

A set of independent selectable markers for transfection of the human malaria parasite *Plasmodium falciparum*

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ABSTRACT Genomic information is rapidly accumulating for the human malaria pathogen, *Plasmodium falciparum*. Our ability to perform genetic manipulations to understand *Plasmodium* gene function is limited. Dihydrofolate reductase is the only selectable marker presently available for transfection of *P. falciparum*. Additional markers are needed for complementation and for expression of mutated forms of essential genes. We tested parasite sensitivity to different drugs for which selectable markers are available. Two of these drugs that were very effective as antiparasitic inhibitors in culture, blasticidin and geneticin (G418), were selected for further study. The genes *BSD*, encoding blasticidin S deaminase of *Aspergillus terreus*, and *NEO*, encoding neomycin phosphotransferase II from transposon Tn 5, were expressed under the histidine-rich protein III (*HRP*III) gene promoter and tested for their ability to confer resistance to blasticidin or G418, respectively. After transfection, blasticidin and G418-resistant parasites tested positive for plasmid replication and *BSD* or *NEO* expression. Cross-resistance assays indicate that these markers are independent. The plasmid copy number and the enzymatic activity depended directly on the concentration of the drug used for selection. These markers set the stage for new methods of functional analysis of the *P. falciparum* genome.

Malaria is responsible yearly for about 2 million deaths—mostly children under the age of 5 (1). Appearance of widespread resistance to currently used drugs such as chloroquine, fansidar, and mefloquine has stimulated efforts to identify new antimalarial drug and vaccine targets. The *Plasmodium falciparum* genome is ≈30 megabases in size, has a base composition of 82% A+T, and contains 14 chromosomes. Genome sequencing is underway, and genomic as well as expressed-sequence-tag databases have been generated (2). Recently the 0.95-megabase chromosome II of this protozoan has been fully sequenced and is predicted to encode 209 proteins (3). Database analyses indicated that 43% of these proteins have no detectable homologs. On this chromosome, 18 ORFs called rifins have been found, and no function has been assigned to them yet. The paucity of genetic tools to study the parasite's most important pathways, such as mechanisms of drug resistance, parasite invasion, differentiation, and cell cycle, leaves us with a growing database of genes but difficulty in determining their actual functions.

Genetic manipulation of the malaria parasite has taken major strides forward. Early transfection success with *Plasmodium* species causing rodent malaria (4, 5) was followed by a breakthrough in transient transfection of *P. falciparum* (6). Subsequently, electroporation-based transfection of ring-stage *P. falciparum* has been used for stable expression and disruption

of malaria genes (7–11). When these early intraerythrocytic parasites are electroporated in the presence of plasmid, DNA seems to be able to cross the erythrocytic membrane, the double membrane surrounding the parasite, and the nuclear membrane to gain entry into the nucleus. There, episomal plasmid can be maintained indefinitely under selective pressure. At a low frequency, homologous integration occurs, and events can be selected following drug withdrawal and subsequent reintroduction (7, 8). Integration has been shown to occur by single-crossover homologous-recombination events that can be exploited for allelic exchange or gene disruption (9, 11). Transfection of the malaria parasite has relied on a single marker, the human or *Toxoplasma gondii* dihydrofolate reductase genes that confer resistance to methotrexate or pyrimethamine (6, 12). This paucity of selectable markers limits our ability to carry out disruption of essential genes, complementation of knockouts, or execution of plasmid-shift experiments to select specific mutants in important biological pathways.

In an attempt to prepare tools that can facilitate systematic analysis of the malaria parasite genome, we sought additional genetic markers for *P. falciparum* transfection. We tested different drugs for their ability to inhibit the growth of the parasite in culture. Based on these results, two drugs were selected, blasticidin S and G418. We sought to construct vectors that would confer stable, episomal resistance to these agents. The present work describes the use of *BSD* (encoding blasticidin S deaminase of *Aspergillus terreus*) and *NEO* (encoding neomycin phosphotransferase II from transposon Tn 5) genes as positive selectable markers for *P. falciparum* transfection. These markers will facilitate functional analysis of the malaria genome and understanding of the biology of the parasite, which are crucial for developing chemotherapies and vaccines.

EXPERIMENTAL PROCEDURES

Strains. The clones 3D7 (The Netherlands), HB3 (Honduras), Dd2 (Indochina), and W2 (Indochina) were used in this study. These parasites were kindly provided by Tom Wellems (National Institutes of Health, Bethesda, MD; HB3 and Dd2) and Pradip Rathod (Catholic University, Washington, DC; 3D7 and W2).

Cell Culture and Materials. All enzyme reactions and DNA preparations were performed as described by Maniatis *et al.* (13). Parasites were cultured by the method of Trager and Jensen (14) by using a gas mixture of 3% O₂, 3% CO₂, and 94% N₂. RPMI medium 1640 was supplemented with 30 mg/liter hypoxanthine (Sigma), 25 mM Hepes (Sigma), 0.225% NaHCO₃ (Sigma), 0.5% Albumax I (Life Technologies, Grand Island, NY), and 10 μg/ml gentamycin (Life Technologies).

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Blasticidin S and zeocin were from Invitrogen. Phosphinothricin, geneticin, kanamycin, hygromycin, and paromomycin were purchased from Sigma. Puromycin was from CLONTECH; nourseothricin from Hans-Knoll-Institut for Natural Products Research (Jena, Germany); and phleomycin from Cayla (Toulouse, France).

Plasmid Constructs. PCR with the primer pair 5'-GGA AGA TGC ATG CCA AGC CTT TGT CTC AAG AAG AAT CCA CCC TC-3' and 5'-GAC GGG AAG CTT TGC TCC TCG GCC ACG AAG TGC-3' was used to amplify the *BSD* gene of *A. terreus* from pcDNA6/V5-His plasmid (Invitrogen). The neomycin phosphotransferase gene of transposon Tn 5 (15) was amplified from the pXG plasmid (16) by using the primer pair 5'-GGA AGA TGC ATG GAT CGG CCA TTG AAC AAG-3' and 5'-GAC GGG AAG CTT CTG TCT TTT TAT TGC CGA-3'. Amplified inserts were digested with *Nsi*I and *Hind*III and used to replace the *T. gondii* *DHFR-TS* in the pDT-Tg23 vector (6), yielding constructs pCBM-*BSD* and pCBM-NEO (Fig. 1).

Drug Response Assays. The susceptibility of parasites to different compounds was assessed by tritiated hypoxanthine uptake as described by Desjardins and colleagues (17).

***P. falciparum* Transfection and Selection of Transfectants.** Electroporation settings and parasite sample preparations have been described by Fidock and Wellem's (12, 18) with the following modifications: 12 ml of infected erythrocyte ring-stage culture at 10% parasitemia and 2% hematocrit was split into two conical tubes. After centrifugation, the cells were washed twice with cold PBS and resuspended in 800 μ l of cytomix buffer (6); 100 μ g of pCBM-*BSD* or pCBM-NEO plasmid DNA was added, and the cells were electroporated. Each electroporation mix was split into three plates. Fresh red blood cells (450 μ l; 50% hematocrit) were added, and the cells were grown at 2% hematocrit for 48 h at 37°C without drugs in tissue culture flasks gassed with 3% CO₂, 3% O₂, and 94% N₂. Varied amounts (1, 2, or 5 μ g/ml) of blasticidin S were added to the cells transfected with pCBM-*BSD*. Similarly, varied amounts (3, 5, or 10 μ g/ml) of G418 were added to the cells transfected with pCBM-NEO. Drug pressure was maintained during the following weeks. The medium was changed twice a week, and a one-to-one subculture with fresh red blood cells was performed every week.

Southern Hybridization and Plasmid Recovery Analyses. Cultures of *P. falciparum* clone 3D7 or 3D7 transfected with pCBM-NEO and pCBM-*BSD* plasmids were harvested at 10% parasitemia and 2% hematocrit (2×10^7 parasites). The cell pellets were lysed for 5 min in five volumes of 0.2% saponin prepared in 1 \times PBS. The parasite pellets were washed in PBS

and resuspended in 600 μ l of TSK buffer [567 μ l of TE buffer (10 mM Tris/1 mM EDTA, pH 7.5), 30 μ l of 10% SDS, and 3 μ l of 20 mg/ml proteinase K] and incubated at 50°C overnight; 114 μ l of 5 M NaCl and 91 μ l of 1 M NaCl/10% (vol/vol) hexadecyltrimethylammonium bromide were then added. After a 15-min incubation at 65°C, DNA was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol) and then once with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol). After precipitation with 900 μ l of isopropanol, the DNA was washed with 70% (vol/vol) ethanol and resuspended in TE buffer. DNA (5 μ g) was digested with *Sca*I or *Sca*I + *Dpn*I. Digested products were separated by electrophoresis on 1% agarose gels, transferred to Hybond N⁺ Nylon membrane (Amersham Pharmacia), and hybridized with ³²P-labeled pBluescript II SK(+) probe (Stratagene). For plasmid recovery, 500 ng of genomic DNA was electroporated into *Escherichia coli* cells.

BSD Enzyme Assay. Cultured parasitized red blood cells (12 μ l) at 10% parasitemia and 2% hematocrit were washed with assay buffer (10 mM Tris-HCl, pH 7.5), suspended in 0.75 ml of assay buffer, and lysed by sonication on ice. The supernatants were recovered by centrifugation at 25,000 \times g for 10 min at 4°C and subsequently used for measurement of enzyme activity. A spectrophotometric assay based on the differential absorbance of the cytosine nucleus in the substrate (blasticidin S) and uracil in the product (deaminohydroxyblasticidin S) was used (19). The assay mixture contained 6 μ l of 10 mM blasticidin S and an appropriate amount of assay buffer to adjust the total volume to 600 μ l. The reaction was initiated by adding 200 μ g of crude enzyme extract to the mixture. The reaction was carried out at 30°C, after which it was stopped by adding 4.4 ml of 0.1 M HCl, and the decrease in absorbance at 282 nm was monitored (20). We determined that an OD₂₈₂ of 0.1 corresponded to 30 nmol of blasticidin S cleaved. Samples were normalized to protein concentration, measured by using a bicinchoninic acid protein assay kit (Sigma).

Neomycin Phosphotransferase Assay. To assay neomycin phosphotransferase, we used the procedure developed by Ramesh and Osborne (21) with the following adjustments. The parasitized red blood cells were washed with PBS; resuspended in 0.4 ml of the assay buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 25 mM NH₄Cl, and 0.6 mM β -mercaptoethanol; and lysed by sonication on ice. The supernatants were recovered by centrifugation at 25,000 \times g for 10 min at 4°C. In a 60- μ l final volume, 10 μ l of cell extracts were added to 40 μ l of the reaction mix [3.3 μ l of 1 M Tris-Maleate, pH 7.1/2.1 μ l of 1 M MgCl₂/20 μ l of 0.4 M NH₄Cl/2.2 μ l (20 μ Ci) of [γ -³²P]ATP (6,000 Ci/mmol)/12.4 μ l H₂O] and 10 μ l of 5

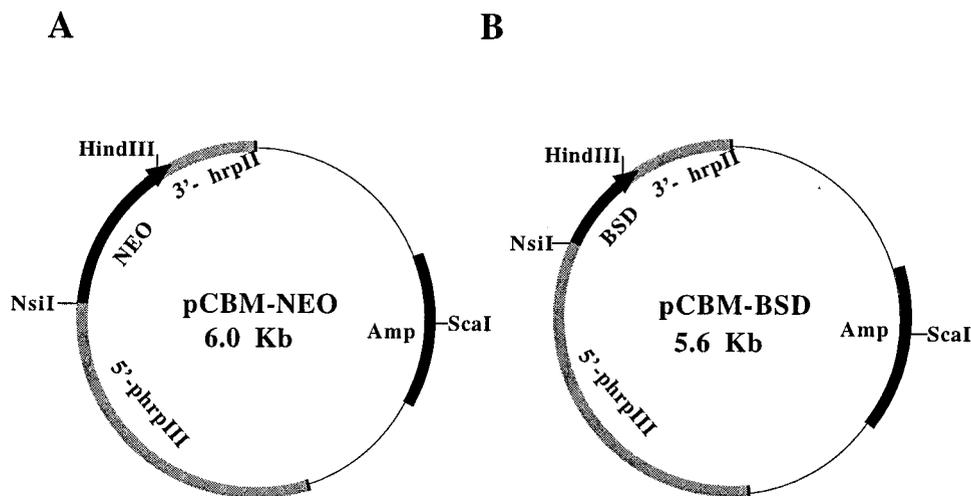


FIG. 1. Maps of pCBM-*BSD* and pCBM-NEO vectors.

mM neomycin sulfate or kanamycin sulfate (Sigma). The reaction was carried out at 37°C for 60 min, after which it was stopped by the addition of an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol). A 30- μ l aliquot of the aqueous phase was applied to P81 paper, washed twice with water for 10 min each, and then washed twice with 50 mM sodium phosphate (pH 7.4) for 2 h. The radioactivity was then determined by liquid scintillation counter. Samples were normalized to protein concentration, measured by using a bicinchoninic acid protein assay kit (Sigma). One unit of activity is defined as 5 fmol (62,500 cpm) of product formed per milligram of protein.

RESULTS

P. falciparum Growth Inhibition by Blasticidin S and G418.

In an effort to develop tools for genomic analysis of the human malaria parasite *P. falciparum*, we tested different drugs for their ability to inhibit the growth of the 3D7 parasite clone in culture. Because the parasite lives in an anucleate host erythrocyte, the range of drugs that can be used for selection is not limited generally by toxicity to the host cell. There were 10 drugs tested at concentrations up to 1 mg/ml (Table 1). Puromycin, nourseothricin, zeocin, phleomycin, phosphinothricin, blasticidin S, and G418 were found to be inhibitory.

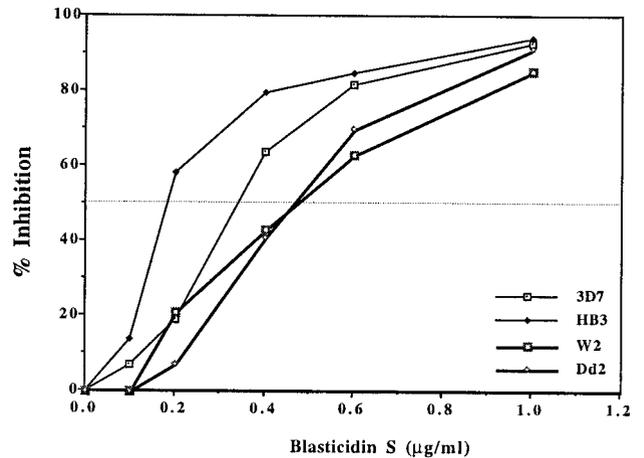
For the purpose of this study, we focused our work on two drugs, blasticidin S and G418. Both drugs had an antiplasmodial effect with IC₅₀ values for *P. falciparum* clone 3D7 of 0.35 μ g/ml and 150 μ g/ml, respectively (Table 1). Blasticidin S was chosen, because it is potent in culture and its resistance determinant is encoded by a small (399 bp) ORF, enhancing the probability of efficient expression and leaving room for cloning of larger DNA fragments. G418 was chosen, because it has been used successfully as a selectable marker in a broad range of eukaryotic organisms. We tested the effect of both drugs on *P. falciparum* lines with different chloroquine sensitivity. Four different parasite lines (3D7, HB3, W2, and Dd2) ranged in sensitivity (IC₅₀) to blasticidin S from 0.15 to 0.45 μ g/ml (Fig. 2A) and to G418 from 150 to 380 μ g/ml (Fig. 2B). There was no correlation with chloroquine sensitivity.

BSD and NEO: Selectable Markers for *P. falciparum* Transfection. The transfection vectors pCBM-BSD and pCBM-NEO contain the BSD or NEO [also called APH(3') II] genes under the regulatory control of *P. falciparum* promoter and terminator sequences (Fig. 1). After individual electroporation of these vectors into *P. falciparum*, the pCBM-BSD culture was split into three lines (BS1, BS2, and BS5), and blasticidin S (1 μ g/ml, 2 μ g/ml, and 5 μ g/ml, respectively) was added 48 h later. The pCBM-NEO culture was also split into three lines (G3, G5, and G10) and G418 (300 μ g/ml, 500 μ g/ml, and 1 mg/ml, respectively) was added 48 h later. Parasites were detected in all three pCBM-BSD plates by microscopy 4 weeks after transfection. Parasites were detected in the G3 and G5 lines at 4 weeks, but no parasites were obtained from the G10 culture up to 7 weeks. Drug pressure was maintained through-

Table 1. IC₅₀ values for clone 3D7

Gene	Drug	IC ₅₀ , μ g/ml
NEO	Geneticin (G418)	150
	Paromomycin	>1,000
	Kanamycin	>1,000
BSD	Blasticidin	0.35
BLE	Phleomycin	200
	Zeocin	500
HYG	Hygromycin	>1,000
SAT	Nourseothricin	75
BAR	Phosphinothricin	600
PAC	Puromycin	0.02

A



B

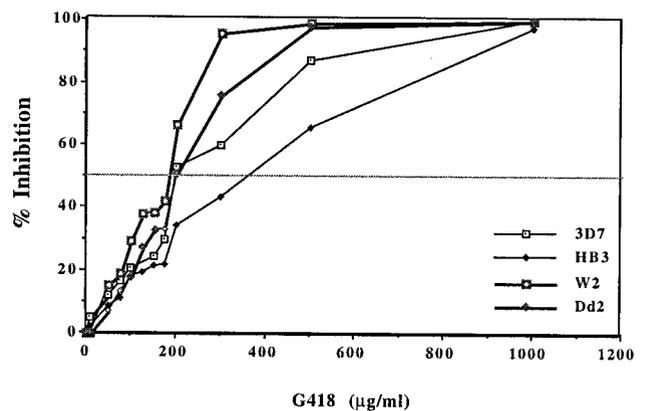


FIG. 2. Inhibition of 3D7, HB3, W2, and Dd2 parasite clones as a function of blasticidin S (A) or G418 (B) concentrations.

out the culture period. As a control, the 3D7 strain was transfected with the pBluescript vector and grown under the same conditions. No resistant parasites were detected at any of the drug concentrations used.

To confirm that the transfected plasmids had been replicated by *P. falciparum*, we tested susceptibility of the episomes to *DpnI*, an enzyme that cleaves GATC sequences only when the adenine is methylated. Because the necessary methylation activity (Dam methylase) is absent from eukaryotes but present in *E. coli*, replication of plasmid DNA in *P. falciparum* would lead to resistance to *DpnI* digestion. There was a failure of *DpnI* cleavage of plasmid sequences present in the parasite genomic DNA preparations, whereas this enzyme produced multiple fragments from *E. coli*-replicated DNA (Fig. 3). The absence of methylation identified by *DpnI* resistance indicates that the transfected plasmid DNA had been replicating episomally in *P. falciparum*. *ScaI* digestion alone gave the expected size of the linearized construct (Fig. 3).

The intensity of the bands corresponding to plasmid increased with the concentration of the drug, which indicates an increase in the copy number of the plasmid in response to the drug. To determine the plasmid copy number in resistant isolates, 0.5 μ g of total DNA was slotted onto nylon membrane and hybridized with the BSD or NEO probe (Fig. 4). Varying amounts of either gene were slotted onto the blot, corresponding to the amount of BSD DNA that would be present per microgram of total DNA if there were 1–35 copies of the construct per cell. The data show an increase in copy number

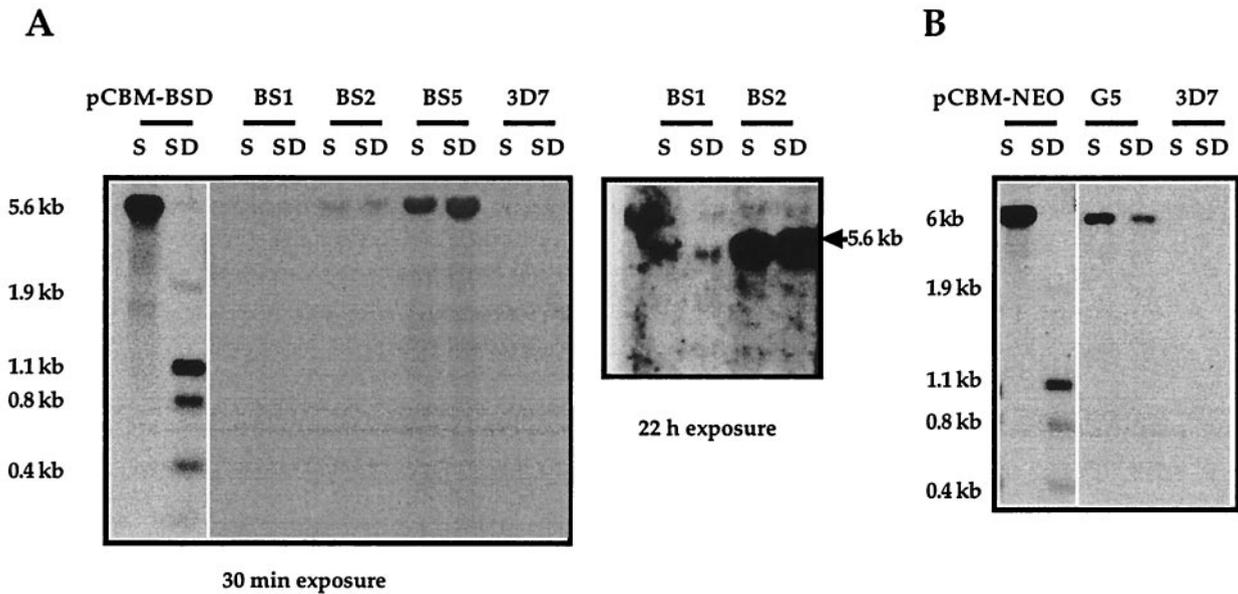


FIG. 3. (A) Detection of pCBM-BSD DNA from parasites selected on 1 $\mu\text{g/ml}$ (BS1), 2 $\mu\text{g/ml}$ (BS2), or 5 $\mu\text{g/ml}$ (BS5) of blasticidin S. Genomic DNA (prepared 7 weeks posttransfection) and DNA from the original plasmid (pCBM-BSD) were restricted with *ScaI* (S, which cuts once in the vector) or *ScaI* + *DpnI* (SD, with 15 pBluescript recognition sites), hybridized with a ^{32}P -labeled pBluescript probe, and detected by autoradiography. A longer exposure of the autoradiograph is also shown. (B) Detection of pCBM-NEO DNA from parasites selected on 500 $\mu\text{g/ml}$ G418 (G5). DNA was prepared as described for *A*. Untransfected 3D7 served as a control in both *A* and *B*.

and IC_{90} with increased drug concentration (Table 2). Plasmid rescue performed on genomic DNA prepared 7 weeks after transfection showed a correlation between numbers of *E. coli* transformants (reflecting episome copy number) and concentration of drug used for selection. Restriction analysis of a dozen plasmid clones from each rescued sample showed no rearrangements of the transfected DNAs (data not shown).

Enzyme Assays. To monitor the expression of the *BSD* gene product, extracts from 3D7 alone or from 3D7-blasticidin S-resistant parasites (BS1, BS2, and BS5) were tested for BSD enzymatic activity. The *BSD* gene encodes a deaminase that converts the potent inhibitor blasticidin S into a nontoxic deaminohydroxy derivative (20). As shown in Fig. 5A, extracts from BS2 and BS5 produced a significant and time-dependent decrease in the absorbance at 282 nm, indicative of BSD activity within these parasites. No significant activity was detected in BS1 extracts, and no activity was detected in nontransfected 3D7 parasites. The level of the activity was proportional to the concentration of the drug used to select for the transfectants (Fig. 5A), indicating once again that higher

drug concentration increases the copy number of the episome and thus the expression of the *BSD* gene. To monitor the expression of the *NEO* gene in G3 and G5 cells, we assayed the aminoglycoside phosphotransferase activity of its gene product *in vitro* by using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as a phosphate donor and kanamycin as a phosphate acceptor. Both G3 and G5 express activity (Fig. 5B). Here again, the data show a correlation between the drug concentration, plasmid copy number, and enzyme activity. Similar results were obtained by using neomycin sulfate as a phosphate acceptor (not shown).

Independence of *BSD*, *NEO*, and *DHFR* Markers. To be used for gene replacement or for simultaneous expression of multiple gene products from expression vectors, markers need to be independent, and the resistance conferred by one marker should not interfere with the activity of the second marker. To test this possibility, we measured the IC_{50} of the clones BS5 and G5 on blasticidin S, G418, and pyrimethamine and compared them to the IC_{50} s of the untransfected 3D7 strain. Other drugs that have the potential to be used in positive selection of *P. falciparum* transfection (puromycin and nourseothricin) were also tested in this study. No cross-resistance was detected between these markers; IC_{50} values for parental and transfected strains were indistinguishable with each drug (not shown).

DISCUSSION

We have developed two markers for *P. falciparum* transfection. *NEO* and *BSD* genes can be expressed in the human malaria

Table 2. Summary of IC_{90} and plasmid copy number experiments for control (3D7) and transfected lines BS1, BS2, BS5, G3, and G5

Line	IC_{90}	Copy number per cell
Blasticidin		
3D7	0.4 $\mu\text{g/ml}$	0
BS1	3 $\mu\text{g/ml}$	1
BS2	4 $\mu\text{g/ml}$	3
BS5	25 $\mu\text{g/ml}$	15
G418		
3D7	400 $\mu\text{g/ml}$	0
G3	1 mg/ml	3
G5	>1 mg/ml	12

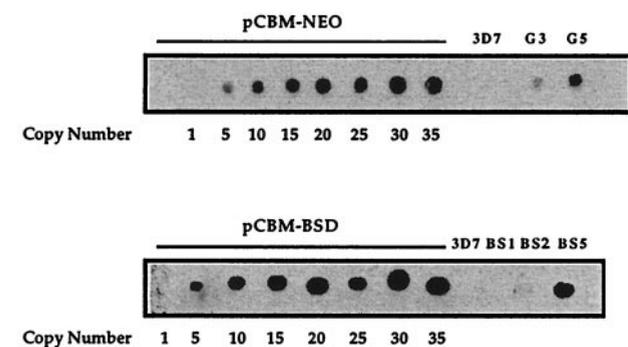


FIG. 4. Plasmid copy number in G3 and G5 clones (Upper) and BS1, BS2, and BS5 (Lower). By using the genome size of 3×10^7 bp, the amount of *NEO* (800 bp) or *BSD* (390 bp) signal that would represent one copy per *P. falciparum* genome was calculated; 0.5 μg of DNA from 3D7 and the various transformants were used for comparison.

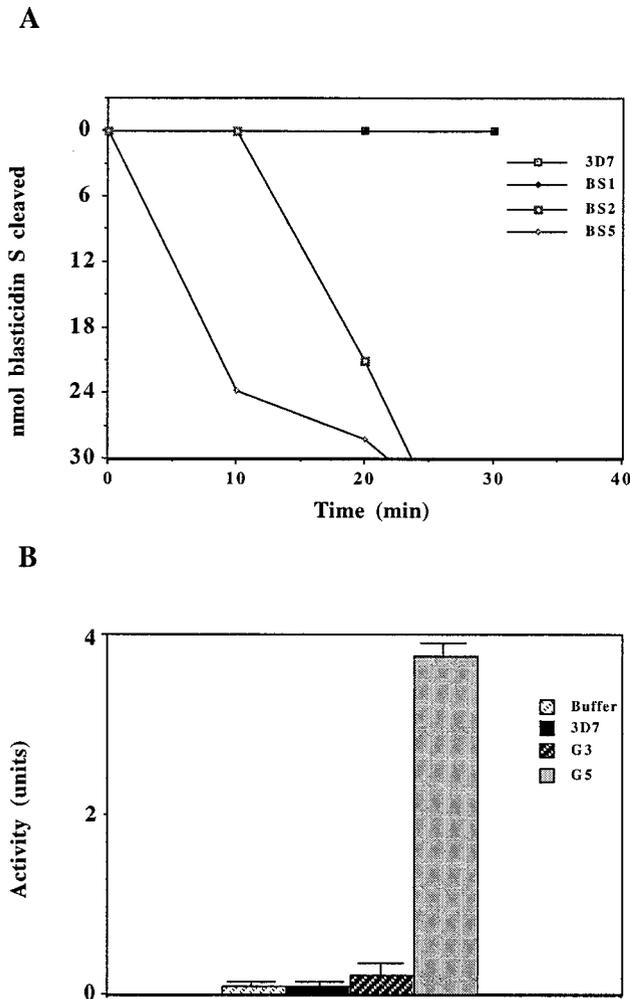


FIG. 5. Enzyme assays. (A) BSD activity. Crude cell extracts from 3D7 alone or 3D7 transfected parasites selected on 1 $\mu\text{g}/\text{ml}$ (BS1), 2 $\mu\text{g}/\text{ml}$ (BS2), or 5 $\mu\text{g}/\text{ml}$ (BS5) of blasticidin S were added to substrate in the assay buffer. Samples were taken at 0, 10, 20, and 30 min after incubation. The differential UV absorbance between the substrate (blasticidin S) and the deaminated product was recorded. (B) Neomycin phosphotransferase II activity. Crude cell extracts from 3D7 alone or 3D7 transfected parasites selected on 300 $\mu\text{g}/\text{ml}$ (G3) or 500 (G5) $\mu\text{g}/\text{ml}$ G418 were incubated with [γ - ^{32}P]ATP and kanamycin sulfate in the assay buffer at 37°C for 60 min. The buffer alone was used as a control.

parasite *P. falciparum* and confer resistance to G418 and blasticidin S, respectively. *NEO* is a widely used marker for eukaryotic transfection, whereas *BSD* has not been used previously for transfection of a parasite (we have now developed it for use in *Leishmania*). For both markers, there is a correlation between the level of enzymatic activity, the episome copy number, and the concentration of the drug used to select for parasite transfectants. This correlation is valuable for heterologous gene expression where the level of expression can be changed by controlling the drug concentration. Parasites harboring the *BSD* plasmid were able to grow in blasticidin S at concentrations up to 25 $\mu\text{g}/\text{ml}$ (70 \times the IC_{50} , data not shown). However, at 1 $\mu\text{g}/\text{ml}$, there is no background resistance in the control culture. Therefore, there is a wide range of drug to use for titration of expression levels.

From our data, we believe that other markers, such as *PAC*, *SAT*, and *BLE*, that confer resistance to puromycin (IC_{50} = 20 ng/ml), nourseothricin (IC_{50} = 75 $\mu\text{g}/\text{ml}$), and phleomycin (IC_{50} = 200 $\mu\text{g}/\text{ml}$) also have the potential to serve as markers for *Plasmodium* transfection. These markers have been used

successfully for transfection of other protozoan parasites such as *Leishmania* (22, 23). Neither *NEO* nor *BSD* induces cross-resistance to other drugs that are or might be used for positive selection in *P. falciparum* transfection, suggesting that these independent markers will be useful for gene replacement or for simultaneous expression of multiple gene products from expression vectors.

The ability to use *BSD* and *NEO* markers for malaria transfection will set the stage for rescue of disrupted essential genes, for making gene libraries to complement naturally occurring or induced mutants, or for other techniques that require the presence of multiple independent markers. The development of these resistance markers will allow better exploitation of the accumulating *P. falciparum* genomic information, lead to better understanding of the biology of the parasite, and facilitate development of drug and vaccine targets.

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