

# Stable molecular transformation of *Toxoplasma gondii*: A selectable dihydrofolate reductase-thymidylate synthase marker based on drug-resistance mutations in malaria

(antifolates/transfection/*Plasmodium falciparum*/molecular parasitology)

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Communicated by Robert T. Schimke, September 13, 1993

**ABSTRACT** To facilitate genetic analysis of the protozoan parasite *Toxoplasma gondii*, sequences derived from the parasite's fused dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene have been used to produce vectors suitable for stable molecular transformation. Mutations introduced into the DHFR coding region by analogy with pyrimethamine-resistant malaria confer drug resistance to *Toxoplasma*, providing useful information on the structure of fused DHFR-TS enzymes and a powerful selectable marker for molecular genetic studies. Depending on the particular drug-resistance allele employed and the conditions of selection, stable resistance can be generated either by single copy nonhomologous insertion into chromosomal DNA or by massively amplified transgenes. Frequencies of integration are independent of selection, and transgenes are stable without continued selection. Cointegration of a reporter gene adjacent to the selectable marker (under the control of an independent promoter) shows no loss of the cointegrated sequences over many parasite generations. By bringing the full power of molecular genetic analysis to bear on *Toxoplasma*, these studies should greatly facilitate the development of a model genetic system for Apicomplexan parasites.

The protozoan parasite *Toxoplasma gondii* is a ubiquitous human pathogen long known for its importance in congenital infections. More recently, this organism has achieved considerable notoriety as an opportunistic infection afflicting AIDS patients (1). Traditional treatment of toxoplasmosis involves blocking the folate metabolic pathway (2), but the persistence of latent parasite cysts requires that AIDS patients receive chronic therapy—a protocol often impractical due to poor patient tolerance of traditional antifolate/sulfonamide mixtures (3, 4). The urgent need for new treatment strategies for AIDS-toxoplasmosis has stimulated interest in the design of better antifolates and the identification of novel drug targets (5).

Aside from its medical importance, *Toxoplasma* provides a promising system for the genetic analysis of intracellular parasites. The organism is convenient and safe to grow in culture, and its unusually wide host range permits a variety of cell genetic approaches (6). *T. gondii* parasites also undergo a sexual cycle in cats, making classical genetic crosses feasible (7). In this report we describe a transformation system suitable for stable expression of functional recombinant dihydrofolate reductase-thymidylate synthase (DHFR-TS) in *Toxoplasma*, based on point mutations within the DHFR coding region. In conjunction with a recently devised transient expression system (8), these studies pave the way for further genetic investigation of *Toxoplasma*.

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## MATERIALS AND METHODS

**Parasite Growth and Transformation.** *T. gondii* tachyzoites (RH strain) were maintained by serial passage in primary cultures of human foreskin fibroblasts (HFFs) as described (9). Parasite metabolism was measured by uptake of [<sup>3</sup>H]-uracil (10, 11). Replication was determined by infecting HFFs with parasites and scoring the average number of parasite divisions in at least 50 parasitophorous vacuoles as a function of time (each vacuole contains the progeny of a single parasite). Plaque assays were carried out by inoculating confluent monolayers of HFFs with various parasite dilutions, leaving the infected cultures to incubate undisturbed until plaques appeared, and staining with crystal violet. Individual parasites were cloned by limiting dilution in 96-well microtiter plates containing HFFs.

For electroporation, 50 μg of plasmid DNA was added to 10<sup>7</sup> extracellular parasites in "intracellular" buffer (8, 12) freshly supplemented with 2 mM ATP and 5 mM glutathione (total volume, 0.4 ml) and transferred to 2-mm gap cuvettes. Optimal electroporation parameters of 1.5 kEV, 24 Ω (pulse time, ≈0.25 msec) were determined on a model 600 electroporator (BTX, San Diego) using the DHFR-TS promoter linked to a chloramphenicol acetyltransferase (CAT) reporter gene. Electroporated parasites were inoculated into confluent monolayers of HFF cells grown in 25-cm<sup>2</sup> T flasks.

**Molecular Methods.** Recombinant vectors used in this report are shown in Fig. 1. Sequence data covering the entire *T. gondii* DHFR-TS gene have been deposited with GenBank (accession no. L08489). A synthetic minigene containing the entire DHFR-TS coding region and flanking 5' and 3' sequences was created by ligating the following fragments between the *Hind*III and *Eco*RI sites of pKS- (Stratagene): (i) a genomic *Hind*III/*Nru* I fragment extending from 1.4 kb upstream of the translational initiation codon into the first exon, (ii) an *Nru* I/*Eco*RI fragment of a cDNA-derived PCR product covering the entire DHFR and junctional domains (clone PCR 31-13; ref. 9), (iii) the *Eco*RI insert from a partial cDNA clone covering the TS domain (clone λgt11-c6; ref. 9). As described in the text, various point mutations suspected to confer pyrimethamine resistance were introduced by oligonucleotide-directed mutagenesis (13) and confirmed by sequencing. Mutant constructs are identified as follows: M0, Met<sup>1</sup> → Leu (null mutation); M2, Ser<sup>36</sup> → Arg; M3, Thr<sup>83</sup> → Asn; M4, Phe<sup>245</sup> → Ser. Double mutants are identified as M2M3, etc. Vectors containing DHFR-TS and CAT coding sequences were constructed by ligating the filled *Hind*III/*Bam*HI insert of the SAG-CAT vector recently reported by Soldati and Boothroyd (8) into the filled *Hind*III site of pDHFR-TS/M2M4.

Abbreviations: CAT, chloramphenicol acetyltransferase; DHFR, dihydrofolate reductase; HFF, human foreskin fibroblast; TS, thymidylate synthase.

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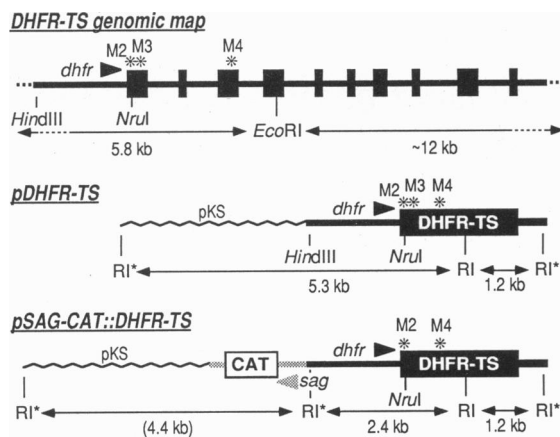


FIG. 1. Organization of the *T. gondii* DHFR-TS locus and recombinant plasmids. Genomic sequences are indicated by heavy lines (black for DHFR, shaded for SAG1; ref. 8); plasmid DNA is shown as wavy lines. Circular plasmids are depicted in linearized form for legibility. RI\* indicates plasmid-derived *EcoRI* sites. Promoter sequences are shown in italics; arrowheads indicate orientation. Protein coding regions are shown as boxes (filled for DHFR-TS, open for CAT). The locations of codons altered to produce pyrimethamine-resistant mutants are shown.

Parasite chromosomes were separated by transverse alternating field electrophoresis on a Geneline system I (Beckman) (14). Intact tachyzoites from freshly lysed cultures were embedded in 0.5% Seaplaque agarose (FMC) at  $5 \times 10^8$  parasites per ml and incubated overnight at 56°C in 0.5 M EDTA/1% sarcosyl/2 mg of proteinase K per ml. Plugs were cast directly into 0.5% agarose (Fast-Lane; FMC), and gels were run under the following conditions: 36 hr at 40 V, 30-min switch times; 24 hr at 50 V, 25 min; 24 hr at 60 V, 22 min; 36 hr at 70 V, 15 min. *Toxoplasma* chromosomes were identified by comparison with published data (14) and *Schizosaccharomyces pombe* DNA run in parallel.

## RESULTS

**Identification of Mutations Conferring Pyrimethamine Resistance to *T. gondii*.** In the related Apicomplexan parasite *Plasmodium falciparum* (malaria), resistance to pyrimethamine in field isolates and laboratory studies has been correlated with point mutations in DHFR (15–19). Sequencing the DHFR and junctional domains of three independently isolated pyrimethamine-resistant mutants of *Toxoplasma* failed to reveal any such mutations, but several of the amino acid residues altered in drug-resistant malaria are conserved in the *T. gondii* sequence (9), suggesting mutations that could potentially confer drug resistance. Three mutations were introduced into the DHFR domain of an intronless *T. gondii* “minigene” (Table 1). Mutation M3 substitutes Asn for Thr at position 83 in the predicted amino acid sequence (9), corresponding to the most frequent mutation in pyrimethamine-resistant *P. falciparum* (Ser/Thr<sup>108</sup> → Asn; refs. 15–17). Mutation M2 (Ser<sup>36</sup> → Arg) is probably analogous to Cys<sup>59</sup> → Arg in malaria, also associated with drug resistance. M4 replaces Phe<sup>245</sup> with Ser—an alteration possibly analogous to the Phe<sup>223</sup> → Ser mutation identified in laboratory screens for pyrimethamine-resistant *P. falciparum* (18). All three point mutations and the double mutants M2M3 and M2M4 retain DHFR and TS activity in the *folA* *E. coli* mutant LH18 (20). M3M4 does not complement folate auxotrophy in *E. coli* and is presumed to inactivate the DHFR enzyme.

*Toxoplasma* tachyzoites electroporated with the wild-type DHFR-TS gene failed to grow in 1  $\mu$ M pyrimethamine ( $\approx$ 8 times the IC<sub>50</sub> for controls), but it was immediately apparent

Table 1. DHFR-TS mutations associated with pyrimethamine resistance

Organism and strain	Residue (locus)	<i>E. coli</i> *	Drug resistance*
<i>P. falciparum</i>			
Strains 3D7, FCR3 Isolates	Cys <sup>59</sup> Thr/Ser <sup>108</sup> Phe <sup>223</sup>		–†
HB3, Camp	Asn		++
V-1, K1	Arg Asn		+++
Mutants			
FCR3/D4-D7	Ser		+
<i>T. gondii</i>			
Strain RH	Ser <sup>36</sup> Thr <sup>83</sup> Phe <sup>245</sup>	+	–†
Mutant			
M2	Arg	+	–
M3	Asn	+	+
M4	Ser	+	+
M2M3	Arg Asn	+	+++
M2M4	Arg Ser	+	+++
M3M4	Asn Ser	–	–

\*Function measured by *in vitro* bioassay of isolates for *P. falciparum* (15–19), complementation of *Escherichia coli* strain LH18 (20), or *in vitro* assay of *T. gondii* transformants (this report).

†Wild type.

from microscopic examination of infected cultures that the double mutations M2M3 and M2M4 confer profound resistance. Parasites transfected with these mutant genes lyse the entire host cell monolayer under drug selection nearly as rapidly as wild-type parasites grown without pyrimethamine. As shown in Fig. 2A, metabolic labeling of cultures 24 hr after transformation with M2M3 or M2M4 plasmids showed, respectively, 4.5- and 4.0-fold greater uracil incorporation than observed in mock transfected controls (average of three experiments; SD,  $\pm$ 0.4), approximately one-third of the incorporation seen without drug.

To directly assess the frequency of pyrimethamine resistance, transfected parasites were scored by plaque assay with or without 1  $\mu$ M pyrimethamine. Fifty-six percent of viable parasites transfected with M2M3 or M2M4 mutants were resistant to pyrimethamine (average of six experiments; SD,  $\pm$ 14%). This frequency is comparable to the level of expression seen by immunofluorescence in parasites transfected with a CAT reporter gene under control of the DHFR-TS promoter (not shown). Mock-transfected parasites and parasites transfected with mutant M0 (in which the ATG initiation codon was destroyed) or mutant M3M4 (which failed to complement DHFR<sup>–</sup> bacteria; Table 1) showed no drug resistance by either uracil uptake or plaque assay.

Parasites transfected with wild-type DHFR-TS or single point mutations were inhibited by pyrimethamine, but small parasitophorous vacuoles and motile extracellular parasites were observed in the M3- and M4-transfected samples, suggesting partial protection. Although no significant uracil uptake was seen at 24 hr posttransfection, incorporation at 72 hr was 2.1 times background for both mutants (average of four experiments; SD,  $\pm$ 0.4). Because of the extremely slow growth rate of these parasites, plaque assays under drug selection were impractical. Viability and proliferation were therefore assessed by removing aliquots of the supernatant medium at various times after transfection and performing plaque assays without drug. As shown in Fig. 2B, increasing numbers of viable progeny were found in M3- and M4-transfected cultures. In contrast, after 4 days in pyrimethamine virtually no viable parasites could be detected in either mock-transfected controls or parasites transfected with the null mutant M0. Small numbers of viable parasites could also be isolated from parasites transfected with wild-

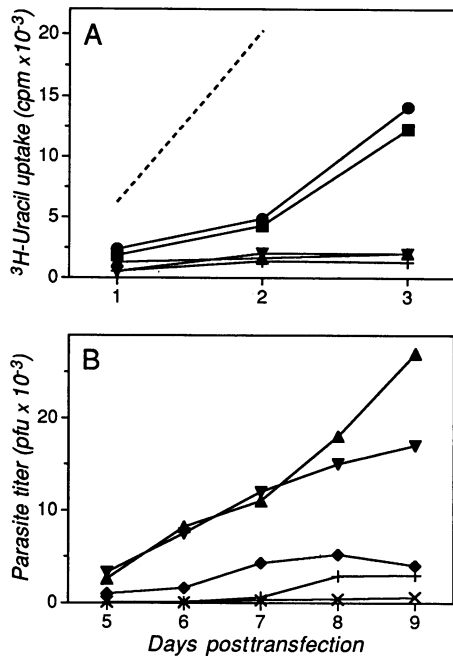


FIG. 2. DHFR-TS mutations confer pyrimethamine resistance to *Toxoplasma*. Parasites were transfected with mutant DHFR-TS minigenes and inoculated into HFF cells in the presence of 1  $\mu$ M pyrimethamine. (A) Double mutants M2M3 (●) and M2M4 (■) readily incorporated the parasite-specific marker [<sup>3</sup>H]uracil, whereas uptake in mutants M3 (▼) and M4 (▲) was only slightly above the background levels observed in mock-transfected controls, the null mutant M0, mutant M2, and double mutant M3M4 (+). The dashed line indicates uptake in mock-transfected parasites grown without drug. (B) Parasite titers were measured by plaque assay (without drug) at various times after transfection. Increasing numbers of parasites were observed in M3 (▼) and M4 (▲) transfected samples after longer incubations. Low numbers of parasites were detected at 8 days in cultures inoculated with wild-type DHFR-TS (+) or M2 (◆) constructs and declined thereafter. No viable parasites were found in cultures transfected with the null mutant M0 (×). pfu, Plaque-forming units.

type DHFR-TS or with the M2 mutant after 7–8 days in pyrimethamine (declining thereafter), suggesting a prolongation of survival due to transient DHFR-TS expression.

**Stable Transformation of *Toxoplasma* with Pyrimethamine-Resistant DHFR-TS.** Because transfected plasmids decline below detectable levels within days after electroporation (8), the continued proliferation of parasites transfected with mutant DHFR-TS suggested stable replication of the transgene. To determine the frequency of stable pyrimethamine resistance and to isolate drug-resistant clonal lines, the progeny of transfected parasites were tested after primary passage in 1  $\mu$ M pyrimethamine, using parallel plaque assays in the presence or absence of drug. M2M3- and M2M4-transfected parasites yielded 12% and 13% (respectively) drug-resistant progeny in the second passage, and all parasites resistant at this stage maintained their resistance upon further culture, even when grown without drug. When transfected parasites were carried for the initial passage without pyrimethamine and then tested for drug sensitivity in the second passage, 6% of the emerging parasites were resistant. As approximately half of the transfected parasites are killed by pyrimethamine in the first passage (see above), these results indicate that expression of drug-resistant DHFR-TS in *Toxoplasma* is not detrimental to the parasite and that selection has little if any effect on the frequency of stable transformation.

Clonal progeny of stably resistant parasites were isolated by limiting dilution in 96-well microtiter plates in the contin-

ued presence of pyrimethamine. Multiple isolates were obtained from independent cultures transfected with M2M3 and M2M4, but, as noted above, M3- and M4-transfected parasites grew extremely slowly and never completely lysed the initial HFF monolayer in the presence of 1  $\mu$ M drug. However, by inoculating those parasites that did emerge into fresh cultures at reduced pyrimethamine concentration (600 nM) it was possible to isolate stable drug-resistant clones. Clones of M3- and M4-transfected parasites were also isolated by inoculation of the initial HFF culture in 600 nM pyrimethamine.

Growth rates for several drug resistant clones in 1  $\mu$ M pyrimethamine are presented in Fig. 3. Resistant lines derived from double mutants M2M3 and M2M4 replicate at the same rate as wild-type parasites grown in the absence of drug (doubling time,  $\approx$ 6 hr). These mutants are unfazed by pyrimethamine concentrations up to 5  $\mu$ M (not shown). The M3 and M4 mutants isolated through initial passage in 1  $\mu$ M pyrimethamine followed by cloning in 600 nM drug grew substantially slower (doubling time,  $\approx$ 9 hr in 1  $\mu$ M pyrimethamine). An M4 clone isolated by selection in 600 nM pyrimethamine from the time of transfection showed a doubling time of >15 hr in 1  $\mu$ M drug.

A hybrid vector was constructed containing DHFR-TS/M2M4 and CAT genes under the control of divergent promoters to assess the flexibility of pyrimethamine selection as a marker for carrying additional genes and to confirm the stability of transgene expression in the absence of selection. Transient CAT expression from the composite vector (pSAG-CAT::DHFR-TS/M2M4 in Fig. 1) was 38% lower than from transfected SAG-CAT plasmid (8) alone, and the frequency of drug resistance was likewise slightly reduced relative to the M2M4 plasmid alone (30% of the cotransfectants showed transient resistance vs. 57% for M2M4; 3% vs. 6% were stably resistant). Interestingly, CAT expression was diminished a further 3-fold when SAG-CAT was introduced in the same orientation as DHFR-TS. Transfected parasites were cloned by limiting dilution under pyrimethamine, and CAT expression followed for 10 serial passages in culture ( $\approx$ 100 parasite generations) in the absence of pyrimethamine. No decline in CAT activity was observed over this period (123%  $\pm$  29% of activity in the original clone).

**Genomic Organization of Transgenic Sequences.** To investigate the physical structure of transgenic DHFR-TS sequences, DNA was purified from multiple independently cloned pyrimethamine-resistant lines, digested with various restriction enzymes, and examined by DNA hybridization. The results of one such analysis are shown in Fig. 4. Restriction with *Eco*RI produces genomic DHFR-TS bands of 5.8 and  $\approx$ 12 kb in size, as indicated by solid arrowheads (see Fig. 1 for map). Restriction of pDHFR-TS yields two bands, indicated by the open

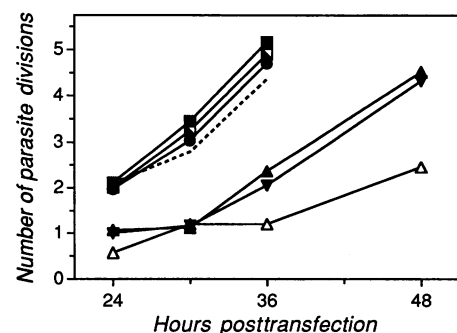


FIG. 3. Growth of pyrimethamine-resistant clones. Double mutant clones M2M3-6.1 (●), M2M4-6.3 (■), and M2M4::SAG-CAT-2 (▣) grew as rapidly in 1  $\mu$ M pyrimethamine as control parasites in the absence of drug (dashed line). Clones M3-2.1 (▼) and M4-2.2 (▲) grew more slowly (under drug selection), and M4-1.2 (Δ) grew slower still.

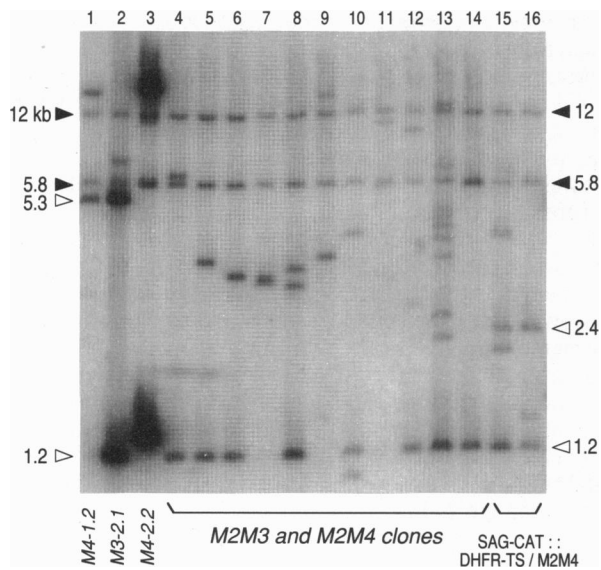


FIG. 4. Genomic organization of transgenic DHFR-TS. DNA was isolated from drug-resistant parasites, digested with *EcoRI*, and probed with DHFR-TS coding sequences. Each lane represents a different, independently cloned parasite line. Lanes 1–3 were isolated from clones transfected with single mutants M3 or M4; lanes 4–14 were transfected with double mutants M2M3 or M2M4; lanes 15 and 16 were transfected with the hybrid DHFR-TS/M2M4::SAG-CAT construct (Fig. 1). The expected 12- and 5.8-kb genomic fragments are indicated by solid arrowheads, and were unaltered in any of these clones. Expected plasmid-specific bands are indicated by open arrowheads (5.3 and 1.2 kb for clones 1–14; 2.4 and 1.2 kb for clones 15 and 16). Most of the double mutants appear to have integrated into the genome as single copy nonhomologous recombinants, whereas mutants M3-2.1 and M4-2.2 are highly amplified.

arrowheads at the left: a 1.2-kb fragment containing the TS domain with 331 nt of genomic 3' flanking sequence and a 5.3-kb band containing the DHFR and junctional domains along with 1.4 kb of 5' genomic flanking sequence and the 3.0-kb pKS plasmid vector (see Fig. 1). Restriction of the hybrid plasmid pSAG-CAT::DHFR-TS produces the same TS fragment as above and a 2.4-kb DHFR plus 5' flanking region fragment, indicated by the open arrowheads at the right. An additional 4.4-kb SAG-CAT plus pKS fragment is not recognized by the DHFR-TS probe.

Several conclusions can be drawn from these experiments. As the transfected DHFR-TS genes are derived from cDNA sequences lacking introns, the unaltered presence of both genomic bands in all clones indicates that no homologous recombination occurred. [*Toxoplasma* tachyzoites are haploid (7) and appear to contain only a single copy of the DHFR-TS gene (9).]

Lanes 4–14 contain DNA from parasites transformed with double mutants M2M3 and M2M4. Labeling intensity suggests that nine of these clones are single copy insertions. This interpretation is supported by the presence of exactly one novel fragment (in addition to the expected genomic and plasmid bands) in most cases. Clone 8 appears to contain two independent transgenes, and clone 13 contains multiple insertions. None of the double mutant clones (4–14) contains the intact 5.3-kb DHFR plus pKS fragment, indicating that integration always involves rearrangement within this region. Additional digests (not shown) demonstrate that recombination took place within the pKS vector sequences in all cases except clone 10—where integration presumably occurred in the 5' noncoding region, upstream of any essential promoter elements. Three clones (lanes 7, 9, and 11) lack the 1.2-kb TS fragment, indicating that integration occasionally involves this domain as well—presumably in the 3' noncoding region.

Parasites electroporated with the DHFR-TS/CAT cotransfection construct (lanes 15 and 16) fit the same pattern. Both clones examined contain bands expected for intact, plasmid-derived DHFR and TS (righthand arrowheads) and CAT (not shown), indicating that plasmid integration involved pKS vector sequences only. Clone 16 appears to have involved a single integration, whereas clone 15 probably contains two transgenes.

Lanes 1–3 represent parasite clones transformed with DHFR-TS genes carrying single mutations. Lane 1 is the minimally pyrimethamine-resistant M4 clone selected in 600 nM drug (clone M4-1.2 in Fig. 3). This mutant appears to contain two copies of the transgene, both integrated within the plasmid DHFR-TS 3' end. Lanes 2 and 3 contain DNA from moderately pyrimethamine-resistant clones (M3-2.1 and M4-2.2 in Fig. 3). These clones exhibit massively amplified transgenes. In lane 2, a 7-kb band is present as a single copy, in addition to multiple copies of both plasmid-specific bands. This pattern suggests that all copies of the transgene are amplified at a single locus as tandem head-to-tail repeats. Lane 3 lacks both expected plasmid-derived bands but contains two novel amplified bands (discussed further below).

To map transgenic sequences within the *T. gondii* molecular karyotype (14), pulsed-field gel analysis was carried out on several parasite clones. As shown in Fig. 5, the endogenous DHFR gene is located on a large chromosome that does not enter the gel. Transgenic DHFR-TS genes comigrate with several chromosomes, such as the apparent localization of clone 11 on chromosome V. Perhaps the most convincing indication of chromosomal integration derives from analysis of lane 2, in which the multiple tandemly arrayed transgenes identified in Fig. 4 migrate at a position slightly ahead of chromosome VI. Comparison with the ethidium bromide-stained gel, however, suggests that this is in fact an aberrant chromosome V, increased in size by the integration of  $\approx 60$  copies of the 6.5-kb plasmid.

As noted above, we were unable to define the nature of genomic integration for lane 3 (clone M4-2.2) by analysis of restriction patterns. In pulsed-field gels this clone also shows peculiar transgene organization. A minority of the label comigrates with chromosome IV, but the bulk of hybridization labels a more rapidly migrating smear. This does not appear to be degraded DNA, as (i) no corresponding smear

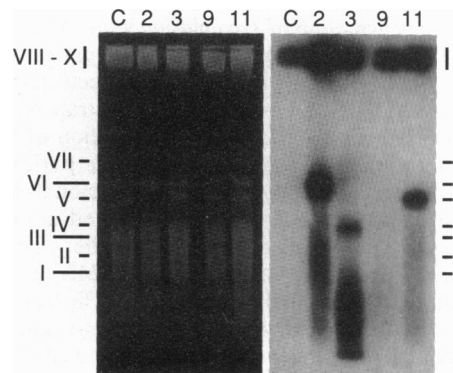


FIG. 5. Chromosomal/episomal localization of transgenes. Pulsed-field gels of parasite chromosomes were blotted and probed to identify the location of DHFR-TS sequences. Lanes are numbered to correspond with Fig. 4; lane C contains DNA from untransfected parasites. The endogenous DHFR-TS gene and the transgene in clone 9 remain in the well (chromosomes VIII–X). Transgene 11 comigrates with chromosome V. The tandemly amplified transgenes in clone 2 appear to have integrated into chromosome V in sufficient copy number to shift the mobility of the chromosome (compare with the ethidium bromide-stained gel at the left). Clone 3 contains multiple transgenes in a more complex pattern as discussed in the text.

is visible in the stained gel, (ii) labeling terminates at a specific rapidly migrating band (which does not correspond to any compression zone in the gel), and (iii) this labeling pattern is reproducible between several independent chromosome preparations. The M4-2.2 transgene may be maintained as an extrachromosomal episome, but this possibility has not been rigorously tested.

## DISCUSSION

By exploiting the knowledge of DHFR-TS mutations conferring pyrimethamine resistance in *P. falciparum* (15–19) in combination with protein alignments (9), we have identified analogous amino acid substitutions in the *Toxoplasma* enzyme that confer drug resistance to transfected parasites. In addition to the value of these mutations as selectable markers for transformation, they also provide structural, pharmacological, and evolutionary information on the DHFR-TS enzyme. For example, the malaria mutation Cys<sup>59</sup> → Arg is known from field isolates only in the context of Ser/Thr<sup>108</sup> → Asn. The analogous M2 and M3 mutations in *Toxoplasma* clearly demonstrate the functional importance of this correlation: M2 alone is ineffective in producing pyrimethamine resistance, but the double mutant M2M3 is at least 10-fold more resistant than M3 alone. The *P. falciparum* mutant Phe<sup>223</sup> → Ser has been difficult to explain, as this locus is generally thought to reside within the DHFR-TS junctional domain (17, 18). Alignment with the *T. gondii* sequence suggested an alternative location, however, within  $\beta$ -strand vii (9). Mutation of the predicted homolog in *Toxoplasma* (Phe<sup>245</sup> → Ser; designated M4) confers pyrimethamine resistance, lending support to this alignment. Structural modeling of *P. falciparum* and *T. gondii* DHFR-TS enzymes (T. Kieber-Emmons and D.S.R., unpublished) indicates that mutation M4 probably acts indirectly: the large aromatic group of Phe<sup>245</sup> is predicted to stabilize His<sup>34</sup> in the  $\alpha_B$  helix. The double mutant M2M4, unknown from either field or laboratory isolates, is dramatically more resistant to pyrimethamine than M4 alone.

Stable cloned parasite lines harboring single copy genomic transgenes can readily be isolated using DHFR-TS plasmids bearing either M2M3 or M2M4 double mutations. These mutants are >80-fold resistant to pyrimethamine relative to untransfected controls (IC<sub>50</sub> > 10  $\mu$ M vs. 125 nM). In 1  $\mu$ M pyrimethamine, mutant parasites grow as rapidly as the wild-type RH strain in nonselective medium. At these levels of selection, spontaneous parasite resistance arises at a frequency of <10<sup>-8</sup>. For genetic studies this extremely tight selection provides an alternative to markers such as neomycin or hygromycin, which are ineffective against *Toxoplasma*. Selection for chloramphenicol resistance is also possible but provides somewhat less satisfactory results in our hands. CAT selection may be more practical in certain studies, however, for reasons of safety. Although normally harmless to otherwise healthy individuals, a large inoculum of a virulent strain such as RH can be dangerous to those previously seronegative (21). In the event of an accidental infection, the presence of a pyrimethamine-resistant DHFR-TS allele would probably render classical pyrimethamine/sulfa therapy ineffective, although alternative treatments are available (5).

It is difficult to determine the precise frequency of genomic integration or exactly when integration occurs, as we have not methodically examined the diversity of stable progeny from a single transfected parasite. Nevertheless, it is clear that the frequency must be extraordinarily high, as progeny bearing stable transgenes arise from 6% of all parasites emerging after a single passage without selection. Integration appears to be independent of selection, and integrated trans-

genes (including genes linked to the selectable marker) are stable without continued selection. No evidence of homologous recombination was detected, perhaps because of insufficient contiguous homology between the cDNA-derived transformation vectors and the parasite genome. These results differ markedly from stable transformation in the Kinetoplastida, where episomal replication or homologous recombination is the norm (22–27). To a first approximation, the distribution of transgenic insertions appears to be random in *Toxoplasma*. These vectors should therefore permit insertional tagging experiments. For expression studies it may be helpful to employ the less potent drug-resistance alleles M3 and M4 (independently), as selection conditions can be manipulated to induce massive amplification rather than single copy insertion.

We thank Drs. D. Soldati and J. C. Boothroyd (Stanford) for discussing the results of parallel experiments prior to publication and for providing the SAG-CAT vector. Dr. E. R. Pfefferkorn (Dartmouth) supplied a pyrimethamine-resistant *T. gondii* isolate and frequent helpful advice. *E. coli* strains deficient in DHFR and/or TS were provided by Dr. E. E. Howell (University of Tennessee, Knoxville). Structural modeling of the DHFR-TS enzyme was carried out with the assistance of Dr. T. Kieber-Emmons (Wistar Institute). This work was supported by National Institutes of Health Grants AI-28724 and AI-31808. D.S.R. is a Presidential Young Investigator of the National Science Foundation, with support from the MacArthur Foundation and Merck Sharp & Dohme Research Laboratories.

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