Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 1130–1134, February 1996
Genetics

Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine

(transfection/dihydrofolate reductase-thymidylate synthase/episomes/drug resistance)

YIMIN WU, LAURA A. KIRKMAN, AND THOMAS E. WELLEMS

Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892-0425

Communicated by William Trager, The Rockefeller University, New York, NY, October 19, 1995

ABSTRACT ***Plasmodium falciparum*** **malaria parasites were transformed with plasmids containing *P. falciparum* or *Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) coding sequences that confer resistance to pyrimethamine. Under pyrimethamine pressure, transformed parasites were obtained that maintained the transfected plasmids as unrearranged episomes for several weeks. These parasite populations were replaced after 2 to 3 months by parasites that had incorporated the transfected DNA into nuclear chromosomes. Depending upon the particular construct used for transformation, homologous integration was detected in the *P. falciparum dhfr-ts* locus (chromosome 4) or in *hrp3* and *hrp2* sequences that were used in the plasmid constructs as gene control regions (chromosomes 13 and 8, respectively). Transformation by homologous integration sets the stage for targeted gene alterations and knock-outs that will advance understanding of *P. falciparum*.**

Methods of incorporating genes into *Plasmodium falciparum* will provide a foundation for genetically manipulating this major human pathogen. Previous reports have shown that transfected plasmids can be transiently expressed in this and other parasites of the Apicomplexan group (1–4). In the case of *Toxoplasma gondii* parasites, both homologous and random integration of exogenous DNA have also been obtained (5–8). These advances have pointed to the next steps for *P. falciparum* transfection research: integration of exogenous DNA into the parasite chromosomes and the development of targeting methods for functional studies of genes.

Transient expression of reporter constructs in *P. falciparum* first required the identification of gene control regions that could be cloned intact in *Escherichia coli*, as many of these A+T-rich regions are unstable in bacterial plasmids (3). With the isolation of suitable regions from two histidine-rich protein genes (*hrp2* and *hrp3*) and from a *P. falciparum* heat shock protein 90 gene (*hsp86*), chloramphenicol acetyltransferase (CAT) and luciferase were successfully expressed (3), suggesting that constructs expressing selectable markers could succeed in stably transforming erythrocyte-stage parasites. Prime selectable markers for this purpose include forms of *P. falciparum* dihydrofolate reductase-thymidylate synthase genes (*dhfr-ts*) that produce pyrimethamine and cycloguanil resistance (9–13). Forms of *dhfr-ts* that produce pyrimethamine resistance are also available from the *T. gondii* sequence (14), thus providing functionally similar markers with primary structures that are highly divergent from *P. falciparum dhfr-ts*. Here we report the transformation of *P. falciparum* by constructs that express these *dhfr-ts* markers under control of the *P. falciparum hrp2* and *hrp3* flanking regions.

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.

MATERIALS AND METHODS

DNA Constructs. PCR with the primer pair 5′-CCT-TTT-TAT-GCA-TGA-ACA-AGT-CGT-CGA-C-3′ and 5′-AAT-TTC-AAG-CTT-AAG-CAG-CCA-TAT-CC-3′ was used to amplify the *dhfr-ts* coding sequences of three *P. falciparum* lines (9): the wild-type, pyrimethamine-sensitive 3D7 sequence; the pyrimethamine-resistant HB3 sequence, which encodes a single mutation (S108N); and the highly pyrimethamine-resistant Dd2 sequence, which encodes three mutations (S108N, N51I, and C59R). The coding sequence of the *T. gondii dhfr-ts* M2M3 sequence was amplified from pDHFR-TS/M2M3 (14) by using the primers 5′-GGA-AGA-TGC-ATA-AAC-CGG-TG-3′ and 5′-GAC-GGG-AAG-CTT-CTG-TAT-TTC-3′. Amplified inserts were digested with *Nsi* I and *Hin*dIII restriction endonucleases and used to replace the CAT-coding sequence in the pHRPCAT vector (3), yielding constructs pDT.3D7, pDT.HB3, pDT.Dd2, and pDT.Tg23 (Fig. 1).

P. falciparum **Transfection, Selection of Transformants, and Drug Response Assays.** *P. falciparum* parasites from the 3D7, HB3, and Dd2 lines were cultivated as described (15). Clones from transformed lines were obtained by limiting dilution (16). Electroporation settings and parasite sample preparations were as reported (3). Following transfection, the samples were immediately mixed with 10 ml of culture medium and cultivated in 25-cm^2 culture flasks. Pyrimethamine responses of the *P. falciparum* lines were determined in standard RPMI 1640 medium (GIBCO/BRL) as previously described (9, 12), except that the parasites were maintained under drug pressure for 5 days.

PCR Amplification of Integration Sites. Homologous integration at the *dhfr-ts, hrp3*, and *hrp2* sites was detected by amplification across insertion breakpoints with the following oligonucleotide primer pairs: 5′-CCA-ACA-TTT-CAA-GAT-TGA-TAC-ATA-AAG-G-3′ (#1) and 5′-GGA-AAC-AGC-ATA-GAC-CAT-G-3′ (#2) at the *dhfr-ts* site; 5′-GTA-AAA-CGA-CGG-CCA-GT-3′ (#3) and 5′-GTA-CCG-AAG-CAA-AAA-CGG-CAG-CGG-3′ (#4) at the *hrp3* site; and 5′-TCT-TGA-CAT-ATT-GTG-TTC-ATG-TAT-3′ (#5) and 5′-CAA-TAC-AAA-ATT-ATG-TTC-ATC-ATG-3′ (#6a, for transformed lines 28a and 28b) or 5′-GCT-ACG-TCC-CGC-ACG-GAC-3′ (#6b, for transformed line 28c) at the *hrp2* site. For quick DNA preparations, erythrocytes from 0.1–0.2 ml of parasite culture (0.2–1% parasitemia) were pelleted by centrifugation, treated with 0.15% saponin in phosphate-buffered saline, washed twice with phosphate-buffered saline, and lysed with a Triton/sucrose solution (17). Ten microliters of the recovered DNA lysate was placed in a PCR mixture of 30 μl and subjected to 45

Abbreviations: *dhfr-ts*, gene encoding dihydrofolate reductase-thymidylate synthase (DHFR-TS); *hrp2* and *hrp3*, genes encoding *P. falciparum* histidine-rich proteins HRP2 and HRP3; CAT, chloramphenicol acetyltransferase; OFAGE, orthogonal-field alternation gel electrophoresis.

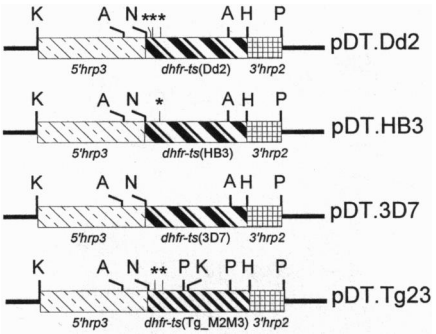

FIG. 1. Schematic depiction of plasmid constructs used for transformation. Plasmids pDT.Dd2, pDT.HB3, pDT.3D7, and pDT.Tg23 contain *dhfr-ts* coding regions from the *P. falciparum* Dd2, HB3, and 3D7 cloned lines and from the *T. gondii* M2M3 mutant gene, respectively. The *dhfr-ts* coding regions are flanked by *hrp3* and *hrp2* gene control elements in the pBluescript vector. Sites of point mutations conferring pyrimethamine resistance are marked with stars. Restriction sites: A, *Pac* I; H, *Hin*dIII; K, *Kpn* I; N, *Nsi* I; P, *Pst* I.

cycles of amplification (each cycle consisted of 50 sec at 94°C, 50 sec at 53°C, and 120 sec at 65°C).

RESULTS

Transformation of *P. falciparum* with *dhfr-ts* Sequences. Pyrimethamine-sensitive malaria parasites (*P. falciparum* line 3D7) were transfected and selected by the strategies indicated in Fig. 2. Transfection with the plasmids encoding pyrimethamine-resistant forms of *P. falciparum* or *T. gondii* DHFR-TS (pDT.Dd2, pDT.HB3, and pDT.Tg23) yielded drug-resistant parasites that were detectable by Giemsa-stained thin films after 15–18 days. Control experiments in which parasites were mock-transfected with no plasmid or were transfected with the pDT.3D7 plasmid encoding pyrimethamine-sensitive DHFR-TS did not produce drug-resistant transformants.

After 5 weeks of continuous pyrimethamine pressure (15 ng/ml), lines 28a, 28b, and 28c were recovered from parasites transformed by pDT.Dd2, pDT.HB3, and pDT.Tg23, respectively. Fingerprint typing with an interspersed repetitive sequence (18) confirmed that all transformants were derived from 3D7 parasites and not from other contaminating parasites selected by drug pressure. Drug response assays indicated that parasites in the 28a and 28b lines had levels of resistance expected of forms carrying the Dd2 or HB3 mutations (16,000 or 1000 ng/ml; Table 1) whereas the 28c line contained parasites with resistance levels of at least 16,000 ng/ml.

Replication of Transfected Plasmids as Episomes. Chromosomal DNA preparations from the 28a, 28b, and 28c lines were separated by orthogonal-field alternation gel electrophoresis (OFAGE) and probed for the presence of pBluescript sequences. A band of hybridization was identified that exhibited similar mobility over a wide range of pulse times and did not track with any *P. falciparum* chromosome (data not shown). This behavior was consistent with that expected of a circular episome (19). The organization of this DNA was further examined by restriction enzyme digestion and hybridization with probes specific for the *P. falciparum dhfr-ts, T. gondii dhfr-ts*, and pBluescript sequences. Results from all three transformed lines showed a 7.5-kb *Nsi* I fragment that corresponded to the transfected plasmid and was distinct from the 23-kb *Nsi* I fragment carrying the endogenous *dhfr-ts* gene (data not shown).

To confirm that the transfected plasmids had indeed been replicated by *P. falciparum*, we tested susceptibility of the episomes to *Dpn* I, an enzyme that cleaves GATC sequences only when the adenine is methylated. Since the necessary methylation activity (Dam methylase) is absent from eukaryotes but present in *E. coli*, resistance of plasmid DNAs replicated by *P. falciparum* was expected. The plasmid DNAs from the transformed *P. falciparum* lines exhibited this expected resistance to *Dpn* I while control plasmids from *E. coli* were efficiently cleaved (Fig. 3*a*). The structure of the replicated circular forms was also examined by restriction of plasmids recovered from *E. coli* that had been transfected with genomic DNA from each of the *P. falciparum* transformants. The recovered plasmids all showed restriction patterns that were identical to those of the original plasmids used for transfection (Fig. 3*b*).

Integration of Plasmids into *P. falciparum* Chromosomes. Episome-containing transformants were slow to propagate in culture and produced high numbers of pyknotic forms under pyrimethamine pressure. These observations suggested that the episomes were unstably maintained, perhaps because of random segregation and losses during cell division. This hypothesis was confirmed by experiments showing that drug-sensitive parasites could be recovered from the transformants after pyrimethamine pressure was removed (data not shown). Such findings are reminiscent of transformation studies of *Plasmodium berghei* rodent parasites, in which *dhfr-ts* plasmids were maintained as episomes for >6 weeks under pyrimethamine selection; these episomes were lost after removal of drug pressure, and their chromosomal incorporation was not detected (4).

The strategies outlined in Fig. 2 were therefore devised to obtain transformants in which plasmid constructs were stably incorporated into chromosomes. After the additional periods of cultivation and selection (7 weeks), the transformed lines had become devoid of pyknotic forms and showed propagation rates comparable to that of the 3D7 clone without drug pressure. Pyrimethamine concentrations of 16,000 ng/ml and 1000 ng/ml were required to kill the lines transformed by the

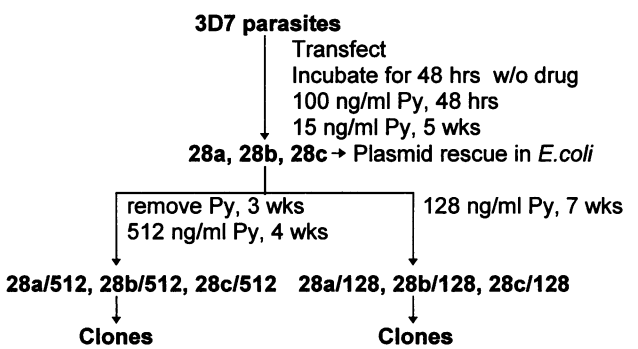

FIG. 2. Strategies for the selection of *P. falciparum* parasites transformed by plasmids that confer resistance to pyrimethamine. The pyrimethamine (Py) concentration during the first 5 weeks was chosen as a low level sufficient to select pyrimethamine-resistant lines.

Table 1. Pyrimethamine susceptibilities of *P. falciparum* control lines and transformants

Line	*dhfr-ts* mutations present	Sensitivity*
Control		
3D7	None: drug-sensitive	10
Dd2	S108N, N51I, C59R	16,000
HB3	S108N	1,000
Transformant		
28a/128	S108N, N51I, C59R (pDT.Dd2)	16,000
28b/128	S108N (pDT.HB3)	1,000
28c/128	*T.g.* T83N, S36R (pDT.Tg23)†	16,000

*Lowest pyrimethamine concentration (ng/ml) at which parasites were killed in a 5-day assay.

†Mutations in *T. gondii (T.g.) dhfr-ts* analogous to S108N and C59R in *P. falciparum* pyrimethamine-resistant forms.

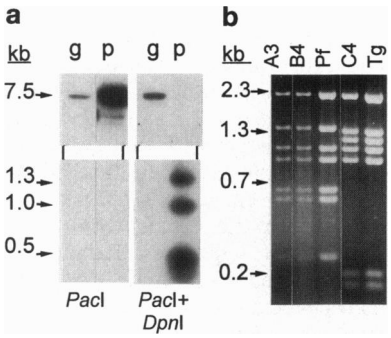

FIG. 3. Detection of transfected DNA replicated as episomes in *P. falciparum*. (*a*) *Dpn* I susceptibility analysis of plasmids replicated by *E. coli* and *P. falciparum*. DNA from the 28c parasite line (lanes g) and the pDT.Tg23 bacterial plasmid (lanes p) were digested with *Pac* I or *Pac* I/*Dpn* I and probed with the pBluescript sequence. Upper part of the panel shows the 7.5-kb *Pac* I bands; bottom part of the panel shows the fragments from *Dpn* I restriction (no hybridization signals were present in the middle part of the panel removed from the display). Similar results were obtained for episomes from the 28a and 28b lines transformed by pDT.Dd2 and pDT.HB3 (data not shown). *Pac* I was included in the digests to avoid plasmid trapping effects from genomic DNA in the agarose gel. (*b*) *Rsa* I digestions of episomal forms recovered from transformed *P. falciparum* lines. Plasmids were recovered by transfecting *E. coli* with 100 ng of DNA prepared from the parasite lines 28a, 28b, and 28c. Lanes: A3 and B4, plasmid DNAs recovered from the 28a and 28b lines; Pf, original pDT.Dd2 plasmid from *E. coli*; C4, plasmid DNA recovered from the 28c line; Tg, original pDT.Tg23 plasmid from *E. coli*. Analysis of eight additional recovered episomes and additional digestions with *Bam*HI and *Ssp* I also showed no evidence of rearrangement.

pDT.Dd2 and pDT.HB3 plasmids, respectively (the same concentrations as those required to kill native Dd2 and HB3 resistant parasites; Table 1). Pyrimethamine concentrations of at least 16,000 ng/ml were also required to kill parasites transformed by the pDT.Tg23 plasmid, which carries the *T. gondii* M2M3 *dhfr-ts* coding sequence.

To determine whether the transformed lines at the end of the selection process had stably integrated plasmid DNA, we separated their chromosomal DNAs by OFAGE and probed for transfected sequences using probes from the pBluescript vector and from the *P. falciparum* and *T. gondii dhfr-ts* coding sequences. Hybridization results identified integration events in three different chromosomes. Integration into chromosome 4 (which carries the *dhfr-ts* locus) was observed in the lines transformed by pDT.Dd2 (Fig. 4) and by pDT.HB3 (data not shown). By contrast, lines transformed with pDT.Tg23 showed no signal from chromosome 4 but instead showed a strong signal from chromosome 13, which carries the *hrp3* locus, and a relatively weaker signal from chromosome 8, which carries the *hrp2* locus (data not shown). Examination of cloned parasites from these transformed lines showed that the hybridization signals were the result of separate chromosomal integration events in different parasite subpopulations (Fig. 4). No parasite clones were identified in which plasmid DNA was integrated into more than one chromosome.

We also explored the efficiency of transformations with linear DNAs from all three plasmids (pDT.Dd2, pDT.HB3, and pDT.Tg23) digested by *Bam*HI, which cuts at a single site 16 bp downstream from the 3′ flanking region of *hrp2* (3). Pyrimethamine-resistant parasites from these transformations were detected in Giemsa-stained smears after 4 weeks of drug selection (about 2 weeks later than in transformation experiments with circular DNA). The transfected DNAs in these parasites were found to be episomes (probably recircularized), as determined by Southern blot analysis and plasmid rescue experiments (data not shown). These findings indicated that

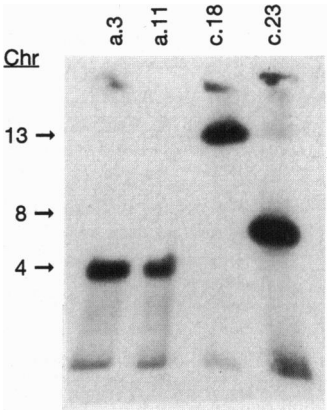

FIG. 4. Detection of integrated DNA in the chromosomes of transformed *P. falciparum* parasites. Chromosomal DNAs of transformed parasites were separated by OFAGE, transferred to nylon membrane, and probed with a pBluescript sequence as described (20). Hybridization signals identify chromosome 4 of the 28a/512.3 and 28a/512.11 parasite clones (lanes a.3 and a.11), chromosome 13 of the 28c/512.18 clone (lane c.18), and chromosome 8 of the 28c/512.23 clone (lane c.23).

linearization of the plasmids had not resulted in an enhanced rate of detectable integration events.

Confirmation of Homologous Integration Events by PCR. Incorporation of pDT.Dd2 and pDT.HB3 into chromosome 4, and of pDT.Tg23 into chromosomes 13 and 8, suggested that the plasmids had undergone homologous integration at the *dhfr-ts, hrp3*, and *hrp2* loci. These observations also suggested a recombination model involving a single-site crossover mechanism (Fig. 5*a*). To confirm homologous integration by this

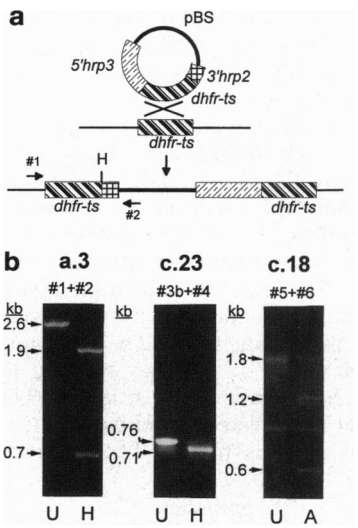

FIG. 5. Schematic diagram of single-site crossover mechanism and confirmation of homologous integration by PCR. (*a*) Crossover diagram for the *dhfr-ts* site. Positions of PCR primers #1+#2 and the diagnostic *Hin*dIII site are indicated on the chromosome line. Analogous models were constructed for the *hrp2* and *hrp3* loci (chromosomes 13 and 8) and tested with primers to the sites indicated in Fig. 6*b*. Note that for each model one primer is specific to the plasmid construct (#2, #3, #6b) while the second is specific for chromosome sequence flanking the integration site (#1, #4, #5). (*b*) Amplification products obtained from integration events in the transformed lines. Results are shown from clones having recombination events at the *dhfr-ts* site (28a/512.3, panel a.3), *hrp3* site (28c/512.23, panel c.23), and *hrp2* site (28c/512.18, panel c.18). Sizes of the amplified products and presence of the expected *Hin*dIII and *Pac* I sites confirm the single-site crossover models of homologous integration. U, unrestricted PCR product; H, *Hin*dIII-digested PCR product; A, *Pac* I-digested PCR product.

model, we designed the specific primer pairs #1+#2, #3+#4, and #5+#6a/b (Figs. 5*a* and 6*b*) and amplified fragments expected from single-crossover integration events in the different transformants. Fig. 5*b* shows that parasites in which the pDT.HB3 or pDT.Dd2 plasmid was integrated into the *dhfr-ts* locus yielded a 2.6-kb product that was cut by *Hin*dIII into the expected 1.9- and 0.7-kb fragments. Parasites in which pDT.Tg23 was integrated into the *hrp2* site yielded a 0.76-kb product that was cut by *Hin*dIII into the expected 0.71- and 0.05-kb fragments. And parasites in which pDT.Tg23 was integrated into the *hrp3* site yielded a 1.8-kb product that was cut by *Pac* I into the expected 1.2- and 0.6-kb fragments. Control PCRs with DNA from untransformed parasites, from early transformed lines containing episomes (lines 28a, 28b, and 28c; Fig. 2), or from bacterial plasmid alone did not yield any of these amplification products.

Since the PCR primers would not have detected residual episomes or nonhomologous (random) integration events, quick DNA preparations of 14 clones from the pDT.Dd2-transformed lines (28a/512 and 28a/128) and 57 clones from the pDT.Tg23-transformed lines (28c/512 and 28c/128) were also examined for amplification products. Eleven of the 14 pDT.Dd2-transformed clones and 53 of the 57 pDT.Tg23-transformed clones gave DNA preparations that yielded PCR products indicating homologous integration events. Repeat DNA preparations, PCR, and chromosome mapping analysis remain to determine whether the 7 samples that did not yield amplification products represented failed PCRs, residual episome-containing transformants, or clones with different recombination events. The conclusion from the results is nevertheless clear—homologous integration was the predominant mode of DNA incorporation in the stable transformants.

Genomic Organization of the Integrated DNA. Southern hybridization analysis of DNA from lines 28a/512 (Fig. 6*a*) and 28b/512 (data not shown) showed that the 23-kb *Nsi* I band carrying the endogenous *dhfr-ts* gene was replaced by two major bands (18 and 13 kb) upon integration of the plasmid DNA. Major bands obtained with *Bcl* I (18 kb) and *Eco*RV (15 kb) indicated the presence of predominant population of "pseudodiploid" forms containing the native and integrated *dhfr-ts* coding regions, separated by the pBluescript and the *hrp* gene control sequences. Relatively weak bands of hybridization in these digests (7.5-kb *Nsi* I, 25-kb *Bgl* I, and 22-kb *Eco*RV fragments) reflected subpopulations of parasites having additional copies of integrated DNA. Cloned parasites from these subpopulations have recently been isolated and have been found to have two copies of plasmid DNA in the *dhfr-ts* locus (data not shown).

Restriction analysis of cloned parasites from the 28c/512 line showed that the integration events involving pDT.Tg23 resulted in incorporation of two copies of plasmid DNA at the chromosome 13 *hrp3* site or at the chromosome 8 *hrp2* site (Fig. 6). Exclusive integration of pDT.Tg23 into these sites reflects the efficiency of homologous targeting and the inability of the divergent *T. gondii dhfr-ts* sequence to recombine with *P. falciparum dhfr-ts* on chromosome 4. In the case of the 3′-flanking region of *hrp2*, this targeting efficiency directed homologous integration to a chromosome segment of 0.57 kb.

DISCUSSION

Stably transformed parasites that had incorporated exogenous DNA into their nuclear chromosomes were obtained 2–3 months after transfection, a period that corresponds to 30–50 generations of erythrocyte-stage parasites. This period evidently provided the opportunity for some parasites to undergo integration events and replace unstable populations containing episomes. Though the chromosomal integration events have yet to be understood in detail, it is likely that persistent episome contact with the chromosomes, the activity of the *P.*

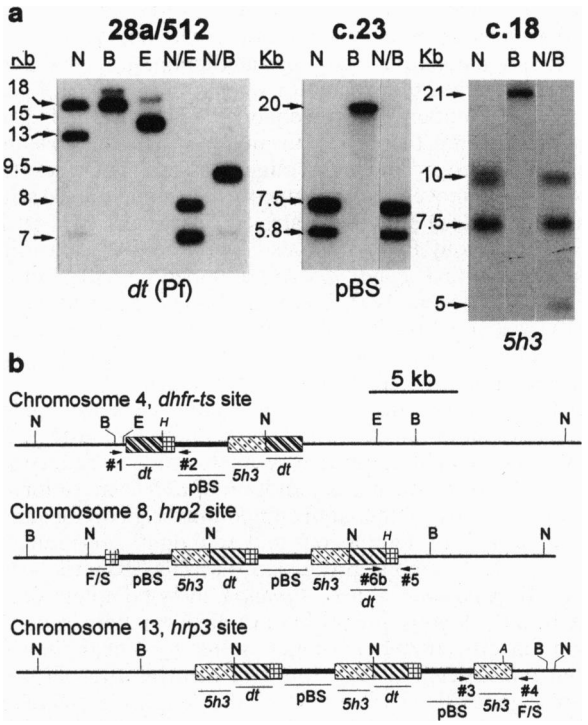

FIG. 6. Restriction analysis of chromosomally integrated plasmid sequences in the transformed *P. falciparum* lines. (*a*) Some representative hybridization signals from restricted DNAs of the transformed 28a/512 line and of clones 28c/512.23 (c.23) and 28c/512.18 (c.18) from the 28c/512 line. Probes used for hybridization: *dt*(Pf), *P. falciparum dhfr-ts* coding region; pBS, pBluescript sequence; *5h3*, 5′ *hrp3* flanking sequence. Restriction enzymes: B, *Bcl* I; E, *Eco*RV; N, *Nsi* I; N/E, *Nsi* I/*Eco*RV; N/B, *Nsi* I/*Bcl* I. The relatively weak 7.5-kb *Nsi* I, 25-kb *Bgl* I, and 22-kb *Eco*RV bands in the 28a/512 DNA restriction digests are from a subpopulation of parasites having two copies of integrated DNA (see text). (*b*) Restriction maps showing the organization of integrated DNA at the *dhfr-ts, hrp2*, and *hrp3* sites of parasites from line 28a/512, clone 28c/512.23 (c.23), and clone 28c/512.18 (c.18), respectively. Patterns within the boxes correspond to construct sequences as indicated in Fig. 1. Letters B, E, and N mark the *Bcl* I, *Eco*RV, and *Nsi* I sites used in developing the restriction maps; *H* and *A* mark *Hin*dIII and *Pac* I sites in the regions between the PCR primers used to confirm homologous integration. Sequences detected by hybridization probes: *dt, dhfr-ts* coding region; pBS, pBluescript; *5h3*, 5′ *hrp3* flanking region; F/S, 544-bp coding sequence from *hrp2* exon II that cross-hybridizes with *hrp3* (21).

falciparum DNA recombination and repair systems, the invasion efficiency of transformed merozoites, and the 2-day parasite generation cycle are important factors that affect the time required for the appearance of stably transformed parasites.

Integration of plasmid DNA into the chromosomes evidently involved single-site crossover events between homologous regions. Interestingly, transformations with plasmids containing the *P. falciparum dhfr-ts* coding region (pDT.HB3 and pDT.Dd2) strongly favored integration at the *dhfr-ts* site (chromosome 4), whereas transformation with a plasmid containing the *T. gondii dhfr-ts* sequence (pDT.Tg23) forced integration to the chromosome 13 *hrp3* site or to the chromosome 8 *hrp2* site. The frequent finding of two copies of plasmid DNA at these sites may reflect a compensation for *hrp* gene flanking regions in the control of *dhfr-ts* expression. No individual parasites identified in this work were found to have integration events in more than one chromosome.

It was important to confirm that the pyrimethamine-resistant transformants had not developed spontaneous *dhfr-ts* mutations under drug pressure, as studies have shown that the S108N point mutation can arise in *P. falciparum* parasites

cultivated in pyrimethamine (22). Several observations indicate that such spontaneous mutations did not arise in the transformed parasite lines: (*i*) pyrimethamine-sensitive parasites that were not transfected or were transfected with plasmids lacking mutant *dhfr-ts* sequences did not yield drug-resistant parasites; (*ii*) in transformed parasites the episomes were maintained by pyrimethamine pressure, but when this pressure was removed the episomes were lost and the parasites reverted to a drug-sensitive state; (*iii*) determination of the codon 108 type by PCR methods (23) showed that parasites transformed with *T. gondii dhfr-ts* contained no S108N mutation in the endogenous *P. falciparum dhfr-ts* gene (data not shown); and (*iv*) parasites transformed with the different *T. gondii* and *P. falciparum* genes exhibited pyrimethamine resistance levels that correlated with those expected of the different mutant forms. In addition to providing evidence against spontaneous mutations, these results formally demonstrate the role of *dhfr-ts* point mutations in pyrimethamine resistance and provide a basis for further exploration of *dhfr-ts* mutations in resistance to other antimalarial compounds (e.g., cycloguanil).

Relative rates of homologous and nonhomologous integration vary widely among protozoan parasites. Kinetoplastids such as *Trypanosoma* and *Leishmania* show complete dominance of homologous integration in transformations, with the integration efficiency being much better for linear than for circular constructs (24–29). Rates of incorporation in these parasites generally decrease when the lengths of matching DNA sequence are <2 kb, although sequences of a few hundred nucleotides do support homologous integration (28). By contrast, the integration of circular and linear DNAs in *Toxoplasma gondii* has been reported to occur at similar rates (6, 7) and relative frequencies of homologous and nonhomologous integration can be manipulated by the choice of plasmid construct. Homologous integration of a *T. gondii dhfr-ts* construct, for example, was favored over nonhomologous recombination by longer segments of genomic DNA, occurring in 50% of transformants with an 8-kb segment and >80% of transformants with a 16-kb segment (7). By contrast, a cDNA-derived *dhfr-ts* construct containing no introns produced random integration at rates high enough for gene identification in *T. gondii* by insertional mutagenesis (8). *P. falciparum* and *T. gondii*, both parasites of the Apicomplexan group, appear to be alike in that the integration efficiency of linear constructs has not been found to be enhanced over that of circular constructs. However, in *P. falciparum*, homologous integration has thus far shown itself to be strongly favored over nonhomologous integration. The detection in this work of homologous recombination within a region of <0.6 kb demonstrates the specificity with which targeted integration can be achieved in *P. falciparum*. This specificity will provide an effective basis for knocking out and modifying genes that are of interest in malaria research.

Note Added in Proof. In further development of their work, M. R. van Dijk, C. J. Janse, and A. P. Waters recently targeted subtelomeric repetitive sequences of the rodent malaria parasite *Plasmodium berghei* and obtained integration of *dhfr-ts* constructs into three different chromosomes (30).

We thank David Roos for the M2M3 coding sequence of *T. gondii dhfr-ts*.

1. Soldati, D. & Boothroyd, J. C. (1993) *Science* **260,** 349–352.
2. Goonewardene, R., Daily, J., Kaslow, D., Sullivan, T. J., Duffy, P., Carter, R., Mendis, K. & Wirth, D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5234–5236.
3. Wu, Y., Sifri, C. D., Lei, H., Su, X. & Wellems, T. E. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 973–977.
4. van Dijk, M. R., Waters, A. P. & Janse, C. J. (1995) *Science* **268,** 1358–1362.
5. Kim, K., Soldati, D. & Boothroyd, J. C. (1993) *Science* **262,** 911–914.
6. Sibley, L. D., Messina, M. & Niesman, I. R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5508–5512.
7. Donald, R. G. K. & Roos, D. S. (1994) *Mol. Biochem. Parasitol.* **63,** 243–253.
8. Donald, R. G. K. & Roos, D. S. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 5749–5753.
9. Peterson, D. S., Walliker, D. & Wellems, T. E. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9114–9118.
10. Cowman, A. F., Morry, M. J., Biggs, B., Cross, G. A. & Foote, S. J. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9109–9113.
11. Zolg, J. W., Plitt, J. R., Chen, G. & Palmer, S. (1989) *Mol. Biochem. Parasitol.* **36,** 253–262.
12. Peterson, D. S., Milhous, W. K. & Wellems, T. E. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3018–3022.
13. Foote, S. J., Galatis, D. & Cowman, A. F. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3014–3017.
14. Donald, R. G. K. & Roos, D. S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11703–11707.
15. Trager, W. & Jensen, J. B. (1976) *Science* **193,** 673–675.
16. Rosario, V. (1981) *Science* **212,** 1037–1038.
17. Zimmerman, P. A., Phadke, P. M., Lee, A., Elson, L. H., Edmundo, A. N., Guderian, R. & Nutman, T. B. (1995) *Hum. Immunol.* **42,** 233–240.
18. Dolan, S. A., Herrfeldt, J. A. & Wellems, T. E. (1993) *Mol. Biochem. Parasitol.* **61,** 137–142.
19. Hightower, R. C., Metge, D. W. & Santi, D. V. (1987) *Nucleic Acids Res.* **15,** 8387–8398.
20. Wellems, T. E., Walliker, D., Smith, C. L., do Rosario, V. E., Maloy, W. L., Howard, R. J., Carter, R. & McCutchan, T. F. (1987) *Cell* **49,** 633–642.
21. Wellems, T. E. & Howard, R. J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 6065–6069.
22. Thaithong, S., Chan, S. W., Songsomboon, S., Wilairat, P., Seesod, N., Sueblinwong, T., Goman, M., Ridley, R. & Beale, G. (1992) *Mol. Biochem. Parasitol.* **52,** 149–158.
23. Plowe, C. V., Djimde, A., Bouare, M., Doumbo, O. & Wellems, T. E. (1995) *Am. J. Trop. Med. Hyg.* **52,** 565–568.
24. Cruz, A. & Beverley, S. M. (1990) *Nature (London)* **348,** 171–173.
25. Anneloor, L. M. A., ten Asbroek, M. O. & Borst, P. (1990) *Nature (London)* **348,** 174–175.
26. Lee, M. G. & van der Ploeg, L. H. T. (1990) *Science* **250,** 1583–1587.
27. Eid, J. & Sollner-Webb, B. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2118–2121.
28. Tobin, J. F. & Wirth, D. F. (1992) *J. Biol. Chem.* **267,** 4752–4758.
29. ten Asbroek, A. L., Mol, C. A., Kieft, R. & Borst, P. (1993) *Mol. Biochem. Parasitol.* **59,** 133–142.
30. van Dijk, M. R., Janse, C. J. & Waters, A. P., *Science*, in press.