Amplified DNAs in Laboratory Stocks of Leishmania tarentolae: Extrachromosomal Circles Structurally and Functionally Similar to the Inverted-H-Region Amplification of Methotrexate-Resistant Leishmania major

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We describe the structure of amplified DNA that was discovered in two laboratory stocks of the protozoan parasite Leishmania tarentolae. Restriction mapping and molecular cloning revealed that a region of 42 kilobases was amplified 8- to 30-fold in these lines. Southern blot analyses of digested DNAs or chromosomes separated by pulsed-field electrophoresis showed that the amplified DNA corresponded to the H region, a locus defined originally by its amplification in methotrexate-resistant Leishmania major (S. M. Beverley, J. A. Coderre, D. V. Santi, and R. T. Schimke, Cell 38:431-439, 1984). Similarities between the amplified DNA of the two species included (i) extensive cross-hybridization; (ii) approximate conservation of sequence order; (iii) extrachromosomal localization; (iv) an overall inverted, head-to-head configuration as a circular 140-kilobase tetrameric molecule; (v) two regions of DNA sequence rearrangement, each of which was closely associated with the two centers of the inverted repeats; (vi) association with methotrexate resistance; and (vii) phenotypically conservative amplification, in which the wild-type chromosomal arrangement was retained without apparent modification. Our data showed that amplified DNA mediating drug resistance arose in unselected L. tarentolae, although the pressures leading to apparently spontaneous amplification and maintenance of the H region are not known. The simple structure and limited extent of DNA amplified in these and other Leishmania lines suggests that the study of gene amplification in Leishmania spp. offers an attractive model system for the study of amplification in cultured mammalian cells and tumors. We also introduced a method for measuring the size of large circular DNAs, using gamma-irradiation to introduce limited double-strand breaks followed by sizing of the linear DNAs by pulsed-field electrophoresis.

Parasites of the genus Leishmania (order Kinetoplastida, family Trypanosomatidae) are a widespread protozoan group which infect a variety of vertebrates (45), Leishmania spp. which infect humans cause a spectrum of diseases collectively termed leishmaniasis. These range in severity from mild cutaneous lesions to fatal visceral infections, depending on the infecting species (45). Because of the significance of Leishmania spp. to world public health, there has been increasing attention focused on the novel biochemical, molecular, and immunological mechanisms used by these parasites to survive within the host.

In addition to the Leishmania spp. infecting mammals, there exists another related group of Leishmania spp. which infect lizards (22, 44). One of these species, Leishmania tarentolae, has been used extensively as a nonpathogenic representative of Leishmania spp. for many laboratory studies (44). For example, there has been extensive research on the molecular features of the distinctive mitochondrial DNA of this organism, the kinetoplast DNA, which exhibits a number of unusual properties such as catenated DNA networks (36) and mRNA editing (35). In this report we describe the phenomenon of specific gene amplification and drug resistance in this parasite. Apparently, these occur spontaneously in two independent laboratory stocks that were not selected for drug resistance in the laboratory. As described below, this study has significance to the study of the occurrence and mechanism of specific gene amplification and drug resistance in the Leishmania spp. that are pathogenic to humans. Moreover, the study of amplification in Leishmania spp. offers a simple model system for the study of gene amplifications previously characterized in tumors and in both transformed and drug-resistant mammalian cell lines. An abstract of the results of that study has been presented [M. Petrillo-Peixoto and S. M. Beverley, Memorias do Instituto Oswaldo Cruz Rio de Janeiro, 81(Suppl.):36, 1986]. Gene amplification has been demonstrated in three different species of Leishmania, in response to selective pressure with five different drugs in the laboratory (2, 3a, 18, 26). The prototypic amplifications have been described in promastigotes of the human parasite Leishmania major that was selected for resistance to the antifolate methotrexate (MTX) (2, 3a, 7; S. M. Beverley, T. E. Ellenberger, and M. Petrillo-Peixoto, in D. T. Hart, ed., Leishmaniasis: The First Century 1885-1985, New Strategies for Control, in press). These cells can exhibit amplification of two different regions of DNA, following stepwise selection in vitro for high levels of resistance. One of these amplified regions of DNA, termed the R region, contains the gene encoding the novel bifunctional dihydrofolate reductase-thymidylate synthase (3, 20), the presumed cellular target of antifolates such as MTX and 5,8-dideaza-10-propargyl folate (18). Amplification of the dihydrofolate reductase-thymidylate synthase gene was anticipated, as the monofunctional dihydrofolate reductase gene is often amplified in MTX-resistant mammalian...
cells (31, 32). The R-region amplification frequently consists of a simple 30-kilobase (kb) circular DNA that is topologically equivalent to a direct repeat (2, 3a).

The other region of DNA, termed the H region, exists as an 85-kb extrachromosomal circular DNA containing two copies of the H region in an inverted repeat configuration (2, 3a; Beverley et al., *Leishmania*, in press). Amplification of a second region of DNA has not been reported previously in MTX-resistant cultured mammalian cells. Interestingly, amplification of the H region has been observed in lines of *L. major* selected for resistance to structurally and mechanistically unrelated drugs (3a; Beverley et al., *Leishmania*, in press; T. E. Ellenberger and S. M. Beverley, manuscript in preparation). These lines also exhibit MTX resistance, and current data suggest that H-region amplification may encode a form of multidrug resistance. The H-region amplification of *L. major* does not appear to mediate decreased MTX uptake, accumulation, or increased MTX efflux (11, 12) and is thus distinct from the amplification-associated multidrug resistance described in cultured mammalian cells (15, 30). As shown here, the amplified DNA of *L. tarentolae* appears to be an amplification of the H-region homolog of this species. *Leishmania* parasites are digenetic, alternating between the infective amastigote stage residing within the phagolysosome of the macrophage and the promastigote stage within the digestive tract of the phlebotomine sand fly vector (44, 45). *Leishmania* spp. are not currently known to undergo a sexual cycle in vitro or at any point in the natural life cycle (39). The ploidy of this organism is thought to be approximately diploid, based on comparisons of total DNA and kinetic renaturation experiments (27), a view that has been confirmed by biochemical or molecular studies of three distinct genetic loci (3a, 25; D. M. Iovannisci and S. M. Beverley, manuscript in preparation).

**MATERIALS AND METHODS**

**Cell lines and culture.** A line of *Leishmania* sp. derived from a parasite stock maintained at the Universidade Federal de Minas Gerais was identified in our laboratory by comparisons of nuclear DNA restriction fragment patterns (4) as *L. tarentolae* and was designated the MG strain. The origins in Brazil of *L. tarentolae*, a species normally found in lizards in the old world (44), are unknown. This strain had been maintained in the laboratory for at least 1 year. Clonal derivatives of the MG line (such as MG-C2) were also used. Other strains of *L. tarentolae* examined were two isolates of the Trager line (ATCC 30267), which were obtained from L. Simpson (this line is named Trager-S here) and independently from G. Holz (this line is named Trager-H here), LTC-1 (L. Simpson); LV-414 (J. Rioux); LEM-87 (G. Holz); and ATCC 30143 (G. Holz).

Recently, it has been shown that parasites identified as *Trypanosoma platydictylii* were identical by isoenzyme profiles to existing laboratory stocks of *L. tarentolae* (43). The nomenclature and identity of these strains have been called into question (19a, 43), and in this report we refer to these species as *L. tarentolae*. The results of other investigators (19a) as well as our own unpublished data, which we obtained by comparing chromosomes and nuclear DNA restriction fragments (cf. reference 4), indicate that regardless of nomenclature, all strains of *L. tarentolae* are closely related.

Promastigotes were grown at 26°C in medium 199-based medium, as described previously (7). Cultures were maintained by passage from late-log-phase cultures and were used within 10 passages (about 60 generations), after which they thawed from frozen stocks. Clonal derivatives were obtained by plating the cultures on 1% agar containing medium 199 and incubating them at 26°C in a 5% CO₂ atmosphere.

For studies of drug sensitivity, promastigotes from late-log-phase cultures were diluted to a concentration of 10⁷/ml in a final volume of 10 ml, and their growth was monitored using a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.).

**DNA manipulations.** Many of the procedures used for the isolation and analysis of genomic and cloned DNAs were performed as described previously (2, 7). Total genomic DNA was isolated from promastigotes in the late log phase; no differences in restriction pattern or amplification were observed with DNAs isolated from stationary-phase organisms. Radiolabeling of DNA fragments with [³²P]dCTP was done by random priming (14). DNA fragments isolated from agarose gels were occasionally purified further by chromatography with Elutip-d columns by the instructions of the manufacturer (Schleicher & Schuell, Inc., Keene, N.H.). Blot hybridization was performed as described previously (12), with the DNA transferred to nylon membranes (Gene Screen Plus; Dupont, NEN Research Products, Boston, Mass.) by the instructions of the manufacturer, except that the filters were baked for 2 h at 80°C prior to prehybridization. The hybridization buffer was 0.36 M NaCl; 20 mM NaPO₄; 2 mM EDTA; 0.04% each of bovine serum albumin, Ficoll (Pharmacia Fine Chemicals, Piscataway, N. J.), and polyvinylpyrrolidone; 0.5% sodium dodecyl sulfate; and 20 µg of denatured sonicated salmon sperm DNA (pH 7.5) per ml; the temperature was 67°C unless indicated otherwise. The copy number of amplified DNAs was estimated by cell dot blot hybridization (12, 17).

**Isolation of molecular recombinants of amplified *L. tarentolae* DNA.** The amplified 2.5-, 3.2-, and 4.6-kb *KpnI* fragments of the MG-C2 line (see Fig. 1 and 2) were isolated and inserted into the plasmid vector pUC8c2 (a modified form of pUC8 into which a *KpnI* site was added to the polylinker; provided by G. F. Crouse, Emory University, Atlanta, Ga.). Putative recombinants were radiolabeled and hybridized to appropriate digests of the genomic DNA, to verify the successful cloning of the amplified DNA fragment. The desired recombinants were termed pLTAR-K25, pLTAR-K32, and pLTAR-K46, respectively.

**Library construction and screening.** A genomic library of *L. tarentolae* MG-C2 DNA was constructed in the phage vector Charon 4A as described previously (13). Briefly, hydrodynamically sheared fragments of total cellular DNA of 15 to 20 kb were ligated to purified vector arms through EcoRI linkers and packaged in vitro. A total of 3 x 10⁶ independent recombinants were obtained, and the library was amplified once by using *Escherichia coli* LE392 as the host cell. The library was screened by the method of Benton and Davis (as described by Davis et al. [8]) by using the three cloned *KpnI* fragments described above as hybridization probes. To obtain the right end of the amplified region, two DNA fragments mapping to the right end of the amplified H region of *L. major* (2) were used. In total, 41 overlapping phase were obtained and subjected to restriction mapping by using enzymes singly or in appropriate combinations. The phages shown in Fig. 2C are representative of the total set of phages analyzed. Every region of the amplified DNA was obtained and analyzed in at least five independent phases.

**Pulsed-field electrophoresis.** Samples for pulséeld field electrophoresis were prepared as described previously (1). Each
The obtained fragments of lambda c1857 Sam7 (New England BioLabs, Inc., Beverly, Mass.) The DNA whose apparent size was 0.8 kb corresponded to free minicircle DNA, which was present at variable levels in different DNA preparations. (B) Southern blot hybridization of the gel shown in panel A. The radiolabeled hybridization probe was the 2.5-kb KpnI fragment isolated from pLTR-TAR-K25.

sample contained 10^7 cells from a late-log-phase culture. Pulsed-field electrophoresis was performed in a gel apparatus similar to that of Schwartz and Cantor (34), as described previously (1), or in a contour-clamped homogeneous electric field (CHEF) apparatus similar to that described previously (7). The temperature was 4°C and the voltage gradient was 6 V/cm for CHEF electrophoresis. Gamma-irradiation of sample plugs was performed using a gamma-iradiator (GR9; ICN Pharmaceuticals, Inc., Irvine, Calif.), which uses a ^60Co source.

RESULTS

Amplified sequences in L. tarentolae. Following digestion with restriction endonucleases, total DNA from the clonal MG-C2 line of L. tarentolae exhibited intensely staining fragments superimposed on the heterogeneous smear of fragments obtained from the Leishmania genome, as shown with the enzyme KpnI (Fig. 1A). A similar KpnI restriction pattern was observed in the original MG parental line and 12 other clonal derivatives, while the intensely staining fragments were not visible in the LTC-1 or Trager-S lines (Fig. 1A) or in any of the other lines examined (data not shown). The extent of DNA amplified in the MG-C2 line was estimated from the sum of the sizes of the intensely staining fragments obtained with different restriction enzymes. These values ranged from 32 to 42 kb by using the enzymes KpnI, SalI, SacI, XbaI, Apal, EcoRI, HindIII, and BamHI separately (data not shown).

Several of the intensely staining KpnI fragments present in the DNA of the MG-C2 line (Fig. 1A) were isolated and inserted into plasmid vectors. Southern blot analysis, with the recombinant 2.5-kb KpnI fragment used as a probe to

Leishmania DNA, revealed hybridization to a 2.5-kb fragment in all lines, with strong hybridization to the MG and MG-C2 DNAs, moderate hybridization to the LTC-1 DNA, and weak hybridization to the Trager-S DNA (Fig. 1B) and all other L. tarentolae DNAs examined (data not shown). The data in Fig. 1B and quantitative cell dot blots indicated that the relative copy number of this DNA was approximately 16 to 32 in both the MG and MG-C2 lines and 8 in the LTC-1 line, relative to 1 for the Trager-S and other lines (Table 1). Similar results were obtained with other amplified fragments throughout the amplified region (data not shown).

The DNA amplification in the MG and MG-C2 lines was stable, as it was maintained for more than 1 year in continuous culture in vitro. Attempts to infect lizards (geckos) with the MG-C2 line were unsuccessful.

Molecular cloning of the amplified DNA. Molecular recombinant phages encompassing the amplified DNA of the MG-C2 line were obtained and mapped as described above. In total, more than 40 phages were analyzed, yielding the integrated restriction map for the amplified DNA shown in Fig. 2A and B. All amplified DNA fragments visualized by restriction endonuclease cleavage of total DNA were predicted by the recombinant phage restriction map, and conversely, all fragments predicted from the phage restriction map were observed and amplified in restriction digests of total DNA. It is therefore likely that the entire amplified region was isolated in the form of molecular recombinants. The restriction map of the L. tarentolae amplified DNA revealed that 42 kb of DNA was amplified, which was in good agreement with the estimates from restriction analysis of total DNA.

There were two regions located at either end of the restriction map of the amplified DNA which occurred in an inverted, head-to-head configuration. Because of the problems often associated with the maintenance of inverted repeats in certain cloning vectors, we describe in detail results of the analysis of the Leishmania DNA insert within phage LTAR-29 (Fig. 2C), which includes the DNA corresponding to the inversion center located at the left end of the restriction map of the amplified DNA (Fig. 2A). The restriction map of the LTAR-29 insert contained an 8.2-kb region demarcated by EcoRI sites, which separates inverted repeats of 4 kb, each of which spanned seven restriction sites (Fig. 2D). The restriction map of the central region suggests that at least some portion of the central region was not in an inverted orientation, although the exact boundaries were not

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**FIG. 1.** Amplified DNA in L. tarentolae. (A) Total DNA (1 μg) was digested with KpnI, separated by electrophoresis on an 0.8% agarose gel, and stained with ethidium bromide. C2 + MTX indicates L. tarentolae MG-C2 after propagation in 10 μM MTX (nine passages). Molecular weight markers (in kilobases) are HindIII fragments of lambda c1857 Sam7 (New England BioLabs, Inc., Beverly, Mass.). The DNA whose apparent size was 0.8 kb corresponded to free minicircle DNA, which was present at variable levels in different DNA preparations. (B) Southern blot hybridization of the gel shown in panel A. The radiolabeled hybridization probe was the 2.5-kb KpnI fragment isolated from pLTR-TAR-K25.

<table>
<thead>
<tr>
<th>Line</th>
<th>Amplification</th>
<th>MTX resistance (EC_{50} [μM])^b</th>
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<tbody>
<tr>
<td>Trager-S</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>LTC-1</td>
<td>8</td>
<td>&gt;10</td>
</tr>
<tr>
<td>MG-C2</td>
<td>16-32</td>
<td>8</td>
</tr>
<tr>
<td>MG-C2 (10 μM MTX)^c</td>
<td>&gt;100</td>
<td>&gt;100</td>
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^a Amplification was measured by cell blot hybridization as described in the text. In this method the hybridization of the probe pK30 relative to that obtained with beta-tubulin was compared over a series of twofold dilutions. Amplification is expressed as the fold amplification relative to that obtained in the Trager-S line.

^b The EC_{50} for MTX inhibition is defined as that concentration which reduced the initial rate of growth by 50% during the initial passage in MTX.

^c This line was derived from MG-C2 by nine successive passages in 10 μM MTX, as described in the text; similar results were obtained with the line obtained following four passages in 1 μM MTX followed by five passages in 10 μM MTX.

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H-REGION AMPLIFICATION IN L. TARENTOLAE

FIG. 2. Restriction site map of the amplified H region of L. tarentolae. (A) Summary of the map. The locations of restriction enzyme cleavage sites mapped as described below for panel B are shown. The large arrows above the restriction map refer to the locations of the inverted repeats inferred by restriction mapping. The boxes below the restriction map indicate the location of the DNA rearrangements in the MG-C2 amplified-H-region DNA relative to the structure observed in the nonamplified Trager-S line. The two boxes at the right end indicate the site of rearrangement if the 5.5-kb EcoRI site was the rearrangement fragment (open box) or if it was identical to the wild-type fragment (dark stippled box; see text). Abbreviations: H, HindIII; B, BamHI; R, EcoRI; K, KpnI; BglII. (B) Restriction fragment map of the amplified H region. Restriction enzyme cleavage sites for HindIII (H), EcoRI (R), BamHI (B), and KpnI (K) and the predicted DNA fragments from analysis of 41 recombinant phage are shown (the location of the single BglII site is not shown in this map). This map was verified by blot hybridization analysis of MG-C2 DNAs. The numbers represent the sizes of the fragments, in kilobases. The 2.5-, 3.2-, and 4.6-kb KpnI fragments are those that were isolated as molecular recombinants in plasmids. (C) Representative recombinant phage used in developing the maps shown in panels A and B. Note that for both centers of inversion several phages were obtained which overlapped the central noninverted region symmetrically as well as asymmetrically on both sides. (D) Restriction map of phage LTAR-29. The Leishmania DNA insert of this phage contained the inverted repeats found in the left portion of the map shown in panel A and is shown in greater detail in this panel. Because of the method of library construction, an EcoRI site was located at the boundary of the vector and the Leishmania DNA insert; the sizes of the fragments obtained with each enzyme alone are shown, except for the outermost fragments, for which the size corresponds to that associated with the EcoRI site of the Charon 4A vector. Some of the restriction digests used in the construction of this map are shown in Fig. 3A. Fragments whose sizes are circled are identified by the SMB87 probe described in Fig. 3B. The locations of the inverted repeats present within this phage are shown by the small arrow maps at the bottom of the panel. (E) Restriction map of phage LTAR-123. The map of the leishmania DNA insert of this phage is presented as determined. The presence of doublet restriction fragments in the digests of LTAR-29 DNA showed the close similarity of the inverted repeats; these included 0.9- and 1.6-kb KpnI, 1.4-kb KpnI-BamHI, 0.7-kb HindIII-KpnI, and 1.7-kb BamHI-HindIII fragments (Fig. 3A), among others. A 0.8-kb DNA fragment (SMB87) from the wild-type H region of L. major (2), which is known to be present in a single copy in this species, specifically identified the duplicated restriction fragments in a Southern blot (Fig. 3B) of the gel shown in Fig. 3A (the relationship of the L. tarentolae amplification to the H region of L. major is discussed below). The hybridization pattern of this probe to digests with enzymes which cut only once or not at all within the central, noninverted region, such as HindIII (22, 12.2, and 7.3 kb; Fig. 3B, lane 5) and HindIII-BamHI (1.8, 0.3, 3.8, and 8 kb; Fig. 3B, lane 6), confirmed that the inverted region was present twice within the phage. The hybridization pattern obtained in the KpnI digests (singly or in combination with EcoRI, BamHI, or HindIII) confirmed that the central 3.2- and 5.5-kb fragments were not identified by the SMB87 probe, demonstrating that the duplicated sequences are separated within the phage (Fig. 3B, lanes 1 to 4). The presence of the inverted repeats was further confirmed by the mapping of phages LTAR-24 and LTAR-17, which overlapped the central noninverted region from different sides and thereby yielded simple restriction maps which lacked duplicated regions (Fig. 2C).

Similarly, at the right end of the restriction map shown in Fig. 2A there was a 5.5-kb region, demarcated by EcoRI sites, which was also flanked by inverted repetition of the flanking sequences. Analysis of phage LTAR-123, which contained this region of DNA, revealed the presence of inverted repeats, each of which consisted of five restriction sites spanning 3 kb (Fig. 2E). Duplicated restriction fragments were evident in restriction digests of phage LTAR-123 DNA, such as the 2.5-kb SsrI, 1.6-kb HindIII-EcoRI, 2.2-kb SsrI-KpnI, and 1.1-kb SsrI-HindIII fragments (Fig. 3C). These fragments were identified by a 2.0-kb fragment from the H region of L. major(pLTS108-T20), which is known to be present in a single copy in this species (2) (Fig. 3D). The hybridization pattern obtained with HindIII, which cut only once within the noninverted region, showed that this region is found twice in phage LTAR-123 (Fig. 3D, lane 6; summarized in Fig. 2E); and the hybridization patterns obtained with EcoRI, SsrI, and KpnI revealed that there is a central region separating the repeated DNA fragments (Fig. 3D; summarized in Fig. 2E). As discussed above, the restriction maps of phage LTAR-128 and LTAR-73, which overlapped the central, noninverted region from either side of the map of phage 123, confirmed the presence of the inverted region (Fig. 2C). Interestingly, phages LTAR-29 and LTAR-23 have been stable thus far during propagation in the recombinant-proficient bacterial strain LE392.

For both regions of the map shown in Fig. 2A which occurred at the center of inverted repeats, the true limits of the inverted repeats may extend further into the central regions, which are currently defined solely by the sites mapped thus far (EcoRI sites in both instances). Nonethe-
FIG. 3. Restriction digestion and Southern blot hybridizations of phage bearing inverted repeat sequences. DNAs from each phage were digested with the enzymes indicated below and were separated on 0.8% agarose gels. Restriction enzyme abbreviations: B, BamH1; H, HindIII; K, KpnI; R, EcoRI; T, SstI. The positions of molecular size markers (in kilobases) are shown on the left of each panel. (A) Phage LTAR-29 (left end of map shown in Fig. 2A) on an ethidium bromide-stained gel. Lanes: 1, R+K; 2, B+K; 3, K; 4, H+K; 5, H; 6, H+B. (B) Phage LTAR-29. The gel shown in panel A was blotted onto a nylon membrane and hybridized with the SMB87 probe, which was derived from the H region of L. major and is a single copy in the wild-type chromosome (2). (C) Phage LTAR-123 (right end of map shown in Fig. 2B), ethidium bromide-stained gel. Lanes: 1, R+T; 2, B+T; 3, R+B; 4, R; 5, H+R; 6, H; 7, T+H; 8, T; 9, R+K; 10, T+K. (D) Phage LTAR-123. The gel shown in panel C was blotted onto a nylon membrane and hybridized with the pLTS108-T20 probe, which was derived from the H region of L. major and is a single copy in the wild-type chromosome (2).

less, the restriction map of the amplified DNA present in L. tarentolae MG-C2 showed that it exists in an inverted repeat configuration, thereby resembling the configuration of the amplified H region of L. major (2).

Homology of the amplified DNA of L. tarentolae with the H region of L. major. We asked whether the amplified sequences of L. tarentolae were related to the inverted-H-region amplification of L. major (2). Separate blots of digested L. tarentolae DNA were hybridized with four overlapping recombinant phages whose Leishmania DNA inserts spanned the entire amplified H region of L. major (2) (see Fig. 5B for diagrams of these phages). Each of the L. major phages hybridized intensely to one or more amplified DNA fragments in L. tarentolae MG-C2 DNA (Fig. 4). In total, the four L. major probes recognized all of the amplified DNA fragments of L. tarentolae, and no other intensely hybridizing fragments were identified by these probes.

The organization of sequences in the L. tarentolae and L. major H-region amplifications were also related, as shown by blot hybridization experiments in which the four overlapping L. major phages described above (probes A to D in Fig. 5B; see also Fig. 4) and four smaller DNA fragments from L. major (probes 1 to 4 in Fig. 5B) were used. The results of these hybridization experiments are depicted on the restriction map of the L. tarentolae amplification in Fig. 5. The leftmost L. major probes (A and 1 in Fig. 5B) hybridized specifically to the leftmost fragments of the L. tarentolae restriction map (Fig. 5A and C). Probes which mapped farther to the right of probes A and 1 tended to identify fragments which were located progressively farther to the

FIG. 4. Hybridization of the amplified DNA of L. tarentolae with probes from the H region of L. major. L. tarentolae MG-C2 total DNA was digested with the indicated restriction endonucleases, separated by electrophoresis in agarose gels, and transferred to nylon filters. Restriction enzyme abbreviations: T, SstI; K, KpnI; S, SalI; R, EcoRI; B, BamH1; H, HindIII. The hybridization probes were overlapping recombinant phages or plasmids encompassing the entire L. major H-region DNA (2) (see Fig. 5B). The hybridization probes were pLTS-R130 (A; left end), lambda LT-HC3 (B; left central), lambda LTS-106 (C; right central), and lambda LTS-108 (D; right end). The molecular size markers (in kilobases) are lambda cut with HindIII as described in the legend to Fig. 1. The hybridization temperature was 58°C; identical results were obtained at 67°C (data not shown).
right in the *L. tarentolae* map. This was especially evident when the smaller probes 1 to 4 were used. These probes showed a clear ordering in the restricted maps of both species (Fig. 5A and C). The larger probes also showed clear ordering from left to right, although some uncertainty in the ordering of probes C and D was evident because of the presence of repeated sequences in both *L. major* and *L. tarentolae* in these regions (data not shown). Thus, amplification in the MG-C2 line of *L. tarentolae* was similar in structure, sequence, and order to the amplified H region of *L. major*, despite the fact that it lacked overall conservation in the restriction map and was somewhat smaller in size (42 versus 48 kb, respectively).

MTX resistance of H-region-amplified *L. tarentolae*. To assess potential MTX resistance conferred by the H-region amplification of *L. tarentolae*, we compared the MTX sensitivity of the MG-C2 and LTC-1 lines relative to that of the Trager-S line. Initial experiments indicated that the two lines bearing the amplified-H region DNA were capable of continued growth in 10 μM MTX (albeit initially slowly), whereas the Trager-S line could not be maintained in 0.5 μM MTX. Attempts to measure the concentration of drug which inhibited the rate of growth by 50% (EC₅₀) were complicated by the fact that even with very high MTX concentrations, the Trager-S line grew slowly, to approximately 10-fold the initial cell density after about 80 h, prior to the cessation of growth. Nonetheless, when the growth rates during this period were used to calculate an EC₅₀, values of 0.4 μM for the nonamplified Trager-S line versus 8 μM for MG-C2 and >10 μM for LTC-1, the H-region-amplified lines, were revealed (Table 1). It was possible to eliminate the apparent lag in growth inhibition by taking the cells after 60 h of growth in MTX and repassing them in the same concentration of MTX. Under these conditions the EC₅₀ calculated for the Trager-S line was 0.5 μM and that for the MG-C2 line was 25 μM. It is clear that the H-region-amplified lines of *L. tarentolae* exhibited resistance to MTX on the order of 20-fold. The correlation with MTX resistance and gene amplification was not exact, however, since the LTC-1 line was more resistant to MTX than the MG-C2 line, while it possessed less amplified H-region DNA.

A second approach further implicated the *L. tarentolae* H region in MTX resistance. Lines of the MG-C2 line were exposed to MTX, either by four passages in 1 μM MTX followed by five passages in 10 μM or by nine passages in 10 μM. These lines exhibited increased levels of H-region amplification (Fig. 1A and B) and increased resistance to MTX, with EC₅₀ of greater than 100 μM MTX (Table 1). These results are similar to those obtained when revertant lines of *L. major* bearing low levels of the H region (12; unpublished data) or both R- and H-region amplification (7) were reexposed to MTX. It is possible that some portion of the increased MTX resistance may represent the rapid acquisition of other MTX resistance mechanisms, such as decreased MTX accumulation which is unrelated to H-region amplification in *L. major* (10, 11, 12). However, our data suggest that the H region of *L. tarentolae* encodes a functional MTX resistance determinant.

**H-region amplification and extrachromosomal circular DNA.** We next asked whether the amplified H-region DNA of *L. tarentolae* was found as extra-chromosomal circular DNA. We were unable to obtain satisfactory separation of the amplified DNA by electrophoresis of *L. tarentolae* DNA in low-percent agarose gels or by centrifugation in CsCl-ethidium bromide gradients (data not shown), and instead, we used pulsed-field electrophoresis techniques.

Figure 6A shows the separation of DNAs of the MG-C2 and Trager-S lines by using CHEF electrophoresis. A number of DNAs were resolved, which are commonly referred to as chromosomes in trypanosomes (40, 41). These chromosomes ranged in size from 300 to >1,500 kb, when compared with size standards consisting of oligomers of bacteriophage lambda DNA or yeast chromosomes. Depending on the pulse time, there was also a region of compression in which higher-molecular-weight DNAs were collected (C in Fig. 6). The MG-C2 and Trager-S lines appeared very similar in their overall molecular karyotypes.

Figure 6B shows the hybridization of a *L. tarentolae* H-region probe to a blot of the gel shown in Fig. 6A. The MG-C2 line showed strong hybridization to the well and additional hybridization to two DNAs (marked by arrows in Fig. 6A). In contrast, only the lower-molecular-weight DNA was identified by the H-region probe in the Trager-S line (marked by the thin arrow). The lower-molecular-weight
DNA in both the Trager-S and MG-C2 lines was also identified by an H-region chromosome-specific hybridization probe (Fig. 6C) derived from nonamplified DNA closely flanking the H region in wild-type *L. major* (2). These data suggest that the lower-molecular-weight DNAs identified by the thin arrows in Fig. 6B and C corresponded to the normal, wild-type chromosome in each lane. Comparison of the hybridization results shown in Fig. 6B and C with the ethidium bromide-stained gel shown in Fig. 6A and control hybridizations with a beta-tubulin probe (data not shown) suggests that this chromosome is present at comparable levels within the two lines.

The DNA in the MG-C2 line identified exclusively by the amplified region probe (Fig. 6B) exhibited the unusual mobility properties characteristic of supercoiled circular DNAs, such as pulse time-dependent mobility (relative to those of linear DNA size markers [1, 19, 22]). Using pulse times of 90 to 150 s, this DNA had an apparent molecular size of about 1,000 kb (Fig. 6B). In contrast, with a pulse time of 30 s, this DNA had an apparent molecular size of about 300 kb (Fig. 7C). This DNA also exhibited resistance to exonucleases (data not shown).

The major fraction of the amplified DNA, however, remained within the sample well following CHEF or pulsed-field gel electrophoresis (PFGE) (Fig. 6B and 7C). Recently, it has been shown that large circular DNAs remain within the sample well in PFGE, a finding that was also observed in conventional electrophoresis when voltage gradients comparable to those used in PFGE were used (1). To examine whether the amplified DNA remaining within the well could be circular, we used a new method for estimating the size of the circular DNA in which we gamma-irradiated cellular DNAs cast in agarose. Gamma-irradiation introduces a spectrum of structural lesions into DNA, including base modifications and strand breaks, with single-stranded breaks occurring 10 to 20 times more frequently than double-stranded breaks (24). After an appropriate dose of gamma-irradiation, linear molecules yield a heterogeneous collection of fragments of various lengths because of the introduction of random double-stranded breaks. In contrast, a circular molecule initially gives rise to a discrete linear molecule whose length corresponds to that of the original circular form. The appearance of a discrete molecule is therefore diagnostic of circular DNA. When combined with PFGE, this method is suitable for demonstrating and measuring the size of very large circular DNAs and is experimentally convenient (S. M. Beverley, unpublished data).

Titrations of the gamma-irradiation dose were performed, followed by hybridization of a *L. tarentolae* H-region probe to blots of the irradiated samples separated by PFGE or CHEF electrophoresis by using differing pulse times (Fig. 7). With increasing radiation dose, the intensity of hybridization to the supercoiled circular DNA decreased, as did the hybridization to the presumptive chromosome (marked by heavy and thin arrows, respectively, in Fig. 7A). Significantly, a new component of about 140 kb appeared and increased with increasing radiation (Fig. 7A; a faintly hybridizing species of about 280 kb was also evident at the highest radiation dose used in Fig. 7A, although this species was not evident in every preparation). Gamma-irradiation of both well-hybridizing and supercoiled circular DNA which was purified following separation by PFGE yielded only the 140-kb DNA (data not shown). The 140-kb DNA was linear, as judged by its mobility characteristics (lack of pulse-time dependence; compare Fig. 7A, B, and C) and sensitivity to exonuclease III (data not shown). By the reasoning given above, the 140-kb linear molecule must correspond to a circular form of the H-region amplification in *L. tarentolae*.

At the highest radiation dosage used (400 krads), the hybridization signal present in the well was completely removed, showing that the well-hybridizing material is not nonspecifically trapped (Fig. 7C, lane receiving 400 krads). Comparison of the hybridization of the 140-kb linear DNA to that remaining in the sample well following 100 krads of irradiation suggests that a substantial portion of the amplified DNA must correspond to the 140-kb circle (Fig. 7C, lane receiving 100 krads). Moreover, the hybridization intensity of the 140-kb linear DNA that was evident after 50 to 200 krads of gamma-irradiation exceeded that of the supercoiled circular amplified DNA (Fig. 7C, lanes receiving 50, 100, and 200 krads versus the lane receiving no radiation). While these data alone cannot determine whether all of the well-hybridizing material was circular, they suggest that a significant portion of the well-hybridizing material consists of extrachromosomal, open circular DNA.

As the amplified unit in *L. tarentolae* determined by molecular cloning was 35 kb (measured from the centers of inversion shown in the restriction map in Fig. 2), the 140-kb circular DNA probably consisted of a molecule bearing four
copies of the central amplified region arranged in an inverted configuration (Fig. 8A). The 140-kb species was also present in the LTC-1 lane (Fig. 7B) and elevated in the MG-C2 lanes, which had increased levels of amplified DNA following growth in 10 μM MTX (Fig. 7B, last two lanes). As expected, the 140-kb DNA was absent from the Trager-S lane, which lacked amplified DNA (Fig. 7B).

Rearrangements in amplified DNA. In the amplified H region of L. major, the centers of the inverted repeats marked the regions of DNA in which rearrangements of wild-type DNA occurred, yielding the final amplified structure (1). To examine any DNA rearrangements generated by the L. tarentolae H-region amplification, we compared the DNA of the amplified MG-C2 line with that of the nonamplified Trager-S line, thereby using this line as an approximate wild-type parent of the MG-C2 line.

We first used fragments mapping to the left end of the H-region restriction map, such as the 3.2-kb KpnI fragment (Fig. 2B). In the Trager-S DNA, this probe identified only nonamplified 2.5-kb and 5.5-kb KpnI fragments (Fig. 9A, lane 2). In contrast, this probe identified a predominant novel amplified 3.2-kb fragment as well as an amplified 5.5-kb KpnI fragment and a nonamplified 2.5-kb fragment in the MG-C2 DNA; other, fainter novel fragments possibly representing minor DNA rearrangements were also evident (Fig. 9A, lane 1). The novel 3.2-kb fragment must contain the predominant site of DNA rearrangement. A similar analysis of BamHI-digested DNAs revealed the presence of a novel, amplified 3.6-kb fragment in the MG-C2 line (Fig. 9A, lanes 3 and 4) and novel 12.2-kb HindIII and 8.2-kb EcoRI fragments (data not shown; Fig. 2B). These data localized the region of the predominant rearrangement at the left end to a 2-kb region (Fig. 2A).

Hybridization of the 4.6-kb KpnI fragment located within the right end of the L. tarentolae restriction map similarly revealed the presence of novel DNA fragments in the MG-C2 line relative to that in the Trager-S line. BamHI digestion revealed the presence of a predominant novel 5.7-kb fragment (Fig. 9C, lanes 1 and 2), HindIII digestion revealed a novel amplified 2.9-kb fragment (Fig. 9C, lanes 3 and 4); the wild-type 14.9-kb fragment was not clearly evident in this exposure but was clearly present in others; data not shown), and KpnI digestion revealed a predominant novel fragment of 1.5 kb; EcoRI digestion did not reveal a novel amplified fragment. Assuming that the amplified 5.5-kb

FIG. 7. PFGE analysis of gamma-irradiated L. tarentolae DNAs. (A) Titration of the radiation dose with PFGE. Chromosomes of the MG-C2 line were subjected to gamma-irradiation and then PFGE. The gel was blotted onto a nylon membrane and hybridized with a pLTAR-K32 probe. Lane 1, No irradiation; lane 2, 10,000 rads; lane 3, 50,000 rads. The position of the supercoiled circular amplified DNA is marked by a heavy arrow, and the H-region chromosome is indicated by a thin arrow, as discussed in the legends to Fig. 6B and C. The pulse time was 50 s, and the electrophoresis time was 17 h. The positions of lambda oligomer molecular weight markers are indicated. (B) Analysis of L. tarentolae strains. Chromosomes of the indicated strains were gamma-irradiated (50,000 rads) and subjected to PFGE. The pulse time was 10 s for 20 h. Southern blot hybridization was done as described in above for panel A. Lanes: LTC-1, line LTC-1, C2, line MG-C2; Trager, line Trager-S; unlabeled lane, lambda oligomers; C2+MTXa, M6-C2 propagated for four passages in 1 μM MTX followed by five passages in 10 μM MTX; C2+MTXb, M6-C2 propagated for nine passages in 10 μM MTX. The arrowheads mark the positions of the new 140-kb linear DNA. (C) Titration of gamma-irradiation dose by CHEF electrophoresis. MG-C2 chromosomes were subjected to the indicated doses of gamma-irradiation (in kilorads) and then separated by CHEF electrophoresis by using a pulse time of 30 s for 30 h. sc, Supercoiled circular DNA; 1, linear DNA.

FIG. 8. Summary of H-region structures in L. tarentolae and L. major. The stippled regions indicate the left and right inversion centers, as shown in Fig. 2A. The arrows designate the orientation for the intervening DNA. (A) L. tarentolae tetrameric inverted amplification. (B) L. major dimeric inverted amplification. (C) Wild-type gene structure determined in L. major (1) and inferred for L. tarentolae.
L. tarentolae

which probe fragments described above. and fragment the Trager-S line) rearrangement of the wild-type H-region DNA structure, in addition to the inverted amplification (Fig. 9). This conclusion was also supported by the PFGE analysis described above, which demonstrated the continued presence of a chromosome similar in size and abundance to the wild-type chromosome of the Trager-S line. Third, the wild-type DNA fragment that was present in the MG-C2 DNA appeared to be retained at the same level as that observed in Trager-S DNA, suggesting that no quantitative loss of DNA occurred (Fig. 9). Fourth, as novel, nonamplified fragments identified by the closely flanking probes were not evident in the MG-C2 line, we infer that no deletions or other rearrangements occurred in the wild-type DNA. Thus, amplification of the L. tarentolae H region appears to be conservative, retaining the wild-type DNA structure and chromosome (1, 3a). This may not necessarily imply a conservative mechanism of amplification.

**DISCUSSION**

We showed that two lines of L. tarentolae exhibit amplification of a region of chromosomal DNA which resembles the H-region amplification previously described in MTX-resistant lines of L. major (2, 3a, 12) by the following criteria. First, molecular cloning in conjunction with restriction enzyme mapping revealed that the L. tarentolae DNA amplification is of the inverted repeat type, containing two regions of the DNA that are rearranged to yield the prototypic head-to-head structure found within the L. major H-region amplification. Second, hybridization analysis with an ordered series of probes spanning the H region of L. major revealed a similar order of hybridization to DNA fragments from the L. tarentolae amplification. Although the restriction maps were different and small differences in the ordering of some fragments may have been present, the overall structure was conserved. Third, electrophoretic analysis of chromosome-sized DNAs revealed that a significant fraction of the amplified DNA in the MG-C2 line consisted of extrachromosomal circular DNA whose length was 140 kb. Comparison of this value with the value obtained by restriction mapping for the basic repeat, 35 kb (measured from the center of the inverted repeats flanking the map shown in Fig. 2), suggests that the circular form present in L. tarentolae consists of an extrachromosomal circular dimer of the basic inverted repeat, similar to the amplified region in L. major, which is a monomeric extrachromosomal inverted repeat (Fig. 8). Finally, the L. tarentolae lines bearing H-region amplifications exhibited significant MTX resistance, on the order of 20-fold. These data indicate that the amplification of L. tarentolae is an amplification of the H-region locus of this species.

It is pertinent to ask what are the origin(s) and role(s) of the H-region amplification in L. tarentolae, a parasite found...
naturally in lizards. Neither the MG-C2 line nor the LTC-1 line underwent any drug pressure in the laboratory, and as MTX is not used for the treatment of either lizards or infectious diseases, it seems unlikely that strains of *L. tarentolae* have undergone specific MTX pressure at any time. Results of recent studies in our laboratory may provide a clue to this puzzle, however, as we have demonstrated that the H region may encode a form of multidrug resistance (3a, T. E. Ellenberger and S. M. Beverley, unpublished data).

We speculate that it is possible that the amplification in *L. tarentolae* may have been selected or induced by exposure to a compound other than MTX. Potential sources of this hypothetical selective pressure could include natural ones, such as secondary plant or insect compounds toxic to *Leishmania* spp.; artificial ones, such as herbicides or pesticides applied by humans; or compounds encountered during laboratory culture, such as plastics or media contaminants. Another, less likely, possibility is that amplification occurs in a nondirected manner. Perhaps the H region is especially susceptible to amplification in a manner reminiscent of transposable elements or parasitic DNAs and only coincidentally happens to carry MTX resistance determinants. Results of studies of the frequency of H-region amplification in *L. major* do not favor this view (unpublished data); however, amplification of the H or other regions of DNA may be more common in *L. tarentolae*. Interestingly, we have recently shown that another region of DNA, provisionally named the T region, is amplified in other lines of *L. tarentolae* (unpublished data). We favor the view that the occurrence of amplified H-region DNA bearing a drug resistance determinant indicates a functional role for this DNA in *L. tarentolae* at some point in time.

An important question concerning gene amplification in parasites is the occurrence and maintenance of amplified DNAs during the natural infectious cycle of *Leishmania* spp., alternating between the amastigote stage in vertebrates and the promastigote stage in the sand fly. Amplified DNAs present in laboratory-selected promastigotes of *L. major* (B. J. Sina, G. M. Kapler, and S. M. Beverley, manuscript in preparation) or *Leishmania mexicana* (26) can be maintained following passage through the amastigote stage in vivo. Regardless of the causes leading to H-region amplification in *L. tarentolae*, our data show that amplification can occur in populations of *Leishmania* that are not overtly exposed to laboratory drug pressure. In combination, these data indicate that amplified DNAs conferring drug resistance can be successfully maintained throughout the infectious cycle of *Leishmania*, and are thus poised as a potential resistance mechanism to prospective antileishmanial chemotherapy.

The H-region amplification within the MG-C2 line appears to be conservative, as both restriction mapping and PFGE analysis revealed that the original wild-type chromosome structure was qualitatively and quantitatively retained. In contrast, a nonconservative model would require some alteration in the structure of the wild-type H-region chromosome, which was not observed. Similar data have been presented for the H- and R-region amplifications observed in certain lines of *L. major* (1, 2, 19). It is tempting to speculate that the amplification mechanism itself was conservative, perhaps occurring by a process involving localized overreplication followed by recombinations among the overrelicated DNAs. Such a mechanism has been hypothesized for amplification events in cultured mammalian cells (31-33, 42). Unfortunately, the origins of the amplified H-region DNA within the MG-C2 line are unknown, as the amplification occurred in an apparently spontaneous manner at some point in nature or during laboratory cultivation. Consequently, we cannot tell whether the apparently conservative amplification was the initial product of the amplification mechanism or, alternatively, whether it represents a secondary result of a nonconservative mechanism which has resolved into a conservative phenotype. The occurrence of less abundant DNA rearrangements at both ends of the restriction map serve to further emphasize this uncertainty. Such questions may be more clearly resolved in MTX-resistant lines of *L. major*, as the first immediately resistant lines bearing the initial amplification structures are available (S. M. Beverley, manuscript in preparation). Interestingly, for both *Leishmania* spp. and cultured mammalian cells, evidence has been presented suggesting that amplified DNAs in cultured mammalian cells can arise by a deletional mechanism as well (3a, 6).

It is evident that *Leishmania* spp. offer an excellent setting for investigating the molecular basis of specific gene amplification, which has been most intensively studied in tumor cells and cultured mammalian cells that have been selected for resistance to chemotherapeutic agents (21, 31-33, 37, 38). Many of the characteristic features of gene amplification in mammalian cells are observed in *Leishmania* spp., including unstable, stable, extrachromosomal, and chromosomal amplifications (2, 3a, 9, 18, 19; Beverley et al., *Leishmaniasis*, in press; S. M. Beverley, unpublished data). *Leishmania* spp. offer an especially attractive system for examining the structure of amplified DNA, as the amplified structures are simple and relatively homogeneous and only a small region of DNA (<100 kb) is amplified (2, 3a, 9, 18). In contrast, the structure of amplified DNA in cultured mammalian cells is often complex, heterogeneous, and changeable over time and can include thousands of kilobases of DNA (13, 31, 32, 37, 38), although smaller amplifications have been reported (5, 28, 29). The utility and relevance of the study of gene amplification in *Leishmania* spp. are illustrated by the fact that amplified DNA has been shown to exist in either direct or inverted configurations (head to tail versus head to head), first in *Leishmania* spp. (2) and subsequently in cultured mammalian cells (16, 28).

The small extrachromosomal elements such as the drug resistance-carrying amplifications in *L. tarentolae*, *L. mexicana* (9), and *L. major* (2) and the small DNAs found in certain isolates of *Leishmania donovani* (R. Hamers, N. Gajendran, J.-C. Dujardin, and K. Stuart, in *D. T. Hart*, ed., *Leishmaniasis: The First Centenary 1885–1985*, New Strategies for Control, in press; K. Stuart, S. Karp, R. Aline, Jr., B. Smiley, J. Scholler, and J. Keithley, in *D. T. Hart*, ed., *Leishmaniasis: The First Centenary 1885–1985*, New Strategies for Control, in press) may have origins in natural infectious cycle of *Leishmania* spp. offer an especially attractive system for examining the structure of amplified DNA, as the amplified structures are simple and relatively homogeneous and only a small region of DNA (<100 kb) is amplified (2, 3a, 9, 18). In contrast, the structure of amplified DNA in cultured mammalian cells is often complex, heterogeneous, and changeable over time and can include thousands of kilobases of DNA (13, 31, 32, 37, 38), although smaller amplifications have been reported (5, 28, 29). The utility and relevance of the study of gene amplification in *Leishmania* spp. are illustrated by the fact that amplified DNA has been shown to exist in either direct or inverted configurations (head to tail versus head to head), first in *Leishmania* spp. (2) and subsequently in cultured mammalian cells (16, 28).

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establishing a system of DNA-mediated transformation and
developing a molecular understanding of basic genetic ele-
ments in Leishmania and other members of the family
Trypanosomatidae.

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