identification of moment-to-moment changes in the activity of the underlying "factor" defined by the PC. For example, we constructed the record in Fig. 1B by (i) dividing the 1813-s experiment into 181,300 10-ms time samples; (ii) measuring the spiking of each of the 48 simultaneously recorded neurons within each time sample; (iii) standardizing each data sample by subtracting the mean and dividing by the standard deviation; (iv) using these samples to construct a 48 by 48 covariance matrix; (v) performing PCA on this matrix; and (vi) using the PC1 eigenvector to weight-sum all the standardized data samples into a continuous population function, consisting of a time series containing a single data point for each 10-ms time bin in the experiment.

11. In contrast to the information derived from PC1, higher-order components (with successively lower eigenvalues) identified local sources of covariance, including phase differences between oscillations at different levels of the pathway and specific sensory information.
12. Chi-square analysis rejected the null hypothesis that there is no temporal relation between WT and the oscillations. This analysis involved comparing the experimentally derived distribution of WT onset times (quantized in 100-ms bins after the onset of oscillation) with the theoretical distribution of onset times that would be predicted by the null hypothesis (that is, a homogeneous distribution whose sum of counts equals the experimental sum). Because the experimental distribution was markedly skewed toward very short time delays after the onset of oscillation, a very high χ^2 was obtained (658; df = 32 bins), allowing rejection of the null hypothesis ($P < 10^{-6}$).
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Stable Transfection of Malaria Parasite Blood Stages

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Genetic manipulation of malaria parasites would revolutionize the study of this group of pathogens and have implications for vaccine and drug development. This report describes the stable, drug-selectable genetic transformation of the clinically relevant intracellular blood stages of a malaria parasite. A plasmid transfection vector carrying the gene locus that encodes a drug-resistant form of the bifunctional enzyme dihydrofolate reductase–thymidylate synthase from the rodent malaria parasite *Plasmodium berghei* was constructed. Derivatives of this vector were introduced into merozoites of *P. berghei* by electroporation, and parasites were selected for successful transformation in the rodent host on the basis of resistance to pyrimethamine. The plasmids were present in a circular, unrearranged form that replicated episomally to an observed maximum of 15 copies per cell in drug-resistant populations.

Malaria is caused by parasitic protozoa of the genus *Plasmodium* and is annually responsible for millions of deaths, predominantly as a result of infection with *Plasmodium falciparum*. Medical research has recently concentrated on attempts to generate synthetic vaccines with the application of recombinant DNA technology or polypeptide synthesis (*1*). It is anticipated that basic research on the cellular and molecular biology of *Plasmodium* will reveal characteristics that may be exploited in disease interdiction. Recently, both transient and stable transfection protocols for genetic transformation have been described for several unicellular parasites (*2*, *3*). Although transient transfection, which has been achieved in *Plasmodium* (*4*), is useful for the investigation of the control of gene expression, it does not permit elucidation of protein function nor can it be used at present for the study of the clinically relevant blood stages.

We now describe the plasmid-mediated genetic transformation of asexual blood stages of the rodent malaria parasite *Plasmodium berghei*, based on a homologous selectable marker, the dihydrofolate reductase–thymidylate synthase (DHFR-TS) bifunctional enzyme in a drug-resistant configuration. Plasmid transfection vectors containing the gene encoding this enzyme were introduced into the parasite by electroporation. Merozoites were chosen as the initial targets because they represent the only form in the blood that exists transiently outside the erythrocyte. The use of merozoites circumvents the potential problem of electroporation-induced damage to the host erythrocyte, on which other blood stages are completely dependent for survival. Another advantage of merozoites of *P. berghei* is that they are readily collectable in large numbers and appear to be more stable than merozoites of other malaria species. Conditions of electroporation (800 V, 25 μF) similar to those used for free-living forms of unicellular parasites, including *Plasmodium* (*4*), proved effective for the introduction of DNA into *P. berghei*.

Genomic DNA (6.1 kb) from *P. berghei* containing the coding region of the DHFR-TS gene as well as 3.5 kb of upstream DNA and 1.0 kb of downstream DNA was cloned into PUC18, thereby creating the parental plasmid pMD200 (*5*). In this construct, a portion of the protein-coding region of the DHFR-TS gene was isolated from a pyrimethamine-resistant clone of *P. berghei* (*6*) that contains a point mutation that results in an amino acid replacement ($Ser^{110} \rightarrow Asn$) associated with a high level of resistance to this folic acid antagonist in both human and rodent species of malaria (*7*). A second vector, pMD204, containing only 2.5 kb of upstream DNA but identical in all other aspects of the DHFR-TS locus was also constructed in pBSKS (*5*). We assumed that the additional DNA flanking the DHFR-TS gene would provide the necessary information for the correct temporal and quantitative expression of the gene.

Two additional plasmids based on the parental plasmid pMD200 were constructed (*5*). The first, pMD221, contained a 1.8-kb fragment of the Pbs21 gene, which encodes a surface antigen of *P. berghei* ookinetes (*8*). The second plasmid, pMD223, contained 2.2 kb of the nontranscribed 2.3-kb repetitive DNA sequence specific to *P. berghei* (*9*); this repeat is present in 200 to 300 copies in the genome and is exclusively located in subtelomeric regions. These plasmids were constructed to allow for possible site-directed integration by homologous recombination into the parasite genome. The 2.3-kb repeat provides a target with a high copy number, whereas the Pbs21 gene is single copy but not transcribed as part of the asexual blood stage cycle.

In two experiments (R1 and R2), plas-

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mids pMD221 and pMD223 were introduced by electroporation in circular and linear form into 10^9 merozoites of *P. berghei*, which were then transferred into rats by intravenous injection (*10*). In R1, both plasmids were introduced together, and in R2 only plasmid pMD221 was used. In each experiment, ~20% of the merozoites invaded erythrocytes, resulting in ~1% parasitemia. These parasites were allowed to complete one cycle of asexual multiplication before rats were administered a single intraperitoneal injection of pyrimethamine (10 mg per kilogram of body mass) on four consecutive days. This regimen effectively inhibits asexual multiplication of drug-sensitive parasites (*6*). As expected, parasitemia decreased rapidly during the treatment period, but, within 5 days after drug treatment, it recovered to 0.3%. These parasites constituted the parental T1 and T2 populations on which all subsequent experiments and manipulations were performed. Analysis of DNA synthesis by flow cytometry (*11*) revealed that the extent of drug resistance of the parental T1 and T2 populations was 10 and 1350 times, respectively, that of the original sensitive parasite clone (Table 1).

Table 1. Susceptibility of asexual multiplication of transformed lines of *P. berghei* to pyrimethamine in vitro. Susceptibility of parasite lines to different concentrations of pyrimethamine was determined in short-term in vitro cultures of the blood stages of *P. berghei* as described (*6*). Inhibition of DNA synthesis as a measure of asexual multiplication was determined by flow cytometry (T1 and T2 experiments) or on the basis of schizont maturation (T3 and T4 experiments) (*11*), resulting in dose-response curves (Fig. 1). From these data, the drug concentrations that resulted in 50% inhibition (IC_{50} values) were calculated by nonlinear regression analysis. In each experiment, the sensitive clone 15cy1 was used as a control. In experiments 1 and 4, the transformed parent populations were tested. In experiment 2, parasites of the three T1 clones obtained from infected mice after pyrimethamine challenge were tested. In experiment 3, two T1 clones were tested after 6 weeks of asexual multiplication in mice without pyrimethamine treatment.

Line	IC_{50} (μg/ml)	Relative resistance (fold)
	Experiment 1	
15cy1	8.8×10^{-4}	1
T1	8.0×10^{-3}	10
T2	1.1	1350
	Experiment 2	
15cy1	7.1×10^{-4}	1
T1.1	3.1×10^{-2}	43
T1.2	4.2×10^{-2}	59
T1.3	5.8×10^{-2}	81
	Experiment 3	
15cy1	1.6×10^{-4}	1
$T1.2^-$	1.5×10^{-4}	0.9
$T1.3^-$	1.8×10^{-4}	1.1
	Experiment 4	
15cy1	2.5×10^{-4}	1
T3	2.3×10^{-2}	90
T4	1.5×10^{-2}	60

Parasites from the T1 and T2 populations were cloned by limiting dilution and then grown in mice that were challenged with pyrimethamine [a single intraperitoneal (10 mg per kilogram of body mass) injection on three consecutive days]. We obtained three clones from the T1 population and four from the T2 population. The pyrimethamine resistance in T1 population clones was shown to be 43 to 81 times that of the original sensitive clone (Table 1). In contrast to the distinct threshold of susceptibility to pyrimethamine shown by the sensitive clone and the T2 population, the dose-response curves of both the cloned and parental T1 parasites showed a gradual transition to drug susceptibility (Fig. 1A). This result suggested that the T1 populations were nonhomogeneous, consisting of parasites with different susceptibilities to pyrimethamine.

The observed pyrimethamine resistance of the parasite populations and clones could have been attributable either to selection of parasites with spontaneous mutations associated with the normal genomic DHFR-TS gene (*7*) or to selection of successfully transformed parasites. To discriminate between these possibilities, we first tested parental resistant populations and progeny clones for the presence of plasmid DNA by the polymerase chain reaction (PCR) (*5*) (Fig. 1B). The T1 population was transfected with both pMD221 and pMD223, and both were detected in the parental T1 DNA. Of the three clones derived from T1, two (T1.1 and T1.3) were positive for pMD221 and one (T1.2) for pMD223. No clone has yet been analyzed that possesses both plasmids. The parental T2 population was transfected with only pMD221 and was positive for this plasmid and negative for pMD223 by PCR. Despite normal multiplication under drug selection, all isolated clones from the T2 population were negative for pMD221 and are thought to have been cured of the plasmid. The T1 clones were grown for a further 6 weeks (representing ~40 generations, with an asexual cycle of 22 to 24 hours) under drug selection and remained positive by PCR for plasmid DNA at the end of this period (*12*). The results of the R1 and R2 experiments show that, on both occasions, plasmid DNA was successfully introduced into the parasites.

A second series of experiments (R3 to

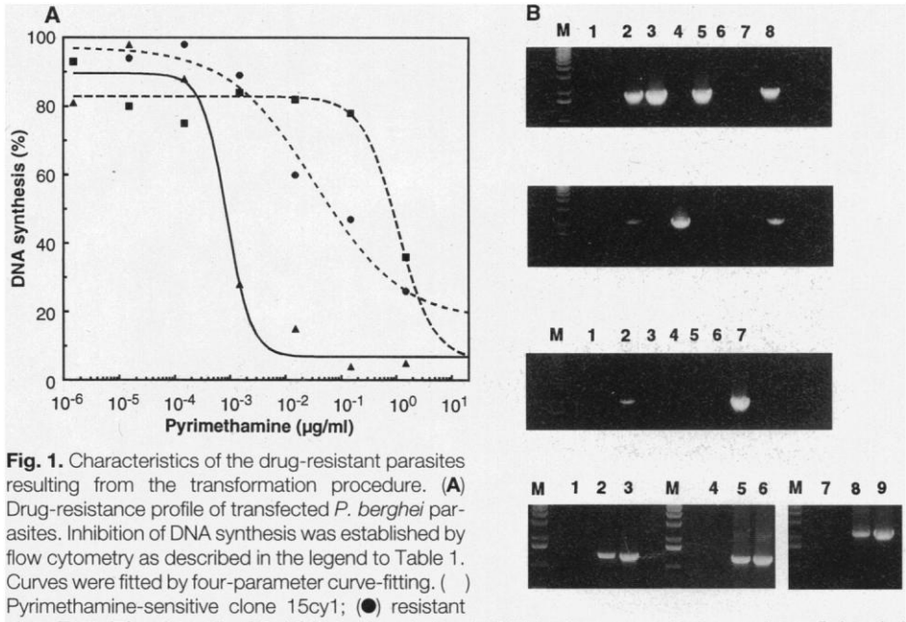

Fig. 1. Characteristics of the drug-resistant parasites resulting from the transformation procedure. (**A**) Drug-resistance profile of transfected *P. berghei* parasites. Inhibition of DNA synthesis was established by flow cytometry as described in the legend to Table 1. Curves were fitted by four-parameter curve-fitting. ( ) Pyrimethamine-sensitive clone 15cy1; (●) resistant clone T1.3; (■) resistant line T2. (**B**) Detection of plasmid DNA in electroporated drug-resistant *P. berghei* parasites. Plasmid and parasite DNA juxtaposed in the transfection vectors was amplified from total parasite DNA isolated from parasite lines and clones (*5*). (Top panel) Detection of pMD221 in T1 parasites by means of the PUC plasmid forward sequencing primer and L80 as primers for PCR. (Second panel) Detection of pMD223 in T1 parasites with the PUC primer and L43 as primers. Lanes for first two panels: 1, clone 15cy1; 2, T1 uncloned; 3, T1.1; 4, T1.2; 5, T1.3; 6, $T1.2^-$; 7, $T1.3^-$; and 8, pMD221 (top panel) or pMD223 (second panel) positive controls. (Third panel) Detection of pMD221 in T2 parasites by PCR. Lanes: 1, clone 15cy1; 2, T2 uncloned; 3, T2.1; 4, T2.2; 5, T2.3; 6, T2.4; and 7, pMD221 positive control. (Bottom panel) Detection by PCR of pMD200 with the primers PUC and 530 in T3 parasites (lanes 1 to 3), of pMD204 with the SK and 530 primers in T4 parasites (lanes 4 to 6), and of pMD223 in T5 parasites (lanes 7 to 9). Lanes: 1, 4, and 7, clone 15cy1; 2, T3 uncloned; 3, pMD200 positive control; 5, T4 uncloned; 6, pMD204 positive control; 8, T5 uncloned; 9, pMD223 positive control. In each panel, M lanes contain molecular size markers.

R6) was performed to test the reproducibility of introducing plasmid DNA into the parasites. Plasmids pMD200 (R3), pMD204 (R4), pMD223 (R5), and pMD221 (R6) were introduced into merozoites, which were then selected for pyrimethamine resistance according to the same protocol as for R1 and R2. In three (R3 to R5) of the four experiments, parental populations (T3 to T5) were obtained that were resistant to pyrimethamine in vivo and which contained plasmid DNA as detected by either Southern (DNA) blot analysis of total genomic DNA or PCR (Figs. 1B and 2B). Experiment R6 exhibited aberrant electroporation parameters and no resistant parasites were obtained. The in vitro pyrimethamine susceptibilities of the T3 and T4 parent populations were comparable to those of the cloned T1 parasite lines, with median inhibitory concentration (IC_{50}) values 90 and 60 times, respectively, that of the sensitive 15cy1 clone (Table 1) (*10*).

Restriction analysis of genomic DNA from the T1 and T2 parasites indicated that neither of the electroporated transfection vectors had integrated with a detectable frequency into the genome at their respective target sites of homologous recombination (Fig. 2A). Therefore, the vectors were either integrated into the DHFR-TS locus by means of a single crossover event or maintained as a plasmid in circular form within the cell in an apparently unrearranged state. The plasmid copy number in the parasites varied from <1 per cell, in transformed populations that had not been grown continuously under pyrimethamine selection, to 7 to 15 per cell, in clones T1.1 to T1.3 grown under drug selection (Fig. 2A). These data suggested that the vector was not integrated and presumably replicated episomally, and that copy number was related to drug exposure. Pulsed-field gel electrophoretic analysis of the chromosomes of T1 and T2 parental populations and clones T1.1 to T1.3 revealed the presence of DNA molecules that migrated with a mobility identical to that of pure plasmid DNA isolated from *Escherichia coli* and distinct from that of any of the *P. berghei* chromosomes (*12*). The circular, episomal nature of the transforming DNA element was confirmed by plasmid rescue, transformation of *E. coli* with total parasite DNA from the parental transformed populations (T1 to T5) and from clones T1.1 to T1.3. Preparations of plasmid DNA from the transformed *E. coli* revealed that only the appropriate plasmid could be rescued from each parasite DNA preparation and that the plasmid DNA was totally unrearranged and replicating in single monomer form (Fig. 2C).

Further analyses were performed on the clones of the T1 and T2 transfected parasites. To determine whether drug resistance was exclusively due to the introduced DHFR-TS gene, we sequenced the rescued plasmids and confirmed the presence of the point mutation that results in the amino acid substitution ($Ser^{110} \rightarrow Asn$) in the DHFR-TS enzyme. No additional alterations in the DHFR-TS gene sequence were observed (*12*). Plasmids are often only maintained in host cells under conditions in which the plasmid provides a selective advantage—in this instance, drug resistance. Therefore, we expected that, if the transformed parasites were grown for prolonged periods in the absence of drug selection, the plasmid would be lost and the parasite would revert to a drug-sensitive phenotype. Indeed, clones T1.2 and T1.3, which contain pMD223 and pMD221, re-

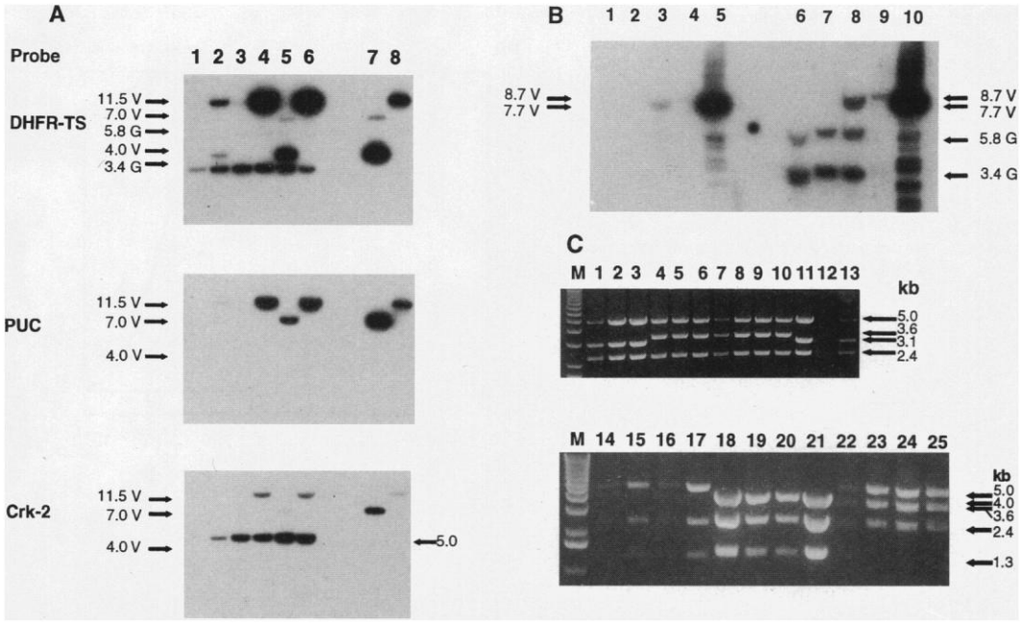

Fig. 2. Status of transfection vector DNA in transformed *P. berghei*. (**A**) Restriction analysis of the genomic DNA of transformed *P. berghei*. Genomic DNA was isolated from parental T1 and T2 lines and from the clones T1.1 to T1.3 and digested with Cla I, which cuts once in pMD221 within the DHFR-TS region and twice in pMD223, with the additional site in the 2.3-kb region (nt 1447 with respect to the Hind III site). DNA was fractionated on agarose gels and blotted onto nylon membranes (Hybond N^+, Amersham); identical blots were probed with either the DHFR-TS gene (top panel) or PUC18 (middle panel). One blot was reprobed with a single-copy *P. berghei* gene that encodes Cdc-2–related kinase–2 (Crk-2) (bottom panel) (*16*) to provide an internal standard for the measurement of plasmid copy number; additional plasmid bands on this blot result from the previous PUC hybridization. Quantitation was performed with a PhosphorImager (Molecular Dynamics) and the associated Image Quant software. Lanes: 1, clone 15cy1; 2, T1; 3, T2; 4, T1.1; 5, T1.2; 6, T1.3; 7, pMD223; 8, pMD221. (**B**) Analysis of the genomic DNA from uncloned populations from experiments T3 and T4. Total parasite DNA was digested with Cla I, which cuts once in both pMD200 (8.7-kb band) and pMD204 (7.7-kb band), and duplicate blots were performed as in (A). Lanes 1 to 5 were probed with PUC19, and lanes 6 to 10 with the entire DHFR-TS locus so that the 5.8-kb genomic DNA band is clearly visible. The plasmid 8.7-kb band in T3 parasites is only visible with prolonged exposure. PhosphorImager quantitation of the blot indicated that the relative abundance of plasmid in T3 and T4 was 1:20. Lanes: 1 and 6, clone 15cy1; 2 and 7, T3 uncloned parasites; 3 and 8, T4 uncloned parasites; 4 and 9, pMD200; 5 and 10, pMD204. (**C**) Plasmid rescue of the transfection vectors. Total parasite DNA was isolated from the T1 to T5 uncloned lines and used to transform *Escherichia coli*. Plasmid DNA was isolated from 30 randomly picked colonies, digested with Pvu II, and compared with that from the original pMD221, pMD223, pMD200, and pMD204 vectors. Both pMD221 and pMD223 could be rescued from the T1 uncloned line but only the appropriate plasmid was rescued from the various cloned transformed T1 parasites. Only pMD221 could be rescued from the uncloned T2 line and plasmid rescue was not possible from the T2 cloned parasite DNA. Only appropriate plasmids could be rescued from T3, T4, and T5 parasite populations. Lanes: 1 to 3, plasmid DNA rescued from total DNA isolated from T2 uncloned parasites; 4 to 11, plasmid DNA rescued from total DNA from T1 uncloned parasites; 12 and 25, pMD223; 13, pMD221; 14 to 16, plasmid DNA rescued from T3 uncloned parasites; 17, pMD200; 18 to 20, plasmid DNA rescued from T4 uncloned parasites; 21, pMD204; 22 to 24, plasmid DNA rescued from T5 uncloned parasites. Molecular sizes (arrows) are shown in kilobases. V, vector; G, genomic.

spectively, were cured of their plasmid when grown in mice for six mechanical passages (a period of 6 weeks) in the absence of pyrimethamine. The resulting clones were designated T1.2$^-$ and T1.3$^-$, respectively, and their in vitro sensitivities to the drug were comparable to the original sensitive parasite clone (Table 1).

The presence of the point mutation in the plasmids within the transformed parasites was also confirmed by PCR amplification of the DHFR-TS locus from total genomic DNA. The PCR products were hybridized to oligonucleotides (L40 and L41) designed to hybridize across the region containing the point mutation and detect either the resistant (L40) or sensitive (L41) forms of the DHFR-TS gene (5) (Fig. 3). We tested the T1 parental population and progeny resistant clones (T1.1 to T1.3) as well as the clones (T1.2$^-$ and T1.3$^-$) that were cured of the plasmid. All parasites hybridized in a consistent fashion to the oligonucleotides L40 or L41. We conclude that the T1 line and resistant clones contain a chromosomal copy of the DHFR-TS gene in a sensitive configuration and a second copy on a plasmid in a resistant configuration. The presence of a plasmid in these parasite clones correlated with drug resistance.

The T2 clones that were shown to lack pMD221 yet remain drug resistant were also hybridized to the oligonucleotides L40 and L41 (Fig. 3). Three of the clones hybridized exclusively to the oligonucleotide defining a pyrimethamine-resistant form of the DHFR-TS gene. One, T2.3, hybridized to both oligonucleotides and requires further analysis. Naturally resistant mutants of *P. berghei* arise infrequently (6). The resistant T2 clones that did not contain plasmid DNA might be natural mutants selected from the parental population. However, we cannot exclude the possibility that the parental copy of the DHFR-TS gene was replaced by a double crossover event with the plasmid. The frequency of gene replacement may be high in this system given the potentially large copy number of the plasmid per parasite.

Our results indicate that the transfection vector replicates episomally as a monomer and therefore must possess an origin of DNA replication that is effective in *Plasmodium*. For other unicellular parasites, the presence of functional replication origins has been shown in transfecting plasmids, but they need not derive from the parasite and the same may hold true in our experimental system (*13*). Our calculations, based on the rate of DNA replication in *P. berghei*, suggest that an origin of DNA replication should occur every 18 kb in *Plasmodium* DNA (*14*). Given that our original vectors (pMD221 and pMD223) contained >7.5 kb of parasite DNA, it is possible that they also included a *Plasmodium* origin of replication. Successful transfection with vector pMD204, which lacks 1 kb of the parasite DNA at the 5′ end of pMD200, indicated that, if the origin is derived from *Plasmodium* DNA, it lies within the 5.1 kb of the DHFR-TS locus. Our data do not support the maintenance of the plasmid in the form of large oligomeric circles consisting of multiple copies of the monomeric unit, as was observed in *Leishmania*, *Trypanosoma*, and *Leptomonas* (*15*). Both the plasmid copy number and drug resistance in cloned T1 parasites were variable, and plasmids were lost during growth in the absence of drug. These observations suggest that the copy number can fluctuate rapidly in response to the presence of pyrimethamine.

We have not yet observed proven integration of the transfection vectors into the *P. berghei* genome, despite the inclusion of potential target sequences and introduction of the vectors in both linear and supercoiled forms. Possible explanations for this failure to detect integration include the following: (i) Integration will not occur with these vectors. (ii) Episomal maintenance of the vectors is so much more efficient than any form of integration that integration is effectively not seen. (iii) Integration has occurred in the T2 clones at the DHFR-TS locus and has resulted in gene replacement. However, with the current transfection vector configuration, we are unable to identify such events. (iv) Integration has occurred but results in a transcriptionally silenced DHFR-TS gene that cannot confer a selective advantage. It is possible that successful gene disruption by homologous recombination may only be successful in our system when the size of the DHFR-TS locus is greatly reduced to the minimal functional unit.

Note added in proof: Since submitting this report, we have learned that transient transfection of blood stage forms of *P. falciparum* has been achieved (*17*).

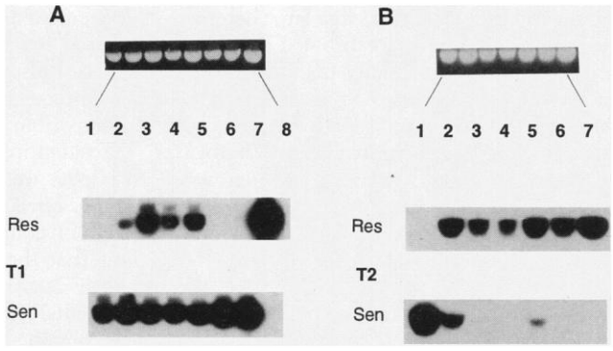

Fig. 3. Detection of the DHFR-TS gene in the drug-sensitive and drug-resistant configurations in the transformed parasites. The coding region of the DHFR-TS gene was amplified by PCR from total DNA of various transformed parasites, fractionated on an agarose gel, and blotted onto nylon (Hybond N$^+$), and separate panels were hybridized to oligonucleotides L40 (resistant) and L41 (sensitive) (*5*). (**A**) The amplified DHFR-TS gene from T1 parasites. Lanes: 1, clone 15cy1; 2, uncloned T1; 3, clone T1.1; 4, T1.2; 5, T1.3; 6, T1.2$^-$; 7, T1.3$^-$; 8, pMD221. (**B**) The amplified DHFR-TS gene from T2 parasites. Lanes: 1, clone 15cy1; 2, uncloned T2; 3, clone T2.1; 4, T2.2; 5, T2.3; 6, T2.4; 7, pMD221. Bands corresponding to resistant (Res) and sensitive (Sen) configurations are indicated.

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5. Construction of the transfection vectors was as follows. For pMD200, a 5-kb Hind III fragment of genomic DNA from the pyrimethamine-sensitive *P. berghei* clone 8417 was isolated from a minilibrary of size-fractionated DNA and cloned into PUC18. This fragment contained 3.5 kb of upstream DNA and most of the open reading frame (ORF) of the DHFR-TS gene, ending at the Hind III site at nucleotide (nt) 1611 of the ORF. The resulting plasmid, pMD20, was digested with Cla I and Xba I to remove a small fragment from nt 149 to nt 388, which spanned the codon for residue Ser^{110}. The gel-purified large fragment was ligated to the equivalent Cla I–Xba I fragment from a PCR clone for the entire DHFR-TS gene that had been amplified from the pyrimethamine-resistant *P. berghei* clone R16$_3$. This sequenced fragment contained a codon for Asn^{110}, which is known to associate with pyrimethamine resistance (*6, 7*). The resulting clone, pMD20R, was verified by sequencing. The remaining portion of the gene was isolated as a 1.3-kb Sau 3A fragment from the drug-sensitive *P. berghei* clone and extended from the Sau 3A site at nt 1332 of the ORF through the stop codon at nt 1764 and 1.0 kb into the 3′ untranslated region. The fragment was digested with Bgl II and Sau 3A and inserted into similarly digested pMD20R. Clones with the fragment in the correct orientation were verified by sequencing and designated pMD200. Plasmid pMD221 was created by PCR amplification of 1.8 kb of the Pbs21 gene (*8*) locus, which introduced unique Eco RI sites at either end, and cloning into the unique Eco RI site in pMD200. Plasmid pMD223 was created by a similar strategy, cloning 2.2 kb of the 2.3-kb repetitive DNA element of the *P. berghei* genome (*9*) into the Eco RI site of pMD200. Plasmid pMD204 was created by subcloning the 5.1-kb Hinc II–Eco RI fragment of pMD200 into pBSKS (Stratagene) digested with Hinc II and Eco RI. Oligonucleotides used for PCR amplification of the Pbs21 gene were L79R (5′-CCA GAA TTC TAT ATG TAA TAT ACA CAG CTA GC-3′) at nt −363 with respect to the initiation codon, and L80R (5′-CCG GAA TTC ATT TCT TAA CAG CAA CCG GC-3′) at nt 1428 to 1447 with respect to the initiation codon. Oligonucleotides for amplification of the 2.3-

kb repeat were L43D (5′-CCG GAA TTC AGA ATT ATA TAT GAG GCT GCG-3′) at nt 66 to 86 with respect to the Hind III site, and L44D (5′-CCG GAA TTC CAA ATA AAG AAA CAT ATT GCG-3′) at nt 2228 to 2248. PCR reactions were performed on total parasite DNA. For detection of the transfection vectors, the PUC forward sequencing primer (5′-TTT TCC CAG TCA CGA CGT-3′) was used in conjunction with either L80R for pMD221, L43D for pMD223, or 530 (5′-GGG GTT CCA TTC AAT ATT GC-3′, which ends at nt 1505 of the DHFR-TS ORF) for pMD200. Oligonucleotide 530 was used in conjunction with the SK primer (5′-TCT AGA ACT AGT GGA TC-3′) designed for pBSKS (Stratagene) for PCR detection of pMD204. The entire DHFR-TS coding region was amplified with the oligonucleotides 644D (5′-GCAGCGGATCC ATG GAA GAC TTA TCT GAA AC-3′, with nt 1 in bold) and 645D (5′-GGGCCGGATCC TTA AGC TGC CAT ATC CAT ATT-3′, which ends at nt 1764). The reaction conditions for all amplifications, except that of the DHFR-TS–pBSKS hybrid (experiment R4), were 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. For amplification of DHFR-TS–pBSKS, conditions were identical except that primers were annealed to the template at 45°C. *Taq* DNA polymerase was obtained from Life Technologies. Oligonucleotides L40 (5′-GAA TAC TTT CCC AA**T** TTT TTT TTC-3′, resistant) and L41 (5′-GAA TAC TTT CCC AA**C** TTT TTT TTC-3′, sensitive) were used in hybridization experiments to determine the presence of the resistant and sensitive configurations of the DHFR-TS gene in total parasite DNA. The residues in bold encode the point mutations observed at position 110 in the DHFR-TS protein which determine parasite resistance (L40) or sensitivity (L41) to the drug pyrimethamine. Oligonucleotides were hybridized in 5× standard saline citrate containing 1% SDS at 42°C, and washing was performed at 56°C with 1× standard saline citrate containing 1% SDS for 5 min.

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10. Synchronized erythrocytic merozoites were obtained from mature schizonts, which were isolated from 5 ml of blood obtained by cardiac puncture from a Wistar rat (body mass, 250 g) 5 days after intraperitoneal injection of 10^8 *P. berghei* blood stage parasites (ANKA strain, clone 15cy1, pyrimethamine sensitive). This blood, containing mainly ring- and trophozoite-infected erythrocytes at 3% parasitemia, was incubated under standard culture conditions (RPMI 1640 medium, 20% fetal bovine serum) in a gently shaking Erlenmeyer flask for 20 hours at 37°C. During this time, parasites mature into schizonts that do not rupture and remain viable for prolonged periods. The mature schizonts were separated from uninfected cells by density-gradient centrifugation; the culture was layered on top of a 58% Nycodenz (Nycomed, Oslo, Norway) and phosphate-buffered saline gradient and centrifuged for 30 min at 200*g*. The schizonts accumulate at the interface and were collected and washed once in culture medium. The resulting preparation consisted of 90% mature schizonts and 10% uninfected cells, with a total of 5×10^8 schizonts. These cells were concentrated into 100 μl of RPMI 1640 culture medium and used for electroporation. In experiment R1, 75 μg of plasmid pMD221 and 15 μg of pMD223 (each in the proportion 1:1 of circular to linear forms) were dissolved in 75 μl of TNE buffer (pH 7.2), and the volume was adjusted to 400 μl with phosphate-buffered saline. The DNA and the parasites were mixed, placed in a 0.4-cm electroporation cuvette (Bio-Rad), and subjected to a single pulse (800 V, 25 μF; time constant, 0.8 ms) at room temperature. Earlier experiments showed a 10 to 40% survival rate of merozoites under these conditions. The mixture was then placed on ice for 10 min before intravenous injection into a Wistar rat (250 g). Thirty hours after injection, the rats were treated with a single dose of pyrimethamine (10 mg/kg, intraperitoneally) on each of four consecutive days. In experiment R2, 75 μg of plasmid pMD221 (1:1, supercoiled:linear) was mixed with parasites and the same procedure was followed as for R1. In experiments R3 to R6, 100 μg of pMD200 (R3), 100 μg of pMD204 (R4), 10 μg of pMD223 (R5), or 40 μg of pMD221 (R6) was introduced into merozoites as for R1. Parasites surviving the pyrimethamine treatment were mechanically passaged to naïve mice and rechallenged with pyrimethamine for 3 days as described for rats. Resistant parasites were passaged again to naïve mice for the collection of parasites for nucleic acid isolation and for in vitro drug susceptibility tests. Clones of resistant parasites from experiments R1 and R2 were obtained by limiting dilution and all proved resistant to pyrimethamine when challenged in vivo or in vitro. These clones were grown in naïve mice as before for nucleic acid isolation. Between electroporation of the T1 and T2 parasites and the testing of the resultant drug-resistant T1 clones, a period of 49 days of parasite multiplication had elapsed (including five mechanical passages). The resistance level was established by determination of schizont development in short-term cultures of *P. berghei* (*11*). B. Mons, C. J. Janse, E. G. Boorsma, H. J. van der Kaay, *Parasitology* **91**, 423 (1985); C. J. Janse, B. Mons, J. J. A. B. Croon, H. J. van der Kaay, *Int. J. Parasitol.* **14**, 317 (1984); C. J. Janse, E. G. Boorsma, J. Ramesar, M. J. Grobbee, B. Mons, *ibid.* **19**, 509 (1989).
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Role of the Protein Chaperone YDJ1 in Establishing Hsp90-Mediated Signal Transduction Pathways

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The substrate-specific protein chaperone Hsp90 (heat shock protein 90) from *Saccharomyces cerevisiae* functions in diverse signal transduction pathways. A mutation in *YDJ1*, a member of the DnaJ chaperone family, was recovered in a synthetic-lethal screen with Hsp90 mutants. In an otherwise wild-type background, the *ydj1* mutation exerted strong and specific effects on three Hsp90 substrates, derepressing two (the estrogen and glucocorticoid receptors) and reducing the function of the third (the tyrosine kinase $p60^{v\text{-}src}$). Analysis of one of these substrates, the glucocorticoid receptor, indicated that Ydj1 exerts its effects through physical interaction with Hsp90 substrates.

In vitro, purified Hsp90 can function as a general protein chaperone (*1*). In vivo, however, Hsp90 functions in a large heteroligomeric complex and exhibits a high degree of substrate specificity (*2*). The chaperone activities of Hsp90 participate in a wide range of signal transduction pathways, including pathways controlled by steroid receptors, tyrosine kinases, and serine-threonine kinases (*2*–*7*). These structural and functional properties are highly conserved: Mammalian and yeast Hsp90s are found in similar macromolecular complexes (*2*, *8*), mammalian Hsp90 complements the essential functions of yeast Hsp90 (*3*, *9*), and yeast Hsp90 promotes the activation of steroid-receptor and tyrosine kinase target proteins from vertebrate cells (*3*, *6*, *10*).

To identify proteins functionally related to Hsp90, we searched (Fig. 1) for *Saccharomyces cerevisiae* mutations that would be lethal in combination with Hsp90 mutations that themselves have little effect on growth at 30°C (synthetic-lethal, or *SL*, mutations) (*11*, *12*). To identify the *SL* gene obtained in this search, we screened yeast libraries for plasmids that would complement the *SL* phenotype. All plasmids recovered encoded either Hsp90 or one of two members of the DnaJ chaperone family,

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