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Unstable Amplification of Two Extrachromosomal Elements in α-Difluoromethylornithine-Resistant *Leishmania donovani*

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We describe the first example of unstable gene amplification consisting of linear extrachromosomal DNAs in drug-resistant eukaryotic cells. α-Difluoromethylornithine (DFMO)-resistant *Leishmania donovani* with an amplified ornithine decarboxylase (ODC) gene copy number contained two new extrachromosomal DNAs, both present in 10 to 20 copies. One of these was a 140-kb linear DNA (ODC140-L) on which all of the amplified copies of the *odc* gene were located. The second was a 70-kb circular DNA (ODC70-C) containing an inverted repeat but lacking the *odc* gene. Both ODC140-L and ODC70-C were derived from a preexisting wild-type chromosome, probably by a conservative amplification mechanism. Both elements were unstable in the absence of DFMO, and their disappearance coincided with a decrease in ODC activity and an increase in DFMO growth sensitivity. These results suggest the possibility that ODC70-C may play a role in DFMO resistance. These data expand the diversity of known amplification mechanisms in eukaryotes to include the simultaneous unstable amplification of both linear and circular DNAs. Further characterization of these molecules will provide insights into the molecular mechanisms underlying gene amplification, including the ability of linear amplified DNAs to acquire telomeres and the determinants of chromosomal stability.

Drug resistance in parasitic protozoa is a major barrier to the treatment and control of parasitic diseases (13, 18, 40, 56, 61). *Leishmania* spp., the causative agents of leishmaniasis, have served as a useful paradigm for analyzing mechanisms of drug resistance in parasitic protozoa, since this genus of parasites can be cultivated continuously in defined growth media. One mechanism by which *Leishmania* spp. become refractory to drugs in vitro is gene amplification (5). Gene amplification has been observed in *Leishmania* spp. selected for their resistance to a variety of drugs, and these gene sequences appear to be amplified as multicopy circular DNAs (7, 17, 29, 57). Similarly, drug resistance in mammalian cells in which gene amplification is unstable is associated with the appearance of acentric circular DNA molecules known as double-minute chromosomes (46, 49, 55). The extrachromosomal DNA molecules in drug-resistant *Leishmania* spp. can be maintained either in a stable or in an unstable fashion, and the stability appears to be proportional to the length of time that cells are propagated in the presence of the drug (7, 30). Moreover, circular (26, 42) and small linear (6, 50, 51) DNAs have also been observed in *Leishmania* stocks that have not been exposed to selective pressure.

Coons et al. (14) have isolated strains of *Leishmania donovani* that are resistant to DL-α-difluoromethylornithine (DFMO), an enzyme-catalyzed irreversible inhibitor of ornithine decarboxylase (ODC) activity (34) that has been successfully used as a chemotherapeutic agent in the treatment of African sleeping sickness (2, 39). These DFMO-resistant *L. donovani* strains express augmented amounts of ODC as a consequence of a 10- to 20-fold increase in *odc* gene copy number (24). The *odc* gene amplification, ODC overexpression, and the DFMO resistance phenotype are all unstable in the absence of selective pressure. In this report, we demonstrate by pulsed-field gel electrophoresis (PFGE) that the DFMO-resistant parasites contain two extrachromosomal DNAs, a 140-kb linear DNA, ODC140-L, which encodes all of the amplified copies of the *odc* gene, and a 70-kb circular DNA, ODC70-C, whose function is unknown. This is the first report of unstable DNA amplification in drug-resistant *Leishmania* spp. in which the amplified gene is localized to an amplified linear extrachromosomal DNA.

MATERIALS AND METHODS

Chemicals and reagents. DFMO was a generous gift from Peter P. McCann of Merrell Dow Research Institute (Cincinnati, Ohio). [α-^{32}P]dCTP (3,000 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, Mass.). InCert agarose was purchased from FMC BioProducts (Rockland, Maine). All other materials, reagents, and chemicals were of the highest quality commercially available.

Cell culture. *L. donovani* promastigotes were cultivated in DME-L culture medium as originally described (28). DI700 is the wild-type strain. DFMO16 cells (14) were propagated in DME-L containing 16 mM DFMO, except in the stability studies, in which DFMO16 cells were grown for 2, 4, or 8 weeks in the absence of DFMO.

DNA probes. The origins and locations of the 1.2-kb *Hin*cII and the 3.9-kb *Sal*I fragments of the *L. donovani odc* gene have been reported (24). A 1.8-kb *Sal*I fragment, ODC70-CS4b, a 1.4-kb *Sal*I fragment, ODC70-CS5b, and a 2.0-kb *Eco*RI-*Bam*HI fragment, ODC70-CEB2, were derived from ODC70-C, a 70-kb circular extrachromosomal DNA found in DFMO16 cells, and individually cloned into pBluescript. pK25 was originally derived from an unselected stock of *L. tarentolae* (41) and consists of a 2.5-kb *Kpn*I fragment of *ltpgpA*, a gene that is homologous to the P-glycoprotein gene family and that is located within the H region of *L. tarentolae* DNA (36). pHM3 is a 2.0-kb *Hin*dIII

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fragment derived from the H region of *L. major* (7). p7R50-P19 is a 1.9-kb *PstI* fragment from the small linear DNAs found in multiple *Leishmania* species (6). The *L. donovani* IMP dehydrogenase (60) and *ldmdr1* (25) genes were isolated as described previously. pTT6 is a probe derived from the telomere repeat of *Trypanosoma brucei* (53).

PFGE. InCert agarose plugs (0.5%) containing 2×10^7 *L. donovani* promastigotes were prepared as described previously (54). Chromosomes of wild-type and drug-selected *L. donovani* were fractionated by PFGE with a uniform field and gel rotation apparatus (1). Chromosomes were separated in 1.5% agarose gels at 15°C for 30 h with a 60-s pulse time and a voltage gradient of 5 V/cm, unless otherwise noted. To determine the migration of extrachromosomal elements in nonhomogeneous electric fields, we fractionated DFMO16 chromosomes by PFGE on a contour-clamped homogenous electric field (CHEF) electrophoresis apparatus as described previously (4).

Isolation of extrachromosomal DNAs. ODC140-L was separated from the large linear chromosomes by PFGE, excised, and purified as reported previously (47). ODC70-C was isolated from 1×10^9 to 2×10^9 DFMO16 cells by the alkaline lysis procedure for the extraction of plasmid DNA as described previously (16), except that the final DNA preparation was not sieved over a Sepharose CL-4B column.

γ-Irradiation of leishmanial chromosomes. Limited γ-irradiation of *L. donovani* chromosomes in agarose plugs was performed either with a JL Shepard and Associates Mark 1 model 68 irradiator containing a ^{137}Cs source in the absence of a buffer or with an ICN GR9 irradiator containing a ^{60}Co source and 0.2 M Tris-HCl–0.1 M EDTA (pH 7.4) as the buffer (4). DFMO16 chromosomes were exposed in the absence of a buffer to the ^{137}Cs source for 3 to 100 min, a dose of 0.6 to 20 krads of γ-irradiation.

λ Exonuclease digestion of extrachromosomal DNA. About 1.0 μg of either ODC140-L or ODC70-C was incubated with 3 U of λ exonuclease at 37°C for various times in a volume of 9.5 μl, using the assay conditions and protocol suggested by Bethesda Research Laboratories (Gaithersburg, Md.). The reactions were stopped by the addition of 1 μl of 0.5 M EDTA, and the DNAs were analyzed by PFGE.

Southern blot hybridization. DI700 and DFMO16 genomic DNAs, as well as purified ODC70-C DNA, were digested with the appropriate restriction enzymes under the conditions recommended by the suppliers, electrophoresed on 0.4% agarose gels, and transferred to Nytran membranes (Schleicher & Schuell, Keene, N.H.) by the method of Southern (48). DI700 and DFM016 chromosomes were separated by PFGE and subjected to Southern blot hybridization as described previously (24), except that depurination was performed with 0.25 N HCl for 30 min and transfer to the Nytran membranes was carried out with an alkaline denaturation solution.

RESULTS

Relative electrophoretic mobility and stability of extrachromosomal elements in DFMO16 cells. PFGE demonstrated that DFMO16 cells possessed two extrachromosomal DNAs that were not present in wild-type parental cells (Fig. 1A). The first extrachromosome, ODC140-L (indicated by the lower arrow in Fig. 1A), migrated faster than the wild-type chromosomes, with an apparent molecular size of 140 kb. The migration of ODC140-L relative to those of the large linear chromosomes and yeast standards was the same at pulse times of 60 s (Fig. 1A) and 30 s (Fig. 1B). Pulse time-independent relative electrophoretic mobility is a characteristic of linear DNA molecules (3). The mobility of the second DNA element, ODC70-C (indicated by the upper arrow in Fig. 1A), relative to those of chromosomal and standard DNAs was much faster at a pulse time of 30 s (Fig. 1B) than at a pulse time of 60 s (Fig. 1A). This pulse time-dependent relative mobility of ODC70-C in PFGE is a property of supercoiled DNA molecules (3).

Examination of the gel depicted in Fig. 1A revealed several other karyotypic changes among the various chromosomal preparations. Although the greatest variation was observed between the DI700 and DFMO16 chromosomes, fluctuations in karyotype were also detected in DFMO16 cells removed from drug for various lengths of time. For instance, a prominent band at 650 kb was only observed in the chromosomes prepared from DFMO16 cells removed from drug for 8 weeks (Fig. 1A). Although the origins and functional significance of these chromosomal anomalies are unknown, chromosomal plasticity has been described for the genomes of *Trypanosoma* (52) and *Leishmania* (9, 10) spp., including stocks subjected to drug pressure (27). It is conceivable that some of the distinctions observed in Fig. 1A were induced by fluctuations in the cellular polyamine pools during and after DFMO selection.

Southern blot analysis of the gel shown in Fig. 1A and probed with the *L. donovani odc* fragment revealed that all of the amplified copies of the *odc* gene in DFMO16 cells were localized on ODC140-L (Fig. 1C). The *odc* hybridization intensity decreased as a function of the length of time that DFMO16 cells were grown in the absence of DFMO. The *odc* probe also hybridized to a 745-kb chromosome in DI700 and DFMO16 cells to equivalent extents. The signal for the hybridization to the 745-kb chromosome remained unaltered in DFMO16 cells after growth in the absence of the drug for 8 weeks (Fig. 1C).

Rehybridization of the blotted gel shown in Fig. 1C with the ODC70-CS4b probe indicated that ODC70-C was not present in DI700 cells and disappeared from DFMO16 cells in proportion to the length of time that DFMO16 cells were cultivated without DFMO (Fig. 1D). Therefore, neither ODC140-L nor ODC70-C was stably maintained in DFMO16 cells in the absence of selective pressure (Fig. 1A, C, and D). In addition, the ODC70-CS4b probe hybridized to a 680-kb chromosome at a similar intensity in both DI700 and DFMO16 cells. The fact that the *odc* and ODC70-CS4b probes hybridized to different DI700 chromosomes demonstrates that the two amplified extrachromosomal elements originated from different chromosomes.

ODC70-C copy number. To ascertain the copy number of ODC70-C in DFMO16 cells, we probed Southern blots of DI700 and various concentrations of DFMO16 DNA with ODC70-CS4b. Dilution analysis revealed that there exist ~20 copies of ODC70-C per DFMO16 cell (data not shown).

Isolation of ODC70-C by the alkaline lysis procedure. One diagnostic technique for ascertaining whether a DNA molecule is circular or linear in nature is to determine whether it can be extracted from chromosomal DNA by the alkaline lysis procedure for plasmid isolation. This protocol was exploited by Detke and coworkers to isolate circular DNA molecules from tunicamycin-resistant *L. mexicana* (17). With this procedure, 0.5 to 1.0 mg of ODC70-C DNA could be purified from 1.0×10^9 DFMO16 cells (Fig. 1B). ODC140-L DNA could not be separated from the linear chromosomes by this technique.

Migration of ODC140-L and ODC70-C in nonhomogeneous electric fields. To further examine the topology of ODC140-L

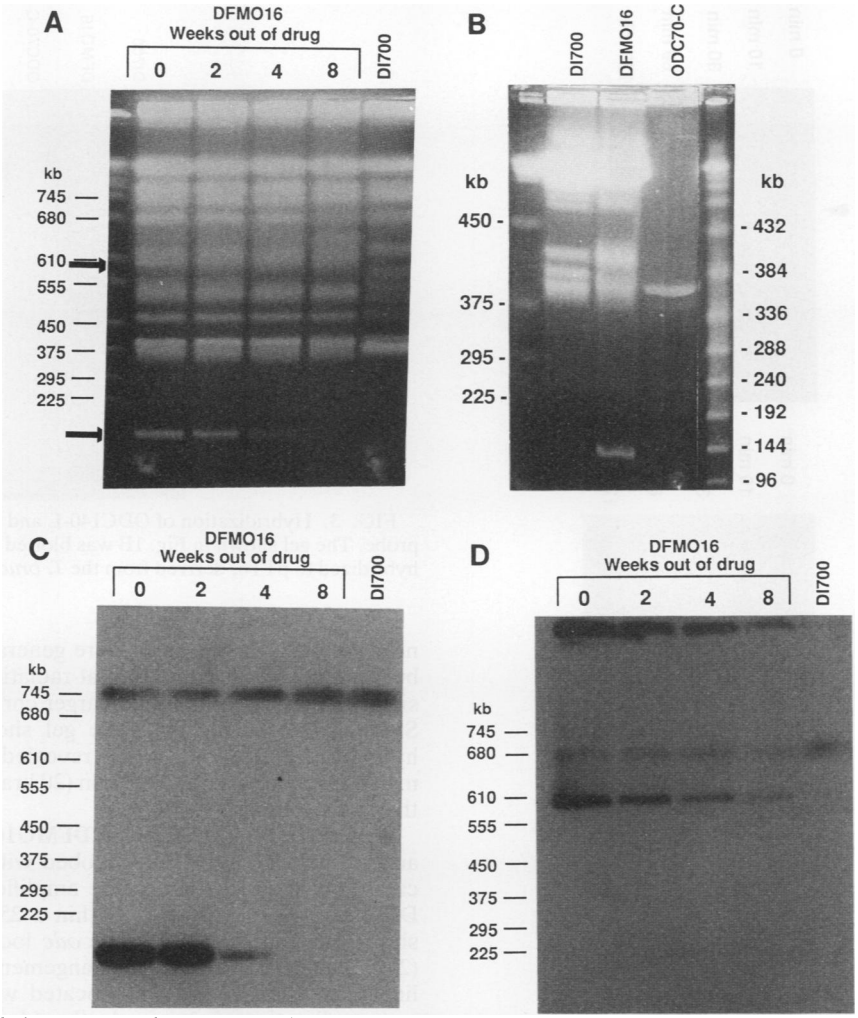


FIG. 1. (A) PFGE of chromosomes from DI700 cells and DFMO16 cells removed from drug for 5 days, 2 weeks, 4 weeks, or 8 weeks. Chromosomes were electrophoresed for 30 h with a 60-s pulse time and stained with ethidium bromide as described in Materials and Methods. ODC70-C and ODC140-L are designated by the upper and lower arrows, respectively. (B) PFGE of chromosomes from DI700 and DFMO16 cells and of ODC70-C was performed for 28 h with a 30-s pulse time. ODC70-C was separated from the linear chromosomes by subjecting DFMO16 cells to the alkaline lysis procedure for plasmid isolation (16). (C) The gel depicted in panel A was blotted onto a Nytran filter and probed with a fragment of the *L. donovani odc* gene (24). (D) The blot in panel C was rehybridized to ODC70-CS4b, a subcloned fragment of ODC70-C.

and ODC70-C, we performed PFGE on DFMO16 chromosomes under conditions in which circular DNA molecules in outside lanes manifest an altered directionality of migration compared with the linear chromosomes as a consequence of the differential effects of nonhomogeneous electric fields on the relative mobilities of circular and linear DNAs (3, 4). When DFMO16 chromosomes were fractionated in a CHEF gel under these nonhomogeneous conditions, ODC70-C migrated outside the lane of the linear chromosomes, whereas ODC140-L migrated within the chromosomal lane (data not shown).

Sensitivity of ODC70-C and ODC140-L to λ exonuclease digestion. λ Exonuclease catalyzes a processive, stepwise release of mononucleotides from the ends of linear double-stranded DNA molecules. Purified ODC70-C was refractory to λ exonuclease digestion after 120 min of incubation, whereas linear lambda *Hin*dIII standard DNAs were totally digested after 10 min (Fig. 2A and B). Conversely, ODC140-L was completely degraded after 60 min of incubation with the same amount of λ exonuclease (Fig. 2C).

Hybridization of ODC70-C and ODC140-L to a telomere probe. For the establishment of whether either of the two amplicons in DFMO16 cells contained telomeric sequences, the gel depicted in Fig. 1B, which contained fractionated DI700 and DFMO16 chromosomes and purified ODC70-C, was hybridized to pTT6, derived from the *T. brucei* telomere repeat unit. pTT6 recognized all linear DNAs in the DFMO16 genome, including ODC140-L, whereas no hybridization signal was observed with purified ODC70-C (Fig. 3). These data indicate that ODC140-L contains telomeric sequences, whereas circular ODC70-C does not.

Effects of γ-irradiation on ODC70-C and ODC140-L mobility in PFGE. γ-Irradiation in limited doses can introduce a single random double-strand break into circular DNA molecules, yielding a discretely sized molecule whose M_r can be accurately assessed by PFGE (4). γ-Irradiation induced the

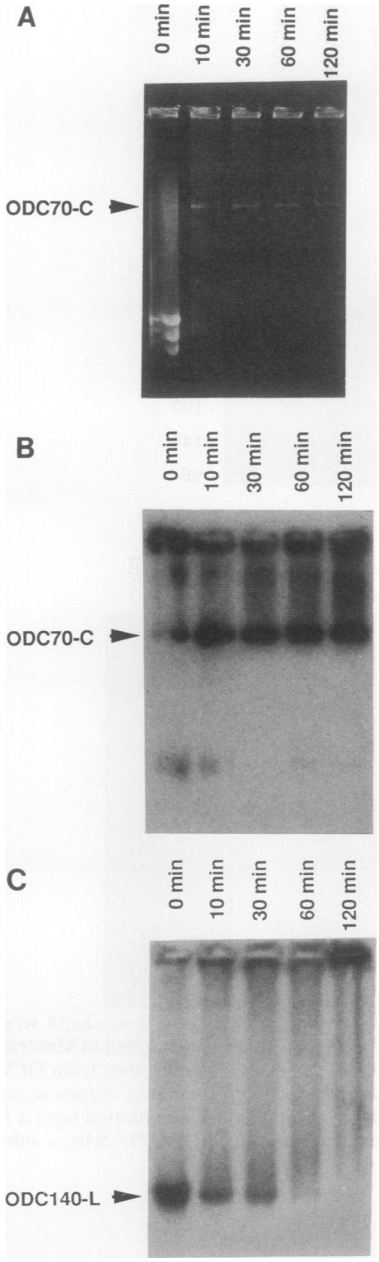


FIG. 2. Effect of λ exonuclease digestion on ODC70-C and ODC140-L. (A) Purified ODC70-C (1.0 μg) was added to 1.0 μg of *Hin*dIII-digested lambda DNA and incubated with 3.0 U of λ exonuclease for various times up to 120 min. The effects of the incubation were assessed by PFGE and then by ethidium bromide staining. PFGE was performed for 18 h with a 60-s pulse time. (B) The gel from panel A was blotted onto a Nytran filter and probed with ODC70-CS4b. (C) ODC140-L isolated from a gel was incubated under the same conditions as those described for the digestion of ODC70-C in panel A. The products were electrophoresed on a gel with the same parameters as those described for the gel shown in panel A, and the gel was blotted onto a Nytran filter and probed with a fragment of the *L. donovani odc* gene.

time-dependent disappearance of ODC70-C and generated a new discrete 70-kb band (Fig. 4A). That the 70-kb band was a linearized derivative of ODC70-C was ascertained by Southern blot hybridization to ODC70-CS4b (Fig. 4B). No

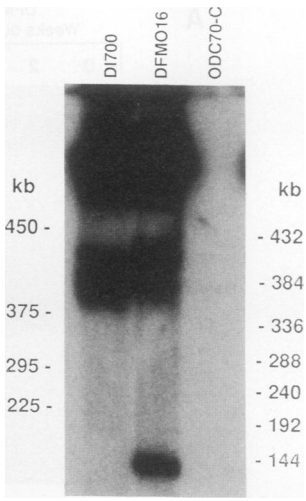


FIG. 3. Hybridization of ODC140-L and ODC70-C to a telomere probe. The gel shown in Fig. 1B was blotted onto a Nytran filter and hybridized to pTT6, derived from the *T. brucei* telomere repeat unit.

new discrete DNA species were generated from ODC140-L by limited γ-irradiation, even at radiation doses that caused significant degradation of the larger chromosomes (Fig. 4A). Southern blot analysis of the gel shown in Fig. 4A and hybridized to the *odc* probe revealed that ODC140-L remained intact even after 100 min (20 krads) of γ-irradiation in the ^{137}Cs source (Fig. 4C).

Structure of the *odc* locus in DFMO16 cells. Southern blot analysis of DFMO16 DNA probed with the *odc* gene indicated that the structure of the amplified *odc* gene locus in DFMO16 cells is identical within a 25-kb region (data not shown) to that of the genomic *odc* locus of wild-type cells (24). Therefore, the DNA rearrangements that generated this linear amplification are not located within a 25-kb region surrounding the *odc* locus. As the *odc* gene copy number is amplified ~10- to 20-fold in DFMO16 cells and ODC140-L has a molecular size of 140 kb, mapping analysis will be necessary to determine the number of amplification units present in each ODC140-L molecule and the ODC140-L copy number.

Physical mapping of the ODC70-C amplicon and chromosomal region. A physical map of the circular extrachromosomal ODC70-C element was constructed by digestion of purified ODC70-C with infrequently cutting restriction enzymes and Southern blot analysis. As expected, we obtained a circular restriction map, which additionally showed that the amplicon comprised two unique DNA segments separated by a repeated DNA sequence arranged in an inverted fashion (Fig. 5A). The leftmost unique region (as shown in Fig. 5A) is delimited by *Bam*HI sites and comprises about 15.5 kb of DNA. The rightmost unique region is flanked by *Eco*RI sites (Fig. 5A). However, additional mapping of the 9.8-kb *Eco*RI-*Bam*HI and 7.0-kb *Eco*RI-*Hin*dIII fragments located to the right of the *Eco*RI sites with the restriction enzymes *Sac*II and *Pst*I suggested that the border of the unique region actually extends nearly 7 kb further to the right, approaching the *Hin*dIII site shown to the right of the upper inverted repeat of ODC70-C (Fig. 5A). These data defined the limits of the central inverted repeats of ~24.5 kb (Fig. 5A). The mapping data predict a circular molecule of 71 kb, in good agreement with the M_r determined by PFGE after limited γ-irradiation (Fig. 4). These data also indicate

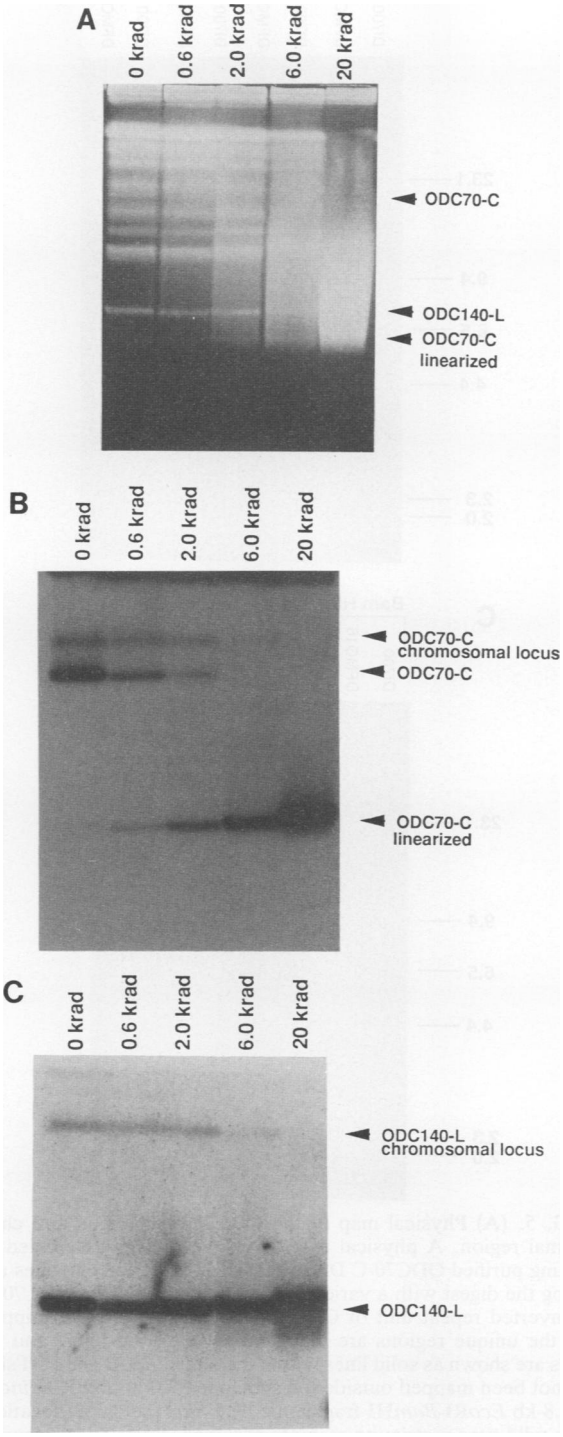


FIG. 4. γ-Irradiation of DFMO16 chromosomes. (A) Agarose plugs containing DFMO16 chromosomes were exposed to increasing doses of γ-irradiation as indicated. The products of the γ-irradiation experiment were fractionated by PFGE for 30 h with a 60-s pulse time and visualized with ethidium bromide. (B) The gel in panel A was blotted onto a Nytran filter and probed with ODC70-CS4b. (C) The filter used in panel B was rehybridized to a fragment of the *L. donovani odc* gene.

that ODC70-C does not exist as higher-order multimers, as has been observed in certain other DNA amplifications for *Leishmania* spp. (41, 57).

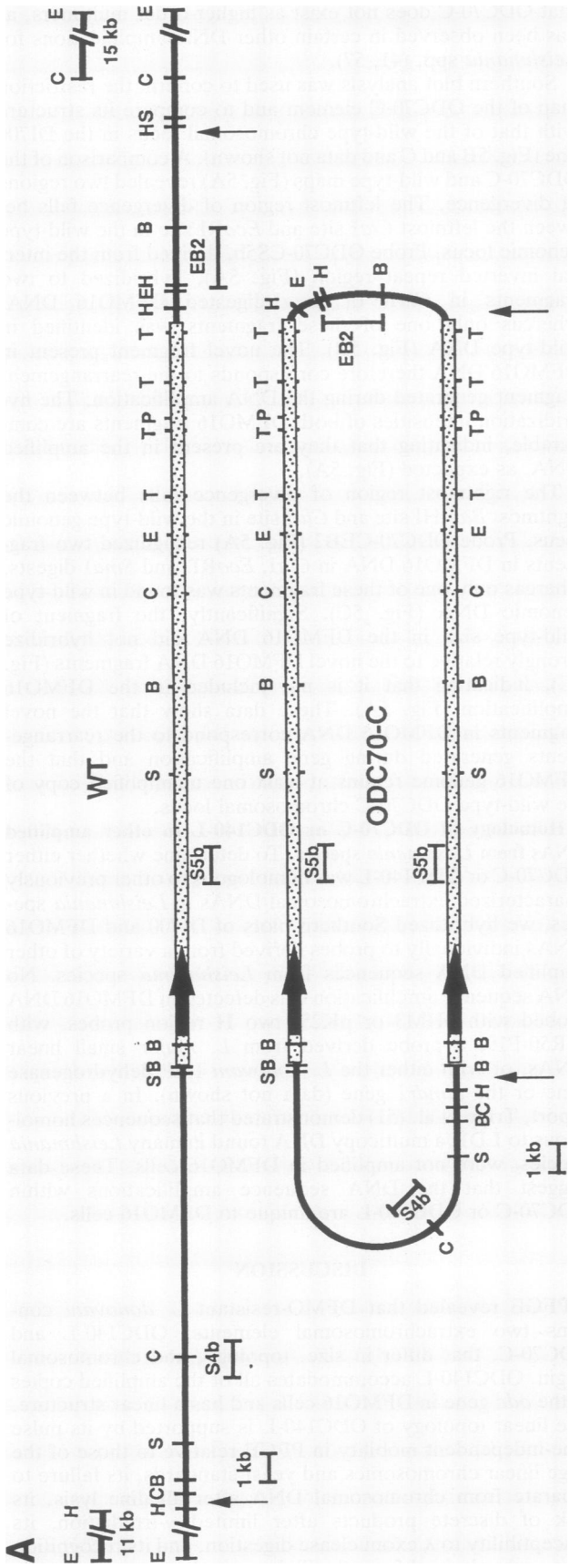
Southern blot analysis was used to confirm the restriction map of the ODC70-C element and to compare its structure with that of the wild-type chromosomal locus in the DI700 line (Fig. 5B and C and data not shown). A comparison of the ODC70-C and wild-type maps (Fig. 5A) revealed two regions of divergence. The leftmost region of divergence falls between the leftmost *Cla*I site and *Eco*RI site in the wild-type genomic locus. Probe ODC70-CS5b, derived from the internal inverted repeat region (Fig. 5A), hybridized to two fragments in *Cla*I- or *Sma*I-digested DFMO16 DNA, whereas only one of these fragments was identified in wild-type DNA (Fig. 5B). The novel fragment present in DFMO16 DNA therefore corresponds to the rearrangement fragment generated during the DNA amplification. The hybridization intensities of both DFMO16 fragments are comparable, indicating that they are present in the amplified DNA, as expected (Fig. 5A).

The rightmost region of divergence falls between the rightmost *Bam*HI site and *Cla*I site in the wild-type genomic locus. Probe ODC70-CEB2 (Fig. 5A) recognized two fragments in DFMO16 DNA in *Cla*I, *Eco*RI, and *Sma*I digests, whereas only one of these fragments was found in wild-type genomic DNA (Fig. 5C). Significantly, the fragment of wild-type size in the DFMO16 DNA did not hybridize strongly relative to the novel DFMO16 DNA fragments (Fig. 5C), indicating that it is not included in the DFMO16 amplification (Fig. 5A). These data show that the novel fragments in DFMO16 DNA correspond to the rearrangements generated during gene amplification and that the DFMO16 genome retains at least one unamplified copy of the wild-type ODC70-C chromosomal locus.

Homology of ODC70-C or ODC140-L to other amplified DNAs from *Leishmania* species. To determine whether either ODC70-C or ODC140-L was homologous to other previously characterized extrachromosomal DNAs in *Leishmania* species, we hybridized Southern blots of DI700 and DFMO16 DNAs individually to probes derived from a variety of other amplified DNA sequences from *Leishmania* species. No DNA sequence amplification was detected in DFMO16 DNA probed with pHM3 or pK25, two H region probes, with p7R50-P19, a probe derived from *L. major* small linear DNAs, or with either the *L. donovani* IMP dehydrogenase gene or the *ldmdr1* gene (data not shown). In a previous report, Tripp et al. (51) demonstrated that sequences homologous to LD1, a multicopy DNA found in many *Leishmania* species, were not amplified in DFMO16 cells. These data suggest that the DNA sequence amplifications within ODC70-C or ODC140-L are unique to DFMO16 cells.

DISCUSSION

PFGE revealed that DFMO-resistant *L. donovani* contains two extrachromosomal elements, ODC140-L and ODC70-C, that differ in size, topology, and chromosomal origin. ODC140-L accommodates all of the amplified copies of the *odc* gene in DFMO16 cells and has a linear structure. The linear topology of ODC140-L is supported by its pulse time-independent mobility in PFGE relative to those of the large linear chromosomes and yeast standards, its failure to separate from chromosomal DNA after alkaline lysis, its lack of discrete products after limited γ-irradiation, its susceptibility to λ exonuclease digestion, and its recognition by a probe derived from the *T. brucei* telomere repeat unit.



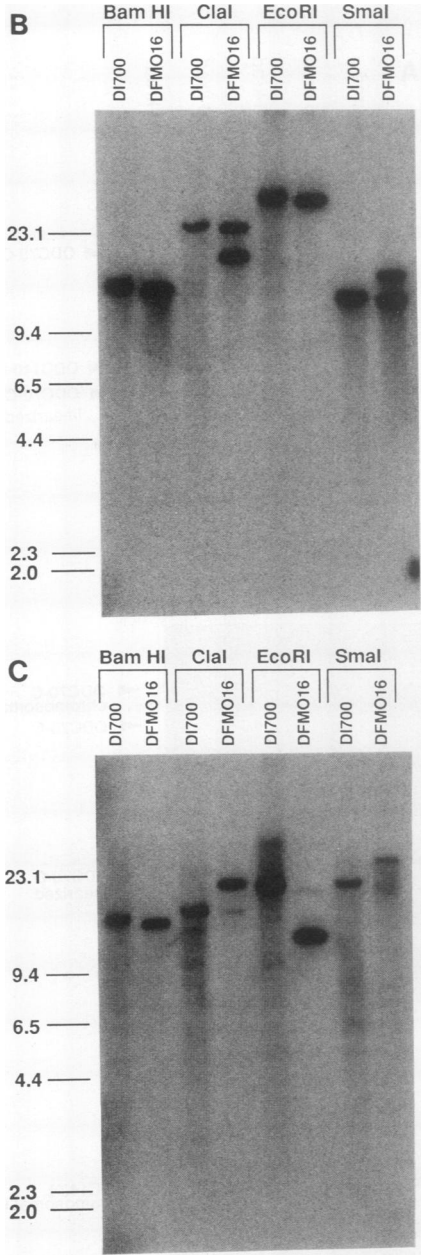


FIG. 5. (A) Physical map of the ODC70-C amplicon and chromosomal region. A physical map of ODC70-C was assembled by digesting purified ODC70-C DNA with five restriction enzymes and probing the digest with a variety of probes derived from ODC70-C. The inverted repeat unit of ODC70-C is indicated by the stippled bars, the unique regions are indicated by the solid lines, and the probes are shown as solid lines within the circle. *Sac*II and *Pst*I sites have not been mapped outside the subcloned 7.0-kb *Eco*RI-*Hin*dIII and 9.8-kb *Eco*RI-*Bam*HI fragments. The *Sac*II and *Pst*I locations on the wild-type restriction map are assumed. Restriction enzymes used for these maps are abbreviated as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sma*I; T, *Sac*II; P, *Pst*I. A restriction map of the chromosomal region from which ODC70-C was derived was compiled from the experiments described in panel B. The direction of the wild-type orientation was arbitrarily designated and is indicated by the thick arrows. The approximate points at which the wild-type and ODC70-C maps diverge are designated by thin arrows. (B) DI700 (1.6 μg) or DFMO16 (0.16 μg) genomic DNAs were digested with four of the same restriction enzymes as those used to construct the physical map of the isolated ODC70-C extrachromo-

Conversely, the ODC70-C amplicon in DFMO16 cells appears to be a circular molecule, as evidenced by its pulse time-dependent mobility relative to those of large linear DNAs in PFGE, its segregation from chromosomal DNA after alkaline lysis, its conversion to a distinct 70-kb linear molecule subsequent to γ-irradiation, its resistance to degradation by λ exonuclease, its failure to hybridize with the *T. brucei* telomere probe, and its circular physical map. The presence of both linear and circular extrachromosomal DNAs in DFMO16 cells is unique among drug-resistant *Leishmania* spp. that carry amplified DNA sequences.

The appearance of more than one amplicon within a single stock of drug-selected *Leishmania* spp. has been observed, as methotrexate-resistant *L. major* containing both R and H region circular DNAs can be derived (7). Whereas ODC140-L, like the amplified R region observed in methotrexate-resistant cells, encodes the gene targeted by drug selection, neither the H region nor ODC70-C encodes the gene specifically targeted by the selective pressure. H region amplification can be induced by exposure of *Leishmania* spp. to a variety of structurally and mechanistically unrelated drugs (3, 11, 20, 36) and has been shown to contain a homolog of a P-glycoprotein gene, *lmpgpA*, whose amplification after transfection confers resistance to arsenite and certain antimonial compounds, but not to methotrexate (11). Methotrexate resistance, however, is bestowed by another locus on the H region, one that is genetically distinct from both *lmpgpA* and the dihydrofolate reductase-thymidylate synthase gene (12, 37). The function of ODC70-C is not known, since it does not contain *odc* sequences. Moreover, ODC70-C is not recognized by either *ltpgpA*, the *L. tarentolae* homolog of *lmpgpA*, or *ldmdr1*, a gene that confers cross-resistance to multiple hydrophobic drugs in *L. donovani* (25). However, its instability in the absence of selective pressure argues that exposure to drug is essential for its maintenance. Therefore, ODC70-C may contribute to the drug resistance phenotype observed in DFMO16 cells by a novel mechanism. Whether ODC70-C contains novel elements of resistance to DFMO or other drugs can be determined by phenotypic dissection of wild-type *L. donovani* transfected with fragments of ODC70-C.

The structure of ODC70-C is somewhat similar to that of the previously characterized H (7) and D (26) region circular amplified DNAs. All three multicopy DNAs encompass two unique sequences flanked by duplicated regions arranged in opposite orientations, suggesting a common mechanism of DNA sequence amplification in *Leishmania* strains containing these amplicons. ODC70-C and the H region do not share substantial sequence homology, however, since H region probes fail to recognize ODC70-C on Southern blots. The relationship of the D region, a DNA with no evident association with drug resistance, to ODC70-C is unknown because of the unavailability of D region probes.

The chromosomal region from which ODC70-C originated, like that of the H region, only contains a single copy of the inverted repeat unit, indicating that the ODC70-C amplicon must have arisen by duplication of a region of chromosomal DNA. Thus, similar to previous observations for the H region, it appears that ODC70-C was generated with a minimal number of DNA rearrangements. In contrast, in drug-resistant mammalian cells, DNA sequence amplifications are usually accompanied by multiple heterogeneous DNA rearrangements (46, 49). Although the amplification mechanism for the origins of ODC140-L and ODC70-C cannot be unambiguously defined, Southern blots of gels implied that the chromosomal regions of both ODC70-C and the *odc* gene in DI700 and DFMO16 cells were identical in structure and ploidy, indicating that both extrachromosomal elements originated from a conservative amplification (5). Conservative amplification is defined as one in which the copy number and structure of the original wild-type chromosome are unaltered. In support of this model, no other novel DNA fragments that could potentially represent deletion-associated rearrangements were observed (Fig. 1C and D). However, a deletional amplification mechanism cannot be eliminated without probing Southern blots of gels with chromosomal DNAs flanking the amplified sequences.

The appearance of an amplicon with a linear structure is unusual among the extrachromosomal DNAs that have been characterized in drug-resistant *Leishmania* species. Most of the amplified DNAs that have been examined in these drug-resistant parasites have been shown to exhibit a circular topology. These circular DNAs include the 30-kb R region observed in methotrexate- and 10-propargyl-5,8-dideazafolate-selected *L. major* (7, 21), the 68- to 70-kb H region amplified in several *Leishmania* species selected for their resistance to one of a variety of structurally unrelated drugs (7, 20, 36), and an extrachromosome of heterogeneous size in tunicamycin-resistant strains (29). Recently, however, Wilson et al. (59) described a 280-kb linear amplified extrachromosomal DNA, IMPDH-280, that encompasses the IMP dehydrogenase gene in mycophenolic acid-selected *L. donovani*. The amplification of ODC140-L and the amplification of IMPDH-280 represent the first two examples in which linear extrachromosomal DNAs have been associated with a drug-resistant phenotype in *Leishmania* species. Previously described linear multicopy DNAs in *Leishmania* species do not appear to be related to drug resistance phenomena in these parasites (6).

Linear extrachromosomal elements are not characteristic of DNA sequence amplifications observed in drug-resistant mammalian cells. Amplified genes in mammalian cells are associated with two types of aberrant DNA structures. Double-minute chromosomes are observed in cells in which the drug resistance phenotype is unstable. These double-minute chromosomes are acentric extrachromosomal molecules of 120 kb to 5.0 Mb in size and are considered to be circular on the basis of a variety of physicochemical criteria, including electron microscopy (22, 23, 44) and PFGE (38). The second class of DNA amplifications in mammalian cells is characterized by extended chromosomal regions, known as homogeneously staining regions. These homogeneously staining regions consist of either direct or inverted tandem repeats of DNA that are between 200 and 500 kb in size and that are stably integrated into chromosomes in the same or different sites as the gene targeted by drug selection (32, 35, 45, 46).

The linear extrachromosomal DNA molecules found in drug-resistant *L. donovani*, IMPDH-280 and ODC140-L, can be distinguished on the basis of their stabilities in the absence of selective pressure. Whereas the removal of DFMO16 cells from DFMO provoked the loss of both of their extrachromosomes within several weeks, IMPDH-280

some and electrophoresed on 0.4% agarose gels. The DNA was transferred to Nytran filters and probed with ODC70-CS5b, a probe for the inverted repeat unit of ODC70-C. (C) The Nytran filter used in the audioradiogram depicted in panel B was stripped of radiolabeled ODC70-CS5b probe and rehybridized to ODC70-CEB2, a probe derived from the 6.2-kb unique region.

was completely stable in the absence of drug for 23 months (59). The kinetics of ODC140-L and ODC70-C disappearance correlated with a decline in ODC activity and a restoration of DFMO growth sensitivity to levels exhibited by *L. donovani* DI700 (24). Future characterization of the linear amplified DNAs present in the two drug-resistant *L. donovani* strains will provide new insights into the processes involved in both gene amplification and normal chromosomal metabolism. The replication of linear DNAs in most eukaryotes is dependent on specialized telomeric DNA, and it is evident that the linear amplified DNAs in DFMO16 cells and mycophenolic acid-resistant *L. donovani* (60) have acquired at least one new telomere during their formation. The acquisition of new telomeres has been observed for several species, including *Plasmodium falciparum* (43), *Drosophila melanogaster* (8), yeasts (19), and mammals (58), and the characterization of this process in *Leishmania* spp. is relevant to chromosomal maintenance and evolution, as well as gene amplification. Since the linear IMPDH-280 and ODC140-L DNAs differ in stability in the absence of drug pressure, we infer that IMPDH-280 DNA must carry chromosomal determinants that mediate mitotic segregation and that are lacking in ODC140-L DNA. Thus, ODC140-L DNA may be considered to lack a functional centromere. By introducing segments of stable IMPDH-280 DNA into unstable ODC140-L DNA and scoring for stability in vivo, researchers should be able to identify DNA segments mediating mitotic chromosomal stability, as first shown in *Saccharomyces cerevisiae* (33). The advent of methods for the integration of transfected DNAs, a process that occurs exclusively by homologous recombination in *Leishmania* spp. (15, 31), now permits the contemplation of such experiments.

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