

# Plasticity in chromosome number and testing of essential genes in *Leishmania* by targeting

(tetraploid/population biology/aneuploidy/dihydrofolate reductase-thymidylate synthase/protozoan parasite)

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**ABSTRACT** We attempted to generate homozygous *dhfr-ts* (dihydrofolate reductase-thymidylate synthase) knockouts in virulent *Leishmania major*, an asexual diploid protozoan parasite. Transfection of a *neo* (neomycin phosphotransferase) targeting fragment yielded heterozygous replacement lines with high efficiency. However, second transfections with a *hyg* (hygromycin B phosphotransferase) targeting fragment in the presence of metabolites shown to rescue homozygous knockouts in attenuated *Leishmania* did not yield the expected *dhfr-ts*<sup>-</sup> thymidine auxotrophs obtained previously with attenuated lines. Molecular karyotype, Southern blot, and flow cytometric DNA content analysis of clonal transfectants revealed three classes: (i) genomic tetraploids, containing two wild-type *dhfr-ts* chromosomes and one *neo* and one *hyg* replacement chromosome; (ii) aneuploid trisomic lines with one wild-type *dhfr-ts* and one *neo* and one *hyg* replacement chromosome; (iii) diploids bearing homologous integration of the targeting fragment without replacement. Aneuploid and tetraploid lines predominated. This confirms the common impression that natural populations of *Leishmania* are often aneuploid. The remarkable ability of these parasites to undergo and tolerate changes in chromosome number suggests a general method for testing whether genes are essential for growth *in vitro*, as the ability of *Leishmania* to simultaneously undergo homologous gene replacement while retaining wild-type genes by increasing chromosome number provides a diagnostic and positive experimental result. Our results show that virulent *Leishmania* require at least one copy of *dhfr-ts* and argue that DHFR-TS plays an unanticipated role in addition to its role in the *de novo* synthesis of thymidine. These results also have implications for genetic tests of the organization of *Leishmania* populations.

It is now possible by DNA transfection to engineer trypanosomatid parasites that overexpress or lack a given gene product. Since trypanosomatids are generally diploid and it is currently either impossible (*Leishmania*) or difficult (*Trypanosoma brucei*) to perform sexual crosses, homozygous gene knockouts are usually obtained by the process of double gene replacement with independent selectable markers (1). This approach is limited to genes whose function is not essential during *in vitro* culture; however, many interesting parasite-specific molecules are known or likely to be nonessential for growth *in vitro* (2, 3).

One approach to the study of essential genes is to use situations in which knockouts are conditionally lethal. We have shown that homozygous knockouts of the *Leishmania major dhfr-ts* (dihydrofolate reductase-thymidylate synthase) locus can be obtained if parasites are provided with thymidine (1, 4), in keeping with the expected role of DHFR-TS in intermediary metabolism. The *dhfr-ts* knockout will facilitate

studies of folate metabolism and antifolate chemotherapy, and conditionally lethal mutants like these *thy*<sup>-</sup> (thymidine negative) auxotrophs may have important applications in the development of safe, avirulent lines that could be used as potential vaccination strains, as could parasites lacking molecules essential for infectivity *in vivo* but nonessential for growth *in vitro* (1). Since virulent *Leishmania* may provide superior vaccination potential relative to attenuated lines, we attempted to develop homozygous knockouts of *dhfr-ts* in virulent lines. To our surprise, we were unable to obtain the desired mutants, despite the ease with which such knockouts were obtained with attenuated lines (1). Instead, virulent *Leishmania* parasites possess a remarkable ability to undergo changes in chromosome number, permitting retention of one or more chromosomes containing *dhfr-ts* despite the success of the double targeting.

## MATERIALS AND METHODS

Virulent *L. major* were LV39 clone 5 (Rho/SU/59/P) and Friedlin (MHOM/JL/80/Friedlin); attenuated lines were LT252 clone CC-1 (MHOM/IR/83/IR; ref. 5) and CB rev3 (6, 7). Cells were grown in M199 medium and transfected by electroporation and plating as described (1, 4, 5). KS supplements were added in transfections expected to yield *dhfr-ts*<sup>-</sup> lines (4). Virulent parasites recovered from lesions were cultured in NNN medium (7), transformed into promastigotes, and grown <2 weeks prior to transfection. Stationary-phase parasites ( $5 \times 10^6$ ) were injected subcutaneously into the foot of BALB/c mice in triplicate, and development of lesions followed (7). General molecular methods were performed as described (1, 4, 5, 8). Gene-specific hybridization probes were as follows: *dhfr-ts*, 0.8-kb *Xho* I fragment (nt 658–1483; ref. 8); *neo* (neomycin phosphotransferase), 0.9-kb *Spe* I fragment from pSpeNEOA (5); *hyg* (hygromycin B phosphotransferase), a PCR-derived cassette (1); probe P, 1.6-kb *EcoRI/EcoRV* fragment (8). DNA content was measured by flow cytometry using ethanol-fixed cells (9). Heat-inactivated RNase (150  $\mu$ l; 10 mg/ml) was added to fixed cells in 1 ml of phosphate-buffered saline (PBS) and incubated 30 min at 37°C. Cells were washed, resuspended for 1 h in 0.5 ml of propidium diiodide (50 mg/ml in PBS), and washed once before flow cytometry.

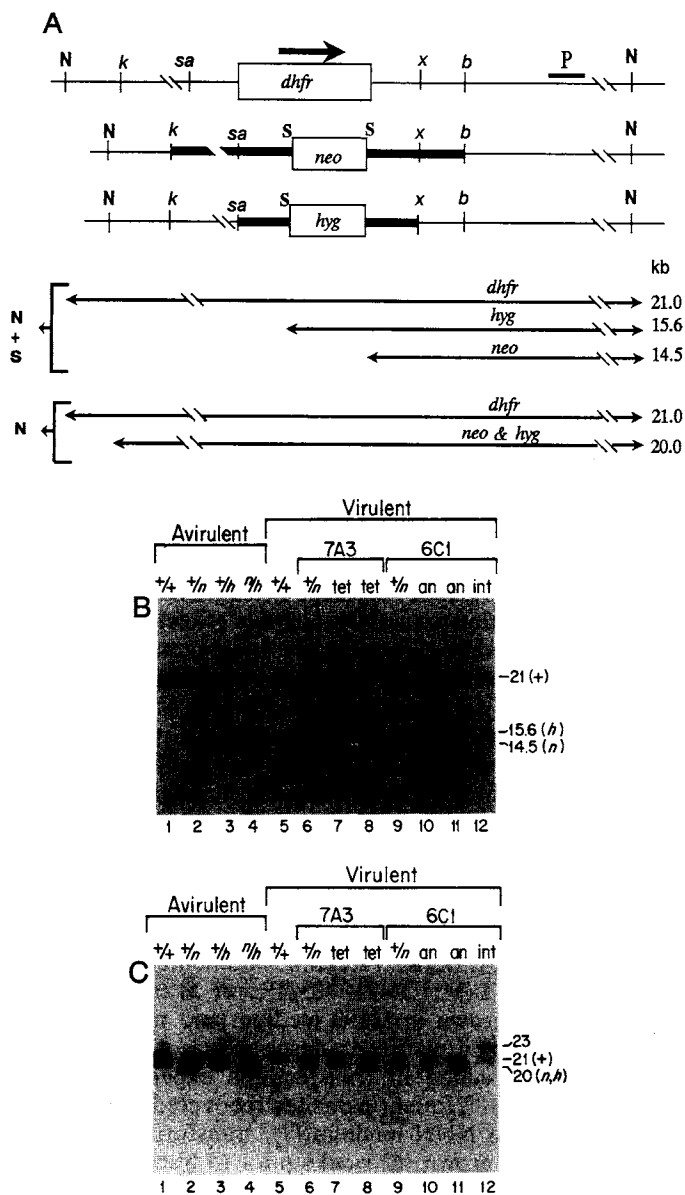
## RESULTS

**Replacement of One Allele of *dhfr-ts* in Virulent *Leishmania*.** Virulent *L. major* (LV39 clone 5) were transfected with a linear targeting fragment containing the *neo* selectable marker joined to DHFR-TS flanking sequences (Fig. 1A), as described for attenuated lines (4). Eight of nine clones

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**FIG. 1.** Southern blot analysis of tetraploid and aneuploid *L. major*. (A) Structure of the *dhfr-ts* locus and planned replacements. Thick lines indicate *neo*- and *hyg*-containing fragments that were used in gene targeting. Sizes of DNA fragments expected in Southern blot analysis after digestion with *Not* I/*Spe* I or *Not* I and hybridization with probe P are shown below. All restriction sites for *Not* I (N) and *Spe* I (S) are shown; only relevant sites for *Kpn* I (k), *Sma* I (x), *Bgl* II (b), and *Sal* I (sa) are shown. (B) Southern blot analysis of *Leishmania* DNAs after digestion with *Not* I/*Spe* I and hybridization with probe P. Lanes 1-4, derivatives of the attenuated parental clone CC-1: lane 1, CC-1 (+/+); lane 2, *neo*/+ replacement clone E2-7D2 (+/n); lane 3, *hyg*/+ replacement clone E8-5B4 (+/h); lane 4, *neo/hyg* replacement clone E10-5B5 (n/h). Lanes 5-12, derivatives of the virulent LV39 clone 5: lane 5, LV39 clone 5 (+/+); lane 6, *neo*/+ clone E18-7A3 (+/n); lanes 7 and 8, tetraploid twice-targeted derivatives of E18-7A3, clones E23-7A3L1 and E23-7A3L'2 (*tet*); lane 9, *neo*/+ clone E18-6C1 (+/n); lanes 10 and 11, aneuploid twice-targeted derivatives of E18-6C1, clones E33-5A1 and E33-5B2 (*an*); lane 12, twice-targeted derivative of E18-6C1 (E23-6C1L1) bearing insertion (without replacement) of the *hyg* targeting fragment (*int*). DNAs were separated by field inversion; 9 V/cm forward pulse, 6 V/cm reverse pulse, and switch times from 0.39 to 0.46 sec in a linear ramp, for 28.6 h. Markers (not shown) were  $\lambda$  DNA digested with *Apa*LI, *Xho* I, or *Hind*III. (C) Southern blot analysis of *Leishmania* DNAs after digestion with *Not* I, separation by field inversion, and hybridization with probe P. Samples are described in B.

possessed the planned homologous replacement, while the remaining clone contained unintegrated extrachromosomal circular DNA (Table 1).

**Table 1.** Targeting of *dhfr-ts* in virulent and attenuated *L. major*

Round	Line	Colonies tested	Replacements	<i>thy</i> <sup>-</sup>	% replacements
1	LV39 (V)	9	8	NA	89
	Friedlin (V)	7	3	NA	43
	CC-1 (AV)	27	21	NA	78
	CBrevA (AV)	4	3	NA	75
2	LV39 (E18-7A3; V)	5	5*	0	100
	LV39 (E18-6C1; V)	6	4†	0	67
	Friedlin (6B4; V)	4	ND	0	ND
	CC-1‡ (AV)	20	12	12	60

ND, not done; NA, not applicable; V, virulent line; AV, attenuated line.

\*All are tetraploids.

†All are aneuploids.

‡Only 6 lines were analyzed molecularly; 5 were the expected homozygous replacements (1). The other 14 lines were analyzed only for thymidine auxotrophy; 7 were *thy*<sup>-</sup> and presumed to be replacements.

The molecular karyotype of the *neo*/+ heterozygous replacement was the same as the parent line (Fig. 2A, lanes 5 and 9 vs. lane 5; others not shown). A *neo* probe identified a 500-kb chromosome only in the *neo*/+ lines (Fig. 2C, lanes 6 and 9), and a *dhfr-ts* probe identified a similarly sized chromosome in these and the parental line (Fig. 2B, lanes 6 and 9 vs. lane 5). Southern blots of DNAs digested with *Not* I/*Spe* I and hybridized with a flanking probe showed the presence of the expected 14.5-kb replacement fragment (Fig. 1A) in *neo*/+ lines (Fig. 1B, lanes 6 and 9), as well as the 21-kb wild-type *dhfr-ts* fragment (Fig. 1B, lanes 6 and 9 vs. lane 5). Southern blots of *Not* I-digested DNAs similarly revealed a 20-kb replacement fragment in the *neo*/+ lines with the flanking probe (Fig. 1C, lanes 6 and 9) in addition to the 21-kb wild-type *dhfr-ts* fragment (Fig. 1C, lanes 6 and 9 vs. lane 5). Similar results were obtained with attenuated *Leishmania* replacement lines (Figs. 1B and C and 2A-C, lanes 1 and 2; ref. 4). These data confirmed the presence of the planned heterozygous replacement in the virulent transfectants.

**Virulence of the Heterozygous Lines.** The infectivity of the eight heterozygous *neo*/+ replacement lines was assessed in susceptible mice. Three lines failed to give a lesion after 2.5 months, while the remaining five lines developed lesions that progressed somewhat less rapidly than the parental virulent lines (data not shown). This shows that the loss of one copy of *dhfr-ts* does not drastically reduce infectivity. *Leishmania* are known to lose virulence during culture *in vitro*, accounting for its loss in some transfectants (unpublished data).

**Second-Round Targeting.** Three virulent heterozygous *neo*/+ replacement clones (E18-6C1, E18-7C5, and E18-7A3) were chosen for a second round of transfection with a *hyg* targeting fragment (Fig. 1A), with protocols and medium supplements that had been used successfully in *dhfr-ts* knockouts in attenuated lines (1).

From clone E18-7C5 hygromycin-resistant colonies were not obtained, although circular plasmid transfections gave several hundred colonies. From clones E18-6C1 and E18-7A3 a total of 11 hygromycin-resistant lines were obtained. The transfection efficiency (colonies per  $\mu$ g of DNA) was  $\approx$ 5% that of control circular DNAs, comparable to that obtained previously (1). All 11 lines were prototrophic for thymidine (Table 1). In contrast, analogous transfections of an attenuated *neo*/+ replacement line yielded many thymidine auxotrophs (12/20; Table 1; ref. 1).

**Tetraploids.** The molecular karyotypes of the five hygromycin-resistant clonal lines from clone E18-7A3 (termed twice-targeted lines) were the same as the parental lines (Fig. 2A, lanes 7 and 8 vs. lanes 5 and 6). Southern blots showed

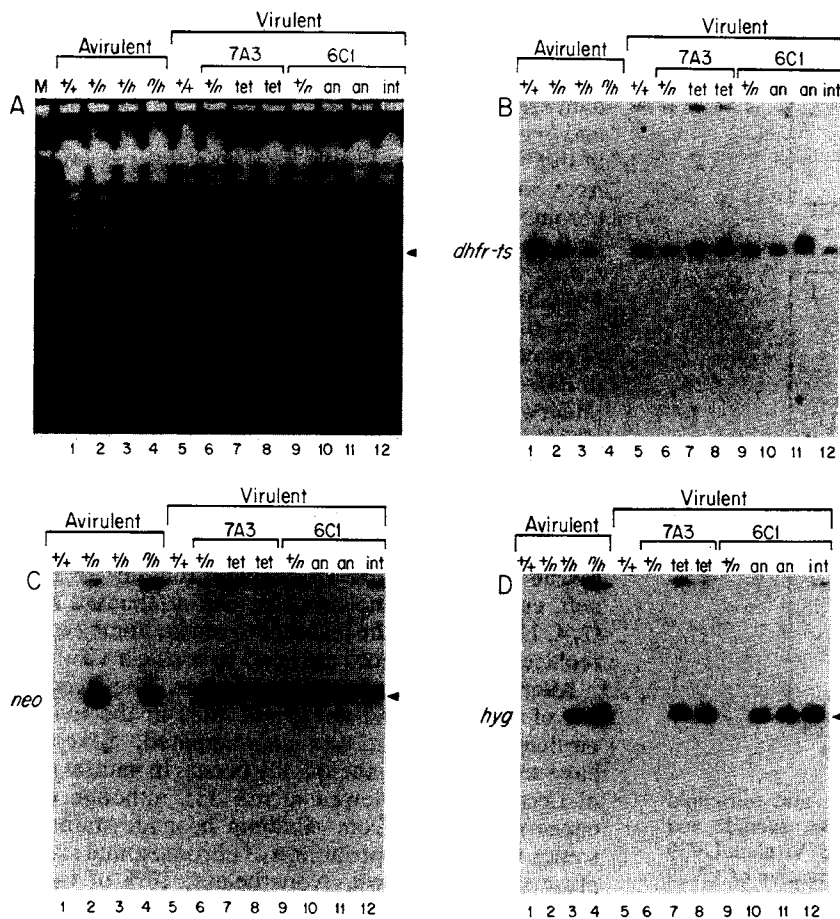


FIG. 2. Molecular karyotype of tetraploid and aneuploid *Leishmania*. Chromosomes were separated by pulsed-field electrophoresis using a Bio-Rad CHEF mapper apparatus at 6 V/cm, 120° separation angle, with switch times varying from 35.4 to 73.6 sec in a linear ramp, for 35.4 h. Arrowheads mark position of *dhfr-ts* chromosome (or derivatives). (A) Ethidium bromide-stained gel. Lane M,  $\lambda$  ladder marker. (B–D) Southern blots of gel shown in A (or duplicates) hybridized with a *dhfr-ts* coding region probe (B), a *neo*-specific probe (C), or a *hyg*-specific probe (D). Samples are described in the legend to Fig. 1B.

that the twice-targeted lines had identically sized chromosome(s) that hybridized with *dhfr-ts*-, *neo*-, and *hyg*-specific probes (Fig. 2 B–D, lanes 7 and 8), which were the same as the wild-type *dhfr-ts* chromosome (lane 5). A twice-targeted attenuated *thy*<sup>-</sup> auxotroph showed no hybridization with the *dhfr-ts* probe (Fig. 2B, lane 4; ref. 1).

Southern blots showed that the twice-targeted E18-7A3 lines contained both expected *hyg* and *neo* replacements plus wild-type *dhfr-ts*. Southern blots of *Not* I/*Spe* I-digested DNAs and flanking probe P (Fig. 1A) yielded the 21-kb wild-type *dhfr-ts* fragment (Fig. 1B, lanes 7 and 8 vs. lanes 1 and 5), the 14.5-kb *neo* replacement fragment (Fig. 1B, lanes 7 and 8 vs. lanes 2 and 6), and the 15.6-kb *hyg* replacement fragment (Fig. 1B, lanes 7 and 8 vs. lane 3). Similar results were obtained with other digests and probes. The intensity of the 21-kb *dhfr-ts* band was greater than that of the *neo* or *hyg* replacement bands, suggesting that there may be more than one copy of this chromosome. Southern blot analysis of *Not* I-digested DNAs with flanking probe P ruled out the possibility that these fragments arose by homologous integration of the targeting fragment into the *dhfr-ts* locus, an event that would give rise to a fragment larger than the normal 21-kb *Not* I fragment, which was not observed (Fig. 1A; Fig. 1C, lanes 7 and 8 vs. lanes 2, 3, and 6).

This showed that the twice-targeted derivatives of clone E18-7A3 contained one *neo* replacement, one *hyg* replacement, and one or more wild-type *dhfr-ts* chromosomes. One possibility was that these cells had undergone alterations in either chromosome number or ploidy, and the total cellular DNA content was measured by flow cytometry of fixed cells stained with propidium diiodide. Logarithmic-phase attenuated CC-1 or virulent LV39 clone 5 cells showed the expected pattern of G<sub>1</sub>, S, and G<sub>2</sub> phase cells (Fig. 3 A and B), with G<sub>1</sub> and G<sub>2</sub> DNA contents of about 130 and 260 fluorescence units (FU), respectively. Stationary-phase cells showed the expected G<sub>1</sub> staining pattern (data not shown). All heterozygous

*neo*/+ replacement lines showed similar profiles, both before and after passage in the mouse (Fig. 3 C and D). However, all of the twice-targeted E18-7A3 lines showed a doubling of total DNA content, with a G<sub>1</sub> peak of about 270 FU and a G<sub>2</sub> peak of 520 FU (Fig. 3E, solid line; other data not shown). Since we previously showed that the attenuated CC-1 line of *L. major* is diploid for both the *dhfr-ts* and minixon chromosomes (1, 4, 10), the DNA content and blotting data confirm that the twice-targeted derivatives of clone E18-7A3 are genomic tetraploids.

Only one of the five tetraploid lines (E23-7A3L'1) was infective to mice. Parasites recovered from this lesion retained the tetraploid DNA profile (Fig. 3F), showing that tetraploidy is stable during the amastigote stage. The tetraploid DNA content was also maintained during 3 months of culture *in vitro* as promastigotes (data not shown).

**Aneuploidy.** The molecular karyotypes of the twice-targeted lines derived from the heterozygous *neo*/+ replacement line E18-6C1 showed no obvious differences (Fig. 2A, lanes 10–12), and Southern blots showed chromosomes hybridizing with the *dhfr-ts*, *neo*, and *hyg* probes whose sizes were similar to each other and the *dhfr-ts* chromosomes present in the parental lines (Fig. 2, lanes 10–12 vs. lanes 5 and 9). Southern blots of digested DNAs revealed two different phenotypes: group I (lines E33-5A1, -5B2, -5C1, and -6B4) exhibited *Not* I/*Spe* I fragments diagnostic for wild-type *dhfr-ts* and both the planned *neo* and *hyg* replacements (Fig. 1A; Fig. 1B, lanes 10 and 11; data not shown). *Not* I digestion showed that insertion (without replacement) of the *hyg* targeting fragment had not occurred (Fig. 1C, lanes 10 and 11). In contrast, in the two group II lines (E23-6C1L1, E33-7C5) the 3.3-kb *hyg* targeting fragment had inserted into homologous sequences 5' of the first-round *neo* replacement. This yielded a larger 23-kb *Not* I fragment and retention of the 21-kb *dhfr-ts* fragment (Fig. 1C, lane 12).

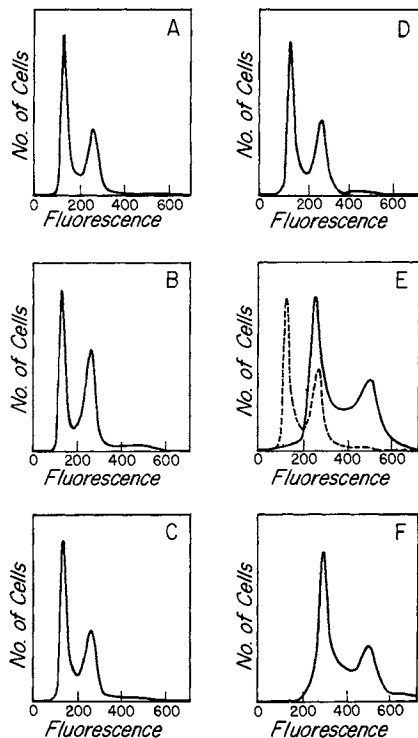


FIG. 3. DNA content of attenuated, virulent, and tetraploid *Leishmania*. Parasites were stained with propidium diiodide and subjected to flow cytometry. (A) Attenuated CC-1. (B) Virulent LV39 clone 5. (C) Heterozygous *neo*/*+* replacement derivative of LV39 clone 5 (E18-7A3). (D) E18-7A3 after passage through a mouse. (E) Tetraploid twice-targeted derivative (E23-7A3L'1) of *neo*/*+* replacement E18-7A3 (solid line) and aneuploid twice-targeted derivative (E33-5C1) of *neo*/*+* replacement E18-6C1 (dashed line). (F) E23-7A3L'1 after passage through a mouse. The y axis shows relative cell number and the x axis shows relative fluorescence (linear scales).

Flow cytometry of lines from both groups showed that all had the standard amount of DNA (Fig. 3E, dashed line; other data not shown). Since the 500-kb *dhfr-ts* chromosome constitutes only 1% of the entire parasite genome, an extra chromosome would not be detectable. This indicated that the group I derivatives were aneuploid, containing one or more normal *dhfr-ts* chromosomes and two modified chromosomes bearing *neo* and *hyg* replacements. We expected that aneuploidy of the *dhfr-ts* chromosome would cause increased staining in the gel shown in Fig. 2A. However, chromosome size polymorphisms between the LV39 and CC-1 lines have resulted in comigration of one or more CC-1 chromosomes with the *dhfr-ts* chromosome of the LV39 line (Fig. 2A, lanes 1–4 vs. lane 5). The copy number of the LV39 *dhfr-ts* chromosome is thereby obscured. All three of the aneuploid clones tested showed virulence comparable to the parental E18-6C1 line (data not shown).

**Double Gene Targeting in a Second Virulent Line.** The studies described above failed to yield the desired homozygous knockout of *dhfr-ts*. This was not unique to the LV39 line, as transfections of a second virulent line of *L. major* (Friedlin) yielded similar results. After the first round of transfection with the *neo* targeting fragment (Fig. 1A), three of seven lines contained the planned heterozygous replacement (Table 1). One of these clones (E43-6B4) was passed through mice and transfected with the linear *hyg* targeting fragment (Fig. 1A), yielding four hygromycin-resistant lines. None was auxotrophic for thymidine (Table 1).

## DISCUSSION

We were unable to obtain complete inactivation of the *dhfr-ts* locus in virulent *L. major* by methods that were successful in

attenuated lines (1). This could happen if either *dhfr-ts* was essential in virulent *Leishmania*, or if methodological problems associated with gene targeting prevented success. Our data argue against procedural difficulties: first, both the *neo* and *hyg* targeting fragments functioned well in the first round in the virulent lines (Table 1). This rules out effects arising from polymorphisms between the targeting fragment and the chromosomal target. Second, the exclusive recovery of *thy*<sup>+</sup> lines in the second-round transfections of virulent *Leishmania* was statistically significant (0 *thy*<sup>-</sup>/15 twice-targeted virulent lines vs. 12 *thy*<sup>-</sup>/20 twice-targeted attenuated lines;  $P < 0.005$ ; Table 1). Third, the twice-targeted *L. major* resorted to extreme genetic measures to avoid complete loss of *dhfr-ts*. The majority of the lines analyzed (9/11; Table 1) underwent alterations in chromosomal number, either aneuploidy or tetraploidy. One would more commonly expect integration without replacement or formation of extrachromosomal circular DNAs by self-ligation (4). Fourth, transfections expected to yield homozygous *dhfr-ts* knockouts were performed in the presence of metabolites known to rescue DHFR- and/or TS-deficient mutants of *Escherichia coli*, cultured mammalian cells, and attenuated *Leishmania* (1, 4, 11, 12). Last, the inability to obtain homozygous *dhfr-ts* replacements was not restricted to a single virulent line.

**Alterations in Chromosome Number.** The frequent recovery of tetraploid and aneuploid lines in the twice-targeted virulent *Leishmania* was unanticipated. Several workers have suspected that aneuploidy occurs in natural populations of *Leishmania* (reviewed in ref. 13), although these inferences were made from ethidium bromide-stained chromosomes where the contribution of chromosomal size polymorphisms was unknown. Nonetheless, 10% of random lines show evidence suggestive of aneuploidy for one or more chromosomes (ref. 13; unpublished data). Our work using genetic manipulations to mark sister chromosomes proves this for the *dhfr-ts* chromosome, and, in combination with previous studies, confirms the prevailing impression of widespread aneuploidy in *Leishmania*. In contrast, tetraploid *Leishmania* have not been reported previously but could occur in nature. Hybrid parasites with increased DNA contents have been reported in sexual crosses of *T. brucei* in tsetse flies, including triploids (reviewed in refs. 14 and 15). Spontaneous fusion of *Leishmania* cells has been observed (16); however, these cells have not been further characterized. Experimental sexual crosses have not been successful in *Leishmania* (17), although natural hybrids are occasionally reported (18, 19); the ploidy of these has not been determined. Natural tetraploids could arise by fusion or by rare and as yet unidentified sexual processes.

Several questions remain unanswered. For example, why were the clones so different in the second round of targeting? Potential explanations are adventitious genetic differences among the *+/neo* clones or environmental factors such as small differences in culture conditions or growth phase. Given the frequency of tetraploids arising from clone E17-7A3, one expects that preexisting aneuploids and tetraploids should be common. However, only  $\approx 1\%$  of the virulent wild-type or first-round *Leishmania* have tetraploid DNA contents (Fig. 3 B–D). Both tetraploid and aneuploid lines exhibited  $\approx 2:1:1$  ratios of the *+*, *neo*, and *hyg* alleles (Fig. 1B); yet, since one copy of *dhfr-ts* is sufficient for viability and normal growth, 1:2:1 or 1:1:2 parasites should also have been recovered. This asymmetry may reflect a bias for replacement of a *neo* allele by the *hyg* targeting fragment, which could occur after tetraploidization via endoreduplication, or in a *+/neo* heterozygote followed immediately by fusion with another *+/neo* cell (this latter model could account for the lack of correlation between the number of preexisting tetraploids and targeting frequencies). It seems unlikely that biased replacement could preclude the recovery

of double knockouts, as the frequency of replacement was similar in both rounds of gene targeting.

**Role of DHFR-TS in Virulent *Leishmania*.** As the lack of virulent *dhfr-ts* knockouts cannot be attributed to procedural or chromosomal sources, we conclude that virulent *Leishmania* cannot tolerate the loss of DHFR-TS. One may imagine that this could arise from trivial sources, such as reduced transport of the key metabolite thymidine. However, virulent parasites transport and incorporate thymidine into DNA (20). This argues that DHFR-TS plays an additional unanticipated role in virulent lines directly or in combination with other enzymes.

Currently, we can only speculate about new roles for DHFR-TS in virulent parasites. Previous biochemical characterizations of attenuated and virulent *Leishmania* have shown many differences, including reduced synthesis and/or alterations in the structure of the major surface carbohydrate lipophosphoglycan (LPG), which plays an important role in parasite infectivity (reviewed in ref. 21). LPG and other *Leishmania* surface glycolipids are anchored to the surface by ether-linked lipids (21, 22), which are known to turn over in *Leishmania* (21–24). In some organisms, cleavage of this family of lipids requires a reduced pterin cofactor (25, 26), and one may speculate that this step could link the DHFR-TS pathway with LPG and virulence. Accumulation of ether-linked lysophospholipids is known to be toxic to *Leishmania* (23, 24), and perhaps failure to cleave these lipids is toxic in virulent but not attenuated parasites. Other possibilities may arise from unique aspects of folate and pterin metabolism in *Leishmania*, which differ significantly from other organisms (27–29).

**Chromosome Number Plasticity May Permit Tests of Essential Genes.** The general ability of *Leishmania* to tolerate changes in chromosome number suggests a unique approach for testing whether genes are essential, which would be useful since *Leishmania* lacks an experimentally manipulable sexual cycle. First, heterozygous replacement lines are developed [the order in which the *neo* and *hyg* markers are used is unimportant (1)]. If a gene is nonessential, or conditionally so, a second round of targeting will yield the desired homozygous knockout (1). If the gene is essential, homozygous knockouts will be lethal; however, the remarkable ability of *Leishmania* to undergo changes in chromosome number will permit the recovery of cells simultaneously bearing wild-type and both planned *neo* and *hyg* replacement chromosomes. We propose that this is a diagnostic criterion for essentiality of gene function, as one is scoring a positive result and not just failure to obtain knockouts. Since *Leishmania* appear to be generally tolerant of chromosome number changes (28), this approach should be applicable at loci located on other chromosomes. Other manipulations that exploit chromosome number plasticity, such as parasexual fusions or crosses may eventually be feasible.

**Chromosome Number Variability and the Genetic Structure of *Leishmania* Populations.** One common finding in genetic surveys of natural populations of *Leishmania* is that the frequency of heterozygotes is much less than expected for randomly mating populations (30, 31). These and other data have led to a clonal, asexual model for the genetic structure of *Leishmania* populations. However, the ability of virulent *Leishmania* to undergo changes in chromosome number suggests another mechanism for generating homozygosity. Consider a chromosomal heterozygote A/B in which aneuploidy occurs, yielding A/B/B progeny. If aneuploidy persists only transiently, the A/B/B progeny will eventually revert to diploidy and generate either A/B or B/B offspring. The B/B offspring will now be homozygous throughout this chromosome regardless of the occurrence of sexual crossing. Over evolutionary time and different chromosomes, this process would affect most of the genome. Since most population genetic tests assume constant diploidy, their appli-

cation to “facultative” or “transient” aneuploids such as *Leishmania* must be viewed with caution. In this regard, linkage disequilibrium criteria may be less sensitive to uncertainty arising from chromosome number variability (31).

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