GENE AMPLIFICATION IN LEISHMANIA

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INTRODUCTION

Trypanosomatid protozoa provide amazingly fertile ground for the discovery of novel molecular phenomena such as RNA editing (102), trans-splicing (16), and bent DNA (77) that have subsequently been observed in metazoans. Studies of drug-resistant Leishmania spp. have similarly yielded new perspectives on the mechanism of gene amplification, in which a limited portion of the genome selectively increases in copy number. De novo circular amplifications mediating drug resistance, including direct and inverted amplification, were first discovered in Leishmania spp. (7). These parasites continue to offer simple model systems for the study of gene amplification in higher eukaryotes, including cultured mammalian cells (reviewed in 51, 93–96, 105, 106).

Since gene amplification in Leishmania spp. was last reviewed (10), progress in this field has been rapid. Amplified DNAs have been modified to generate prototypic DNA transfection vectors for Leishmania spp. (60), which can be used to directly test gene function. This approach has permitted the identification of the drug resistance genes contained within amplified DNA (H. L. Callahan & S. M. Beverley, in preparation) and will allow identification of cis-acting elements mediating gene expression, replication, and maintenance. Current data indicate that gene amplification is widespread in drug-resistant Leishmania, occurring in many different species in response to a wide spectrum of compounds. One amplification, the H region, is a complex drug-resistance element widely conserved during Leishmania spp. evolution, encoding at least two different drug-resistance genes. The region appears to be an amplification-prone segment of the Leishmania spp. genome that combines elements of prokaryotic drug resistance factors and gene amplification. Surprisingly, gene amplification is a common phenomenon in unselected laboratory stocks, although the role of most of these amplifications is often unknown.

A Leishmania Primer

The eukaryotic Leishmania is a genus of pathogenic protozoan parasites belonging to the family Trypanosomatidae (Order Kinetoplastida). Depending upon the particular species, Leishmania infection can result in a mild cutaneous lesion, a disfiguring mucocutaneous disease, or a fatal visceral infection. Approximately 10 million cases are estimated worldwide, but this figure is probably an underestimate (119). Current methods for treatment of the parasite involve pentavalent antimony complexes; however, these drugs are antiquated and better chemotherapeutic agents are urgently needed (2, 30, 119). Several studies of gene amplification in fact arose from investigations focused upon proteins that may prove to be excellent targets for selective chemotherapy in the future.
Leishmania are digenetic parasites with two basic life stages. An insect vector, the phlebotomine sand fly, transmits the flagellated promastigote stage. After introduction by the bite of the fly, promastigotes are taken up into the phagolysosome of macrophages. Despite the hostile cellular environment, the infective promastigotes resist the action of complement, hydrolytic enzymes, and oxidizing agents, and in some manner mitigate the response of the immune system. Differentiation into the aflagellate amastigote stage ensues, followed by growth and cell division. The developmental cycle is complete when a feeding sand fly takes up cells containing amastigotes, which then differentiate back into promastigotes in the fly gut. Promastigotes are grown in defined or semidefined media in vitro, and quantities are generally not limiting (58). Colonies can readily be obtained on the surface of semisolid media, greatly facilitating genetic analysis and the recovery of drug resistant mutants.

The Leishmania Genome and Circular DNAs

One of the attractive features of Leishmania spp. is the small size of its genome, approximately 50,000 kb (75); current data indicate that the parasite is diploid at most loci (10, 31, 56, 57, 75). Because amplified DNA often constitutes as much as 5–10% of total parasite DNA (27), amplified DNA fragments are readily detected as abundant DNA fragments in restriction-enzyme digests of total genomic DNA and can be directly isolated and molecularly cloned (10, 27). Many Leishmania spp. amplifications arise as extrachromosomal circular DNAs, so preparative quantities can be obtained by biochemical fractionations such as CsCl density gradient centrifugation, alkaline lysis, or differential NaCl/SDS precipitation (7, 32, 117).

Leishmania spp. contain 25–30 small chromosomes that are readily separable by pulsed field electrophoresis (28, 42, 44, 91, 98, 113). Amplified supercoiled circular DNAs exhibit migration properties distinct from those of linear DNAs, such as pulse time–independent absolute mobility (pulse time–dependent relative to linear markers) (4, 42). In many apparatuses, supercoiled circular DNAs tend to migrate along a somewhat different track than the linear chromosomes because of the variable response of DNAs of differing topology under alternating electric fields of different strengths (4). These diagnostic properties have been used to establish the circularity of Leishmania DNA amplifications, and can be manipulated to purify circular DNAs that are free of the chromosomes (4, 22, 42). Large (> 200 kb), nicked, relaxed, or concatenated circular DNAs are frequently trapped in the sample well and must be distinguished from amplified DNA that has integrated into larger linear chromosomes. One can use γ-irradiation to introduce limited numbers of double-strand breaks, thereby releasing the circular molecules as linear DNAs whose size can readily be measured (5, 88, 90, 112).
MULTIPLE MECHANISMS OF DRUG RESISTANCE

Most amplifications studied in *Leishmania* spp. arose in response to multiple rounds of stepwise drug selection. Schimke et al (96) have suggested that, in mammalian cells, the stepwise selection protocol, which employs relatively small increases in drug pressure, greatly increases the likelihood of gene amplification. No data addressing this point have been obtained for *Leishmania* species. Lines derived by multiple serial steps often contain multiple alterations contributing to drug resistance, although this effect depends greatly upon the particular drug and cell line studied (96). Selection with methotrexate (MTX) has yielded clonal lines of *Leishmania major* possessing two different functional gene amplifications [dihydrofolate reductase-thymidylate synthase (DHFR-TS) and the H region] and alterations in MTX uptake, as well as lines exhibiting various combinations of these three mutations (36, 38). These data suggest that even when gene amplification is a potential resistance mechanism, it is not necessarily the favored event. For example, selections of clonal derivatives of *L. major* have yielded DHFR-TS or H amplifications in about 30–40% of the MTX-resistant lines (S. M. Beverley, J. Cordingley, & D. D. Rogers, in preparation). MTX-resistant lines of *Leishmania donovani* and *Leishmania mexicana amazonensis* did not exhibit gene amplification (64, 100), nor did MTX-resistant *Leishmania tarentolae* selected from a line lacking pre-existing H amplification (117). Thus, any given resistant line may fail to include amplification in its spectrum of resistance mechanisms.

One often-overlooked factor is the effect of the specific culture medium utilized on the spectrum of drug-resistance mutations obtained. In MTX selections, the external folate concentration can modulate the potency of MTX over a factor of 100,000 in *Leishmania* spp., whereas the effect of external folate is small in mammalian cells (64, 89). Whether this potency modulation affects the type of resistance mechanisms elicited is unknown. However, the recovery of mutants exhibiting severe deficiencies in MTX and folate uptake could probably be enhanced in several commonly utilized media that contain folate levels nearly 1000 times normal physiological concentrations (64, 117). Similarly, to obtain sensitivity to low concentrations of mycophenolic acid, an inhibitor of inosine monophosphate dehydrogenase (IMPDH), Wilson et al (118) used a defined medium containing hypoxanthine as the sole source of purine (trypanosomatids are obligatory purine auxotrophs); resistant mutants then arose by amplification of the IMPDH structural gene.

The occurrence of multiple resistance mechanisms means that one must demonstrate a causal association between molecular alterations such as gene amplification and inferred biochemical mechanism of resistance. Prior to the availability of genetic tests, most workers relied upon correlative studies,
associating amplifications with phenotypes in resistant lines obtained by
different methods, with different drugs, or in different species (33, 37, 53,
62, 63, 88, 117). Surprisingly, this kind of analysis also revealed the presence
of several amplifications whose presence does not correlate with drug resist-
ance. These apparently nonfunctional amplifications are discussed in a later
section and underscore the need for functional analysis.

The advent of stable transfection and expression vectors for *Leishmania*
spp. (26, 60, 69, 71) and methods of specific gene targeting (31) will enable
functional tests of the genes encoded within amplifications. These techniques
have already enabled direct demonstration of the functional role of the H
region in drug resistance in both *L. major* and *L. tarentolae* (H. L. Callahan
& S. M. Beverley, in preparation). As the use of this powerful technology is
explored, progress will be rapid on many of the questions raised in this
review.

**GENES AMPLIFIED IN RESPONSE TO
DRUG PRESSURE**

Several drug-resistant *Leishmania* spp. have been obtained, but for many of
these the causal biochemical mechanism or molecular changes associated with
resistance have not been characterized. This section considers only those
drug-selected lines in which amplification of genes known or strongly sus-
pected to mediate resistance has been observed.

*Dihydrofolate Reductase–Thymidylate Synthase*

MTX is a stoichiometric inhibitor of DHFR from most sources, and MTX-
resistant mutants in many species frequently exhibit overproduction of DHFR
as well as structural alterations and reductions in MTX accumulation. In
eukaryotes, overproduction is mediated by amplification of the DHFR struct-
ural gene. Many MTX-resistant lines of *L. major* selected for resistance to
MTX show elevated DHFR activity and amplification of the structural gene
contained within a segment of DNA termed the R region (7, 9, 10, 27; S. M.
Beverley, J. Cordingley, & D. D. Rogers, in preparation). In *Leishmania*
spp. and all protozoan parasites studied to date, DHFR is encoded in a fusion
polypeptide that contains the structural gene for thymidylate synthase (TS)
appended to the carboxy terminus (9, 49). DHFR-TS amplification has thus
been observed in lines resistant to the TS inhibitor 10-propargyl-5,8-
deazafolate (CB3717) (41). Because MTX-resistant lines always exhibit addi-
tional metabolic alterations, a transfection-based approach was employed to
test whether DHFR-TS amplification alone was sufficient to confer MTX
resistance. A multicopy molecular construct containing the DHFR-TS struc-
tural gene was introduced into wild-type cells without employing MTX
treatment. DHFR-TS transfectants were MTX-resistant, while control transfectants were not (86).

Depending upon the specific amplification, upwards of 30 kb of DNA flanking the DHFR-TS structural gene are additionally co-amplified (7, 10, 54; S. M. Beverley, J. Cordingley, & D. D. Rogers, in preparation). The DNAs found within the most commonly amplified 30-kb segment (the prototypic R region) (7, 10) are extensively transcribed into at least nine polysomal polyadenylated RNAs, but sequencing of the genomic copy of several of these has not revealed significant open reading frames (59, 61). No function nor role in drug resistance for these additional RNAs has been shown.

The H Region: a Complex Multiple Drug–Resistance Element

The first MTX-resistant line of *L. major* (R1000) contained another DNA amplification in addition to the DHFR-TS/R region amplification, termed the H region amplification (7). Analysis of clonal derivatives revealed that the H amplification occurred within the same cells bearing DHFR-TS/R region amplification, the first example of cells bearing two unrelated amplifications. Initially, the H region was a puzzle: amplification of genes other than DHFR-TS in antifolate-resistant mutants from other species had not been reported; no data causally implicated this amplification in MTX resistance; and it might have represented an amplification not functionally mediating drug resistance (examples of which are discussed in a later section).

Subsequent studies showed a correlation between drug resistance and H region amplification in numerous independent lines and species. First, independent selections of *L. major* with the drugs primaquine and terbinafine, which are structurally and mechanistically unrelated to MTX, yielded lines bearing only H amplification that were 10- to 20-fold cross-resistant to MTX (38). Second, independent MTX selections of *L. major* and *Leishmania tropica* (including clonal derivatives) have yielded lines bearing H but not DHFR-TS amplification (36, 38, 53; S. M. Beverley, J. Cordingley, & D. D. Rogers, in preparation; Beverley, S. M. Iovannisci, P. F. Kamitsuka, J. Manning, & N. Mukhopadhyay, in preparation). Third, several unselected laboratory stocks of the lizard parasite *L. tarentolae* contained an unselected amplification that turned out to be the equivalent of the *L. major* H region amplification, with extensive sequence homology and the characteristic inverted repeat structure (88, 117) (Figure 1). These lines exhibited up to 20-fold resistance to MTX, and the H region copy number could be elevated by MTX pressure. Fourth, selection of *L. mexicana amazonensis* with sodium arsenite yielded lines bearing amplification of the H region of this species. These lines were highly cross-resistant to MTX (33, 62). Correspondingly, H-amplified *L. major* exhibit varying levels of arsenite resistance (37; H. L. Callahan & S. M. Beverley, in preparation).
Direct Amplification

Inverted Amplification

Figure 1  Amplified DNA structures characterized in Leishmania species. The boxes indicate repetitive DNA sequences whose orientations are indicated by small arrows and the heavy lines flanking chromosomal DNA.

The data summarized above reveal a clear correlation of H amplification with drug resistance, especially MTX resistance. However, the use of lines obtained by serial stepwise selection introduced an element of uncertainty resulting from the possibility of multiple mechanisms of MTX resistance discussed earlier. One approach to this problem was to focus on lines selected with structurally unrelated drugs because the MTX resistance of these lines could probably be attributed solely to the H region amplification. Biochemical studies of the MTX resistant, H-amplified L. major obtained by terbinafine and primaquine selection revealed no alterations in MTX uptake, accumulation, or efflux (37) or increased MTX hydrolase activity (38). In contrast, alterations in MTX uptake were observed in all MTX-selected lines including those lacking H region amplification (36, 64). Thus far, no biochemical change likely to mediate MTX resistance has been associated with H region amplification, suggesting that this region of DNA encodes a novel mode of resistance.

Molecular studies of the H-amplified lines of L. major have shown that the 45-kb H region is transcribed into at least 20 polyadenylated RNAs and encodes at least one protein (35, 83). The relative levels of these molecules do not vary in response to heat shock or drug pressure (35). Interestingly, a homologue of the P-glycoprotein or multiple drug-resistance (MDR) genes implicated in multiple drug resistance in cultured mammalian cells (47) has been identified within the H amplification of both L. tarentolae (ltpgA) (83) and L. major (lmpgpA) (H. L. Callahan & S. M. Beverley, in preparation).
Some P-glycoproteins mediate resistance by increasing drug export, and P-glycoprotein relatives in *Drosophila melanogaster* control the accumulation of pterin derivatives (34, 82) (recall that MTX is a pterin derivative). However, a comparable role for P-glycoprotein genes in MTX resistance seems unlikely because (a) MTX accumulation was not altered in the H-amplified lines selected by unrelated drugs (37); (b) MTX resistance was not reversed by verapamil, which does reverse the toxicity of many P-glycoprotein substrates (37; H. L. Callahan & S. M. Beverley, in preparation); and (c) the H-amplified *L. tarentolae* was not resistant to a variety of compounds normally considered to be substrates of the mammalian P-glycoprotein, such as puromycin, vincristine, or adriamycin (83).

H REGION AMPLIFICATION CONFERS DRUG RESISTANCE Recently DNA transfection approaches have been used to assess the coding potential of the H region (H. L. Callahan & S. M. Beverley, in preparation). A series of DNA fragments encompassing the wild-type *L. major* H region were inserted into multicopy transfection vectors bearing the neomycin (NEO)-resistance gene, introduced into *Leishmania* by electroporation and G418 selection, and amplified by increasing G418 pressure (60). When tested for drug resistance, a single segment of the H region was shown to confer resistance to arsenite by this protocol, and deletion studies localized the resistance gene to the MDR gene homologue *lmpgpA* mentioned previously. Consistent with previous work, *lmpgpA* constructs did not confer MTX resistance, and preliminary data indicate that the MTX resistance gene maps to another part of the H region (H. Callahan and S. M. Beverley, unpublished data). These data provide the first evidence that the H region encodes drug resistance determinants that are functional following gene amplification. Moreover, it suggests that the *lmpgpA* gene is functionally divergent from P-glycoprotein genes involved in classical multiple drug resistance (49). Further studies are required to determine (a) if *lmpgpA* mediates arsenite resistance through reductions in arsenite accumulation, as expected for members of the P-glycoprotein gene family and (b) if point mutations in *lmpgpA* or other genes confer altered drug resistance specificities as observed in mammalian MDR genes (24). Studies of the MTX-resistance element encoded by the H region are currently underway.

Interestingly, reports of a parallel for the association of arsenite and MTX resistance with gene amplification in *Leishmania* spp. have emerged for metazoans. Treatment of cultured mammalian cells with arsenite increases the frequency of DHFR amplification (73) and induces the expression of P-glycoprotein mRNAs (23); however, P-glycoprotein expression alone does not confer MTX resistance. Whether these associations are functional or fortuitous is unknown.
While pre-existing amplification of the H region is clearly evident in unselected laboratory stocks of *L. tarentolae* (88, 117), some controversy surrounds the occurrence of pre-existing H region amplifications in the human pathogen *L. major*. One group (53) has reported that two independent stabilates of the unselected LT252 line possess H region amplification. However, our (37) studies of an early-passage stabilate of this line, prepared prior to selection of the first MTX-resistant line bearing R and H amplification (R1000) (7), have failed to detect any H amplification. Recent work employed a sensitive PCR amplification method to detect H amplification-specific rearrangements (S. M. Beverley, D. M. Iovannisci, P. F. Kamitsuka, J. Manning, & N. Mukhopadhyay, in preparation). It is possible that the instability of the H amplification may account for the discrepant findings (7). However, at present the existence of H-region amplification in unselected *L. major* is uncertain.

*Tunicamycin and Glycosyltransferase*

Chang and coworkers have studied a series of *Leishmania* spp. lines selected with tunicamycin (TUN), an inhibitor of the microsomal N-acetylglucosamine-1-phosphate transferase. In most species, this enzyme is the first step in the dolichol pathway for protein N-glycosylation. In other systems, TUN resistance results from altered uptake, structural alterations, or amplifications of the glycosyltransferase (68, 74, 99, 108). Although the first two mechanisms have not been tested in *Leishmania* spp., every TUN-resistant line of *Leishmania* obtained thus far contains a DNA amplification and elevated levels of a TUN-sensitive glycosyl transferase activity, regardless of whether mutagenesis was included in the selection process (32, 63, 65, 86). Examination of a variety of independent amplified lines from several species revealed the presence of a 20-kb homologous region common to all amplifications (63). The common region is transcribed into at least five abundant RNAs, which translate in vitro into several protein bands (63; K.-P. Chang, personal communication). The common region may contain the glycosyltransferase structural gene, but heterologous probing with the yeast gene has proven inconclusive despite early reports (63). The coding potential of the common region is currently being tested using DNA sequencing and transfection studies. This amplification is interesting because it appears to be associated with a small but significant increase in parasite virulence (65, 66), although it has not been encountered in unselected virulent strains.

*ODC, IMPDH, and Classic MDR*

Recent work by Ullman and coworkers suggests that gene amplification has been obtained in response to at least three different agents in *L. donovani*. They (118) have reported amplification of the *L. donovani* IMPDH gene in
response to mycophenolic acid selection. Vinblastine-resistant lines contain an amplification of a P-glycoprotein homologue distinct from the ImpgpA gene present on the H region and show collateral resistance to many of the classical mammalian P-glycoprotein substrates (47; B. Ullman, personal communication). A line selected for resistance to α-difluoromethylornithine has been obtained that exhibits unstable overproduction of ornithine decarboxylase (ODC), a phenotype highly suggestive of gene amplification (29). These new amplifications are currently being characterized. Their occurrence emphasizes the fact that, as in mammalian cells, gene amplification in *Leishmania* spp. is probably a widespread phenomenon underlying many instances of drug resistance and is not restricted to any specific structural or functional class of compounds.

**MISCELLANEOUS AMPLIFIED GENES**

The powerful methods available for electrophoretic separation of *Leishmania* spp. chromosomes have revealed a variety of chromosomal alterations involving gene amplification that are distinct from those reported above. These include the T amplification of *L. tarentolae*, the D amplification of *L. tropica*, subchromosomal amplifications of the LD-1/715 class, and amplification of the miniexon. Current data suggest that such chromosomal changes may be common (6, 43, 56, 85; S. M. Beverley, J. Cordingley, & D. D. Rogers, in preparation). The four kinds of amplifications discussed below are those that have been characterized to date. These amplifications often do not involve DNAs of known function, and in most cases have not been associated with any phenotype.

**T and D DNAs**

The Trager line of *L. tarentolae* contains an amplified DNA, designated the T region (87). Hybridization studies revealed that the T amplification was distinct from other known amplifications, including the H region amplified in other lines of *L. tarentolae* (87). The amplified T region contains about 20 kb of DNA, amplified as a circular dimeric molecule. This amplification is unusually ephemeral in that examination of the Trager line obtained from other laboratories or following various periods of culture showed widely varying levels. T-amplified lines do not show drug resistance.

Electrophoretic surveys revealed the presence of an amplification termed D DNA in an unselected laboratory stock of *L. tropica* (53). This DNA contained approximately 75 kb of DNA and possessed an inverted repeat structure. Hybridization studies revealed that this DNA was distinct from both the H and R regions, and selection of these cells with MTX led to the emergence of H amplification but no changes in D DNA levels. Thus far, no phenotype has been ascribed to either T or D DNA amplification.
Miniexon

Studies of *L. major* revealed alterations in karyotype arising from amplifications occurring within the locus encoding the miniexon donor RNA (56). This RNA provides the 39-nucleotide leader sequence added by *trans*-splicing onto the 5' end of all known protein-coding mRNAs in trypanosomatids (16) and is encoded in the genome as a tandem repetitive array of 0.45-kb units in *Leishmania* spp. (56, 78). Chromosome 2 in *L. major* contains the miniexon array, and several lines exhibiting alterations in this chromosome contained expansions of the miniexon array. Clonal derivatives of the LT252Δ, in which the miniexon amplification was first observed, exhibit a growth advantage (56), although genetic tests are required to determine whether the miniexon amplification itself is responsible for the growth phenotype. Interestingly, amplification of the miniexon array has been observed in several *L. major* isolates (56; S. Beverley, unpublished data). Whether this amplification occurs in nature or represents an adaptation to in vitro culture is currently unknown.

Subchromosomal Amplifications

Karyotypic surveys of numerous *Leishmania* species have revealed the occasional occurrence of relatively small chromosomes (250 kb or less) absent in other isolates of the same species (6, 15, 40, 98, 107). These small linear DNAs (SLDs) can be distinguished from normal chromosomes by the fact that they occur in multiple copies, up to 40 per cell. In *L. donovani*, they were termed the “HU-3” minichromosome (15) or LD-1 (98), and in *L. major* they were designated the 715-class of SLDs (6). Hybridization studies revealed that these DNAs from different strains or species are similar but not identical in size or sequence content (6). Because SLDs occur in some but not all isolates from numerous *Leishmania* species, researchers initially proposed that they could represent the result of some form of horizontal transmission. Subsequent studies have revealed that all species of *Leishmania* contain a reservoir copy of the sequences present on the new small chromosomes (6, 14). Current data, though limited, are most consistent with the idea that the SLDs represent large, subchromosomal amplifications; as such they must have acquired at least one new telomere during their formation. Their greatly increased copy number suggests that they replicate autonomously, and somehow escape mechanisms normally limiting chromosome number. Interestingly, Stuart and coworkers (6, 40, 50, 107) have also described a multicopy circular DNA, CD-1, which exhibits a complex relationship with certain SLDs.

Although the antiquity of the SLDs detected in laboratory stocks of *L. donovani*, *L. braziliensis*, and *L. mexicana* (6, 15, 40, 107) is unknown, the de novo generation of SLDs may have occurred in *L. major* and *L. braziliensis* (including a WHO reference strain) during routine culture in vitro and in
two clonal derivatives of *L. major* undergoing MTX selection. In lines not
known to have undergone drug pressure that bear SLDs, drug resistance has
not been observed, and to date no biological role for these SLDs has been
demonstrated. Their recurrent emergence throughout most *Leishmania* spe-
cies is provocative.

**What Leads to the Emergence of Apparently Nonfunctional Amplified DNAs?**

Why are amplified DNAs commonly observed in unselected laboratory stocks
of *Leishmania* spp., and what is their significance to the parasite? This section
discusses two models.

**FUNCTIONAL SELECTION** These amplifications may be functional but con-
fer a phenotype not yet tested. For example, they may provide some subtle
growth advantage during adaptation to culture in vitro or conceivably in
nature. This could provide a directed selective force responsible for the
multiple independent occurrence of events such as amplification of the
miniexon in *L. major* or SLDs in many *Leishmania* species. Recall that in the
1970s a variety of amplified DNAs of unknown function and origins were
similarly observed in cultured mammalian tumor cells (93). Subsequently,
many of these amplifications were shown to contain functional oncogenes.

A variant of this model is that these amplifications are part of more
complex biochemical resistance mechanisms, obligatorily requiring the pres-
ence of another unlinked resistance gene. Segregation in the absence of
selective pressure could produce cells bearing only the amplification, which
would appear to be nonfunctional. The best candidates for this kind of model
would be those amplifications discovered in unselected laboratory stocks,
such as T and D DNA and certain SLDs. Studies of chloroquine-resistant
malaria postulated an analogous two-step model (39, 81, 115); however, at
present this model remains only a possibility for *Leishmania* species.

**NEUTRAL CHROMOSOMAL MUTATIONS** Some chromosomal alterations
and amplifications may be approximately functionally neutral, constituting
chromosomal mutations with no functional significance. Because this model
postulates no intrinsic selective force promoting fixation of these mutations,
other mechanisms such as recurrent directed mutation and population forces
involving genetic drift must be invoked. Although other creatures exhibit
examples of these forces, their role and presence in *Leishmania* is unknown.
Founder effects contributing to the emergence of variant phenotypes could
arise from multiple serial passages or cloning (1). Another possibility is
hitchhiking, i.e. the occurrence of an unlinked advantageous mutation (such
as drug resistance) in a line containing a neutral chromosomal alteration or
amplification. A similar phenomenon, termed periodic selection, has been reported in bacterial chemostat experiments (52, 67).

FREQUENCY OF GENE AMPLIFICATION

In cultured mammalian cells, the frequency of gene amplification in drug-selected cells has been estimated to be from $10^{-3}$ to less than $10^{-8}$; this value depends on the specific cell line utilized (primary, established, or tumor), the methods of measurement, and whether positive or negative selection methods are utilized (96, 110, 120). In *Leishmania* spp., studies of 20 independent clonal lines of *L. major* undergoing MTX selection revealed a frequency of DHFR-TS or H region amplification of about $5 \times 10^{-8}$ (S. M. Beverley, J. Cordingley, & D. D. Rogers, in preparation), a value considerably lower than estimates for DHFR amplification in tumorigenic or cultured mammalian cells using comparable methods (about $10^{-5}$). The value for *Leishmania* spp. is consistent with studies showing that the frequency of gene amplification in normal human cells is very low (120). Gene amplification does not generally reduce the infectivity of *Leishmania* spp. to animals, cultured macrophages, and sand flies (10, 65, 86), and appears quite compatible with the normal infectious cycle.

Many of the amplifications currently thought to be unrelated to drug resistance have been detected in drug-selected lines. For example, in the 20 clonal derivatives of *L. major* described above that were selected for methotrexate resistance, 3 independent mutations apparently unrelated to methotrexate resistance were observed, 2 of which belonged to the 715-class of subchromosomal amplifications (6, 56; S. Beverley, unpublished data). Similarly, in 10 lines maintained over a period of at least 6 months, 2 mutations unrelated to drug resistance were observed, 1 involving the miniexon and 1 a SLD (6, 56). Although these data are not directly comparable (since hitchhiking effects will be greater in the drug-selected lines because of the low frequency of drug resistance), they provide no evidence for an elevated frequency of the apparently nonfunctional amplifications in *L. major* undergoing drug pressure.

STRUCTURE OF EXTRACHROMOSOMAL AMPLIFIED DNAs

The structures and properties of several *Leishmania* spp. amplifications are known in detail. The general features emerging from these studies are described below.
Simple, Time-Invariant Direct and Inverted Amplifications

Amplified DNA segments can be organized in a direct, head-to-tail orientation, or alternatively, in an inverted, head-to-head manner (Figure 1). To date, the DHFR-TS and TUN amplifications reported fall into the direct class, while the D and prototypic H region amplifications are inverted. Unlike mammalian amplifications, Leishmania spp. amplifications appear to be quite homogeneous and possess only the minimum number of DNA rearrangements necessary to generate each class from a chromosomal reservoir: one for direct amplification, two for inverted amplification (see Figure 1). Although oligomers of the amplified DNA may arise during in vitro culture (7, 42, 54), additional DNA rearrangements do not generally occur. This finding is quite important, for it means that the rearrangements observed are probably primary events associated with the initial formation of the amplified DNA (unlike most mammalian amplifications, discussed below).

Circular and Linear Amplifications

Most DNA amplifications appear to consist of extrachromosomal circular DNAs, ranging in size from 30 kb to greater than 200 kb. However, SLDs are linear, subchromosomal amplifications, and MTX- and difluoromethylornithine (DFMO)-resistant Leishmania spp. have recently been developed that contain linear amplifications (S. M. Beverley, J. Cordingley, & D. D. Rogers, in preparation; B. Ullman, personal communication). Because linear DNAs are expected to contain telomeric ends, their emergence suggests the de novo formation of the characteristic telomeric DNA (114), presumably by the action of the leishmanial telomerase.

Unstable and Stable Amplification

As in cultured mammalian cells, many Leishmania drug resistance amplifications are initially unstable, rapidly declining in copy number in the absence of continued drug pressure (7, 29, 65, 118). After lengthy propagation in the presence of drug, amplified DNAs can become stable upon drug removal (7). Not surprisingly, most of the amplifications observed in unselected laboratory stocks of Leishmania spp. are stable. In cultured mammalian cells, the transition from unstable to stable amplification is usually associated with a relocalization of the extra-chromosomal amplified sequences into the chromosome (94, 106). In Leishmania spp., once extrachromosomal amplified DNAs are established, they do not appear to reinsert back into chromosomal DNA during the stabilization process (42; S. Beverley, unpublished data). Occasionally the number of amplification units per circle increases, but otherwise no structural alterations have been shown. This finding suggests that subtle mutations in the initially unstable circular DNA can confer...
stability, or that stability is conferred by factors or mutations acting in trans (11).

The lack of knowledge concerning chromosomal elements mediating karyotypic stability in *Leishmania* hinders studies of the stability of amplified *Leishmania* DNA. Mitotic chromosomes do not condense, and although structures resembling kinetochores have been reported in other trypanosomatids their role is dubious because their number is considerably less than the number of chromosomes detected using pulsed-field electrophoresis (45, 104). Whether *Leishmania* chromosomes possess functional localized centromeres or whether they are holocentric, with centromeric activity dispersed over the entire chromosome as in nematodes and some arthropods (116) is unknown.

**Recurrence of Rearrangements Involving Repetitive DNA Sequences**

Frequently, the same amplified DNA structures are observed in independent lines. Six of nine independent amplifications of the DHFR-TS region utilized the same site, and six of six independent amplifications of the H region of *L. major* have the same structure (10, 37; S. M. Beverley, J. Cordingley, & D. D. Rogers, in preparation; Beverley, S. M. Iovannisci, P. F. Kamitsuka, J. Manning, & N. Mukhopadhyay, in preparation). Current data indicate that these rearrangements occur within repetitive DNA sequences. The DHFR-TS gene is flanked by two 600-bp elements separated by 30 kb, and the circular amplified genes are preferentially formed by joining of these two elements (10; S. M. Beverley, J. Cordingley, & D. D. Rogers, in preparation) (Figure 1). Similarly, the H region of *L. major* is flanked by two different pairs of inverted repeats (Figure 1), which are joined during amplification to form the two new rearrangements required (S. M. Beverley, D. M. Iovannisci, P. F. Kamitsuka, J. Manning, & N. Mukhopadhyay, in preparation). Similar data were recently reported for *L. tarentolae* (84).

**Functional Genetic Elements Within Amplified DNAs**

The ability of the extrachromosomal amplified DNAs to persist and direct the synthesis of encoded gene products has led many workers to conclude that these are autonomous elements. Kapler et al (60) explicitly tested this conclusion in the R region amplification of *L. major*, where a modified version of this region introduced by DNA transfection replicated autonomously and directed the efficient formation of a correctly trans-spliced RNA. Smaller derivatives of this plasmid are also equivalently functional (71), and as these DNAs are dissected further the identification of cis-acting elements involved in DNA replication, segregation, initiation of transcription, and RNA processing should follow. Origins of replication would be especially interesting
to study in an amplification context. Curiously, many *Leishmania* DNA segments can apparently replicate autonomously (60, 69, 71). Because linear amplifications may have acquired telomeres de novo, characterization of these should provide some insight into the structure and mechanism of telomere formation in *Leishmania* species.

**Comparisons with Amplified DNAs in Cultured Mammalian Cells**

Comparisons of the structural features of amplified *Leishmania* genes with those observed in cultured mammalian cells, summarized in several recent reviews (95, 96, 105), are interesting. One is immediately struck by the complexity of amplified mammalian DNA: large tracts of DNA can be amplified, possibly as much as 10 megabases. The structures of these amplified DNAs are usually quite heterogeneous, containing multiple rearrangements or novel joints. The structure of the amplified DNA also varies, both within and between different cell lines. As a function of increased drug pressure and time, additional DNA rearrangements accumulate, and occasionally smaller amplification units predominate. In contrast, secondary rearrangements are generally not observed in *Leishmania* species.

The simplicity of amplification in *Leishmania* spp. could be attributed to the small size of the *Leishmania* genome and chromosomes; a 30-kb amplification in *Leishmania* is proportionally 1.8 Mb in mammalian cells. Another source could be the propensity of *Leishmania* spp. to undergo homologous recombination (31, 111), accounting for the frequent occurrence of rearrangements joining homologous repetitive DNAs. Regardless of the cause, the relative simplicity of amplified DNAs in *Leishmania* spp. greatly facilitates their analysis.

**Three Chromosomal Types of Amplification**

Pulsed-field methods of chromosomal analysis have permitted an examination of the fate of the wild-type chromosomal genes during generation of the amplified DNAs. Thus far, three outcomes have been characterized (Figures 2 and 3):

1. In deletional amplification, a copy of the wild-type chromosomal locus is deleted, yielding a smaller chromosome. Because *Leishmania* spp. are diploid, the result is a heterozygous deletion line (10, 31; S. M. Beverley, J. Cordingley, & D. D. Rogers, in preparation).
2. In conservative amplification, no alterations in chromosomal structure or ploidy are detected (7, 10, 37, 88; S. M. Beverley, J. Cordingley, & D. D. Rogers, in preparation).
3. In duplicative amplification, several additional gene copies are found
LEISHMANIA AMPLIFICATION

Figure 2  Three chromosomal types of amplification. The thick bars represent flanking chromosomal DNA, and the thin lines are the DNA segments that give rise to the extrachromosomal circular DNA in addition to being either deleted, retained, or duplicated.

inserted into the wild-type locus, yielding a larger chromosome in addition to the amplified extrachromosomal copies (S. M. Beverley, J. Cordingley, & D. D. Rogers, in preparation).

Thus far, the majority of events characterized fall into the conservative class; only one example each of the deletional and duplicative classes has been observed, both involving DHFR-TS (10; S. M. Beverley, J. Cordingley, & D. D. Rogers, in preparation). Chromosomal changes are evident in some arsenite-resistant L. mexicana amazonensis, but these have not been further characterized (33). Some cases of deletional amplification may have been missed because hybridization probes flanking the amplified region are not always employed to reveal deletion chromosomes obscured in ethidium bromide-stained gels.

MECHANISM OF GENE AMPLIFICATION

Several models have emerged from studies of amplification in cultured mammalian cells, including: (a) unscheduled rereplication of the wild-type chromosomal locus during a single cell cycle, followed by resolution into the final amplified DNA (51, 92, 94, 106); (b) duplications formed by unequal sister-chromatid exchange (46, 105, 106); and (c) models invoking recombinations in the absence of rereplication, such as the deletion plus episome model in which the wild-type chromosomal locus is converted into an ex-
Figure 3  Formation of the onionskin intermediate and potential products. Overreplication gives rise to the characteristic onionskin structure; both DNA strands are shown in the upper half of this figure. The vertical black bars indicate repetitive DNAs that are the sites of DNA rearrangement (see Figure 1 for an expanded view). Potential products formed by resolution of the onionskin intermediate by recombination among the repetitive DNAs or by branch migration and telomere addition are shown.

trachromosomal plasmid by intrachromosomal recombination (21, 97). The diversity of amplified structures has led some workers to postulate that mammalian cells may employ several different mechanisms that vary among loci or during different amplification steps (105).

If one considers only the Leishmania spp. amplifications that mediate drug resistance whose structures have been characterized, the majority appear conservative: changes in the wild-type chromosomal structure or ploidy are seen only rarely. This observation probably rules out models requiring alteration of the parental chromosome structure, such as sister chromatid exchange or recombination in the absence of rereplication (among others). Therefore, the amplified DNAs are likely generated from extra copies of the chromosomal locus, which are formed by some kind of rereplication occurring within a...
single cell cycle (92). One model for the initial structure of the rereplicated DNA is the “onionskin” model, in which one or more extra rounds of replication generate a hypothetical structure shown in Figure 3 (for another representation of the onionskin, see 105).

The onionskin model is quite flexible (97); by postulating different combinations of DNA rearrangements, one can obtain virtually all types of amplification structures. Examination of the onionskin intermediate reveals that rearrangements involving only the overreplicated DNA segments could lead to either direct or inverted amplification structures (factors affecting this choice are discussed below). In this case, DNA strands retaining the unrearranged wild-type information would be retained and could subsequently resolve into normal chromosome structure when the onionskin collapses, and so lead to conservative amplification. However, other structural outcomes of the onionskin are possible. Rearrangements within the wild-type chromosomal strand would lead to deletion plus episome formation, while rearrangements between the chromosomal strand and the overreplicated segment could lead to integrational or duplicative amplification. Although both of these structures could also arise by one or more of the other models discussed above, the only two cases of duplicative/integrational amplification observed in *Leishmania* spp. (at DHFR-TS) involved simultaneous formation of an episomal gene copy (10; S. M. Beverley, J. Cordingley, & D. D. Rogers, in preparation). Because many extra gene copies are generated in the onionskin intermediate, this model can easily accommodate the simultaneous formation of both integrated and circular amplified DNA. Another potential outcome of the onionskin intermediate is resolution by branch migrations, leading to the extrusion of linear gene copies that could either undergo rearrangements leading to circle formation or alternatively acquire telomeres by the action of a leishmanial telomerase; this process may be the source of linear amplifications (6). Thus, the onionskin model can readily account for all amplified structures observed in *Leishmania* spp. thus far, and, conversely, all types of possible structures postulated to arise from an onionskin intermediate have been observed. Although these data do not unambiguously rule out other mechanisms or combinations of mechanisms, they constitute perhaps the best evidence in support of overreplication and the onionskin intermediate in gene amplification. The preferential recovery of the conservative class of amplifications may signify that the rearrangements involving the overreplicated DNA are favored, perhaps because of its elevated copy number or the presence of nicks, gaps, and ends known to stimulate recombination in fungal systems.

However the nascent amplified DNAs are formed, they must ultimately resolve into amplified DNAs containing rearrangements. In *Leishmania* spp., the sites of DNA rearrangement are commonly within homologous repetitive
sequences, “hotspots” often used in multiple independent lines; in contrast, in mammalian cells amplification rearrangements are not generally recurrent nor preferentially associated with homologous repetitive DNAs. This finding may reflect intrinsic differences in the activity of recombination pathways: *Leishmania* spp. and other unicellular eukaryotes appear to possess powerful systems mediating homologous recombination among DNAs as much as 1,000 times more active than those observed in mammalian cells when measured by the relative frequencies of homologous gene targeting (20, 31, 109). These systems could direct DNA rearrangements towards homologous repeated DNA sequences in *Leishmania* spp. and give rise to hotspots for DNA rearrangement. One prediction of this model is that genes flanked by direct repeats should undergo direct-type amplification, while genes flanked by inverted repeats should undergo inverted amplification. The amplifications characterized thus far have upheld this prediction: direct amplifications of the R region usually join flanking direct repeats, while inverted amplifications of the H region join two flanking sets of inverted repeats (Figure 1), and direct amplifications of DNA overlapping a portion of the H region join two directly repeating P-glycoprotein genes (84).

Thus, amplification in *Leishmania* spp. likely occurs by the over-replication/recombination model (3, 92, 94, 95), in which homologous repetitive DNA sequences usually direct the sites of recombination. White et al (117) proposed a similar model in which overreplication of the amplified region is initiated by DNA synthesis primed by the repetitive elements themselves (as opposed to initiating from an internal element as depicted in Figure 3—possibly a chromosomal origin of replication). Because most recombinational mechanisms require strand invasion and subsequent DNA resynthesis, the amplified DNAs are probably ultimately formed by a combination of these two mechanisms. The final step in the amplification mechanism is that the circular DNA, once formed, must increase in copy number. Whether this increase results from unequal segregation or an escape from normal mechanisms regulating copy number is currently unknown.

**ROLE OF GENE AMPLIFICATION IN *LEISHMANIA* BIOLOGY AND EVOLUTION**

**Clinical Drug Resistance**

Most of the agents used in laboratory studies of drug resistance and gene amplification are not employed in clinical treatment of leishmaniasis, although proteins such as DHFR-TS are likely to be targeted in the future (103). Recent studies of the H region amplification suggest that this region could be involved in the parasite’s response to clinically utilized organic antimonial compounds. In addition to conferring resistance to arsenite, over-
expression of the H region \textit{impgpA} gene confers resistance to trivalent antimonials [Sb(III)] when tested using DNA transfection (H. L. Callahan & S. M. Beverley, in preparation). Many workers believe that the pentavalent antimonial derivatives are not the active form and are metabolically altered into an active species, possibly into Sb(III); however, definitive evidence has not been presented. If the Sb(III) model is correct, the \textit{impgpA} gene could modulate the toxicity of clinical antimonials, perhaps by altering Sb(III) accumulation. In a manner consistent with this model, agents such as verapamil that are known to inhibit the action of P-glycoprotein family members have been shown to reverse antimonial resistance in \textit{L. donovani} (79). Moreover, monoclonal antibodies directed against the mammalian P-glycoprotein recognize proteins in \textit{Leishmania} spp.—these proteins are more abundant in some antimonial-resistant lines (48). One might further speculate that the reportedly pre-existing H region amplifications in human-infecting \textit{Leishmania} spp. may have been induced by clinical antimony treatment, although this idea has not been proven. Future studies are required to definitively address the role of the \textit{impgpA} gene in antimony sensitivity and \textit{ImpgpA} amplification in clinical antimony resistance.

\textbf{Amplified Genes in Leishmania: a Bridge Between Prokaryotic Resistance Factors and Mammalian Gene Amplifications}

In many ways, amplified \textit{Leishmania} DNAs occupy a position intermediate between the drug-resistance plasmids of prokaryotes and the amplified DNAs of cultured mammalian cells. Like the prokaryotic plasmids (and some mammalian amplifications), they are generally extrachromosomal circular DNAs, occasionally bearing more than one drug-resistance element. Like mammalian amplifications, they arise from a chromosomal reservoir de novo, and can arise as unstable or stable forms. However, the differences are also instructive: unlike prokaryotic resistance factors, amplified \textit{Leishmania} DNAs do not appear to contain gene functions that facilitate their transfer amongst cells. Unlike mammalian amplifications, frequently the same structure is observed in independent amplifications in many \textit{Leishmania} species.

One model for \textit{Leishmania} DNA amplification is that these organisms contain within their genome amplification-prone cassettes that participate in an amplification and loss cycle. When subjected to selective stresses, information built into the wild-type genome (such as flanking repeats) directs the formation of characteristic amplified DNA structures; although most cells perish, the small proportion undergoing amplification survive. Once the selective pressure abates, the extrachromosomal DNAs are lost, leaving behind the original locus. This kind of amplification and loss model incorporates features of both prokaryotic and eukaryotic systems discussed
above. However, for most loci we have little information about the obligatory role of specific flanking elements in directing the site of DNA amplification, and the amplification structures probably arise from utilization of fortuitously placed repeated sequences.

The H region seems like an excellent candidate for the amplification cassette model, as this model suggests a reason for the conservation of the unique wild-type structure (shown in Figure 1) in both *L. major* and *L. tarentolae* (84; S. M. Beverley, D. M. Iovannisci, P. F. Kamitsuka, J. Manning, & N. Mukhopadhyay, in preparation), which diverged as much as 50–100 million years ago (13; K. Nelson & S. M. Beverley, in preparation). The flanking inverted-repeat pairs evolve more rapidly than internal H region loci and have no known coding potential (S. M. Beverley, D. M. Iovannisci, P. F. Kamitsuka, J. Manning, & N. Mukhopadhyay, in preparation). Thus, their sole role may be in directing the site of H region amplification. The H region may then be seen as a reservoir of amplification-based drug resistance mechanisms, encoding at least two functional drug resistance genes (mediating resistance to certain metals and antifolates) and having the coding potential for many more (35) (genes mediating resistance to other agents known to induce H region amplification have not yet been sought). Because H region genes have been retained as readily amplified DNA segments during evolution, one can postulate that this persistence provides some evolutionary advantage to the parasite population. Thus, *Leishmania* spp. may be seen as possessing composite drug resistance factors analogous to those of prokaryotes.

**Role of Amplification in Shaping the Parasite Genome**

Gene amplification is a mechanism commonly employed during evolution, and it undoubtedly plays a similar role in shaping the parasite genome. Many workers have noted that most genes in trypanosomatid protozoa are present in multiple linked copies, including many genes normally present in one or a few copies in other taxa (25). A surprising fact is that in the current literature single-copy genes are the exception. In *Leishmania* spp., examples of repeated gene families include the miniexon and ribosomal RNA clusters (56, 75, 78), α- and β-tubulins (55, 70), a putative transporter (18), and proteins recognized by immune sera such as the surface antigens gp63 (17) and gp46 (76), and the 70- and 83-kilodalton (kd) heat shock proteins (72, 101). Amplification of genes provides not only abundant quantitative changes, but also new substrates for evolution because extra amplified copies can receive point mutations and evolve new functions while leaving the original copies unchanged.

Given the widespread occurrence of amplification in drug-resistant *Leishmania* spp., and the widespread occurrence of gene families in trypanosoma-
tids, it is interesting that thus far gene amplification in drug-resistant African trypanosomes has not been reported. This fact may reflect fundamental molecular differences between these two genera of trypanosomatid protozoa or possibly the focus of many workers on drug-resistance mechanisms of *Leishmania* species.

**PROSPECTUS**

Originally, gene amplifications in *Leishmania* were sought as a simple approach towards protein and gene isolation, which remains an important application of this phenomenon. Although transfection-based methods now threaten to supplant amplification for this purpose, these genetic methods will aid future work on the mechanism of amplification as well as the functional elements within amplified DNA. The widespread occurrence of gene amplification in both drug resistant lines and unselected laboratory stocks, the advantages of studying simple amplifications that nonetheless resemble those of higher eukaryotes, and the existence of complex amplification-prone cassettes suggest that the study of this phenomenon will occupy the attention of molecular parasitologists for some time to come.

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