Electron Microscopy of Amplified DNA Forms in Antifolate-resistant Leishmania

Robin C. Hightower, Mei Lie Wong, Luis Ruiz-Perez, and Daniel V. Santi

From the Departments of Biochemistry and Biophysics and of Pharmaceutical Chemistry, University of California, San Francisco, California 94143 and the Howard Hughes Institute, University of California, San Francisco, California 94143

Three independently derived antifolate-resistant Leishmania major cell lines overproduce the bifunctional protein thymidylate synthase-dihydrofolate reductase (TS-DHFR) by amplification of a region of DNA (R-region DNA) that contains the gene for TS-DHFR. On orthogonal-field-alternation gel electrophoresis (OFAGE), the extrachromosomal R-region DNAs are circular molecules, and different forms of R-region DNA within these cell lines are resolved. The R-region DNAs migrate aberrantly on OFAGE with respect to linear DNA and supercoiled plasmid standards. We describe a method for the isolation of these R-region DNA forms from OFAGE. By electron microscopy, we show that the extrachromosomal elements are single supercoiled circular DNA molecules, and are predominantly circular monomers and dimers of the original R-region DNA amplification unit. Using OFAGE, an analysis of cloned isolates shows that individual cells may contain multiple forms of R-region DNA. Furthermore, within a given cell line, certain distinguishable forms appear to have the same size and restriction map, suggesting they may be topoisomers. The multiple forms of R-region DNA are in a dynamic state in the antifolate-resistant populations, and the relative amount of DNA in each form as well as the number of forms within each cell line change through time. As currently understood, the generation of amplified R-region DNA in L. major is summarized.

Drug resistance in parasitic protozoa is a serious problem, and the mechanisms by which such resistance develops remain poorly understood. This laboratory has developed a system for studying the molecular basis of antifolate resistance in Leishmania, the causative agent of leishmaniasis, which may serve as a model for drug resistance in pathogenic protozoa. Three independently derived antifolate-resistant Leishmania promastigote cell lines have been shown to overproduce the bifunctional protein thymidylate synthase-dihydrofolate reductase (TS-DHFR)1 by amplification of a region of DNA from chromosome four (R-region DNA) that contains the gene for TS-DHFR (1–3). The R1000 (4) and D7BR10062 cell lines were developed by selecting for resistance to methotrexate (MTX), a potent inhibitor of DHFR whereas the CB line was generated by selecting for resistance to 10-propargyl-5,8-dideazafolate (CB3717), a potent inhibitor of TS (5). The unit size of the amplified R-region DNA is ~30 kilobases (kb) in the R1000 and CB cell lines and is ~42 kb in the D7BR1000 line. Cells grown in 1 mM MTX for 3 months (R1000–3) and CB cells resistant to low levels of CB3717 (0.8 and 2.0 μM) initially contained amplified R-region DNA in the form of 30-kb extrachromosomal circles which was unstable in the absence of drug selection (5, 6). R1000 cells grown in 1 mM MTX for 11 months (R1000–11 cells) possessed R-region DNA that was stable when MTX was removed from the medium. Originally, it was believed that this DNA was stable because it had integrated into chromosomal DNA as a tandem array of the 30-kb DNA amplification unit (6). Subsequently, using orthogonal-field-alternation gel electrophoresis (OFAGE), the amplified R-region DNAs from all of these lines were shown to be extrachromosomal elements which migrated aberrantly on OFAGE and were resistant to exonuclease III (7). From these results, we presumed that the R-region DNA molecules were individual supercoiled circles, catenated circles, or an unrecognized structure containing closed circular DNA. Furthermore, different R-region DNA forms within cell lines were resolved on OFAGE (7).

In this report, we describe studies directed at the identification of structural features of the R-region DNAs. We have developed a method for isolating intact extrachromosomal R-region DNAs from OFAGE and have determined their size and structure directly by electron microscopy (EM). We show that these R-region DNAs are individual supercoiled circular DNAs and that they are either monomers or directly repeating dimers of the original DNA amplification unit. Some cloned cell lines possess multiple forms of R-region DNA which are separable on OFAGE; these forms may exist as topoisomers since the resolved forms apparently have the same size and restriction map. Combining these data with results from previous studies, we summarize our current understanding of the development of amplified R-region DNA in Leishmania.

EXPERIMENTAL PROCEDURES

[32P]dCTP was obtained from Amersham Corp., and nick translations were performed using a kit obtained from Bethesda Research Laboratories (BRL). Restriction endonucleases were purchased from New England Biolabs. Low melt agarose gels were prepared from SeaPlaque agarose (FMC BioProducts). Calf thymus topoisomerase I and the supercoiled plasmid ladder (containing 2.067, 2.972, 3.990, 5.012, 6.030, 7.048, 8.066, 10.102, 12.138, 14.174, and 16.210-kb plasmids) were purchased from BRL. T4 topoisomerase II is a gift from D. V. Santi, unpublished results.

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1 Present address: Instituto "Lopez Neyra" of Parasitology, Consejo Superior de Investigaciones Científicas, C/ Ventanilla, 11, 18001 Granada, Spain.

2 Burroughs Wellcome Scholar in Molecular Parasitology. To whom correspondence should be addressed.

3 The abbreviations used are: TS-DHFR, thymidylate synthase-dihydrofolate reductase; kb, kilobase(s); MTX, methotrexate; OFAGE, orthogonal-field-alternation gel electrophoresis; EM, electron microscopy.

4 Robin C. Hightower, Mei Lie Wong, Luis Ruiz-Perez, and Daniel V. Santi.
OFAGE—Agarose blocks containing DNA from *Leishmania* were prepared as described (7), and total DNA from *Leishmania* was prepared by phenol extraction as described (5). OFAGE was performed essentially as described (8). Samples were applied to 1.5% agarose gels electrophoresis performed in 0.6 X TBE (53 mM Tris, 53 mM boric acid, 1.2 mM EDTA, pH 8.4) at 12 °C for 22 h at a 120-s pulse time. The recirculated buffer was changed approximately midway during the run for optimal resolution of the DNA samples.

**Recovery of Amplified DNAs from Low Melt Agarose Gels**—OFAGE electrophoresis of total DNA was performed in 1.5% low melt agarose. Recovery of amplified DNA (10) was performed in 1 X TAE buffer. The final volume was determined and the solution adjusted to a 0.5 X TAE buffer containing 0.3 M NaCl, 0.1 M Tris-HCl adjusted to 0.3 M NaCl. Slices were heated at 65 °C for 15 min. An equal volume of phenol saturated with 0.3 M NaCl, 0.1 M Tris-HCl pH 7.5 was added to the melted sample, and the sample was vortexed at the highest setting for 10 s. This phenol extraction step was repeated (no vortexing), and a third extraction was performed with phenol/chloroform (1:1) saturated with TE (10 mM Tris, 1 mM EDTA, pH 8.3), blotted to nitrocellulose (12), and hybridized with pooled 32P-labeled pKGW plasmids which contain the R-region DNA from the CB line (1). The filter was hybridized with 11 pBR322 derivatives ranging in size from 2.1 to 16.2 kb (BRL) (Fig. 1), and the relative mobilities (Rf) of these plasmids are not affected by variations in pulse times ranging from 45 to 120 seconds (data not shown). The plasmids migrate in proportion to their size and can be used as size standards.

**Restriction Analysis—**DNA recovered from low melt agarose OFAGE was restricted with BglII as recommended by the supplier. Undigested and BglII-digested samples were fractionated on a 0.8% agarose gel in 1 X TAE buffer. The final volume was determined and the solution adjusted to a 0.5 X TAE buffer containing 0.3 M NaCl, 0.1 M Tris-HCl pH 7.5 was added to the melted sample, and the sample was vortexed at the highest setting for 10 s. This phenol extraction step was repeated (no vortexing), and a third extraction was performed with phenol/chloroform (1:1) saturated with TE (10 mM Tris, 1 mM EDTA, pH 8.3), blotted to nitrocellulose (12), and hybridized with pooled 32P-labeled pKGW plasmids which contain the BglII fragments encompassing the R-region DNA from the CB line (1). The filter hybridization and washing conditions used were as described (1).

**Cell Cloning—**Clones of the R1000-3 (+10 months) and CB50 cell lines were prepared by agar plating (10) in collaboration with Dr. Bruce Buerkle at the Oregon Health Sciences University.

**Topoisomerase I Treatment of Amplified DNA**—Reactions (20 μl) were incubated for 30 min at 37 °C in 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl2, 0.5 mM dithiothreitol, 0.1 mM Na3EDTA, 30 μg/ml bovine serum albumin and contained 2.5 μg of purified total DNA from the CB50 cell line, and 2-fold dilutions of topoisomerase I (14). Control samples contained 0.5 μg of pBR322 DNA. Reactions were stopped by addition of EDTA to 20 mM; after 1 min sodium dodecyl sulfate was added to 0.2% and proteinase K was added to 100 μg/ml; samples were incubated at 37 °C for 1 h. Untreated and treated samples of CB50 DNA (1.25 μg) and pBR322 DNA (0.65 μg) were electrophoresed on OFAGE. Electrophoresis was performed as described in the preceding section on OFAGE. After visualizing DNA samples with ethidium bromide, gels were soaked in 0.25 M HCl (2 X 15 min), rinsed with distilled H2O, soaked in 0.5 M NaOH, 1.5 M NaCl (2 X 30 min), and 1 M Tris-HCl, pH 8.0, 1.5 M NaCl (2 X 30 min). DNA was transferred from the gel to nitrocellulose (12), and the filter was hybridized with 32P-labeled plasmid pC1, the *Leishmania* TS-DHFR cDNA cloned in pUC9 (3). The hybridization and washing conditions used are as described (1).

**Topoisomerase II Treatment of Amplified DNA**—Reactions (20 μl) were incubated for 20 min at 30 °C in 40 mM Tris-HCl, pH 7.8, 6.0 mM KCl, 10 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM ATP, 30 μg/ml bovine serum albumin and contained 2.5 μg of purified total DNA from CB50 cell line and 2-fold dilutions of topoisomerase II (15). Control samples contained 0.5 μg of pBR322 DNA. The conditions used for the rest of this experiment are as described above for topoisomerase I.

**RESULTS**

**Multiple Forms of R-Region DNA Are Resolved on OFAGE**—Fig. 1 shows the OFAGE separation of phenol-extracted DNA that was isolated from several antifolate-resistant *Leishmania major* cell lines. The three lines used in this study, R1000, CB50, and D7/BR1000, each contain at least two R-region DNA bands which are resolved on OFAGE. The amplified R-region DNAs from these lines are circular and do not migrate as a function of pulse time (7); therefore, their mobilities and sizes cannot be compared to those of pulse time-dependent linear DNAs, such as *Leishmania* chromosomes.

We have used a plasmid ladder as a reference for the mobility of these circular DNAs. The supercoiled plasmid ladder contains 11 pBR322 derivatives ranging in size from 2.1 to 16.2 kb (BRL) (Fig. 1), and the relative mobilities (Rf) of these plasmids are not affected by variations in pulse times ranging from 45 to 120 seconds (data not shown). The plasmids migrate in proportion to their size and can be used as size standards.
standards with which to compare other pulse time-independent DNAs such as the R-region DNAs. In this paper, the mobility of the R-region DNAs on OFAGE is described as an $R_F$ value which is defined as the distance of the molecule’s migration divided by the migration distance of the 4-kb plasmid in the ladder. Plots of plasmid size in log kb versus $R_F$ indicated that plasmids larger than approximately 30 kb should not enter OFAGE gels. In addition, DNA supercoiling strongly affects mobility, for example, supercoiled pBR322 (4.3 kb) migrates at $R_F = 0.83$ whereas the relaxed form migrates at $R_F = 0.45$ (data not shown). As shown below, the circular DNA forms in Leishmania migrate anomalously with respect to size even though the migration of these forms is not affected by pulse time.

The amplified DNAs in the antifolate-resistant cell lines migrate as unusual, highly compressed bands on OFAGE as detected by ethidium bromide staining. The R-region DNAs hybridize to probes such as the TS-DHFR gene which is found within this amplified unit (Fig. 1). CB50 cells contain two R-region DNA bands; the upper, minor band ($R_F = 0.36$) and the lower, major band ($R_F = 0.41$) are present in a ratio of 30:70 (as determined by densitometric scans of Southern transfer autoradiograms) (Fig. 1, lane A). In the D7BR1000 line, there are two bands ($R_F = 0.37; R_F = 0.39$) which are present in roughly equimolar amounts (upper/lower = 0.45:0.55) (Fig. 1, lane B). The R1000-3 (+1 month) cell line (cells from the original R1000-3 line grown in 1 mM MTX for 1 additional month) contains three species of R-region DNA which are present at relative levels of 60:30:10; a major broad, slowly migrating band ($R_F = 0.35$), a sharp band ($R_F = 0.38$), and a minor band ($R_F = 0.41$) (Fig. 1, lane C). When these cells were grown an additional 10 months in 1 mM MTX, only the two slower migrating bands were detected (Fig. 2, lane b). The stable R1000-11 (+4 months) cells (cells from the original R1000-11 line grown in 1 mM MTX for 4 additional months) contain one predominant R-region band ($R_F = 0.41$) (Fig. 1, lane D), which migrates at the same position as the fastest moving, minor band in the R1000-3 (+1 month) line (Fig. 1, lane C).

Multiple Forms of R-Region DNA Are Present in Cloned Isolates—The multiple species of R-region DNA observed in the antifolate-resistant populations arise from either: (a) a homogeneous population of cells in which each cell possesses multiple species of amplified DNAs, or (b) a heterogeneous population of cells in which individual cells contain single or multiple species of amplified DNA. To address this question, cell lines were cloned and amplified DNA patterns from the cloned cells and the parental line were compared. Cell blocks of uncloned and cloned isolates were prepared and electrophoresed on OFAGE gels, and Southern analysis was performed using the TS-DHFR cDNA as a probe. Fig. 2 shows some of the clones that were analyzed, in particular two clones (clones f and g) from the CB50 population and four clones (clones a–d) from the R1000-3 (+10 months) population.

The parental CB50 cells were cloned previously; one such clone, CB50a, contained equimolar levels of the two R-region DNA bands ($R_F = 0.36, 0.41$) (7). In the present work, CB50a was subcloned 1 year after its generation. Of six subclones analyzed (clones b–g), four contained the two R-region DNA bands (present at equimolar levels in one clone and at 30:70 levels in three clones), whereas the other two clones contained only the faster migrating R-region DNA band ($R_F = 0.41$).

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4 Cloned isolates from each cell line have been designated by a lower case letter following the name of the parent line. The time a cell line was grown in culture after receiving a designation is indicated in parenthesis following the name of the cell line.

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FIG. 2. OFAGE analysis of intact chromosomal and R-region DNAs in cloned isolates from the R1000-3 (+10 months) and CB50 populations. The R1000 line used for cloning was the R1000-3 line grown for 10 months in 1 mM MTX, R1000-3 (+10 months), and the CB50 line used had been cloned 1 year prior to this subcloning. Refer to Fig. 1, lane A for the R-region DNA pattern of the CB50 population. A, ethidium bromide-stained gel containing 2 

$\mu$g of intact chromosomal DNA/lane from cell block preparations (7) of: a, CB50 clone f; b, R1000-3 (+10 months) uncloned population; c, R1000-3 (+10 months) clone a; d, R1000-3 (+10 months) clone b; e, R1000-3 (+10 months) clone c; f, R1000-3 (+10 months) clone d; g, CB50, clone g. In B, lane e, the arrows indicate the positions of the R-region DNA circular forms, and the corresponding forms in A, lane e, are marked by the two outer arrows. In A, lane e, the central arrow points to extrachromosomal H-DNA which is the prominent band found in lanes b–f. B, Southern transfer of gel in A hybridized with the TS-DHFR cDNA probe; the correspondence of the blot to panel A is indicated by brackets at the right of each panel. The faint, lowermost signal indicates chromosome four which hybridizes to the TS-DHFR cDNA probe (signals in the outermost lanes may not be apparent in this reproduction).

The parental R1000 cell line used for cloning was the R1000-3 line grown for an additional 10 months in 1 mM MTX, R1000-3 (+10 months). Eight clones of R1000-3 (+10 months) (clones a–h) were compared to the uncloned population from which they were derived. Six contained the same R-region DNA bands present in roughly the same proportions as the uncloned population ($R_F = 0.33, 0.38$), whereas two contained only the $R_F = 0.38$ R-region DNA band. No clones were isolated which had only the slower moving band. Therefore, individual cells may contain either single species or multiple species of R-region DNAs, but most of the clones contain multiple species of amplified DNAs.

Clones that contain a single R-region DNA species may have originated from cells containing multiple species which have been lost. Propagation of unstable R1000-3 cells for an additional 8 months in 1 mM MTX yielded the stable R1000-11 population. This transition was accompanied by a change
in the banding pattern in R1000-3 (+1 month) cells, from three bands in a 6:3:1 ratio, to the pattern in R1000-11 (+4 months) cells, which contains only the single fastest migrating band (RF = 0.41) (7). No further changes in the mobility of the R-region DNA have been observed in this line during an additional 12 months in the presence or absence of MTX. During CB500 growth in 50 μM CB3717 for 1 year, two R-region DNA bands (RF = 0.36, 0.41) that were originally equimolar became represented at a 30:70 ratio. Similarly, when D7BR1000 was grown in 1 mM MTX for 1 year, the faster migrating R-region DNA band (RF = 0.39) was lost. Thus, the amplified R-region DNAs appear to be in a dynamic state in these populations, and the number and relative amounts of distinguishable R-region DNA forms can change through time.

Stability of TS-DHFR Locus—Southern analysis of all cloned isolates and populations showed that the TS-DHFR cDNA probe hybridized with chromosome four. This result shows that the TS-DHFR locus remains present on chromosome four in all populations and cloned isolates described in this paper and that the locus is not lost in cells that are maintained under drug selection pressure.

Purification of Intact R-Region DNA—Previously described methods for the preparation of R-region DNAs, including CsCl-ethidium bromide (EtBr) equilibrium sedimentation and low percentage agarose gel electrophoresis, did not separate R-region DNA species within a given cell line (5, 6). However, it is possible to resolve the R-region DNA species on OFAGE. Therefore, it was necessary to develop a method for recovering purified amplified DNAs from OFAGE gels for further analysis of their structure. OFAGE of intact chromosomal DNA preparations, obtained by lysis of Leishmania cells embedded in agarose (7), separates the amplified DNAs, but they are superimposed on the chromosomal DNA profile. When total DNA from Leishmania is isolated by phenol extraction and electrophoresis on OFAGE, the large linear chromosomies are sheared and are well separated from the intact amplified DNAs which appear unaffected by the extraction procedure (Fig. 1). Control experiments in which DNA was recovered from background regions of these gels, i.e. gel sections in front of and behind the amplified R-region DNA bands, revealed very low levels of variable length linear DNA molecules by EM (data not shown). Therefore, the amplified DNAs can be separated from the bulk of genomic DNA on OFAGE.

Recovery of intact R-region DNA from excised OFAGE bands was attempted initially by electrophoresis in dialysis membranes using conventional electrophoresis and the OFAGE apparatus (12). Eluates were analyzed by agarose gel electrophoresis, but recoveries were low. Therefore, we developed a method for direct recovery of DNA from low melt agarose OFAGE gel slices using a modification of the procedure of Wieslander (9). Banding patterns of the R-region DNAs are identical on low melt and regular agarose gels, and dissolution of the low melt agarose in excised bands followed by phenol extraction steps consistently provided samples of intact amplified DNA molecules which were suitable for EM.

Electron Microscopy of R-Region DNA—The amplified R-region DNAs examined by EM are single circular molecules of approximately 60 kb. The R-region DNA band from R1000-11 cells which were grown in the absence of MTX for an additional 5 months [R1000-11 (+5 months, minus MTX)], also contained only 60-kb molecules. Therefore, R-region DNAs from R1000-11 cells grown in the presence or absence of MTX are the same size. Measurements of DNA molecules from the two R-region bands in the CB50 cells (RF = 0.36, 0.41) revealed sizes of approximately 60 kb. DNA molecules measured from the two R-region bands in D7BR1000 cells (RF = 0.37, 0.39) showed that both bands contained molecules of approximately 45 kb.

The circular DNA molecules of approximately 30 kb found in the two upper bands of unstable amplified R1000-3 (+1 month) cells (RF = 0.33, 0.38) correspond in size to the first detectable amplified unit of the R-region DNA in the R1000 line; they are presumably the species observed by electrophoresis in low percentage agarose and sedimentation centrifugation in earlier studies (5, 6). Similarly, the 43-kb circles of the D7BR1000 cells correspond in size to the original amplified unit in this cell line as determined by restriction mapping analysis. The 60-kb circular DNA molecules observed in the R-region DNA bands from R1000-11 (+4 months) cells (RF = 0.41), from R1000-3 (+1 month) cells (RF = 0.41), and from both bands from CB50 cells (RF = 0.36, 0.41) are, within error, twice the size of the ~30-kb amplification unit of each cell line. A few higher molecular weight multimeric forms of R-region DNA were observed; the upper band (RF = 0.37) from D7BR1000 cells showed two 83-kb circular molecules that we believe to be dimers of this R-DNA unit. We also detected two 118-kb circular DNAs from the upper band (RF = 0.36) in CB50 cells which represent tetramers of the R-region DNA unit.

Approximately 60% of the molecules observed by EM were open circular forms, which appear to result from nicking during the recovery procedure. As a control for the extent of nicking in the EM recovery method, a 16-kb supercoiled plasmid was electrophoresed in a separate lane and recovered from low melt agarose OFAGE. By agarose gel electrophoresis, the relative amount of supercoiled and nicked plasmid in this sample was compared to the amount in the original sample. We detected about 10% open circular molecules in the original sample, and about 30–40% open circular molecules in the sample recovered from the gel. Therefore, it seems that our isolation procedure results in some nicking of the DNA.

Restriction Endonuclease Analysis of R-Region DNA—Restriction maps of total amplified R-region DNA in the antifolate-resistant cell lines are homogeneous within each cell line throughout selection (5, 6). Furthermore, the amplified DNA restriction maps are nearly identical in the CB50, D7BR1000, and R1000 cell lines except for the differences in size of the fragment containing the junctional region where DNA rearrangements occur during the initial amplification event (5, 6).

In order to confirm that the DNA recovered for EM analysis was R-region DNA, each purified sample was restricted with BglII, and Southern analysis was performed using the entire amplified R-region DNA as a hybridization probe (data not shown). All R-region DNA species within any cell line revealed a restriction map that was identical to that of the original amplified R-region DNA unit from that cell line. These results indicate that the R-region DNA species within each cell line are comprised of monomers and/or dimers of the original amplification unit. Furthermore, together with the EM results, these data are consistent only with the 60-kb circles being dimeric molecules, representing single direct repeats of the amplification unit.

H-Region DNA.—By EM analysis, a population of 72-kb

\[^{6}\text{D. V. Santi, unpublished results.}\]
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**FIG. 3. Analysis of DNA amplification by electron microscopy.** R-region DNAs were recovered from OFAGE gels and prepared for EM as described under “Experimental Procedures.” The panels represent R-region DNA molecules from the following bands: A, CB50 (RF = 0.36); B, R1000-3 (+1 month) (RF = 0.33); C, R1000-3 (+1 month) (RF = 0.41); D, R1000-11 (+4 months) (RF = 0.41); E, R1000-11 (+5 months, minus MTX) (RF = 0.41). The magnification bar in each panel represents 2 kb. Supercoiled and open circular R-region DNAs have been shown; the open circular forms are the result of nicking during sample recovery for EM.

| Cell line, recovery band | RF | Mean kb | S.D. kb | No. molecules measured |
|--------------------------|----|---------|---------|-----------------------|
| CB50                     | 0.36 | 59.2    | 1.54    | 22                    |
| CB50                     | 0.41 | 58.0    | 1.22    | 22                    |
| D7BR1000                 | 0.37 | 43.8    | 1.23    | 20                    |
| D7BR1000                 | 0.39 | 42.0    | 1.30    | 24                    |
| R1000-3 (+1 month)       | 0.33 | 30.1    | 1.65    | 14                    |
| R1000-3 (+1 month)       | 0.38 | 30.9    | 1.66    | 20                    |
| R1000-3 (+1 month)       | 0.41 | 59.7    | 2.84    | 19                    |
| R1000-3 (+1 month)       | 0.41 | 30.7    | 2.52    | 3                     |
| R1000-11 (+4 months)     | 0.41 | 58.0    | 1.81    | 20                    |
| R1000-11 (+5 months, minus MTX) | 0.41 | 59.8    | 1.82    | 22                    |

Circular DNA molecules was also observed in the R-region DNA band (RF = 0.33) from R1000-3 (+1 month). We concluded from the following evidence that these molecules are H-region DNA, an amplified DNA of unknown function that is found in these MTX-resistant cells (6); (i) the EM data agree with sizes of H-region DNA calculated from restriction mapping analysis (6), (ii) these molecules are seen only in cell lines that contain H-region DNA, R1000 but not CB50 or D7BR1000 (5, 6), and (iii) Southern blot analysis of restricted excised DNA from the R1000-3 (+1 month) RF = 0.33 band indicates the presence of both R-region and H-region DNA (data not shown).

Topoisomerase Treatment of R-Region DNA—CB50 DNA was treated separately with topoisomerase I and II. Topoisomerase I relaxes positively and negatively supercoiled DNA (14), and topoisomerase II relaxes positively and negatively supercoiled circles and releases catenated circles (15). After treatment of total CB50 DNA with either enzyme, R-region DNAs were no longer detected at RF = 0.36 and 0.41, and hybridization appeared to be only in the gel slot; genomic DNA was unaffected, as detected by ethidium bromide staining and Southern hybridization analysis with the TS-DHFR cDNA probe (data not shown). With pBR322 (4.3 kb) as a positive control plasmid, a ladder of topoisomers was resolved between the supercoiled (RF = 0.83) and relaxed (RF = 0.45) forms. This result shows that relaxed circular DNA migrates significantly slower on OFAGE than the corresponding supercoiled form and that topoisomers were generated and resolved in the experiment. These results suggest that the R-region DNAs are relaxed by both topoisomerase I and II. However, the topoisomerase-treated R-region DNA forms are not resolved on OFAGE, presumably because the relaxed forms of these DNAs do not enter into the gel. Although this experiment does not distinguish between single circular or catenated molecules, all R-region DNA molecules examined by EM were single circles. Therefore, based on the collective results of R-region DNA treatment with limited deoxyribonuclease (DNase) I (6), exonuclease III (7), topoisomerase I, and topoisomerase II, as well as results from the EM analysis, we conclude that the amplified R-region DNAs are individual supercoiled circular molecules.
Discussion

Structure of R-Region DNA—Recent studies using OFAGE indicated that extrachromosomal R-region DNAs of Leishmania major were circular forms that were either singular or catenated molecules, possibly having unusual higher order structure (7). In the present study, we have developed a method for gentle isolation of these amplified R-region DNAs from OFAGE gels and have observed them by electron microscopy. These amplified R-region DNAs have now been definitively shown to be individual, supercoiled circles. Furthermore, most of the circles that were observed correspond to the monomer (30 kb for R1000 and CB50, 43 kb for D7BRI000) and dimer forms (60 kb for R1000 and CB50) of the original amplified DNA sequence unit. The dimer forms represent single direct repeats of the amplification unit because all R-region DNA forms within any cell line have a restriction map that is identical to the original amplified R-region DNA unit from that cell line. We have also observed a small number of higher multimeric forms of R-region DNA which represent dimers in the D7BRI000 line and tetramers in the CB50 line.

Migration of R-Region DNA and Supercoiled Plasmids on OFAGE—Relative to linear DNAs, R-region DNA is relatively insensitive to pulse times used in OFAGE (7). For example, at a 40-s pulse time, linear DNAs of about 500 kb comigrate with R-region DNA, whereas at a 120-s pulse time, ~1,500-kb linear DNA migrates at the same position as R-region DNA. These differences in relative mobility are due to the pulse time-dependent nature of the linear DNA and to the pulse time-independent migration of the R-region DNA (7). In this work, we show that a plasmid ladder can be used as a reference for the mobility of pulse time-independent DNAs such as the R-region DNAs. Interestingly, when we compared the mobility of R-region DNAs to these standards, we found that all R-region DNAs (30–60 kb) migrate similarly to supercoiled circles of about 7–8 kb. It is striking that the relative mobilities of R-region DNAs are not directly related to their sizes. For example, different forms of 60-kb R-region DNAs are observed to move slower than or faster than 30-kb forms, while different forms of 43-kb R-region DNAs move nearly the same as, or faster than 30-kb forms of R-region DNA (see Table I, Rf values). We conclude that there are unknown features of the R-region DNAs, superimposed on their size and circular structures, which affect their mobility on OFAGE. We have considered several possibilities. First, there may be components associated with the R-region DNA which affect its mobility. Since our preparations have been extensively denatured and extracted with phenol, these components are not likely to be proteins. The mobility is also not affected by RNase A treatment (7), and we see no evidence of other nucleic acid components by EM. Second, there may be sequences within the R-region DNA which impart the unusual mobility to it. For example, certain DNA structures, such as a region of the kinetoplast minicircle DNA from Leishmania tarentolae, bend naturally in the absence of protein due to the nucleotide sequence of the fragment (16, 17). Third, there may be a higher order structure, unobserved by our EM analysis, which results in the unusual mobility of the R-region DNAs. For example, superhelical density of knotting might result in the aberrant migration on OFAGE. In this regard, we have observed that topoisomers of small plasmids resolve on OFAGE to form a ladder extending between the supercoiled and relaxed forms, so linking number does exert an influence on OFAGE migration.

As a final point, we noted that the characteristics which cause this unusual migration are not restricted to R-region DNA. The H-region DNA, found in the R1000 cell line and the wild-type strain from which it was derived (6, 7), also migrates as a condensed band with an anomalously low apparent size. In R1000–3 (+1 month), H-region DNA (Rf = 0.365, 72 kb) migrates between R-region DNAs (Rf = 0.33, 30 kb) and (Rf = 0.38, 30 kb; Rf = 0.41, 60 kb). We have also observed extrachromosomal circular DNA in another Leishmania cell line which migrates as a condensed band on OFAGE. It will be interesting to determine whether the structures associated with these amplified extrachromosomal DNAs are unique to these organisms.

Individual Cells Can Possess Multiple Forms of R-Region DNA—We observed multiple R-region DNA bands in 19 of 14 cloned isolates from the CB50 and R1000–3 (+10 months) cells, confirming that multiple forms of R-region DNA may exist within single cells. We have also isolated four clones containing single R-region DNA species which indicates that the multiplicity of forms is not always seen. These clones with a single band could represent a subpopulation in the parental line or they may be derived from cells which originally contained multiple forms that have been lost. We have observed that the number of R-region DNA species within each cell line decreases through time in all cell lines. Three forms convert to one in R1000, two forms convert to one in D7BRI000, and one of two R-region DNA forms has decreased in CB50. We presume that these changes represent either an advantageous outgrowth of cells containing these final R-region DNA forms, or that these forms are preferentially maintained in the drug-resistant populations. The multiple forms within each cell line are monomers and/or dimers of the original amplified DNA sequence unit. Furthermore, some of the cell lines contain two forms of R-region DNA that are indistinguishable in size and restriction pattern. It is possible that small size differences are not detected by our methods, but it is unlikely that these would result in the striking separation of R-region DNAs that we observe on OFAGE. We believe that particular sequences in the R-region DNA or topological differences between these forms may result in their aberrant migration on OFAGE.

Stability of R-Region DNA—In the present work, we show that the stable extrachromosomal R-region DNA is comprised of dimeric molecules, representing a single direct repeat of the original amplified DNA sequence unit. However, stability cannot be due solely to the formation of dimeric molecules because dimers also are observed in the unstable R1000–3 and CB50 lines. Therefore, the process of forming dimers can precede the acquisition of stability. Interestingly, we are observing now that antifolate resistance in the CB50 cell lines is becoming relatively stable in cells that have been propagated extensively under selective conditions, and that the faster migrating R-region DNA form is becoming more predominant in the population. This cell line may be in the process of acquiring stability and could provide the tools for understanding this phenomenon. The stable phenotype has not been found in cells containing only monomers of the amplified DNA sequence unit, and the formation of multimers may be necessary but not sufficient for the acquisition of stability.

The DNA Amplification Process—Based on our current understanding of the amplified unit in the R1000 and CB50 cell lines, we can distinguish several steps in the generation of antifolate resistance in Leishmania (Fig. 4). As shown in this scheme, the original amplified R-region DNA sequence unit is derived from a 30 kb or larger region of chromosome four (7) which includes the TS-DHFR gene (1–3). It first appears as a transient, supercoiled form that overproduces...
The light and dark shaded boxes represent the junction sequence which is joined to form the original DNA sequence unit is converted to a circular dimer representing a direct repeat of the amplified unit and the original monomer is lost. In some cases, larger multimers are also formed corresponding to tetramers of the amplified unit, albeit in small amounts. Regardless of size, multiple forms of R-region DNAs can exist within cells and may represent topoisomerases. The various forms appear to be dynamic and, with time, ultimately convert to a predominant R-region DNA form in the cell lines. During this process, and presumably after the formation of multimers, mitotic stability of the extrachromosomal R-region DNA may be acquired. With our current understanding of the steps in the amplification process, we can now address more specific questions about the individual events (19-21).

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