Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 7170–7174, August 1991
Genetics

Double targeted gene replacement for creating null mutants

(*Leishmania*/Trypanosomatidae/parasitic protozoa/asexual diploid cells/vaccine)

ANGELA CRUZ*, CARA M. COBURN, AND STEPHEN M. BEVERLEY†

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 250 Longwood Avenue, Boston, MA 02115

Communicated by Paul C. Zamecnik, April 17, 1991

ABSTRACT **We have used double gene targeting to create homozygous gene replacements in the protozoan parasite *Leishmania major*, an asexual diploid. This method uses two independent selectable markers in successive rounds of gene targeting to replace both alleles of an endogenous gene. We developed an improved hygromycin B-resistance cassette encoding hygromycin phosphotransferase (HYG) for use as a selectable marker for *Leishmania*. HYG-containing vectors functioned equivalently to those containing the neomycin phosphotransferase (NEO) cassette previously used for extrachromosomal transformation or gene targeting. Drug resistances conferred by the NEO and HYG markers were independent, allowing simultaneous selection for both markers. A HYG targeting vector was utilized to replace the single dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene remaining in a line heterozygous for a NEO replacement at the *dhfr-ts* locus (+/*neo*), with a targeting efficiency comparable to that seen with wild-type recipients. The resultant *dhfr-ts*$^-$ line (*hyg*/*neo*) was auxotrophic for thymidine. The double targeted replacement method will enable functional genetic testing in a variety of asexual diploids, including cultured mammalian cells and fungi such as *Candida albicans*. Additionally, it may be possible to use *Leishmania* bearing conditionally auxotrophic gene replacements as safe, improved live vaccines for leishmaniasis.**

Protozoan parasites of the genus *Leishmania* are responsible for a spectrum of tropical diseases, ranging from mild, self-healing cutaneous lesions to lethal visceral forms (1). Controlled vaccination with living strains of Old World cutaneous leishmaniasis has met with some success (2–4). Unfortunately, virulent strains provide the best immunization, and the infections incurred often prove as troublesome as the naturally acquired disease. Thus, current work is devoted to developing biochemically defined preparations, such as parasite extracts or purified molecules. However, the live-vaccine strategy would be viable if safe immunizing strains could be developed. Here we present a general method for engineering conditionally viable *Leishmania*, by targeted deletion of essential metabolic genes.

Homologous gene replacement offers a powerful method for altering and testing gene function (5). In diploid organisms with a sexual cycle, a single heterozygous replacement is first obtained, which is then rendered homozygous by sexual crossing. Although *Leishmania* are diploid at most loci (6–10), they appear to be predominantly or exclusively asexual in nature and in the laboratory (11–13). This situation is not unusual, as many diploid unicellular organisms lack a sexual cycle (14) or possess one that is not readily manipulable, and cultured mammalian cells similarly lack a sexual cycle. Since many cell lines offer superb systems for studying phenomena not readily accessible in whole organisms, directed methods for obtaining homozygous gene replacement in asexual diploid cells would be generally applicable.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

In some eukaryotes the experimental frequency of homologous gene replacement is naturally high or can be increased by specific targeting vectors and counter-selections. In the yeast *Saccharomyces cerevisiae* and in *Leishmania major* the frequency of homologous gene replacement approaches 100% (6, 15), and in cultured mammalian cells efficiencies of >10% have been obtained (16–20). These high frequencies suggested an alternative method for creating homozygous gene replacements in asexual lines, by sequentially targeting one allele and then the second with constructs bearing two different selectable markers. In this work we report the success of this strategy in *Leishmania major* at the *dhfr-ts* [dihydrofolate reductase-thymidylate synthase (DHFR-TS)] locus. Previous stable transfections of *Leishmania* have used the *aph* [neomycin phosphotransferase (NEO)] gene, which confers resistance to aminoglycosides such as G418 (6, 21–23). As a second selectable marker we have now employed the *hph* [hygromycin phosphotransferase (HYG)] gene, which confers resistance to hygromycin B (24). A similar strategy was recently applied in cultured mammalian cells by te Riele *et al.* (17).

METHODS

Construction of pX63HYG. The HYG gene within pUC-HYGR (25) was used as a template for PCR amplification with the 5′ oligonucleotide primer CGGGATCCACTAGTG/ATGAAAAAGCCTGAA (which contains synthetic *Bam*HI and *Spe* I sites joined to the first 15 base pairs of the *hph* coding region; ref. 24) and the M13 reverse sequencing primer (New England Biolabs). The predicted 1.05-kilobase (kb) product was obtained, digested with *Spe* I (5′ site) and *Xba* I (3′ site, arising from the polylinker in pUC-HYGR), and isolated after agarose gel electrophoresis. It was then inserted into pX63-NEO, a modified version of the expression vector pX differing only in the bacterial vector portion (23, 26). The 0.9-kb *Spe* I NEO fragment of pX63-NEO was removed by *Spe* I digestion and replaced with the HYG cassette, yielding pX63-HYG (Fig. 1). Constructs in which the HYG cassette was inserted in the opposite orientation to normal *Leishmania* transcription were also obtained (pX63-GYH; not shown).

Cell Culture and Transfection. All lines were derived from the *L. major* line LT252. CC-1 is a diploid wild-type clonal line (designated +/+; ref. 21), and E2-7′C3 is a heterozygous line formed by replacement of one *dhfr-ts* allele with NEO (designated +/*neo*; ref. 6). Cells were cultured in M199 medium and transfected by electroporation (500 μF, 2.25 kV/cm; ref. 21). G418 (Geneticin; BRL) was present at 16 μg/ml in plates and 6 μg/ml in liquid culture; corresponding concentrations of hygromycin B (Sigma) were 32 and 16

Abbreviations: DHFR-TS, dihydrofolate reductase-thymidylate synthase; NEO, neomycin phosphotransferase; HYG, hygromycin phosphotransferase.

*Permanent address: Faculdade de Odontologia, Ribeirao Preto Universidade de Sao Paulo, Sao Paulo, Brazil.
†To whom reprint requests should be addressed.

μg/ml. KS supplements (thymidine, 10 μg/ml; glycine, 25 μg/ml; biopterin, 0.6 μg/ml; folate, 4 μg/ml; putrescine, 50 μM) were added to media in transfections expected to yield *dhfr-ts* null mutants (6). For nutritional tests, cells were cultivated in DMENF medium [FD + B medium (27) in which serum albumin is replaced by 10% heat-inactivated fetal bovine serum]. This semidefined medium contains biopterin, lacks exogenous folate and thymidine, and can support parasite growth indefinitely.

Karyotype and Southern Analysis. The following were performed as described: preparation of *Leishmania* chromosomes (28); separation of chromosomes by pulsed-field electrophoresis with a contour-clamped homogeneous electric field (CHEF) apparatus (29); digestion of chromosomes (30); Southern blot hybridization using GeneScreen*Plus* membranes (DuPont/NEN; ref. 31); radiolabeling of probes by random priming (32); and γ-irradiation to linearize circular DNAs (33).

RESULTS

Hygromycin Resistance as a Selectable Marker in *Leishmania*. Although a number of hygromycin-resistance cassettes are available, most retain one or more out-of-frame ATG codons immediately upstream of the presumptive initiation codon of the *Escherichia coli hph* gene (24). Since their potential effect on HYG expression in *Leishmania* was unknown, we used PCR amplification to remove these upstream ATG codons. This HYG coding-region cassette was then substituted for the NEO cassette in the vector pX63-NEO (23) to yield pX63-HYG (Fig. 1*A*).

We determined the concentration of hygromycin B that inhibited the rate of *Leishmania* growth by 50% (EC_{50}) to be 10 μg/ml in liquid culture. Since the G418 concentration required in plating studies is about 2–4 times the EC_{50} (21, 30), pX63-HYG-transfected *L. major* were plated on semi-solid medium containing hygromycin B at 32 μg/ml. Within 2 weeks after transfection 345 colonies were obtained per 10 μg of pX63-HYG, comparable to the results obtained with G418 selection and the plasmids pR-NEO and pX (15–60 colonies per μg of DNA; refs. 21 and 23). pX63-GYH, which contains the HYG cassette in the antisense orientation, did not yield any colonies on hygromycin B-containing plates.

The molecular karyotypes of several clonal pX63-HYG transfectants that had been maintained in hygromycin selective medium for >100 cell doublings were identical to that of the parental CC-1 line (Fig. 2*A*). Hybridization to a HYG probe was observed in the transfectant lines but not in the CC-1 line (Fig. 2*B*, lanes 1 and 2 vs. 3). The predominant hybridizing DNAs were at 280 and 400 kb; some hybridization to the sample well was also observed. These DNAs exhibited pulse-time-dependent relative mobility, a hallmark of circular DNAs (compare Fig. 2*B*, lanes 1 and 2, with Fig. 2*D*, lane 0). γ-Irradiation was used to introduce a limited number of double-strand breaks, as circular but not chromosomal DNAs yield new discrete linear fragments after this treatment (33). As expected, γ-irradiation of DNA from line E26-pX63-HYG clone B yielded two new linear DNA fragments, labeled A and C in Fig. 2*B*. We estimated the sizes of these new fragments at the lowest dose of γ-irradiation, where the effect of comigrating broken chromosomal DNAs was minimal (lane 60 in Fig. 2*D*). Fragments A and C were estimated to be 28 and 52 kb, respectively, suggesting that this transfectant contains tetrameric and octameric extrachromosomal circular forms of the 6.2-kb pX63-HYG plasmid. Hybridization with a DHFR-TS probe revealed only the wild-type *dhfr-ts* chromosome in all three lines (Fig. 2*C*), suggesting that integration of pX63-HYG DNA had not occurred. These data established that the HYG cassette functioned extrachromosomally as expected in *Leishmania*.

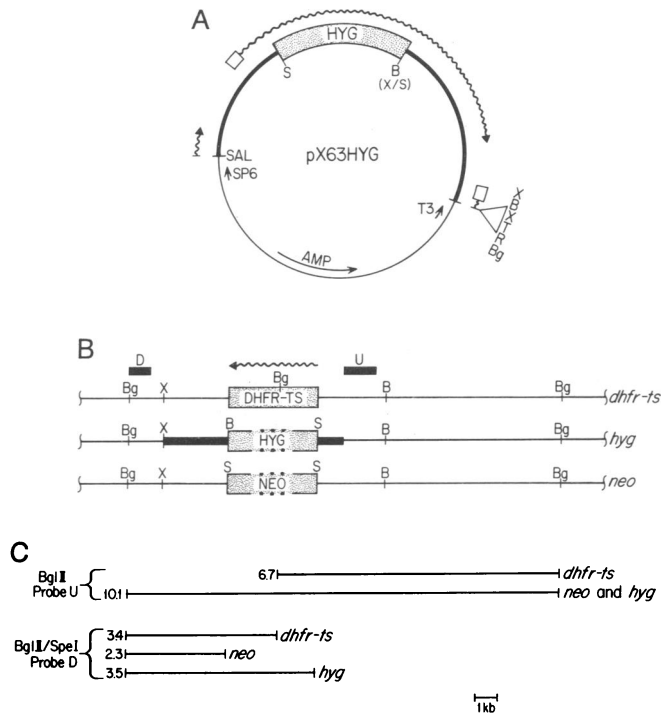

FIG. 1. Map of pX63-HYG, targeting fragments, and predicted gene replacements. (*A*) Map of pX63-HYG. The plasmid was constructed as described in *Methods*. Thin line represents the bacterial vector sequences (pSP6/T3); orientations of the SP6 and T3 phage promoters are shown by small arrows. *Leishmania*-derived sequences are marked by heavy lines and are the same ones present in pX (20). Stippled box represents the *hph* gene cassette (HYG). Small open boxes and arrowheads attached to wavy lines represent sites of miniexon addition and polyadenylylation present in normal *Leishmania* DNA, sequences that are functional in pX (20); wavy line represents the expected chimeric RNA. S, *Spe* I; B, *Bam*HI; Bg, *Bgl* II; X, *Xma*(*Sma*) I; T, *Sst* I; R, *Eco*RI; SAL, *Sal* I. (*B*) Structure of the *dhfr-ts* locus and planned NEO and HYG replacements. Top line (*dhfr-ts*), wild-type chromosome; the DHFR-TS coding region is shown by the box. Second line (*hyg*), planned HYG replacement chromosome. Third line (*neo*), planned NEO replacement chromosome. HYG and NEO coding regions are shown by boxes with dashed lines, as they are ≈1 kb smaller than that of DHFR-TS. The 3.3-kb HYG targeting fragment is shown by heavier lines on the *hyg* map. Hybridization probes U and D used in Fig. 4 are shown above the *dhfr-ts* map. Wavy line represents the direction of transcription. (*C*) Predicted fragments in Southern analysis. Fragment sizes (kb) expected with the indicated restriction digests and hybridization probes depicted in *B* are shown at left, for the chromosomes indicated at right and depicted in *B*.

Independence of HYG and NEO Resistance Markers. Preliminary tests showed that in liquid culture, *Leishmania* transfected with pX63-HYG remained sensitive to G418 while those bearing pX (carrying NEO) remained sensitive to hygromycin B. In plating tests of line E26-pX63-HYG clone 1, 121 ± 15 colonies (mean ± SD) were obtained on plates containing hygromycin B (32 μg/ml) whereas none was obtained on those containing G418 (16 μg/ml). Correspondingly, plating of line E15-pX clone A1 yielded 162 ± 52 colonies on G418 plates whereas none was obtained on hygromycin B plates.

One experiment was performed to test whether both HYG and NEO vectors could be introduced simultaneously. pX63-HYG and pBg94NEOA (6) were mixed (5 μg of each) and transfected into *Leishmania*, and the cells were divided and plated on media containing G418, hygromycin B, or both drugs. On single-drug plates 87 colonies were obtained, while on double-drug plates 15 colonies were obtained (17%). This shows that transfected *Leishmania* frequently take up more

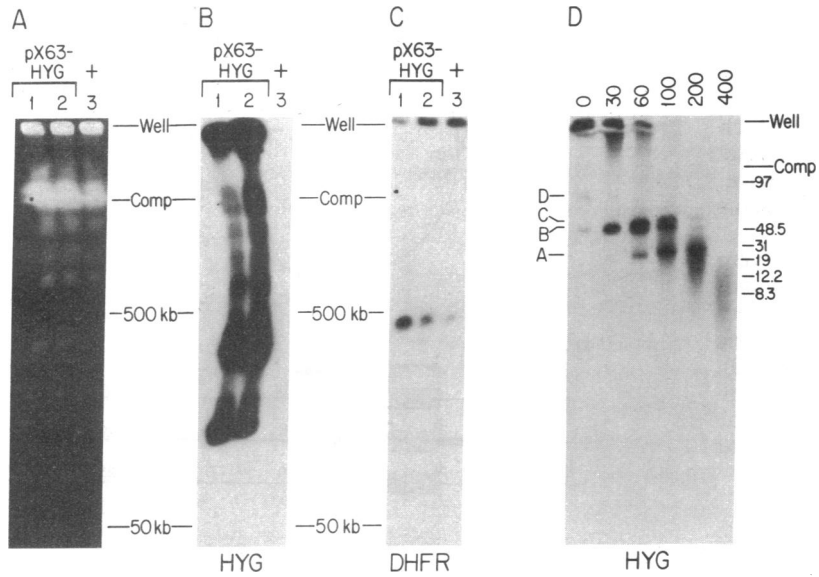

FIG. 2. Analysis of pX63-HYG transfectants. (*A*) Chromosomes from the indicated lines were separated by pulsed-field electrophoresis (55-sec pulse, 48 hr). The ethidium bromide-stained gel is shown. Lane 1, line E26-pX63-HYG clone A; lane 2, line E26-pX63-HYG clone B; lane 3, wild-type (+) *L. major*. Molecular size markers were concatemers of λ phage DNA. Comp, compression. (*B* and *C*) Gel shown in *A* was subjected to Southern blot hybridization successively with coding-region probes for HYG (*B*; 1.05-kb PCR product) or DHFR [*C*; 1.2-kb *Pst* I–*Eco*RV fragment of the *L. major* DHFR-TS gene isolated by cloning of this fragment into *Pst* I/*Eco*RV-digested pIC19H (34)]. (*D*) γ-Irradiation analysis. Chromosome samples from line E26-pX63-HYG clone B were washed in 10 mM Tris/1 mM EDTA, pH 7.4, and subjected to γ-irradiation (33). DNAs were separated by pulsed-field electrophoresis (4-sec pulse, 18 hr), and analyzed by Southern blot hybridization with the HYG probe used in *B*. Dosage of γ-irradiation (in kilorads; 1 rad = 0.01 Gy) for each sample is indicated above the autoradiogram. Molecular size markers were λ oligomers and the BRL high molecular weight marker.

than one DNA molecule. Subsequent studies have shown that clonal lines transfected with extrachromosomal NEO constructs can be subsequently transfected with pX63-HYG derivatives by plating on medium containing both drugs, with no change in plating efficiency (data not shown).

HYG as a Selectable Marker for Gene Replacement. To test whether HYG was suitable as a marker for gene replacement, we transfected the 3.3-kb *Sal* I–*Sma* I fragment of pX63-HYG (Fig. 1 *A* and *B*). This fragment contains only 0.9 and 1.4 kb of 5′ and 3′ DNA that normally flanks the *L. major dhfr-ts* coding region, whereas a previously utilized targeting fragment contained 5 and 2 kb, respectively, of *dhfr-ts* 5′ and 3′ flanking DNA (6). We used <5 μg of the targeting DNA fragment because simple replacement events in *Leishmania* are favored by relatively low DNA amounts (6). Two to four colonies were obtained per μg of targeting fragment, 5–10% of the yield obtained with circular pX63-HYG. A similar result was obtained with the analogous 3.3-kb fragment from pX (containing the NEO marker) after plating on G418-containing medium. In contrast, the larger NEO targeting fragment yielded colonies at 20–100% the efficiency of the circular controls (6), consistent with studies in other species showing increased targeting frequencies with fragments bearing longer homologous sequences (35, 36).

Molecular karyotype analysis of nine clonal derivatives arising from these experiments suggested that all possessed the planned targeted gene replacement (data for two clones are in Fig. 3). Hybridization with a HYG probe revealed a single linear chromosome of about 500 kb, as did hybridization with a DHFR-TS probe (Fig. 3 *A* and *B*, lanes 3 and 4). This suggested that these lines now contained one wild-type and one HYG-replacement chromosome (+/*hyg*); this was tested by Southern blot analysis (Fig. 4). With hybridization probe U and *Bgl* II digests (Fig. 1 *B* and *C*), a 6.7-kb fragment arises from the wild-type *dhfr-ts* locus, while a 10.1-kb fragment is predicted from the planned replacement. Accordingly, wild-type (+/+) *Leishmania* showed only the 6.7-kb fragment (Fig. 4*A*, lane 3), whereas the HYG transfectants contained both the 6.7- and 10.1-kb fragments (lane 1). Analogous results were obtained in *Bam*HI/*Spe* I digests with probe D (data not shown). This confirms that these lines contain the planned replacement and are now genetically +/*hyg* at the *dhfr-ts* locus.

Replacement of the Second Allele of the DHFR-TS Gene. By gene targeting we previously created lines heterozygous at the *dhfr-ts* locus, containing one chromosome with NEO replacing *dhfr-ts* (+/*neo*; ref. 6). To create lines completely lacking *dhfr-ts*, one of these lines was employed as a recipient for the 3.3-kb HYG targeting fragment. After electroporation, cells were plated on medium containing both drugs as well as nutritional supplements that support the growth of *dhfr-ts*$^-$ cells (6). Two to four colonies were obtained per μg of HYG targeting fragment, a frequency comparable to that obtained with the wild-type recipient. Five of six transfectants analyzed exhibited a karyotype consistent with the planned replacement (data for two lines are in Fig. 3). In these

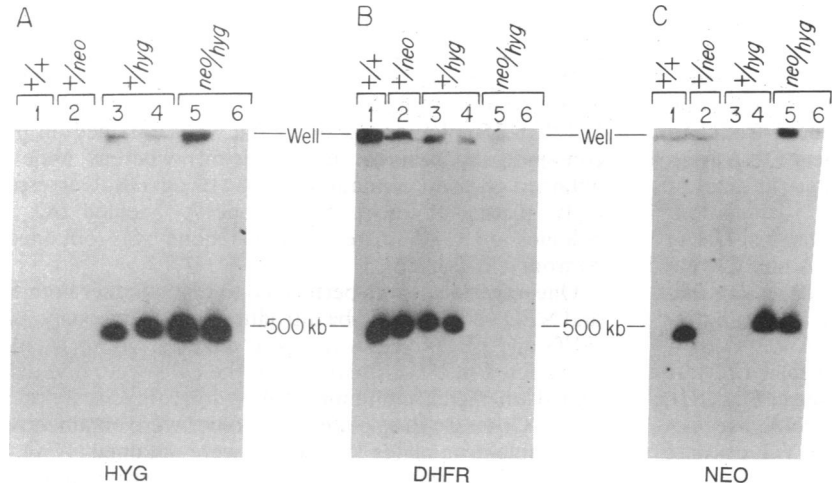

FIG. 3. Molecular karyotype analysis of targeted gene replacements. Chromosomes from the indicated clonal lines were separated and analyzed by Southern blot hybridization as described for Fig. 2. Hybridization probes were the coding regions for HYG (*A*), DHFR-TS (*B*), and NEO [*C*; 0.9-kb *Spe* I fragment from pSpeNEOA (21)]. Different blots were used for each probe. The genotype inferred is indicated. Lane 1, wild-type *L. major*; lane 2, E2-7D2 (transfected with NEO targeting fragment only; ref. 4); lanes 3 and 4, lines E8-5C5 and E8-5′A1 (transfected with HYG targeting fragment only); lanes 5 and 6, E10-5B5 and E10-5A3 [line E2-7′C3 (+/*neo*) transfected with the HYG targeting fragment].

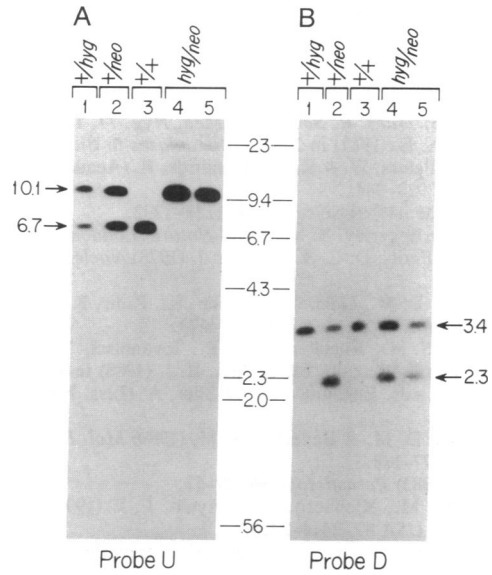

FIG. 4. Southern blot analysis of targeted gene replacements. DNAs from the indicated lines were digested with either *Bgl* II (*A*) or *Bgl* II/*Spe* I (*B*), electrophoresed in 0.8% agarose gels, and subjected to Southern blot hybridization with probe U (*A*) or D (*B*) depicted in Fig. 1*B*. The predicted fragments depicted in Fig. 1*C* are marked by arrows; the positions of molecular size markers (kb) are shown between *A* and *B*.

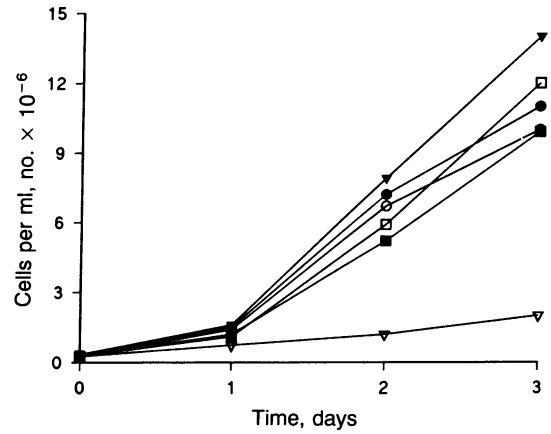

FIG. 5. Growth of +/+, +/*hyg*, and *neo/hyg Leishmania* in the presence and absence of thymidine. *Leishmania* were grown in DMENF medium, except that the medium for the *neo/hyg* line additionally contained thymidine (10 μg/ml). Stationary-phase cells were inoculated into fresh DMENF medium without (open symbols) or with (filled symbols) thymidine, and cell density was monitored with a Coulter counter. Squares, +/+ *L. major*; circles, +/*hyg* line E8-5′A1; triangles, *neo/hyg* line E10-5C5. The *neo/hyg* line did not exhibit additional growth at increased times or after resuspension in fresh medium (data not shown).

cells the HYG hybridization probe identified a single 500-kb chromosome (Fig. 3*A*, lanes 5 and 6), as did the NEO probe (Fig. 3*C*, lanes 5 and 6). In contrast, the DHFR-TS probe did not identify any chromosome in these lines (Fig. 3*B*, lanes 5 and 6). These data showed that *dhfr-ts* had been lost.

Southern blot analysis confirmed that these lines contained only the planned NEO and HYG replacements. With *Bgl* II digests and probe U, only the 10.1-kb fragment expected for both the NEO and HYG replacements was observed (Fig. 4*A*, lanes 4 and 5); in contrast, a +/*neo* line additionally exhibited the 6.7-kb wild-type fragment (lane 2). With *Bgl* II/*Spe* I digests and probe D (Fig. 1 *B* and *C*), both the 2.3-kb NEO replacement fragment (Fig. 4*B*, lane 2) and the 3.5-kb HYG replacement fragment (lanes 4 and 5) were observed. Analogous data were obtained with probe U and *Bam*HI/*Spe* I digests, which test the structure from the 5′ side (data not shown). These data indicated that the planned replacement had occurred, yielding transfectants that were now *neo/hyg* at the *dhfr-ts* locus.

We attempted to simultaneously disrupt both alleles by mixed transfection of HYG and NEO targeting fragments into wild-type cells but were unsuccessful.

Phenotype of *dhfr-ts*⁻ Disruptants. Clonal lines that were either +/+, +/*hyg*, or *neo/hyg* were inoculated into a defined medium in the presence or absence of thymidine (Fig. 5). The +/+ or +/*hyg* lines grew at comparable rates in both media. In contrast, the *neo/hyg* line grew only in the presence of thymidine (Fig. 5). These data suggested that the sole essential role of DHFR-TS in the defined medium was in provision of thymidine.

DISCUSSION

We have developed HYG as a second selectable marker for stable DNA transfection of *Leishmania*. The properties of transfection vectors containing this marker are similar to those bearing the NEO marker previously utilized in *Leishmania*: the efficiency of DNA transfection is comparably high, previously developed extrachromosomal vectors now bearing the HYG marker remain extrachromosomal, and targeting fragments employing HYG can be used for gene replacement at the *dhfr-ts* locus. As in other organisms (37), the mechanisms of resistance mediated by HYG and NEO are independent in *Leishmania*. This permits the simultaneous introduction and maintenance in *Leishmania* of constructs bearing both markers, in either extrachromosomal or chromosomal locations. The availability of a second, independent selectable marker will expand the kinds of tests of gene function that can be pursued in this organism.

The fact that *Leishmania* is a functionally asexual diploid requires that both alleles be modified prior to functional testing. The availability of a second selectable marker allowed us to overcome this problem. Null mutants at *dhfr-ts* were obtained by two rounds of gene targeting, first with NEO-containing and then with HYG-containing targeting vectors (the order is unimportant; unpublished data). The efficiency of each step was comparable. Although previous studies suggested that it should be possible to obtain simultaneous replacement of both *dhfr-ts* alleles (6), we have not been able to accomplish this by transfection of the NEO and HYG targeting fragments simultaneously. We presume that this reflects the low cumulative frequency expected for simultaneous transfection and replacement. Improved procedures may remove this limitation at the *dhfr-ts* locus in the future; however, for loci in which the viability of the null mutant is unknown the two-step procedure may be preferable.

The *dhfr-ts*⁻ *Leishmania* developed by double gene targeting will constitute a powerful tool for probing some of the unusual aspects of folate metabolism and methotrexate toxicity in *Leishmania* (27, 38). For these kinds of studies the *hyg/neo* line will be preferable to the *dhfr-ts*⁻ line described previously (6), which is heterozygous for a *neo* replacement chromosome and a 30-kb deletion including *dhfr-ts*. The line originally bearing the *dhfr-ts* deletion arose from a complex procedure including multiple rounds of stepwise selection with the antifolate 5,8-dideaza-10-propargylfolate accompanied by deletional gene amplification (9, 39). Lines obtained by stepwise antifolate selection frequently exhibit multiple uncharacterized alterations (40), whereas lines obtained by transfection and aminoglycoside selection are unlikely to contain mutations specifically involved in folate metabolism.

Double Gene Replacement and Functional Genetic Testing in Other Organisms. Other methods could be used to obtain homozygous mutant lines from a heterozygous parent, in the absence of a manipulable sexual cycle. Parasexual crossing has been utilized in some organisms, but parasex has not been demonstrated in *Leishmania*. Another approach is the use of UV radiation or other agents to induce mitotic recombination following transformation, as shown in *Candida albicans* (41). The disadvantage is that mutagenic agents may create secondary mutations that cannot be removed by back-crossing. Double gene replacement avoids this problem, if the DNA transfection protocol itself does not induce general mutagenesis.

The requirements of this method are straightforward and accessible in most organisms: two independent selectable markers and a frequency of gene replacement sufficient to permit two successive rounds of gene targeting. This method should be widely applicable to many diploid organisms that lack a sexual cycle or possess one that is experimentally difficult to use [e.g., trypanosomes, fungi such as *C. albicans* (14), and cultured mammalian cells (17)].

Application to *Leishmania* Vaccination Strategies. To develop appropriate *Leishmania* vaccine strains, it will be necessary to conduct double gene targeting directly within infective strains. The constructs and methods for transfection used in this work can be readily applied to infective lines from several species complexes of *Leishmania*; significantly, the transfected lines can maintain virulence (ref. 30 and unpublished data). In theory, inactivation of any locus essential for growth *in vivo* but not *in vitro* could be a suitable target for development of attenuated strains. One potential problem is that many trypanosomatid proteins are encoded by arrays of multiple genes (42), although future technical advances may permit inactivation of such loci.

As an example, infective *dhfr-ts*$^-$ deletion strains may possess many of the characteristics desired of prospective vaccine strains, including conditional auxotrophy and inability to revert. The role of DHFR-TS in the synthesis of precursors for DNA replication suggests that it will be essential for growth *in vivo*, and DHFR-TS is expressed in the amastigote stage (43). One potential problem is that thymidine levels *in vivo* may prove sufficient to rescue *dhfr-ts*$^-$ *Leishmania*. Although the success of antifolate chemotherapy against other species suggests that this may not be a problem, it will be necessary to determine this empirically.

Even if gene targeting (or any other method) can be utilized to make safe vaccine strains, there remain significant problems associated with the preparation, storage, and use of live parasite vaccines (2–4). Stable, biochemically defined preparations or proteins overproduced by recombinant DNA methods may ultimately prove superior in vaccination strategies. However, the extensive experience with live parasite vaccination for leishmaniasis suggests that this approach should remain under consideration. Moreover, it may prove possible to combine these strategies and further engineer the attenuated strains to effectively overproduce or present immunoprotective molecules.

We thank Tom Egelhoff for providing pUC-HYGR; D. Coen, R. Kolodner, and R. Titus for discussions; and D. Dobson and the members of our lab for reading the manuscript. This work was supported by grants from the National Institutes of Health (S.M.B.), Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior–Fulbright and Fundação de Amparo à Pesquisa do Estado de São Paulo (A.C.), and the Lucille P. Markey Charitable Trust (Harvard Medical School).

1. World Health Organization Expert Committee (1984) in *The Leishmaniasis* (World Health Organization, Geneva), pp. 1–146.
2. Greenblatt, C. L. (1980) in *New Developments with Human and Veterinary Vaccines* (Liss, New York), pp. 259–285.
3. Green, M. S., Kark, J. D., Witztum, E., Greenblatt, C. L. & Spira, D. T. (1983) *Trans. R. Soc. Trop. Med. Hyg.* **77**, 152–159.
4. Gunders, A. E. (1987) in *The Leishmaniases in Biology and Medicine*, eds. Peters, W. & Killick-Kendrick, R. (Academic, London), Vol. 2, pp. 929–944.
5. Capecchi, M. (1989) *Science* **244**, 1288–1292.
6. Cruz, A. & Beverley, S. M. (1990) *Nature (London)* **348**, 171–174.
7. Leon, W., Fouts, D. L. & Manning, J. (1978) *Nucleic Acids Res.* **5**, 491–503.
8. Iovannisci, D. M., Goebel, D., Allen, K., Kaur, K. & Ullman, B. (1984) *J. Biol. Chem.* **259**, 14617–14623.
9. Beverley, S. M., Ellenberger, T. E., Iovannisci, D. M., Kapler, G. M., Petrillo-Peixoto, M. & Sina, B. J. (1988) in *The Biology of Parasitism*, eds. Englund, P. T. & Sher, A. (Liss, New York), pp. 431–448.
10. Iovannisci, D. M. & Beverley, S. M. (1989) *Mol. Biochem. Parasitol.* **34**, 177–188.
11. Tait, A. (1983) *Parasitology* **86**, 29–57.
12. Tibayrenc, M., Kjellberg, F. & Ayala, F. J. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2414–2418.
13. Panton, L. J., Tesh, R. B., Nadeau, K. & Beverley, S. M. (1991) *J. Protozool.* **38**, 224–228.
14. Whelan, W. L. (1987) *CRC Crit. Rev.* **14**, 99–170.
15. Rothstein, R. J. (1983) *Methods Enzymol.* **101**, 202–211.
16. Charron, J., Malynn, B. A., Robertson, E. J., Goff, S. P. & Alt, F. W. (1990) *Mol. Cell. Biol.* **10**, 1799–1804.
17. te Riele, H., Maandag, E. R., Clarke, A., Hooper, M. & Berns, A. (1990) *Nature (London)* **348**, 649–651.
18. Johnson, R. S., Sheng, M., Greenberg, M. E., Kolodner, R. D., Papaioannou, V. E. & Spiegelman, B. M. (1989) *Science* **245**, 1234–1236.
19. Doetschman, T., Gregg, R. G., Maeda, N., Hooper, M. L., Melton, D. W., Thompson, S. & Smithies, O. (1987) *Nature (London)* **330**, 576–578.
20. Mansour, S. L., Thomas, K. R. & Capecchi, M. R. (1988) *Nature (London)* **336**, 348–352.
21. Kapler, G. M., Coburn, C. M. & Beverley, S. M. (1990) *Mol. Cell. Biol.* **10**, 1084–1094.
22. Laban, A., Tobin, J. F., de Lafaille, M. A. C. & Wirth, D. F. (1990) *Nature (London)* **343**, 572–574.
23. LeBowitz, J. H., Coburn, C. M., McMahon-Pratt, D. & Beverley, S. M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 9736–9740.
24. Gritz, L. & Davies, J. (1983) *Gene* **25**, 179–188.
25. Egelhoff, T. T., Brown, S. S., Manstein, D. J. & Spudich, J. A. (1989) *Mol. Cell. Biol.* **9**, 1965–1968.
26. LeBowitz, J. H., Coburn, C. M. & Beverley, S. M. (1991) *Gene*, in press.
27. Petrillo-Peixoto, M. P. & Beverley, S. M. (1987) *Antimicrob. Agents Chemother.* **31**, 1575–1578.
28. Beverley, S. M. (1988) *Nucleic Acids Res.* **16**, 925–938.
29. Chu, G., Vollrath, D. & Davis, R. W. (1986) *Science* **234**, 1582–1585.
30. Coburn, C. M., Otteman, K., McNeely, T., Turco, S. & Beverley, S. M. (1991) *Mol. Biochem. Parasitol.* **46**, 169–179.
31. Ellenberger, T. E. & Beverley, S. M. (1989) *J. Biol. Chem.* **264**, 15094–15103.
32. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13.
33. Beverley, S. M. (1989) *Anal. Biochem.* **177**, 110–114.
34. Marsh, J. L., Erfle, M. & Wykes, E. J. (1984) *Gene* **32**, 481–485.
35. Shulman, M. J., Nissen, L. & Collins, C. (1990) *Mol. Cell. Biol.* **10**, 4466–4472.
36. Thomas, K. R. & Capecchi, M. R. (1987) *Cell* **51**, 503–512.
37. Blochlinger, K. & Diggelmann, H. (1984) *Mol. Cell. Biol.* **4**, 2929–2931.
38. Kaur, K., Coons, T., Emmett, K. & Ullman, B. (1988) *J. Biol. Chem.* **263**, 7020–7028.
39. Garvey, E. P., Coderre, J. A. & Santi, D. V. (1985) *Mol. Biochem. Parasitol.* **17**, 79–91.
40. Ellenberger, T. E. & Beverley, S. M. (1987) *J. Biol. Chem.* **262**, 13501–13506.
41. Kelley, S. L., Basu, A., Teicher, B. A., Hacker, M. P., Hamer, D. H. & Lazo, J. S. (1988) *Science* **241**, 1813–1815.
42. Clayton, C. E. (1988) *Genet. Eng. (London)* **7**, 1–56.
43. Kapler, G. M., Zhang, K. & Beverley, S. M. (1989) *Mol. Cell. Biol.* **9**, 3959–3972.