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Selection against the Dihydrofolate Reductase-Thymidylate Synthase (*DHFR-TS*) Locus as a Probe of Genetic Alterations in *Leishmania major*

FREDERICO J. GUEIROS-FILHO AND STEPHEN M. BEVERLEY*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

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The genome of the trypanosomatid protozoan genus *Leishmania* has been shown to undergo a number of changes relevant to drug resistance and virulence, such as gene amplification, chromosomal rearrangement, and variation in ploidy. Experimental approaches to the study of genomic changes have in some cases been limited by the fact that *Leishmania* cells are asexual diploids, as are some other trypanosomatids, pathogenic fungi, and cultured mammalian cells. Here we report upon a system which permits the measurement of several types of genomic change occurring at the dihydrofolate reductase-thymidylate synthase (*DHFR-TS*) locus. First, we show that *DHFR-TS* can function as a positive/negative marker. We used selection against *DHFR-TS* on a heterozygous line (+/*HYG*) to generate colonies exhibiting both loss of heterozygosity and structural mutations in *DHFR-TS*, permitting the first measurement of mutation frequencies in this parasite. Loss of heterozygosity occurred at a frequency ranging from 10^{-4} to 10^{-6} and was elevated 24-fold by treatment with γ-irradiation, while the frequency of other events was less than 10^{-6} and was increased more than 1,000-fold by nitrosoguanidine treatment. The frequency of loss of heterozygosity relative to other processes such as mutation and gene replacement has important implications for genetic variability in natural *Leishmania* populations and the generation of both targeted and random mutations. We also developed a protocol for null targeting of diploid cells, in which transfection of a *DHFR-TS* deletion construct into *Leishmania* cells followed by negative selection yielded parasites lacking *DHFR-TS* or foreign sequences. The null-targeting method can be applied to any diploid cell, at any locus for which a negative selection exists. Such marker-free auxotrophic *Leishmania* cells show potential as an attenuated vaccine, and the methods developed here provide a new approach for manipulating and characterizing the plasticity of the *Leishmania* genome.

The genus *Leishmania* comprises a group of parasitic protozoan species which infect over 10 million people worldwide (70). The parasite undergoes several developmental transitions, alternating between an extracellular transmissible form, which is carried in the gut of phlebotomine sand flies, and an intracellular form, which resides within the phagolysosome of vertebrate macrophages. The genome of the parasite has been shown to be quite plastic, undergoing a number of mutational and recombinational events including deletions, gene amplifications, chromosomal rearrangements, and alterations in ploidy (2, 4, 7, 20, 47, 54, 59, 66). Many of these have been implicated in biological phenomena such as drug resistance and virulence, making their study relevant to the understanding and control of this important disease.

To undertake laboratory studies of genomic change, one usually begins with the identification of mutants exhibiting the desired phenotype. This has been readily accomplished for dominant mutations, especially those mediating drug resistance by gene amplification (7, 54). However, our understanding of genomic changes conferring recessive phenotypes has been limited by the fact that this primitive eukaryote is effectively an asexual diploid (4, 18, 38, 64). While the discovery of occasional hybrid leishmanias in field isolates suggests at least the possibility of genetic exchange (reviewed in reference 51), neither we nor others have been able to demonstrate genetic exchange in experimental crosses in vitro or in the sand fly vector (reference 55 and references therein).

Diploidy without sex poses considerable challenges to those seeking to obtain mutants, a problem noted in previous studies of other asexual diploids including cultured mammalian cells and some *Candida* species (61, 69). Since two events are required, recessive mutants rarely occur, and thus selective rather than screening methods must be used to identify them. For diploid *Leishmania* cells, the frequency of recovery of recessive mutants is estimated to be less than 10^{-7} after mutagenesis (38, 40). When strong selective methods are available, this barrier can be overcome, as shown for drug resistance (7, 9, 17, 38, 40, 54) and lectin selections against an important surface glycolipid of *Leishmania* species, lipophosphoglycan (24, 41). For lipophosphoglycan mutants, it has proven possible to apply functional rescue approaches to identify the defective gene (23, 60). However, in studies of pathogens, a most desired mutant class affects virulence and survival within the host, and selections for avirulence are often not readily devised. Improved methods for generating *Leishmania* mutants would prove invaluable in genetic studies of pathogenesis in this organism, and basic studies of the frequency, type, and induction of mutations in the *Leishmania* genome would be helpful in this regard.

Targeted mutagenesis of asexual diploids also requires the inactivation of two alleles. This was accomplished in *Leishmania* spp. and other species by conducting two rounds of targeting with independent selectable markers (19, 44, 63). This places a burden on the number of selectable markers required if one wishes to inactivate several loci and is experimentally

* Corresponding author. Mailing address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 250 Longwood Ave., Boston, MA 02115. Phone: (617) 432-0611. Fax: (617) 432-1476. Electronic mail address: sbeverle@warren.med.harvard.edu.

tedious, because one must apply two different targeting vectors for each locus. Moreover, once inserted, the markers themselves may compromise further manipulations or have undesirable consequences (32). For this reason, a number of schemes have been developed to permit conservation or recycling of selectable markers (1, 12, 31, 36, 52).

Here we have developed a system that permits the measurement of several types of genomic change occurring at the *DHFR-TS* locus in *Leishmania major*. In *L. major*, as in all protozoan and plant species known, the genes encoding dihydrofolate reductase (DHFR) and thymidylate synthase (TS) have been joined to generate a bifunctional fusion protein (8, 45). First, we show that it is possible to select against the activity of the DHFR-TS protein by plating parasites in the presence of methotrexate plus thymidine (MTX+TdR). Since DHFR-TS activity can be selected for in the absence of exogenous TdR (18), *DHFR-TS* thus may be used as a positive-negative marker in *L. major*. We used the negative *DHFR-TS* selection in several ways: to characterize several types of genomic change in *L. major*, including loss of heterozygosity (LOH) and the induction of defective DHFR-TS proteins; to probe and quantitate the effect of mutagenic treatments on *L. major*; and to measure the emergence of LOH in heterozygous replacement parasites (+/*HYG*) subjected to elevated hygromycin B pressure, a method which conserves markers and is applicable at loci not amenable to negative selections (52). Finally, we used negative *DHFR-TS* selection in a protocol we term null targeting, which simultaneously deletes both copies of the chromosomal *DHFR-TS* gene without inserting any selectable marker. This approach, which could potentially be applied to other diploid organisms, was used here to generate a marker-free *dhfr-ts*$^-$ mutant parasite that has some potential as an attenuated vaccine for leishmaniasis (65). In total, our findings have important implications for our understanding of targeted and random mutations within the *Leishmania* genome and other asexual diploids.

MATERIALS AND METHODS

Cell lines, culture, and transfection. All cell lines studied were derivatives of the *L. major* clonal line CC-1 (39). CC-1 is diploid and homozygous at *DHFR-TS* (+/+); clone E8-5C7 has a heterozygous replacement of the *DHFR-TS* gene with a hygromycin B resistance marker (+/*HYG*), and E10-5A3 is a clonal *dhfr-ts*$^-$ knockout that is heterozygous for replacements of *DHFR-TS* with a G418 resistance marker (*NEO*) and *HYG* (*NEO*/*HYG*) (19). Promastigotes were cultivated in M199 medium containing 10% heat-inactivated fetal bovine serum (39) or, for tests of thymidine prototrophy, in a completely defined M199 medium lacking thymidine and supplemented with 0.66% bovine serum albumin (Cohn fraction V), 4 μg of folate per ml, 1.5 μg of biopterin per ml, 100 μM adenine, 10 μg of heme per ml, 40 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4), 50 U of penicillin per ml, 50 μg of streptomycin per ml, and 1 μg of biotin per ml. As indicated, liquid medium contained G418 (8 μg/ml), hygromycin B (16 μg/ml), or thymidine (10 μg/ml). Cells were enumerated with a Coulter Counter (model Zf). The concentration of MTX required to inhibit cell growth by 50% was termed the EC_{50}. Log-phase promastigotes were transfected by electroporation, and colonies were obtained by plating cells on semisolid M199 medium (39). In MTX selections, colonies appeared after 8 to 20 days. As indicated, the plates were supplemented with G418 (16 μg/ml), hygromycin B (32 μg/ml), thymidine (TdR; 10 μg/ml), or methotrexate (MTX; 100 μM). The plating efficiency was determined in parallel on medium containing only hygromycin B and TdR.

Mutagenesis. For mutagenic treatments, log-phase cells (4×10^6 to 8×10^6/ml) were suspended at 5×10^6/ml in M199 medium. MNNG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine [Sigma Chemical Co]) was added to a final concentration of 0.6 μg/ml, and the cells were incubated for 4 h at 25°C. γ irradiation was carried out with a ^{60}Co source for times designed to yield doses of 500 or 5,000 rads. Immediately after mutagenesis, viability was determined by plating aliquots of control and mutagenized cells on nonselective medium. The remaining cells were diluted to 10^6/ml in fresh medium and allowed to grow for 2 days before being plated on MTX-TdR. The fraction of LOH cells was determined by cell slot blots (described below).

DNA manipulations. *Leishmania* genomic DNA was prepared by the Triton-LiCl miniprep method (49) and used for Southern blot analysis as described previously (27). Cell slot blots were made as follows. Aliquots (0.1 ml) of stationary-phase cultures were applied to nylon membranes (GeneScreen Plus), prewetted with 2× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA), with a slot blotter apparatus (Schleicher & Schuell). The cells were lysed by treatment in 0.4 N NaOH–1.5 M NaCl for 10 min and then neutralized in 0.5 M Tris HCl (pH 7.5)–3 M NaCl for 10 min. The DNAs were immobilized by baking, and hybridization was carried out as for the Southern blots. Final washes were performed at 67°C in 0.1× SSPE–0.5% sodium dodecyl sulfate. The *DHFR-TS* coding-region probe fragment was generated by PCR with genomic DNA as a template and the following primers: sense, 5′-cctctagaggtaccATATG TCCAGGGCAGCTGCGA; antisense, 5′-CCCACGCCGGTGCGGTCCTCCT T-3′ (lowercase letters represent nucleotides added to generate restriction sites and are not present in *Leishmania* DNA). The *HYG* gene probe was a 1.05-kb *Bam*HI-*Spe*I fragment from pX63HYG (19). The *DHFR-TS* locus "near flanking" probe was a 2.0-kb *Eco*RI fragment from pK300 (39), and the "far flanking" probe was a 5.7-kb *Kpn*I fragment from plasmid pLTS-D4AJ11-K57 (6). The *Leishmania* actin probe was a 1.7-kb *Sac*I fragment derived from genomic DNA subcloned in pBluescript (21). All DNA probes were labeled with [α-^{32}P]dCTP by the random-primer method (29).

Enzyme and transport assays. Crude extracts were prepared by sonication of cells in phosphate-buffered saline (137 mM NaCl, 15 mM KCl, 10 mM Na_2HPO_4, 32.6 mM KH_2PO_4) containing 1 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 150 μg of benzamidine per ml, 20 μg of leupeptin per ml, 200 μg of 1,10-phenanthroline per ml, and 50 μg of soybean trypsin inhibitor per ml. Extracts were centrifuged at 13,000 × *g* for 20 to 30 min at 4°C, and the supernatants were taken for enzyme assay. DHFR activity was measured with [^{3}H]H_2-folate (Moravek Biochemicals) and NADPH (37). The concentration of MTX required to inhibit DHFR activity by 50% was termed the IC_{50}. TS was measured by the transfer of tritium from 5-[^{3}H]dUMP (Moravek Biochemicals) to H_2O (58). [^{3}H]MTX (5 μM; Moravek Biochemicals) uptake was measured following centrifugation through oil (26). Total soluble protein was determined by the dye-binding method (10). Pteridine reductase 1 (PTR1) protein levels were determined by Western blot (immunoblot) analysis (35) with a rabbit polyclonal antiserum (53).

RESULTS

Selection against *DHFR-TS* in *L. major*. In prokaryotes, loss of TS activity confers transient resistance to antifolates if thymidine is provided (5, 62). This stems from a sparing of reduced-folate pools, since TS is the only significant enzyme whose activity oxidizes tetrahydrofolates. We predicted that the absence of the bifunctional DHFR-TS would confer a similar if not stronger phenotype in *Leishmania* spp. as a result of linkage of the two enzymatic activities. We tested this with a panel of cells, obtained by homologous gene replacement, which contained either one (+/*HYG*) or no (*NEO*/*HYG*) copies of *DHFR-TS*. In liquid media, wild-type or heterozygous cells show typical sensitivities to MTX (EC_{50}s, about 1 μM), while the *dhfr-ts*$^-$ *NEO*/*HYG* knockout grew well at 100 μM MTX (Fig. 1). Permanent MTX resistance may arise from the provision of reduced folates by the alternate pteridine reductase PTR1 in *L. major* (4a).

These data suggested that MTX-TdR could be used as a selection against *Leishmania* DHFR-TS. Accordingly, 2×10^6 20×10^6 +/*HYG Leishmania* cells were plated on medium containing MTX-TdR, and colonies appeared at a frequency of 0.7×10^{-5} to 2.5×10^{-5} per cell plated (Table 1). In contrast, wild-type cells bearing two copies of *DHFR-TS* (+/+) did not give rise to any colonies. Omission of TdR also gave no colonies, suggesting that those obtained with TdR did not arise from common MTX resistance mechanisms, such as amplification of *DHFR-TS* or *PTR1*, mutations in DHFR, or from altered MTX accumulation, as described previously for numerous *Leishmania* lines and species (2, 7, 9, 25).

Cell blot hybridizations were performed to determine whether the colonies obtained by MTX-TdR selection of the +/*HYG* line contained *DHFR-TS* sequences. Remarkably, 75% (18 of 24) of the colonies lacked *DHFR-TS*, while the remaining 25% (6 of 24) were positive and thus retained the gene in some form (Table 1; Fig. 2). In the following discussion, we will refer to these two colony types as DTSΔ and

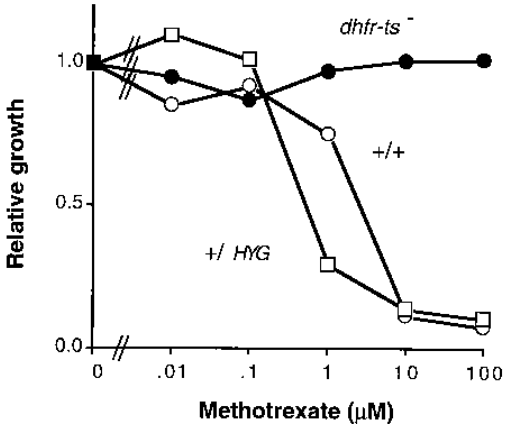

FIG. 1. MTX sensitivity of *DHFR-TS*$^+$ and *dhfr-ts*$^-$ *L. major* in the presence of TdR. Promastigotes were seeded at 10^5/ml in M199 medium supplemented with 10 μg of TdR per ml containing MTX as indicated. Relative growth was measured when the control cultures lacking MTX had attained late log phase (0.5×10^7 to 1×10^7 cells per ml). One representative experiment (of several) is shown. Symbols: ○, +/+ (clone CC-1); □, +/*HYG* (clone E8-5C5); ●, *NEO*/*HYG* (*dhfr-ts*$^-$, clone E10-5A3).

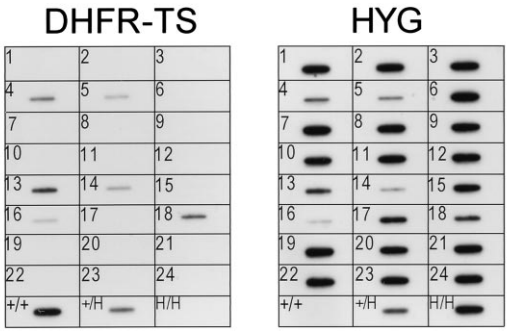

FIG. 2. Cell slot blot of colonies obtained after negative *DHFR-TS* selection of a +/*HYG* heterozygote. Cells from 24 independent colonies arising in experiment 1 from plating of the +/*HYG* line on medium containing MTX-TdR (Table 1) were analyzed by cell blot hybridization with either a *DHFR-TS* or *HYG* probe. Control lines were +/+ (wild type), +/H (+/*HYG*), and H/H (*HYG*/*HYG* homozygous replacement). The *HYG*/*HYG* clone (H250.1) was obtained by selection of the +/*HYG* line with elevated hygromycin B treatment as described in Results.

DTS+, respectively. As expected, all DTSΔ lines were functionally *thy*$^-$, and we show below that many of the DTS+ lines have reduced TS activity (Table 2). Thus, as in prokaryotes, this selection yields mutations affecting TS.

DTSΔ lines have undergone a LOH event. We used Southern blot hybridization to probe the structure of the *DHFR-TS* locus in the DTSΔ lines. A *DHFR-TS* coding-region probe (Fig. 3C, probe C) confirmed the absence of *DHFR-TS* (Fig. 3A, lanes LH1, LOH 5-1, LOH 5-2, and LOH 5-3). A flanking probe (Fig. 3C, probe N) showed no alterations in the structure of either the *DHFR-TS* or *HYG* replacement allele (Fig. 3B), other than loss of the *DHFR-TS* allele in the DTSΔ lines (Fig. 3B). A wild-type structure was also found in Southern blots with a probe (pK300 [39]) encompassing 30 kb of DNA spanning *DHFR-TS* (data not shown). Moreover, no changes in the size of the 500-kb *DHFR-TS* chromosome could be detected in contour-clamped homogeneous electric field gel electrophoresis blots hybridized with a probe located 100 kb 5′ of *DHFR-TS* (Fig. 3C, probe F, and data not shown). Thus, the DTS+ and DTSΔ lines have not undergone deletion or DNA rearrangements.

We measured the *HYG* copy number in several DTS+ and DTSΔ lines in Southern blot (Fig. 3) and slot blot analysis, using hybridization with a *Leishmania* actin probe to control for DNA loading. Assuming the *HYG* copy number in the

TABLE 1. Colony formation on MTX-TdR plates[a]

Expt	Supplements	Cells[b]	Total no. of MTX^r colonies[c]	Frequency of colonies	Plating efficiency	Corrected frequency	% DTSΔ (no. tested)
1	MTX+TdR	pop.	375	1.6×10^{-5}	ND[d]		75 (24)
	MTX	pop.	0	0 ($<10^{-7}$)		0	
	MTX+TdR	+/+	0	0 ($<10^{-7}$)		0	
2	MTX+TdR	pop.	476	2.2×10^{-5}	0.23	9.7×10^{-5}	80 (48)
3	MTX+TdR	Clone 3A	120	4×10^{-6}	0.04	1.0×10^{-4}	ND
		Clone 3B	183	6×10^{-6}	0.11	5.5×10^{-5}	
		Clone 3C	126	4×10^{-6}	0.02	2.0×10^{-4}	
4	MTX+TdR	Clone 4A	168	2.2×10^{-5}	0.07	3.2×10^{-4}	100 (48)[e]
		Clone 4B	300	2.9×10^{-5}	0.11	2.7×10^{-4}	
		Clone 4C	156	1.5×10^{-5}	0.04	3.8×10^{-4}	
5	MTX+TdR	pop.	264	4.4×10^{-5}	2.0	2.2×10^{-5}	100 (24)
		Clone 5A	10	1.7×10^{-6}	1.4	1.2×10^{-6}	100 (19)[e]
		Clone 5B	4	0.7×10^{-6}	1.15	0.6×10^{-6}	
		Clone 5C	6	1.0×10^{-6}	1.04	1.0×10^{-6}	
		Clone 5D	7	1.2×10^{-6}	0.70	1.2×10^{-6}	

[a] A total of 1.5×10^6 to 10×10^6 cells were plated on selective medium. For plating efficiency control, 500 to 1,000 cells were plated on nonselective medium.
[b] All tests involved the +/*HYG* heterozygote, except for one test with the wild type (+/+) in experiment 1. Plates for the +/*HYG* lines included 16 μg of hygromycin B per ml, much less than the EC_{50} (200 μg/ml). The clones indicate freshly isolated colonies obtained by plating the +/*HYG* line on standard hygromycin B concentrations (16 μg/ml). pop. indicates the parental +/HYG line without recloning.
[c] Sum of three or four replicates.
[d] ND, not done.
[e] A sampling of colonies from every clone was analyzed.

TABLE 2. Characterization of DTS+ colonies arising from MTX-TdR selection of +/*HYG L. major*

Line[a]	MTX EC_{50} (μM)		DHFR activity[b]	TS activity[c]	MTX uptake[d]	Growth (−TdR)
	(+TdR)	(−TdR)				
+/*HYG*	0.7	0.07	730	80	38.5	+
S7	0.5	0.04	866	126	51.0	+
S8	0.4	0.05	ND[e]	149	45.9	+
PM13	3.7	0.06	870	128	50.5	+
F22	3.7	0.1	810	121	49.5	+
PM23	100	0.008	130	0[f]	55.5	+
MNNG9	78	ND	180	0[f]	37.5	−
F4	>100	0.007	50	0[f]	35.6	+
MNNG1	>100	ND	900	0[f]	44.0	−
MNNG17	>100	ND	0	0[f]	30.6	−

[a] PM13 was obtained in experiment 1, and S7, S8, F4, and F22 were obtained in experiment 2 (Table 1); PM23 was obtained from the untreated control, and MNNG1, MNNG9, and MNNG17 were obtained from the treated cells in the MNNG mutagenesis experiment shown in Table 3.

[b] Expressed as picomoles of tetrahydrofolate per milligram of protein per minute.

[c] Expressed as picomoles of thymidine 5-phosphate per milligram of protein per minute.

[d] Expressed as picomoles of MTX/10^8 cells per minute.

[e] ND, not done.

[f] In these experiments, wild-type cells gave activity 10-fold over background; 0 indicates that only background levels were observed.

parent +/*HYG* to be 1, we found values approaching 2 for the DTSΔ lines (1.7 ± 0.2, 1.7 ± 0.3, 1.6 ± 0.3, and 1.5 ± 0.2 [n = 4] for colonies LH1, LOH 5-1, LOH 5-2, and LOH 5-3, respectively) and close to 1 for the DTS+ lines (0.7 ± 0.2 and 0.8 ± 0.2 for colonies F22 and PM13, respectively) (Table 2). In combination with the Southern blot data described above, it appeared that the LOH event in the DTSΔ lines did not occur by chromosome loss but arose from an event where loss of the *DHFR-TS* allele was coupled to duplication of the *HYG* allele. Potential mechanisms include gene conversion, mitotic crossing over, or some form of chromosome mis-segregation.

Characterization of DTS+ colonies. We examined several of the DTS+ colonies arising from MTX-TdR selection of the +/*HYG* line for their sensitivity to MTX (with or without TdR) and for factors associated with MTX resistance in *Leishmania* spp. These included DHFR or TS activity, PTR1 protein levels, and MTX uptake (Table 2). Some DTS+ lines obtained after mutagenesis of +/*HYG* and selection in MTX-TdR (see below) were also included. No alterations in MTX uptake (Table 2) or PTR1 levels (data not shown) were found.

The DTS+ lines exhibited a diverse array of phenotypes. All lines exhibiting high levels of MTX resistance (>50 μM) showed dramatic reductions in TS activity, not different from background levels (Table 2). One of these lines showed normal DHFR activity (MNNG1), while the remaining four lines showed reduced DHFR activity ranging from 0 to 25% of the wild-type activity (Table 2). As biochemical studies and the

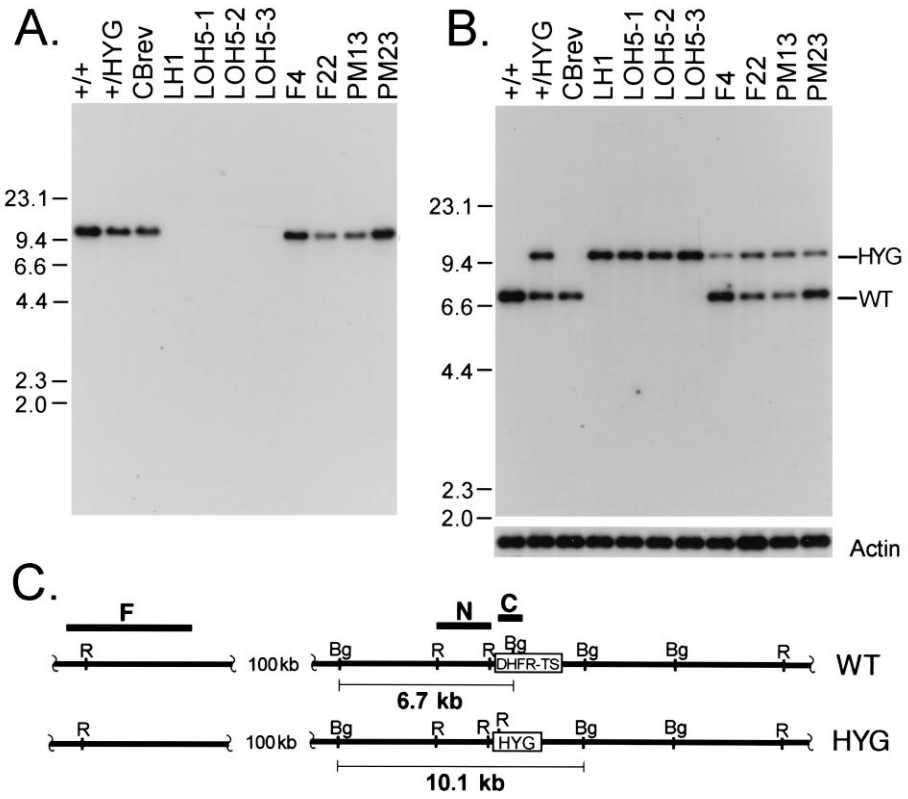

FIG. 3. Structure of the *DHFR-TS* locus in DTSΔ and DTS+ clones. Total DNA of several DTSΔ, DTS+, and control lines was prepared, digested with the indicated restriction enzymes, electrophoresed through 0.8% agarose gels, and transferred to nylon membranes for Southern blot analysis. Control cell lines were wild type (+/+); +/*HYG*, heterozygous *DHFR-TS* replacement clone E8-5C7; and CBrev, *DHFR-TS* "hemizygote" (line CBrev3, containing a 30-kb deletion of one *DHFR-TS* allele [19]). Lines LH1, LOH 5-1, LOH 5-2 and LOH 5-3 represent the DTSΔ class and arose from experiments 1 and 5 (Table 1). Lines F4, F22, PM13, and PM23 represent the DTS+ class (see Table 2 footnotes). (A) *Eco*RI-digested DNAs hybridized to a *DHFR-TS* coding region probe. (B) *Bgl*II-digested DNAs hybridized to near flanking (N) probe. Hybridization of this blot with the *Leishmania* actin probe is shown at the bottom of the panel. (C) Schematic representation of wild-type (WT) and *HYG*-targeted *DHFR-TS* alleles with location of the probes used: Bg, *Bgl*II; R, *Eco*RI. The size of the *Bgl*II fragments recognized by the near flanking (N) probe in the wild-type and *HYG*-targeted allele is shown. C marks the *DHFR-TS* coding region probe, and F marks the "far-flanking" probe.

TABLE 3. Effect of mutagenic treatments on LOH frequency

Treatment	Viability[a]	MTX^r frequency[b]	LOH fraction (DTSΔ/total)	LOH frequency[c]	Non-LOH frequency
MNNG					
0 μg/ml	1	1.4×10^{-5}	0.96 (23/24)	1.4×10^{-5}	$\sim 6 \times 10^{-7}$
0.6 μg/ml	0.56	1.0×10^{-3}	0.07 (5/69)	7.3×10^{-5} (5×)	9.3×10^{-4} (~1,500×)
γ irradiation					
0 kilorads	1	1.2×10^{-5}	0.96 (23/24)	1.2×10^{-5}	$\sim 5 \times 10^{-7}$
0.5 kilorad	0.66	2.4×10^{-4}	0.98 (47/48)	2.4×10^{-4} (20×)	$\sim 5 \times 10^{-6}$ (~10×)
5 kilorads	0.09	2.9×10^{-4}	0.98 (47/48)	2.9×10^{-4} (24×)	$\sim 6 \times 10^{-6}$ (~12×)

[a] Viability was set to 1 for control cultures. The plating efficiency was 0.18 for the MNNG experiment and 0.29 for the γ-irradiation experiment.
[b] Corrected for plating efficiency.
[c] LOH frequency was calculated as the product of the MTX^r frequency and the LOH fraction. Non-LOH frequency was calculated as (1 − LOH fraction) times the MTX^r frequency. The fold increase over the untreated control is given in parentheses.

three-dimensional structure of the DHFR-TS predict that the DHFR and TS domains can function relatively independently (42, 50), it is possible that mutations affecting both activities arise from termination codons in the N-terminal DHFR domain or effects on protein stability.

Despite reductions in TS activity, several of these lines did not require TdR in defined media (Table 2). This was surprising since extragenic *thy*$^+$ revertants of *Leishmania dhfr-ts*$^-$ knockouts have not been observed (33) and TS-independent pathways for de novo synthesis of thymidylate have not been described (43). One possible explanation arises from the fact that in other organisms, only 1% of normal TS activity is sufficient for survival (16). We suspect that in vivo, low levels of TS undetectable by in vitro assays could provide sufficient activity for growth.

Another group of DTS+ lines (colonies PM13 and F22) exhibited moderate MTX resistance (around 10-fold in the presence of TdR), but were otherwise wild type (Table 2). The MTX sensitivity of DHFR activity in lines PM13 and F22 was comparable to that in the wild type (IC_{50}s of 1.5 to 3.0 nM for PM13 and F22 versus 1.5 to 2.0 nM for the wild type), and no evidence of gene amplification of *DHFR-TS*, *PTR1* or any other region of DNA was found in restriction digests of total genomic DNA (reference 17 and data not shown). These lines may represent an unanticipated class of MTX resistance mutations not involving the activities measured here. Finally, some lines, like S7 and S8, were indistinguishable from the +/*HYG* parent in terms of MTX sensitivity, DHFR and TS activities, and MTX uptake (Table 2). These lines were originally scored as having a small-colony phenotype on MTX-TdR plates and probably were "breakthroughs" escaping the selection. These studies thus confirm that most of the DTS+ *Leishmania* strains arising from selection with MTX-TdR bear the expected mutations affecting TS activity.

Spontaneous frequency of LOH in *Leishmania* populations and clones. To determine the spontaneous frequency of LOH at the *DHFR-TS* locus of *L. major*, we carried out four experiments, using the original +/*HYG* line as well as several clonal derivatives obtained immediately after plating on hygromycin-containing solid medium. The frequency of colonies on MTX-TdR plates ranged from 0.7×10^{-6} to 4.4×10^{-5}, or from 0.6×10^{-6} to 3.8×10^{-4} after correcting for plating efficiency (Table 1). Within an experiment, the uncorrected frequency for different +/*HYG* clones varied by less than 2-fold, yet among experiments, the uncorrected frequency differed by more than 40-fold (experiments 4 and 5). In experiment 5, the original +/*HYG* line gave a 20-fold higher frequency than its recloned descendants, although this trend is not evident in other similar comparisons in Table 1. We cannot account for the wide range observed, and no trend was evident over the course of our experiments. An intriguing speculation is that the variability may reflect the presence of trace levels of parasites aneuploid for the *DHFR-TS* chromosome. The occurrence of aneuploid parasites and their potential effects on homozygosity in *Leishmania* species have been discussed previously (20).

Examination of representative colonies from each experiment showed that as in the first, most of the colonies lacked *DHFR-TS* and thus belonged to the DTSΔ class (Table 1). In experiments 4 and 5, all of the colonies were DTSΔ. Assuming that 30 cell doublings were required to obtain sufficient cell numbers following plating of single cells for our studies of fresh clones, that the majority of colonies obtained have lost *DHFR-TS*, and that these events occurred de novo, we calculated a rate of LOH (events per cell generation) ranging from 0.2×10^{-7} (Table 1, experiment 5) to 1.3×10^{-5} (experiment 4). This range overlaps that of 10^{-5} to 10^{-6} observed in mammalian cells (52).

Effects of mutagenic treatments. Although mutagenic treatments have been used successfully to generate a number of interesting *Leishmania* mutants (24, 38, 40, 41, 48), their effects and potency have not been well characterized. We reasoned that the frequency and nature of the events induced by mutagenic treatment of *Leishmania* cells could be analyzed following MTX-TdR selection of the +/*HYG* heterozygote. We examined the alkylating agent MNNG and γ irradiation with a ^{60}Co source. The cells were treated with mutagen, allowed to recover for 2 days in M199 medium supplemented with TdR (about four cell doublings), and then plated on MTX-TdR media to score for the effects.

Treatment with 0.6 μg of MNNG per ml resulted in only a small decrease in viability (44%) but led to a 70-fold increase in the frequency of colonies arising in selective plates (Table 3). This reflected primarily an increase in the frequency of DTS+ colonies of about 1,500-fold (this value is approximate because of the very small number of DTS+ colonies observed in the control and the potential occurrence of events not affecting DHFR-TS). The frequency of DTSΔ colonies increased fivefold as well. Similarly, exposure to 500 and 5,000 rads of γ irradiation resulted in a 20- and 24-fold increase, respectively, in the frequency of colonies on selective plates, although the higher dose caused a considerable loss in viability (>90%) (Table 3). Unlike the effects noted with MNNG, the increase from γ irradiation arose primarily from an increase in the DTSΔ class (Table 3).

These studies showed (i) that the spontaneous frequency of the DTS+ class is about 10^{-7} to 10^{-6}, which is in the range expected from studies in other organisms, and (ii) that MNNG acts primarily to increase the number of point mutations (DTS+ colonies) whereas γ irradiation acts primarily to increase the frequency of LOH at the *DHFR-TS* locus.

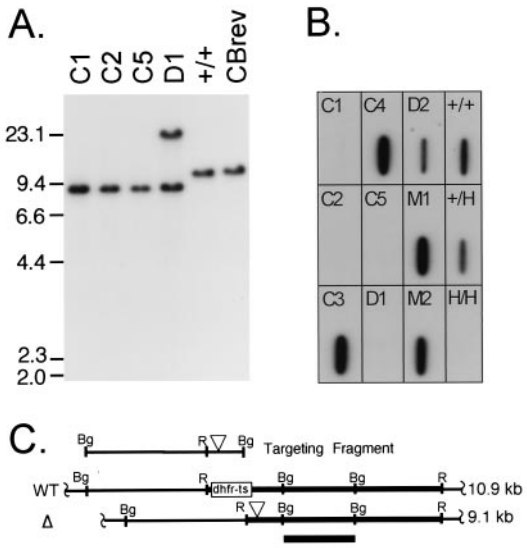

FIG. 4. Negative selection against *DHFR-TS* in primary transfections. (A) Southern blot analysis of *dhfr-ts*$^-$ cells obtained from the wild type after transfection with the *dhfr-ts*-deleted targeting fragment (C) and plating directly on MTX-TdR medium. DNAs were digested with *Eco*RI. Lines +/+ and CBrev are described in the legend to Fig. 3. Clones C1, C2, C5, and D1 were *thy*$^-$ clones obtained in targeting of wild-type *L. major*. (A) Hybridization to the 3.4-kb *Bgl*II fragment depicted in panel C. Molecular size markers (in kilobases) are shown on the left. (B) Hybridization of a cell blot to the *DHFR-TS* coding-region probe. The +/+, +/H, and H/H lines are described in the legend to Fig. 2; lines C3, C4, and D2 were *thy*$^+$ colonies arising from null-targeting transfections, and lines M1 and M2 were *thy*$^+$ colonies arising on mock-transfected control plates. (C) Schematic representation of the targeting fragment and the wild-type (WT) and planned deletion alleles. Predicted sizes of *Eco*RI fragments (thick lines) are shown on the right of each allele. The black box shows the probe used in panel A.

***DHFR-TS* as a negative marker in primary transfections.** We reasoned that it might be possible to use selection against *DHFR-TS* in primary transfection experiments. To test this, we used a 7.8-kb *Bgl*II targeting fragment derived from plasmid pR, which contains a deletion of the whole *DHFR-TS* coding region (Fig. 4C) (39). Homologous replacement at *DHFR-TS* by this fragment would be expected to yield a deletion of the gene (Fig. 4C). To exhibit the desired *dhfr-ts*$^-$ phenotype following transfection of diploid wild-type cells, two events were required, either two independent replacements or one replacement combined with LOH.

Ten micrograms of the purified targeting fragment or an equimolar amount of *Bgl*II-digested pR was electroporated into wild-type *L. major*, and the cells were plated on MTX-TdR. Seven colonies were obtained in the transfected cells, four of which were *thy*$^-$. In contrast, two colonies were obtained in mock-transfected controls, both of which were *thy*$^+$. All five *thy*$^+$ colonies were noticeably smaller than the *thy*$^-$ colonies, and we presume that they represent the expected background of events involving known MTX resistance mechanisms, structural alterations of TS, or breakthroughs (Table 2). In contrast, cell and Southern blot analysis of the four *thy*$^-$ colonies showed that three contained the planned homozygous deletion (clones C1, C2, and C5 [Fig. 4A]). Hybridization with a *DHFR-TS* probe confirmed the loss of *DHFR-TS* (Fig. 4B). The fourth (D1) contained one deletion allele plus another bearing a more complex event which also resulted in deletion of *DHFR-TS* (Fig. 4A and B and data not shown). Similar complex events have been described previously at *DHFR-TS* in cells transfected with large amounts of targeting fragment (18). The presence of two distinct alleles in this clone suggests that they arose from separate replacement events. For the other three *thy*$^-$ colonies, we cannot presently discern whether two replacements or a single replacement followed by LOH occurred. Regardless of the mechanism, these data demonstrate the feasibility of direct selection against *DHFR-TS* in diploid wild-type *L. major.*

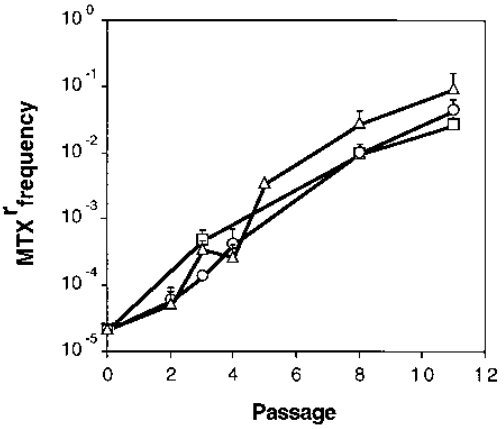

FIG. 5. Selection for LOH by increased drug pressure. Three independent cultures of +/*HYG* were propagated as described in the text in medium containing TdR plus 250 (□) 500 (○) or 750 (△) μg of hygromycin per ml. Periodically, aliquots were taken and plated on MTX-TdR or TdR plates. The mean corrected frequencies and standard deviations are shown.

Positive selection for LOH. Mortensen et al. (52) showed that selection of a +/*NEO* heterozygote mouse embryonic stem cell with elevated G418 pressure yielded *NEO*/*NEO* homozygotes. We tested whether a similar approach would be feasible in *L. major*, because selection against the wild-type gene would not be possible for most loci.

Normally, the +/*HYG* parasite was propagated in medium lacking TdR and containing 16 μg of hygromycin B per ml. Several independent cultures of this line were serially passaged in medium containing TdR and either 250 or 500 μg of hygromycin B per ml (for the +/*HYG* line, 200 μg of hygromycin B per ml results in a 50% reduction in growth). At the higher drug concentrations, a decreased rate of growth was observed initially but the cultures rapidly adapted and grew at near-normal rates thereafter. After 9 to 12 passages (each at a 1:100 dilution, for a total of 60 to 80 cell doublings), the frequency of colony formation on MTX-TdR plates rose dramatically, ranging from 0.001 to 0.95 in different cultures. Both concentrations of hygromycin B worked equally well. Similar results were obtained by selecting the *NEO*/*HYG dhfr-ts*$^-$ knockout in an analogous fashion, which yielded a *HYG*/*HYG* knockout homozygote (data not shown).

We measured the rate of increase of colony formation on MTX-TdR plates as a function of the period of growth at higher hygromycin B concentrations. On average, every three passages resulted in about a 10-fold increase in colony formation (Fig. 5). Again, no correlation was found between hygromycin B concentration and the frequency of colonies on MTX-TdR plates. We did not systematically examine the *DHFR-TS* phenotype in the colonies arising from these studies, but those tested always belonged to the DTSΔ class (data not shown). Thus, elevated drug pressure can be used effectively to induce homozygosity from heterozygous replacements in *L. major.*

DISCUSSION

Diploid organisms with an asexual life cycle such as *Leishmania* spp. pose a challenge for the development of genetic

systems. In this work, we have used studies of the *DHFR-TS* locus to develop a system for probing basic mechanisms of genomic change and to develop new approaches for genetic manipulation in this important pathogen.

DHFR-TS as a positive/negative marker in *Leishmania* spp. Our data show that under proper conditions, selection against *DHFR-TS* can be performed in *Leishmania* spp. Using several protocols involving plating on selective media containing the antifolate MTX plus TdR, we obtained numerous parasite colonies, virtually all of which showed loss of the *DHFR-TS* structural gene or alterations in the TS enzyme (Tables 1 to 3). Thus, the "classical" anti-TS selection works in *Leishmania* spp. as well as it has in bacteria, yeasts, and cultured mammalian cells (3, 5, 11, 62). In *Leishmania* spp. and other taxa with a bifunctional DHFR-TS protein, such as protozoa and plants, the selection may actually be more stringent, because inhibition of DHFR would be absolute following genetic inactivation or deletion (Table 2).

The facile generation of null *dhfr-ts*$^-$ mutants by a single transfection step with a null deletion targeting construct permits the generation of auxotrophic *thy*$^-$ *L. major*. Direct selection for auxotrophs in *Leishmania* spp. was not reported previously because of the difficulty in simultaneously inactivating both alleles in this asexual diploid organism. In *thy*$^-$ auxotrophs, *DHFR-TS* may be used as a positive selectable marker (18, 33). Thus, *DHFR-TS* can be manipulated as a positive/negative marker in a way similar to the *URA3* gene of *Saccharomyces cerevisiae* and potentially has many uses in genetic analysis of this parasite. One caveat is that selection against *DHFR-TS* is restricted to chromosomally borne genes, because *DHFR-TS* overexpression mediated by multicopy plasmids confers MTX resistance even in the absence of thymidine (14). Another caveat is that it may not be possible to eliminate *DHFR-TS* in all strains (20). In these situations, the herpes simplex virus thymidine kinase gene would be a more appropriate negative marker (46).

The nature of LOH in *Leishmania* spp. The most common event observed following plating of a +/*HYG* heterozygote *L. major* on anti-*DHFR-TS* selective media is LOH, resulting in a complete loss of the *DHFR-TS* gene (DTSΔ class [Table 1; Fig. 2]). These colonies showed no alterations in the 30-kb region surrounding the *DHFR-TS* locus or in the size of the *DHFR-TS* chromosome, thereby ruling out models involving gene deletion. Quantitative Southern and cell slot blot analysis showed that DTSΔ colonies contained two copies of the *HYG* allele, suggesting that simple chromosome loss is not responsible (it should be noted that our data do not exclude deletion or loss events, because these could represent minor classes).

There are several possible models for the origin of the DTSΔ phenotype. These include gene conversion, mitotic crossover, or chromosome mis-segregation, and all three have been invoked in previous studies of LOH (15, 52, 57, 68). To discriminate amongst these models, flanking markers capable of distinguishing the two *DHFR-TS* chromosomes would be required. In general, the degree of heterozygosity in cultured *Leishmania* cells is low (4, 51), perhaps because of the phenomenon of LOH described here. In any event, we have not found a heterozygous marker in the CC-1 line of *L. major* used here (6), and thus the flanking markers necessary to probe the mechanism of LOH are not presently available.

The occurrence of LOH in random and targeted mutagenesis in *Leishmania* spp. Our data show the spontaneous occurrence of LOH at the *DHFR-TS* locus with a frequency of up to 10^{-4} (Tables 1 and 3), corresponding to a rate of up to 10^{-5} cell per generation. This frequency and rate are similar to those reported for other organisms (52). The magnitude of this effect relative to the frequency of spontaneous mutation (less than 10^{-6}/allele) and DNA transfection (less than 10^{-4}) suggests that LOH may play an important role in shaping the parasite genome both in nature and in the laboratory. Moreover, it is likely that the mechanisms leading to LOH contribute to the low frequency of heterozygotes observed in natural parasite populations (4, 51), especially if the frequency of genetic exchange is low.

Current data suggest that the contribution of LOH to both targeted and random mutagenesis in *Leishmania* spp. may be quite significant. In numerous targeted-replacement experiments, alteration of only one allele is commonly found, thus mandating a second targeting round to obtain the desired knockout. Occasionally, alterations at both alleles have been noted; these can be heterozygous or homozygous (Fig. 4) (18, 56). Both classes of events have been attributed to the generally high frequency of targeted replacement in *Leishmania* spp. (18, 56), although only the heterozygous class unambiguously arises from independent events. Our studies now suggest the possibility that the single-step homozygous replacements arose by a single replacement followed by LOH. The fortuitous occurrence of LOH at heterozygous replacements was noted previously by Mortensen et al. (52), and our data suggest that a similar process could occur in *Leishmania* spp. (Fig. 5), especially if high selective drug concentrations are used.

It is commonly believed that the majority of recessive mutations in mammalian cells arise from mechanisms involving some form of LOH (15, 52, 57, 68). Random mutagenesis with MNNG has been used to induce a number of mutations in diploid *Leishmania* cells (24, 38, 40, 41), four of which have been characterized at the molecular level (22, 23). Three of these are homozygous deletions, and only one is heterozygous. Thus, the emerging data set suggests that mutants often arise through mechanisms involving LOH in *Leishmania* spp. as well.

The prevalence of LOH-type events in the generation of mutants recovered from diploid organisms may simply reflect the relative frequencies of point mutagenesis and LOH. For example, from the data in Table 3, we calculated that in diploid *Leishmania* cells, mutants arising from a point mutation and LOH should occur more than 20-fold more frequently than mutants requiring two independent point mutations. However, recent studies suggest that mutational and LOH mechanisms may not be independent, since the frequency of homozygous mutants in diploid *S. cerevisiae* is more than 1,000-fold higher than expected (28). Interestingly, the frequency of recovery of homozygous mutants in one-step selections of transfected *Leishmania* cells (ca. 10^{-7} [Fig. 4] [56]) is greater than that calculated for independent replacements (less than 10^{-8}). This may signal a similar lack of independence in the events affecting both alleles.

Applications to mutagenesis and genetics of *Leishmania* spp. Our studies provide the first estimate of the frequency of point mutations at the *DHFR-TS* locus in *L. major*, somewhat less than 10^{-6} (Table 3). This value is comparable to that observed in a wide range of other organisms for loss-of-function mutations (30) and may be compared to the mutation rate of 1×10^{-7} to 9×10^{-7} observed in *Trypanosoma brucei* for inactivation of an integrated thymidine kinase (*TK*) gene (67a). Interestingly, these studies did not yield trypanosomes exhibiting LOH. However, the trypanosomes tested also bore a cointegrated *NEO* resistance marker, and G418 selection was maintained during negative selection against thymidine kinase. LOH events affecting *TK* but not *NEO* would be much less common than those affecting *TK* alone, and the failure to recover them is not surprising.

The frequency of point mutants (DTS+ class) was elevated greatly following mutagenic treatments, more than 1,500-fold by the alkylating agent MNNG and 10-fold following irradiation (Table 3). In contrast, the frequency of LOH rose 10-fold in response to MNNG and more than 20-fold after γ irradiation (Table 3). Although limited, these studies suggest that the mutational spectrum induced by these classic mutagens in *L. major* resembles that observed in other organisms (30). Quantitative analysis of the frequency and types of mutations recovered in response to different mutagenic treatments, in combination with the appreciation of the role of LOH, may prove helpful in the design of protocols for increasing the yield of *Leishmania* mutants. This is important since the general unavailability of recessive mutants is currently a limitation in the application of functional complementation approaches in this organism.

The system established here has several direct applications. First, the defective DHFR-TS mutants (Table 2) may prove useful in studies exploring the role of particular amino acids in the activity or stability of this key metabolic protein. Second, it will be possible to characterize the type and frequency of mutations induced by other mutagenic treatments and to incorporate these data into improved mutant recovery protocols as outlined above. It would be particularly interesting to examine the effects of oxidative stress on mutagenesis, since the entry of *Leishmania* cells into and propagation within the phagolysosome of the vertebrate macrophage may expose the parasite to this class of DNA-damaging agents as part of the normal infectious cycle. Interestingly, studies of *Salmonella* spp. have stressed the importance of DNA repair in the expression of virulence (13). Potentially, measurement of alterations at *DHFR-TS* within the *DHFR-TS*/*HYG* heterozygote could be used as a probe of the exposure of *Leishmania* spp. to mutagenic stress during the natural infectious cycle.

The occurrence of LOH has important uses in the conservation of genetic markers in transfectional manipulations of the *Leishmania* genome. Following the protocol of Mortensen et al. (52), we showed that selection of a +/*HYG DHFR-TS* heterozygote with elevated hygromycin B levels rapidly gave rise to *thy*$^-$ parasites completely lacking *DHFR-TS* (Fig. 5). This permits the use of a single targeting construct and transfection to obtain null mutants for genes for which negative selections are unavailable. Of course, the LOH-based approach can be accelerated by the application of negative selections, and we have generated homozygous *HYG*/*HYG* replacements at the *LPG2* locus of *L. donovani* by lectin selection against lipophosphoglycan expression in this manner (23). Similar results have been obtained at the hypoxanthine-guanine phosphoribosyltransferase (*HGPRT*) and adenine phosphoribosyltransferase (*APRT*) loci of *L. donovani* (67).

Null-targeting strategies as a general tool. Using the negative *DHFR-TS* selection and transfection of a null-targeting fragment, we were able in one step to generate *Leishmania* spp. deleted for both copies of *DHFR-TS* (Fig. 4). This null-targeting approach could be applied at any locus for which an appropriate negative selection can be devised, without sacrificing any selectable marker. A large number of loci are potentially suitable targets for this purpose in different species, including *TK*, the orotidine 5′-phosphate-decarboxylase locus, *HGPRT*, *APRT*, *TS*, or *DHFR-TS*. This protocol could also be used to introduce any given coding region directly into the genome, by construction of a null targeting fragment in which the negative selectable gene (*DHFR-TS* here) was replaced with another coding region. This would be advantageous in many circumstances, and these approaches should be feasible with cells from any diploid species.

Marker-free *dhfr-ts*$^-$ *Leishmania* knockouts as attenuated live vaccine lines. In many situations, the introduction of selectable markers may not be desirable, as in organisms destined for use outside the laboratory. For example, we have shown previously that *dhfr-ts*$^-$ parasites have potential as live, attenuated vaccines against cutaneous leishmaniasis in a susceptible mouse model (65). Unfortunately, the *NEO* resistance marker present in this line can inactivate the aminoglycoside paromomycin, which shows some efficacy in antileishmanial chemotherapy (32). It is remotely possible that *NEO* genes from auxotrophic vaccine lines will find their way into natural field populations, thereby compromising paromomycin therapy. The use of marker-free knockouts described here circumvents this problem, because they lack any *DHFR-TS* or selectable marker coding sequences. Preliminary studies of this parasite show that its efficacy as a live vaccine is uncompromised and comparable to that of the previously studied *NEO*/*HYG dhfr-ts*$^-$ null mutant (34). The methods developed in this work thus permit the construction of marker-free parasites that may have clinical applications.

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