Homozygous null mutants of the hypoxanthine-guanine phosphoribosyltransferase (hgppt) and adenine phosphoribosyltransferase (aprt) loci were created in *Leishmania donovani* in which both alleles were eliminated using only a single targeting construct. Functional heterozygotes were first generated by homologous recombination after transfection with vectors containing 5' and 3' flanking regions of either the hgppt or the aprt gene encompassing drug resistance markers. Homozygous null mutants were then isolated from the heterozygotes by negative selection in media containing subversive substrates of the encoded proteins, i.e. allopurinol for HGPRT and 4-aminopyrazolopyrimidine for APRT. The novel alleles created by homologous recombination were verified by Southern blotting, and the effects of gene replacement upon gene expression in intact parasites were evaluated by direct enzymatic assay and by immunoblotting. All mutant strains were viable under the selection conditions and exhibited appropriate drug resistance phenotypes. The ability to generate homozygous knockouts with single targeting constructs greatly facilitates the genetic dissection and subsequent biochemical investigations of the purine pathway in *Leishmania* and has important general implications for the genetic manipulation and analysis of the leishmanial genome.

Improved methods for the chemotherapeutic manipulation of parasitic diseases depends upon the exploitation of fundamental metabolic or biochemical differences between the parasite and the mammalian host. Basic investigations of metabolic pathways in parasitic protozoa have been hampered by the inability to select directly for mutants genetically defective in some component of the metabolic pathway. This impediment has been circumvented in part by the implementation of DNA transfection methods that now permit the genetic engineering of trypanosomatid chromosomes by targeted gene replacement (1–7). As parasites of the *Leishmania* and *Trypanosoma* genera are generally thought to be diploid at most loci (1, 8–11) and do not readily undergo sexual crosses in the laboratory (12–14), the finding that genes can be efficiently targeted by homologous recombination has created new avenues for mutationally altering parasites that lack specific gene sequences, thereby enabling direct assessment of gene function. The protocols for the generation of knockout parasites have routinely involved the sequential replacement of both alleles of a genetic locus with two different targeting constructs, each containing flanking regions from the targeted gene encompassing independent selectable markers. Although double targeted gene replacement has permitted the construction of a variety of knockout mutants in both *Leishmania* (6, 15, 16) and *Trypanosoma* (11, 17), the process for generating these strains is both time-consuming and laborious, and the restricted number of available drug resistance markers limits the introduction of multiple homozygous mutations within a single parasite population for biochemical and cellular studies. These barriers can be surmounted in part by the creation of homozygous knockout strains using only a single targeting construct. Such a strategy has been exploited in mammalian cell lines by selecting for loss-of-heterozygosity after a single round of gene targeting by increased drug pressure (18).

The purine salvage pathway offers an excellent opportunity to examine whether *Leishmania* express the metabolic machinery to undergo loss-of-heterozygosity, because several purine salvage enzymes metabolize cytoxic purine analogs, thereby affording a powerful negative selection scheme to detect the phenomenon. These enzymes include hypoxanthine-guanine phosphoribosyltransferase (HGPRT)1 and adenine phosphoribosyltransferase (APRT), which initiate the metabolism of 4-hydroxypyrazolopyrimidine (HPP, allopurinol) and 4-aminopyrazolopyrimidine (APP), respectively (19). Both HPP and APP are toxic to *Leishmania* spp. at relatively low concentrations (9, 20). As both hgppt (21) and aprt (22) and their respective flanking sequences have been isolated from cosmid libraries, the molecular reagents to construct appropriate knockout vectors are now available.

We now describe the generation of homozygous null mutants of both hgppt and aprt in *Leishmania donovani* by targeted gene replacement using only a single targeting construct. The construct is used to target the first allele by transfection, and the loss of the wild type allele from the functional heterozygote is selected directly in a subversive substrate of the encoded gene product. Recently, Guerios-Filho and Beverley have demonstrated that loss-of-heterozygosity can also occur at the dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) locus of *Leishmania major* (23).

**EXPERIMENTAL PROCEDURES**

*Materials, Chemicals, and Reagents—[8-14C]Hypoxanthine, [8-14C]guanine, and [8-14C]adenine, all at 45–60 mCi/mmol, were purchased from Moravek Biochemicals (Brea, CA). [α-32P]dCTP (3000 Ci/mmol), procured from New England Nuclear (Boston, MA), was diluted with an equal volume of nonradioactive dCTP.*

1 The abbreviations used are: HGPRT, hypoxanthine-guanine phosphoribosyltransferase; APRT, adenine phosphoribosyltransferase; neo', neomycin phosphotransferase gene; bgp', hygromycin phosphotransferase gene; HPP, 4-hydroxypyrazolopyrimidine or allopurinol; APP, 4-aminopyrazolopyrimidine; PCR, polymerase chain reaction; kb, kilobase pair(s).
mmol) and [α-32P]dATP (1320 Ci/mmol) were acquired from DuPont NEN. Restriction and DNA modifying enzymes were from either Life Technologies, Inc. or New England Biolabs, and Thermus flavus DNA polymerase was obtained from Epicentre Technologies (Madison, WI). The pX63-NEO and pX63-HYG vectors that encompass the neoR (neor) and hygromycin phosphotransferase (hygR) genes, respectively, were generously provided by Dr. Stephen Beverley (Harvard Medical School). Probes encompassing neoR and hygR were obtained by excision of pX63-NEO and pX63-HYG with BamHI/SpeI and SpeI alone, respectively. A probe for the α-tubulin gene (α-tubulin) from Leishmania enriettii was kindly provided by Dr. Scott Landfeer of the Oregon Health Sciences University (Portland, OR). Genetin (G418), hygromycin, HPP, and APP were all obtained from Sigma. The sources of all other materials, chemicals, and reagents employed in these experiments have been reported previously and were of the highest purity commercially available.

Cell Culture—L. donovani promastigotes were cultivated in completely defined DME-L culture medium (24). Single cell cloning of parasites was carried out in semi-solid DME-L medium as reported (25). The D700 cell line is a wild type clone of the Sudanese 1S strain of L. donovani that was used as the initial recipient strain for transfection. For the purposes of the present reports pX63-NEO and pX63-HYG were digested with appropriate restriction enzymes, and purified using the GeneClean Kit (Intermountain Scientific Corp., Bountiful, UT), digested with appropriate restriction enzymes, and ligated into the pertinent sites of the pX63-NEO and pX63-HYG plasmids, respectively. Each of the two flanking regions of both loci were ligated sequentially into the appropriate vectors containing the drug resistance markers. The final vector constructs employed for allelic replacement were designated pX63-NEO-Δhypert and pX63-HYG-Δaprt.

**Fig. 1. Lineage of L. donovani cell lines derived by targeted gene replacement and selection.** The pedigree of the L. donovani strains generated during the course of these studies is indicated. Uppercase letter designations indicate the locus, either HGPRT (H) or APRT (A), while superscripts specify the allele, either wild type (+) or those replaced by X63-NEO-Δhypert (n) or X63-HYG-Δaprt (h). The five strains designated are wild type (H+/A+/), the hypert heterozygote in an hypert background (H+/A−/h), and the hypert/lprt double homozygote (H+/A−/h).
allele was disrupted by targeted gene replacement using the X63-NEO–\textit{hgprt} construct (shown in Fig. 2B) to generate the \textit{hgprt} heterozygote, H^{+/A^{-/+}}. The homozygous null mutant of \textit{hgprt}, H^{+/A^{-/-}}, was then obtained from the H^{+/A^{-/-}} heterozygote line by direct negative selection in 1.0 mM HPP, a subversive substrate of the \textit{L. donovani} HGPRT (19) with amply demonstrated antileishmanial properties (20). The H^{+/A^{-/-}} cell line was then transfected with the \textit{aprt} knockout construct X63-HYG–\textit{aprt} (shown in Fig. 2E) to produce the \textit{aprt} heterozygote, H^{+/A^{-/-}}h, within the \textit{hgprt}− genetic background. Finally, the \textit{aprt} heterozygote was subjected to selective pressure in 100 μM APP, a cytotoxic substrate of the \textit{L. donovani} APRT (9, 19), to generate the \textit{hgprt}−, \textit{aprt}− double knockout mutant H^{+/A^{-/-}}h.

\textbf{Southern Blot Analysis—} Southern blot analysis of genomic DNA prepared from wild type and mutant strains confirmed the existence of the new alleles that had been created after a single round of gene targeting followed by direct selection for loss of the second wild type allele (Figs. 3–5). The maps of the genomic loci of \textit{hgprt} and \textit{aprt} and the novel alleles created by
homologous recombination of the X63-NEO-neor and X63-HYG-Δaprt constructs into the hgprt and aprt loci, as well as the restriction fragments employed in the Southern blot experiments, are shown in Fig. 2. The differences in the new hgprt alleles can be observed in Fig. 3. The disrupted hgprt allele could be distinguished from the wild type allele by an altered EcoRI restriction pattern (Fig. 3). After digestion of genomic DNA with EcoRI and hybridization to probe B (shown in Fig. 2A) corresponding to a portion of the 3'-flanking region of hgprt, only the expected 4.6-kb EcoRI fragment from the wild type allele was observed in the wild type H\(^{+/+}\)A\(^{+/+}\) cell line (Fig. 3A, probe B). It should be noted that the 4.6-kb EcoRI restriction fragment corresponds to the expected size of the wild type hgprt allele even though the original hgprt clone was isolated as a 3.5-kb EcoRI fragment from a cosmid (21). However, fine mapping of the cosmid indicated that the 3' EcoRI restriction site of the original 3.5-kb EcoRI insert was located within the cosmid DNA sequence. In contrast, a band of 6.4-kb (Fig. 3A, probe B) was observed in all strains in which the wild type hgprt allele had been disrupted by X63-NEO-Δhgprt. The cell line heterozygous for hgprt, H\(^{+/+}\)A\(^{+/+}\), also retained a copy of the wild type hgprt allele, as illustrated by the presence of the 4.6-kb EcoRI fragment derived from the wild type locus (Fig. 3A, probe B). The H\(^{+/+}\)A\(^{+/+}\) strain generated by selection of H\(^{+/+}\)A\(^{+/+}\) cells in HPP (Fig. 1), as well as its progeny, i.e. the H\(^{+/+}\)A\(^{+/+}\) and H\(^{+/+}\)A\(^{+/+}\) cell lines, did not display the wild type 4.6-kb EcoRI band and revealed only the expected 6.4-kb displacement by X63-NEO-Δhgprt (Fig. 3A, probe B). The losses of the wild type hgprt alleles by targeting and then selection were verified further by hybridization of genomic DNA from the five cell lines to probe A encompassing the protein coding portion of hgprt (Fig. 3A, probe A). The 4.6-kb EcoRI fragment from the wild type allele was observed only in the H\(^{+/+}\)A\(^{+/+}\) and H\(^{+/+}\)A\(^{+/+}\) cells and not in the H\(^{+/+}\)A\(^{+/+}\) and H\(^{+/+}\)A\(^{+/+}\) lines (Fig. 3, probe A).

Normalization of the signal intensities of the bands in the genomic DNA that hybridized to either probe A or probe B to that obtained with α-tubulin (Fig. 3A, α-tubulin probe) generally supported the idea that the first wild type hgprt allele was replaced by homologous recombination of the targeting construct used in the transfection, while loss-of-heterozygosity accounted for the elimination of the remaining wild type allele (Table I). Densitometric ratios of the signal intensities from the 4.6 EcoRI band hybridized to either probe A (A/T) or probe B (B/T) to that obtained with genomic DNA hybridized to α-tubulin (see Fig. 3A) were 0.273 and 0.115 (A/T) and 0.105 and 0.044 (B/T) for the H\(^{+/+}\)A\(^{+/+}\) and H\(^{+/+}\)A\(^{+/+}\) cell lines, respectively. Det

### Table I

| Cell line | A/T | B/T | C/T | D/T | N/T | H/T |
|-----------|-----|-----|-----|-----|-----|-----|
| H\(^{+/+}\)A\(^{+/+}\) | 0.273 | 0.105 | 0 | 0.684 | 0.005 | 0.677 | 0.048 | 0 |
| H\(^{+/+}\)A\(^{+/+}\) | 0.115 | 0.044 | 0.132 | 0.802 | 0 | 0.816 | 1.106 | 0 |
| H\(^{+/+}\)A\(^{+/+}\) | 0 | 0 | 0.170 | 0.785 | 0 | 0.709 | 1.081 | 0 |
| H\(^{+/+}\)A\(^{+/+}\) | 0 | 0 | 0.153 | 0.502 | 0.263 | 0.452 | 1.016 | 0.118 |
| H\(^{+/+}\)A\(^{+/+}\) | 0 | 0 | 0.157 | 0.016 | 0.643 | 0 | 1.329 | 0.251 |

FIG. 3. Southern blot analysis of hgprt and aprt loci in wild type and mutant strains. Genomic DNAs from H\(^{+/+}\)A\(^{+/+}\), H\(^{+/+}\)A\(^{+/+}\), H\(^{+/+}\)A\(^{+/+}\), and H\(^{+/+}\)A\(^{+/+}\) cells were digested with EcoRI and subjected to Southern blot hybridization using probes A and B from the hgprt locus (panel A). The same genomic DNAs were also digested with BamHI and SalI and hybridized to probes C and D from the aprt locus (panel B) and to the neo' and hyg' probes (panel C). Each set of Southern blots was normalized by hybridization to the L. enriettii α-tubulin. The locations of probes A, B, C, and D are shown in Fig. 2. The positions of molecular size markers are indicated on the left in base pairs.
Baprt served in all cell lines, except H
region. The 3.5-kb wild type neor ascertained as described under "Experimental Procedures." Extracts to phosphoribosylate 20 H fragment is detected in with Bam fragments and with the Aprt protein was detected in extracts of H

The ability of H
parental line. Drug Resistance Phenotypes—No significant differences in growth rate or morphology were observed among the H
aprt alleles evaluated by Western blotting with polyclonal antibodies prepared against the pure recombinant HGPRT and APRT proteins. As observed in Fig. 5A, HGPRT protein was detected in extracts of H

Enzymatic Activities and Immunoblotting—To establish that elimination of wild type hgprt and aprt alleles conferred the appropriate enzymatic deficiencies, the activities of HGPRT and APRT were determined for the H
A

Quantitative densitometry of the autoradiograms probed with aprt fragments and with the a-tubulin probe (Fig. 3B, a-tubulin probe) confirmed the sequential allelic replacements (Table I). The ratio of the signal intensities of the 3.5- and 1.4-kb bands (Fig. 3B, probe D) was 64% of that obtained with DNA from the H

FIG. 5. Western blot analysis of HGPRT and APRT expression in wild type and mutant cell lines. Total cell lysates of exponentially growing H

Enzymatic Activities and Immunoblotting—To establish that elimination of wild type hgprt and aprt alleles conferred the appropriate enzymatic deficiencies, the activities of HGPRT and APRT were determined for the H
A

To confirm that the double knockout mutants did not express their corresponding gene products, extracts of wild type and mutant L. donovani were evaluated by Western blotting with polyclonal antibodies prepared against the pure recombinant HGPRT and APRT proteins. As observed in Fig. 5A, HGPRT protein was detected in extracts of H

Drug Resistance Phenotypes—No significant differences in growth rate or morphology were observed among the H
A

The five cell lines were also evaluated for their sensitivities to the subversive substrates of the two purine PRTs, HPP and
APP. All mutants in which both copies of the relevant wild type gene have been obliterated expressed a high degree of growth resistance to the germane purine base analog, i.e. the EC_{50} values obtained with the mutant strain were 2–3 orders of magnitude greater than the EC_{50} values obtained with H^{+/+} A^{+/+} cells. The H^{+/+} A^{+/+} cell line exhibited intermediate resistance to HPP, while the H^{+/+} A^{+/+} cell line displayed the same modest increase in APP resistance previously observed for an L. dono\-vari cell line obtained by direct selection that expressed only 50% of wild type APRT activity (9).

**DISCUSSION**

Homozygous null mutants of L. dono\-vari in which both wild type alleles of a genetic locus have been replaced using only a single targeting construct have been created at two independent loci, *hgprt* and *aprt*. The strategy for the generation of homozygous null mutants of both genetic loci required elimination of the first wild type allele after transcription and integration of a linear drug resistance cassette encompassing 5’ and 3’ untranslated regions of the *hgprt* and *aprt* loci by homologous recombination, which *Leishmania* accomplish at high frequency (1, 4). The second step in the generation of knockout mutants entailed selection for loss-of-heterozygosity by exposure of the heterozygote to selective pressure using subversive substrates of the encoded gene products. That *Leishmania* exhibit the capacity to undergo this loss-of-heterozygosity is a general phenomenon for any gene for which a strong negative selection can be devised is supported by the applicability of this strategy for creating null mutants to two different genetic loci. This approach is further substantiated by the recent observations of Guieros-Filho and Beverley that *dhfr-ts* heterozygotes of *L. major* can be selected for loss-of-heterozygosity at the *dhfr-ts* locus by plating in neomycin and thymidine (23).

Our data do not, however, support a general mechanism for selecting or distinguishing homozygous null mutants from heterozygotes on the basis of positive selection, as no differences in the growth sensitivities of H^{+/+} A^{+/+} and H^{+/+} A^{+/+} to G418 or of H^{+/+} A^{+/+} and H^{+/+} A^{+/+} to hygromycin could be discerned (Table II). Conversely, it should be noted that loss-of-heterozygosity at multiple loci could be obtained by positive selection in murine ES cells by exposure to high concentrations of the selectable marker (18), and the frequency of loss-of-heterozygosity observed at the *dhfr-ts* locus of *L. major* could be increased by selection in hygromycin (23).

The mechanisms by which loss-of-heterozygosity occurs at the *hgprt* and *aprt* loci in *Leishmania* remain to be investigated. The genesis of H^{+/+} A^{+/+} and H^{+/+} A^{+/+} from H^{+/+} A^{+/+} and H^{+/+} A^{+/+} cells, respectively, could have arisen as a consequence of a number of genetic events including chromosome loss, gene deletion, chromosome nondisjunction, mitotic recombination, gene conversion, or homologous recombination of episomal DNAs derived either from the chromosome or the original targeting construct. Although none of these mechanisms has been described for *Leishmania*, the karyotypic plasticity of the leishmanial genome under selective (10, 32) or stress (33) conditions and the ability of the parasite to undergo conservative amplifications of chromosomal segments (34), to maintain these amplified segments episomally as extrachromosomal elements (35), and to perform homologous recombination (36, 37).

In the two homozygous strains generated in this study, the mechanisms for loss-of-heterozygosity may very well be different. Normalization of the densitometric tracings of the Southern blot data imply that H^{+/+} A^{+/+} and H^{+/+} A^{+/+} cells have a single copy of the neo’ gene, while H^{+/+} A^{+/+} and H^{+/+} A^{+/+} cells have one and two copies of *hygr*, respectively. Thus, one could conjecture that loss-of-heterozygosity at the *hgprt* locus could be ascribed to a simple chromosome loss or deletion of DNA sequences encompassing *hgprt*, while the mechanism of allelic replacement in the H^{+/+} A^{+/+} *aprt* homozygote appears to involve a duplication of the *hygr* allele in the heterozygote. In the *L. major* selected for loss-of-heterozygosity at the *dhfr-ts* locus, Southern and slot blot analyses indicated that the knockouts contained two copies of the *hygr* marker, suggesting that the null mutants did not originate as a consequence of simple chromosome loss or *dhfr-ts* deletion (23). As no differences in hygromycin sensitivity were observed between H^{+/+} A^{+/+} and H^{+/+} A^{+/+}, these data provide further support that positive selection, at least using hygromycin resistance as a selection, cannot be used to isolate homozygous null mutants from heterozygous populations. This result contrasts with analogous experiments performed on the murine ES cells in which cells containing one or two copies of *neo’* can be distinguished on the basis of G418 sensitivity (18).

Targeted gene replacement has become a powerful tool to evaluate gene function in *Leishmania* and related parasites (1, 4, 7, 11, 15–17). In diploid organisms with a sexual cycle, the general strategy for obtaining null mutants is to target the first allele with a specific construct to generate the heterozygote, followed by sexual crossing to generate the homozygote. As *Leishmania* spp. are ostensibly asexual in nature, at least under laboratory conditions, and are presumed to be diploid at most loci, elimination of a wild type diploid locus has required two independent drug resistance markers, e.g. *neo’* and *hygr*. Moreover, a third independent marker is theoretically required to verify gene function by complementation of an observed phenotype. The ability to create null mutants, therefore, with only a single targeting construct greatly facilitates genetic investigations into complex metabolic pathways, such as that for purine salvage, because the number of drug resistance markers available for gene targeting in *Leishmania* is limited (6, 15, 38, 39). As a consequence, other drug resistance markers are preserved for subsequent genetic manipulations, such as the creation of strains with multiple homozygous mutations, e.g. H^{+/+} A^{+/+}. Indeed, the H^{+/+} A^{+/+} cell line is the first *Leishmania* strain constructed by targeted gene replacement to contain more than one homozygous mutation. Given the availability of preexisting *L. dono\-vari* mutants genetically deficient in either *APRT* (9) or adenosine kinase (40), one can envision a strategy to generate all the requisite strains with multiple muta-
tions in purine genes to thoroughly dissect the multifarious and divergent purine acquisition pathway in Leishmania. This purine salvage pathway is of particular interest, because of the purine auxotrophy established in all protozoan parasites studied to date (41). Thus, the pathway may provide selective targets for antiparasitic therapeutic strategies. It should also be noted that the ability to obliterate both wild-type alleles of a genetic locus with only a single targeting construct also significantly expedites the construction of mutants in this genus of protozoan parasite. As vector construction and strain generation and characterization are laborious and time-consuming endeavors, the ability to generate mutant lines of L. donovani after one round of transfection substantially conserves both time and financial expenditures.

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