Nucleic Acids Research, 1993, Vol. 21, No. 18 **4305–4312**

Frequent amplification of a short chain dehydrogenase gene as part of circular and linear amplicons in methotrexate resistant *Leishmania*

Barbara Papadopoulou, Gaétan Roy and Marc Ouellette*
Infectiologie, Centre de Recherche du CHUL et Département de Microbiologie, Faculté de Médecine, Université Laval, Québec, Canada

Received May 19, 1993; Revised and Accepted August 2, 1993

ABSTRACT

The H locus of *Leishmania* codes for a short chain dehydrogenase gene (*ltdh*) that is involved in antifolate resistance. *Leishmania tarentolae* cells, selected in a step by step fashion for resistance to the antifolate methotrexate (MTX), frequently amplified *ltdh* in response to drug selection. Both circular and linear extrachromosomal amplicons were generated *de novo* from the chromosomal H locus and several contained inverted duplications. At least four different rearrangement points were used during the formation of amplicons, with one of them used preferentially. All mutants highly resistant to MTX, whether or not they have the H locus amplified, showed a decreased steady-state accumulation of MTX. Nevertheless, two types of transport mutants were clearly discernable. In the first type, accumulation was reduced four to five-fold, whereas in the second class of mutants, accumulation was reduced more than 50-fold. The *ltdh* gene was amplified in all the mutants with the transport mutation of the first type, but not in all the mutants with a more pronounced decrease in the steady-state accumulation of MTX. Both types of transport mutation, leading to the reduction in MTX accumulation, arose early during the selection process and were stable even when cells were grown in absence of the drug for prolonged period.

INTRODUCTION

The flagellated protozoan parasite *Leishmania* often responds to drug selection by gene amplification and several different loci have been found to be amplified (reviewed in 1–3). In general, the amplified DNA is part of extrachromosomal circular DNA with direct or inverted repeats (4–8) but linear amplicons have also recently been observed in drug selected *Leishmania* (9–11). However, intrachromosomal amplification has not yet been described and it is usually accepted that in *Leishmania*, in contrast to cancer cells (12), secondary rearrangements are rare once amplicons are formed (1). The amplicons so far described in *Leishmania* were generated by intrachromosomal homologous recombination between direct or inverted repeats, presumably during replication (8, 11, 13).

The H locus of *Leishmania* is often amplified during drug selection. This locus encodes a P-glycoprotein-related gene *ltpgpA* (14) involved in low level oxyanion resistance (15, unpublished) and a short chain dehydrogenase *ltdh* (16, 17) involved in methotrexate resistance. Not surprisingly, both arsenite (an oxyanion) and methotrexate are potent inducers of the amplification of the H locus (4–8, 11, 18). In addition to the amplification of the H locus, amplification of the gene for the target enzyme dihydrofolate reductase-thymidylate synthase (DHFR-TS) (19) and reduced uptake of the drug (20–22) have also been described in MTX resistant *Leishmania*. Every cell line selected for MTX resistance, whether or not it had DNA amplification, exhibited a reduced accumulation of the drug (20, 21). This decrease was attributed to mutations in the high affinity folate carrier (21, 22). In every *L.major* MTX resistant cell line studied, a two to four-fold decrease of the V_{max} of the MTX influx was observed, but did not always correlate with the level of MTX resistance (21). In contrast, a clone of a mutagenized *L.donovani* population that was selected for high level MTX resistance, showed a pronounced decrease in MTX accumulation (22).

Amplification of the *dhfr-ts* gene has been reported in *L.major* (19, 21) whereas amplification of the *ltdh* gene present on the H locus was reported in several different species of *Leishmania* selected for MTX resistance (4–7). The *ltdh* gene is part of extrachromosomal circles with long inverted duplications, 30 kb and greater, separated by unique DNA segments (4–8). In *L.major* the circles were apparently always formed at the same rearrangement point (23) whereas some variation in the sequences was observed in the two amplicons so far studied in *L.tarentolae* (8). In this study, we present a detailed analysis of both circular and linear amplicons derived from the chromosomal H locus of *Leishmania* after stepwise selection with MTX. The contribution of gene amplification in the emergence and establishment of MTX resistance was also studied in relation to transport mutations.

* To whom correspondence should be addressed at: Service d'Infectiologie du Centre de Recherche du CHUL, 2705 boul. Laurier, Ste-Foy, Québec G1V 4G2, Canada

MATERIALS AND METHODS

Cell lines and cultures

The wild type *L. tarentolae* TarII and TarVIa strains have been described previously (5) and were obtained either from the ATCC, or from F. Opperdoes (via L.Simpson), respectively. The unselected TarVIa wild type strain contains free H circles whereas no circle is detectable in TarII (5). Apparently TarVIa has also been called TarII as determined by its culture history, and analysis of restriction digests of kinetoplast DNA has suggested that both TarVIa and TarII are the same (L.Simpson, personal communication) and are very similar to the UC and T strains of *L. tarentolae* (24). From now on we shall therefore call our TarVIa cell TarII' to differentiate it from the TarII cell line that is free of H circle. The mutants TarII'MTX1000, TarIIMTX1000.1 and TarIIMTX1000.3 have been described previously (5, 14). Mutants of a nonclonal population of TarII or TarIIAs20.3 were adapted to MTX and clones of TarII were adapted to 1000 μM MTX and to 20 μM arsenite in SDM-79 by passaging them in increasing drug concentrations as described previously (14). Revertants were obtained by growing the mutants in SDM-79 for several passages in absence of the selecting drug.

DNA manipulations

Genomic parasite DNA was prepared as described (25). Intact chromosome DNA of *in situ* lysed *Leishmania* cells in agarose blocks was prepared as described (26). Chromosomes were resolved by TAFE gel (Beckman) as described previously (11). Isolation of the linear amplicon in mutant TarIIMTX1000.4 was performed by using a Sephaglas BandPrep Kit (Pharmacia). Southern blots, hybridization and washing conditions were done following standard protocols (27). Most DNA fragments used as probes in this study are shown in Figure 4. Probe 1 is a 1.2 kb *Bam*HI−*Hin*dIII fragment derived from pMAC3 (5); probe 2 is a 850 bp *Pst*I−*Pst*I fragment covering the second nucleotide binding site of *ltpgpA* (14); probe 3 is the complete *ltdh* gene made by PCR and probe 4 is an 1.4 kb *Eco*RV−*Eco*RV fragment derived from pMAC14 (5). The neo probe, containing the whole coding sequence of the neomycin phosphotransferase gene, was also obtained by PCR and the probe recognizing telomeric sequences was derived from pT4 (28). The construct made to target the *neo* gene on the *ltpgpA* gene of the linear amplicon in mutant TarIIMTX1000.4 was done by inserting a 2.5 kb *Bam*HI−*Bam*HI neo expression cassette flanked by the α-tubulin intergenic regions (16, 29) in the unique *Bgl*II site of a 9.5 kb *Hin*dIII−*Hin*dIII fragment containing the *ltpgpA* gene (pMAC6-neo). Transfections were carried out as described (16).

Transport assays

Cells in exponential growth phase were pelleted and washed twice in HEPES-NaCl buffer (29). An equal volume of cells and folate deficient defined medium (30) were layered over 100 μl of dibutylphthalate (Sigma). Transport assays were initiated by adding ^{3}H-MTX (Amersham 14 mCi/mmol) and were stopped

Table 1. Properties of MTX resistant *Leishmania* mutants

Mutants	*ltdh* copy number[a]	types of amplicon			decreased in MTX accumulation[b]
		c/l[c]	IR[d]	A−D[e]	
TarII'MTX1000	86	c	+	A	>50
TarIIMTX1000.1	72	c	+	B	>50
1000.3	2	−	−	−	>50
100.4	ND[f]	l	+	B	2
1000.4	46	l	+	B	4.5
100.5	2	−	−	−	2
300.5	ND	c	?	D	4.5
1000.5	63	c	?	D	4.5
1000.5rev	2	−	−	−	4.5
100.6	2	−	−	−	>50
1000.6	2	−	−	−	>50
100.7	2	−	−	−	>50
1000.7	2	−	−	−	>50
1000.8	18	l	?	D	ND
TarIIAsMTX1000.1	96	c	+	B	ND
1000.2	128	c	+	B	ND
1000.3	2	−	−	−	ND
1000.4	2	−	−	−	ND
1000.5	55	c+l[g]	?	C	ND
As20.3MTX1000.1	78	c	?	D	ND
1000.2	38	c	?	D	ND
1000.3	152	c	+	A	ND

[a]The copy number of *ltdh* was determined by quantitative Southern blot analysis as described (8).
[b]The relative fold decrease in labeled MTX accumulation in mutants was compared to the accumulation in TarIIWT cells.
[c]Presence of circular (c) or linear (l) amplicons or absence (−) of amplicons in mutants.
[d]Presence (+) or absence (−) of inverted duplications in the amplicons. A question mark indicates that it is not known whether the amplicon contains direct or inverted repeats.
[e]The letters A to D refer to the four types of rearrangement points identified in the various amplicons as schematically illustrated in Figure 4.
[f]ND: not done
[g]Only the circular amplicon encodes *ltdh*. The linear amplicon hybridizes to a probe derived from the 50 kb linear amplicon frequently observed in arsenite resistant *Leishmania* (11).

at various time by spinning the cells through the inert dibutylphthalate layer (31) in an Eppendorf centrifuge. Unincorporated MTX was removed by aspiration and cells were washed once in HEPES-NaCl buffer and counted in liquid scintillation counter (Beckman LS6000TA). The quantity of incorporated radioactivity was normalized with protein concentration that was measured using a Bradford reagent (Bio-Rad).

RESULTS

Leishmania MTX resistant mutants

Amplification of a gene coding for an enzyme that shows similarities with the family of short chain dehydrogenases is a novel mechanism of antifolate resistance described in a protozoan parasite. To look at the frequency of this resistance mechanism in *Leishmania* we have generated several MTX resistant mutants. The *Leishmania tarentolae* strain TarII (5) was adapted to 1000 μM MTX by increasing step by step (50, 100, 300, 500 and 1000 μM) the concentration of MTX. Mutants TarIIMTX1000.1 and 1000.3 have already been described (14) and five novel mutants, TarIIMTX1000.4 to 1000.8 have been generated for this study. Mutants selected at lower drug concentration, from which the highly resistant mutants were derived, were also analyzed when appropriate. In addition to *ltdh*, the H region encodes *ltpgpA* (14), a P-glycoprotein-related gene involved in low level oxyanion resistance (15; unpublished). Arsenite (an oxyanion) is a potent inducer of *ltpgpA* gene amplification (8, 11, 18). Selection with two different drugs has been shown in cancer cells to increase the rate of gene amplification (32, 33). To see whether double selection could also increased the frequency of gene amplification in *Leishmania*, five clones of TarII were selected in a step by step fashion to high level resistance to both arsenite (As) and MTX (TarIIAsMTX1000.1 to 1000.5). The mutant TarIIAs20.3 has been described previously and was shown to contain a circular amplicon generated by homologous recombination between *ltpgpA* and *ltpgpB* (8, 11). The *ltdh* gene was absent from this amplicon. Three independent cell lines of this mutant were selected for 1000 μM MTX resistance in the same step by step selection procedure. These mutants as well as some of their properties are listed in Table 1.

Frequent amplification of the H locus in MTX resistant mutants

Three out of five MTX- or MTX-As-selected *L.tarentolae* TarII strains and three out of three TarIIAs20.3 mutants selected for MTX resistance had their *ltdh* gene amplified *de novo* from the chromosomal copy (Table 1). Double selection with MTX and arsenite, two drugs known to induce the amplification of the H locus, did not increase appreciably the frequency of gene amplification. Blots of TAFE chromosome gels indicated that most amplicons were circular (Table 1). This conclusion was reached since the migration of the *ltdh* hybridizing band was independent of pulse time (not shown); a large amount of hybridizing material (most probably open circles) remained in the slot (Figure 1D); a characteristic intense hybridizing smear, representing different topoisomers of the closed circles (5), was extending from the slot to the circle band (Figure 1D); and finally a probe recognizing telomeric sequences failed to hybridize to the circles (not shown). All these properties are characteristics of circles migrating under alternating field electrophoresis conditions (5, 34–36). However, in mutants TarIIMTX1000.4 and TarIIMTX1000.8, the amplicons derived from the H locus were linear (Figure 1A–B, lanes 2 and 4) since their migrations were dependent on pulse (not shown), and they hybridized to a probe recognizing telomeric sequences (Figure 1C). This represents the first instance where the H locus is amplified as part of linear amplicons. Both linear amplicons were lost rapidly when the mutants TarIIMTX1000.4 and TarIIMTX1000.8 were grown in absence of MTX (Figure 1A–B, lanes 3 and 5).

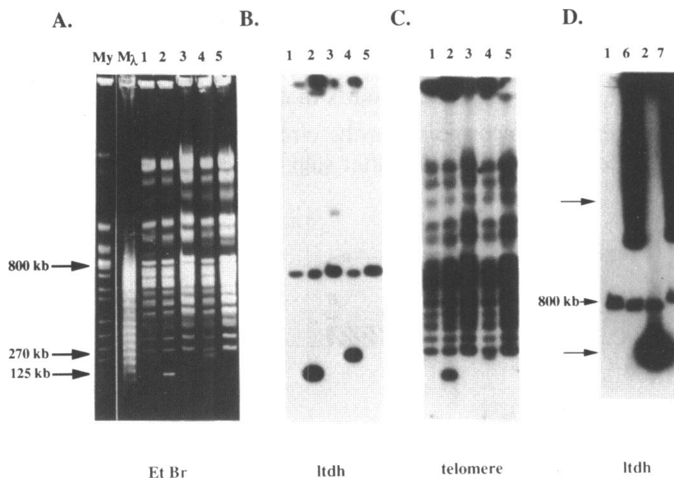

Figure 1. Analysis of amplicons in MTX resistant *Leishmania* by TAFE chromosome gels. The chromosomes of *Leishmania* cells were resolved by TAFE gels and stained with ethidium bromide (EtBr) (**A**) or transferred to nitrocellulose and hybridized with the probes indicated below the autoradiograms (**B, C** and **D**). The arrow at 800 kb indicates the chromosomal H locus, whereas the arrows at 270 and 125 kb indicate the size of the linear amplicons. My: *S.cerevisiae* chromosome marker (Bio-Rad); Mλ: lambda PFGE marker (Pharmacia); 1: TarIIWT; 2: TarIIMTX1000.4; 3: TarIIMTX1000.4 rev (rev for revertant); 4: TarIIMTX1000.8; 5: TarIIMTX1000.8 rev; 6: TarIIAsMTX1000.1; 7: TarIIAsMTX1000.2.

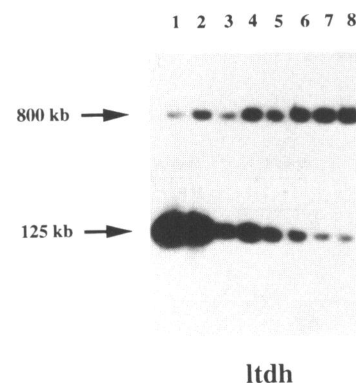

Figure 2. Instability of the linear amplicon in mutant TarIIMTX1000.4. The stability of the linear amplicon present in TarIIMTX1000.4 grown in the presence of 1 mM MTX was studied according to time (from 1 to 13 weeks). Chromosomes were resolved by TAFE, transferred to nitrocellulose and hybridized to *ltdh* probe (probe 3, see Figure 4). The chromosomal 800 kb locus and the 125 kb linear amplicon are indicated with arrows. Lanes 1: TarIIMTX1000.4; 2 to 8, TarIIMTX1000.4 grown in the presence of 1 mM MTX taken at time intervals from 1 to 13 weeks. More cells were present in agarose blocks of the latest passages to permit the visualisation of the linear amplicon at 125 kb.

The linear amplicon in TarIIMTX1000.4 was unstable since even when grown in the presence of 1 mM MTX its copy number was decreasing (Figure 2), suggesting that the amplicon was now dispensable for growth. A neomycin phosphotransferase marker (*neo*) was introduced in the amplicon by gene targeting (37–40) to measure the contribution of the amplicon in resistance (Figure 3). The *neo* marker was introduced in the unique *BglII* site of a 9.5 kb *HindIII–HindIII* fragment containing the *ltpgpA* gene which is present on the linear amplicon of mutant TarIIMTX1000.4 (see Figure 4 and Table 1). *The linearized HindIII* construct was introduced by electroporation. Cells growing in G418 (drug to which *neo* confers resistance) were analysed by Southern blots of digested DNA (not shown) and of chromosome sized TAFE blots (Figure 3). The *neo* gene was indeed found integrated in the linear amplicon but also in the chromosomal copy of *ltpgpA* (Figure 3B, lane 3). Two populations of linear amplicons were detected after electroporation and the largest one was estimated to be twice the size of the initial 125 kb amplicon, possibly representing a dimerization product. Hybridization profiles with probes 1 to 4 (see Figure 4) were indistinguishable in mutants having two or one linear amplicons (not shown). The transfection of the linear amplicon has been reproduced once with similar results. The multimerization of linear amplicons following transfection seems to be a frequent event since we also observed it after transfection of an unrelated linear amplicon isolated from an arsenite resistant mutant (K.Grondin, M.Ouellette, unpublished). The linear amplicons in TarIIMTX1000.4-neo were isolated and reintroduced by electroporation in a TarII WT strain selected for G418 resistance. Southern blot analysis of chromosome sized TAFE gels of transfectants indicated that only the largest linear amplicon was maintained as an 'extrachromosomal' linear element in TarII WT, possibly selected because of its higher neo copy number, but it had also integrated in its homologous chromosome (Figure 3A–B, lane 4). During the integration a portion of the amplicon with its novel junction was probably inserted, hence explaining the increase in size of one allele of the chromosomal locus (Figure 3A–B, lane 4). This result demonstrates that it is possible to transfer large linear amplicons in *Leishmania* and it should therefore be conceivable to transfer whole chromosomes, the smallest *Leishmania* chromosome being close to 250 kb (see Figure 1A). The TarII WT transfectant containing the linear amplicon derived from TarIIMTX1000.4-neo was eight-fold more resistant to MTX than the wild type cells, showing that the formation of amplicons encoding *ltdh* in MTX resistant mutants plays an important role in resistance.

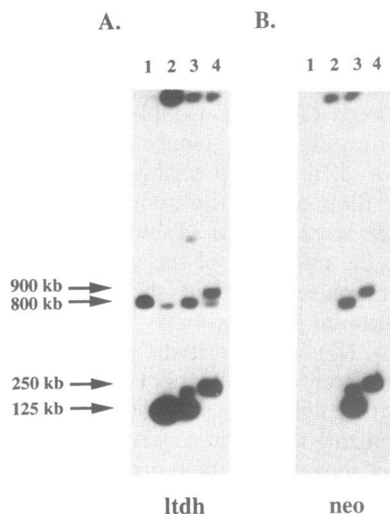

Figure 3. Transfection of a linear amplicon in a wild type strain. A construct with the *ltpgpA* gene interrupted with the neomycin phosphotransferase marker (pMAC6-neo) (see Materials and methods) was introduced in the mutant TarIIMTX1000.4 by electroporation. Two linear amplicons of 125 and 250 kb were detected. Both hybridized with *ltdh* (probe 3 in Figure 4) and a *neo* specific probes (lane 3A–B). The linear amplicons with the *neo* gene inserted by homologous recombination were isolated and reintroduced in a TarII WT cell (lane 4A–B). Arrows indicate either the *ltdh* chromosomal locus at 800 kb, one allele containing the H locus that has increased in size during the transfection (900 kb), or linear amplicons at 125–250 kb. Lanes 1: TarIIWT; 2: TarIIMTX1000.4; 3: TarIIMTX1000.4-neo (TarIIMTX1000.4 transfected with pMAC6-neo); 4: TarIIWT transfected with the linear amplicons isolated from TarIIMTX1000.4-neo.

Multiple rearrangement points in *ltdh*-containing amplicons

The rearrangement points in the circular and linear amplicons derived from the H locus, after step by step MTX or MTX-As

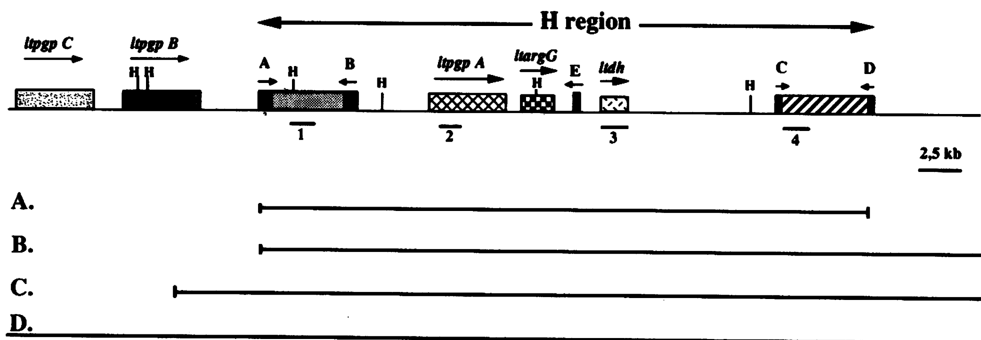

Figure 4. Diversity of amplicons generated *from the H locus in methotrexate resistant Leishmania.* The H locus (5, 6) is represented between arrows and is delimited by a dotted (left) and cross-hatched (right) rectangles that are flanked by the inverted repeats A–B and C–D, respectively (8). A sequence related to B is present within the H locus and is indicated by the letter E (11). The P-glycoprotein gene *ltpgpA* (14, 15), a homolog of the argininosuccinate synthetase *ltargG* (unpublished) and the short chain dehydrogenase *ltdh* (16, 17) are indicated. Two nearby linked P-glycoprotein genes *ltpgpB* and *ltpgpC* (8, 42) are also indicated. The numbers 1 to 4 below bars under the H locus indicate the location of some of the probes used in this study. Bars below the map with the letters A to D are representatives of the respective regions amplified. A vertical line interrupting the bars indicates that the rearrangement points were precisely mapped.

selection, were studied by Southern blot analysis and at least four different rearrangement points were used during amplification (Figure 4, and Table 1). We have described recently the rearrangement points in mutant TarII'MTX1000 (8). These were included in an amplified 5.8 kb *Bam*HI fragment hybridizing with probe 1 and an amplified 8.5 kb *Eco*RI fragment hybridizing with probe 4 (Figure 4), (see Figure 5A–B, lane 2). The presence of these rearranged amplified fragments is consistent with the formation of a circular amplicon having inverted repeats created by the annealing of the inverted repeat sequences A-B and C-D, respectively (Figure 4) (8, 41). These two rearrangement points were used in only one instance during the *de novo* generation of an extrachromosomal circular amplicon from the chromosomal copy (Figure 5A-B, lane 12). None of the other mutants used the sequences C and D to generate the inverted duplications since in all these mutants, the amplified *Eco*RI band comigrates with the 12.5 kb genomic *Eco*RI fragment indicating that the rearrangement point is further away (Figure 5B). We are currently making a chromosomal walk in that region to try to see whether the amplicons found in the different mutants were formed at the level of the same sequences.

The sequences A and B have been used more often during the formation of amplicons containing inverted duplications, including the linear amplicon found in TarIIMTX1000.4 (see Figure 5A, lane 4), and represent therefore a preferred rearrangement point. Several mutants nevertheless did not rearrange at the level of sequences A and B since they had an amplified 17 kb *Bam*HI band comigrating with the genomic fragment (Figure 5A lanes 5, 6, 10 and 11 and Table 1). In all these mutants, the ltpgpB and *ltpgp*C genes (8, 42) linked to *ltpgpA* (see Figure 4) are co-amplified, as determined by Southern blot hybridization with probe 2 (not shown), with the exception of mutant TarIIAsMTX1000.5 (Table 1). In this mutant the *ltpgpC* is not co-amplified and mapping data are consistent with the hypothesis that one rearrangement has occurred at the level of *ltpgpB* sequences (Figure 4, and not shown). At least two amplicons seem present in mutants TarIIAsMTX1000.5, TarIIAs20.3MTX1000.1 and TarIIAs20.3MTX1000.2 since in addition to the amplified band at 10 kb in lane 9 or at 17 kb in lanes 10 and 11 (Figure 5A), a 5.8 kb band was detected with probe 1. This could suggest either that two amplicons containing similar sequences were formed independently in the same cell or that the largest amplicon with large inverted duplications was rearranged by internal recombination at the level of the inverted repeats A and B to yield a secondary rearrangement point as suggested previously (41).

Amplicons in *Leishmania* have so far been described, or searched for, in mutants adapted to the highest concentration of the selecting drug. We examined the rate of appearance of amplicons in two mutants selected for MTX resistance (Figure 6). In mutant TarIIMTX1000.5 we observed the appearance of an extrachromosomal amplicon only when cells were adapted to 300 μM of MTX. The copy number of the circle increased with higher drug concentration. Another mechanism than *ltdh* amplification should be responsible for resistance in mutants adapted to 50 and 100 μM MTX. The formation of amplicons derived from the H locus in MTX resistant cell lines is usually conservative with

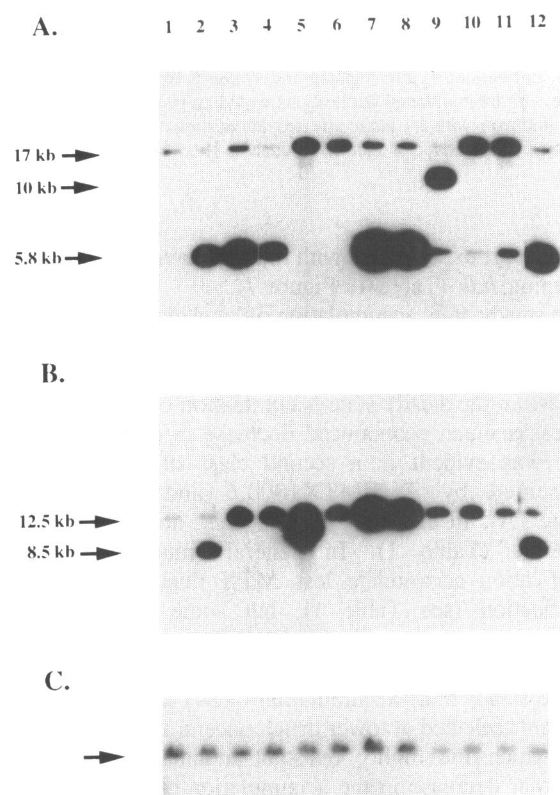

Figure 5. Mapping of rearrangement points of amplicons present in MTX resistant *Leishmania*. DNAs of *Leishmania* mutants were isolated and digested with *Bam*HI (A) or *Eco*RI (B) and hybridized with probes 1 and 4, respectively (see Figure 4). Panel C is the same Southern blot than in B which was rehybridized, after stripping off the probe 4, with a *ltpgpE* specific probe (D.Légaré, unpublished) to monitor the amount of DNA loaded. Lanes 1: TarIIWT; 2: TarII'MTX1000; 3: TarIIMTX1000.1; 4: TarIIMTX1000.4; 5: TarIIMTX1000.5; 6: TarIIMTX1000.8; 7: TarIIAsMTX1000.1; 8: TarIIAsMTX1000.2; 9: TarIIAsMTX1000.5; 10: TarIIAs20.3MTX1000.1; 11: TarIIAs20.3MTX1000.2; 12: TarIIAs20.3MTX1000.3.

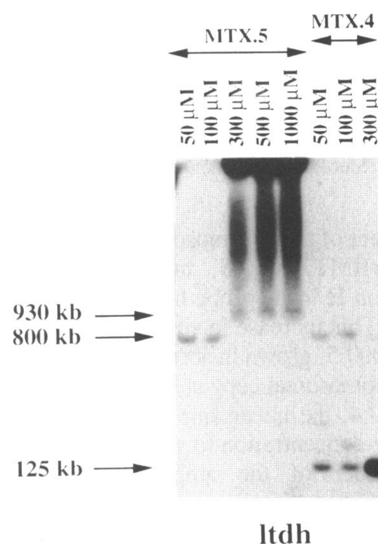

Figure 6. Appearance of *ltdh*-amplicons during MTX stepwise selection. Chromosomes of mutants TarIIMTX1000.5 (MTX.5) and TarIIMTX1000.4 (MTX.4), adapted to the concentration of MTX indicated, were resolved on TAFE gel, transferred and hybridized to probe 3 (see Figure 4). *Ltdh* amplification in mutant TarIIMTX1000.5 occured at 300 μM of MTX and was associated with an increase in the size of the chromosomal locus from 800 to 930 kb. The amplicon in TarIIMTX1000.4 (arrow at 125 kb) appeared at the first drug concentration to which parasites were adapted (50 μM).

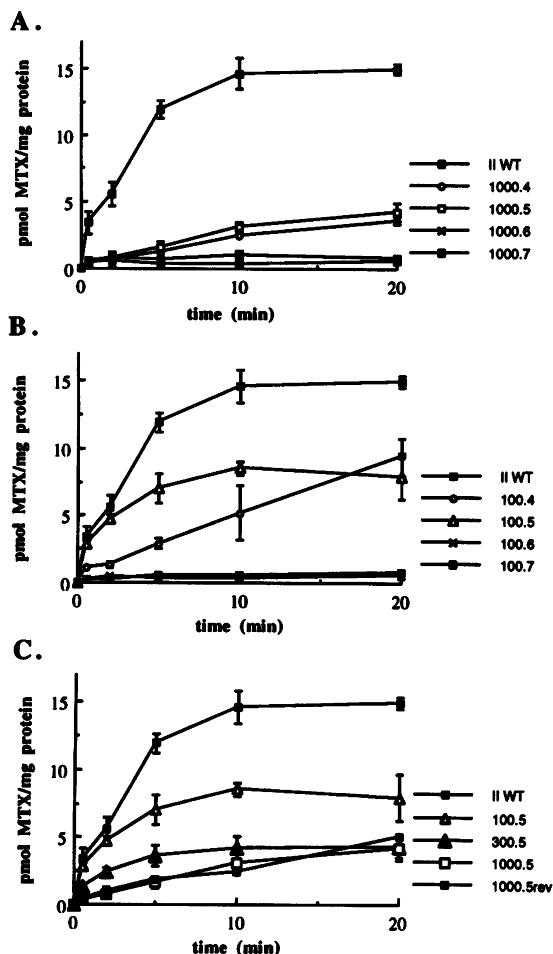

Figure 7. Steady-state accumulation of labeled MTX in *Leishmania* cells. Transport studies were carried out as indicated in Materials and methods. A). Accumulation studies in highly resistant MTX mutants with (TarIIMTX1000.4 and TarIIMTX1000.5) and without (TarIIMTX1000.6 and TarIIMTX1000.7) *ltdh* amplification. B). Accumulation studies in mutants adapted to 100 μM MTX, early during the selection process. C). Accumulation studies in a mutant adapting to increasing concentrations of MTX and the absence of correlation between *ltdh* copy number and reduced accumulation of MTX.

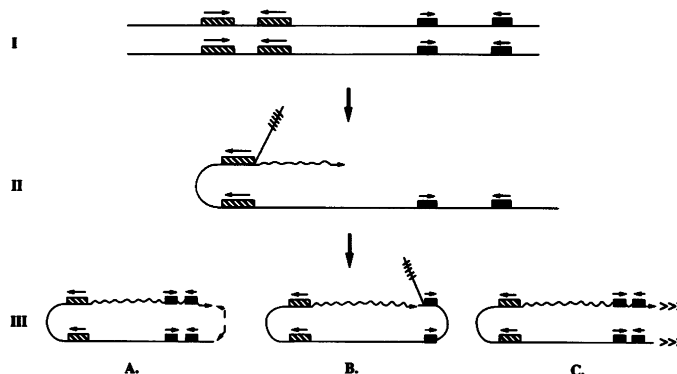

Figure 8. Proposed model for the formation of both circular and linear amplicons derived from the same genomic region. A putative genomic region with pairs of inverted repeated sequences (black and hatched rectangles) is shown in I. In II we show only part of a newly replicated strand displaced from its replication 'bubble' (not drawn, see also 8, 41). We postulate that during a block in DNA replication, DNA is partially displaced in the replication 'bubble' to allow the annealing of inverted repeats (II). The annealing could serve as a primer for a DNA polymerase to synthesize another copy of the inverted duplication while displacing the remainder of the DNA. The displaced single stranded DNA could be eliminated by nucleases as schematically illustrated by a line interrupted by several small perpendicular bars (II). At least two scenarios could explain the formation of circles with large inverted duplications. Either the polymerase could synthesized the novel copy of the inverted duplication until it reaches the end of the recently replicated strand (III A), or that the progression of the polymerase would be blocked by the annealing of another (unrelated) pair of inverted repeats (III B). In both cases, after ligation and synthesis of a complementary strand, a circle with large inverted duplications would be generated. Alternatively, the novel amplicon with its large inverted duplications could be protected from nucleases by the addition of telomeric sequences (> > >) instead of circularization (III C).

no rearrangement of the chromosomal copies (8, 23). However, in mutant TarIIMTX1000.5, the size of the chromosome hybridizing to an H locus probe has increased from 800 to 930 kb (Figure 6). This increase in size was stable since a revertant of TarIIMTX1000.5, grown in absence of the drug, lost the circle but kept the chromosomal copy at 930 kb (not shown). In mutant TarIIMTX1000.4, the linear amplicon encoding *ltdh*, emerged at the first drug concentration to which cells were adapted, and the copy number of the amplicon increased with drug concentration (Figure 6).

MTX transport studies in MTX resistant mutants

Although H locus amplification is a frequent event during MTX selection, several MTX resistant mutants did not have *ltdh* gene amplification (Table 1). None of these mutants showed *neither* dhfr-ts amplification as determined by hybridization experiments (not shown), nor any other sign of gene amplification as observed by ethidium bromide stained gels of digested total DNA of various mutants (not shown). Since several *Leishmania* cells can resist MTX by decreased uptake of the drug (20−22) we have carried out transport experiments with mutants having or not amplicons containing *ltdh* (Table 1, Figure 7).

The steady-state accumulation of labeled MTX was measured in a selection of mutants resistant to 1000 μM MTX. The mutants TarIIMTX1000.4 and TarIIMTX1000.5 showed a 4.5-fold decrease in the steady-state accumulation of MTX (Figure 7A) whereas a much pronounced decrease in the accumulation of MTX was evident in a second class of transport mutants, represented by TarIIMTX1000.6 and TarIIMTX1000.7 (Figure 7A). In the latter two mutants, no amplified *ltdh* was detectable (Table 1). In general, mutants without *ltdh* amplification accumulate less MTX than mutants with *ltdh* amplification (see Table 1), but some mutants with *ltdh* amplification, such as TarII'MTX1000 and TarIIMTX1000.1 demonstrated a marked decrease in MTX accumulation (Table 1). The steady-state accumulation of MTX was also measured in mutants selected at lower drug concentration (100 μM MTX) from which the highly resistant mutants were derived. A significant decrease in the accumulation of MTX was already observed in TarIIMTX100.6 and TarIIMTX100.7, whereas only a two-fold decrease was observed in TarIIMTX100.4 and TarIIMTX100.5 (Figure 7B). The mutation responsible for the decreased accumulation of MTX in mutants TarIIMTX100.6 and TarIIMTX100.7 was retained in cells grown in absence of the drug for 120 generations.

MTX accumulation was also studied in mutants adapting to higher drug concentration to look at the relation between the degree of resistance and the level of MTX accumulation. Although there was a significant difference between the

TarIIMTX100.5 and 1000.5 mutants, the maximum reduced accumulation was reached rapidly in mutants resistant to 300 μM (Figure 7C). The revertant TarIIMTX1000.5rev has been grown in absence of MTX and has lost the *ltdh*-containing amplicon (Table 1). Its accumulation phenotype was similar to the mutant TarIIMTX1000.5 (Figure 7C) hence confirming that *ltdh* amplification has no role to play in the decreased accumulation of MTX (21, 43). Moreover, it indicated that this transport mutation, which is different to the mutation found in mutants TarIIMTX1000.6 and TarIIMTX1000.7, was also stable since it was retained in a mutant grown in absence of the drug for prolonged period.

DISCUSSION

Amplicon formation

De novo amplification of a short chain dehydrogenase gene is a frequent event in *L.tarentolae* cells selected for MTX resistance. Amplification of the H locus as part of a linear amplicon is described here for the first time in Figure 1. Southern blot analysis of the linear amplicon found in mutant TarIIMTX1000.4 indicates that it contains large inverted duplications apparently created by annealing of the 1.2 kb inverted repeat sequences A and B (Figure 4) during replication, as previously proposed for the circular amplicon found in strain TarII'MTX1000 (8). The annealing of the inverted repeats A and B (Figure 8-I) could be used as a primer for a polymerase to synthesize inverted duplication using the copy recently replicated as the template (Figure 8-II). Circles could be formed by ligation of the two inverted duplications. The length of the duplication could differ depending on whether the polymerase would reach the end of the replicated strand being used as the template (Figure 8III-A) or if the annealing of another pair of inverted repeats would stop the progression of the polymerase (Figure 8III-B, see also 8, 41). Linear amplicons, as found in mutant TarIIMTX1000.4, could be formed similarly. However, the amplicons would be stabilized and protected from cellular nucleases by the addition of telomeric sequences, probably by a telomerase like enzyme (44), instead of circularization (Figure 8-IIIC). Several factors such as culture and selection procedures could influence whether amplicons would be linear or circular.

The DNA sequences amplified varied considerably in the different mutants (results summarized in Figure 4) and mapping data indicated that some amplicons had more than 80 kb of unique sequences, representing therefore more than 10% of the whole chromosome. The variety of amplicons implies that different rearrangement points were used during amplicon formation, if we take into account that secondary rearrangements are rare in *Leishmania* (1). Mapping data will eventually reveal whether the amplicons that have long genomic sequences amplified share common rearrangement points. The diversity of amplicons generated from the H locus of *L.tarentolae* is in contrast with results reported previously in *L.major* strain LT252, where apparently the same rearrangement points were used in three uncloned populations selected for resistance to different drugs (23). If more mutants were studied more rearrangement points might have been found in *L.major*. Alternatively, sequences could be used preferentially because the repeated sequences necessary for the amplification of the *ltdh* gene might be confined to the H locus in *L.major*. Finally, since conflicting results exist on whether LT252 contains a low level of H circles or not (1, 7), it is difficult to exclude that the circles found in *L.major* were already present in a minority of the parental population. Nevertheless, it is clear that in *L.tarentolae*, as in *L.major*, there are also preferred rearrangement points. The majority of *ltdh*-containing amplicons presented here has inverted duplications possibly created by the annealing of the inverted repeat sequences A and B flanking the chromosomal H locus. These inverted repeats are much longer than the inverted repeats C and D (1.2 kb versus 198 bp; 8) and this might explain why rearrangements occurred more frequently at the level of repeats A and B. In yeast and in mammalian cells there is an increase in the frequency of gene targeting by homologous recombination with the length of homologous sequences (45, 46) and it is possible that the frequency of annealing of the inverted repeats is related to their length. The analysis of several amplicons derived from the H locus in arsenite selected mutants indicates that all were generated by homologous recombination between various direct repeat sequences and that all amplicons, except one, were formed where the direct repeats were the longest (11).

Resistance to MTX

Genes of the H locus of *L.tarentolae* are poorly expressed and when amplified they give only low resistance to methotrexate and or to arsenite (8, 14, 16). However, the *ltdh* gene confers high level resistance in transfectants when it is properly expressed (16). The difference between mutants and transfectants does seem less important in *L.major* (17, 23). *Ltdh* is frequently amplified in *L.tarentolae* despite that it only gives a three to four-fold increase in MTX resistance (8, 16), with the exception of the transfectant that has received the linear amplicon of TarIIMTX1000.4, which exhibits an eight-fold MTX resistance. In contrast, amplification of the *dhfr-ts* gene has never been observed in *L.tarentolae* MTX resistant mutants (not shown). One possible explanation for the absence of *dhfr-ts* containing amplicons in *L.tarentolae* could be that direct or inverted repeated sequences are not in the vicinity of the *dhfr-ts* gene hence reducing its likelihood of being amplified (3).

The mechanism by which *ltdh* confers resistance to MTX is not known, but it is clear that it is not involved in MTX transport (23, 43; see also Figure 7) and probably not in MTX modification (16, 47). We think that LTDH is capable to provide cells with reduced folates even if DHFR is blocked (3, 16, 41, 43). *Ltdh* amplification is not the only mechanism by which *Leishmania* can resist MTX and it often co-exists with transport mutations. Multiple co-existing mechanisms of MTX resistance have also been reported in mammalian cells (48, 49). In every mutant studied, a decrease in the steady-state accumulation of MTX was observed (Table 1). In *Leishmania* this decreased accumulation is due to a reduced uptake of the drug (21, 22). Two different transport mutant phenotypes were clearly discernable in our *Leishmania* MTX resistant mutants. The first category exhibited a 4.5-fold decrease in the accumulation of MTX whereas the reduction in accumulation was more pronounced in the second category (Figure 7, Table 1). Obviously, a 4.5-fold decrease in the accumulation of MTX is probably not sufficient to confer resistance to 1 mM MTX in TarIIMTX1000.4 and TarIIMTX1000.5 (20 fold the EC_{50} in SDM-79 medium). Indeed, in addition to this transport mutation, the *ltdh* gene was amplified in these mutants. Transfection of a linear amplicon containing *ltdh* in a wild type cell indicated that the amplicon is involved in drug resistance. *Ltdh* amplification can probably becomes dispensable with time if mutants are capable to resist MTX by other (more efficient) means (see Figure 2). This result

is in agreement with our previous proposal suggesting that the ability to amplify the H locus rapidly allow the cell to survive until it activates more efficient mechanism of MTX resistance (16, 41).

The second type of transport mutants showed a greater than 50-fold decrease in accumulation of MTX. The mutation responsible for this phenotype in the mutants studied arose early during the selection process. This mutation is possibly so efficient that the mutants do not require to amplify the *ltdh* gene to adapt to higher drug concentration. The *ltdh* gene is nevertheless amplified in mutants TarII'MTX1000 and TarIIMTX1000.1 despite the defect in MTX accumulation of these two mutants (Table 1). It is possible that prolonged culture in the presence of MTX (>4 years) has eventually selected for mutations that abolished MTX accumulation in these mutants. Nevertheless, these two strains have kept their amplicons, even if they seem dispensable. This could be attributed to the surprising stability of large circular amplicons in *Leishmania* (manuscript in preparation).

In conclusion, two types of transport mutation are found in the MTX resistant *Leishmania* cells studied. The type of transport mutation can partly influence whether gene amplification will be observed or not. Indeed, if the transport mutation can confer only a modest decrease in the steady-state accumulation of the drug, cells will have to find other ways (gene amplification) to survive when they are selected for resistance to higher concentration of the drug. Biochemical analysis has revealed that only one common folate-MTX transporter is operative in *Leishmania* (50). Therefore the mutations leading to the observed difference in MTX accumulation phenotypes in *Leishmania* might influence the same locus. The cloning of the folate-MTX transporter should help in elucidating the molecular mechanism of reduced accumulation of MTX in *Leishmania*.

ACKNOWLEDGEMENTS

We thank Katherine Grondin for help with experiments shown in Figure 3 and Dr Larry Simpson (UCLA) for further characterization of the *L. tarentolae* strains. We thank Dr L.Poulin for her critical reading of the manuscript. This work was supported in part by MRC, NSERC and an Etablissement de nouveau chercheur from FCAR to MO. This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. MO is a chercheur boursier junior of the Fonds de Recherche en Santé du Québec.

REFERENCES

1. Beverley, S.M. (1991). Ann. Rev. Microbiol. **45**:417–444.
2. Stuart, K.D. (1991). Parasitol. Today **7**:158–159.
3. Ouellette, M., and Papadopoulou, B. (1993). Parasitol. Today **9**: 150–153.
4. Beverley, S.M., Coderre, J.A., Santi D.V., and Schimke R.T. (1984). Cell **38**:431–439.
5. White, T.C., Fase-Fowler, F., van Luenen, H., Calafat, J., and Borst, P. (1988). J. Biol. Chem. **263**:16977–16983.
6. Petrillo-Peixoto, M.L., and Beverley, S.M. (1988). Mol. Cell. Biol. **8**: 5188–5199.
7. Hightower, R.C., Ruiz-Perez, L.M., Lie Wong, M., and Santi, D.V. (1988). J. Biol. Chem. **263**:16970–16976.
8. Ouellette, M., Hettema, E., Wust, D., Fase-Fowler, F., and Borst, P. (1991). EMBO J. **10**:1009–1016.
9. Wilson, K., Beverley, S.M., and Ullman, B. (1992). Mol. Biochem. Parasitol. **55**:197–206.
10. Hanson, S., Beverley, S.M., Wagner, W., and Ullman, B. (1992). Mol. Cell. Biol. **12**:5499–5507.
11. Grondin, K., Papadopoulou, B., and Ouellette, M. (1993). Nucleic Acids Res., **21**:1895–1901.
12. Stark, G.R., Debatisse, M., Giulotto, E., and Wahl, G.M. (1989). Cell **57**:901–908.
13. Liu, J., Salinas, G., Gajendran, N., Muthui, D., Muyldermans, S., and Hamers R. (1992). Mol. Biochem. Parasitol. **50**:351–354.
14. Ouellette, M., Fase-Fowler, F., and Borst, P. (1990). EMBO J. **9**:1027–1033.
15. Callahan, H.L., and Beverley, S.M. (1991). J. Biol. Chem. **266**:18427–18430.
16. Papadopoulou, B., Roy, G., and Ouellette, M., (1992). EMBO J. **11**:3601–3608.
17. Callahan, H.L., and Beverley, S.M. (1992). J. Biol. Chem. **267**:24165–24168.
18. Detke, S., Katakura K., and Chang K.-P. (1989). Exp. Cell Res. **180**:161–170.
19. Coderre, J.A., Beverley, S.M., Schimke, R.T., and Santi, D.V. (1983). Proc. Natl. Acad. Sci. USA **80**:2132–2136.
20. Dewes, H., Ostergaard, H.L., and Simpson, L. (1986). Mol. Biochem. Parasitol. **19**:149–161.
21. Ellenberger, T.E., and Beverley, S.M. (1987b). J. Biol. Chem. **262**:13501–13506.
22. Kaur K., Coons, T., Emmet, K., and Ullman, B. (1988). J. Biol. Chem. **263**:7020–7028.
23. Ellenberger, T.E., and Beverley, S.M. (1989). J. Biol. Chem. **264**:15094–15103.
24. Gomez-Eichelmann, M.C., Holz, G., Jr., Beach, D., Simpson, A.M., and Simpson, L. (1988). Mol. Biochem. Parasitol. **27**:143–158.
25. Bernards, A., van der Ploeg, L.H.T., Frasch, A.C.C., Borst, P., Boothroyd, J.C., Coleman, S., and Cross, G.A.M. (1981). Cell **27**:497–505.
26. Van der Ploeg L.H.T., Schwartz D.C., Cantor C.R., and Borst P. (1984). Cell **37**: 77–84.
27. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press.
28. Van der Ploeg, L.H.T., Liu, A.Y.C., and Borst, P. (1984). Cell **36**:459–468.
29. Laban, A., Finbarr Tobin, J., Curotto de Lafaille, M.A., and Wirth D.F. (1990). Nature **343**:572–574.
30. Iovannisci, D.M., and Ullman, B. (1983). J. Parasitol. **69**:633–636.
31. Aronow, B., Kaur, K., McCartan, K., and Ullman, B. (1987). Mol. Biochem. Parasitol. **22**:29–37.
32. Giulotto, E., Knights C., and Stark G.R. (1987). Cell **48**: 837–845.
33. Rice, G.C., Ling, V., and Schimke, R T. (1987). Proc. Natl. Acad. Sci. USA. **84**:9261–9264.
34. Hightower, R.C., Metge, D.W., and Santi, D.V. (1987). Nucleic Acids Res. **15**:8387–8398.
35. Beverley, S.M. (1988). Nucleic Acids Res. **16**:925–939.
36. Van der Bliek, A.M., Lincke, C.R., and Borst, P. (1988). Nucleic Acids Res. **16**: 4841–4851.
37. Cruz, A., and Beverley, S.M. (1990). Nature **348**:171–173.
38. Lee, M.G.-S., and van der Ploeg, L.H.T. 1990. Science **250**:1583–1586.
39. Ten Asbroek, A.L.M.A., Ouellette, M., and Borst, P. (1990). Nature **348**:174–175.
40. Eid, J.E., and Sollner-Webb, B. 1991. Proc. Natl. Acad. Sci. USA **88**:2118–2121.
41. Ouellette M., and Borst, P. (1991). Res. Microbiol. **142**:737–746.
42. Borst P., Baas F., Lincke C., Ouellette M., Schinkel A., Smit J. (1992). In proceedings Bristol-Myers Squibb Symposium on Cancer Research: Drug resistance as a biochemical target in cancer chemotherapy. **14**:11–26. Academic Press, New York, NY.
43. Papadopoulou, B., and Ouellette, M. (1993). In 'Chemistry and Biology of pteridines and folates' in Ayling, J.E., Nair MG., and Baugh, C.M. eds. Plenum Publishing Corporation, New York, in press.
44. Blackburn, E.H. (1991). Nature **350**: 569–573.
45. Rothstein, R. (1991). Meth. Enzymol. **194**:281–301.
46. Thomas, K.R., and Cappechi, M.R. (1987). Cell **51**: 503–512.
47. Ellenberger, T.E., Wright J.E., Rosowsky, A., and Beverley, S.M. (1989) J. Biol. Chem. **264**:15960–15966.
48. Assaraf, Z.I., Feder, J.N., Sharmal, R.C., Wright, J.E., Rosowsky, A., Shane, B., and Schimke, R.T. (1992). J. Biol. Chem. **267**:5776–5784.
49. Li, W.W., Lin, J.J., Tong, W.P.,Trippett T.M., Brennan M.F., and Bertino J.R. (1992). Cancer Res. **52**: 1434–1438.
50. Ellenberger, T.E., and Beverley, S.M. (1987). J. Biol. Chem. **262**:10053–10058.