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Co-existence of circular and multiple linear amplicons in methotrexate-resistant *Leishmania*

Asunción Olmo, Rosalía Arrebola, Victor Bernier, Dolores González-Pacanowska and Luis M. Ruiz-Pérez*

Instituto de Parasitología y Biomedicina, Consejo Superior de Investigaciones Científicas, C/ Ventanilla 11, Granada 18001, Spain

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ABSTRACT

Circular and linear amplicons were analyzed in detail in *Leishmania tropica* cells resistant to methotrexate (MTX). Both types of elements presented sequences related to the H locus and coexisted in resistant cells. The linear amplicons appeared first during the selection process (at 10 μM MTX) and varied with regard to size and structure in cells exposed to increasing concentrations of drug. The circular element was evident at higher concentrations (50 μM) but was the major amplified DNA in cells resistant to 1000 μM MTX while the level of amplification of the linear elements remained low. The extrachromosomal DNAs were unstable in the absence of drug and their disappearance coincided with an increase in sensitivity to MTX. Mapping of the minichromosomes and the circular element showed that they were all constituted by inverted duplications. The circular amplicon contained an inverted repeat derived from the H locus that encompassed the pteridine reductase gene (*PTR1*) responsible for MTX resistance. The amplified segment in the linear amplicons was longer and included the *pgpB* and *pgpC* genes that encode P-glycoproteins of unknown function previously characterized in different *Leishmania* species.

INTRODUCTION

Methotrexate (MTX) is a stoichiometric inhibitor of the enzyme dihydrofolate reductase (DHFR) and different species mutants resistant to the drug exhibit overproduction of DHFR (1,2), structural alterations in the target protein (3) or reductions in drug accumulation (4,5). In *Leishmania*, the mechanism of MTX resistance has been analyzed in detail (6). Several MTX-resistant lines of *Leishmania major* showed elevated DHFR activity and amplification of the dihydrofolate reductase-thymidylate synthase gene (*DHFR-TS*) contained within a segment of DNA termed the R region (1,7). The extrachromosomal DNA H region has been more frequently identified in MTX resistant *Leishmania* cells (8). Amplification of the H locus has been described in *Leishmania* resistant to MTX (8–10), arsenite (11–13), primaquine or terbinafine (14), as well as in unselected laboratory stocks (15–17). In all cases the amplification occurred as an extrachromosomal circular element that was unstable in the absence of drug.

Certain variations exist with regard to the structure of the circular amplicons derived from the H locus. Ouellette *et al.* (18) have reported that circles obtained after selection of *Leishmania tarentolae* for MTX resistance contained a 30 kb inverted duplication separated by two unique DNA sequences, while certain mutants obtained by selection for arsenite resistance contained H locus derived plasmids without such duplications. At present, two H locus genes involved in drug resistance have been identified: a P-glycoprotein homologous gene (*pgpA*) associated with heavy metal resistance (9,12,19) and *PTR1* which encodes an enzyme responsible for antifolate resistance as demonstrated by transfection experiments (20–23). The exact mechanism by which the H locus gene confers resistance to MTX has been recently identified. *PTR1* is the gene for pteridine reductase, an enzyme involved in a folate salvage pathway (24).

In addition to the existence of extrachromosomal circular DNA molecules, another phenomenon related to the karyotypic variability associated with different cell lines of species of *Leishmania* is the appearance of small linear DNA elements (25–27). The occurrence of such elements has been associated to nutrient stress or subcloning (28) although a few cases have been described where linear extrachromosomal DNAs contain genes responsible for drug resistance (29,30). *Leishmania donovani* strains resistant to DL-α-difluoromethylornithine (DFMO) contain two independent gene amplifications: a linear 140 kb element on which all of the amplified copies of the ornithine decarboxylase gene (*odc*) were located and a second 70 kb circular DNA, of unknown function. A 280 kb linear amplified extrachromosomal DNA, IMPDH-280, has been identified in *L.donovani*. This linear amplicon contains the gene for inositol monophosphate dehydrogenase (*IMPDH*) and confers resistance to mycophenolic acid (30). Amplification of the H locus as linear elements has also been described in mutants resistant to MTX (31).

Elements that present extensive homology with H locus DNA have been previously found in MTX-resistant *L.tropica* (15). Now we show that MTX-resistant *L.tropica* cells derived from a cloned cell line lacking extrachromosomal amplicons contained

* To whom correspondence should be addressed

two types of extrachromosomal elements: several linear multicopy DNAs and a circular element of 83 kb which both show a high degree of homology with H region DNA. This constitutes to our knowledge the first time that amplification of the same locus occurs as both a linear and circular element in the same cell line. We have characterized in detail the amplicons derived from the H locus and determined the degree of homology between them. We have also determined the variability in size and structure of the linear amplicons that occurred upon prolonged exposure to MTX.

MATERIALS AND METHODS

Cell culture and MTX resistance induction

The *L.tropica* LRC-L39 strain was originally isolated and typified by Dr L. F. Schnur, Hebrew University (Hadassah Medical School, Jerusalem, Israel). Promastigotes of LRC-L39 were grown at 28°C in RPMI 1640 modified medium (Gibco, Middlesex, UK) as previously described (32) supplemented with 20% heat-inactivated fetal bovine serum (Flow Laboratories, UK) and, when specified, MTX. The cloned cell line was derived from the LRC-L39 cell population by dilution and plating on 1% agar prepared with RPMI and 20% fetal bovine serum (33). *L.tropica* cell lines resistant to 1000 μM MTX were obtained by using a stepwise selection process as described (1). Starting at an initial drug concentration of 5 μM, cells were seeded into the next higher concentrations (10, 20, 50, 500 and 1000 μM MTX) when the doubling time was stabilized, usually after five passages. In the stability studies, revertants were obtained by growing the MTX resistant lines for several passages in the absence of the selecting drug.

CHEF gel electrophoresis

The procedure for preparing intact *Leishmania* chromosomal DNA in agarose blocks was adapted from Garvey and Santi (7). Contour-clamped homogeneous electric field (CHEF) electrophoresis was performed using a commercially available apparatus (Pharmacia) in 0.5× TBE buffer (40 mM Tris, 45 mM boric acid, 1 mM EDTA pH 8.3) at 13°C. The voltages, pulse frequencies, time and agarose percentages of the electrophoresis were chosen as appropriate for the DNA size range to be separated. Total chromosomal separation was carried out with 1–1.5% agarose gels at 160 V for 36 h 30 min, including a 1 s pulse time for 30 min and 75 s pulse time for 36 h. Extrachromosomal circular DNA was resolved free from genomic contaminants using 1.5% agarose gels and pulse frequencies of alternating 300–900 s times for 12 h and 900–300 s for 12 h at 160 V and 13°C (10). Molecular weights of the chromosomal DNA bands were determined by comparison with DNA standards from *Saccharomyces cerevisiae* strain S13 and concatamers of the λ genoma (Pharmacia).

Isolation and characterization of extrachromosomal DNAs

The extrachromosomal linear H region was separated from the large linear chromosomes by CHEF electrophoresis and excised from 1% Seakem-GTG agarose (FMC BioProducts). The blocks were washed twice for 10 min with 10 mM Tris–HCl, pH 7.4 and equilibrated in 2 ml of the appropriate restriction buffer (34). Each block was incubated for 4 h in 200 μl of fresh restriction buffer containing 100 μg of BSA (Sigma, Molecular Biology) and the appropriate amount of the restriction enzyme, *Eco*RI or *Eco*RV. Restricted DNA bands were separated in CHEF electrophoresis at 300 V for 12 h with 4 s pulses. The extrachromosomal circular DNA was isolated by an isopycnic CsCl gradient as described (35). The structure of the circular H region was analyzed by restriction mapping. In order to obtain large amounts of purified DNA, two unique *Eco*RI fragments from the H region, purified by CsCl gradient, were isolated from agarose gels by electroelution and cloned into the pWE15 cosmid vector (Stratagene Kit). Both fragments were digested with the restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, *Xba*I and *Xho*I.

DNA probes

Extrachromosomal circular and linear DNAs to be used as hybridization probes were electrophoresed on CHEF and recovered by electroelution. In order to determine the circular H region structure, the following probes were used: (i) a 2.3 kb *Bam*HI–*Bam*HI and (ii) a 2.3 kb *Xho*I–*Hind*III fragment from the isolated circular amplicon; (iii) pHM3 which is a 1.9 kb *Hind*III fragment derived from the H region of *L.major* (8); (iv) the *PTR1* gene isolated from *L.major* 252 by PCR and (v) a *Bam*HI–*Pst*I 400 bp fragment of the *ltpgpA* gene of *L.tarentolae* (36). The structure of H locus linear amplicons was determined using the same probes as for the circular element plus the following: (i) a 4.3 kb *Eco*RV–*Xho*I fragment and (ii) a 0.9 kb *Bam*HI–*Hind*III fragment from the rearrangement points of the circular amplicon; (iii) nbsA which is a 850 bp *Pst*I–*Pst*I fragment covering the first nucleotide binding site of the *ltpgpA* gene from the H region of *L.tarentolae* (9); (iv) a 150 bp *Hind*III–*Eco*RV fragment specific for the P-glycoprotein genes *ltpgpB* and *ltpgpC* from *L.tarentolae* (36) and (v) the telomeric repeat 5′-$(CCCTAA)_{20}$-3′ (27,37).

Exonuclease III digestion of extrachromosomal DNA

Agarose blocks with ~2 μg of total DNA were equilibrated (three times for 3 h each) with exonuclease III buffer (66 mM Tris–HCl, pH 7.6; 1 mM DTT; 0.66 mM $MgCl_2$). Blocks were then added to 0.5 ml of buffer containing 0–280 U exonuclease III (Boehringer Mannheim) and incubated at 37°C for 130 min. The reaction was stopped with EDTA 0.2 M pH 8.0 and the DNAs were analyzed by CHEF electrophoresis.

Southern blotting and hybridization

Total DNA from *L.tropica* promastigotes was isolated by phenol extraction as described (38). Southern blotting was performed with nylon filters (Hybond N, Amersham Corp.) by the method of Southern (39). Blots were hybridized in: 5× SSC, 50% formamide, 5% Denhardt's solution, 5 mM EDTA and 100 μg/ml salmon sperm DNA at 42°C for 16 h. Labelling of probes was performed by the random priming method (40). Filters were washed at a stringency of 2× SSC, 0.1% SDS at 42°C twice for 20 min each. Blots were visualized by autoradiography and the relative intensities of the bands and the copy number of extrachromosomal DNAs were quantitated by scanning densitometry (BioRad Model 620 Video Densitometer).

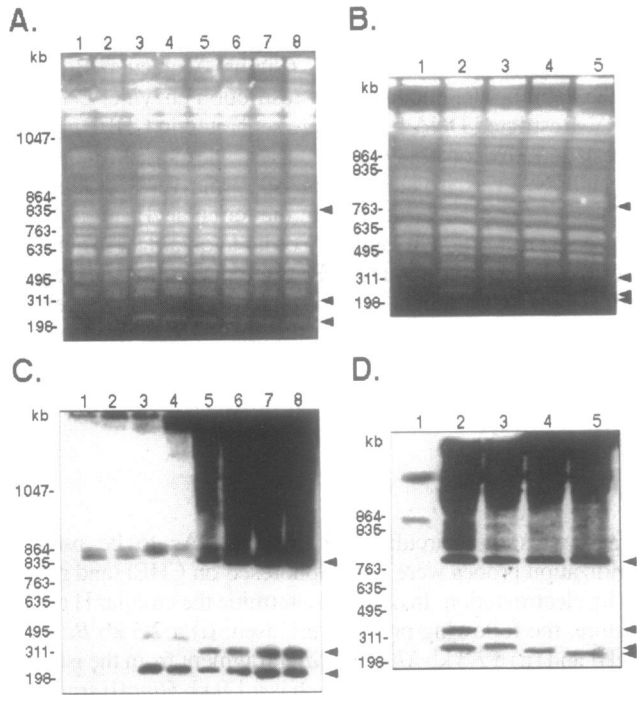

Figure 1. Molecular karyotype of MTX-resistant *L.tropica* LRC-L39. (**A**) Chromosomes were separated by CHEF electrophoresis for 36 h 30 min, including a 1 s pulse time for 30 min and 75 s pulse time for 36 h, and stained with ethidium bromide. 1, L39M; 2, M5; 3, M10; 4, M20; 5, M50; 6, M100; 7, M500; 8, M1000. (**B**) Evolution of the molecular karyotype of M1000 cells after exposure to 1 mM MTX for 5, 7 and 10 months. 1, L39M; 2, M1000; 3, M10005; 4, M10007; 5, M100010. (**C**) Southern blot hybridization of the gel shown in panel A with the *PTR1* probe from the *L.major* H region. (**D**) The gel shown in panel B was blotted and probed with a fragment of the H region from *L.major* (pHM3). The extrachromosomal H DNAs are indicated by arrows.

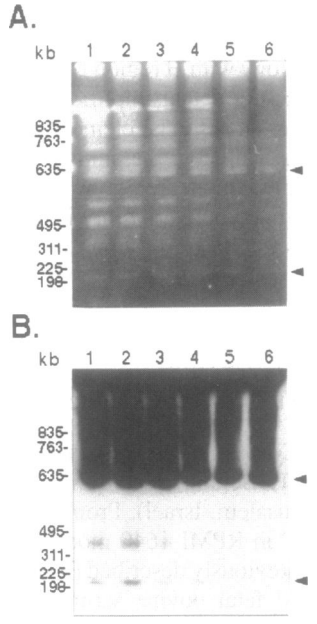

Figure 2. Effect of exonuclease III digestion on circular and linear elements of M100010 cells. (**A**) Ethidium bromide stained gel of chromosomes digested with different amounts of exonuclease III. 1, 0 U; 2, 80 U; 3, 130 U; 4, 180 U; 5, 230 U; 6, 280 U. (**B**) Southern blot hybridization of the gel shown in panel A with the *PTR1* probe. The location of the circular and linear H elements is shown by arrows.

Drug sensitivity determinations

The effects of MTX on the growth of the LRC-L39 wild type, resistant and revertant lines were determined by measuring the rate of exponential growth in liquid culture over a range of different drug concentrations as described previously for MTX (14). The EC_{50} was defined as the drug concentration (in μM) which decreased the rate of exponential cell growth by 50%. The resistance index is the ratio of EC_{50} values for resistant and wild-type lines.

RESULTS

Identification of amplified DNAs in a MTX-resistant cell line of *L.tropica*

In the present study, we have induced resistance to MTX using a cloned cell line of *L.tropica* LRC-L39 (L39M) devoid of extrachromosomal elements. It is evident that under these conditions, amplicons that would arise as a mechanism of drug resistance would be generated *de novo* and not as a result of a gradual selection of a minor population of organisms containing amplified DNAs. Cells resistant to 5, 10, 20, 50, 100, 500 and 1000 μM MTX (M5, M10, M20, M50, M100, M500 and M1000, respectively) obtained by stepwise selection were examined for the presence of extrachromosomal elements by CHEF electrophoresis (Fig. 1A). At 10 μM, a band appeared with an apparent molecular size of 300 kb which was undetectable in wild-type cells. Additionally, at 50 μM MTX, two new DNA bands were present; a small DNA of ~360 kb and an element whose apparent molecular size changed with pulse frequency and had properties characteristic of supercoiled DNA.

Cells resistant to 1000 μM MTX grown for 10 months in the presence of drug (M100010), contained the band whose migration was characteristic of supercoiled DNA, and a low molecular weight element of ~280 kb while the 300 and 360 kb elements were no longer detected (Fig. 1B). The migration of the 360, 300 and 280 kb bands relative to that of the yeast standards was independent of pulse time, a characteristic of linear DNAs. These linear and circular DNAs of new appearance could be separated from the chromosomal DNA using particular pulse frequencies as described in Materials and Methods. We therefore chose to use CHEF electrophoresis to isolate the extrachromosomal DNAs free of genomic contaminations.

Southern blot analysis probing with sequences of other known extrachromosomal elements established the identity of the amplicons observed in the MTX-resistant *L.tropica* cell lines. Figure 1C is a blot of the gel shown in Figure 1A probed with the PCR amplified *PTR1* gene of *L.major*. There was a hybridization signal in the region of the supercoiled element which was clearly evident in cells resistant to 50 μM MTX and increased in intensity with drug concentration. Hence, the supercoiled DNA molecule appeared to be circular H region similar to what had been previously described in *L.tropica* as a mechanism of resistance to MTX (15).

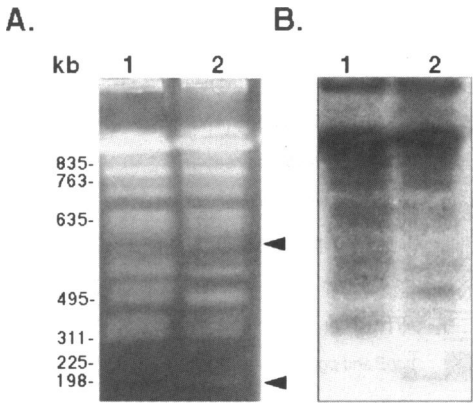

Figure 3. Hybridization of the circular and H280-L elements to a telomeric probe. (**A**) Chromosomes separated by CHEF electrophoresis. 1, L39M; 2, M100010. (**B**) Southern blot hybridization of the gel shown in panel A with a telomeric repeat unit. The location of the circular and linear H elements is shown by arrows.

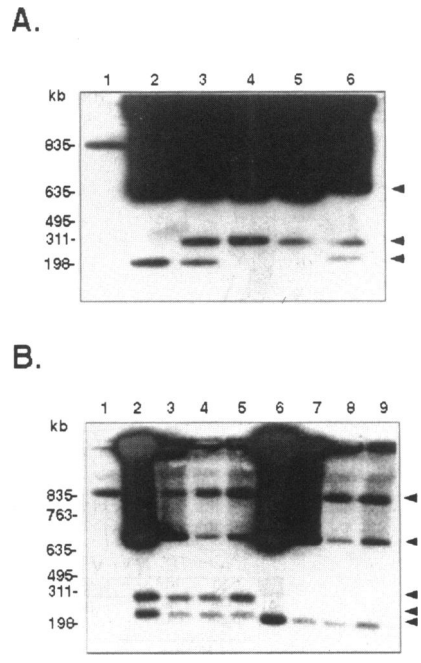

Figure 4. Analysis of the variability and stability of amplicons in MTX-resistant *L.tropica* lines. (**A**) Molecular karyotypes of several clonal lines derived from the resistant line M100010. Chromosomes were resolved by CHEF electrophoresis and hybridized to the *PTR1* probe. 1, L39M; 2, M100010; 3, M100010.1; 4, M100010.2; 5, M100010.3; 6, M100010.4. (**B**) The stability of circular and linear elements from M1000 and M100010 cells was studied by removing the drug from 3 to 7 months. Chromosomes were separated by CHEF electrophoresis and hybridized with the *PTR1* probe. 1, L39M; 2, M1000; 3, M1000.RV3; 4, M1000.RV5; 5, M1000.RV7; 6, M100010; 7, M100010.RV3; 8, M100010.RV5; 9, M100010.RV7. The chromosomal locus and the circular and linear H amplicons are indicated with arrows.

The *PTR1* probe also hybridized strongly to the new linear DNA elements which were present in the MTX resistant lines (Fig. 1C). Similar results were obtained when the pHM3 and *ltpgpA* probes were used (results not shown). The linear amplicon of 280 kb that arose after prolonged exposure to MTX also presented homology with sequences of the H locus (Fig. 1D). All the minichromosomes presented an increased hybridization signal compared to the two bands of 864 and 835 kb which we have assumed are the chromosomal locus from which the amplicons are derived. The existence of two chromosomal bands exhibiting faint hybridization can be explained on the basis of the size polymorphism exhibited by certain chromosomes which are homologous (41,42). Using the chromosomal copy as a reference for single copy sequences, densitometric analysis of the Southern blot revealed that all extrachromosomal elements are in multicopy. We have determined that in 1000 μM MTX resistant cells, there are ~60 copies of the supercoiled circular element per cell and between 3 and 6 copies of each of the three linear elements of 360, 300 and 280 kb. Due to their homology with H region DNA, these linear amplicons have been denominated H360-L, H300-L and H280-L respectively.

Further evidence was obtained in order to confirm the nature of the amplicons. First, agarose blocks from M100010 cells were subjected to digestion with exonuclease III using different enzyme concentrations and analyzed subsequently by CHEF. Figure 2 shows the ethidium bromide stained gel (panel A) and the corresponding Southern blot probed with the *PTR1* gene (panel B). As can be observed, H280-L readily disappears throughout the incubation while the supercoiled amplicon was resistant to digestion. Additionally, blots of CHEF gels were hybridized with a probe specific for telomeric repeat sequences (37). The linear amplicon hybridized clearly while no strong bands were evident in the area of the supercoiled element (Fig. 3).

Variability and stability of extrachromosomal elements

A considerable variability in the size of the linear elements was observed under different experimental conditions. For instance, prolonged exposure to MTX of the M1000 cell line resulted in a loss of the H360-L and H300-L elements. As can be seen in Figure 4A, M100010 cells present high levels of circular H region DNA but only H280-L is evident after Southern blot analysis using *PTR1* DNA as the probe. Curiously, when M100010 cells are cloned (Fig. 4A), a polymorphic situation is obtained. All of the clones examined contained the high molecular weight element H360-L while only some of them (lanes 3 and 6), contained additionally H280-L. None of the clones examined presented characteristics similar to the uncloned heterogeneous population. Cloning of the M100010 population confirms the presence of both circular and linear amplicons derived from the H locus within the same cell.

Circular H region was unstable in the absence of drug. A Southern blot analysis of M1000 and M100010 cells grown without drug for 3 (RV3), 5 (RV5) and 7 (RV7) months is shown in Figure 4B. The copy number of the circular extrachromosomal element decreases gradually when cells are grown in the absence of MTX although it is still present even after 7 months of culture (M1000.RV7 and M100010.RV7 cell lines). The hybridization intensities of H360-L, H300-L and H280-L also decrease although, after 7 months, a certain hybridization signal is obtained at the level of linear amplicons similar to that of the genomic H region locus of wild type cells suggesting that both the circular and linear elements are maintained at low or single copy number after prolonged culture in the absence of MTX (Fig. 4B).

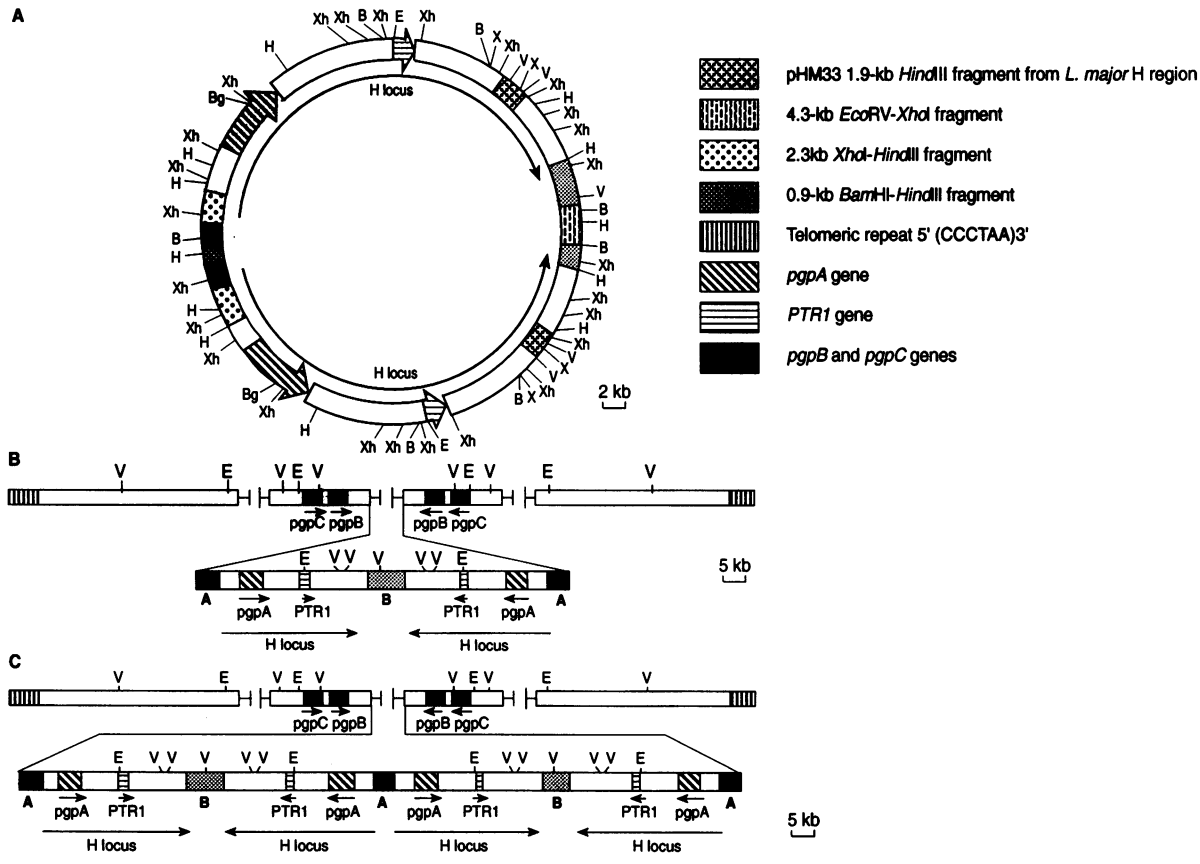

Figure 5. Restriction map of the circular and linear H elements from *L.tropica*. (**A**) A physical map of the circular H region was assembled by digesting two recombinant H fragments (H1 and H2) cloned in pWE15 with the restriction enzymes *Bam*HI (B); *Bgl*II (Bg); *Eco*RI (E); *Eco*RV (V); *Hin*dIII (H); *Xho*I (Xh) and *Xba*I (X), and hybridizing with a variety of probes derived from the circular amplicon and with fragments from the *L.major* and *L.tarentolae* H regions. The restriction maps of H280-L and H300-L (**B**) and H360-L (**C**) were constructed by digestion with *Eco*RI (E) and *Eco*RV (V) and hybridization with the different probes indicated in the table. The large arrows correspond to the inverted repeats of ~36 kb (locus H), separated by two unique regions of 4.6 kb (segment A) and 6.7 kb (segment B) flanking the left and right ends of the H locus respectively. The position of the *pgpA*, *pgpB*, *pgpC* and *PTR1* genes is indicated by thick arrows.

Mapping of circular H-region DNA in M1000 cells

For physical mapping and analysis of the structure of the extrachromosomal circular elements, fragments of the circular amplicon were cloned into the cosmid pWE15. Digestion of H region DNA of *L.tropica* with *Eco*RI gave two fragments of ~39 and 44 kb which could be resolved by CHEF. These two fragments were cloned into pWE15 to give pWEH1 and pWEH2 and the resulting constructs were used for mapping the entire amplicon. The restriction map was constructed by using the enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Xba*I and *Xho*I and confirmed by Southern blot analysis. As other H regions (8,17), H DNA from M1000 cells is an inverted repeat of a DNA segment of ~36 kb separated by two unique DNA sequences of ~6 kb. The complete restriction map is shown in Figure 5A. Although the exact size of the unique segments is yet to be defined, we have delimited that one of the unique DNA segments (segment A represented at the left of the figure) is comprised within two *Xho*I sites and is of ~4.6 kb in length. The other unique sequence (segment B represented on the right hand side of the figure) includes 6.7 kb and is also flanked by two *Xho*I sites. The data put together give a M_r of 83 kb for the circular amplicon which is similar to the mean contour length that has been previously determined by EM analysis for L region DNA from *L.tropica* (15); a supercoiled element that presents extensive homology with the H region of *L. major*. The position of the *pgpA* and *PTR1* genes has also been determined and is indicated in Figure 5A.

Mapping of H region related linear elements in M1000 cells

We have analyzed the structure of the H280-L, H300-L and H360-L minichromosomes present in the resistant lines. A physical map of the H region organization in the H280-L amplicon was constructed by digestion of isolated agarose blocks containing the linear element. The enzymes used were those with infrequently cutting restriction sites in the circular H region DNA such as *Eco*RI and *Eco*RV. Southern blot analysis was used to complete the restriction map. Results obtained using the *L.tropica* circular H region as probe (Fig. 6A) showed that not all the restriction fragments that hybridized when the entire mini-chromosome was used as probe were recognized (Fig. 6A), indicating that the linear amplicon had sequences not related to the H locus. Hybridization with different probes derived from the H locus gave fragment patterns identical to those obtained with the circular H region. Identical hybridization patterns for the circular element and H280-L were also obtained when the probe

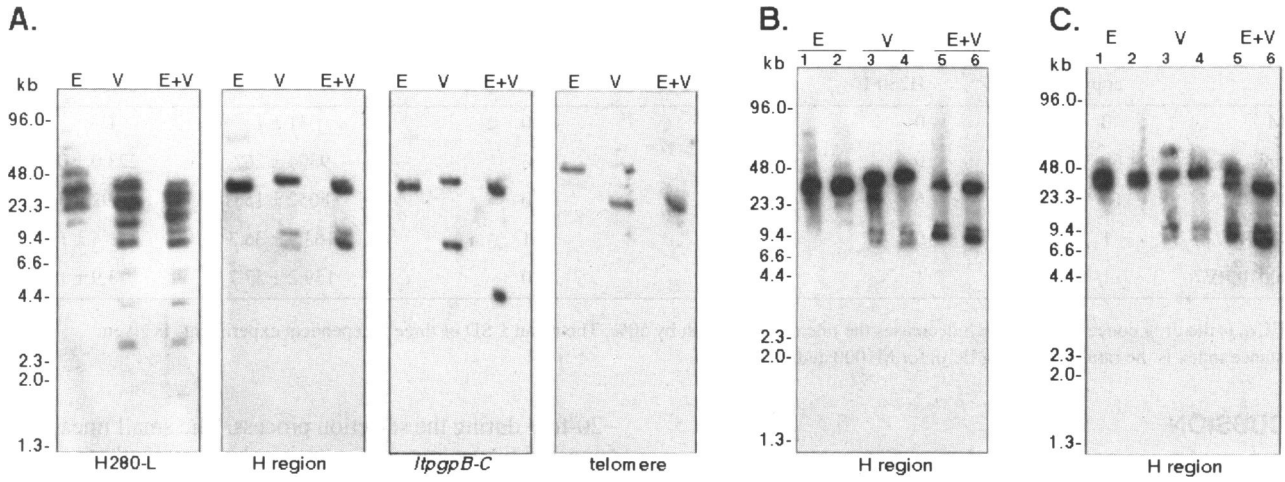

Figure 6. Restriction digestion of the linear amplicons present in MTX resistant lines. After digestion of CHEF purified linear elements with *Eco*RI (E) and *Eco*RV (V), the fragments were separated by a second electrophoresis using 4 s pulses, blotted and hybridized. (**A**) Hybridization of digested H280-L with gel isolated minichromosome, circular H region, a 150 bp *Hin*dIII–*Eco*RV fragment specific for the *ltpgpB* and *ltpgpC* genes from *L.tarentolae* and the telomeric repeat 5′-(CCCTAA)$_{20}$-3′ probe. (**B**) Southern blot of digested H280-L and H300-L probed with gel isolated circular H region. (1,3,5) H280-L; (2,4,6) H300-L. (**C**) Southern blot of digested H280-L and H360-L probed with gel isolated circular H region. (1,3,5) H360-L; (2,4,6) H280-L.

used was a fragment from the right unique flanking region (segment B) showing that this linear amplicon contained an inverted duplication with rearrangements occurring at the right end of the inverted repeat (data not shown). An analysis of a hybridization with a probe specific for both the *ltpgpB* and *ltpgpC* genes located at the 5′ end of the H locus of *L.tarentolae* (36), revealed that these two genes were co-amplified and duplicated at the right and left ends of the inverted duplication (Fig. 6A). The telomeric repeat 5′-(CCCTAA)-3′ was assayed to analyze whether the minichromosome was a complete duplication or if just the H locus and the adjacent 5′ region were duplicated. The probe recognized a unique band in both *Eco*RI and *Eco*RV digestions (Fig. 6A) suggesting that the two telomeric ends were identical in size and therefore we have assumed that the amplicon H280-L is a complete inverted duplication (Fig. 5B).

The structure of H300-L from M1000 cells was determined in the same way as with H280-L. Analysis of the digestion fragments and the Southern blot hybridizations indicated that its structure is similar to that of H280-L (Fig. 5B). Figure 6B is a comparison of a digestion of H280-L and H300-L probed with the circular H region, and no new bands were observed in H300-L that correspond to H locus sequences. The size variation observed between both elements (~20 kb) mapped in a region between the H locus and the telomeric end. In this sense, differences in size attributed to variations in repetitive sequences located in subtelomeric regions have been previously described (37,42).

The H360-L amplicon was digested with the same restriction enzymes, *Eco*RI and *Eco*RV, and the fragments obtained were compared with those of H280-L. Novel fragments of 57 and 40 kb, which were not present in the H280-L, appeared in the *Eco*RV and double *Eco*RI–*Eco*RV digestions respectively. Southern blot analysis (Fig. 6C) showed that the complete circular H region hybridized with identical bands as in H280-L, and also with the new extra bands of the *Eco*RV and *Eco*RI–*Eco*RV digestions. Apparently, H360-L contained at least an extra copy of the H locus, apart from the inverted duplication characterized in H280-L.

Several probes from the H circular DNA were used in order to determine the organization of the extra copy. The 57 kb EcoRV fragment was recognized by the *PTR1* and *ltpgpA* probes and it also hybridized with the 0.9 kb *Bam*HI–*Hin*dIII fragment included in the left rearrangement point of the circular H region. The 40 kb *Eco*RI–*Eco*RV fragment hybridized with the 400 bp *ltpgpA* and 0.9 kb *Bam*HI–*Hin*dIII probes. Neither bands were recognized by the 4.3 kb *Eco*RV–*Xho*I fragment from the rightmost unique region nor by the 150 bp fragment specific for the *ltpgpB* and *ltpgpC* genes. The data allow us to conclude that the H360-L element contains a double inverted duplication of the H region and that two different rearrangements could have generated this organization (Fig. 5C). The first rearrangement would have occurred at the right repetitive sequences and during the duplication process, the *pgpB* and *pgpC* genes were co-amplified. The second duplication, which is at an inverted orientation related to the first one, could have arisen by a rearrangement at the left repetitive sequences. The resulting structure was a chromosome containing four copies of the H locus disposed in an inverted orientation to each other.

Drug sensitivity associated with H region amplification

The resistance index of wild-type, M1000, M100010 and revertant cell lines was determined using the EC_{50} values as described in Materials and Methods and is shown in Table 1.

There is a direct relationship between H region copy number and the level of MTX resistance. The revertant lines showed significantly increased sensitivity to the drug compared to the MTX-resistant lines, however, they were 4-fold more resistant than L39M cells which could be attributed to the presence of low copy H region amplicons.

Table 1. Gene amplification and drug resistance in *L.tropica* LRC-L39

Line	Circular H region copy number	Linear H amplicons copy number			EC_{50}[a]	Resistance index[b]
		H280-L	H300-L	H360-L		
L39M	0	0	0	0	41 ± 1	1
M1000	60	0	3	3	9304 ± 267	223.0 ± 0.3
M100010	60	6	0	0	8052 ± 1551	192 ± 31
M1000.RV7	1	0	1	1	163.9 ± 36.3	4 ± 1
M100010.RV7	1	1	0	0	159.2 ± 57.7	3.9 ± 1.5

[a]The EC_{50} is the drug concentration which decreases the rate of cell growth by 50%. The mean ± SD of three independent experiments is given.
[b]Resistance index is the ratio of the drug EC_{50} for M1000 and L39M cells.

DISCUSSION

In the present study, *L.tropica* LRC-L39 cells undergo amplification of the H locus in response to MTX selection. The amplification occurred in the form of different types of elements when analyzed by CHEF electrophoresis. One of the amplicons exhibits characteristics of supercoiled circular DNA and is present at high copy number. Experimental evidence supporting this observation includes typical migration in CHEF electrophoresis independent of pulse length, insensitivity to digestion with exonuclease III and the absence of telomeric sequences that characterize linear amplicons. The second group of amplicons characterized in resistant cells was a series of small linear DNAs that appear at low copy number, are sensitive to digestion with exoIII and hybridize with a probe specific for telomeric sequences.

Hybridization with probes derived from the H locus of both *L.tarentolae* and *L.tropica* proved that both amplicons present extensive homology between them and with sequences previously described in the H region of other species of *Leishmania* (9,20,21). The mechanism by which H region confers resistance to MTX has been well characterized (23,24). The H locus contains the *PTR1* gene which encodes for an enzyme that possesses an oxidized pteridine reductase activity responsible for pteridine salvage. When overproduced, *PTR1* could mediate MTX resistance by providing an alternative source of tetrahydrofolate, increasing dihydrofolate levels or by increasing the H_4-biopterin pools (24).

In addition to amplification of the H region, we examined the possible occurrence of other mechanisms of resistance such as variations in MTX accumulation (results not shown). However, alterations in the transport of the drug do not appear to exist in *L.tropica* cells contrary to what has been described in *L.tarentolae* where a decrease in drug accumulation co-existed in most cases with linear or circular amplicons (31). Hence, gene amplification appears to be the only mechanism responsible for the resistance phenotype. This observation would be supported by the fact that when resistant parasites are grown in the absence of drug, the circular amplicon decreases in number and, in a proportional fashion, cells are newly susceptible to MTX.

The co-existence of circular and linear elements within one same cell line is an infrequent phenomenon (24,29), and the present study constitutes the first description of cells that contain linear and circular amplicons derived from the same locus. Even though both are in multicopy, the number and intensity of the linear amplicons remains low even at high MTX concentrations while the copy number of the circular amplicon increases ~20-fold during the selection process. The small linear elements appeared first at low concentrations but apparently the amplification of the circular H region is favored versus the increase in number of the linear elements. Since resistance was induced in a cloned population, it is clear that both elements have originated *de novo* and are not a result of being present in a minor number of cells of the parental population. In addition, cloning of M100010 cells supports the observation that both types of elements are present within one cell and not in a mixed population of cells containing one or another. The existence of multiple copy linear elements in *L.tarentolae* resistant to MTX has been previously described (31). The linear elements in these cells were at high copy number and presented a large inverted repeat structure. Nevertheless, in MTX-resistant *L.tropica* cells, circular amplified elements in multicopy would be preferably involved in MTX resistance while the copy of linear elements is maintained low.

All the amplicons contain inverted duplications derived from the H locus. In the circular element, the duplication is shorter and does not include the *pgpB* and *pgpC* genes present in linear amplicons. In H360-L, there is a double inverted duplication, but only the copies located at the ends of the linear amplicon contain the *pgpB* and *pgpC* genes. Both H280-L and H360-L appear to have homologous end sequences located at similar distances from the telomere and present structures of a complete inverted duplication (Fig. 5B–C). The difference in size between H300-L and H280-L is located at certain fragments which correspond to subtelomeric sequences which have not been mapped in detail in the present study (Fig. 5B).

It is not clear whether the different amplicons correspond to separate recombination events occurring at the chromosomal H locus. The generation of both circular and linear elements can be explained by the existence of inverted repeats flanking the H locus. Thus following the model suggested by Papadopoulou *et al.* (31), the annealing of inverted repeats would render a primer for a polymerase to synthesize an inverted duplication. Both the circle and linear elements could arise by recombination events occurring at the same rearrangement point and the size of the duplication is dependant on how far the polymerase advances along the duplication strand (19). The nature of the amplicon would depend on the existence of another pair of annealing repeats and how the amplicon is stabilized; either by circularization, in the case of circular amplicons or by the addition of telomeric sequences (43), in the case of the minichromosomes. We have demonstrated that the different linear elements all present telomeric repeats. Whether these correspond to the original telomeric sequences that have been co-amplified, and the H locus presents a subtelomeric localization or, if they are added

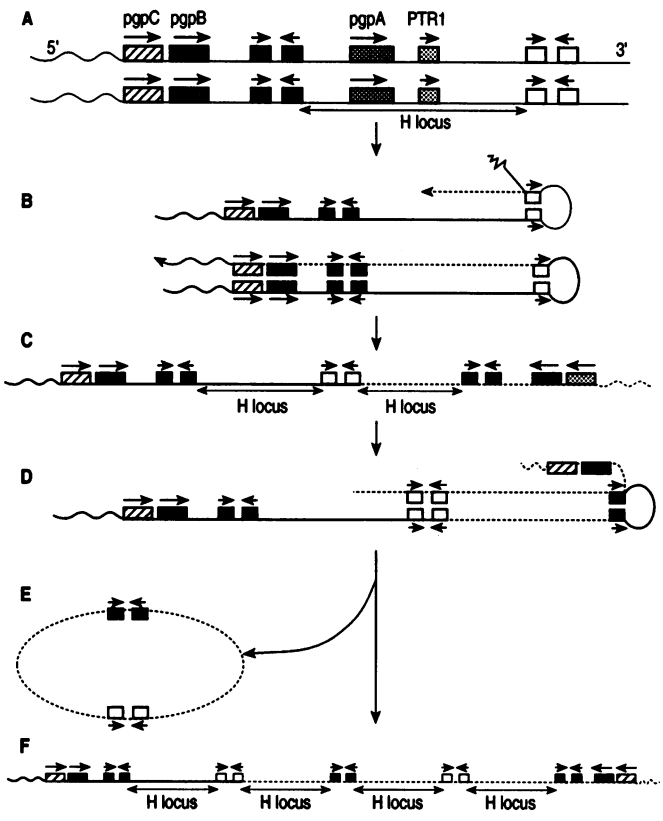

Figure 7. Model suggested for the generation of the co-existing linear and circular amplicons. (**A**) Chromosomal H locus. The inverted repeats are indicated by arrows. (**B**) The annealing of two inverted repeats renders a primer which is used in the synthesis of another copy of the original locus. The polymerase advances along the duplication strand and the *pgpB* and *pgpC* genes are also co-amplified. (**C**) The linear amplified segment (H300-L) could be stabilized by the addition of telomeric sequences. (**D**) New recombination events occurring on H300-L, at the 5′ end of the H locus, could give rise to a duplication of sequences contained within the minichromosome. (**E**) The existence of another pair of inverted repeats could stabilize a circular amplicon. (**F**) The resulting linear element (H360-L) would be a double inverted duplication.

after the excision of the inverted duplication remains unclear. In the present case, considering that the linear elements appear first during the selection process and, of them, H300-L is the first detected, it is possible to speculate with the idea that H300-L represents an intermediate state in the generation of both H360-L and the circular H region. Figure 7 is a model representing the possibility that both H360-L and the circular element originated by recombination events that occurred on H300-L. In *Leishmania* all the circular amplified sequences characterized so far have originated by excision of a chromosomal copy via homologous recombination. Nevertheless, the structure of H360-L implies that at least two recombination events take place in order for the double inverted repeat to originate. The nature of the amplicons described in the present study suggests that the formation of extrachromosomal elements is a dynamic process where certain amplicons may be the precursors of others and the nature of the predominant amplicon at a given stage of the selection process depends on certain advantages that remain to be characterized.

Prolonged culture in the presence of MTX results in a population (M100010) that apparently no longer contains H360-L, and where H280-L is the only linear amplicon. However, cloning gives rise to organisms that contain either the 360 kb amplicon or both H280-L and H360-L. It is possible that the stress associated to the cloning procedure is responsible for these striking observations. Different cell types co-existing in the original population would be selected, or alternatively, H360-L is newly originated during the cloning process. The recurrent emergence of small linear extrachromosomal DNAs in association with nutrient stress or cell cloning has been previously described (28,29) although these elements had no relationship with drug resistance.

When the drug is withdrawn, the size and type of amplicons is maintained, although seven months of culture in the absence of MTX are not sufficient for the entire reversion of the resistance phenotype. While in previous studies amplicons were lost rapidly when the resistant cells were grown without drug (29,31), *L.tropica* cells, as determined by the values of EC_{50} for M1000.RV7 cells, were still slightly resistant compared to wild-type after 7 months in culture without MTX.

The implications of gene amplification in clinical resistance has not been determined although sodium arsenite, an oxyanion related to antimony, is a potent inducer of H region amplification in *Leishmania* cells cultured *in vitro* (13). In the present study, five weeks were sufficient to induce the appearance of the linear element present in cells resistant to 10 μM MTX. It is possible that gene amplification occurs in resistance to antimonials especially in cases where prolonged treatment has occurred. A detailed analysis of isolates obtained from documented cases of resistance using procedures that allow for the identification of amplicons would be required to clearly determine the role of gene amplification in clinical drug resistance.

In summary, *L.tropica* cells resistant to MTX exhibit amplification of the H locus as the single mechanism involved in drug survival. Variable linear and circular amplicons co-existed in resistant cells although the circular H region element appeared to be preferentially selected. It is possible that circular elements present increased stability and are prone to an increase in copy number compared to linear minichromosomes or that they present some advantage in conferring resistance and are therefore positively selected at increasing concentrations of drug.

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REFERENCES

1 Coderre, J. A., Beverley, S. M., Schimke, R. T., and Santi, D. V. (1983). *Proc. Natl. Acad. Sci. USA* **80**, 2132–2136.
2 Washtien, W. L., Grumont, R., and Santi, D. V. (1985). *J. Biol. Chem.* **260**, 7809–7812.
3 Arrebola, R., Olmo, A., Reche, P., Garvey, E. P., Santi, D. V., Ruiz-Perez, L. M., and Gonzalez-Pacanowska, D. (1994). *J. Biol. Chem.* **269**, 10590–10596.
4 Ellenberger, T. E., and Beverley, S. M. (1987). *J. Biol. Chem.* **262**, 13501–13506.

5 Kaur, K., Coons, T., Emmett, K., and Ullman, B. (1988). *J. Biol. Chem.* **263**, 7020–7028.
6 Ouellette, M., and Papadopoulou, B. (1993). *Parasitology Today* **9**, 150–153.
7 Garvey, E. P., and Santi, D. V. (1986). *Science* **233**, 535–540.
8 Beverley, S. M., Coderre, J. A., Santi, D. V., and Schimke, R. T. (1984). *Cell* **38**, 431–439.
9 Ouellette, M., Fase-Fowler, F., and Borst, P. (1990). *EMBO J.* **9**, 1027–1033.
10 Chiquero, M. J., Olmo, A., Navarro, P., Ruiz-Perez, L. M., Castanys, S., Gonzalez-Pacanowska, D., and Gamarro, F. (1994). *Biochim. Biophys. Acta* **1227**, 188–194.
11 Katakura, K., and Chang, K. P. (1989). *Mol. Biochem. Parasitol.* **34**, 189–192.
12 Callahan, H. L., and Beverley, S. M. (1991). *J. Biol. Chem.* **266**, 18427–18430.
13 Grondin, K., Papadopoulou, B., and Ouellette, M. (1993). *Nucleic Acids Res.* **21**, 1895–1901.
14 Ellenberger, T. E., and Beverley, S. M. (1989). *J. Biol. Chem.* **264**, 15094–15103.
15 Hightower, R. C., Ruiz-Perez, L. M., Wong, M. L., and Santi, D. V. (1988). *J. Biol. Chem.* **263**, 16970–16976.
16 Petrillo-Peixoto, M. L., and Beverley, S. M. (1988). *Mol. Cell. Biol.* **8**, 5188–5199.
17 White, T. C., Fase-Fowler, F., van Luenen, H., Calafat, J., and Borst, P. (1988). *J. Biol. Chem.* **263**, 16977–16983.
18 Ouellette, M., Hettema, E., WYst, D., Fase-Fowler, F., and Borst, P. (1991). *EMBO J.* **10**, 1009–1016.
19 Ouellette, M., and Borst, P. (1991). *Res. Microbiol.* **142**, 737–746.
20 Papadopoulou, B., Roy, G., and Ouellette, M. (1992). *EMBO J.* **11**, 3601–3608.
21 Callahan, H. L., and Beverley, S. M. (1992). *J. Biol. Chem.* **267**, 24165–24168.
22 Callahan, H. L., Roberts, W. L., Rainey, P. M., and Beverley, S. M. (1994). *Mol. Biochem. Parasitol.* **68**, 145–149.
23 Papadopoulou, B., Roy, G., Mourad, W., Leblanc, E., and Ouellette, M. (1994). *J. Biol. Chem.* **269**, 7310–7315.
24 Bello, A., Nare, B., Freedman, D., Hardy, L., and Beverley, S. M. (1994). *Proc. Natl. Acad. Sci. USA* **91**, 11442–11446.
25 Beverley, S. M., and Coburn, C. M. (1990). *Mol. Biochem. Parasitol.* **42**, 133–142.
26 Stuart, K. D. (1991). *Parasitol. Today* **7**, 158–159.
27 Navarro, M., Maingon, R., Hamers, R., and Segovia, M. (1992). *Mol. Biochem. Parasitol.* **55**, 65–74.
28 Rovai, L., Tripp, C., Stuart, K., and Simpson, L. (1992). *Mol. Biochem. Parasitol.* **50**, 115–126.
29 Hanson, S., Beverley, S. M., Wagner, W., and Ullman, B. (1992). *Mol. Cell. Biol.* **12**, 5499–5507.
30 Wilson, K., Beverley, S. M., and Ullman, B. (1992). *Mol. Biochem. Parasitol.* **55**, 197–206.
31 Papadopoulou, B., Roy, G., and Ouellette, M. (1993). *Nucleic Acids Res.* **21**, 4305–4312.
32 Jackson, P. R., Wohlhieter, J. A., Jackson, J. E., Sayles, P., Diggs, C. L., and Hockmeyer, W. T. (1984). *Am. J. Trop. Med. Hyg.* **33**, 808–819.
33 Iovannisci, D. M., and Ullman, B. (1983). *J. Parasitol.* **169**, 633–636.
34 Ponzi, M., Janse, C. J., Dore, E., Scotti, R., Pace, T., Reterink, T. J. F., van der Berg, F. M., and Mons, B. (1990). *Mol. Biochem. Parasitol.* **41**, 73–82.
35 Tripp, C. A., Wisdom, W. A., Myler, P. J., and Stuart, K. D. (1992). *Mol. Biochem. Parasitol.* **55**, 39–50.
36 Lgare, D., Hettema, E., and Ouellette, M. (1994). *Mol. Biochem. Parasitol.* **68**, 81–91.
37 Ellis, J., and Crampton, J. (1988). *Mol. Biochem. Parasitol.* **29**, 9–18.
38 Garvey, E. P., Coderre, J. A., and Santi, D. V. (1985). *Mol. Biochem. Parasitol.* **17**, 79–91.
39 Southern, E. (1975). *J. Mol. Biol.* **98**, 503–517
40 Feinberg, A. P. and Vogelstein, B. (1983). *Anal. Biochem.* **132**, 6–13.
41 Iovannisci, D. M., and Beverley, S. M. (1989). *Mol. Biochem. Parasitol.* **34**, 177–188.
42 Blaineau, C., Bastien, P., Rioux, J. A., Roizs, G., and Pags, M. (1991). *Mol. Biochem. Parasitol.* **46**, 293–302.
43 Blackburn, E. H. (1991). *Nature* **350**, 569–573.