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Formation of Extrachromosomal Circular Amplicons with Direct or Inverted Duplications in Drug-Resistant *Leishmania tarentolae*

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Selection for methotrexate resistance in *Leishmania* spp. is often associated with amplification of the H locus short-chain dehydrogenase-reductase gene *ptr1* as part of extrachromosomal elements. Extensive sequences are always coamplified and often contain inverted duplications, most likely formed by the annealing of inverted repeats present at the H locus. By gene targeting mediated by homologous recombination, several repeated sequences were introduced in the vicinity of *ptr1*. Selection for methotrexate resistance in these transfectants led to *ptr1* amplification as part of small circles with direct or inverted duplications whether the integrated sequences consisted of direct or inverted repeats. Hence, for a region to be amplified in *L. tarentolae* during drug selection, a drug resistance gene is required and must be flanked by (any) homologous repeated sequences. The distance between these repeats and their orientation will determine the length of the amplicon and whether it contains direct or inverted duplications.

Gene amplification is a common event in *Leishmania* spp. selected for drug resistance. Indeed, several loci on different chromosomes have been amplified in *Leishmania* cells selected for resistance to various drugs (reviewed in references 2 and 30). Amplicons in *Leishmania* spp. can be found either as extrachromosomal circles (reviewed in references 2, 27, and 41) or as linear molecules with telomeric sequences (14, 15, 25, 34, 47). Circular and linear amplicons contain either direct (head-to-tail) or inverted (head-to-head) repeats (3, 16, 19, 23, 29, 34, 43), two types of structures also found in extrachromosomal circular amplicons of tumor cells (reviewed in references 39 and 40). Despite the structural similarities between amplicons of tumor cells and those of *Leishmania* spp. (45), the exact mechanisms of their formation seem to differ. Amplicons are formed at the level of homologous sequences usually ranging from 200 to 1,200 bp in *Leishmania tarentolae* (14, 29), although smaller homologous sequences have also been proposed to be involved in amplicon formation in *Leishmania* spp. (20, 21) and even other mechanisms (26), yet to be defined precisely, may also account for amplicon formation. There is apparently little, if any, need for homologous sequences for the rearrangement of chromosomal sequences to yield circular amplicons in tumor cells (13, 40).

One locus frequently amplified in *Leishmania* spp. is the H locus, a stretch of DNA of about 40 kb that is part of an 800-kb chromosome (Fig. 1). H circles have been found in some wild-type cells (16, 36, 46), but they were also generated de novo from the H-locus chromosomal copy in different *Leishmania* species selected for resistance to several unrelated drugs (2, 27). The H circle found in wild-type cells contained two 30-kb inverted duplications separated by unique segments of 5 and 6 kb (16, 36, 46). To explain how such circles arise de novo from the chromosomal copy, White et al. (46) proposed that each genomic unique segment is flanked by inverted repeats. The annealing of the inverted repeats during a block in replication would serve as a primer for a polymerase to use the newly synthesized strand as a template to generate large inverted duplications (Fig. 1C). After ligation, the single-stranded circle could then replicate into a double-stranded circle. The mapping of amplicons generated after methotrexate (MTX) selection and cloning and sequencing of the rearrangement points were indeed in agreement with the model in which large inverted duplications were formed after annealing of the inverted repeats (29, 34). Selection for arsenite resistance also leads to H-locus amplification (11, 14, 29), but the mapped amplicons contained direct repeats and were generated by homologous recombination between directly repeated sequences (14, 29). In addition to having differences in structure, the regions amplified between MTX- and arsenite-selected cell lines differed considerably in size (Fig. 1A). Indeed, regions amplified in arsenite-resistant cell lines were always small (Fig. 1A), whereas in MTX mutants the regions amplified varied from 40 to >100 kb (Fig. 1A, lines I to III).

The genes on the H locus implicated in resistance have been isolated; a P-glycoprotein-related gene, *pgpA* (28), is involved in arsenite resistance (6, 31), and a short-chain dehydrogenase-reductase gene, *ptr1*, confers MTX resistance (7, 32, 33) by reducing pterins (1, 5). Directly repeated sequences, such as the nucleotide binding sites of *pgpA* and *pgpB* (29) (Fig. 1A) or the sequences B and E (14) (Fig. 1A), surround the *pgpA* gene. No directly repeated sequences seem to be present in the vicinity of *ptr1*, and the closest repetitions, the sequences indicated by A-B and C-D in Fig. 1A, are inverted. Therefore, the size and structure of an amplicon might be related to the presence of repeated sequences surrounding a resistance gene. This report describes how, by gene-targeting experiments, we determined experimentally the role of repeated sequences in the formation, length, and structure of amplicons in *Leishmania* cells selected for MTX resistance.

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MATERIALS AND METHODS

Cell lines and cultures. The *L. tarentolae* cell line TarIIWT has been described previously (45). Cell lines were grown in SDM-79 (46). Mutants derived from the

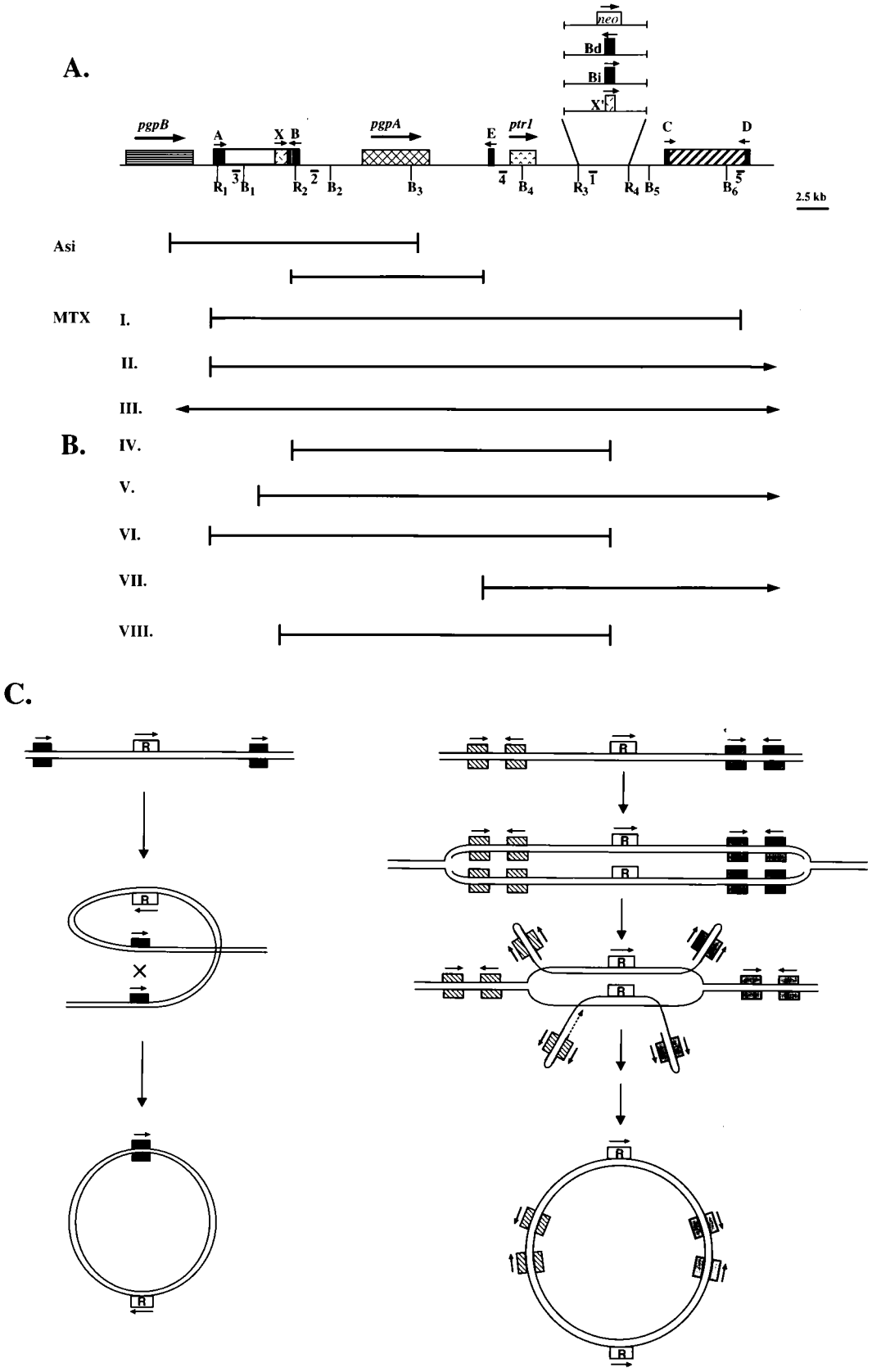

FIG. 1. The H locus of *L. tarentolae* and regions amplified after drug selection. (A) The H locus is delimited by the open and cross-hatched boxes flanked by inverted repeats A-B and C-D, respectively (29, 46). The drug resistance genes *pgpA* (6, 28, 31) and *ptr1* (1, 7, 32, 33) are indicated. The *pgpB* gene closely related to *pgpA* (18) is located just outside the H locus. The repeat E, homologous to the repeats A and B (14), and a single-copy sequence of unknown sequence named X are

also shown. The integration sites for *neo* or for the sequences Bd, Bi, and X′ are shown above the map. The *Eco*RI sites (R_1 to R_4) and the *Bam*HI sites (B_1 to B_6) used throughout this study are indicated. Not all *Eco*RI and *Bam*HI sites are shown. The small numbered bars (bars 1 to 5) below the map correspond to probes used. Bars below the map indicate the regions amplified in previously characterized arsenite (Asi)- and MTX-resistant mutants (14, 29, 34). (B) Bars indicate the regions amplified in wild-type or *neo*, Bd, Bi, and X′ transfectants selected for MTX resistance. A vertical line terminating the bars indicates that the rearrangement points were precisely mapped, whereas an arrow indicates that amplified sequences extend farther in the direction of the arrow. (C) Models for the generation of amplicons with direct or inverted repeats. The upper lines show the chromosomal copies from which the extrachromosomal elements are derived. The boxes with small arrows indicate direct or inverted repeats. Boxes with the letter R indicate a drug resistance gene. We postulated that circles with direct repeats (left) are formed by homologous recombination between direct repeated sequences. The mechanism of formation of circles with inverted duplications is shown on the right. The second diagram from the top on the right shows a replication bubble; following a putative block in replication, a partial displacement of the newly replicated DNA strand occurs. This would allow the inverted repeat to anneal and allow the polymerase to switch strands and synthesize another copy of the inverted duplication (dotted line with arrow). After ligation and a new round of replication, a circle with inverted duplications would be formed.

wild type or of the transfectants *neo*, Bd, Bi, and X′ were obtained by stepwise selection with 50, 100, 250, 500, and 1,000 μM MTX (ICN Biochemicals).

DNA manipulations. Genomic parasite DNA was prepared as described previously (29). Chromosomes in agarose blocks were resolved by transalternative field electrophoresis (TAFE) (Beckman) as described previously (14). Circular amplicons in *L. tarentolae* were isolated by standard alkaline lysis (35). Southern blotting, hybridization, and washing were done by standard protocols (38). All probes were labeled by random priming (12). The probes (Fig. 1 and 2) included a 0.5-kb *Eco*RV-*Eco*RI fragment covering a small portion of the B repeat and derived from pMAC4 (46) and a 1.3-kb *Pvu*II-*Pvu*II fragment containing the whole X repeat and also derived from pMAC4. The *ptr1* and *neo* probes containing the entire coding sequences were obtained by PCR. Finally, probes labeled 1 to 5 in Fig. 1A correspond, respectively, to a 500-bp *Eco*RI-*Eco*RV fragment derived from pMAC40, a 1-kb *Hin*dIII-*Eco*RV fragment derived from pMAC4, a 1-kb *Bam*HI-*Hin*dIII fragment derived from pMAC3 (46), an 850-bp *Bam*HI-*Apa*I fragment derived from pMAC40, and a 650-bp *Sac*I fragment derived from pMAC14 (46).

DNA constructs. A *neo* expression cassette (35) was cloned in a 5-kb *Eco*RV-*Eco*RI fragment located between the R_3 and R_4 sites shown in Fig. 1A. The B and X sequences, as 1.43-kb *Eco*RV-*Pvu*II and 1.3-kb *Pvu*II-*Pvu*II restriction fragments, respectively, were cloned into the unique *Sma*I site of the *neo* construct. Orientation of inserts was determined by restriction digest to yield the Bd, Bi, and X′ constructs.

Transfections. Wild-type *L. tarentolae* promastigotes were transfected by electroporation as reported previously (33). Selections were done with 40 μg of G418 (Gibco-BRL) per ml, and the cells were cloned on agar plates as described elsewhere (32).

RESULTS

Integration of repeats flanking the *ptr1* gene. Several repeated sequences are close to the *ptr1* gene, but none surrounds the gene (Fig. 1A). Repeats A and B are large inverted repeats of 1,241 bp that differ by 10-bp substitutions and a 96-bp deletion in repeat A (29). The E sequence is a direct repeat of 541 bp of sequence B with 22 mismatches (14). The entire sequence B (Fig. 2B and C), as part of a 1.43-kb fragment, was introduced by homologous recombination downstream of *ptr1* in both orientations (Fig. 1A). Upon integration of the construct depicted in Fig. 2B, the novel repeat Bd would be a direct repeat of B, whereas after integration of the construct shown in Fig. 2C, the extra repeat Bi would be an inverted repeat of sequence B (Fig. 1A). A single-copy sequence of 1.3 kb of unknown function and sequence but with no homology to the A, B, or E repeated sequences, here termed X, was also introduced downstream of the *ptr1* gene by using the construct shown in Fig. 2D to yield repeat X′, which is a direct repeat of sequence X (Fig. 1A). Constructs made for the integration of Bd, Bi, and X′ contained *neo* in order to be able to select for their integration into the H locus. As a

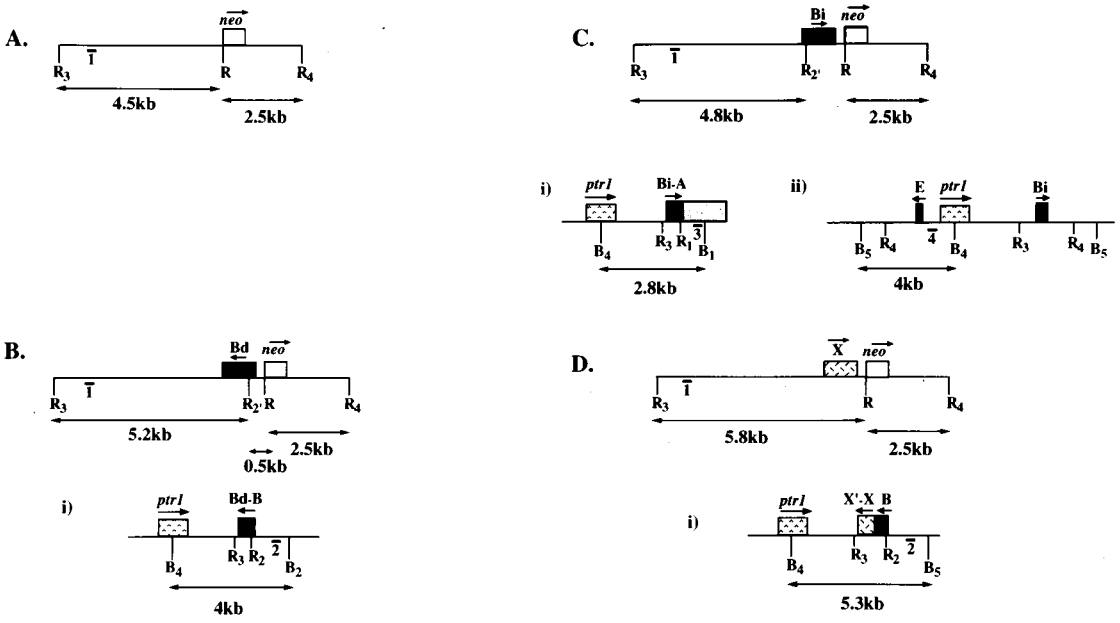

FIG. 2. Restriction maps of the *neo*, Bd, Bi, and X′ transfectants and of the recombination junction of their amplicons. Only the R_3-R_4 fragment of the H locus (Fig. 1A) is shown for each transfectant. Upon the integration of *neo*, Bd, Bi, and X′, novel *Eco*RI sites are introduced. The sizes of the *Eco*RI fragments expected after hybridization with probes 1 to 4 are shown below the maps. (A) Integration of the *neo* gene between the R_3 and R_4 *Eco*RI sites of the H locus. (B) Integration of the repeat B in direct orientation with its chromosomal copy (Bd); i), *Bam*HI rearranged fragment formed by homologous recombination between repeats B and Bd. (C) Integration of repeat B in an inverted orientation with its chromosomal copy (Bi); i), *Bam*HI rearranged fragment formed by homologous recombination between repeats A and Bi; ii), inverted duplications formed by annealing of repeats E and Bi. (D) Integration of the repeat X in direct orientation with its chromosomal copy (X′); i), *Bam*HI rearranged fragment formed by homologous recombination between repeats X and X′.

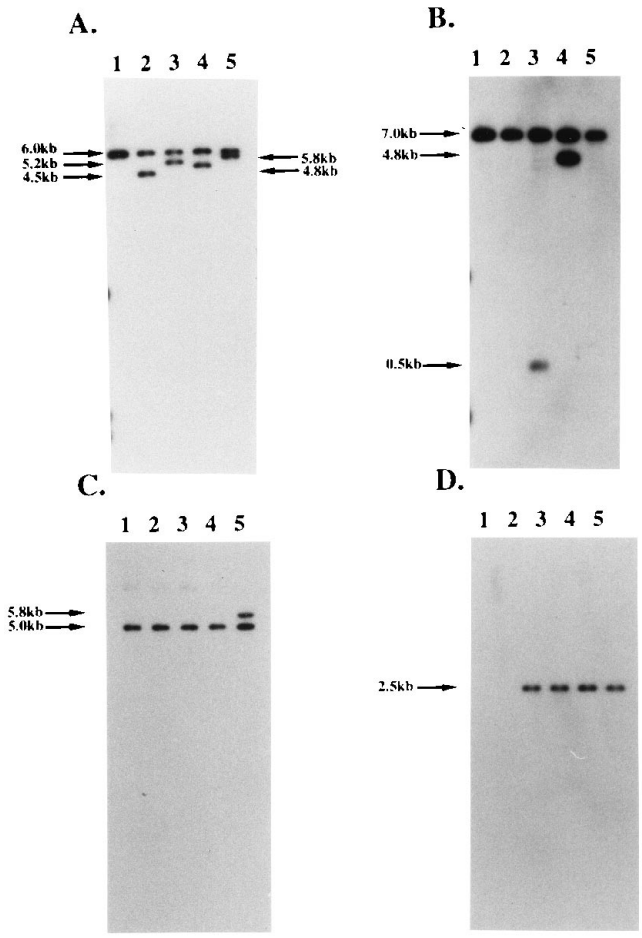

FIG. 3. Mapping of integration of novel repeated sequences downstream of the *ptr1* gene. Total DNAs of *L. tarentolae* TarII wild type and transfectants were digested with *Eco*RI, electrophoresed in a 0.7% agarose gel, and blotted and hybridized to probe 1 (A), to a probe derived from repeat B (B), to a probe derived from repeat X (C), and to a probe derived from the *neo* gene (D). Lanes 1, *L. tarentolae* TarII wild-type strain; 2, TarII*neo*; 3, TarIIBd; 4, TarIIBi; 5, TarIIX′. Molecular sizes were estimated from the 1-kb BRL ladder.

TABLE 1. Characterization of amplicons generated in *L. tarentolae* MTX-resistant mutants

Cells	Mutant no.[a]	MTX concn (μM)[b]	Type of amplicon[c]	Amplicon structure[d]	Nature of duplications[e]
Wild type	1–10	50	III	L	U
		1,000	III	L	U
neo	1–3, 5–10	50	III	L	U
	4	50	II	L	I
Bd	1, 3–5, 10	250	IV	C	D
	2, 6, 8	100	III	L	U
		500	IV	C	D
	7	100	IV	C	D
	9	500	V	C	U
Bi	1	50	VI	C	D
	2, 7	100	III	L	U
		1,000	III	L	U
	3, 8, 10	100	III	L	U
		500	VII	C	I
	4, 5, 6	100	III	L	U
		500	VI	C	D
	9	100	III	L	U
		500	I	C	I
X′	1, 7, 9, 10	100	III	L	U
		500	VIII	C	D
	2, 4, 8	100	III	L	U
		1,000	III	C	U
	3	250	VIII	C	D
	5, 6	250	III	L	U
		1,000	III	L	U

[a] The numbers 1 to 10 correspond to the 10 independent mutants derived from wild-type, *neo*, Bd, Bi, and X′ transfectants.
[b] Concentration of MTX at which *ptr1*-containing amplicons were studied.
[c] The roman numerals I to VIII refer to regions spanning the H locus amplified, as indicated in Fig. 1. The structure of amplicons was determined by Southern blot analysis.
[d] L, linear; C, circular.
[e] U, unknown; I, inverted; D, direct.

control, a construct was made (Fig. 2A) for the integration of the *neo* gene downstream of *ptr1*. Constructs shown in Fig. 2 were transfected in *L. tarentolae* by electroporation, and G418 (the selective drug for *neo*)-resistant transfectants were cloned and tested for the integration of *neo*, Bd, Bi, and X′. Genomic DNA of the wild type and transfectants was cut with *Eco*RI and hybridized to probes that allowed us to test whether the novel repeats had integrated properly. The wild-type *Eco*RI-*Eco*RI fragment (R_3 to R_4 in Fig. 1A) recognized by probe 1 is 6 kb (Fig. 3A, lane 1). Upon the integration of *neo*, Bd, Bi, and X′, the size of the *Eco*RI fragment of one allele recognized by probe 1 changed by the expected number of bases (Fig. 2; Fig. 3A, lanes 2 to 5). The 6-kb band hybridizing to probe 1, comigrating with wild-type sequences and present in all transfectants, is the remaining intact allele. The presence of the Bd and Bi repeats was confirmed by hybridization with a probe derived from the B sequence. Hybridizing bands of the expected sizes of 0.5 and 4.8 kb (Fig. 2B and C) were found in the Bd and Bi transfectants (Fig. 3B, lanes 3 and 4). The presence of the X′ repeat in the *Leishmania* genome was tested similarly with a probe derived from this repeat. A single 5.0-kb *Eco*RI band is present in wild-type cells and in transfectants, but an extra band of the expected size of 5.8 kb (Fig. 2D), corresponding to the extra repeat, also hybridized in the X′ transfectant (Fig. 3C, lane 5). Finally, the presence of the *neo* gene was confirmed by hybridization with a *neo* probe, which gave an expected 2.5-kb *Eco*RI-*Eco*RI fragment (Fig. 2) in the *neo*, Bd, Bi, and X′ transfectants (Fig. 3D, lanes 2 to 5), whereas no signal was observed in the wild-type strain (Fig. 3D, lane 1).

The results showed that transfectants with a modified H locus with novel repeats flanking the *ptr1* gene were now available. These transfectants were used to analyze the role of repeated sequences in determining the length and structure of amplicons. For this purpose, 10 independent mutants of the *neo*, Bd, Bi, and X′ transfectants, as well as of wild-type untransfected parasites, were selected for MTX resistance (see Materials and Methods). Amplification of the *ptr1* gene was monitored by hybridization experiments at every step of the selection.

Gene amplification in the control *neo* transfectants and wild-type cells. Ten *neo* transfectants without extra repeats were selected for MTX resistance. Amplification of the *ptr1* gene was observed with all 10 mutants after the first step of MTX selection at 50 μM (Table 1). Chromosome-sized TAFE blotted gels indicate that all 10 amplicons migrated like linear amplicons (Fig. 4A, lanes 2 and 3, and data not shown), which was further confirmed by their hybridization to a probe containing telomeric sequences (data not shown). Linear amplicons derived from the H locus have been described before (9, 25, 34). Mapping of the rearrangement points of the linear

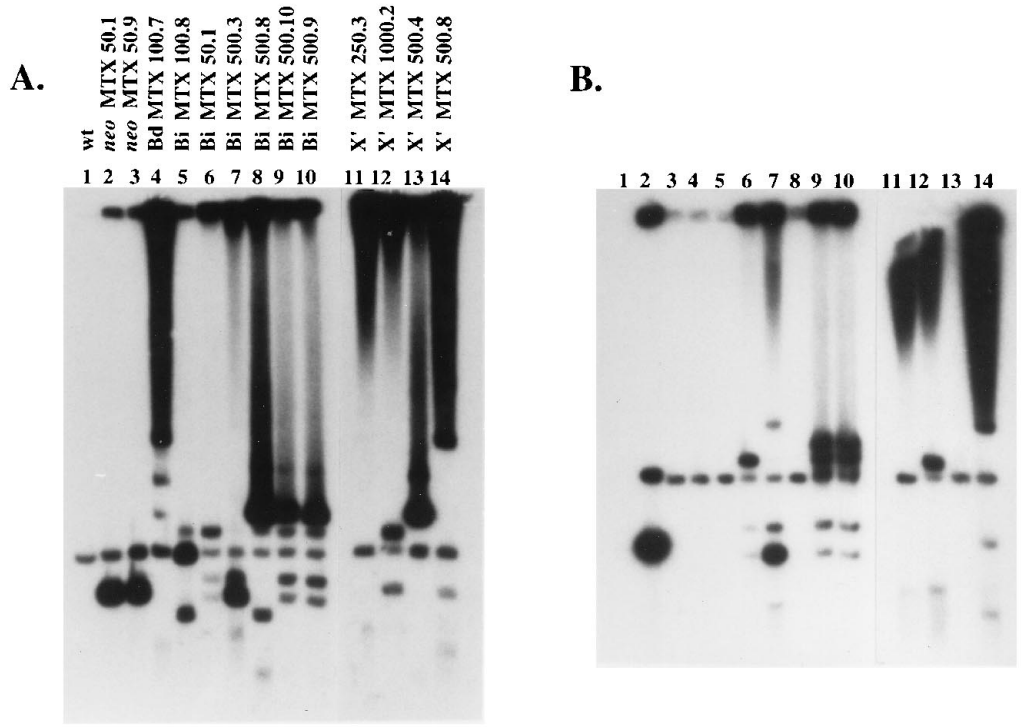

FIG. 4. Selection of amplicons in MTX-resistant mutants generated from the *neo*, Bd, Bi, and X′ transfectants (see also Table 1). Chromosomes were separated by TAFE, and Southern blots were hybridized to a probe derived from the *ptr1* gene (A) and to a probe derived from the *neo* gene (B). The content of each lane is indicated above panel A. wt, wild type.

amplicons with probes 3 and 5 (Fig. 1A) indicated that 9 of 10 contained amplified sequences larger than the H locus (Table 1), defined here as the region between the repeated sequences A and D (27, 45) (Fig. 1A). Analysis of Southern blots of the DNA of the remaining mutant *neo*MTX50.4, hybridized with probe 3, gave results consistent with a linear amplicon containing large inverted duplications formed by the annealing of the inverted repeats A and B (Table 1 and data not shown), a rearrangement observed previously to occur in several circular or linear amplicons (29, 34). The selective pressure was increased for two of these *neo* transfectants, but no circles were observed.

Only one allele of the H locus contains a *neo* marker (Fig. 3A, lane 2). This *neo*-tagging procedure, in combination with the large number of H-locus amplicons derived from *neo* transfectants, provided us with the opportunity to test whether one of the two alleles was preferentially amplified. Half of the linear H-locus-derived amplicons hybridized with *neo* (Fig. 4B, lanes 2 and 3, and data not shown), indicating that linear amplicons in *L. tarentolae* can be derived from both alleles.

In untransfected *L. tarentolae* MTX-resistant mutants analyzed previously (usually at a high drug concentration, after several steps of selection), amplicons were found predominantly in the form of circles (2, 29). The frequent generation of linear amplicons from the *neo* transfectants prompted us to check whether the integration of *neo* per se had changed the amplification of the locus. Ten wild-type *L. tarentolae* cells were selected in a stepwise manner for resistance to MTX. Amplification of the *ptr1* gene as part of a linear amplicon was observed with all 10 mutants after the first step of MTX selection at 50 μM (Fig. 5A). Therefore, the integration of *neo* into the H locus did not perturb in any way the amplification of the H locus after MTX selection. Moreover, all MTX-generated amplicons present in the wild type or the *neo* transfectants without extra repeats contained, in addition to *ptr1*, extensive coamplified sequences (Table 1), corroborating previous observations (29, 34). The selective pressure was increased to 1,000 μM MTX for all 10 mutants derived from the wild type, and the results indicated that the copy number of the linear amplicons was increased, but no circles were detected once cells were adapted to 1,000 μM MTX.

Formation of *ptr1*-containing amplicons with direct repeats. A DNA sequence homologous to sequence B was inserted downstream of *ptr1* (Fig. 1A and 3B). If the formation of amplicons requires only the presence of a drug resistance gene surrounded by repeated sequences, a *ptr1*-containing amplicon, generated after homologous recombination between sequences B and Bd (Fig. 1), should be expected after selection with MTX. Formation of *ptr1*-containing amplicons following recombination between sequences E and Bd could also be possible (Fig. 1). Ten independent Bd transfectants were selected for MTX resistance. Contrary to the situation with the wild-type or the *neo* transfectants, few *ptr1*-containing amplicons in the Bd mutants were linear (Table 1). Most were circles as determined by characteristic migrations (14, 34) on TAFE electrophoresis (Fig. 4A, lane 4, and data not shown) and by the ease of purifying them from *L. tarentolae* by standard plasmid preparation (35). Circles appeared in one mutant at only 100 μM MTX, but most were observed when cells were adapted to 250 μM MTX (Table 1). Mapping of the rearrangement points of the circular amplicons by Southern blot hybridization was consistent with the scenario in which circles were formed by homologous recombination between the B and Bd sequences in 9 of 10 mutants. An example is shown in Fig. 6A. Probe 2 recognized a 6-kb *Bam*HI-*Bam*HI fragment (B_1 to B_2 in Fig. 1A) in wild-type *L. tarentolae* (Fig. 6A, lane 1). A

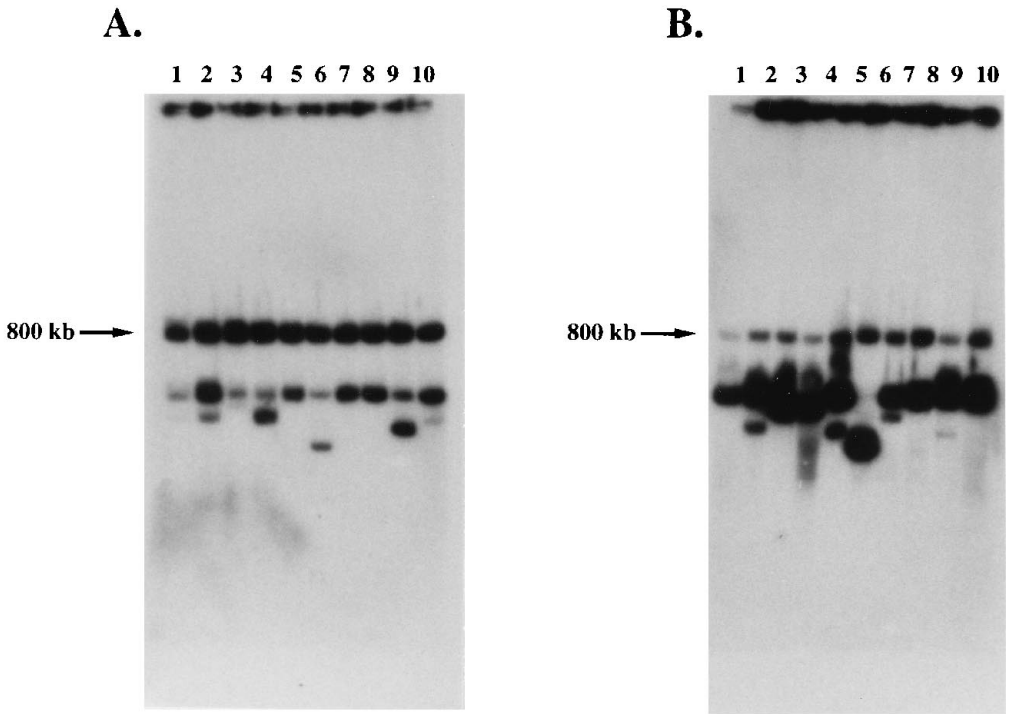

FIG. 5. Amplicons in 10 independent MTX-resistant mutants generated from the TarII wild type. Chromosomes were separated by TAFE, and Southern blots were hybridized to a *ptr1* probe. (A) Mutants resistant to 50 μM MTX; (B) mutants resistant to 1,000 μM MTX. The 800-kb band corresponds to the chromosomal copy of *ptr1*.

circular amplicon formed after the recombination between repeats B and Bd should yield a 4-kb *Bam*HI-*Bam*HI fragment hybridizing with probe 2 (Fig. 2B, panel i). This is exactly the size of the fragment observed in the purified circle isolated from the mutant BdMTX100.7 digested with *Bam*HI (Fig. 6A, lane 2). The intact circle migrates in agarose gels as a 60-kb circle (data not shown), indicating that the region amplified is repeated two times as a head-to-tail repeat. Only one amplicon, in mutant BdMTX500.9, was not rearranged between the B and Bd sequences, and it did not have any previously characterized rearrangement points (Table 1; Fig. 1B). It was not studied further. Apparently no amplicons were generated by homologous recombination between repeats E and Bd.

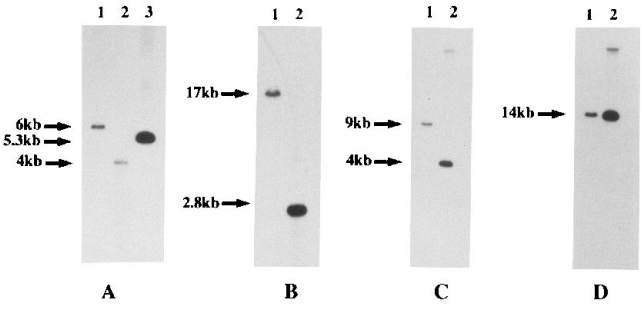

FIG. 6. Mapping the rearrangement points of the novel amplicons (see also Table 1). Total DNA was isolated from the *L. tarentolae* TarII wild-type strain, and circular DNA was isolated by the standard alkaline lysis procedure (35) from the mutants, which explains the absence of hybridization of the genomic fragment. All DNAs were digested with *Bam*HI, except for DNAs shown in panel D, which were digested with *Hin*dIII. Digested DNAs were electrophoresed in 0.7% agarose gels and hybridized to probe 2 to map the recombination between the repeats B and Bd and X and X′ (A); recombination between A and Bi was mapped with probe 3 (B); annealing of the inverted repeats E and Bi and that of C and D were tested by using probes 4 and 5, respectively (C and D). Lanes 1 (all panels), *L. tarentolae* TarII wild-type strain; lanes 2, BdMTX100.7 (A), BiMTX50.1 (B), and BiMTX500.8 (C and D); lane 3, X′MTX500.1. Molecular sizes were estimated from the 1-kb BRL ladder.

Formation of amplicons with inverted duplications. The insertion of Bi downstream of *ptr1* can theoretically yield several different types of amplicons after MTX selection. The Bi repeat is a direct repeat of sequence A but an inverted repeat of the B and E sequences (Fig. 1A). Therefore, amplicons formed by homologous recombination between A and Bi would contain head-to-tail repeats, whereas they should contain head-to-head repeats if the sequence Bi had annealed to sequence E or B. Ten Bi transfectants were selected for MTX resistance, and amplification of *ptr1* was observed to occur in all of them. At a low MTX concentration of 100 μM, only linear amplicons with large regions amplified (Fig. 4A, lane 5, and data not shown) were observed, except in mutant BiMTX50.1, where a circle was present (Fig. 4A, lane 6; Table 1). When the concentration of the selective drug was increased, circular amplicons, in addition to the already present linear ones, were formed in 8 of 10 Bi transfectants (Fig. 4A, lanes 7 to 10, and data not shown). When the selective pressure was kept, populations with the circles were selected and the linear amplicons were lost after a few passages (data not shown). Four of the mutants contained circular amplicons formed by homologous recombination between sequences A and Bi (Table 1). An example is shown in Fig. 6B. Hybridization with probe 3 (Fig. 1A) recognized a 17-kb *Bam*HI-*Bam*HI fragment in wild-type *L. tarentolae* (Fig. 6B, lane 1). The circle isolated from BiMTX50.1 was cut by *Bam*HI, and a 2.8-kb fragment, a size expected if the circle was formed by homologous recombination between sequences A and Bi, hybridized to probe 3 (Fig. 2C, panel i, and 6B, lane 2). Three mutants (BiMTX500.3, BiMTX500.8, and BiMTX500.10) contained circular amplicons (Fig. 4A, lanes 7

to 9) with inverted duplications formed by annealing of the E and Bi inverted-repeat sequences. The wild-type genomic *Bam*HI fragment (B_3 to B_4 in Fig. 1A) recognized by probe 4 is 9 kb (Fig. 6C, lane 1), whereas the isolated amplicon digested with the same enzyme yielded a 4-kb fragment (Fig. 6C, lane 2), a size consistent with the formation of inverted duplications following the annealing of E and Bi sequences (Fig. 2C, panel ii). To search for the second rearrangement point, the blot was rehybridized with probe 5 (Fig. 1A). A 14-kb *Hin*dIII fragment, which comigrates with genomic sequences, hybridized to probe 5 (Fig. 6D), indicating that the second rearrangement point was located farther downstream of the D sequence (Fig. 1B, structure VII). The final circular amplicon in BiMTX500.9 also contained inverted duplications, but its analysis by Southern blotting suggested that it was formed by annealing of sequence A with sequence B and of sequence C with sequence D (Table 1), just like the H circle found in *L. tarentolae* TarVIa wild-type cells (29, 46).

Usage of repeats is sequence independent. The results presented above indicate that the presence and orientation of repeated sequences determine the structure and length of the region amplified. However, the experiments were done with sequences already present in three copies (Fig. 1A) that are naturally used during H-locus amplification (14, 29, 34). To exclude the possibility that the B sequence acts as a specialized sequence for recombination during gene amplification, we have put a novel repeat, X′, downstream of *ptr1* in a direct orientation with the sequence X (Fig. 1A). X is normally single copy, has never been used during amplicon formation, and has no homology with B (Fig. 3B and C). The X-X′ repeats are about the same size as the B-Bd repeats (1.3 versus 1.4 kb) and are separated by similar distances, X and X′ being 1.3 kb farther apart. Ten independent X′ transfectants were selected for MTX resistance. With low-MTX-concentration selection, mainly linear amplicons were observed, similar to the situation with the Bi mutants, but when drug pressure was increased, circular amplicons containing *ptr1* were detected in 8 of the 10 X′ MTX-resistant mutants (Table 1). Five circular amplicons were created by homologous recombination between the X and X′ repeats (Table 1; Fig. 4A, lane 11). Indeed, a 5.3-kb *Bam*HI fragment derived from the circle hybridized to probe 2 (Fig. 6A, lane 3), exactly as predicted if the circle was formed by homologous recombination between X and X′ (Fig. 2D, panel i). The three remaining circular amplicons (Fig. 4A, lanes 12 to 14) derived from X′ transfectants selected for MTX resistance contained extensive genomic sequences coamplified with *ptr1* (Table 1).

DISCUSSION

Gene amplification models proposed after the characterization of rearrangement points in amplicons of drug-resistant *L. tarentolae* (14, 27, 29, 34) were tested experimentally. In the wild-type cells or the *neo* transfectants selected for MTX resistance, the regions coamplified with *ptr1* were always large, often representing more than 10% of the chromosome (29, 34) (Table 1). In contrast, when novel sequences were introduced by homologous recombination 3′ of the *ptr1* gene to create the direct repeats B-Bd, A-Bi, and X-X′, circles with small regions amplified were formed at the level of the novel repeats in MTX-selected mutants (Table 1). The regions amplified were repeated at least twice, as determined by the migration of several intact circles in agarose gel. The formation of dimers may be necessary for stability or replication of circles as small transfected plasmids always oligomerized when maintained stably in *Leishmania* cells (35). No specific sequences seem required for recombination, as similar numbers of circular amplicons (4 and 5 of 10) are generated by recombination between either repeats A and Bi or X and X′. The B and Bd repeats were used more frequently in the generation of circular amplicons (Table 1). This finding could be attributed to the longer stretches of homology in the B-Bd repeats. A 96-bp insertion in the Bi repeat is absent in sequence A (29), and the X-X′ repeat is slightly smaller (1.3 instead of 1.43 kb). The frequency of homologous recombination in yeast and mammalian cells is proportional to the length of homologous sequences (37, 42). The distance between the repeats, B and Bd being the closest together of the three pairs of repeats, might also influence the frequency of use. Finally, a combination of small differences in lengths of the repeats, in the level of homology (there is a 10-bp substitution between A and B), and in the distance between them might explain the differences noted.

No amplicons formed by homologous recombination between the E and Bd sequences were observed, although these are the closest together (Fig. 1A). The repeats are shorter (540 bp), with some mismatches (14), which could be sufficient reasons for their not being used at a high frequency. Another reason could be the absence of an origin of replication between the E and Bd repeats. The region located between the repeated sequence B and the second nucleotide binding site of *pgpA* (Fig. 1) is common to all amplicons derived from the H locus, and this region was suggested to contain an origin of replication (27). Origins have not yet been described for *Leishmania* spp., however. The recent demonstration that transfected bacterial vectors without any *Leishmania* DNA insert replicate well in *L. tarentolae* (32) raises the question of whether an origin of replication is required for selection of the event leading to the formation of extrachromosomal amplicons.

L. tarentolae cells selected for MTX resistance often contain large amplicons with head-to-head repeats (29, 34). Inverted duplications are common in amplicons found in tumor cells (reviewed in reference 13). We have added a repeat, Bi, 3′ of *ptr1* that would be an inverted repeat of sequences E and B (Fig. 1A). In three instances, amplicons with head-to-head repeats created at the level of Bi and E sequences were formed. The results represent, therefore, a strong confirmation of our model (29, 46), in which inverted duplications in *Leishmania* spp. are formed at the level of inverted repeats (Fig. 1C). We postulated previously that a pause in DNA replication would allow partial strand displacement within a replication bubble, which would then permit the inverted repeats to anneal. This would serve as a primer for a DNA polymerase and lead to strand switching. The newly synthesized strand would serve as a template to form the other copy of the inverted duplication (Fig. 1C). After ligation and synthesis of a complementary strand, a circle with two large inverted duplications would be formed. The putative events leading to blocks in DNA replication are yet unknown. Sequences in the vicinity of the repeats, or in the repeats, may form hairpins and cause a pause in replication. Computer analysis of the A and B repeats indeed indicated the presence of several putative hairpin structures, notably one spanning nucleotides 613 to 661 (according to the numbering used in reference 29). The use of MTX, which may unbalance the deoxynucleotide pools, may also contribute to replication pausing. The B and Bi repeats were not used for the formation of inverted duplications. This observation could be explained by the distance between the two repeats, which could be too large to permit annealing. A recent report indicated that the rate of recombination between inverted repeats is indeed strongly reduced when the distance

between the repeats is increased (3). A similar model was proposed for amplification of simian virus 40 sequences in CHO cells (10). In this case, however, the strand switching leading the polymerase to backtrack and continue replication on the newly synthesized strand is probably due to accidental annealing of nonhomologous sequences which could be stabilized by elongation (10). Inverted duplications are widespread in microbial systems as well, and the formation of head-to-head palindromes of rRNA genes in *Tetrahymena thermophila* was shown to require inverted repeats (48). The annealing of inverted repeats during replication is also proposed to yield inverted duplications of genetic elements in bacteria (24). As indicated before, we cannot exclude the possibility that other mechanisms are responsible for the formation of H circles (27, 29). An interesting variation to the foldback model proposed here is the reciprocal-strand-switching model, proposed to explain the formation of inverted duplications from inverted repeats in *Escherichia coli* (4). In this model, a reciprocal switching of the leading and lagging strands of replicating inverted repeats is suggested to occur, leading to the formation of a Holliday junction (4). This junction could be resolved by DNA endonuclease and ligase to lead to a circle with inverted duplications (4). There are no signs of strand switching, however, in the two recombination junctions of the H circle characterized in detail (30), and the distance between the H-locus inverted repeats (5 kb) would suggest that strand switching between the lagging and leading strands would be inefficient.

In previous studies (29, 34), amplicons in *L. tarentolae* cells highly resistant to MTX (1,000 μM) were studied. To study the role of repeated sequences in gene amplification in *L. tarentolae*, we have looked for amplicon formation at every step of MTX selection. We were surprised to note the high frequency of formation of linear amplicons during the first selection steps. With increased selective pressure, linear amplicons in the Bd, Bi, and X′ series were replaced by circles (Table 1). Circles were not observed in wild-type cells (Fig. 5B), indicating that circles are possibly more easily formed when homologous repeats, close to a drug resistance gene, are available. As circles have been detected in *L. tarentolae* in previous studies (29, 34), it is possible that circles would be formed at later passages. At this point it is not possible to determine whether the circles were formed from linear amplicons, but a recent study of *Leishmania tropica* also indicated that the H locus was amplified as part of linear amplicons at low MTX concentrations and circles followed at high MTX concentrations (25). A linear-precursor–circular-product relationship has recently been proposed for the LD1/CD1 locus of *Leishmania* spp. (23). Evidence has been provided that in tumor cells linear extrachromosomal elements could be intermediates in gene amplification (22) and transfection of linear plasmids in yeast cells can yield circles with inverted duplications (17). The advantage of passing through a linear intermediate is not clear but could be related to the type of chromosomal breaks induced which, in turn, could be related to the type of drugs used. Circles would be formed and selected because of their increased stability (34, 35) or simply because their copy number may be more easily increased than that of linear amplicons when selective pressure is increased. In the Bd mutant series, few linear amplicons were observed (Table 1), suggesting that they were either present in small amounts, short-lived, or simply not formed. Some overexposed blots suggested that linear amplicons (less than 1/100 cells) may have indeed been present in additional Bd mutants.

In conclusion, for a region to be amplified after drug selection in *L. tarentolae*, repeated homologous sequences on each side of a drug resistance gene are required. We have proven experimentally that when repeated sequences are direct or inverted repeats, amplicons with head-to-tail or head-to-head repeats can be formed, respectively. Future work will concentrate on the parameters influencing the frequency of usage of repeats, the possibility that circles are formed from linear precursors, and finally whether sequences are required for replication, as for amplicons in mammalian cells (8, 44).

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REFERENCES

1. **Bello, A. R., B. Nare, D. Freedman, L. Hardy, and S. M. Beverley.** 1994. PTR1: a reductase mediating salvage of oxidized pteridines and methotrexate resistance in the protozoan parasite *Leishmania major*. Proc. Natl. Acad. Sci. USA **91:**11442–11446.
2. **Beverley, S. M.** 1991. Gene amplification in *Leishmania*. Annu. Rev. Microbiol. **45:**417–444.
3. **Beverley, S. M., J. A. Coderre, D. V. Santi, and R. T. Schimke.** 1984. Unstable DNA amplifications in methotrexate-resistant *Leishmania* consist of extrachromosomal circles which relocalize during stabilization. Cell **38:**431–439.
4. **Bi, X., and L. F. Liu.** 1996. DNA rearrangement mediated by inverted repeats. Proc. Natl. Acad. Sci. USA **93:**819–823.
5. **Borst, P., and M. Ouellette.** 1995. New mechanisms of drug resistance in parasitic protozoa. Annu. Rev. Microbiol. **49:**427–460.
6. **Callahan, H. L., and S. M. Beverley.** 1991. Heavy metal resistance: a new role for P-glycoproteins in *Leishmania*. J. Biol. Chem. **266:**18427–18430.
7. **Callahan, H. L., and S. M. Beverley.** 1992. A member of the aldoketo reductase family confers methotrexate resistance in *Leishmania*. J. Biol. Chem. **267:**24165–24168.
8. **Carroll, S. M., J. Trotter, and G. M. Wahl.** 1991. Replication timing control can be maintained in extrachromosomally amplified genes. Mol. Cell. Biol. **11:**4779–4785.
9. **Chiquero, M. J., A. Olmo, P. Navarro, L. M. Ruiz-Perez, S. Castanys, D. Gonzalez-Pacanowska, and F. Gamarro.** 1994. Amplification of the H locus in *Leishmania infantum*. Biochim. Biophys. Acta **1227:**188–194.
10. **Cohen, S., D. Hassin, S. Karby, and S. Lavi.** 1994. Hairpin structures are the primary amplification products: a novel mechanism for generation of inverted repeats during gene amplification. Mol. Cell. Biol. **14:**7782–7791.
11. **Detke, S., K. Katakura, and K.-P. Chang.** 1989. DNA amplification in arsenite-resistant *Leishmania*. Exp. Cell Res. **180:**161–170.
12. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
13. **Fried, M., S. Feo, and E. Heard.** 1991. The role of inverted duplications in the generation of gene amplification in mammalian cells. Biochim. Biophys. Acta **1090:**143–155.
14. **Grondin, K., B. Papadopoulou, and M. Ouellette.** 1993. Homologous recombination between direct repeat sequences yields P-glycoprotein containing circular amplicons in arsenite resistant *Leishmania*. Nucleic Acids Res. **21:**1895–1901.
15. **Hanson, S., S. M. Beverley, W. Wagner, and B. Ullman.** 1992. Unstable amplification of two extrachromosomal elements in α-difluoromethylornithine-resistant *Leishmania donovani*. Mol. Cell. Biol. **12:**5499–5507.
16. **Hightower, R. C., L. M. Ruiz-Perez, M. Lie Wong, and D. V. Santi.** 1988. Extrachromosomal element in the lower eukaryote *Leishmania*. J. Biol. Chem. **263:**16970–16976.
17. **Kunes, S., D. Botstein, and M. S. Fox.** 1990. Synapsis-mediated fusion of free DNA ends forms inverted dimer plasmids in yeast. Genetics **124:**67–80.
18. **Légaré, D., E. Hettema, and M. Ouellette.** 1994. The P-glycoprotein related gene family in *Leishmania*. Mol. Biochem. Parasitol. **68:**81–91.
19. **Liu, J., N. Gajendran, D. Muthui, S. Muyldermans, J.-C. Dujardin, S. DeDonker, D. Jacquet, D. Le Ray, F. Mathieu-Daudé, and R. Hamers.** 1991. Chromosome rearrangement in *Leishmania mexicana* M379. Mol. Biochem. Parasitol. **46:**53–60.
20. **Liu, J., G. Salinas, N. Gajendran, D. Muthui, S. Muyldermans, and R. Hamers.** 1992. DNA recombination associated with short direct repeats in *Leishmania mexicana* M379. Mol. Biochem. Parasitol. **50:**351–354.
21. **Lodes, M. J., G. Merlin, T. de Vos, A. Ghosh, R. Madhubala, P. J. Myler, and K. Stuart.** 1995. Increased expression of LD1 genes transcribed by RNA

polymerase I in *Leishmania donovani* as a result of duplication into the rRNA gene locus. Mol. Cell. Biol. **15:**6845–6853.
22. **Meese, E. U., S. D. Horwitz, and J. M. Trent.** 1992. Evidence for linear extrachromosomal elements mediating gene amplification in the multidrug-resistant J774.2 murine cell line. Cancer Genet. Cytogenet. **59:**20–25.
23. **Navarro, M., J. Liu, D. Muthui, D. Ortiz, M. Segovia, and R. Hamers.** 1994. Inverted repeat structure and homologous sequences in the LD1 amplicons of *Leishmania* spp. Mol. Biochem. Parasitol. **68:**69–80.
24. **Ohshima, A., S. Inouye, and M. Inouye.** 1992. *In vivo* duplication of genetic elements by the formation of stem-loop DNA without an RNA intermediate. Proc. Natl. Acad. Sci. USA **89:**1016–1020.
25. **Olmo, A., R. Arrebola, V. Bernier, D. Gonzalez-Pacanowska, and M. Ruiz-Pérez.** 1995. Co-existence of circular and multiple linear amplicons in methotrexate resistant Leishmania. Nucleic Acids Res. **23:**2856–2864.
26. **Ortiz, G., M. Navarro, and M. Segovia.** 1995. Location in the source chromosome of the 180 kb minichromosome of Leishmania major and characterization of the novel junction. Mol. Biochem. Parasitol. **71:**153–161.
27. **Ouellette, M., and P. Borst.** 1991. Drug resistance and P-glycoprotein gene amplification in the protozoan parasite *Leishmania*. Res. Microbiol. **142:** 737–746.
28. **Ouellette, M., F. Fase-Fowler, and P. Borst.** 1990. The amplified H circle of methotrexate-resistant *Leishmania tarentolae* contains a novel P-glycoprotein gene. EMBO J. **9:**1027–1033.
29. **Ouellette, M., E. Hettema, D. Wust, F. Fase-Fowler, and P. Borst.** 1991. Direct and inverted repeats associated with P-glycoprotein gene amplification in drug resistant *Leishmania*. EMBO J. **10:**1009–1016.
30. **Ouellette, M., and B. Papadopoulou.** 1993. Mechanisms of drug resistance in *Leishmania*. Parasitol. Today **9:**150–153.
31. **Papadopoulou, B., G. Roy, S. Dey, B. P. Rosen, and M. Ouellette.** 1994. Contribution of the *Leishmania* P-glycoprotein related gene ltpgpA to oxyanion resistance. J. Biol. Chem. **269:**11980–11986.
32. **Papadopoulou, B., G. Roy, W. Mourad, E. Leblanc, and M. Ouellette.** 1994. Changes in folate and pterin metabolism after disruption of the *Leishmania* H locus short-chain dehydrogenase gene. J. Biol. Chem. **269:**7310–7315.
33. **Papadopoulou, B., G. Roy, and M. Ouellette.** 1992. A novel antifolate resistance gene on the amplified H circle of *Leishmania*. EMBO J. **11:**3601–3608.
34. **Papadopoulou, B., G. Roy, and M. Ouellette.** 1993. Frequent amplification of a short chain dehydrogenase gene as part of circular and linear amplicons in methotrexate resistant *Leishmania*. Nucleic Acids Res. **21:**4305–4312.
35. **Papadopoulou, B., G. Roy, and M. Ouellette.** 1994. Autonomous replication of bacterial DNA plasmid oligomers in *Leishmania*. Mol. Biochem. Parasitol. **65:**39–49.
36. **Petrillo-Peixoto, M. L., and S. M. Beverley.** 1988. 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*. Mol. Cell. Biol. **8:**5188–5199.
37. **Rothstein, R.** 1991. Targeting, disruption, replacement and allele rescue: integrative DNA transformation in yeast. Methods Enzymol. **194:**281–301.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Schimke, R. T.** 1988. Gene amplification in cultured cells. J. Biol. Chem. **263:**5989–5992.
40. **Stark, G. R., M. Debatisse, E. Giulotto, and G. M. Wahl.** 1989. Recent progress in understanding mechanisms of mammalian DNA amplification. Cell **57:**901–908.
41. **Stuart, K. D.** 1991. Circular and linear multicopy DNAs in *Leishmania*. Parasitol. Today **7:**158–159.
42. **Thomas, K. R., and M. R. Capecchi.** 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell **51:**503–512.
43. **Tripp, C. A., P. J. Myler, and K. D. Stuart.** 1991. A DNA sequence (LD1) which occurs in several genomic organizations in *Leishmania*. Mol. Biochem. Parasitol. **47:**151–160.
44. **Vaughn, J. P., P. A. Dijkwel, and J. L. Hamlin.** 1990. Replication initiates in a broad zone in the amplified CHO dihydrofolate reductase domain. Cell **61:**1075–1087.
45. **Wahl, G. M.** 1989. The importance of circular DNA in mammalian gene amplification. Cancer Res. **49:**1333–1340.
46. **White, T. C., F. Fase-Fowler, H. van Luenen, J. Calafat, and P. Borst.** 1988. The H circles of *Leishmania tarentolae* are a unique amplifiable system of oligomeric DNAs associated with drug resistance. J. Biol. Chem. **263:**16977–16983.
47. **Wilson, K., S. M. Beverley, and B. Ullman.** 1992. Stable amplification of a linear extrachromosomal DNA in mycophenolic acid-resistant *Leishmania donovani*. Mol. Biochem. Parasitol. **55:**197–206.
48. **Yasuda, L. E., and M.-C. Yao.** 1991. Short inverted repeats at a free end signal large DNA palindrome formation in Tetrahymena. Cell **67:**505–516.