The EMBO Journal vol.10 no.4 pp.1009 - 1016, 1991

Direct and inverted DNA repeats associated with P-glycoprotein gene amplification in drug resistant *Leishmania*

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Communicated by P.Borst

The H circle of *Leishmania* species contains a 30 kb inverted duplication separated by two unique DNA segments, *a* and *b*. The corresponding H region of chromosomal DNA has only one copy of the duplicated DNA. We show here that the chromosomal segments *a* and *b* are flanked by inverted repeats (198 and 1241 bp) and we discuss how these repeats could lead to formation of H circles from chromosomal DNA. Selection of *Leishmania tarentolae* for methotrexate resistance indeed resulted in the *de novo* formation of circles with long inverted duplications, but two mutants selected for arsenite resistance contained new H region plasmids without such duplications. One of these plasmids appears due to a homologous recombination between two P-glycoprotein genes with a high degree of sequence homology. Our results show how the same DNA region in *Leishmania* may be amplified to give plasmids with or without long inverted duplications and apparently by different mechanisms.

Key words: gene amplification/homologous recombination/inverted repeats/*Leishmania*/P-glycoprotein

Introduction

In the protozoan parasite *Leishmania*, resistance to the dihydrofolate reductase (DHFR) inhibitor methotrexate (MTX) may be accompanied by the amplification of two unrelated DNA regions as extrachromosomal circles, the R and H circles (Coderre *et al.*, 1983). The R circle encodes the bifunctional enzyme DHFR-thymidylate synthetase and has arisen by the circularization of a contiguous stretch of chromosomal DNA (Beverley *et al.*, 1984). The H circle is more complex (Figure 1a) and contains a 30 kb segment in two orientations; the two parts of this inverted duplication are separated by unique DNA segments. The chromosomal H region contains only one copy of the duplicated segment and therefore the circles cannot be formed by simple excision of the chromosomal copy.

The H circle has also been found in wild-type cells of *Leishmania* and its amplification has been noted in several different *Leishmania* species, either selected for resistance to MTX (Hightower *et al.*, 1988; Petrillo-Peixoto and Beverley, 1988; White *et al.*, 1988), or for resistance to other unrelated drugs (Beverley *et al.*, 1988; Ellenberger and Beverley, 1989; Katakura and Chang, 1989; this paper). The relation between the H circle and drug resistance is not yet clear. The only gene found in the H circle thus far appears to encode a P-glycoprotein (Ouellette *et al.*, 1990). P-glycoproteins are large plasma membrane proteins that can extrude hydrophobic drugs from mammalian cells (see Endicott and Ling, 1989; Van der Bliek and Borst, 1989) and probably from protozoa like *Plasmodium* (see Foote *et al.*, 1990; Wellems *et al.*, 1990). P-glycoproteins have never been implicated in resistance against a hydrophilic drug, such as MTX, however.

In this paper we analyse two aspects of *Leishmania* H circle formation in more detail. First, we test whether H circles can arise *de novo* in a *Leishmania tarentolae* strain, previously shown to be devoid of H circles. As many wild-type *Leishmania* strains contain H circles (Hightower *et al.*, 1988; Petrillo-Peixoto and Beverley, 1988; White *et al.*, 1988), it remained possible that all H circles were originally derived from a single unusual event. We show here that the appearance of H type circles can indeed accompany selection for MTX resistance, but that selection for arsenite resistance unexpectedly leads to appearance of novel circles which contain part of the H region but without long inverted repeats.

If H type circles can arise *de novo*, a second aspect of H circle formation becomes of interest. What is the mechanism of circle formation and are there specific features of the chromosomal H region that promote DNA amplification? A speculative model for H circle formation was proposed by White *et al.* (1988) and is schematically summarized in Figure 1b. A key prediction of this model is that the inverted repeats (IRs) of the H circles are already present in the chromosomal copy of the H region, i.e. the unique segments *a* (hatched rectangle) and *b* (dotted rectangle) of the H region should be flanked by terminal IRs as indicated by the solid black blocks in Figure 1a. We have verified this prediction.

Results

Both unique segments of the H circle contain terminal inverted repeats

To test the model of Figure 1b, we used probes overlying the termini of the unique segments *a* and *b* (probes 1 and 3 in Figure 1a) and hybridized these to DNA from strain TarII of *L.tarentolae*, which lacks H circles (White *et al.*, 1988). Figure 2 shows that probes 1 and 3 indeed detect more than one band in restriction digests of TarII DNA, whereas the control probes 2 and 4, derived from the middle of the unique segments, detect only one band. This was confirmed by other digests. Fine-structure mapping confirmed that probe 1 hybridized to segments A and B in Figure 1a (upper line, chromosomal copy of the H region),

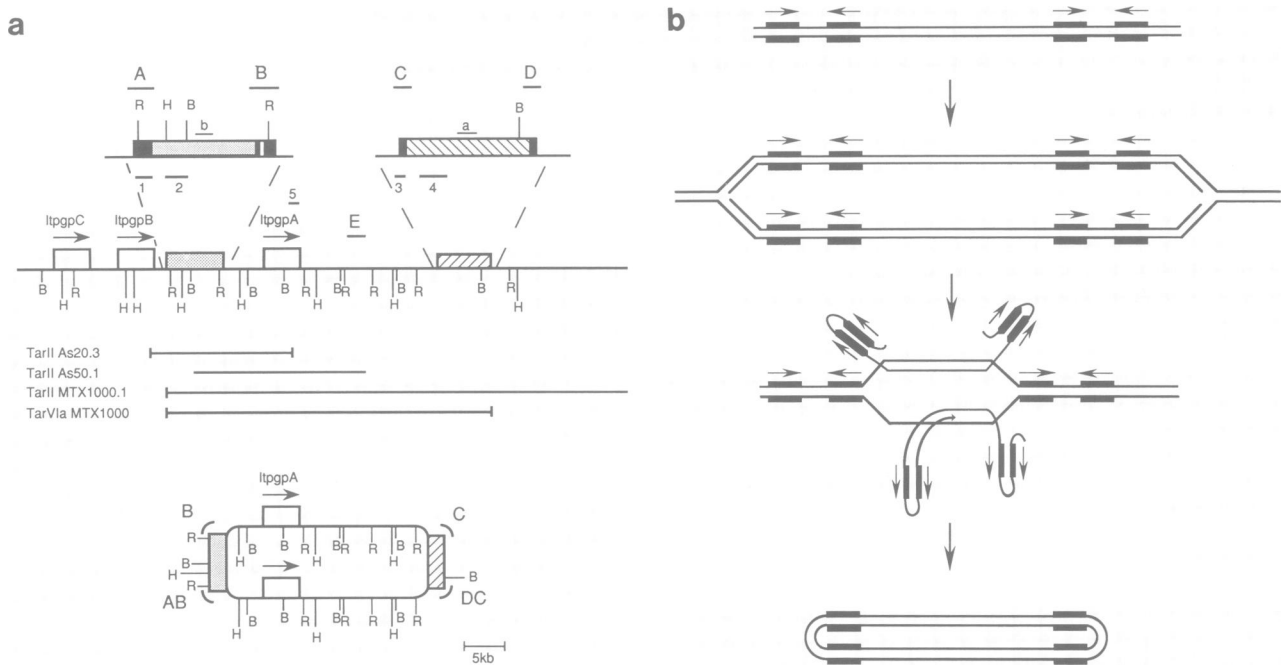

Fig. 1. (**a**) Map of the H locus and regions amplified in wild-type and drug resistant mutants. The map of the H locus, defined here as the chromosomal region present in the 68 kb H circles indicated in the lower part of the figure is from White *et al.* (1988). The unique segments are indicated by the dotted (*b*) and cross-hatched (*a*) rectangles. The P-glycoprotein gene *ltpgpA*, present in the H circle, is described by Ouellette *et al.* (1990). The map of flanking chromosomal sequences was made with overlapping inserts in lambda phages derived from a genomic library of *L.tarentolae* TarII (see Materials and methods). The two new P-glycoprotein genes, *ltpgpB* and *ltpgpC*, have not yet been characterized extensively, but they both have duplicated nucleotide binding sites separated by a distance comparable to that in *ltpgpA* (unpublished). It is therefore likely that *ltpgpB* and *ltpgpC* are genuine P-glycoprotein genes. The bars with numbers (1–5) correspond to the probes used and the bars with capital letters (A–E, AB and DC) indicate schematically the regions sequenced. The black boxes are the terminal IRs. Bars below the map indicate the respective regions amplified in the drug resistant mutants. A vertical line interrupting the bars indicates that the rearrangement points were precisely mapped. B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI. (**b**) Speculative model for the formation of the H circle from the chromosomal H region (after White *et al.*, 1988). The upper line shows the chromosomal copy from which the H circle is thought to be derived. The black boxes marked by arrows indicate short inverted repeats (IRs). The second panel shows a DNA replication 'bubble' formed by bi-directional replication. A block in DNA replication is postulated in the third panel to result in displacement of newly replicated single-stranded DNA. If DNA replication is inhibited the newly synthesized DNA could (partially) be displaced. This would allow the IRs to anneal (after trimming of excess single-stranded DNA) to serve as primer for a DNA polymerase to synthesize another copy of the inverted duplication. After ligation and synthesis of a complementary strand, an H circle would be generated.

whereas probe 3 only hybridized to segments C and D. Probe 1 detected an additional copy of the repeat mapped at position E in Figure 1a. Probes 1 and 3 did not cross-hybridize and no other copies of these repeats were detected in total nuclear DNA, even at relaxed stringency (65°C, 3 × SSC; 1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7).

To further characterize the IRs, the fragments labelled A, B, C and D (see Figure 1a) were cloned and sequenced (see Materials and methods). Figure 3 shows that unique segment *a* is flanked by an almost perfect IR of 198 bp with 7 bp substitutions and a one nucleotide deletion. The unique segment *b* is flanked by a much longer IR of 1241 bp that differs by 10 bp substitutions and a 96 bp deletion in A (Figure 4). This long IR contains at least one open reading frame of >300 amino acids, not interrupted by the 96 bp deletion. No significant homology was noted between the IRs flanking the two unique segments *a* and *b* and the IRs did not share any similarity with sequences present in data banks (GenBank release 63.0, EMBL-modified release 22.0, NBRF-protein 23.0). The long open reading frames may not correspond to genes as the high G+C content of *Leishmania* DNA results in a low frequency of stop codons.

Figures 3 and 4 show that the new junctions created by the formation of the circle found in strain TarVIa occur exactly at the IRs: junctions 'B' and 'C' are identical to

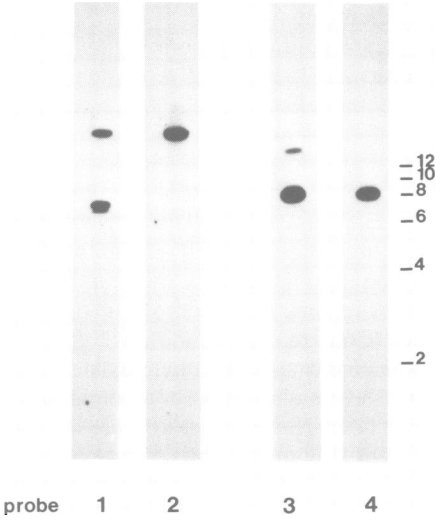

Fig. 2. The unique segments of the H locus are flanked by repeats. Total DNA of wild-type *Leishmania* TarII was digested with *Bam*HI, electrophoresed in an 0.5% agarose gel, blotted and hybridized to probes 1–4 (see Figure 1a). The difference in hybridization intensity of the fragments recognized by probe 3 is due to the fact that part of that probe is outside the IR. The mol. wt marker is the 1 kb BRL ladder.

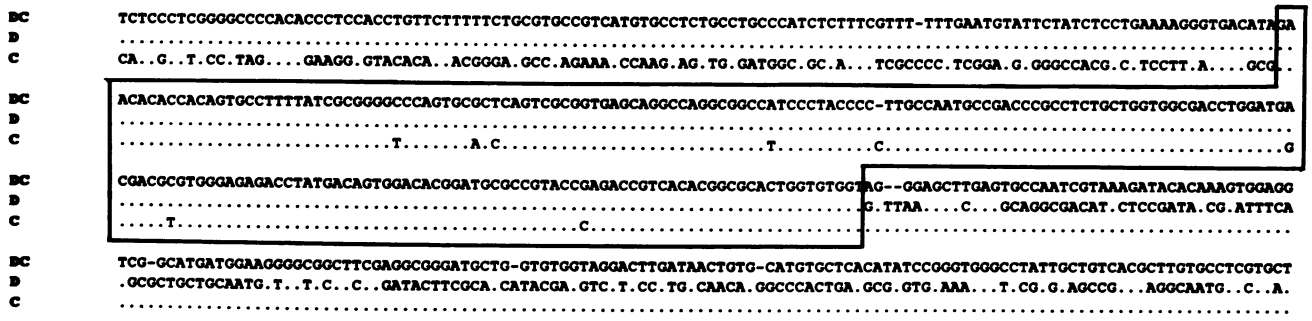

Fig. 3. The nucleotide sequence of the inverted repeats flanking unique segment *a* and of the novel junction DC. The nucleotide sequences of regions DC, D and C (see Figure 1a) were determined. The sequences are listed so that they all start in the unique region and then extend either into the new inverted duplication generated during the formation of the circle (DC), into the flanking chromosomal sequences (D), or into the H locus (C). Identities with the DC sequences are shown by dots. Gaps (dashes) have been introduced to optimize alignment. The region of the IR is boxed.

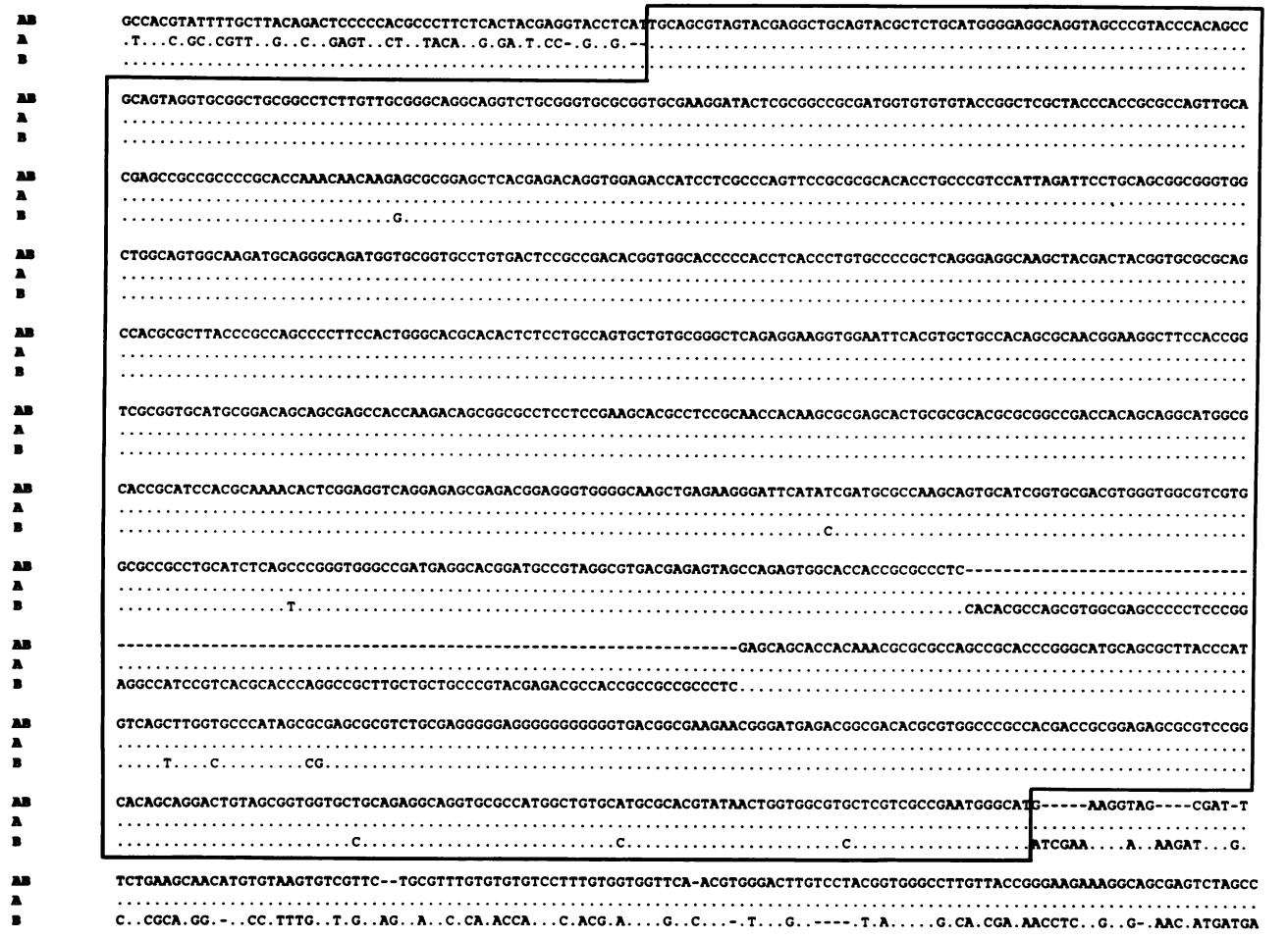

Fig. 4. The nucleotide sequence of the inverted repeats flanking unique segment *b* and of novel junction AB. The nucleotide sequences of regions AB, A and B (see Figure 1a) were determined. The sequences are listed so that they all converge in the unique region. The sequences start either in the new inverted duplication created during the formation of the circle (AB), in the flanking chromosomal sequences (A), or in the H locus (B). Identities to the AB sequence are indicated by dots. Gaps (dashes) have been introduced to optimize alignment. The region of the IR is boxed.

junctions 'AB' and 'DC', respectively. This confirms one major prediction of the model in Figure 1b.

Rearrangement at one of the inverted repeats in a circle generated do novo using MTX selection

L.tarentolae strain TarII has been shown to be free of detectable H circles (White *et al.*, 1988). Using stepwise selection with MTX we have succeeded in generating H circles *de novo* from the chromosomal H region in four independent experiments (twice from cloned populations). In all these mutants the chromosomal copies of the H region were retained, as judged by hybridization intensity of blots of chromosome sized fractionated DNA (not shown). In three mutants the level of amplification proved to be low and the circles generated were lost during continued growth on MTX, complicating their analysis. In one mutant however, TarII MTX1000.1, a relatively high copy number of the circle was stably maintained and we have mapped the

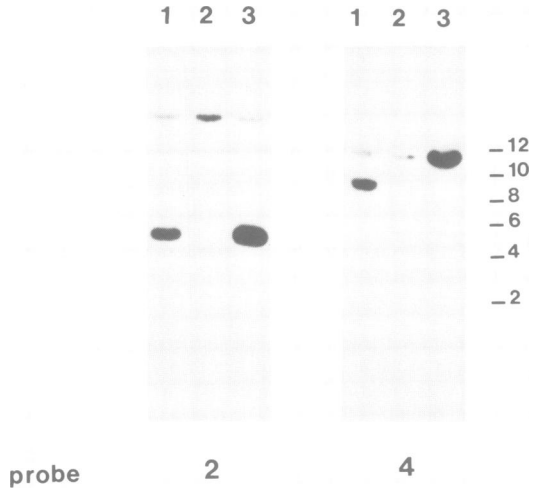

Fig. 5. Characterization of a circle generated *de novo* in the MTX resistant mutant TarII 1000.1. DNAs of *Leishmania* cells were digested with *Bam*HI (**left** panel) or *Eco*RI (**right** panel), electrophoresed through an agarose gel, blotted and hybridized to probes 2 and 4 (see Figure 1) respectively. 1, *L.tarentolae* TarVIa wild-type; 2, *L.tarentolae* TarII wild-type; 3, *L.tarentolae* TarII 1000.1. The mol. wt marker is the 1 kb BRL ladder.

rearrangement points in this circle. One rearrangement point was at the border of the unique segment *b*, at a similar position as in the circle present in wild-type strain TarVIa (Figure 5). The newly formed junction was cloned and sequenced and found to be identical to the junction (AB) of the H circle in strain TarVIa. The other inversion point in the circle of mutant TarII MTX1000.1 was not at the same position as in the circle in strain TarVIa, but the region amplified extended further into the flanking chromosomal sequences (Figures 1a and 5). We have not yet cloned the new rearrangement point, but in transverse alternating field electrophoresis gels, the circle linearized by γ-irradiation (Van der Bliek *et al.*, 1988) was estimated to be 125 kb (not shown) making it substantially larger than the monomeric circle found in TarVIa cells (68 kb).

Formation of circles without long inverted duplications

H circle amplification has been observed in several *Leishmania* species selected for resistance to drugs other than MTX (Ellenberger and Beverley, 1989; Katakura and Chang, 1989). We have used one of these drugs, arsenite, to test whether resistance in this case can also be accompanied by *de novo* H circle formation. In two out of three independent attempts with *L.tarentolae* strain TarII, stepwise arsenite selection resulted in extrachromosomal amplification of *ltpgpA*, the P-glycoprotein gene present in the H locus (Ouellette *et al.*, 1990). The two arsenite generated circles were different and unexpectedly lacked rearranged long inverted duplications. The circle present in mutant TarII As20.3 was analysed in detail by hybridization with H circle probes and found to contain a single stretch of 17 kb of chromosomal DNA covering the unique segment *b* up to *ltpgpA* (results not shown). The size of the circle was estimated, by γ-irradiation, to be 34 kb and by electron microscopy to be 33 kb, indicating that the circle must contain two direct repeats of the 17 kb stretch (unpublished results). We infer that this circle was formed by homologous

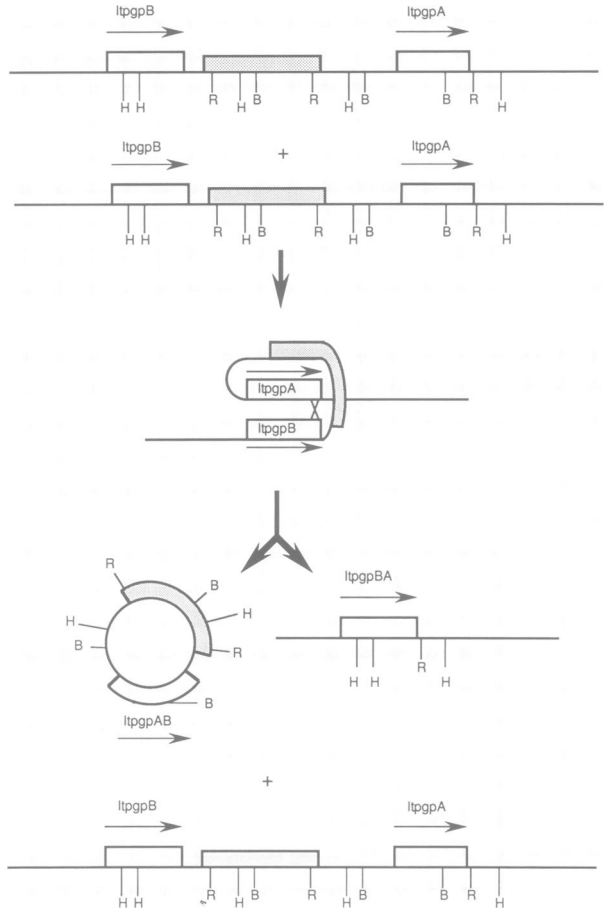

Fig. 6. Interpretative model for the generation of the extrachromosomal DNA circle found in mutant As20.3. The map is a schematic version of the left-hand part of the H region map in Figure 1a. The As20.3 17 kb monomer circle is shown to be generated by a homologous recombination between *ltpgpB* and *A*, resulting in a deleted chromosomal allele. The restriction junction fragments shown in Figure 7 are indicated.

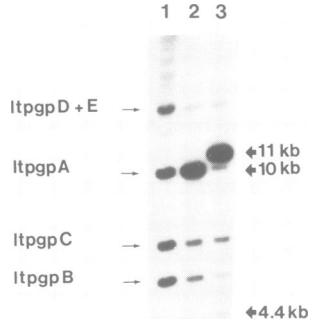

Fig. 7. DNA amplification in arsenite resistant mutants. Total DNA of *Leishmania* strains was digested with *Hin*dIII, electrophoresed in a 0.4% agarose gel, blotted and hybridized to probe 5 (see Figure 1a). This probe covers the second nucleotide binding site of *ltpgpA* and recognizes five different P-glycoprotein genes *ltpgpA*, *B*, *C*, *D* and *E* (see Materials and methods). 1, *L.tarentolae* TarII wild-type; 2, *L.tarentolae* TarII As50.1; 3, *L.tarentolae* TarII As20.3. Mol. wts were estimated from the 1 kb BRL ladder.

intrachromosomal recombination between *ltpgpA* and a second P-glycoprotein gene, *ltpgpB*, with loss of the corresponding region of part of the H locus (Figure 6). One of the blots supporting that scenario is presented in

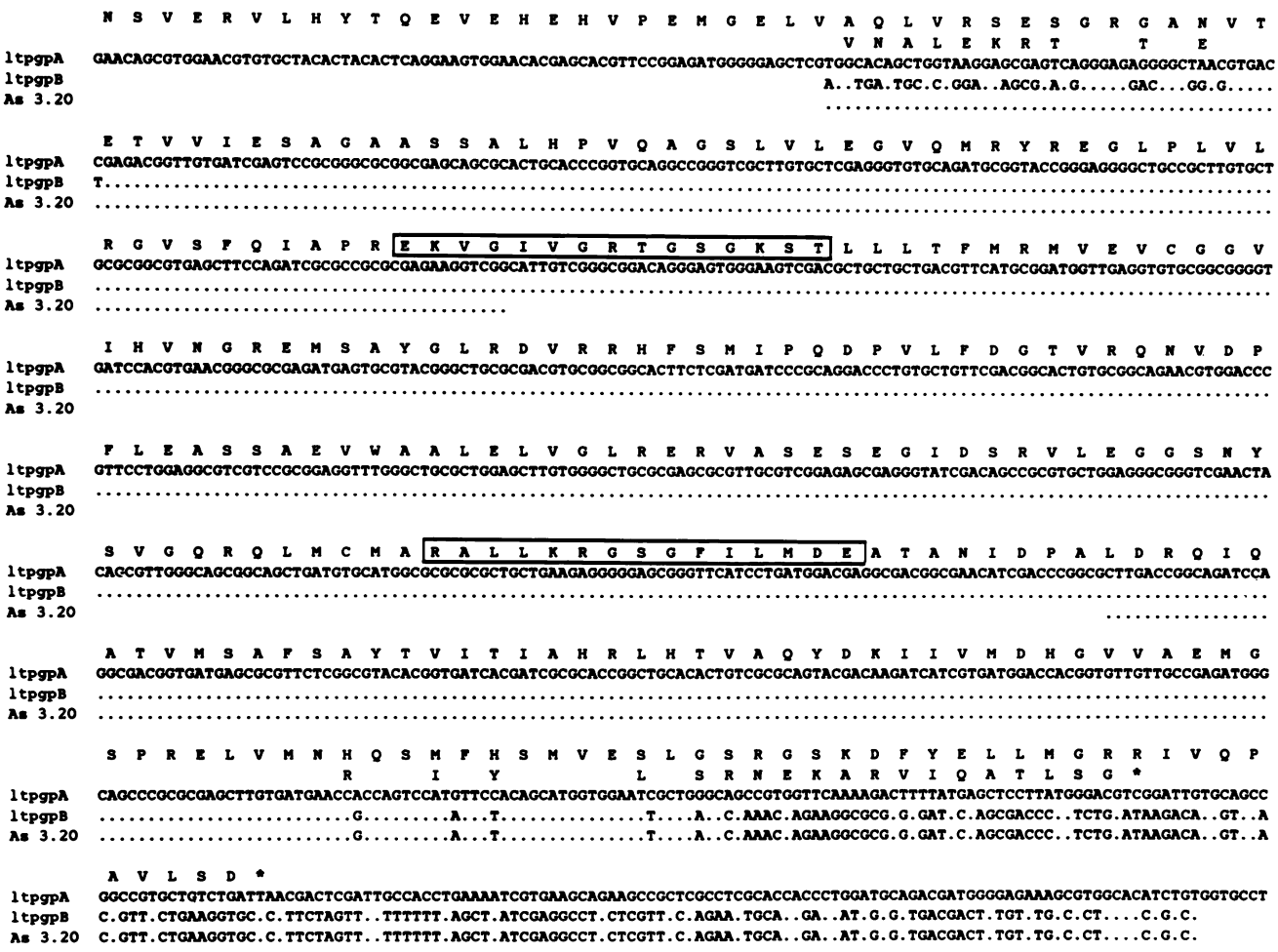

Fig. 8. Nucleotide sequence of the *ltpgpA−ltpgpB* fusion gene present on a 17 kb plasmid in mutant TarII As20.3. The sequence of the *ltpgpA* gene is taken from Ouellette *et al.* (1990) and starts at amino acid 1244 in the C-terminal part of *ltpgpA*. The corresponding C-terminal parts of the *ltpgpB* gene and of the fusion *ltpgpA−ltpgpB* gene are lined up with the *ltpgpA* sequence. Dots indicate identities with *ltpgpA*. Amino acids are indicated in the one letter code and for the ltpgpB protein only the differences with the ltpgpA protein are indicated. Consensus amino acids constituting the conserved nucleotide binding site are boxed.

Figure 7. In this experiment total DNA was digested with *Hin*dIII and hybridized to a probe (probe 5, see Figure 1a) recognizing five P-glycoprotein genes in *Leishmania*. The relative hybridization intensity of bands indicated by *ltpgpA*, *B* and *C* in the wild-type strain (lane 1) and the two resistant mutants, shows that mutant TarII As20.3 (lane 3) has lost half of the *ltpgpA* and *B* copies, i.e. part of one of the two chromosomal alleles of the H region. In addition, lane 3 shows two new (junction) fragments, hybridizing to probe 5. The 4.4 kb fragment has the size and intensity expected for the junction fragment formed in one chromosomal allele when the circle was excised (see *Hin*dIII map in Figure 6). The other junction, present in the 11 kb fragment cut from the As20.3 circle, was cloned and sequenced. Figure 8 shows that the cross-over between *ltpgpB* and *A* falls in a stretch of 750 bp that is identical in *ltpgpA* and *B*, indicating that the circle was formed by homologous intrachromosomal recombination.

H region amplification and drug resistance

Table I summarizes our main new results (see also White *et al.*, 1988; Ouellette *et al.*, 1990) on the relation between H region amplification and drug resistance. Resistance to MTX or arsenite is often associated with H region amplification, but high levels of resistance can also be obtained without. As revertants lose amplification, the extra H region copies appear to contribute to resistance, even in fully adapted strains. The cross-resistance results show that amplification of the complete H region in response to arsenite or vinblastine invariably leads to a 2- to 3-fold resistance to MTX, whereas amplification of part of the H region in the TarII As50.1 and 20.3 mutants is not associated with MTX cross-resistance. This suggests that the gene determining low-level MTX resistance lies in the non-amplified region. The nature of the putative gene remains unknown.

The cross-resistance to unrelated drugs accompanying H region amplification is invariably low in our experiments and not linearly proportional to H circle copy number. A rather low cross-resistance to primaquine, terbenafine and arsenite was also observed in *L.major* (Ellenberger and Beverley, 1989) and a more substantial cross-resistance (10-fold) to MTX in *L.major* (Ellenberger and Beverley, 1989) and in *L.mexicana amazonensis* (Katakura and Chang, 1989). Although this resistance to a wide variety of cytotoxic compounds could be explained by a non-specific general ability to withstand the stress of cytotoxicity, the absence of cross-resistance to MTX in the two arsenite resistant TarII mutants (Table I) argues for a more specific effect, at least on MTX. It is possible that H region genes encode proteins

Table I. H region amplification and drug resistance

Strain	H region amplification[a]			Relative resistance[b]	
	ltpgpA copy number	Complete region?	Increased *ltpgp* RNA	MTX	Arsenite
TarVIa wild-type	7	+	−	−	−
TarVIa As20	>60	+	+	+	+++
TarVIa VBL1000.1	44	+	+	+	ND[c]
TarII wild-type	2	NR[d]	NR	−	−
TarII MTX1000.3	2	NR	−	+++	ND
TarII MTX1000.1	40	+	+	+++	ND
TarII As20.2	2	NR	−	ND	+++
TarII As50.1	21	−	+	−	+++
TarII As20.3	43[e]	−	+	−	+++

[a]The approximate copy number of *ltpgpA* was determined by DNA hybridization. Overexpression of *ltpgp* genes was qualitatively determined by RNA blot (Northern) analysis of total RNA of the different mutants hybridized to probe 5 (see Figure 1a). A + indicates increased hybridization in the mutant compared with the wild-type strain. Probe 5 recognizes all five *ltpgp* genes (see Figure 7) and we have not verified whether the increased *ltpgp* RNA was actually derived from the *ltpgpA* gene.

[b]The relative resistance was measured by comparing the growth rates of mutants with that of wild-type in the presence of MTX or arsenite; −, + and +++ indicate that the mutant is <1.5-fold, 2- to 3-fold, and >10-fold more resistant, respectively, than the wild-type strain.

[c]Not determined.

[d]Not relevant.

[e]This includes the *ltpgpA−B* fusion gene.

that inefficiently extrude or inactivate a broad range of toxic compounds. High-level resistance may then ensue upon selection of a mutated version of a resistance gene or upon selection of other mechanisms not related to H region genes. Several such mechanisms are known to exist in the case of MTX (Ellenberger and Beverley, 1987; Kaur *et al.*, 1987; Ellenberger *et al.*, 1989). The recent development of stable DNA transformation systems for *Leishmania* (Laban *et al.*, 1990; Kapler *et al.*, 1990; Ten Asbroek *et al.*, 1990) now makes it possible to test these speculations.

Discussion

Our results show that the H region of the *Leishmania* genome can give rise to two types of precisely defined plasmids. The type I plasmid studied contains a contiguous stretch of DNA excised from the chromosome by a homologous recombination between two P-glycoprotein genes that share a 750 bp stretch of sequence. The type II plasmids (H circles) contain a long inverted duplication and appear to arise without loss of one of the chromosomal H regions. White *et al.* (1988) have proposed that the formation of type II plasmids involves short IRs, as illustrated in Figure 1b. We have verified that these IRs are present at the positions predicted in Figure 1b, and that H circles can be generated *de novo*. Nevertheless, in the one additional H circle analysed here, only one of the IRs coincides with a circle inversion point. The IRs flanking segment *a* have been skipped and a rearrangement point further down the chromosome is used. Whether this inversion occurs at a third set of (unrelated) IRs remains to be verified. In contrast, Ellenberger and Beverley (1989) concluded from restriction digest analysis that the inversion points used in H circle generation in three independent *L.major* mutants were identical. As the parental *L.major* stock was apparently uncloned and contains a low level of H circles according to Hightower *et al.* (1988), it is difficult to exclude the possibility that the H circles found in the three mutants were already present in a minority parental population.

Arsenite can promote specific gene amplification

Sodium arsenite was shown to promote amplification of the DHFR gene by MTX selection in mouse 3T6 cells (Lee *et al.*, 1988) and of the H region in *Leishmania* (Detke *et al.*, 1989; Katakura and Chang, 1989). Whereas arsenite induced overproduction of circles resembling H circles in *L.mexicana amazonensis* (Detke *et al.*, 1989), we only obtained plasmids without a large duplication in the two arsenite resistant mutants, TarII As50.1 and 20.3, in which amplification of the H region was induced *de novo*. The difference may be due to the presence of a minority population with H circles in the parental *L.mexicana amazonensis* strain, lacking in our TarII *L.tarentolae* strain. In the TarVIa parental strain of *L.tarentolae* that does contain H circles, selection for arsenite resistance led to an increase in H circles rather than formation of excised type I plasmids (Table I). Although the number of independent mutants analysed is small, our results suggest that amplification of pre-existent H circles occurs more frequently during drug selection than *de novo* generation of H circles.

Whereas normal animal cells are highly resistant to gene amplification, normal *Leishmania* species readily respond to drug selection with amplification of relevant genes. In addition to H region amplification we have observed amplification of an uncharacterized DNA segment in our arsenite resistant mutants TarII As20.1 and 20.2 (unpublished). Others have observed amplification of the DHFR-thymidylate synthetase gene in *L.major* on 30 kb plasmids during MTX selection (Beverley *et al.*, 1984) and amplification of a gene for *N*-acetylglucosaminyl transferase on 63 kb plasmids during tunicamycin selection in *L.mexicana amazonensis* (Detke *et al.*, 1988). As the cross-over points in these plasmids have not been determined, it is not known whether the amplification in these cases involves excision by homologous recombination, as in our TarII As20.3 mutant. Special sequences seem involved, however, as we have never been able to induce amplification of the DHFR-thymidylate synthetase gene in *L.tarentolae* stocks. Moreover, we note that the 96 bp sequence, deleted in one of the IRs of unique segment *b* (Figure 4) is flanked by perfect direct repeats of 7 bp (CGCCCTC). Several short direct repeats may therefore promote excision in *Leishmania*, as has been observed in prokaryotes (reviewed by Ehrlich, 1989).

A comparison of DNA amplification in Leishmania and in mammalian cells

Several models have been put forward to explain how inverted duplications arise during DNA amplification in animal cells (Kaufman *et al.*, 1983; Passananti *et al.*, 1987; Ruiz and Wahl, 1988). A recently favoured model involves strand switching in the replication fork (Nalbantoglu and Meuth, 1986; Hyrien *et al.*, 1988) and this may be promoted if the fork moves through a region enriched in palindromic sequences. The mammalian DNA joints sequenced thus far do not contain the type of long IRs found in *Leishmania* H circles (Hyrien *et al.*, 1988; Legouy *et al.*, 1989). This does

not mean that our strand displacement model could not also account for the formation of inverted duplications during DNA amplification in animal cells. Given the inherent tendency of DNA polymerases to backtrack on single-stranded DNA (Kornberg, 1980), short regions of imprecise base-pairing may suffice for reversal. As no single mechanism for gene amplification readily explains all experimental results (see Stark *et al.*, 1989), there is room for additional mechanisms.

Although our experiments are compatible with the model in Figure 1b, it should be stressed that other models could also account for the formation of H circles. Given the presence of IRs flanking the genomic copy of the H region, any large circle containing two copies of regions *a* and *b* can give rise to H circles by internal recombination, e.g. between the left-hand IR of one copy of region *b* and the right-hand IR of another copy and an analogous recombination between the two copies of region *a*. Hence, strand displacement need not occur precisely at the position of regions *a* or *b*, as depicted in Figure 1b and any of the other mechanisms for the formation of long IRs, discussed above, could also result in formation of 68 kb H circles.

The work of Wahl and coworkers has demonstrated that gene amplification in mammalian cells may sometimes involve excision and circularization of DNA (Carroll *et al.*, 1988; Ruiz and Wahl, 1988, 1990). Amplification of N-*myc* sequences in human neuroblastoma cells can also be associated with the loss of one chromosomal copy of the amplified gene (Hunt *et al.*, 1990). The resulting plasmids may oligomerize and serve as precursors for DMs and ECRs (see Wahl, 1989). Whether excision occurs by homologous recombination in these cases is still not known as the cross-over site has not been characterized. The analysis of the junctions of other amplicons in mammalian cells suggests, however, that the generation of amplified DNA usually involves illegitimate recombination between sequences that share at most very short sequence homology (Hyrien *et al.*, 1988; Legouy *et al.*, 1989; Stark *et al.*, 1989). This is also observed for deletions without amplification (see Meuth, 1989), although exceptions have been found (Yen *et al.*, 1990). Analysis of additional junctions is required to test whether excisional amplification in kinetoplastids, like *Leishmania*, always requires more sequence homology than in mammals. Such a difference is not inconceivable, since non-homologous recombination is also much less frequent during transformation of kinetoplastids than of mammals (Cruz and Beverley, 1990; Ten Asbroek *et al.*, 1990).

Materials and methods

Cell lines and culture

The two parental cell lines TarII and TarVIa as well as the MTX resistant mutant TarII 1000.1 have been described previously (White *et al.*, 1988; Ouellette *et al.*, 1990). Cells were grown in SDM-79 medium in the presence or absence of MTX (Emthexate, Pharmachemie B.V., Haarlem, Holland). To obtain clones of TarII, we adapted that strain to BHI medium (Difco) supplemented with hemin. Cells were plated and single colonies were inoculated in BHI and then readapted to SDM-79. In an attempt to induce the formation of H circles from the chromosomal H region, clones of wild-type cells (or uncloned populations) were adapted in 3–6 months to 500–1000 μM MTX by passaging them in increasing drug concentrations at steps of 50, 200, 500 and 1000 μM MTX. The wild-type TarII and TarVIa strains were selected for resistance to sodium arsenite (Detke *et al.*, 1989) by stepwise increase from 400 nM to 20 or 50 μM and the wild-type TarVIa strains for resistance to vinblastine by stepwise increase from 50 μM to 1 mM. Relative drug resistance was measured as previously described (Ouellette *et al.*, 1990).

DNA cloning

High mol. wt total DNA of *L.tarentolae* TarII was partially digested with *Sau*3AI, size-fractionated on low melting point agarose and fragments between 12 and 25 kb were ligated to the lambda vector λ-GEM-11 (Promega), packaged *in vitro* and plated. The genomic border A of the unique segment *b* (see Figure 1a) was obtained by screening the genomic bank with probe 2 (see Figure 1a). The genomic border D of unique segment *a* (see Figure 1a) was obtained by making a plasmid *Hin*dIII bank derived from DNA in the supernatant of a Hirt (1967) extract of mutant TarII 1000.1, which contains this junction in unusual H circles (see Figure 1a). The plasmid of interest was selected with probe 4 (see Figure 1a). The junctions B, AB, C and DC had already been cloned (White *et al.*, 1988). The new junctions created during the *de novo* formation of circles in mutant TarII 1000.1 and TarII As20.3 were obtained as follows. The DNA of the mutants was digested with restriction enzymes and hybridized to probe 1 to determine the size of the rearranged fragment. Fragments of the desired size were isolated from low melting point agarose, ligated to digested pGEM3-Zf (Promega), transformed into *Escherichia coli*, and recombinants with the desired insert were picked up by colony hybridization using probe 1 (see Figure 1a). All probes used were radiolabelled by random priming (Feinberg and Vogelstein, 1983).

Additional clones containing P-glycoprotein genes were identified in the lambda phage genomic DNA bank by hybridization with probe nbsA, which covers one of the nucleotide binding sites of the *ltpgpA* gene (see Ouellette *et al.*, 1990). Thus far, we have obtained four putative genes in addition to the *ltpgpA* gene characterized previously. Two of these genes, *ltpgpB* and *C*, are tandemly linked and just adjacent to the H region in *L.tarentolae* chromosomal DNA (see Figure 1a). The other two, *ltpgpD* and *E*, are linked on a 1400 kb *Leishmania* chromosome (Borst *et al.*, 1990; and unpublished).

DNA sequence analysis

Doubled-stranded plasmid DNAs, derivatives of pGEM3-Zf (Promega), were sequenced using the dideoxy method (Sanger *et al.*, 1977). In addition to the SP6 and T7 sequencing primers, oligonucleotides were synthesized using phosphoramidite chemistry and were used as internal primers to complete the sequence on both strands of at least one version of the IRs flanking the unique segments *a* and *b*. Computer analysis of the nucleotide sequence was performed using the software package of the University of Wisconsin Genetics Computer Group (UWGCG) (Devereux *et al.*, 1984). The upper and lower IR sequences will appear in the EMBL/GenBank/DDBJ nucleotide sequence databases under the accession numbers M55397–M55402.

Acknowledgements

We thank Dr Ted White and Henri van Luenen for several of the plasmids used in this study. We also thank the members of the laboratory for interest and advice throughout the course of this work and for the critical reading of the manuscript. This work was supported in part by a grant from the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (NWO) and by a NATO (Canada) and FRSQ post-doctoral fellowship to M.O.

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Received on December 13, 1990