. However, in Dictyostelium discoideum, neither of the two loci is heat inducible (Giorda and Ennis, 1987). Still more different is the situation found in chicken embryo fibroblasts. In these fibroblasts, one locus shows a three to five-fold increase in expression while the other locus does not appear to generate a transcript (Bond and Schlesinger, 1985). Hence, ubiquitin gene expression is extremely variable, despite its conservation in gene structure.

### D. FUNCTIONS OF UBIQUITIN

Originally isolated from bovine thymus during the purification of thymic polypeptide hormones by Goldstein et al (1974), ubiquitin was thought to play roles in the induction of T

cell differentiation and adenylate cyclase stimulation (Schlesinger et al., 1975). Such claims, however, subsequently failed to be substantiated by other investigators. Rather, the biochemical and genetic evidence showed that ubiquitin functions chiefly as a protein modifier by becoming covalently attached to the  $\epsilon$ -amino groups of various proteins via its C-terminal glycine residue. This process, referred to as ubiquitination, is activated by ATP and then followed by a complex series of enzymatic reactions.

The pathway by which ubiquitin-protein conjugates are formed and dissolved is cyclic as diagrammed in Figure 3. There are four major steps in ubiquitination: (1) ubiquitin carboxy terminal transesterification; (2) thiolester bond formation; (3) transesterification; and (4) conjugation of ubiquitin to the acceptor protein. These steps are mediated by enzymes E1, multiple E2s that differ in substrate specificity, and E3. Once ubiquitinated, the conjugate can be degraded into amino acids by an enzyme, as yet unidentified, or simply deubiquitinated by an isopeptidase to regenerate the protein and ubiquitin monomer.

1. PROTEIN DEGRADATION. Although ubiquitin has numerous and diverse functions, it is best known for its role in selective protein degradation. Studies suggest that ubiquitin may serve as a signal for attack by proteinases specific for ubiquitin conjugates (Finley and Varshavsky, 1985). In addition, work with the mouse cell line ts85, a mutant in the ubiquitin pathway, (Mita et al., 1980) has shown that ubiquitin also serves essential regulatory functions. This cell line is temperature sensitive for the ubiquitin activating enzyme E1 and fails to degrade short-lived proteins at the non-permissive temperature (Finley et al., 1984). These observations indicate that under normal *in vivo* conditions, the turnover of short-lived proteins occurs primarily through nonlysosomal ubiquitin-dependent proteolysis.

2. CELL CYCLE CONTROL. Evidence for ubiquitin's involvement in cell cycle control stems mainly from experiments once again using ts85 mouse cells (Finley et al., 1984). It was previously known that ts85 cells arrest at the G2 phase in the cell cycle (Mita et al., 1980; Yasuda et al., 1981), and this finding now correlates with the inactivation of the enzyme E1. The arrest may be due to the stabilization or accumulation of normally short-lived regulatory proteins of which some may function in regulating cell cycle progression.





3. UBIQUITIN-HISTONE CONJUGATION. The first ubiquitin-protein conjugate described was that of ubiquitin linked to the  $\epsilon$ -amino group of lysine 119 of histone H2A (uH2A) via an isopeptide bond to form a branched molecule (Hunt and Dayhoff, 1977). It was later shown by West and Bonner (1980a, 1980b) using immunochemical techniques that ubiquitin is conjugated in vivo to all subtypes of histone H2A and also to H2B, albeit the latter to a lesser extent. These ubiquitinated histones, unlike many proteins targeted for degradation, contain a single ubiquitin and are among the most abundant ubiquitinated polypeptides in the nucleated mammalian cell (Ciechanover et al., 1984).

Since uH2A and uH2B are not rapidly degraded, these observations further argue that ubiquitin functions other than in proteolysis. Biochemical studies demonstrate that ubiquitinated histones are in rapid equilibrium with the free ubiquitin pool in interphase cells (Ciechanover et al., 1984), consequently suggesting that the reversible binding somehow regulates chromatin structure. In addition, because ubiquitin-histone complexes tend to be localized in regions of gene activity, it is postulated that ubiquitin binding may induce chromatin unfolding which is needed for gene expression (Levinger and Varshavsky, 1982). Therefore, ubiquitin may simply "mark" particular chromosomal regions for repair or transcription.

4. CONJUGATION TO CELL SURFACE RECEPTORS. Ubiquitin has also recently been identified attached to cell surface receptors such as the lymphocyte homing receptor (Siegelman et al., 1986; St. John et al., 1986) and the platelet-derived growth factor receptor (Yarden et al., 1986). Much like the ubiquitinated histones, the ubiquitinated receptors are not destined for degradation. These findings were initially surprising as ubiquitin was not thought to occur at the cell surface because it does not impart the specificity that is required by a receptor molecule (Marx, 1986). Whether or not ubiquitin actually participates directly in receptor function remains to be determined.

5. THE STRESS RESPONSE. Ubiquitin's role in proteolysis appears to be closely connected to the cell's defense against heat and other stresses by targeting for degradation those damaged or abnormal proteins induced by the stress agent (Munro and Pelham, 1985). This

point is demonstrated clearly by the induction of heat shock protein synthesis at 39°C in ts85 cells, but not in the wild-type parental cells (Ciechanover et al., 1984; Ananthan et al., 1986). There is also evidence to indicate that the heat shock response is triggered by the accumulation of abnormal proteins per se (Goff and Goldberg, 1985; Finley et al., 1984). For instance, in certain *Drosophila* actin mutants, the abnormal actin disrupts cellular structures and causes heat shock protein expression in the affected cells (Karlik et al., 1984; Hiromi and Hotta, 1985). Furthermore, it is thought that under stress conditions, ubiquitination rather than proteolysis is the rate limiting process, since the level of ubiquitinated histones are also greatly reduced (Glover, 1982).

Although the complementary mechanisms operating are not fully understood, the favoured hypothesis suggests that the heat shock response occurs when some target protein, such as the heat shock transcription factor itself, fails to be ubiquitinated (Munro and Pelham, 1985). When the HSTF accumulates, it could promote transcription of normally inactive heat shock genes. The network, therefore, would be self-regulating such that the HSTF would be inactivated once the level of free ubiquitin increase either as a result of degradation of abnormal proteins or increased ubiquitin synthesis, the HSTF would become inactivated once again. This model fits well with the observation that heat shock genes are only transiently transcribed even in cells that have been maintained for long periods at elevated temperatures (Ashburner and Bonner, 1979).

6. INVOLVEMENT IN DEVELOPMENT. Ubiquitin has been found to be developmentally expressed in at least two organisms. In Dictyostelium discoideum, the transition from single cell to multicellularity involves differential expression of specific genes whose products may be required for the formation of the fruiting body that contains the spores (Giorda and Ennis, 1987). In *Xenopus*, the most abundant ubiquitin mRNA is found in unfertilized eggs and embryos during the first half day of development (Dworkin-Rastl et al., 1984). Because the latter situation parallels that which occurs for histone mRNAs (Ruderman et al., 1979), it has been suggested that there is a demand for ubiquitin during development. The requirement may be due to the extensive DNA replication occurring at this stage, and therefore,

correlates well with the need for histones. Alternatively, ubiquitin may function to degrade specific maternal proteins that are no longer required by the embryo.

Perhaps the most intriguing aspect of ubiquitin's putative role in development is that the structure of the protein, namely polyubiquitin, offers a means of storing large amounts of the polypeptide in an inactive form (Dworkin-Rastl et al., 1984). With the regulatory control primarily at the translational level, the demand for ubiquitin during development can be met quickly when the appropriate signals for cleavage of the precursor are transduced.

#### IV. RELEVANCE OF STUDYING UBIQUITIN GENE EXPRESSION IN LEISHMANIA

The high level of conservation throughout eukaryotic evolution of the gene structure of ubiquitin and the synthesis of ubiquitin as a polyprotein precursor have prompted many investigators to study ubiquitin gene expression and protein function in divergent organisms. However, the complete amino acid sequence of ubiquitin in the phylum protozoa has been previously unknown. Hence, one objective of this thesis is to identify ubiquitin coding elements in the parasitic protozoan Leishmania major, thereby establishing the existence of ubiquitin in another kingdom. Furthermore, studies involving yeast and chicken embryo fibroblasts have shown that ubiquitin gene expression is heat inducible. Since the trigger for differentiation of leishmania involves a temperature shift, the second objective of this thesis is to determine whether ubiquitin functions under heat shock control during the development of Leishmania major.

## MATERIALS AND METHODS

### I. $\lambda$ EMBL 3 GENOMIC LIBRARY

A. Construction. The  $\lambda$ EMBL 3 genomic library consisting of DNA from Leishmania major (NIH Seidman Strain kindly provided by Dr. N. Reiner, University of British Columbia, Vancouver, B.C.) was prepared by A. E. Wallis and W. R. McMaster (University of British Columbia, Vancouver, B. C.). In brief, the  $\lambda$ EMBL 3 library was constructed by partially digesting genomic DNA with Mbo I, size fractioning the fragments by centrifugation through a sucrose gradient, and then ligating the fragments in an equimolar ratio with Bam HI/Eco RI predigested  $\lambda$ EMBL 3 arms. The ligated DNA was packaged in vitro using commercially prepared packaging extracts (Gigapack, Statagene). The packaged phage were stored in SM buffer [100 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM Magnesium Sulfate ( $\text{MgSO}_4$ )] plus 0.4% Chloroform at 4°C in the dark. This library was screened with a 1120 bp Bgl II-Bcl I insert from the plasmid pUB2 (a gift from A. Varshavsky) containing the yeast ubiquitin gene (Ozkaynak et al., 1984).

B. Screening. The  $\lambda$ EMBL 3 library was screened by plaque hybridization as described by Benton and Davis (1977). *E. coli* cells of the strain P2392 were grown at 37°C to stationary phase in LB medium (Difco Lab Co., Madison, Wisconsin, 20 g/l  $\text{dH}_2\text{O}$ ) containing 10 mM  $\text{MgSO}_4$  and 0.2% maltose. The cells were pelleted at 1600g for 10 minutes then resuspended in 0.4 volumes 10 mM  $\text{MgSO}_4$ . The cells were stored at 4°C for up to 10 days and used as required. Approximately 125,000 recombinant  $\lambda$ EMBL 3 phage were adsorbed to 1.5 ml of magnesium treated P2392 host cells for 20 minutes at 37°C in a 5 ml Falcon polypropylene tube and then plated immediately with 0.6% NZYAM top agarose onto 150mm 1.5% NZYAM agar plates (NZYAM= 5 gm NaCl, 2 gm Magnesium chloride ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ), 10 gm NZ-Amine A, 5 gm yeast extract, 2 gm casamino acids, 2 gm maltose/l  $\text{dH}_2\text{O}$ , pH 7.5). The top agarose was allowed to harden for 15 minutes at room temperature and the plates were incubated at 37°C for 5 hours.

Meanwhile, sterile 150mm circular nitrocellulose filters (BA85 Schleicher and Schuell) were numbered with a ball-point pen and dipped in a 10 fold dilution of an overnight culture of P2392 grown in LB. These filters were placed number side down on the NZYAM plates once the plaques had begun to appear and were allowed to sit at 4°C for 60 minutes. The numbers were traced onto the bottom of the plates, then the filters were lifted and placed on fresh 1.5% NZYAM agar plates with the plaque side up and incubated at 37°C overnight for plaque amplification. The master plates were stored at 4°C.

Filters were removed the following morning and the DNA immobilized by placing each filter on 3MM Whatman paper soaked in 0.5N Sodium Hydroxide (NaOH), 1.5M Sodium Chloride (NaCl) for 20 minutes. This procedure was repeated once, then the filters were transferred to 3MM Whatman paper soaked in 0.5M Tris-HCl pH 7.5, 1.5M NaCl to neutralize. The filters were air-dried for 20 minutes followed by baking at 68°C for 2 hours. They were then placed in a glass pyrex dish containing prewashing mix (1M NaCl, 50mM Tris-HCl pH 7.5, 0.1% SDS, 1mM EDTA) and incubated at 42°C for 1 hour with occasional gentle rubbing. The filters were rinsed in a solution containing 3X SSC (20X SSC=3M NaCl, 0.3M Sodium citrate) and then placed immediately in a petri dish containing 8 ml of prehybridization solution (40% deionized formamide, 3X SSC, 1mM EDTA, 10 mM Tris-HCl pH 7.5, 10X Denhardt solution (100X= 4% polyvinylpyrrolidone/ficoll/BSA), 0.05% sodium pyrophosphate, 0.1% SDS, 100 ug/ml denatured herring testes DNA) per filter and incubated overnight at 37°C with gentle shaking. Hybridization was carried out in a plastic bag at 37°C overnight between 2 glass plates with 2 ml of fresh solution per filter and the DNA probe at 2 million cpm/ml of solution. The filters were washed twice in 2X SSC/1X Denhardt at room temperature for 5 minutes, then twice in 2X SSC/0.1% SDS at 50°C for 30 minutes, and finally once in 2X SSC at 50°C for 30 minutes. The filters were air dried and exposed to x-ray film (Kodak X-OMAT XPR-1) with intensifying screens at -70°C for 24 hours.

Plaques displaying positive signals were located on their respective master plates, then pulled using a sterile Pasteur pipette and placed into 1ml SM buffer. Each positive plaque was plated at 1000 pfu/82mm plate and subjected to a second screening following the procedure

described above. Second screen positives were plaque purified by a third screen and the resulting positives were stored in SM buffer plus 0.4% chloroform at 4°C in the dark.

## II. $\lambda$ GT 11 GENOMIC LIBRARY

A. Construction. The  $\lambda$ gt11 library was prepared as described by Wallis and McMaster (1987, in press). L. major DNA was partially digested with either Hae III or Alu I to produce random fragments which were then size fractionated on an S-1000 Sepharyl column. Fragments of approximately 5 kb were methylated at internal Eco RI sites and annealed to Eco RI linkers. Concatemerized linkers were removed by digestion with excess Eco RI and free linkers were size fractionated from the fragments. The Eco RI fragments were then ligated to commercially prepared  $\lambda$ gt11 arms carrying phosphorylated Eco RI cohesive ends. The DNA was packaged and the phage stored as described for the  $\lambda$ EMBL 3 library. This library was screened with a 507 bp Xba I/Pst I insert of pLUB.C containing one and a half *leishmania* ubiquitin coding elements and partial 3' untranslated sequence.

B. Screening. The  $\lambda$ gt11 library was also screened by plaque hybridization. *E. coli* cells of the strain Y1090(r-) were grown at 42°C to stationary phase in LB supplemented with 10mM  $\text{MgSO}_4$  and 0.2% maltose, and treated with 10mM  $\text{MgSO}_4$  as previously described. Approximately 250,000 recombinant  $\lambda$ gt11 phage were adsorbed with 1.5 mls Y1090(r-) cells for 20 minutes at 37°C, then plated immediately with 0.6% LBMg (LB supplemented with  $\text{Mg}^{++}$ ) top agarose onto 150mm 1.5% LBMg agar plates. The plates were incubated at 42°C for 3.5 hours.

Once plaques had begun to appear, sterile 150mm circular Hybond-N filters (Amersham) were numbered, then placed number side down on the plates and allowed to sit for 1 minute. After tracing the number onto the bottom of the plate, the filter was carefully removed and the DNA immobilized on the filters by denaturation and neutralization. The filters were then prehybridized for one hour at 65°C in 6X SSC, 1% SDS, 50mM Tris-HCl pH 7.5, 10mM EDTA, 5X Denhardt solution, and 100 ug/ml denatured herring testes DNA. Hybridization was carried

out at 65°C overnight using 1.5 ml fresh solution per filter plus the radiolabelled probe. Filters were washed at 65°C twice for 15 minutes each with 2X SSC, twice with 2X SSC/0.1% SDS, and once with 0.1X SSC. The filters were air dried and exposed to x-ray film. Once positive signals were identified on the master plates, the plaques were pulled and subjected to second and third screenings.

### III. PREPARATION OF RADIOACTIVELY LABELLED PROBES

A. Isolation of DNA Fragments. All DNA fragments used for radiolabelling were prepared by trough elution from agarose gels as described by Maniatis (1982). Briefly, 400 ug of plasmid DNA was digested with the appropriate restriction endonuclease to generate the fragment of interest. The digest was then precipitated with 1/10 volume 2.5 M NaAc and 2 volumes 95% EtOH at -70°C for 30 minutes. The DNA was pelleted by centrifugation in a microfuge, vacuum dried, and resuspended in dH<sub>2</sub>O plus 1/5 volume sample buffer (10% Ficoll, 50mM EDTA, 0.04% bromophenol blue). The DNA was electrophoresed on a 1% agarose gel containing EtBr in 1X TAE (50X = 2M Tris, 1M Acetate, 0.1M EDTA) at 140 V until the fragment was well isolated from other DNA species. The gel was removed from the electrophoretic unit, and using short wave UV illumination to visualize the DNA, a trough 0.5 cm in width was excised with a razor blade directly below the desired fragment. The gel was then replaced in the electrophoretic unit and buffer was added to the trough. The voltage was turned on at 200 V for 1 minute intervals to allow DNA to run into the trough. The buffer carrying the DNA was then withdrawn with a pasteur pipette into a separate tube. This process was repeated until all the DNA had been eluted. The pooled eluate was then mixed with an equal volume of butanol, centrifuged, and transferred to a clean tube in which the extraction process was repeated once with phenol, then with 24:1 (v/v) chloroform and isoamyl alcohol. The DNA was precipitated with 1/10 volume 2.5M NaAc and 2 volumes EtOH at -20°C overnight. The DNA was recovered by centrifugation at 15,000 RPM for 30 minutes and finally resuspended in 50 ul dH<sub>2</sub>O. The concentration was determined by analysis of the



absorbance at 260nm wavelength by spectrophotometry. The Molar Extinction Coefficient for double stranded DNA is 20 (Maniatis, 1975) and the concentration, given in mg/ml, was calculated by multiplying the absorbance reading with the dilution factor and then dividing the product by the Molar Extinction coefficient. The concentration was checked by electrophoresing a small sample of the DNA on an agarose gel with a known standard.

B1. Nick translation. Purified DNA fragments were nick translated with P32 radiolabelled dATP and dCTP according to Rigby et al. (1977). First, 10 ul of Deoxyribonuclease I (1 mg/ml in dH<sub>2</sub>O, Boehringer Mannheim, Dorval, Quebec) was activated with 90 ul DNase activation buffer (10mM Tris-HCl pH 7.5, 5mM MgCl<sub>2</sub>, 0.1 mg/ml BSA) for 30-60 minutes on ice, then diluted one thousand fold before use. The reaction was carried out in a 500 ul Eppendorf tube containing 100-200 ng of DNA, 2.5 ul 10X Nick translation buffer (500mM Tris-HCl pH 7.5, 50 mM MgCl<sub>2</sub>), 0.5 ul 50mM DTT, 1 ul BSA (2mg/ml), 1 ul 500mM dTTP, 1 ul 500 mM dGTP, 0.75 ul 35mM dATP, 0.75 ul dCTP, 1.25 ul activated DNase I, 3.5 ul each of  $\alpha^{32}\text{P}$ -dATP and  $\alpha^{32}\text{P}$ -dCTP (3000 Ci/mmol, Amersham), 0.5 ul 10mM Calcium chloride (CaCl<sub>2</sub>), 1.75 ul DNA Polymerase I (7 U/ul Pharmacia), and dH<sub>2</sub>O to 25 ul. The reaction was incubated at 15°C for 60 minutes after which the enzymes were inactivated by heating to 68°C for 5 minutes.

Free nucleotides were separated from the probe by either affinity chromatography on a 10 ml column packed with Sephadex G-50 beads (Pharmacia) or by using a commercially prepared GENECLAN kit (this latter procedure is described under the heading VI.B. Isolation of DNA Fragments for Subcloning). If a column was used, it was necessary to add 30 ug yeast tRNA and ethanol precipitate the fractions containing the radiolabelled probe. DNA pellets were then resuspended in a total volume of 100 ul dH<sub>2</sub>O. If using the GENECLAN procedure, the final elution volume used was 50 ul dH<sub>2</sub>O. All probes were boiled for 10 minutes and placed immediately on ice for 10 minutes prior to being added to the hybridization solution containing the filters. Only probes labelled to a specific activity of  $5 \times 10^7$  cpm/ug or greater were used for hybridizations.

B2. Oligo labelling. Purified DNA fragments were oligo-labelled by the method of Fernberg and Vogelstein (1983).

Solution A. 1000 ul Solution O (1.25M Tris-HCl pH 8, 0.125M MgCl<sub>2</sub>)

18 ul  $\beta$ -mercaptoethanol

5 ul 0.1M dATP in TE (3mM Tris-HCl pH 7, 0.2mM EDTA)

5 ul 0.1M dTTP in TE

5 ul 0.1M dGTP in TE

Solution B. 2M HEPES (titrated to pH 6.6 with 4N NaOH)

Solution C. Hexadeoxynucleotides (final concentration of 90 O.D U/ml in TE)

OLB Buffer. Solutions A:B:C in the ration 10:25:15

The reaction was carried out in a 500 ul Eppendorf tube containing 5 ul OLB buffer, 1 ul BSA (5mg/ml), 13.5 ul DNA (50 ng in dH<sub>2</sub>O; boiled for 10 minutes, then stored at 37°C for 10 minutes prior to use), 5 ul  $\alpha^{32}\text{P}$ -dCTP, and 0.5 ul Klenow Polymerase I (U/ul, Pharmacia Biochemicals), and incubated at room temperature for at least 3 hours. Then 125 ul stop buffer (20mM NaCl, 20mM Tris-HCl pH 7.5, 2mM EDTA, 0.25% SDS, 1 uM dCTP) was added and the free nucleotides removed by affinity chromatography with a Prepac NACS column according to the supplier's protocol (Bethesda Research Laboratories). The probe was denatured by heat as described above prior to use.

#### IV. ISOLATION OF $\lambda$ DNA

$\lambda$  DNA was prepared as described by Maniatis (1982). Briefly, the number of phage needed to give confluent lysis for ten 82mm plates were adsorbed to plating bacteria in 10mM MgSO<sub>4</sub> for 20 minutes at 37°C. After plating with top agarose and incubating the plates at the appropriate temperatures and times, 5 ml of SM buffer was pipetted gently onto each plate and maintained at room temperature with shaking for 2 hours. The buffer was then removed gently with a Pasteur pipette, pooled into 50 ml polypropylene tubes, and centrifuged at 15,000 RPM for 10 minutes in a Sorval RC-5B centrifuge with a GSA rotor to remove bacterial debris. The supernatant was decanted into a graduated cylinder to determine the volume, and then

transferred to a 250 ml polypropylene bottle to which 0.15 volumes 5M NaCl and 0.3 volumes 20% PEG were added. The mixture was placed on ice for 90 to 120 minutes and centrifuged at 10,000 RPM for 5 minutes. Once the supernatant was discarded, the pellet was resuspended in 5 ml DNase I buffer (50mM Tris-HCl pH 7.5, 5mM  $\text{MgSO}_4$ , 0.5mM  $\text{CaCl}_2$ ) and transferred to a 16 ml Falcon polypropylene tube containing 100  $\mu\text{l}$  DNase I (1 mg/ml) and 200  $\mu\text{l}$  RNase A (5 mg/ml, previously boiled for 5 minutes, Sigma). The tubes were incubated at 37°C for 30 minutes and then the mixture was transferred to a 50 ml polypropylene tube and extracted twice with an equal volume of chloroform. The final aqueous layer was transferred to a clean tube to which 0.75 gm Cesium Chloride ( $\text{CsCl}$ ) were added per ml of solution to give a final density of 1.45-1.5 /ml. The solution was vortexed thoroughly and transferred to a 10 ml quick seal centrifuge tube using a Pasteur pipette. The gradients were formed in a Beckman LK8-M Ultracentrifuge with a Type 70.1Ti rotor at 60,000 rpm for at least 16 hours at 20°C.

The phage, appearing as a bluish band, was removed with an 18 gauge needle and 5 ml syringe and placed in dialysis tubing to be dialysed against 100x volume of TE (10mM Tris pH 8, 1mM EDTA) twice for 60 minutes each. The dialysed solution was then transferred to a 16 ml tube and SDS was added to 1%, EDTA to 20mM, and Proteinase K (Boehringer Mannheim) to 100  $\mu\text{g}/\text{ml}$ . The contents were mixed by inversion, followed by incubation at 68°C for 60 minutes. The DNA was then transferred to a clean 50 ml tube and extracted with an equal volume of phenol. The aqueous layer was separated by centrifugation at 1600g for 5 minutes at room temperature and pipetted to a clean tube using a wide-bore pipette. The DNA was extracted with 1/2 (v/v) phenol and chloroform, and finally with an equal volume of 24:1. The aqueous layer was transferred to dialysis tubing and dialysed against 3 changes of 1000X volume TE at 4°C for at least 16 hours.

## V. ISOLATION OF PLASMID DNA

A. Alkaline Lysis Method. Large scale plasmid DNA was isolated by the alkaline lysis method as described by Maniatis (1982). A single bacterial colony containing the plasmid of interest was used to inoculate 10 ml of LB containing antibiotic selection at 20  $\mu\text{g}/\text{ml}$ . This

culture was grown to stationary phase and then used to inoculate one liter of sterile M9 media [6 gm Sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ), 3 gm Potassium phosphate ( $\text{KH}_2\text{PO}_4$ ), 0.5 gm NaCl, 1 gm Ammonium chloride ( $\text{NH}_4\text{Cl}$ )/1 dH<sub>2</sub>O supplemented with 2mM  $\text{MgSO}_4$ , 0.2% glucose, and 0.1mM  $\text{CaCl}_2$ ] with the antibiotic at 20 ug/ml. This large culture was incubated at 37°C with vigorous shaking until the absorbance at 600nm wavelength was between 0.6 and 0.8 at which point chloramphenicol (dissolved in 95% EtOH) was added to a final concentration of 250 ug/ml for plasmid amplification. The culture was then grown overnight at 37°C in a shaking incubator.

Cells were pelleted by centrifugation in four 250 ml polypropylene bottles at 5000 rpm for 10 minutes. The supernatant was decanted, and each pellet resuspended thoroughly in 5 ml sucrose solution (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA, fresh lysozyme added to 5 mg/ml). These were pooled into a single bottle and stored at room temperature for 5 minutes after which 20 ml of freshly prepared lysis solution (0.2M NaOH, 1% SDS) was added. The contents were mixed gently by inversion, and allowed to stand on ice for 10 minutes. Then 30 ml of acetate solution (3M Potassium, 5M Acetate) was added, again gently mixed by inversion, and placed on ice for 10 minutes. The lysed cell debris was pelleted by centrifugation at 10,000 rpm for forty minutes at 4°C.

The supernatant was measured in a graduated cylinder and then transferred to a clean 250 ml bottle to which 0.6 volumes isopropanol were added. The contents were vortexed and stored at room temperature for at least 15 minutes. The DNA was pelleted by centrifugation at 10,000 rpm for 30 minutes at 4°C, dried under vacuum, and resuspended thoroughly in 9.4 ml TE. Then 600 ul RNase A (5 mg/ml) was added and the tube incubated at room temperature for 15 minutes. Enzymes and proteins were extracted by the addition of an equal volume of phenol and centrifugation at 1600g for 5 minutes. The aqueous layer was transferred to a clean tube using a pipette and the extraction procedure repeated with an equal volume of 24:1. The final aqueous phase was transferred to a clean tube and precipitated with 1/2 volume 7.5M  $\text{NH}_4\text{Ac}$  and 2 volumes 95% EtOH for 30 minutes at -70°C.

The DNA was collected by centrifugation at 15,000 rpm for 20 minutes at 4°C and dried under vacuum. The pellet was then resuspended thoroughly in 20 ml TE after which 20 gm CsCl and 10 mg EtBr were added and vortexed. The density of the solution was adjusted to a sucrose density of 36% with a refractometer and then transferred to two 10 ml quick seal centrifuge tubes using a pasteur pipette. The gradients were formed in a Beckman L8-M Ultrafuge with a Type 70.1Ti rotor at 60,000 rpm for at least 16 hours at 20°C. The DNA bands were detected using a hand held UV light and the lower band containing covalently closed circular DNA was removed with an 18 gauge needle and syringe. The recovered DNA was extracted 3X with an equal volume of butanol to remove the EtBr, then transferred to dialysis tubing and dialysed against 3 changes of 1000X volume of TE for at least 16 hours.

B. LiCl Boiling Method. The LiCl boiling method used for mini-preparations of plasmid DNA was exactly as described by (Wilimzig, 1985). Briefly, the cell pellet from a 1 ml overnight culture was resuspended in 100 ul TELT buffer (2.5M LiCl, 62.5mM EDTA, 50mM Tris-HCl pH 8, 0.4% Triton X-100) and then 10 ul freshly mixed lysozyme (10 mg/ml in 10mM Tris-HCl pH 8) was added. The lysate was placed in a boiling water bath for 1 minute before storing on ice for 5 minutes. Bacterial debris was pelleted by centrifugation in a microfuge for 8 minutes at room temperature and the supernatant precipitated with 1/10 volume 2.5M NaAc and 2 volumes 95% EtOH. The DNA was recovered by centrifugation for 30 minutes and the pellet washed thoroughly in 70% EtOH to remove excess salt. The DNA was pelleted again, vacuum dried, and resuspended in 50 ul TE plus RNase A at 20 ug/ml.

## VI. CHARACTERIZATION OF $\lambda$ AND PLASMID DNA

The quantities of DNA obtained from  $\lambda$  and plasmid DNA preparations were determined by spectrophotometric analysis as described earlier. A small sample was always electrophoresed on an agarose gel with known standards to confirm the absorbance readings.

A. Restriction Mapping. Restriction maps of recombinant plasmids were generated by analysing the digestion patterns of plasmid DNA digested with one or more restriction enzymes. In general, 5 U of enzyme was used per ug DNA in a 15 ul reaction volume containing 1/10 volume of the appropriate salt buffer as recommended by the supplier.

B. Isolation of DNA fragments for subcloning. DNA fragments were isolated from agarose gels by two methods. One method involved using the NACS column (BRL) and the protocol was carried out as described in the product's accompanying handbook. Briefly, a restriction enzyme digest was run on a 0.7% gel comprised of low-melting point agarose. The region of the gel containing the DNA of interest was then excised using a razor blade and the slice weighed by difference in a 1.5 ml Eppendorf tube. Four volumes of 0.5M NaCl were added and the tube was heated at 70°C to dissolve the gel. The liquid was then pipetted onto the NACS mini-column and allowed to bind under the low salt conditions. The flow-through was discarded and the column washed with several ml of the low salt buffer. The DNA was eluted in three 100 ul fractions with high salt buffer and precipitated with EtOH as described previously.

The second method involved using a commercially packaged kit called GENECLAN (BIO 101, La Jolla, California). A restriction enzyme digest was electrophoresed on an agarose gel, and the region containing the DNA of interest was excised and weighed by difference in a 1.5 ml Eppendorf tube. Then 2.5 volumes of a Sodium Iodide solution (NaI and Sodium Sulfite dissolved in dH<sub>2</sub>O) were added and the tube was incubated at 50°C for 5 minutes to dissolve the gel matrix. Once the DNA was thoroughly in solution, 5 ul of glassmilk (silica beads) was added, vortexed and stored on ice for 5 minutes. The glassmilk bound with DNA was pelleted by centrifugation in a microfuge for 5 seconds and washed several times with 200 ul of Wash buffer (NaCl, Tris, and EDTA dissolved in EtOH) and pelleted in the same manner. The pellet was then resuspended thoroughly in 5 ul dH<sub>2</sub>O and the tube heated at 50°C for 3 minutes to elute the DNA. The DNA in solution was withdrawn as the supernatant after pelleting the glassmilk by centrifugation for 30 seconds.

C. Ligations. Once DNA fragments of interest were isolated, they were subcloned into various vectors. Fragments carrying different termini were ligated in a ratio of 1:1 insert to vector whereas fragments cloned into a single restriction enzyme site were ligated in a 3:1 ratio of insert to vector. Each ligation was carried out in 250  $\mu$ l tube containing 1  $\mu$ l 10X Ligase buffer (500mM Tris-HCl pH 8.0, 100 mM  $\text{MgCl}_2$ ), 1  $\mu$ l 10mM ATP, 2  $\mu$ l 50mM DTT, 50 ng linearized vector, insert DNA, and  $\text{dH}_2\text{O}$  to 10  $\mu$ l. After mixing thoroughly, 0.5  $\mu$ l T4 ligase (6 U/ $\mu$ l, Pharmacia Biochemicals) was added and the tube incubated overnight at 15°C. The tube was heated to 65°C for 5 minutes prior to use in transformations.

D. Preparation of Competent Cells. E. coli cells of the strains DH5 $\alpha$ , JM83, and JM101 were used to carry pUC19 and Bluescript<sup>+</sup> (Stratagene) recombinant plasmids. They were made competent for transformation with  $\text{CaCl}_2$  as described by Lederberg and Cohen (1974). Briefly, a single colony either from an LB agar plate (DH5 $\alpha$ ) or M9 agar plate (JM83, JM101) was used to inoculate a sterile 16 ml tube containing 10 ml of sterile medium. These cells were grown to stationary phase in a shaking incubator at 37°C, then 500  $\mu$ l was used to inoculate a sterile 500 ml Erlenmeyer flask containing 50 ml of LB. This culture was kept in a shaking incubator at 37°C until the absorbance at 600nm wavelength was 0.3 (about 2 hours). The cells were immediately transferred to sterile 50 ml tubes which had been prechilled on ice and pelleted at 1600g for 10 minutes at 4°C in a precooled centrifuge. After the supernatant was carefully decanted, the cells were washed with 20 ml of ice cold 100mM  $\text{MgCl}_2$  and pelleted as before. The supernatant was removed and the cells gently resuspended in 20 ml of ice cold 100mM  $\text{CaCl}_2$ . After being stored on ice for 30 minutes, the cells were pelleted, resuspended in 5 ml of 100mM  $\text{CaCl}_2$ , and left on ice at 4°C overnight.

E. Transformation of Competent Cells. Each transformation was set up in a 5 ml Falcon polypropylene tube using 200  $\mu$ l competent cells, 47.5  $\mu$ l TEN 7.5 (100mM NaCl, 10 mM Tris-HCl pH 7.5, 1mM EDTA), and 2.5  $\mu$ l of the ligation reaction. The tube was left on ice for forty minutes, then heated at 37°C for 5 minutes after which 2 ml of LB was added. The cells were incubated at 37°C for 60 minutes without shaking. A panel of dilutions were plated on 1.5% LB agar plates containing antibiotic selection, 0.1% X-gal, and 6.7 mM IPTG, and

the plates were incubated overnight at 37°C. Colonies selected by colour were screened for inserts by restriction enzyme analyses of mini-prep plasmid DNA. Using the CaCl<sub>2</sub> method, the transformation frequency was approximately 10<sup>6</sup> CFU/ug.

## VII. DNA SEQUENCE ANALYSES

A. Preparation of competent JM101. E. coli cells of the strain JM101 were also made competent for transformation (with M13mp18 and M13mp19) by the CaCl<sub>2</sub> procedure. A single colony scraped off an M9 plate was used to inoculate a 16 ml sterile Falcon polypropylene tube containing 5 ml M9 media. This culture was grown to stationary phase at 37°C in a shaking incubator, then 600 ul of culture was used to inoculate 60 ml of YT (2X YT=16 gm Bactotryptone, 10 gm yeast extract, 5 gm NaCl/l dH<sub>2</sub>O) media in a sterile 500 ml Erlenmeyer flask. These cells were grown until the absorbance at 660nm wavelength was 0.5 (about 2.5 hours). They were then transferred to prechilled sterile 50 ml tubes and pelleted by centrifugation at 1600g for 10 minutes at 4°C. The supernatant was decanted, and the cells were resuspended gently in 1/2 volume 50mM CaCl<sub>2</sub> then stored on ice for 20 minutes. The cells were pelleted, resuspended in 6 ml 50mM CaCl<sub>2</sub>, and used immediately. Exponentially growing cells were obtained by adding 5 ml 2X YT to the Erlenmeyer flask (after the cells had been transferred to tubes) and the cells grown at 37°C with shaking until ready for use.

B. Transformation of Competent JM101. The transformation procedure used was as follows. In a sterile 1.5 ml microfuge tube on ice, 300 ul competent JM101 cells and 2 ul of the ligation reaction were added and maintained on ice for 40 minutes. The cells were then heat shocked at 45°C for 2 minutes and dispensed immediately into a warmed 5 ml tube containing 4 ml 0.6% YT top agarose, 200 ul exponential cells, 10 ul 100mM IPTG, and 50 ul 2% X-gal. The contents were mixed by inversion and poured immediately onto 1.5% YT agar plates. The plates were allowed to set at room temperature for 30 minutes before incubating at 37°C overnight. In general, the frequency of transformation was greater than 10<sup>6</sup> PFU/ug.



C. Template Preparation. A JM101 culture grown to stationary phase in M9 media (500 ul) was used to inoculate 50 ml of sterile 2X YT in a 500 ml Erlenmeyer flask and the cells were grown for 1 hour at 37°C with vigorous shaking. Then 1.2 ml of this culture was dispensed into 16 ml Falcon polypropylene tubes and the plaque of interest (pulled from a fresh 1.5% YT agar plate) was added to the tube. The culture was grown at 37°C in a shaking incubator for 4 hours. The cells were pelleted by centrifugation at 1600g for 10 minutes, and then 800 ul of supernatant was transferred to a sterile 1.5 ml Eppendorf tube. Two hundred ul of 20% PEG/2.5M NaCl was mixed in thoroughly and the tube stored at room temperature for 15 minutes. Phage were pelleted by centrifugation in a microfuge for 10 minutes and a drawn out pasteur pipette was used to remove all supernatant. The pellet was dissolved in 200 ul NET (10mM NaCl, 10mM Tris-HCl pH 8.0, 0.25mM EDTA) and extracted first with an equal volume of phenol, then with 1/2 (v/v) phenol and chloroform, and lastly with an equal volume of chloroform. The aqueous layer was precipitated with 1/2 volume 7.5M NH<sub>4</sub>Ac and 3 volumes 95% EtOH, and incubated at -70°C for 60 minutes. After pelleting, the ssDNA was resuspended in 500 ul NET and reprecipitated with NH<sub>4</sub>Ac and EtOH. After washing the final pellet with 70% EtOH, template DNA was vacuum dried and resuspended in 20-40 ul dH<sub>2</sub>O.

D. Dideoxy Sequencing. Reactions for M13 sequencing were carried out as described by Sanger (1977). Briefly, in a 1.5 ml microfuge tube, 5 ul of one template were vortexed with 1 ul of M13 sequencing primer (17mer from Pharmacia Biochemicals) and 2 ul 10X Hin FI buffer (600mM NaCl, 100mM Tris-HCl pH8, 70mM MgCl<sub>2</sub>). The tubes were placed in a heating block at 75°C and allowed to cool to room temperature (about 50 minutes) after which 1 ul 15mM dATP and 1.5 ul  $\alpha^{32}$ P-dATP were added. The reactions were vortexed and then 2 ul were dispensed in tubes containing 1.5 ul dd/dATP and 1.5 ul dd/dTTP, and 2.5 ul were dispensed in those containing dd/dGTP and dd/dCTP. (All dd/dNTPs were lab stocks prepared previously.) Klenow fragment (0.2 U diluted in 100mM Tris-HCl pH 8.0, 100 mg/ml BSA, 10mM DTT) was added to each and the tubes spun briefly to mix the contents. The reactions were incubated at 50°C for exactly 15 minutes, then chased with 1 ul 0.5mM dATP and an

additional 0.2 U Klenow fragments. The reactions were incubated for another 15 minutes at 50°C and terminated by the addition of 5 ul sample buffer (98% deionized formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 10mM EDTA). The DNA was denatured at 90°C for 3 minutes and quenched on ice prior to loading 3 ul onto each polyacrylamide gel.

Up to four templates were sequenced per gel and 2-3 gels with the same loading order were electrophoresed in 0.5X TBE (10X=0.9M Tris base, 0.9M Boric acid, 0.25M EDTA) at 35 W per gel as follows: the 8% gel for 60 to 80 minutes, one 6% gel for 2.5 hours, and the other 6% gel for 4.5 hours. After electrophoresis, the gels were transferred from their glass plates to 3MM Whatman filter paper and dried down under vacuum using a gel drier at 80°C for 15 minutes. The gels were exposed to Kodak XRP-1 film overnight at room temperature. DNA sequence analyses were carried out using the computer program of Delaney (1982).

E. Double Stranded Sequencing. Double stranded sequencing was attempted only once following the protocol described by Stratagene for use with their Bluescript vectors (Stratagene).

## VIII. GENOMIC SOUTHERN ANALYSES

Leishmania genomic DNA (10 ug) was digested in a total volume of 200 ul containing 50 U of the desired restriction enzyme(s) and salt conditions as recommended by the supplier. Incubation was carried out overnight at the appropriate temperature after which 0.2 ug of each sample were electrophoresed through a 1% agarose mini-gel and checked for complete digestion. When completely digested, the samples were precipitated with 1/10 volume 2.5M NaAc and 2 volumes of EtOH at -70°C for 30 minutes and centrifuged in a microfuge for 15 minutes. The pelleted DNA was dried under vacuum and resuspended in 10 ul dH<sub>2</sub>O and 2.5 ul sample buffer. The samples were then electrophoresed with known  $\lambda$  standards for 2-3 hours through an agarose gel in 1X TAE at 60 V and 120 V for mini-gels and large gels respectively.

After marking the gel for orientation, the DNA fragments were denatured by soaking the gel twice in 0.5N NaOH, 1.5M NaCl for 20 minutes each and neutralized by soaking the gel twice in 0.5M Tris-HCl pH 7.5, 1.5M NaCl for 20 minutes each. The DNA was then transferred according to Southern (1975) onto nitrocellulose (BA85 Schleicher and Schuell) or

Hybond-N (Amersham) with 20X SSC overnight at room temperature. The membranes were rinsed in 20X SSC with gentle rubbing, placed between Saran wrap and then exposed to shortwave UV light for 3 minutes to crosslink the DNA to the filters. Prehybridization was carried out at 65°C for at least 1 hour in plastic bags containing 4 to 8 ml prehybridization solution (6X SSC, 1% SDS, 50mM Tris-HCl pH 7.5, 1 mM EDTA, 5X Denhardt's solution, and 100 ug/ml denatured herring testes DNA) between two glass plates. Hybridization was carried out using 2 to 4 ml fresh solution and the radiolabelled probe at 65°C overnight.

Membranes were washed twice at 65°C in solutions containing 2X SSC for 15 minutes each, followed by two washes in 2X SSC/0.1% SDS and one wash in 0.1X SSC. Filters were then rinsed 2 times at room temperature in 0.1X SSC, air-dried, and exposed to either Kodak X-OMAT RP-1 or XAR-2 film with an intensifying screen for at least 24 hours at -70°C.

## IX. LEISHMANIA RNA PREPARATION

A. GuHCl-CsCl Method. Total RNA from L. major cells grown at 26°C and 37°C for various time intervals was prepared as described by Chirgwin (1979). Cells were transferred from culture flasks to prechilled 50 ml sterile tubes and pelleted from the PBS media by centrifugation at 1600g for 10 minutes at 4°C. The cells were washed gently with several ml of DEP-treated 0.15M NaCl using a wide-bore pipette and pelleted again. The cells were then lysed in 2-3 ml lysis buffer (7.5M GuHCl, 0.5% sarcosine added fresh, 10mM DTT added fresh) and the viscosity reduced by shearing first through a 22G needle and then an 18G needle. The lysate was layered on a 3 ml CsCl cushion (5.7M CsCl in 0.25mM Sodium Citrate) in a 5.4 ml Beckman cellulose nitrate tube which had been pretreated with 95% EtOH and 0.1% DEP to inactivate RNases. The RNA was pelleted through the gradient by centrifugation in an LC-8M ultrafuge with an SW50.1 swinging bucket rotor at 36,000 rpm for at least 21 hours at 20°C.

The supernatant was carefully pipetted off using a sterile pasteur pipette and the RNA pellet thoroughly resuspended in 200 ul TE/0.1% SDS. The RNA was then transferred to a sterile 1.5 ml Eppendorf tube to which an equal volume of phenol was added immediately and vortexed. The tube was centrifuged in a microfuge for 5 minutes and the aqueous layer was

removed. The phenol layer was back extracted with DEP-treated dH<sub>2</sub>O until an interface layer no longer formed. All aqueous layers were then extracted with an equal volume of 24:1 and precipitated with 1/10 volume 2.5M DEP-treated NaAc and 2.5 volumes 95% EtOH at -20°C overnight.

The RNA was recovered by centrifugation in a microfuge for 30 minutes, washed with 70% EtOH, and the pellet dried under vacuum. The RNA was resuspended in DEP-treated dH<sub>2</sub>O and the concentration determined by spectrophotometric analysis at 260nm wavelength using a Molar Extinction Coefficient of 25 for RNA (Maniatis, 1982). Five ug was heated at 90°C for 5 minutes, quenched on ice, and electrophoresed on a 1% agarose gel in sterile 1X TAE against known quantities of RNA to confirm the concentration and check for integrity of ribosomal RNAs.

B. GuSCN-LiCl Method. Total RNA was prepared from cells grown at 26°C and 42°C using the method described by Cathala et al. (1984). After the cells were washed with DEP-treated 0.15M NaCl, the packed cells were weighed and 7 ml of lysis solution (5M GuSCN, 50mM Tris-HCl pH 7.5, 10mM EDTA, 0.1mM  $\beta$ -mercaptoethanol added fresh) per gm of cells were added. The lysate was vortexed thoroughly and sheared with an 18G needle and syringe. Then 7 volumes of 4M LiCl per volume of homogenate were added and the RNA stored at 4°C for at least 20 hours. The lysate was transferred to 15 ml Corex tubes (DEP treated and autoclaved) and centrifuged at 10,000 rpm for 90 minutes at 4°C. The pellet was dissolved in 7 volumes 3M LiCl per volume of homogenate and centrifuged at 10,000 rpm for a further 60 minutes. The resulting pellet was solubilized in TE/0.1% SDS and then extracted immediately with an equal volume of phenol. The aqueous layer was transferred to a sterile 1.5 ml Eppendorf tube and the phenol layer back extracted with sterile TE until an interface was barely visible. The aqueous layers were extracted once with an equal volume of 24:1, and the RNA precipitated and analysed as described above.

## X. NORTHERN BLOT ANALYSES

Northern blot analyses were carried out as described by Maniatis (1982) and Gerard and Miller (1986). In a 1.5 ml sterile Eppendorf tube, 2 ul of total RNA (5 mg/ml) were mixed thoroughly with 0.5 ul 5X Buffer A (1X Buffer A=0.147M MOPS pH 7.0, 3mM EDTA) and 5.5 ul formaldehyde/formamide solution ( 89 ul of 37% formaldehyde, 250 ul deionized formamide). The solution was heated at 70°C for 10 minutes and quenched on ice for 3 minutes. Then 1.7 ul Gel loading Buffer (322 ul 1X Buffer A, 5 mg xylene cyanol, 5 mg bromocresol green, 400 mg sucrose, 178 ul 37% formaldehyde, 500 ul deionized formamide) was added and mixed. Samples were loaded on a pre-electrophoresed 1.2% agarose gel in 1X MOPS/EDTA (10X MOPS/EDTA= 0.5M MOPS pH 7.0, 0.01 M EDTA pH 7.5). Gel electrophoresis was carried out for 2-3 hours at 120 V with periodic mixing of the buffer in the electrophoretic unit.

The gel was stained with acridine orange at 30 ug/ml in sterile dH<sub>2</sub>O for 30 minutes and destained with several changes of sterile dH<sub>2</sub>O at least for 45 minutes. The RNA was transferred onto Hybond-N with 20X SSC overnight, and the hybridization and washing conditions were exactly as described for Southern blot analysis when using the *Leishmania* ubiquitin gene as a probe. For Northern blots using the 2.0 kb Xba I fragment from p132E3(S) (provided by P. Candido, University of British Columbia, Vancouver, B.C.: Moran et al., 1979) carrying the *Drosophila* Hsp70 gene as the probe, the temperature of hybridization and washing was lowered to 50°C.

## RESULTS

### I. IDENTIFICATION AND CHARACTERIZATION OF UBIQUITIN CODING ELEMENTS

#### A. IDENTIFICATION OF AN UBIQUITIN GENE IN L. MAJOR

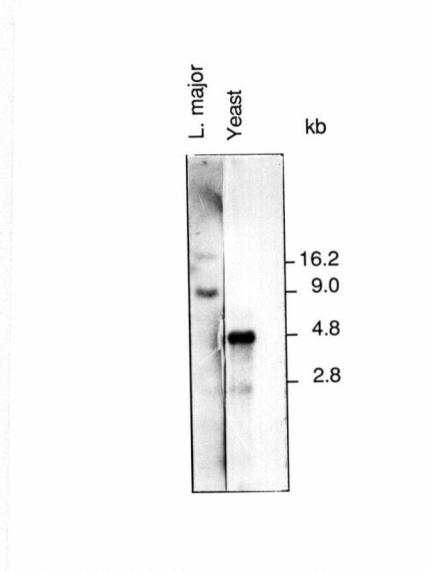
Five ug of L. major DNA and 2.5 ug of yeast DNA (strain AB20 $\alpha$  provided by L. L. Button, UBC; Nasmyth and Tatchell, 1980) were digested with Eco RI and hybridized to the yeast ubiquitin gene (1220 bp Bgl II/Bcl I insert of pUB2 carrying 5 ubiquitin coding elements) as shown in Figure 4. Under low stringency hybridization and washing conditions, a band at 4.8 kb is detected in the lane containing yeast DNA which corresponds to the gene from which the probe was derived (Ozkaynak et al., 1984). The autoradiogram is a 4 hour exposure, and a longer exposure reveals at least 3 other cross-hybridizing bands which denote other ubiquitin loci in this organism (exposure not shown). A single band at approximately 9.0 kb is also detected after 24 hours in the lane containing L. major DNA. This latter finding shows that a homologous gene exists in the parasite and can be detected under the given conditions.

#### B. $\lambda$ EMBL 3 LIBRARY SCREENING REVEALS TWO POSITIVE CLONES

The  $\lambda$ EMBL 3 library was screened with the yeast ubiquitin gene under the same conditions used for the genomic blot. Two positive clones were isolated and named  $\lambda$ LUB.A and  $\lambda$ LUB.C. Due to the loss of one or both of the cloning sites in each of the  $\lambda$  DNAs, the genomic inserts could not be excised from the vector by the predicted restriction enzyme digest. Attempts at constructing a restriction map were not successful due to the large size and complexity of the recombinant DNAs. Consequently, each species of DNA was cut with a panel of restriction enzymes and hybridized to the yeast gene in order to determine which fragments carried ubiquitin coding elements.

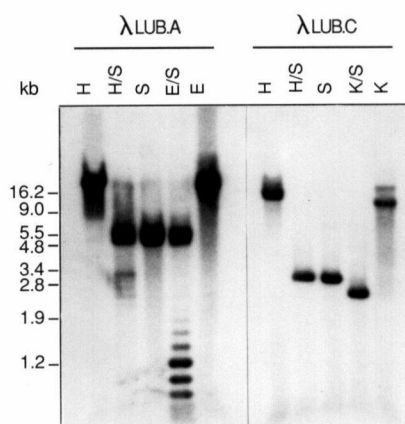
Figure 5 compares the fragments in each clone which hybridized to the probe. Firstly, since  $\lambda$ LUB.A and  $\lambda$ LUB.C do not appear to share any similar bands in equivalent digests, a region of overlap between the two DNAs cannot be assigned. Secondly, an obvious and unusual

**FIGURE 4.** Hybridization of the Bgl II/Bcl I fragment from pUB2 to L. major and Yeast Genomic DNA



Genomic DNA from L. major and yeast was digested to completion with Eco RI, separated on a 0.5% agarose gel, transferred to nitrocellulose and hybridized with the nick-translated 1220 bp Bgl II/Bcl I fragment from pUB2. The autoradiogram for L. major DNA has been exposed 6 times longer than that of yeast DNA. Marker sizes are derived from Bam HI/Eco RI digested phage  $\lambda$  DNA.

**Figure 5.** Hybridization of the Bgl II/Bcl I fragment from pUB2 to  $\lambda$ LUB.A and  $\lambda$ LUB.C.



DNA prepared from  $\lambda$ LUB.A and  $\lambda$ LUB.C was digested with Hind III (H), Eco RI (E), Sal I (S), and Kpn I (K) and separated on a 0.7% agarose gel. The fragments were transferred to nitrocellulose and hybridized to the nick-translated Bgl II/Bcl I fragment from pUB2. Marker sizes are derived from Bam HI/Eco RI digested phage  $\lambda$  DNA.



ladder pattern is observed below the 2 kb region in the Eco RI/Sal I lane of  $\lambda$ LUB.A in addition to the two closely spaced bands in the 5.5 kb region. It was initially thought that this pattern was due to incomplete digestion of the DNA, however, EtBr staining of the gel prior to transfer did not reveal any bands in this region despite the relative intensity of the signals compared with those in the 5.5 kb region. These conflicting data on  $\lambda$ LUB.A suggested that  $\lambda$ LUB.C would be easier to characterize. Thus, the 3 kb Sal I fragment of  $\lambda$ LUB.C was isolated and cloned into the Sal I site of pUC19 which was then transformed into *E. coli* cells of the strain JM83. The recombinant plasmid was named pLUB.C3.

## 1. CHARACTERIZATION OF pLUB.C3

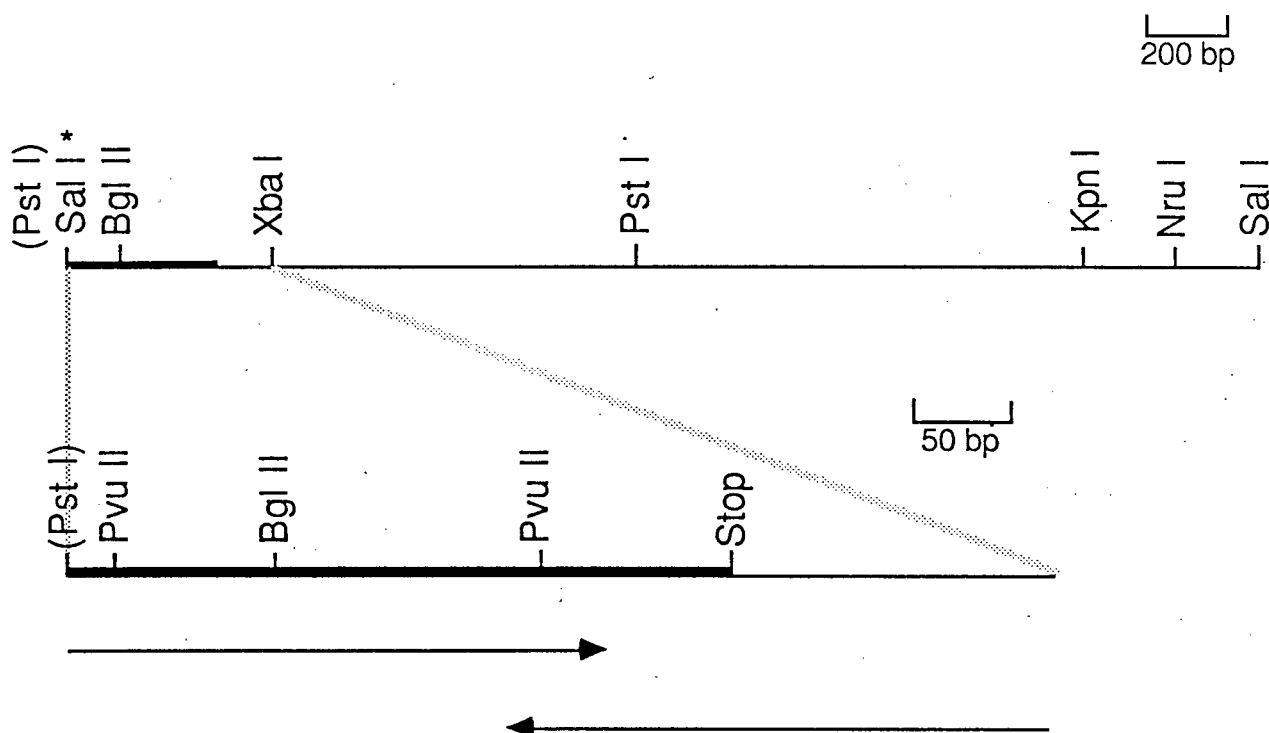
A restriction map of pLUB.C3 generated from analyses of restriction fragments on agarose gels and Southern blots is presented in Figure 6. The sequencing strategy of the Pst I/Xba I fragment which was subcloned into M13mp18 and M13mp19 is shown enlarged on the same figure.

## 2. DNA AND AMINO ACID SEQUENCES OF THE PST I/XBA I FRAGMENT

Templates were made from 4 individual subclones in each vector and the average length of sequence determined per subclone was 250 bp. The sequences were overlapped to obtain the complete nucleotide sequence presented in Figure 7. The fragment is 507 bp in length excluding 16 bp at the 5' end which can be accounted for by  $\lambda$ EMBL 3 and pUC19 vector sequences. The DNA sequence begins in the middle of one ubiquitin coding element and extends, without any intervening sequences, to include a complete ubiquitin unit of 228 bp, 3 bp preceding the TAA stop signal, and 166 bp of 3' untranslated (UT) sequence. There is no evidence of a polyadenylation signal (AATAAA) in the 3' UT regions.

Figure 7 also depicts the translated amino acid sequence of the *L. major* ubiquitin coding elements. The only non-ubiquitin coding residue is a cysteine residue found preceding the stop codon. Sequences common to the two elements share 100% identity at both the protein and DNA levels.

**FIGURE 6.** Restriction Map and Sequencing Strategy of pLUB.C3.



Inserts are oriented such that transcription occurs left to right. The heavy black lines represent the coding regions and the fine lines represent 3' sequences. Parentheses and stars denote restriction sites arising from plasmid and  $\lambda$ EMBL3 vector sequences respectively. Horizontal arrows indicate the direction and extent of sequencing.

**Figure 7.** DNA and Predicted Amino Acid Sequence of the Pst I/Xba I fragment of pLUB.C3.

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54
Ile Phe Ala Gly Lys Gln Leu Glu Glu Gly Arg Thr Leu Ser Asp Tyr Asn
ATC TTC GCC GGC AAG CAG CTG GAG GAG GGC CGC ACG CTC TCG GAC TAC AAC 51

76 -1
Ile Gln Lys Glu Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly Met
ATC CAG AAG GAG TCC ACG CTG CAC CTG GTG CTG CGC CTG CGC GGC GGC ATG 102

Gln Ile Phe Val Lys Thr Leu Thr Gly Lys Thr Ile Ala Leu Glu Val Glu
CAG ATC TTC GTG AAG ACG CTG ACC GGC AAG ACG ATC GCG CTG GAG GTG GAG 153
Bgl II

Pro Ser Asp Thr Ile Glu Asn Val Lys Ala Lys Ile Gln Asp Lys Glu Gly
CCG AGC GAC ACG ATC GAG AAC GTG AAG GCC AAG ATC CAG GAC AAG GAG GGC 204

Ile Pro Pro Asp Gln Gln Arg Leu Ile Phe Ala Gly Lys Gln Leu Glu Glu
ATC CCG CCG GAC CAG CAG CGC CTG ATC TTC GCC GGC AAG CAG CTG GAG GAG 255
Pvu II

Gly Arg Thr Leu Ser Asp Tyr Asn Ile Gln Lys Glu Ser Thr Leu His Leu
GGC CGC ACG CTC TCG GAC TAC AAC ATC CAG AAG GAG TCC ACG CTG CAC CTG 306

76
Val Leu Arg Leu Arg Gly Gly Cys Stop
GTG CTG CGC CTG CGC GGC GGC TGT TAA GCTCTGTTGGCTGAAGCGCTGTAGCCAAGGT 364

GTTTTTGCTTTTTCTGCACTACGATGCGGCGTTTATTGCTAGAGGCTTATCCCTGAATTGTCTCGG 431

AAGCGGGTTAGTGTTGTGGCGTGATGTGAATGATAATGTTTGAAGGTCCGGGCGATTGTTGCGGG 498

AATGGTTGC 507

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The nucleotide sequence begins in the middle of one ubiquitin coding element and is immediately followed by the complete sequence of another element. Bases 341-507 comprise the 3' UT sequence in which there was no evidence of a polyadenylation signal. Positions of amino acids, base pairs, and restriction sites are as indicated.

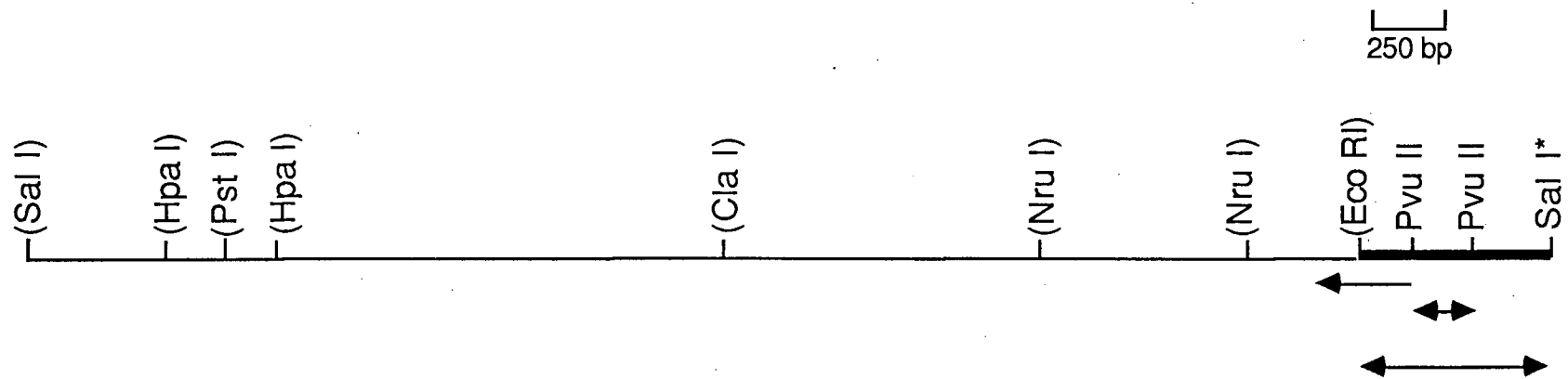
### 3. CHARACTERIZATION OF pLUB.A54

The 5.5 kb region of  $\lambda$ LUB.A generated by a Sal I digest was subsequently isolated and subcloned into the plasmid vector  $\text{Blu}^+$  which was then used to transform JM101. Although both the 5.4 kb and the 6.0 kb fragments indicated in Figure 5 should have had an equal probability of ligation, all successful recombinants carried only the 5.4 kb fragment as determined by mini-plasmid preps. A restriction map of the recombinant plasmid, pLUB.A54, is shown in Figure 8 along with the sequencing strategy of the Eco RI/Sal I fragment. The blunt-ended fragments were subcloned into Sma I cut M13mp18 and the Eco RI/Sal I fragment was force cloned into M13mp18 and M13mp19.

By overlapping the sequences of the various M13 templates, the complete DNA sequence between the Eco RI and 3' Sal I sites was determined as shown in Figure 9. The DNA sequence begins 11 bp after the Eco RI cleavage site and continues 582 bp 3' to include one complete ubiquitin coding element and partial sequence of the flanking elements. The sequence suggests that the ubiquitin gene is comprised of at least 3 elements which share 100% identity at the nucleotide level.

Although informative, the sequence of the Eco RI/Sal I fragment presents many ambiguities as indicated by the underlined area in the figure. Firstly, the 3' region has 13 nucleotides which are believed to be derived from the  $\lambda$ EMBL 3 cloning site. Secondly, the 5' region of the proximal ubiquitin element is interrupted by unfamiliar sequence which was confirmed by sequencing through the Eco RI site in the Pvu II/Nru I fragment shown in Figure 8. There is no evidence of either a cis-splicing acceptor site (AG) to suggest the presence of an intron or the spliced leader acceptor sequence involved in trans-splicing of trypanosomes (Borst, 1986). Therefore, to establish the origin of the sequence 5' of the Eco RI site, the Nru I/Eco RI fragment from pLUB.A54 was isolated and hybridized to Nru I restricted DNA from  $\lambda$ EMBL 3,  $\lambda$ LUB.A and *L. major*. The probe hybridizes to the same fragment in each of the  $\lambda$  DNAs in addition to a high molecular weight species in the genomic DNA (data not shown).

**FIGURE 8.** Restriction Map and Sequencing Strategy of pLUB.A54.



The insert is oriented such that transcription occurs left to right. The bold line indicates ubiquitin coding sequence. The bold line and sites in parentheses denote uncertainty in the origin of these sequences. The site shown with an asterisk is derived from  $\lambda$ EMBL 3. Horizontal arrows indicate the direction and extent of sequencing.

**Figure 9.** The DNA and Amino Acid Sequence of the Eco RI/Sal I Fragment of pLUB.A54.

The nucleotide sequence of a ubiquitin coding element begins 11 bp after the Eco RI cleavage site. Ambiguous sequences at both the 5' and 3' ends are underlined. Positions of base pairs, amino acids, and restriction sites are as indicated.

**Figure 9.** The DNA and Amino Acid Sequence of the  
Eco RI/Sal I Fragment of pLUB.A54.

AGGTATTTTATCGCGCAGCGCAAAAAGCATCAGGTCCTTCCTTCGAAGGGGATCTGGGAATTC 68  
 Eco RI

3

Ile Phe Val Lys Thr Leu Thr Gly Lys Thr Ile Ala Leu Glu Val Glu Pro  
 ATC TTC GTG AAG ACG CTG ACC GGC AAG ACG ATC GCG CTG GAG GTG GAG CCG 119

Ser Asp Thr Ile Glu Asn Val Lys Ala Lys Ile Gln Asp Lys Glu Gly Ile  
 AGC GAC ACG ATC GAG AAC GTG AAG GCC AAG ATC CAG GAC AAG GAG GGC ATC 170

Pro Pro Asp Gln Gln Arg Leu Ile Phe Ala Gly Lys Gln Leu Glu Glu Gly  
 CCG CCG GAC CAG CAG CGC CTG ATC TTC GCC GGC AAG CAG CTG GAG GAG GGC 221  
 Pvu II

Arg Thr Leu Ser Asp Tyr Asn Ile Gln Lys Glu Ser Thr Leu His Leu Val  
 CGC ACG CTC TCG GAC TAC AAC ATC CAG AAG GAG TCC ACG CTG CAC CTG GTG 272

76 1

Leu Arg Leu Arg Gly Gly Met Gln Ile Phe Val Lys Thr Leu Thr Gly Lys  
 CTG CGC CTG CGC GGC GGC ATG CAG ATC TTC GTG AAG ACG CTG ACC GGC AAG 323  
 Bgl II

Thr Ile Ala Leu Glu Val Glu Pro Ser Asp Thr Ile Glu Asn Val Lys Ala  
 ACG ATC GCG CTG GAG GTG GAG CCG AGC GAC ACG ATC GAG AAC GTG AAG GCC 374

Lys Ile Gln Asp Lys Glu Gly Ile Pro Pro Asp Gln Gln Arg Leu Ile Phe  
 AAG ATC CAG GAC AAG GAG GGC ATC CCG CCG GAC CAG CAG CGC CTG ATC TTC 425

Ala Gly Lys Gln Leu Glu Glu Gly Arg Thr Leu Ser Asp Tyr Asn Ile Gln  
 GCC GGC AAG CAG CTG GAG GAG GGC CGC ACG CTC TCG GAC TAC AAC ATC CAG 476  
 Pvu II

76 1

Lys Glu Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly Met Gln Ile  
 AAG GAG TCC ACG CTG CAC CTG GTG CTG CGC CTG CGC GGC GGC ATG CAG ATC 527  
 Bgl II

Phe Val Lys Thr Leu Thr Gly Lys Thr Ile Ala Leu Glu Val Glu Pro Ser  
ITC GTG AAG ACG CTG ACC GGC AAG ACG ATC GCG CTG GAG GTG GAG CCG AGC 578

Asp Thr Ile Glu Asn Val Lys Ala Lys Ile Gln Asp Lys Glu Gly Ile Pro  
 GAC ACG ATC GAG AAC GTG AAG GCC AAG ATC CAG GAC AAG GAG GGC ATC CCG 629

44

Pro Asp Gln Gln Arg Leu Ile  
 CCG GAC CAG CAG CGC CTG ATC CGTTGAGACTGCAG 664  
 Sal I

This finding suggests that a rearrangement had occurred in  $\lambda$ LUB.A either as a recombination event between two clones or possibly as an artifact of the library construction in which two random pieces of genomic DNA were joined by the linker sequence. No further characterization of pLUB.A54 was pursued.

### C. $\lambda$ GT11 LIBRARY SCREENING

The  $\lambda$ gt11 genomic library was screened with the 507 bp Pst I/Xba I sequence from pLUB.C3 in an attempt to isolate clones carrying the full length gene and/or 5' region. Of the 250,000 screened, 7 strong positives were identified and their inserts cloned into the Eco RI site of  $\text{Blu}^+$ . Restriction enzyme analyses of mini-prep plasmid DNAs indicated that all 7 contained the Xba I site, and therefore, the 3' region. The longest clone, designated pLUB.1, was chosen for further characterization.

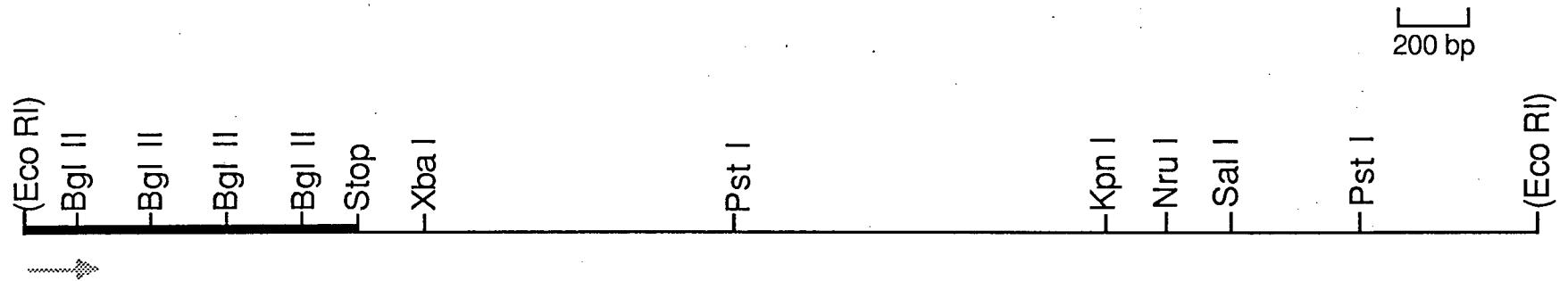
#### 1. CHARACTERIZATION OF pLUB.1

A restriction map of pLUB.1 is presented in Figure 10. The insert is approximately 4.5 kb in length of which approximately 1 kb contains ubiquitin coding sequences. To isolate this coding sequence, the 1.2 kb Eco RI/Xba I fragment was subcloned into  $\text{Blu}^+$  and used to transform DH5 $\alpha$  cells. After performing a large scale plasmid preparation, however, the resulting plasmid actually comprised of a population of plasmids carrying various deletions of the ubiquitin coding elements. This mixed population can be detected easily when the plasmid is digested with Eco RI/Xba I and electrophoresed on an agarose gel containing EtBr. Two additional bands varying by 200 bp are observed below the 1.2 kb insert suggesting that the recombinant plasmid had been highly unstable during its propagation.

Although it is not clear whether the deletions arose by inter- or intra-molecular recombination or both, it is likely that the instability is the result of the elements sharing 100% identity in their nucleotide sequences. Such a interpretation is consistent with the data presented on  $\lambda$ LUB.A in Figure 5 in that the "ladder pattern" can now be attributed to deletions in the  $\lambda$  clone. If this is the case, then the 5.4 kb Sal I fragment may be a derivative of the 6.0



**FIGURE 10.** Restriction Map of pLUB.1.



The insert is oriented such that transcription occurs left to right. The bold and fine lines represent coding and 3' sequences respectively. Parentheses denote sites derived from the Eco RI linkers used during library construction. The hatched arrow denotes an area that was sequenced by double stranded sequencing.

kb fragment minus three coding elements. Likewise, the rearrangement seen in the 5' region of the fragment may also be a manifestation of the deletion event.

pLUB.1 was sequenced by double stranded sequencing methods from the 5' Eco RI linker site into the insert to determine if the clone carried a full length gene. Although only 40 bp around the Bgl II site could be read clearly, sufficient DNA sequence was analysed to show that the clone ends within another ubiquitin coding unit. Thus, pLUB.1 contains 4.5 ubiquitin coding elements arranged in tandem, and no further sequence analyses were carried out.

## II. COMPARISON OF THE AMINO ACID SEQUENCES OF UBIQUITIN IN DIFFERENT ORGANISMS

Figure 11 shows a comparison of ubiquitin amino acid sequences among organisms representing four kingdoms: protista, animal, fungal, and plant. The sequence presented for human ubiquitin is also the sequence of ubiquitin in chicken (Bond and Schlesinger, 1985), *Xenopus* (Dworkin-Rastl et al., 1984), trout (Watson et al., 1978), and *Drosophila* (Gavilanes et al., 1982). By indicating only the amino acid differences between Leishmania ubiquitin and ubiquitin in each of the other organisms, the high level of protein conservation found throughout eukaryotic evolution is underscored. The only amino acid positions that are unique to Leishmania are the alanine and glutamic acid residues at positions 14 and 52 respectively. Otherwise, leishmania ubiquitin is most similar to human ubiquitin differing at only 2 amino acid positions.

## III. IDENTIFICATION OF ANOTHER UBIQUITIN ENCODING LOCUS

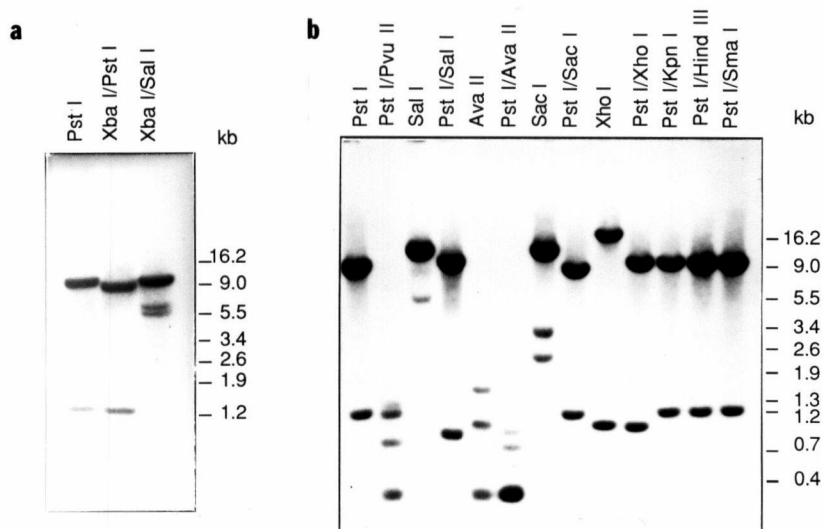
Based on the finding that sites for Pst I and Xba I map in the 3' flanking sequence of many of the  $\lambda$  clones, L.major genomic DNA was digested with these enzymes and hybridized to the 507 bp fragment of pLUB.C3 to show that the  $\lambda$  DNAs could be accounted for in the genome. As shown in Figure 12a, the probe hybridizes to 2 species when the genomic DNA is digested with Pst I alone or in combination with Xba I suggesting that there are 2 loci encoding ubiquitin. Comparisons between the Pst I and Pst I/Xba I digests indicate that all the  $\lambda$  clones

**FIGURE 11.** Amino Acid Sequences of Ubiquitin in Various Kingdoms.

Leishmania	MQIFVKTLTGKTIALEVEPSDTIENVKAKIQDKEGIPP
Human	----- T -----
Yeast	----- T --- S --- D --- S -----
Barley	----- T --- S --- D -----
Leishmania	DQQRLIFAGKQLEEGRTLSDYNIQKESTLHLVLRRLRGG
Human	----- D -----
Yeast	----- D -----
Barley	----- D --- A -----

Sources of data: human (Wiborg et al., 1985), yeast (Ozkaynak et al., 1984), barley (Gausing et al., 1986).

**Figure 12.** Hybridization of the Pst I/Xba I fragment from pLUB.C3 to L. major Genomic DNA.



Genomic DNA from L. major was digested with one or more restriction enzymes, separated on a 0.5% agarose gel, transferred to Hybond-N, and hybridized with the nick-translated PstI/Xba I fragment of pLUB.C3. The autoradiogram in panel A is that of a mini-gel with marker sizes derived from Bam HI/Eco RI digested phage  $\lambda$  DNA. Panel B shows an autoradiogram from a large gel with additional markers derived from Taq I digested pBR322.

which have the Xba I site contain ubiquitin coding elements which map to a 9.0 kb Pst I fragment. The other locus maps to a 1.2 kb Pst I fragment. (These 2 loci are hereafter referred to as the 9.0 kb locus and the 1.2 kb locus.)

Further demonstration of the existence of 2 loci is presented in Figure 12b which consists of genomic DNA digested with a panel of restriction enzymes and again hybridized to the 507 bp fragment of pLUB.C3. A number of significant features are noted as follows. Firstly, the bands appearing in the lanes marked PstI/Sal I and Pst I/Xho I show that the ubiquitin coding element at the 1.2 kb locus can in fact be located within a 1 kb region carrying Xho I and Sal I sites as flanking markers. Also, these 2 enzymes do not have sites within the 9.0 kb locus. Secondly, the Pst I/Pvu II is particularly informative since Pvu II cuts consistently within the ubiquitin coding elements. The smallest band represents all ubiquitin units comprised of 228 bp. The band at 1.1 kb represents the Pvu II/Pst I fragment comprised of DNA from the Pvu II site in the last coding element to the 3' Pst I site (refer to Figure 6). Since the 1.3 kb fragment is larger than the 1.2 kb fragment obtained from a Pst I single digest, it must represent DNA carrying the 5' region of the 9.0 kb locus. Hence, the remaining 2 bands at approximately 700 bp and 500 bp (the latter is faint on the photograph in Figure 12) are derived from the 1.2 kb locus. This locus does not appear to contain more than one ubiquitin coding element or at least not more than one carrying the Pvu II site. Thirdly, it is striking that the 9.0 kb locus appears to be relatively devoid of restriction sites. With the exception of Ava II and Pvu II which cut within the coding element, Sac I and Xba I are the only other markers which define the 9.0 kb locus. Because the Sac I site maps just 3' to the Xba I site in pLUB.C3, the 2 enzymes give similar banding patterns when the genomic DNA is digested in combination with Pst I.

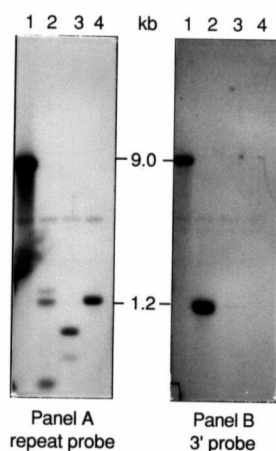
Since trypanosome telomeres have been shown to possess stretches of barren DNA, one explanation for the latter observation is that the 9.0 kb locus exists at the end of a chromosome. Therefore, L. major genomic DNA was treated with Bal 31 for 0, 10, 20, 40, and 60 minutes before being digested with Pst I and hybridized to the 507 bp fragment of pLUB.C3. If the 9.0 kb locus was located at a telomere, the Bal 31 treatment would reduce the size of the Pst I

fragment and cause regions of smearing. Furthermore, the smear would decrease in size with increased Bal 31 digestion time. No change in the 9.0 kb Pst I fragment was observed (data not shown). Since there are no leishmania genes known to occur at a telomere, a positive control could not be included in the experiment. However, the Bal 31 enzyme had been assayed for efficient activity using plasmid DNA. Consequently, the negative result suggests that the 9.0 kb locus is not located at a telomere.

Since the Pvu II/Pst I double digest indicates that the 5' end of the 9.0 kb locus is located on a 1.3 kb Pvu II fragment, attempts were made to isolate this region so that a specific genomic library enriched for fragments in this size range could be made. In order to study this 5' end, the 9.0 kb locus had to be isolated from the 1.2 kb locus. This isolation was achieved by first digesting genomic DNA with Pst I and then using Geneclean to purify the DNA in the 2 regions which were thereafter stored separately. After removing an aliquot from each, the DNA was digested with Pvu II and samples loaded in duplicate were electrophoresed on an agarose gel. The DNA was then transferred to Hybond-N and one filter was hybridized to a nick translated 228 Bgl II fragment prepared from pLUB.1 (designated as the repeat probe). Figure 13 (Panel A) shows the autoradiogram of this blot where lanes 1 and 4 contain DNA isolated from the Pst I digest and lanes 2 and 3 contain the DNA subsequently digested with Pvu II. The bands correspond to the pattern seen in the Pst I/Pvu II digest in Figure 12b except that in this autoradiogram, the bands can be assigned unambiguously to their respective loci. When a nick translated 800 bp Xba I/Pst I fragment from pLUB.C3 containing 3' flanking sequence (designated as the 3' probe) was hybridized to the twin filter, only the 9.0 kb locus hybridized as expected (Figure 13, Panel B). The band at 1.1 kb in lane 2 denotes the piece of DNA from which the probe is derived. The faint band seen in each lane spanning the middle of the autoradiograms appears to be an artifact as its signal is not enhanced with prolonged exposure to film.

Although DNA from the 2 loci could be separated successfully, all attempts to isolate a positive clone from the enriched library were unsuccessful. Likewise, the Pst I fragments in the 9.0 kb and 1.2 kb regions could not be sufficiently represented in a plasmid library. Thus,

**Figure 13.** Hybridization of coding sequences and non-coding sequences to isolated regions of *L. major* genomic DNA



*L. major* genomic DNA was digested with Pst I and the 9.0 kb (lane 1) and 1.2 kb (lane 4) regions were isolated. These regions were then digested to completion with Pvu II (lanes 2 and 3 respectively) and electrophoresed on a 0.7% agarose gel. After being transferred to Hybond-N, the fragments were probed with either the nick-translated 228 bp Bgl II fragment containing one ubiquitin coding element (Panel A) or the nick-translated 800 bp Xba I/Pst I fragment of pLUB.C3 containing 3' flanking sequence (Panel B). Marker sizes are derived from Bam HI/Eco RI digested phage  $\lambda$  DNA.

neither the 5' end of the 9.0 kb locus nor the 1.2 kb locus was characterized at the nucleotide level. The possibility exists that the 5' region of ubiquitin is unstable when grown in *E. coli* such that any plasmids carrying this region consistently deleted the insert.

#### IV. UBIQUITIN GENE EXPRESSION

As Bond and Schlesinger (1986) have demonstrated in chicken embryo fibroblasts, ubiquitin gene expression can be heat inducible. For this reason, *L. major* cultures were grown at 37°C and 42°C to determine the effects of heat on ubiquitin gene expression in these cells. Total RNA was prepared from two batches of *L. major* cultures grown to log phase and treated in the following manner. Batch 1 is comprised of cells grown at 37°C for 2 hours, 12 hours, and 24 hours. Batch 2 is comprised of cells grown at 42°C for 30 minutes, 2 hours, and 4 hours. RNA was also prepared from cells maintained at 26°C for each batch. Once the concentrations of RNA from each sample had been determined accurately, two gels were run with constant amounts of RNA loaded in each lane (10 ug/lane). Figure 14a shows these gels stained with acridine orange prior to transfer. The 3 ribosomal RNAs, 19s, 28s $\alpha$ , and 28s $\beta$  migrate at 2.25, 1.85, and 1.55 kb respectively when compared with an RNA ladder sized in kb. Acridine orange staining of the rRNA bands among the various lanes indicate that all samples contained equal quantities of total RNA. After transferring the RNAs to Hybond-N filters, the gels were stained with EtBr to ensure that no RNA remained in the gel. Then one filter was hybridized to the 228 bp Bgl II "repeat" probe and the other filter was hybridized to the 2 kb Xba I fragment of p132E3(s) containing the Z element of the *Drosophila* Hsp70 gene.

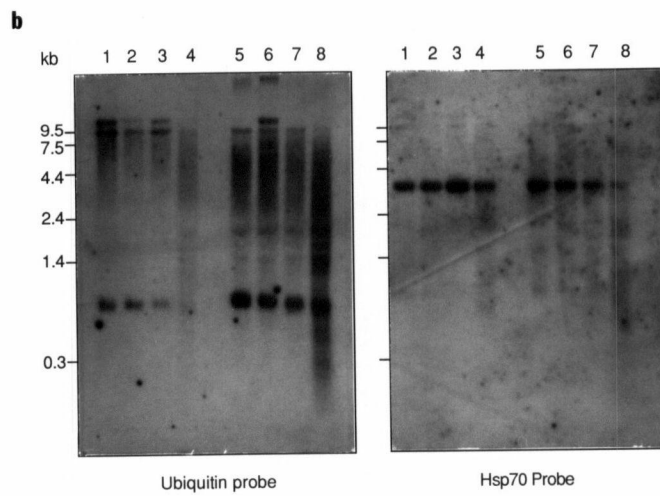
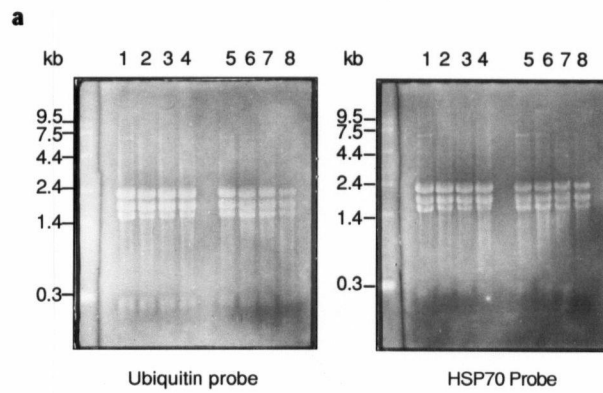
As shown in the left panel of Figure 14b, hybridization to the ubiquitin probe reveals a signal at 1.1 kb which is believed to represent a transcript from the 9.0 kb locus. If the 1.2 kb locus gives a discrete transcript as well, it has not been detected. Instead, RNA species in the high molecular weight region are apparent and may represent aberrant or unprocessed transcripts from the 1.2 kb locus. Alternatively, the 1.1 kb signal may actually represent two RNA transcripts which co-migrate.



**Figure 14.** Hybridization of Leishmania ubiquitin and *Drosophila* Hsp70 to total RNA prepared from L. major grown at different temperatures.

Total RNA prepared from L. major was denatured and equal amounts of RNA (10ug/lane) were separated on an 1.2% agarose gel containing 2.2M Formaldehyde. Panel A shows the RNA stained with acridine orange prior to being transferred onto Hybond-N. Panel B shows the autoradiogram after one filter was hybridized to an oligo-labelled 228 bp Bgl II fragment carrying a Leishmania ubiquitin coding element and the other filter was hybridized to an oligo-labelled 2 kb Xba I fragment carrying the *Drosophila* Hsp70 coding element. Lane 1, 26 °C; lane 2, 37 °C, 2 hr; lane 3, 37 °C, 12 hr; lane 4, 37 °C, 24 hr; lane 5, 26 °C; lane 6, 42 °C, 30 min; lane 7, 42 °C, 2 hr; lane 8, 42 °C, 4 hr. Marker sizes are derived from an RNA ladder.

**Figure 14.** Hybridization of *Leishmania* ubiquitin and *Drosophila* Hsp70 to total RNA prepared from *L. major* grown at different temperatures.



As the autoradiogram shows, gene expression at 37°C decreases with time. Since L. major grown at 37°C for 24 hours were still motile when examined under the light microscope, this decrease in gene expression cannot be attributed to cell death. An equivalent observation at 42°C, however could not be made since cells grown under these conditions could not survive much more than 4 hours. Nonetheless, the ratio in the band intensity between lanes 1 and 2 is comparable to that between lanes 5 and 7 and suggests that high temperatures tend to cause a decrease rather than increase in ubiquitin gene expression.

The Hsp 70 gene was hybridized to the second filter in a control experiment so that the signal in each lane of this blot could be compared quantitatively with its equivalent lane in the ubiquitin blot. As shown in the autoradiogram on the right of Figure 14b, Hsp 70 gene expression does not decrease with time in L. major grown at 37°C. Instead, a slightly enhanced signal at 12 hours (lane 3) can be observed and may represent a maximum level of gene expression. Thus, this finding clearly demonstrates that a decrease in RNA at 37°C is ubiquitin specific. At 42°C, however, the converse situation occurs and a progressive decrease in Hsp 70 gene expression is observed.

## DISCUSSION

### I. UBIQUITIN GENE EVOLUTION

In this study, the existence of ubiquitin coding elements is demonstrated in the protozoan parasite, Leishmania major. Since the kingdom protista is considered to be the most diverse and evolutionarily ancient of the four eukaryotic kingdoms (Whittaker, 1969), these data provide valuable insights into the evolution of ubiquitin as well as its gene structure. As mentioned earlier, ubiquitin is unique in many ways, one being its synthesis as a polyprotein precursor. Because this protein organization appears to have changed little during evolution, its design likely serves a function. Similarly, the high level of conservation of the ubiquitin protein sequence among extremely divergent organisms suggests functional constraints on the precise amino acid sequence.

According to Sharp and Li (1987), ubiquitin is evolving more slowly than any other protein. Such ideal circumstances allow the nucleotide sequences of ubiquitin coding elements within a locus to be more similar to each other than those between species. This phenomenon, referred to as concerted evolution of tandem repeats, is exemplified best by the ubiquitin coding units in Leishmania as these units share 100% identity at the nucleotide level. However, whether gene conversion or unequal crossing-over acts as the primary mechanism in this parasite remains to be determined since the frequency of neither occurrence is known.

### II. UBIQUITIN GENE EXPRESSION IN L. MAJOR

The isolation and characterization of several  $\lambda$  clones carrying Leishmania genomic DNA led to the finding that L. major has two ubiquitin encoding loci. One locus, termed the 9.0 kb locus, contains at least 5 contiguous ubiquitin coding elements arranged head-to-tail. The presumed corresponding transcript migrates at approximately 1.1 kb on a denaturing formaldehyde gel. A transcript of this size suggests that 5 is the maximum number of ubiquitin units at this locus and that the transcript contains very little 3' UT and 5' leader sequences. In contrast, the other locus, termed the 1.2 kb locus, appears to contain only one ubiquitin coding

element. Because sequence analysis on the DNA in this region was not performed, it is not known whether this locus carries a pseudogene, a gene fusion, or a gene encoding a ubiquitin monomer. Interestingly, a transcript smaller than 1.1 kb was not detected which is surprising if the gene encodes a functional protein.

It is unfortunate that plasmid and  $\lambda$  clones carrying ubiquitin coding elements were predisposed to deletions due to the nature of the DNA sequence itself. Since ubiquitin is highly conserved throughout eukaryotic evolution, there was a good possibility that analyses of the 5' region of the 9.0 kb locus might have revealed a heat shock element or some other promoter functionally resembling the eukaryotic PolII promoter. As yet, promoter sequences have not been well characterized in trypanosomatids (Glass et al., 1986). Similarly, it would have been of interest to examine the consensus 3' splice site [(C/U)<sub>n</sub>NNAG] (Sutton and Boothroyd, 1986) which is believed to be involved in the trans-splicing of all leishmania mRNAs.

Despite the lack of data concerning the regulation of ubiquitin, an attempt was made to determine how ubiquitin genes are expressed in leishmania. Cultures of L.major grown at 26°C were heat shocked in time course experiments in hopes of mimicking the conditions under which transmission of the parasites from their insect vector to their mammalian host occurs. The finding that a basal level of ubiquitin exists in promastigotes suggests that ubiquitin gene expression is constitutive and that its regulation can be uncoupled from heat shock. Given the diverse functions of the protein, it is expected that all cells, including leishmania, should express ubiquitin under normal physiological conditions. Upon a temperature shift to 37°C, ubiquitin gene expression decreases substantially whereas Hsp70 gene expression maintains its basal level. The latter observation does not correlate with the data presented by Van der Ploeg et al (1985) which showed that additional Hsp70 transcripts are induced at 37°C. However, the strain of parasites and the heat shock conditions used differ, thus direct comparisons of the data may not be feasible.

The difference in gene expression observed between Hsp70 and ubiquitin is substantial at 37°C (24 hours) and does not correlate with the supposition that transcription of both genes is induced by heat. As the basal level of expression is high, it is possible that induction is

occurring but at such a low level that it passes undetected. The drop in ubiquitin specific mRNA may subsequently be attributed to decreased stability of ubiquitin mRNA versus Hsp70 mRNA. Such an interpretation, however, conflicts with the data concerning gene expression at 42°C in which the converse is observed. It is difficult to reason why ubiquitin transcripts might suddenly be more stable than those of Hsp70 at the higher temperature.

Another interpretation of the data is that ubiquitin gene expression is controlled at the translational level while Hsp70 gene expression is controlled at the transcriptional level. It is possible that the maximum number of ubiquitin transcripts already exists in the parasites at 26°C in preparation for a large demand for ubiquitin upon exposure to an elevated temperature. Accumulation of the protein during heat shock may inhibit further expression of the gene, and this negative regulation is observed over time as a decrease in the level of ubiquitin specific mRNA.

### III. LEISHMANIA DIFFERENTIATION AND THE HEAT SHOCK RESPONSE

There are two key points that must be considered when interpreting the heat shock data presented in this study. First, since parasites such as leishmania incorporate a temperature shift in their normal life cycles, they may have evolved to be particularly well adapted to surviving at elevated temperatures. Since the most critical aspect of parasite survival is its ability to respond quickly to sudden environmental changes, what may be considered as heat shock conditions in other organisms may not necessarily apply to leishmania. Perhaps the term heat shock itself must be refined when dealing with parasitic organisms. Secondly, and perhaps more importantly, the strain of L. major used in these experiments did not show any of the morphological changes characteristic of promastigote to amastigote differentiation. Hence, the conditions created for the heat shock experiments may not have resulted in differentiation of the parasite.

Since neither the heat shock conditions nor the states of parasitic differentiation can be defined with certainty, a correlation between ubiquitin expression and the stages of parasitic

differentiation cannot be established. However, it can be determined that Hsp70 and ubiquitin differ in their levels of gene expression upon exposure of Leishmania major to elevated temperatures.

It is of interest to note here that a role for heat shock proteins during development of certain trypanosome species (Glass et al, 1986, Davis et al., 1987), Plasmodium falciparum (Ardeshir et al, 1987), and Schistosoma mansoni (Hedstrom et al, 1987) has also been suggested. In both Plasmodium falciparum and Schistosoma mansoni, Hsp70 appears to be constitutive and not heat inducible despite the fact that both parasites are exposed to a heat shift in their transmission from the invertebrate to the vertebrate host. Similarly, Hsp83 is constitutively expressed in Trypanosoma cruzi epimastigotes and trypomastigotes. Hence, the data from other investigators support the observations on Hsp70 gene expression in leishmania.

The question arises then as to why ubiquitin gene expression is so different from that of other heat shock proteins if the molecules are thought to function for the same purpose during heat shock. The simplest explanation is that the ubiquitin system does not complement the heat shock system in these parasites. Heat shock may be the trigger for differentiation, but ubiquitin gene expression may be developmentally regulated. As seen in Dictyostelium discoideum (Giorda and Ennis, 1987), regulation at this level is equally complex and intriguing.

#### IV. FUTURE RESEARCH

Since ubiquitin is a member of a gene family and only one locus was examined at the nucleotide level in L. major, it would be of interest to determine the gene product, if any, that is encoded by the other locus. In contrast, pursuing the search for the 5' end of the locus containing many ubiquitin coding elements appears futile given the instability of these elements when they are found in tandem sharing 100% identity at the nucleotide level. With regard to ubiquitin expression studies, it would be worthwhile to examine the same phenomenon proposed in this study using a species of leishmania which can be characterized morphologically during promastigote to amastigote transformation. Conducting protein and transcription analyses might also reveal a more accurate description of the level at which ubiquitin in

regulated. Although any data regarding the regulation of heat shock proteins provides valuable insights into the general mechanisms governing gene expression and regulation, such information is particularly relevant for understanding how parasite development occurs so that the ultimate goal of inhibiting parasite growth in the mammalian host cell can be realized.



## CONCLUSIONS

1. Demonstration of ubiquitin gene expression in protozoa contributes to the evidence suggesting that ubiquitin is the most conserved eukaryotic protein known. This high degree of conservation implies that ubiquitin mediates functions which are vital to cellular survival.
2. Leishmania major has two gene loci encoding ubiquitin. One locus contains at least 5 ubiquitin coding elements arranged head-to-tail without intervening sequences. The other locus appears to contain only 1 ubiquitin coding element.
3. The ubiquitin coding elements share 100% identity at both the nucleotide and amino acid levels. The only non-ubiquitin coding residue determined is a cysteine located at the carboxy terminus of the polyubiquitin precursor preceding the stop codon.
4. Gene expression of ubiquitin in L. major at 37°C and 42°C appears constitutive rather than heat inducible. However, the level of ubiquitin specific mRNA does decrease with prolonged exposure of the cells at the non-lethal temperature.
5. A correlation between ubiquitin gene expression and parasite differentiation cannot be determined as it is not known whether differentiated states of L. major were actually established upon exposure of the parasite to elevated temperatures.

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