Research Article

Stable Amplified DNA in Drug-Resistant *Leishmania* Exists as Extrachromosomal Circles

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The relative stability of amplified DNA in drug-resistant *Leishmania major* was previously reported to be dependent on location, that is, unstable amplified DNA was extrachromosomal and stable amplified DNA was chromosomal. Leishmanial chromosomes have now been directly examined by means of orthogonal-field-alternation gel electrophoresis (OFAGE). The amplified DNA's in three resistant cell lines displayed unusual migration and were clearly extrachromosomal, regardless of whether the amplified DNA's were stable or unstable. Thus, contrary to conclusions from earlier studies of drug resistance in cultured animal cells, stable amplified DNA in *Leishmania* can be extrachromosomal. In addition, these amplified DNA's were shown to be circular on the basis of their resistance to exonuclease III digestion and their behavior on OFAGE. Their mobility was also greatly changed after treatment with topoisomerase II, suggesting that the amplified DNA's were either supercoiled or concatenated circles.

A BASIC TENET OF GENE AMPLIFICATION IN DRUG-RESISTant cultured animal cells is that stability in the absence of continued drug pressure is determined by the location of the amplified gene (*1*). If the amplified genes occur on acentromeric, extrachromosomal elements (double minute chromosomes), they segregate randomly in actively dividing cells and are eventually lost. If the amplified DNA resides on a chromosome, that DNA will be stable in the absence of drug. We have previously reported on the stability of amplified DNA in drug-resistant *Leishmania major*, a parasitic protozoan (*2–4*). The results indicated that the stability of gene amplification in this protozoan mimicked aspects of what had been found in cultured animal cells.

In our initial study of the molecular details of drug resistance in *Leishmania* (*2*), *L. major* promastigotes were selected for resistance against methotrexate (MTX), a potent inhibitor of dihydrofolate reductase. The resistant cell line overproduced the bifunctional protein thymidylate synthase–dihydrofolate reductase (TS-DHFR), with amplification of a 30-kilobase (kb) region of DNA (R-region DNA) (*3*) that contained the gene for TS-DHFR (*5, 6*). The level of R-region DNA was approximately 100 times that of the wild-type copy number. The R-region was apparently generated by the joining of two regions of DNA separated by about 30 kb in wild-type organisms, yielding a rearranged "junctional" region in the amplified DNA. In cells grown in 1 m*M* MTX for 3 months (R1000-3 cells), a significant fraction of amplified R-region DNA existed as a 30-kb extrachromosomal, supercoiled circle, as shown by sedimentation in cesium chloride–ethidium bromide (EtBr) equilibrium gradients, gel electrophoresis in 0.4 percent agarose, and limited deoxyribonuclease I (DNase I) digestion (*3*). When the resistant cells were grown in 1 m*M* MTX for 11 or more months (R1000-11 cells), most of the amplified DNA cosedimented with chromosomal DNA and did not enter a 0.4 percent gel upon electrophoresis; on the basis of restriction site map analysis, the amplified DNA was believed to have been incorporated into chromosomal DNA as a repetitive array of the 30-kb unit (*3*). In support of this was the observation that the amplified DNA in R1000-11 cells persisted when MTX selection pressure was withdrawn. In contrast, the R1000-3 amplified unit was unstable when MTX was removed from the culture medium. Another amplified DNA (H-region DNA) that was found in MTX-resistant cells has yet to be assigned a function. The amplified H-region DNA was also proposed to initially exist as an unstable, extrachromosomal circle in R1000-3 cells, and then to partly relocalize into chromosomes as stable DNA in R1000-11 cells.

Leishmania major promastigotes were also selected for resistance against 10-propargyl-5,8-dideazafolate (CB3717) (*4*), an inhibitor of thymidylate synthase. These cells resembled the R1000 cells in that they overproduced TS-DHFR by amplification of the R-region DNA; however, CB3717-resistant cells did not have amplified H-region DNA. The restriction site map of the R-region DNA in these cells was nearly identical to the R1000 R-region map, except for a slight increase in size of the fragment that contains the rearranged junction of the R-region. When DNA was fractionated by electrophoresis in 0.4 percent agarose, the R-region DNA from cells resistant to low levels of CB3717 (0.8 and 2 μ*M*) migrated with a similar mobility to the extrachromosomal circle found in R1000-3 cells. But in cells grown in 50 μ*M* CB3717 for 2 months (CB50-2 cells), the R-region DNA migrated as higher molecular weight forms of DNA in low percentage agarose gels. Most, but not all, of the CB50-2 amplified DNA was shown to be unstable when CB3717 was removed from the medium; approximately 90 percent of the amplified DNA was lost in cells 125 generations removed from drug. On the basis of the electrophoretic properties, we assumed that the unstable amplified DNA's were high molecular weight, extrachromosomal forms, and that the stable DNA had been integrated into a chromosome or chromosomes.

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We now refine our earlier report concerning the location and nature of the stable amplified DNA's in R1000-11 cells and present our results obtained when DNA from resistant cells was analyzed by orthogonal-field-alternation gel electrophoresis (OFAGE). This technique (*7*) is at present capable of fractionating high molecular weight DNA's up to 4000 kb. In addition, we have taken advantage of a gentle isolation procedure of DNA in which intact cells are embedded in agarose and then lysed in situ (*8*); breakage of DNA is therefore greatly reduced. Enzymatic modifications of the DNA embedded in agarose blocks were possible, thereby allowing the amplified DNA to be characterized.

Leishmanial chromosomes. DNA's from wild-type *L. major* promastigotes and from *Saccharomyces cerevisiae* were subjected to electrophoresis in adjacent lanes (Fig. 1A). Most of the chromosomes of *L. major* are similar in size to those found in yeast. We used a number of different pulse times and were able to resolve 14 different EtBr-staining bands that ranged in size from approximately 300 to 2000 kb (Fig. 1B); this compares well with a recent report in which a similar technique, pulsed-field electrophoresis (*8*), was used to separate 17 leishmanial chromosomes (*9*). Some of the chromosomal bands were noticeably more intense than others, and were believed to represent either unresolved chromosomes or aneuploidism within *Leishmania*. When Southern blots were probed with the complementary DNA (cDNA) for the bifunctional protein TS-DHFR (*6*), a single chromosome (chromosome 4) displayed hybridization; likewise, only chromosome 6 hybridized when the H-region probe (*3*) was used. Therefore, we concluded that these represented the chromosomal locus of the gene for TS-DHFR and the chromosomal location for the H-region DNA, respectively.

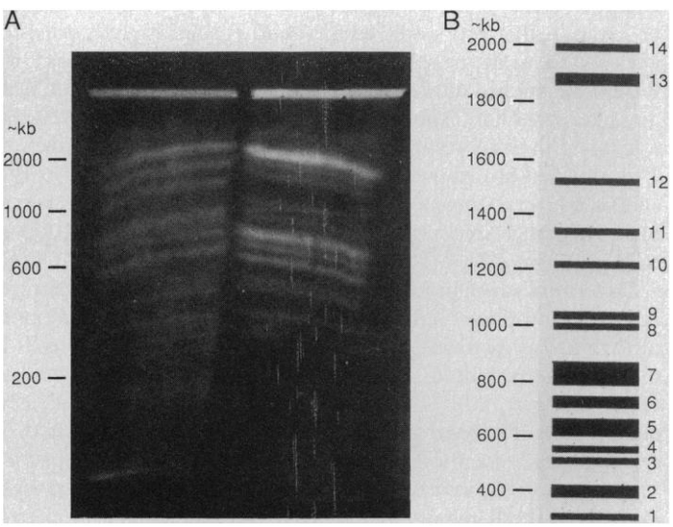

Fig. 1. Chromosomal gel of genomic DNA from *Leishmania*. (A) OFAGE of genomic DNA's (about 6 μg) from *S. cerevisiae* (left lane) and from wild-type *L. major* (right lane). Wild-type *L. major* cells refer to promastigotes (strain 252; Iran) that were cloned on agar plates and then grown continuously in culture for 3 months prior to this study. Agarose blocks containing DNA from *L. major* were prepared by slightly modifying the cell-handling procedure of Schwartz and Cantor (*8*). *Leishmania major* promastigotes grown to late log phase were harvested and washed once with phosphate-buffered saline; the cells (approximately 4×10^7) were then placed in 0.6 ml of 0.6 percent low-melting agarose blocks (agarose from Bio-Rad). The cells were lysed in 0.5*M* EDTA, *p*H 9.0, 1 percent lauroyl sarcosine, and proteinase K at 1 mg/ml for 2 days at 50°C. DNA blocks were washed thoroughly and stored at 4°C in 0.2*M* EDTA, *p*H 8.0. OFAGE was performed essentially as described by Carle and Olson (*7*). Electrophoresis was performed in 0.6× TBE (56 m*M* tris, 56 m*M* boric acid, 1.2 m*M* EDTA), *p*H 8.4, for 22 hours including a 40-second pulse time for 9 hours, an 80-second pulse time for 11 hours, and a 120-second pulse time for 2 hours. In all OFAGE gels described in this article, the buffer was changed at approximately the half-way point of electrophoresis to achieve optimal resolution. (B) A composite of *L. major* chromosomes, drawn from several OFAGE gels in which various pulse times were used. Chromosomes are numbered from 1 to 14, with number 1 being the fastest migrating chromosome. The approximate sizes were taken from the sizes of the yeast chromosomes (*25*).

Amplified DNA in drug-resistant *L. major* is extrachromosomal. When wild-type and R1000-11 DNA's were analyzed by OFAGE, two bands absent in wild-type DNA were readily apparent in R1000-11 DNA (Fig. 2A). When compared with the chromosomal DNA, these two new bands had an unusual banding pattern that was most apparent when they were actually superimposed on chromosomal DNA. The bands were tightly compressed and, in our system, skewed to the right side of the lanes when compared with linear chromosomes. We refer to them as tight bands. Probing the Southern blot with the cDNA for TS-DHFR showed that the faster migrating tight band was the amplified R-region DNA (Fig. 2B). Several minor, slower migrating bands also hybridized to the cDNA probe, although it is not easily seen in this particular Southern blot. The major band is approximately 30 times the wild-type copy number. The remainder of the amplified DNA remained in the gel slots (see below), so that the total R-region copy number was approximately 100. The cDNA probe also showed that the chromosomal locus for the TS-DHFR gene in wild-type cells remained in the R1000-11 cells. When the Southern blot was again probed with an H-region–specific probe (*3*), the slower migrating tight band in the R1000-11 DNA was seen to be the amplified H-region. There is also a tight band in wild-type DNA; it is seen in Fig. 2A, positioned above the tight bands in R1000-11 DNA. When the Southern blot was probed with H-region DNA, this tight band hybridized. We cannot explain this observation at the present time.

R1000-3 genomic DNA, containing the unstable amplified R-region DNA, was directly compared to R1000-11 DNA (Fig. 3A). The amplified R-region DNA's in the R1000-3 cells also appeared as extrachromosomal tight bands. When the Southern blot was probed with the cDNA for TS-DHFR (Fig. 3B), three species of the R-region DNA were apparent. The major R-region tight band in R1000-3 cells moved at a noticeably slower rate than the major tight band in R1000-11 cells and was approximately three times more intense than were either of the two minor bands; the total cDNA hybridization indicated that the R-region copy number was approximately 75 times greater than in wild-type cells. A single H-region tight band was present in both resistant cell lines (Fig. 3C). When this autoradiogram was further exposed, hybridization was also discernible to what we believe is the chromosomal locus of the H-region DNA (chromosome 6). When DNA from R1000-3 cells that had been grown in MTX-free medium for 80 generations was analyzed, each of the three R-region tight bands was approximately 40 percent of the original level, confirming that each of these amplified DNA's was unstable. Specific activity of TS-DHFR was approximately 30 percent of the original at the same point of MTX withdrawal. In contrast, electrophoresis of DNA from R1000-11 cells that were removed from selection pressure for 80 generations showed no differences in the R-region DNA when compared to R1000-11 R-region DNA, and TS and DHFR levels were the same compared to R1000-11 enzyme levels. Thus, we concluded that both the stable amplified DNA in R1000-11 cells and the unstable amplified DNA in R1000-3 cells are extrachromosomal.

To establish similarities and differences between the amplified DNA's in two resistant lines that had been independently selected, DNA's from R1000-11 cells and from CB50-10 cells (cells grown in 50 μ*M* CB3717 for 10 months) were examined by OFAGE (Fig. 4A). The CB50-10 cells showed two tight bands of approximately equal molar amounts; the faster moving band migrated with a mobility similar to the R1000-11 R-region DNA. Both of the

CB50-10 tight bands hybridized to the cDNA for TS-DHFR (Fig. 4B). (The slower migrating CB50-10 tight band moved with a mobility similar to the slowest migrating, major tight band in R1000-3 cells.) The cDNA for TS-DHFR also hybridized to the chromosomal locus of TS-DHFR (chromosome 4). When the filter was washed and again probed with the specific probe for the H-region, chromosome 6 (the chromosomal locus for the H-region DNA) showed hybridization; no H-region tight band was observed in CB50 cells.

We had previously reported that CB50-2 cells (cells grown in 50 μM CB3717 for 2 months) had relatively unstable DNA, although neither the amplified DNA nor TS-DHFR enzyme levels reverted completely back to those of wild-type (approximately 90 percent of the amplified DNA and 90 percent of the overproduced TS-DHFR were lost) (*4*). DNA from CB50-2 cells grown for 250 generations in the absence of CB3717 was examined by OFAGE, and subsequently showed cDNA hybridization to three locations: the chromosomal locus of TS-DHFR, the faster migrating CB50 tight band, and the gel slot. The total cDNA hybridization to the tight band and the gel slot positions was approximately five times that hybridizing to the chromosomal locus, which is in agreement with the R-region DNA copy number reported previously in this cell line (*4*). Even when CB50 cells had been exposed to drug for 10 months, the amplified R-region did not gain significant stability; this is compared with R1000-11 amplified DNA that had acquired stability. The amplified DNA from CB50-10 cells grown 85 generations in the absence of drug showed the same tight band and hybridization patterns as displayed in Fig. 4, but the relative amount was only 50 percent of that found in CB50-10 cells. Both TS and DHFR show similar decreases: levels were approximately 70 percent at 50 generations removed and approximately 30 percent at 100 generations removed from drug.

A third independently selected resistant cell line with amplified R-region DNA has been examined by OFAGE. A cloned *L. major* cell line (D7B *L. major*) was used to select for resistance to MTX, and cells resistant to 1 mM MTX (D7BR1000 cells) have been obtained (*10*). The amplified R region in D7BR1000 cells is considerably larger (about 37 kb) than either the original R1000 or CB50 R-regions (about 30 kb). When DNA from D7BR1000 cells was examined by OFAGE, transferred to nitrocellulose, and probed with the cDNA for TS-DHFR, two R-region tight bands were seen by both EtBr stain and Southern blot analysis (*10*). The mobility of both tight bands was slightly decreased when compared to the R1000-11 R-region tight band. As is the case in all resistant cells, the chromosomal locus of TS-DHFR remained present in D7BR1000 cells. No amplified H-region DNA was apparent in D7BR1000 cells. Finally, the amplified R-region DNA in D7BR1000 cells grown in 1 mM MTX for 1 month was relatively unstable. After 80 generations in drug-free medium, approximately 80 percent of both the R-region copy number and TS-DHFR enzyme had been lost; when the DNA from these cells was examined by OFAGE, both tight bands showed the same decrease in the intensity of hybridization to the cDNA of TS-DHFR.

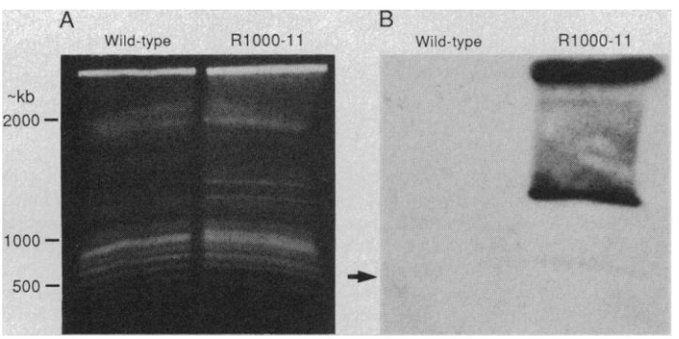

Fig. 2. Demonstration that stable amplified DNA in R1000-11 cells is extrachromosomal. OFAGE of genomic DNA (about 6 μg) isolated from wild-type and R1000-11 *L. major*. (A) EtBr-stained gel; (B) the Southern blot probed with the cDNA for TS-DHFR. The arrow denotes the location of the chromosomal locus for the gene for TS-DHFR. Experimental details same as described in Fig. 1, except that OFAGE gel was run for 22 hours, at a pulse time of 120. After visualization with EtBr, gels were soaked in 0.5N HCl (twice for 20 minutes each time); 1N NaOH, 1.5M NaCl (twice for 20 minutes); and 1M tris, pH 8.0, 1.5M NaCl (three times for 20 minutes). Transfer to nitrocellulose and hybridizations were performed as described (*26*). Plasmid containing the cDNA for TS-DHFR has been described (*4*). Quantitation of autoradiogram was performed by densitometry.

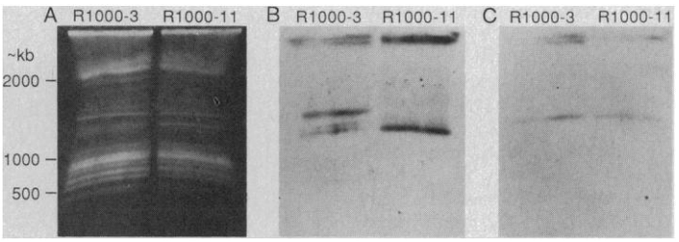

Fig. 3. Comparison of R1000-3 amplified DNA (unstable) and R1000-11 amplified DNA (stable). OFAGE of genomic DNA (about 6 μg) isolated from R1000-3 and R1000-11 cells. (A) The EtBr-stained gel; (B) the Southern transfer probed with the cDNA for TS-DHFR; (C) the transfer probed again with the H-region-specific probe. Experimental details are the same as described in Fig. 2, except that after probing with the cDNA, the filter was placed in boiling water (twice for 10 minutes each) before probing with the H-region-specific probe. The arrow indicates the position of chromosome 4 which hybridizes to TS-DHFR cDNA but may not be apparent in the reproduction.

To summarize these results, we have shown that both stable and unstable amplified DNA's in three resistant cell lines are extrachromosomal, and are observed as bands with unusual migration. In addition, we have shown that on the basis of differing mobility upon OFAGE, each cell line has more than one species of amplified DNA. Even though one predominant species of tight band is present in the R1000-11 DNA, tight bands corresponding to unstable, amplified R-region DNA migrate with a similar mobility. Thus, at this time, we cannot equate mobility of DNA in OFAGE gels with stability (*11*).

Characterization of the tight bands. We investigated the size and structure of the stable amplified DNA in R1000-11 cells that migrated as tight bands. Unusual banding patterns have been observed when double-stranded (ds) RNA is analyzed by OFAGE

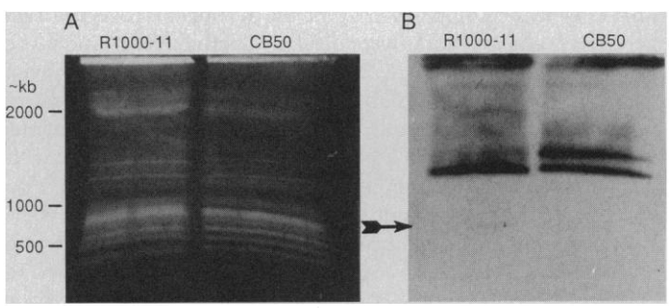

Fig. 4. Comparison of amplified DNA's from two drug-resistant *L. major*: R1000-11 and CB50-10 DNA's. OFAGE of genomic DNA (about 6 μg) isolated from R1000-11 and CB50-10 cells. (A) The EtBr-stained gel and (B) the transfer probed with the cDNA for TS-DHFR. Experimental procedure is the same as described in Fig. 2. The arrow indicates the position of chromosome 4 which hybridizes to TS-DHFR cDNA but may not be apparent in the reproduction.

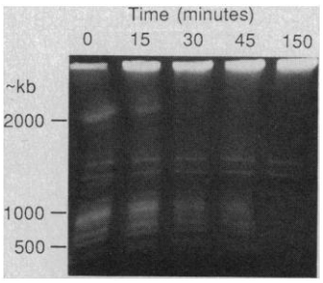

Fig. 5. Demonstration that R1000-11 amplified DNA is circular. OFAGE of indicated time points from a reaction of exonuclease III with R1000-11 DNA blocks. R1000-11 DNA blocks (about 2 μg of DNA per block) were equilibrated (three times for 3 hours each) with exonuclease III buffer (66 m*M* tris, *p*H 8.0, 1 m*M* dithiothreitol, 0.66 m*M* $MgCl_2$). Blocks were then added to 2.5 ml of buffer containing 130 units of exonuclease III (BRL), and incubated at 37°C. Blocks were added to 0.2*M* EDTA, *p*H 8.0 (to stop reaction) at indicated times.

(*12*). The tight bands were not dsRNA, because they were resistant to ribonuclease (RNase); as a positive control, dsRNA (*13*) was embedded in agarose and shown to be RNase-sensitive. We noticed that when pulse times were shifted to resolve different chromosomes, the tight bands migrated aberrantly with respect to the chromosomal bands. At the 120-second pulse time shown in Fig. 1, the amplified DNA containing the TS-DHFR gene migrated between chromosomes 11 and 12 (apparent size of 1300 kb). When electrophoresis was performed at pulse times of 45 seconds and then 90 seconds, this amplified DNA banded between chromosome 7 and 8 (about 800 kb). At a 40-second pulse time, the amplified DNA migrated between chromosome 4 and 5 (about 550 kb). Thus, at pulse times varying between 40 and 120 seconds, the apparent size of the tight band changed some 700 kb. Subsequently, we learned that supercoiled circles are not much affected by a change in pulse times; they migrate as a function of electrophoretic duration and presumably size (*14*). We realized that the tight bands were behaving in a similar manner; that is, they were not responding to the changes in pulse time. The apparent changes of size were in fact due to the differing mobilities of the chromosomes. Therefore, we attempted to demonstrate directly that the tight bands were circular DNA. When DNA agarose blocks from R1000-11 cells were incubated with exonuclease III and then analyzed by OFAGE, there was a time-dependent disappearance of all chromosomal bands but not the two tight bands (Fig. 5). By probing the Southern transfer with the TS-DHFR cDNA or the H-region probe, we confirmed that both amplified DNA's were resistant to exonuclease III activity. It was also apparent on the Southern blots that the amplified DNA that remained in the gel slots was unreactive to exonuclease III digestion. We therefore concluded that the tight bands were circular DNA.

In order to determine the size of the R1000-11 R-region tight band, DNA agarose blocks were incubated with limiting amounts of Eco RI; Eco RI cleaves the 30-kb unit of R-region DNA twice, generating fragments of 28 kb and 2 kb (*3*). If the tight band were the amplified 30-kb supercoiled circle, then the limited digest would produce only the linear 30-kb DNA and the 28-kb fragment. If the tight band were a circular multimer of the 30-kb unit, then a limited digest would show linear multiples of the unit size. If the 100 copies of the R-region DNA had been incorporated into chromosome or chromosomes as a repetitive array (as was previously proposed for R1000-11 amplified DNA), then the digest would produce an extensive ladder of R-region DNA when subjected to OFAGE, and would consist of multiples of 30 kb. There was a progressive decrease in the R-region DNA in the tight band and, although not apparent in Fig. 6, also in the wells. The only significant new R-region DNA generated from the limited digest appears to be the linear 30-kb unit and the 28-kb fragment (Fig. 6); a faint band that appears to be approximately 60 kb in size is seen when the autoradiogram is extensively exposed. (The minor bands of R-region DNA that migrate more slowly than the tight band alluded to previously are more noticeable in Fig. 6.) The final time point shows complete digestion of the R-region DNA; only the 28-kb fragment remains. The above results were confirmed when R1000-11 DNA agarose blocks were digested with limited amounts of Not I (*15*), a restriction enzyme that cleaves the R-region DNA once (*3*); however, slightly different results were observed. In addition to the predominant 30-kb linear DNA expected and a small amount of a 60-kb intermediate, an equally small amount of a second intermediate was produced (slightly larger and estimated to be about 90 kb). The ratio of intermediates to 30-kb R-region was 1 to 1 to 4. The above data suggest that the R1000-11 amplified DNA exists predominantly as a monomer of the 30-kb R-region, with small amounts of multimeric forms (probably dimer and trimer).

There is considerable hybridization of the cDNA for TS-DHFR to the gel slots. Early in these studies, it was considered possible that the amplified DNA had been incorporated into a very large chromosome as a repetitive array, and that the chromosome does not migrate from the origin. However, because the Eco RI–limited digest of R1000-11 DNA did not produce a ladder of R-region DNA, we think this explanation for the gel slot hybridization unlikely. It is more probable that the hybridization results in part

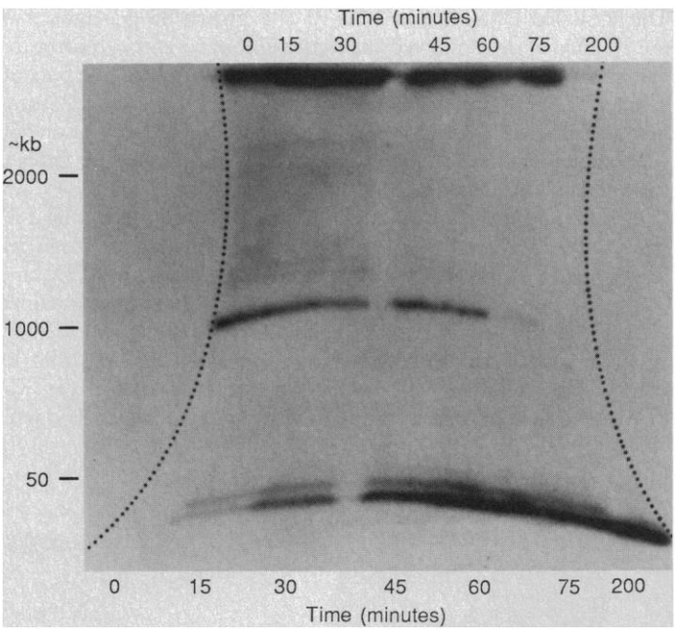

Fig. 6. Determination of the size of the amplified DNA in R1000-11 cells. Indicated time points from a reaction of limiting amounts of Eco RI with R1000-11 DNA blocks were analyzed by OFAGE; the subsequent Southern transfer was probed with the cDNA for TS-DHFR. The times of digestion are indicated both at the top and bottom, and the curvature of migration is outlined to aid in viewing. R1000-11 DNA blocks (about 2 μg of DNA per block) were equilibrated (three times for 3-hour periods) with Eco RI buffer (33 m*M* tris acetate, *p*H 7.9, 66 m*M* potassium acetate, 10 m*M* magnesium acetate). Blocks were then added to 3 ml of buffer containing 30 units of Eco RI (New England Biolabs) and incubated at 37°C. Blocks were placed into 0.2*M* EDTA, *p*H 8.0 (to stop reaction), at indicated times. After 75 minutes, 100 units of Eco RI was added, and the final DNA block was incubated for an additional 2 hours. The fragments generated after 15 minutes of digestion represent less than 5 percent of the total amplified DNA; a criterion that a limited digest was achieved. Densitometric quantitation of the Southern blot also showed a progressive decrease of R-region DNA in the slots (not apparent in the reproduction), demonstrating partial digestion of these DNA's; all DNA was digested after the final time point. Uncut lambda DNA was subjected to electrophoresis in the center lane and migrated slower than both DNA's generated by Eco RI.

from the random entrapment of DNA within the agarose block. [Many reports of results based on either OFAGE (*7, 16*) or pulsed-field electrophoresis (*8, 9, 17*) contain examples of nonspecific sticking of DNA to the gel slots.] In addition, nicked circular DNA has a tendency to be entrapped in the agarose (*18*). The well hybridization, therefore, may represent nicked supercoiled circles (*19*). Finally, this lack of migration may result from a higher order structure of the amplified DNA (concatenated circles or a form unknown) that impedes movement out of the block. However, more information concerning the species of DNA which remain at the origin in this system is needed.

In order to determine whether the structure of the R-region amplified DNA was either supercoiled or concatenated circles, we incubated R1000-11 DNA agarose blocks with topoisomerase II (*20*). This enzyme makes an opening in dsDNA that allows for both the relaxation of supercoiled circles and also the release of concatenated circles. In samples treated with topoisomerase II, both the R-region and H-region tight bands disappeared (by both EtBr and hybridization criteria), with no effect on the chromosomal DNA, and the hybridization intensity at the gel slot increased by the amount that was originally present in the tight bands. This particular experiment unfortunately does not clarify why the amplified R-region sticks to the gel slots. It does, however, suggest that the R- and H-region tight bands represent either supercoiled or concatenated circles.

These enzymatic modifications of the DNA entrapped in agarose blocks were also used to examine the tight bands found in the other resistant cell lines. In all cell lines, the tight bands were resistant to exonuclease III. When CB50-10 DNA blocks were digested with Eco RI, the reaction produced an intermediate (about 60 kb) in addition to the 30-kb and 28-kb R-region DNA's (Fig. 7). The presence of the 60-kb intermediate suggested that some of the amplified R-region DNA exists in a dimeric form, consistent with what had been previously proposed (*4*). When D7BR1000 DNA agarose blocks were digested, the results showed only the pattern expected of the monomeric R region; no intermediate-sized DNA's were observed. And when R1000-3 DNA blocks were digested with Not I, a small amount of a 60-kb intermediate was observed in addition to the linear 30-kb DNA (ratio of 1 to 10 in favor of the monomer). Finally, when DNA blocks from R1000-3, CB50-10, or D7BR1000 cells were incubated with topoisomerase II, the tight bands were no longer visible, with no effect on the linear chromosomes; and the cDNA hybridization to the gel slots increased by the amount originally present in the tight band. We regarded these results as demonstration that the structure and sizes of the amplified DNA's in all three different resistant cells were qualitatively similar.

Finally, the amplified H-region DNA's in the R1000-3 and R1000-11 cells, which migrated as tight bands, were resistant to exonuclease III and remained in the well when treated with topoisomerase II. In addition, the H-region DNA appeared to consist only of a monomeric form. Thus, we have described two independent amplified DNA's in these cells, both of which are extrachromosomal and circular; these characteristics may be a signature for gene amplification in drug-resistant *L. major*.

Additional alteration of chromosomal DNA in MTX-resistant cells. A final observation from the OFAGE analysis of these resistant cells is worth noting; when gels were run at short pulse times, an EtBr-staining band (about 300 kb in size) was visible between chromosomes 1 and 2 in MTX-resistant cells that was absent in both wild-type and CB3717-resistant cells. The intensity of the EtBr stain was equal to the intensity of any single chromosome. Not only was this band present in R1000-3, R1000-11, and D7BR1000 cells, but it was stable in these cells even when grown in the absence of MTX. This band did not have the unusual characteristics of the tight

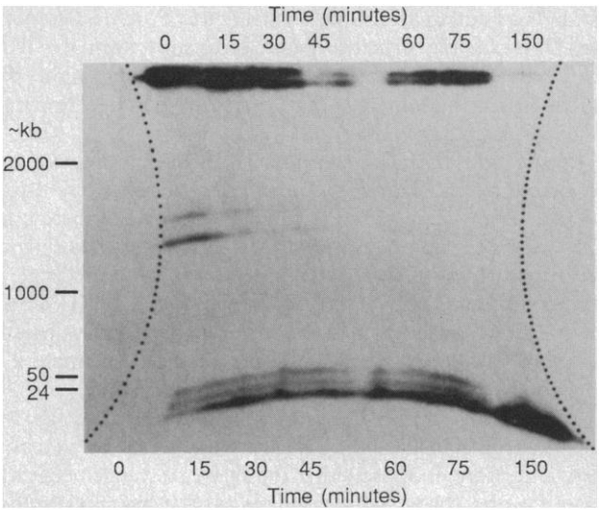

Fig. 7. Determination of the size of the amplified DNA in CB50-10 cells. Experimental procedures and figure explanations are the same as described in Fig. 6, except that the final time point indicates 75 minutes of incubation after 100 units of Eco RI was added to the initial 75 minutes incubation; and, in addition to uncut lambda DNA, a Hind III digest of bacteriophage lambda DNA was subjected to electrophoresis in the center of the gel to aid in the sizing of the DNA's generated by Eco RI.

bands; it was sensitive to exonuclease III, and therefore linear, and its migration was unaffected by topoisomerase II. Also, the band did not hybridize to either the cDNA for TS-DHFR or the H-region–specific probe. In addition, in the MTX-resistant cell lines, assigned bands 5 and 7 have been resolved into two chromosomes each; these bands appear to represent unresolved chromosomes in wild-type cells (Fig. 1B). Thus, it appears that significant chromosomal alterations have occurred in the MTX-resistant cells, in addition to the rearrangements that led to the formation of the R-region DNA. At present, we do not know how these alterations originated or if they are functionally important to resistance.

Possible mechanisms. We have applied the technique of orthogonal-field-alternation gel electrophoresis to fractionate leishmanial chromosomes; this has allowed us to directly examine the amplified DNA in various *L. major* cells which are resistant to antifolates. We have been able to both confirm and extend our previous observations regarding the size, structure, and location of the amplified DNA's. Although many questions still remain, we can justify the following conclusions.

1) Most significantly, both stable and unstable amplified DNA's in drug-resistant *L. major* are extrachromosomal. We had previously concluded that only unstable amplified DNA was extrachromosomal, and that the mechanism of mitotic stability of the amplified R-region DNA was insertion of the amplified DNA into a chromosomal locus as a repetitive array (*3*). This is not the mechanism by which stability is gained. Other possible explanations are explored below.

2) The amplified R-region unit that contains the gene for the bifunctional protein TS-DHFR exists as circular DNA. These circles may exist as individual supercoiled circles, or as concatenated circles, or as an unrecognized structure consisting of circles. Clearly, none of the resistant cells described here have amplified DNA that exists as a large, repetitive array.

3) All resistant cells examined in our study have at least two different species of R-region DNA. R1000-11 cells have one predominant tight band (this DNA is stable in the absence of MTX); the other cell lines, however, have significant amounts of amplified R-region that have slightly different mobilities on

OFAGE. We hope to determine the structure of these different tight bands. One possible structure based on results from the limited endonuclease digests is that the slower migrating tight bands represent circular multimers of the 30-kb R-region unit (existing as a 60- or 90-kb circular DNA).

4) Initially, the amplified R-region DNA has no inherent stability; stability must be acquired. Three independent selection procedures have led to a similar amplified unit, and none of these units is initially stable. In the original study stability was gained only after cells were grown for many months in the presence of the drug. This acquisition of stability was not repeated when CB3717-resistant cells were grown in the presence of CB3717 for a similar length of time. These CB50 cells are being maintained to determine if and when stability of the amplified DNA is acquired.

5) In all the resistant cell lines examined in our study, the wild-type chromosomal locus of TS-DHFR remains present on chromosome 4. Therefore, it appears that the DNA rearrangements which generate the extrachromosomal circles leave the chromosomal locus unchanged.

This appears to be the first demonstration in eukaryotic cultured cells of stable, amplified DNA that is extrachromosomal and circular. As such, factors must be present which permit autonomous replication and, in the stable R1000-11 cells, confer mitotic stability. Although we expect that the details of these factors may prove to be unusual, insight is gained by consideration of better understood systems. As in other systems studied (*21*), the amplified extrachromosomal DNA's in *L. major* probably possess a sequence which is responsible for autonomous replication; the copy number increases during selection and is subsequently maintained through many generations of growth. In other eukaryotic systems studies, stability of plasmids and chromosomes requires mitotic-stabilizing sequences. These are exemplified by sequences of the yeast 2-μm circle (*22*), of the *S. cerevisiae* centromere (CEN) DNA (*23*), and of the bovine papilloma virus (*24*); CEN sequences also control segregation during meiosis. In some systems, copy number and size also play a role in stability of plasmids (*17, 24*).

Regarding the stability of the extrachromosomal circular DNA in *L. major* R1000-11 cells, we can now say the following. (i) The stability is acquired. We have shown that upon continued selection in MTX, unstable amplified DNA changes to a stable form without alteration in physical form. (ii) The stability is probably not exclusively due to high copy number or size. The copy number and size of the amplified DNA's are similar in both the unstable and stable cell lines. (iii) We propose that stability of MTX resistance in *L. major* involves acquisition of mitotic-stabilizing sequences in the extrachromosomal, circular DNA. How similar such sequences would be to mitotic-stabilizing sequences such as CEN is unknown. Because amplified DNA's from both unstable and stable resistant cells are available for us to study, we hope to identify these putative mitotic-stabilizing sequences if they exist. Whatever the mechanism, the acquisition of stability is not as simple as previously envisioned.

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11. It should be noted that the multiplicity of tight bands in the various cells might reflect heterogenous populations; of the resistant cells described in this article, only the CB50-10 have been cloned (CB50 cells were cloned on agar after 7 months of growth in 50 μ*M* CB3717).
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15. Not I reaction: R1000-11 DNA agarose blocks were equilibrated (three washings for 3 hours each) with Not I reaction buffer [150 m*M* NaCl, 6 m*M* tris-HCl (*p*H 7.9), 6 m*M* $MgCl_2$, 6 m*M* 2-mercaptoethanol, bovine serum albumin (BSA) at 100 μg/ml, 0.01 percent Triton X-100]. Blocks were then added to 3 ml of buffer containing 30 units of Not I (New England Biolabs) and incubated at 37°C. Time points were taken by putting the DNA block into 0.2*M* EDTA, *p*H 8.0.
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19. It has been pointed out to us by a reviewer that the exonuclease III (exo III) digestion might provide information concerning the nature of the material in the gel slots. It was argued that if exo III failed to react with the R-region DNA remaining in the slots, as indicated by Southern blot analysis of the gel in Fig. 5, then this material must also be circular, possibly a nicked circle. Two facts obscure the interpretation of these results. First, exo III possesses a nuclease activity toward nicked circles; the end product of this reaction is a single-stranded circle. The second fact is apparent upon close examination of Fig. 4. There is a time-dependent increase in the intensity of EtBr-staining within the gel slots, as if the partially digested linear DNA's were sticking to the slots.
20. Topoisomerase II (Topo II) reaction: R1000-11 DNA agarose blocks were equilibrated (three times for 3 hours each) with Topo II reaction buffer [40 m*M* tris (*p*H 7.8), 60 m*M* KCl, 10 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol (DTT), 0.5 m*M* EDTA), 0.5 m*M* adenosine triphosphate (ATP), and 30 μg/ml of BSA]. Blocks were added to 1.5 ml of buffer containing Topo II at 0.32 μg/ml, and incubated at 30°C for 120 minutes. The reaction was stopped by adding block to 0.2*M* EDTA, *p*H 8.0.
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