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Malaria continues as a major health threat throughout the tropical world and potential demand for antimalarials is higher than for any other medication yet the world faces a crisis-drug resistance is emerging and spreading faster than drugs are being developed and the flow in the pipeline of new drugs has all but stopped. This represents a particular threat to the US Military. In a short time there may be parts of the world where no effective antimalarial drug is available. The recent emergence of multidrug resistant malaria parasites has intensified this problem.

The goal of this work is to use a molecular genetic approach both in the identification of new drug targets and in the investigation of mechanisms of drug resistance. There are two parallel approaches being developed, one the development and characterization of a homologous transformation system and two the development of a heterologous expressions system in yeast for potential drug target enzymes. The yeast expression system should allow rapid screening of new drugs, greatly increasing the rate at which new antimalarials can be tested and developed. Both of these approaches are based on the functional analysis of malaria genes with goal of using this information in the identification and development of new antimalarial drugs.

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ANNUAL REPORT
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5. INTRODUCTION

Malaria - Drugs

Malaria continues as a major health threat throughout the tropical world and potential demand for antimalarials is higher than for any other medication,yet the world faces a crisis-drug resistance is emerging and spreading faster than drugs are being developed and the flow in the pipeline of new drugs has all but stopped. This represents a particular threat to the US Military. In a short time there may be parts of the world where no effective antimalarial drug is available. The recent emergence of multidrug resistant malaria parasites has intensified this problem. Recognizing this emerging crisis, it is necessary to identify new strategies for the identification and development of new antimalarials. The goal of this work is the development of a framework for antimalarial drug development into the 21st century.

A new strategy for drug development is urgently needed. Current drugs are based on a small number of target molecules or lead compounds and in most cases the target of drug action is yet to be identified. Resistance is emerging rapidly and the mechanisms of resistance are poorly understood. The identification of new targets or new candidate drugs based on an understanding of the parasite biology are key elements in this new strategy. Clearly the development of a new antimalarial will require both basic and applied research working in concert with one another.

The goal of this work is to use a molecular genetic approach both in the identification of new drug targets and in the investigation of mechanisms of drug resistance. There are two parallel approaches being developed, one the development and characterization of a homologous transformation system and two the development of a heterologous expressions system in yeast for potential drug target enzymes. The yeast expression system should allow rapid screening of new drugs, greatly increasing the rate at which new antimalarials can be tested and developed. Both of these approaches are based on the functional analysis of malaria genes with goal of using this information in the identification and development of new antimalarial drugs. This is a new strategy and it is being applied because of the crisis facing us in antimalarial drugs. The previous strategy, namely lead directed screening must be supplemented by new strategies or we will be faced with multiresistant *Plasmodium falciparum* and no drugs to treat it. There are two areas where they will be immediate application of this technology to pressing problems in malaria drug development.

Drug resistance and its implications for drug development

One of the major problems in infectious diseases today is that of drug resistance and multidrug resistance and this is a particularly acute problem for *Plasmodium falciparum* because there are only a few drugs available for safe and effective treatment and we are seeing evidence of cross-resistance and parasites with multiple resistant phenotypes. In parts of Southeast Asia and Brazil, there are reports *Plasmodium falciparum* resistant to all drugs which are currently registered for use in malaria treatment in the US and resistance to some

drugs which are still under evaluation and development. Thus, resistance is occurring faster than drugs are being developed.

This problem is not unique to malaria but is common in many infectious microorganisms. For example in HIV, there is rapidly emerging resistance to both inhibitors of reverse transcriptase and protease inhibitors. However, because we already have the tools of transfection and genetic manipulation, we are able to understand at the molecular level the mechanisms of this resistance in the HIV virus and investigators are able to develop strategies to use the available drugs in the most efficient way to prevent or delay the emergence of resistance and thus increase the useful life of the drug. For example, in the case of reverse transcriptase inhibitors in HIV infection, there are two different drugs currently in use, AZT and DDI. Each has a separate target in the protein which has been identified at the genetic level using methods of transfection and genetic engineering. With this knowledge, investigators are developing new lead compounds which are effective against viruses resistant to either AZT or DDI and importantly for today, have developed a strategy of alternating treatment with these drugs thus delaying the emergence of resistant organisms.

There are many similar examples in bacterial diseases as well, but the principle remains the same, the identification of the drug target and the analysis of resistance mechanisms is critical both to drug development and to effective use of drugs to delay the emergence of resistance. This is one of the applications of the new transfection/genetic engineering technology being developed in collaboration with WRAIR and Harvard. The first priority will be the investigation of drug resistance mechanisms with the goal of devising strategies either to prevent or overcome resistance.

In drug development today, one of the critical steps is the validation of the target. In other words, is the proposed target of the drug essential for the survival of the organisms and as a corollary to that, does the organism have alternate pathways to circumvent the inhibition of one enzyme. This is often the case for essential pathways and it is far better to know in the early stages of drug development whether this will be a problem. Malaria has severely lagged behind other diseases with regard to identification of drug targets and that is about to change based on this new transfection/genetic engineering technology.

6. BODY OF PROGRESS REPORT

Importance of drug resistance

Drug resistance has emerged as a major problem in the treatment of all microbial agents and in many cancer chemotherapy's. This has necessitated that continuous development of new chemotherapeutic agents both for treatment of infectious agents and for cancer chemotherapy. Often resistance develops through selection of a mutation in the target enzyme of the drug or in the overexpression of that enzyme. For example, resistance to antifolate drugs is frequently associated with mutations in the dihydrofolate reductase enzyme or in its overexpression. An alternative type of resistance, namely multidrug resistance has

emerged as a major problem in the treatment of many cancers and remains a major obstacle to the successful control of certain neoplasias with chemotherapy. This type of resistance is characterized by several unique features and the molecular basis for this resistance is under extensive investigation (Choi et al. 1991, Gros et al. 1986, Roninson et al. 1986, Raymond et al. 1990, Udea et al. 1987a, 1987b, Guild et al. 1988). In the case of multidrug resistance, resistance is observed to a number of structurally distinct drugs each with a different target. Selection of cells resistant to one drug results in the cross resistance to several structurally and functionally unique drugs. The genes associated with this resistance are the multidrug resistant mdr genes. The mdr gene encode membrane glycoproteins, the P-glycoprotein which mediate the efflux of drugs from the cell. Amplification of the mdr1 gene in resistant cells results in increased expression of the P-glycoprotein and thus increased efflux of drugs. Thus, the cells are resistant because drug is rapidly removed from the cell before significant toxicity occurs. Use of transfection of the mdr1 cDNA has demonstrated that overexpression of this gene is sufficient to confer the multidrug resistance phenotype. Drug resistance can be modulated by the use of several compounds including verapamil which appear to inhibit drug efflux. The current hypothesis is that verapamil and related compounds directly bind the P-glycoprotein molecule and block efflux and evidence for direct binding of radiolabelled verapamil to the P-glycoprotein molecule support this hypothesis.

In the case of malaria, the similarity in the pharmacological features of the chloroquine resistance in *P. falciparum*, namely the proposed efflux mechanism and the reversal of resistance by verapamil, desipramine and related compounds led to the proposal that a similar mechanism for drug resistance was operating in *P. falciparum* (Martin et al. 1987, Krogstad et al. 1992). Both our group under the support of this grant (Wilson et al. 1989, Foote et al. 1989) and David Kemp's group identified genes that had sequence and predicted structural similarity to the mdr genes and have completed the sequence, analyzed the expressed mRNA and protein. These genes and their encoded proteins are indeed related to the family genes in the ATP-Binding Cassette family and have the highest homology with the mdr genes from mouse and human. Thus, the hypothesis was proposed that these genes are involved in drug resistance in *P. falciparum*. Further evidence for this proposal was presented by the Foote et al. (1990) in identifying several polymorphism within the Pfmdr1 gene which appeared to be associated with chloroquine resistance in field isolates. This evidence was in contrast to the analysis by Wellems et al. in performing a genetic cross between a chloroquine resistant and chloroquine sensitive cloned parasite (Wellems et al. 1990, 1991). In the genetic analysis, both the pfmdr1 gene and its assorted polymorphism could be dissociated from chloroquine resistance. This was confirmed by a collaboration between our group and the NIH group in which we sequenced the relevant regions of the polymorphism from the resulting progeny (Wilson et al. submitted). Further evidence to refute the association of polymorphism in the pfmdr1 gene associated with chloroquine resistance was obtained by sequencing recent isolates of drug resistant *P. falciparum* (Wilson et al. submitted). We have completed this and have demonstrated in 12 new isolates of chloroquine resistant parasites, that the pfmdr1 gene sequence is identical to that predicted for the chloroquine sensitive phenotype, thus refuting the original Foote et al claim. Further RFLP analysis of the genetic cross by the Wellems group has determined linkage of the resistant phenotype to a small region of chromosome 7, a location distinct from the known location of either pfmdr1 or

pfmdr2. Thus, the conclusion from this work is that neither the pfmdr1 or pfmdr2 gene is linked to chloroquine resistance.

The mechanisms of chloroquine resistance remains unknown but progress has recently been reported on a putative target for chloroquine drug action. Slater et al. (1992) have reported an enzyme activity, heme synthetase which is hypothesized to be involved in the formation of hemozoan pigment and is a method for detoxification of the heme. This activity in cell extracts is inhibited by chloroquine and related quinones. Interestingly, the activity is equally sensitive to chloroquine whether derived from chloroquine sensitive or chloroquine resistant parasites. Recent work has suggested that heme polymerization may occur through a chemical reaction without the requirement for an enzyme (Bohle et al. 1993). These results indicate progress towards identifying the primary target of chloroquine action and are consistent with the hypothesized importance of efflux of the chloroquine in drug resistance. The increased efflux phenotype remains associated with chloroquine resistance both in the genetic cross experiments and in new chloroquine resistant field isolates (Krogstadt et al. 1992, Wellems et al. 1990 and Watt et al. 1990). In addition reversal of chloroquine resistance with verapamil is observed in all chloroquine resistant strains tested. Thus, the pharmacology of this system remains consistent and has many similarities to the efflux mediated multidrug resistance in mammalian cells. However, the genetic evidence argues strongly that the identified pfmdr1 and pfmdr2 genes are not linked to the chloroquine resistance phenotype.

The role of pfmdr genes in other drug resistance mechanisms remains an open and important question. This is particularly the case for mefloquine resistance in Southeast Asia. In our original work, we demonstrated that in a laboratory selected mefloquine resistant cloned parasite, W2mef, the pfmdr1 gene was amplified when compared to the cloned parent parasite, W2. In subsequent work, Peel et al have demonstrated that under increased mefloquine selection pressure that the pfmdr1 gene is further amplified approximately 8-10 fold. We also demonstrated an increased expression of pfmdr1 mRNA in W2mef compared to W2. This work has now been expanded to include several field isolated of mefloquine resistant parasites and our data suggests that in mefloquine resistant parasites in Southeast Asia, an amplification of the pfmdr1 gene and an increased expression of mRNA is associated with this resistance (Volkman et al. 1992). Further, analysis of the mefloquine resistant strains from Southeast Asia demonstrates that they are cross-resistant, in vitro, to other unrelated drugs, similar to the cross-resistance observed in multidrug resistant mammalian cells (Wilson et al. submitted). Resistance to all drugs can be reversed by penfluoridol and other reversal compounds. Thus, it appears that mefloquine resistant *P. falciparum* has many of the characteristics in common with multidrug resistant mammalian cells, however, definitive proof of this relationship awaits functional analysis.

The protein encoded by the pfmdr1 gene has been identified both by our group and by Cowman and coworkers using antibody raised against fusion proteins. The P-glycoprotein molecule is 160,000 -170,000 MW and is found associated with membranes in fractionation studies. Cowman finds an association of the protein with the parasite food vacuole and proposes that it is involved with transport in and out of that vacuole. Further investigation of its

localization throughout the parasite life cycle and in drug resistant verses drug sensitive parasites is necessary.

HYPOTHESIS

Drug resistance has emerged as a major problem in the treatment of all microbial agents and in many cancer chemotherapy's. Drug resistance has become particularly acute in malaria where resistance to chloroquine, the cheapest and most efficacious antimalaria has spread throughout the endemic parts of the world and resistance to other antimalarials is rapidly developing and spreading. The goal of this work is to understand the mechanism of drug resistance and to eventually use that information to develop new approaches to chemotherapy. The approach we propose is to develop a method for the functional analysis of genes in the malaria parasite. This methodology has proven invaluable in the analysis of drug resistance in other microbial systems, including our own recent work in leishmaniasis and particularly in multidrug resistance neoplastic cells. The initial aim of this work will be to test the function of genes implicated in drug resistance. This will be a multistep process in which we will first develop a method for the introduction and transient expression of foreign DNA into the parasite, we will then develop a method for the stable introduction of DNA using selectable markers and finally, we will test the role of the malaria mdr-like genes in drug resistance and in other parasite functions using methods of overexpression and gene knockout by homologous recombination. In parallel, we will continue development of the functional complementation system in yeast in which we are expressing the pfmdr1 gene. This system could prove extremely useful in testing drugs as potential resistance reversers.

TECHNICAL OBJECTIVES

1. Further development and characterization of the recently discovered transient transfection system in *Plasmodium gallinaceum* sexual stage parasites. This will include identification of putative promoter elements, transcriptional mapping, and testing of these vectors or modified vectors in other species.

2. Development of a system for stable transfection of the malaria parasite using a selectable marker.

3. Use of these transfection systems for the functional analysis of putative drug resistance genes in the malaria parasite.

4. Development of the heterologous yeast expression system for functional analysis of parasite genes and for the development of new testing systems for potential antimalarial drugs.

PROGRESS DURING THE REPORTING PERIOD

Progress has been made in all four technical objectives in the period July 95 - June 96. This progress report covers the period since the previous annual progress report and will focus on the use of our previously established transient transfection system to analyze *Plasmodium gallinaceum* and *Plasmodium falciparum* sexual stage gene expression. The goal of this work is to identify and characterize those DNA sequences which are necessary for gene expression in malaria. We have focused our effort on two genes, the pgs28 gene and the Pfs25 gene, from *P. gallinaceum* and *P. falciparum*, respectively. This work is in the final stages of completion and analysis and a draft manuscript is being prepared. The work is summarized below in sections 1 and 2. Recently, we have been successful in establishing both the transient and the stable transformation system in *P. falciparum*. Our recent results and progress in using this system in the functional analysis of pfmdr1 is summarized in section 3 below. We have also made progress in using the yeast expression system in the development of this system for screening drugs active against pfmdr1 function. This work is summarized in section 4 below.

1) **Identification of Gene Expression Putative Control Elements in *P. gallinaceum*.**
W.F. Mbacham, E. Budge, L. Golightly, J. Daily, T.J. Sullivan, D.F. Wirth. Dept. of Tropical Public Health, Harvard School of Public Health, Boston, MA.

Stage specific expression is important in the malaria parasite and little is known about its control. To understand mechanisms of gene expression in malaria, cis control elements necessary for expression have been characterized using a Plasmodium transient transfection system recently developed in this laboratory (Goonewardene et al, 1993). In this system, a firefly luciferase-pgs28 chimera was expressed in developing ookinetes and used to characterize the control elements within the 5'UTR. The luciferase-pgs28 chimera together with the 5 ' and 3' flanking sequences, was cloned into PBluescript KS+ for this purpose. Nested deletions were performed by application of the exonuclease III-mung bean nuclease protocol (Strategene). Deletion mutants were incubated with T4 DNA ligase under conditions that allowed for ligation of filled-in ends. Constructs were run in agarose gels to determine the apparent molecular sizes, then transfected into purified gametes. Luciferase activities were assayed 24 hours later with luciferine as substrate and normalized against a full length 5'UTR similar construct with β-glucuronidase as reporter gene. Activity decreased sequentially with increased deletion up to 400bp from the ATG start site for the pgs28 gene. Deletions beyond this point showed no detectable activity in this system (See Figure 1).

In a parallel series of experiments, expression of a chimeric pfs25 gene containing the luciferase reporter gene was tested. Again a series of nested deletions was created using the mung bean/ exonuclease method and these deletion constructs were tested for expression in the *P. gallinaceum* system (See Figure 2). Forty independent deletion clones were isolated, however, they fell into two categories, those with deletions of less than 50 bp at the 5' end of the upstream region and those which deleted the entire 5' upstream region. To further characterize this region, additional deletion mutants will be isolated. Now that the entire

sequence of this region is completed, a directed PCR approach to deletion analysis will be used.

The entire 5' region of the pgs28 gene and the pfs25 gene have been sequenced and those sequences are presented in Figure 3a and 3b. It is interesting to note that in both sequences, there is a long stretch of T-rich sequence just 5' to the ATG start codon. This region is present in both mRNAs and deletion of this sequence in the constructs containing the pgs28 gene results in a reduction of expression of the reporter gene. Further investigation of the role of this sequence in expression of these stage specific genes is planned during the next period. Analysis of the 5' flanking sequence has identified several eukaryotic consensus promoter and enhancer elements. This information is of interest because of the potential similarities of gene controlling elements in malaria with other eukaryotic systems. In the pgs 28 sequence, 2 adjacent TATA-like boxes at -368bp and -443 have been identified; 2 contiguous putative start sites for mRNA at -333 and -408, a series of five 8-mer inverted and three 27-mer direct repeats all clustered within -142 to -239 of the coding region start site are identified. All distances were measured from the ATG of pgs28 coding sequence. Comparison with Pfs25, another similar gene in *P. falciparum*; site specific mutagenesis, inversions and DNA binding studies are underway to determine the relevance of these cis elements in stage specific and/or basal regulation of gene expression in *Plasmodium* sp.

Figure legends:

Figure 1: Analysis of pgs28 gene expression using deletion mutants and a transient transfection system. Figure 1a shows the restriction map of a series of deletion mutants in which successively larger fragments of the 5' flanking regions of the pgs28 gene have been deleted. The resultant chimeric plasmids were tested by transient transfection analysis and Figure 1b shows the relative expression of luciferase activity for each deletion construct. Expression was tested in both sexual stage and asexual stage parasites.

Figure 2: Analysis of pfs25 gene expression using deletion mutants and transient transfection. Figure 2a shows the restriction map of a series of deletion mutants in which successively larger fragments of the 5' flanking regions of the pfs25 gene have been deleted. The resultant chimeric plasmids were tested by transient transfection analysis and Figure 2b shows the expression of luciferase activity for each deletion construct. In each experiment, a control plasmid containing the pgs28 /GUS reporter gene was included in the transfection and GUS activity was measured.

Figure 3: Sequence of the pgs28 (a) and the pfs25 (b) 5' flanking regions.

Fig. 1a

Identification of Putative Gene Expression Regulation Elements in *P. gallinaceum* Pgs28

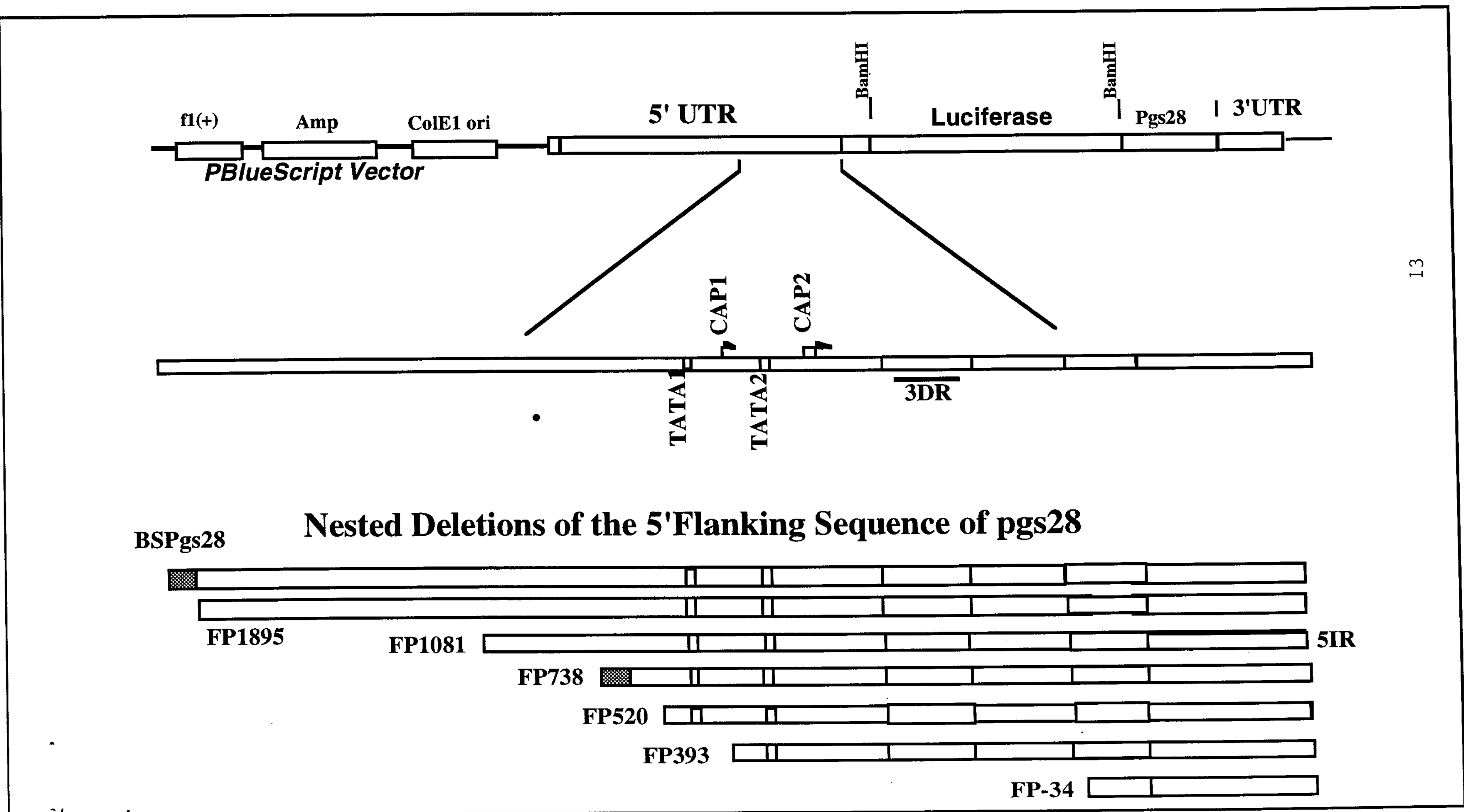


Fig. 1b Expression of Luciferase in the Sexual Stage Parasites of P. gallinaceum

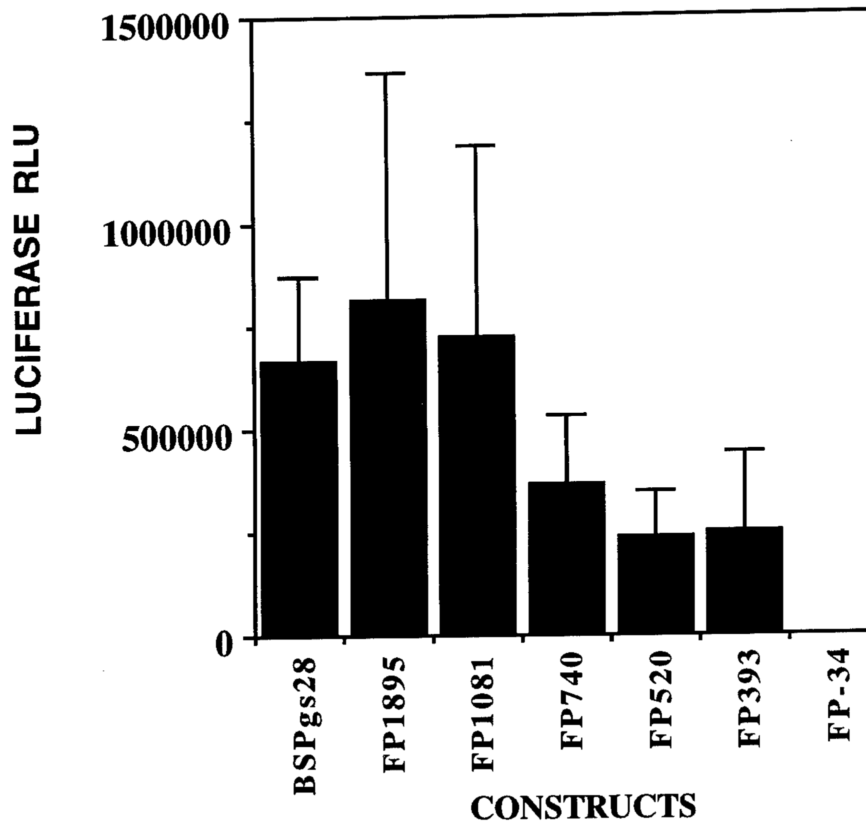


Fig. 2

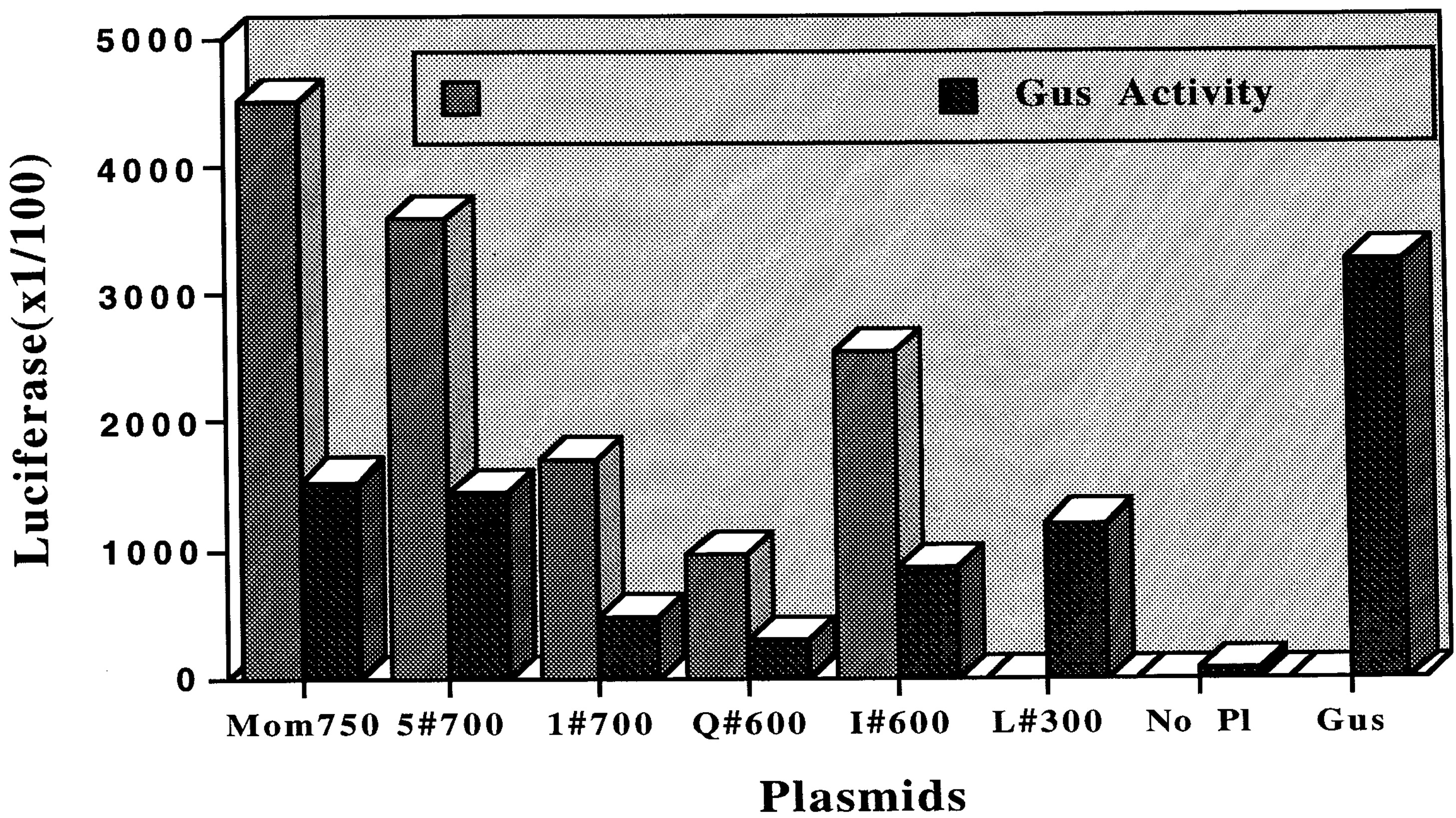


Fig. 3a

Putative Promoter Elements of *P. falciparum*, *Pfs 25*

```
TGATATATTA AATATGTTAC AAAACTTTTT TAAAAAAAAC ATTAATATAT TTTAAACAAG ATTACAAATT
AAAGAAAACA TTATATCATG CAATGATTCA TATTGATAAA AACTCCGATG ACGAAATAAA TCGTTTAAAG
TTAATTTGGA AGGAAACTCC ATTTTTATAT TTAATCATTC AAAAATTTCA TCTTAATAAA ATAATAACGG
ATTGTTCTCA ATTCCTAAAC AAAACATAAT AAATAATAAA AAAAAAAAAA AAAAAAGATA CTTTNCTATG
TACATATATA TATATATATA TATATATATA TATATATATA TATATATGTA TATCTCACAG AGCTTNTNAA
TGTTGCTTGT GTAGAACATA CGTTCCTTTT CCTAACATAA AATATCAAAN NAAAAAAAGT CATCGATTCT
TGAGAAATCA TATTAATTTT TTTTTTTTGC CCCAAAAANC ATATCGATTT ATCTGTATTA ATTCTATTAT
TAATTTAATA TTATTTATAT TATCTATTTT ATTATTATTT TGTTAATTTA TAAAATATTN TATTTCTTTT
TTAAAAACTT CAAAAAAAAG ATTTATTTTT AATTTTTTTT AAAAGAATCT TATTTTATTT CTTTTTTTTC
CTCAAATTAA TCATTTTTTT CGGTATCCTT TTTTTTTTTT TTTTTTTGTC TTATTATTTT ATATTTTATT
TTTCTCATTT GTTAATAAAT TGTTGTGAAA ACAGAAAAAC AAATAAAAAA ACAAAAAAAA CTCATACCTT
ATATTTTTTT ATTACTTTTA AAAAATG
```

Fig. 3b

5'Uptream Flanking Sequence of *P gallinaceum Pgs28*

FP1965 FP1895

AGCTCCACCG CGGTGGCGGC CGCTCTAGAA CTAGTGGATC CCCCGGGCT GCAGAAATTC GATATCAAGC TTAACTTTTT TTGTGTAAAT CTTTGTTAAA TATTTTCACG

TATCTTTTTT TTTTTTTTTT TGTATATATA TATATATATA CATATCATTT TGAAAAGTGT GGACAGATTT TTCGTTTTAC ACTATAATAA TTGGTGCTTA ATAAAAAAAA

ATTATTCAAA AATGAAATAA TGAAAGTTAC TATAAATAGA ATCACAACAA TTTTTATTTA ATAGAACAAA ATAACTAAAA ATAAGTTATG GAAAAAAATA TGTTGTTCTA

GAATAATTCT TTTTTAATTA AAAAAAAAAA AAAAGACTCA AAAGAAAATT TATTA[illegible] [illegible]GAAAT CCCAAATAAG AATTAATTTT GATATAAAAA ATGTTTCTAA

TAAAAAAAGT ATATTTAATA ATTGTTTGTT TTATAAAAAA AAAAAAATTT CCCACAAAAA AAAATATCAG ATAAGTATTG CTAAAATGCC TAATAATTAC TACA[illegible]

AAAAAAGAAT AATCAAAAGA AAGGCGAGAA TACTAAACTA ATGCATATAA TAATTCTTAA AGGATCGAAA AAAAAGAGTA TACAAGAGAA ATTATCTTCA TGATTTTTTT

CTCTNTATGT TATTTTATTT TTTTATTTCT TTTTTTTTTT TTTCTTTTTT TGTGTTTTTT TTTTCTTATA TGAATTGCTT CGAAAAGGTA ATAATATCAT TTTAATATTT

AAAACTTAGT AAAATGTAAT TTTACTATAA TATACAATTT TATCTTTTTC TACTAAAAAA AAAAATTCCT TATACATTTT TTTTTTTTAC ATCTCACAAT CAATTGTATT

FP1081

AGCTCTCTAG TTGTTCCTCC TGGTTAAAAT TGTGACAGTT GATATTTAGT GAATTATGTA GTGGATTTTA TGTACGTATA TGTGATGAAA AATATTCAGT TCAAATGCCC

TTTGGACAAA AAATTATTAT TCAAAGGACA TTATGACGTT CATAAAATTT GTGACATTTT TTCTGTATAT TCAAAGGGTA ATAATTTTAA TAATACCAAA TTTTCTTTCC

ATCAATATTT TATCTTACAT AAAAAAAGCA AAAAGTATAT TCCAAAAAAA TGTAATAATA TTGTCAGCAG TGAAGGCAAA TGATATATAG ATATACAAAT TACATGTGAA

FP740

AAATGTGAAT ATTTTCTTAA TGAATTTTTA CATTAAGAGA ACACACCTGA TTATCGAAGG TTTGTGGACA ATGGAAAAAC TAAAAACATG TTATATTTAA ATGGGTCAGA

ATGTGCAATA AAAAATTATT GTTATAAATA TCCTCACAAT ATAAAACTTC CCAATGGAAG TACCTATGAC GACAATAATA TTCGAGAGCA TGACCACGTC AGTAAGCCAT

FP520

GTATCAGTAT TGTGGTCATT ATGATTACGA TTATTTTTAT CTAAACTTTA ACAAAGTTTC GCAACATATG AAGACATTAA AATACCAAAT AAAAAGAATA AATTATTATC

FP393

TTTAATTATT TAACAGTTAT TTTTACAAAG ATTAAGAAGA TCTTTTTTTT TCTATAAATG TTTTAAGAAT TTTATATTTT AATATTACCA TTTGTACAGA CAGCCATTTG

TTATTGTATA TAAAAAAAAA AACTAGCTAA AAGAAATATG AAAATTTTTT ATGAACTTTT TGTTTTTCAT *TAATTTTATT TTTATTTAAT TTTTATTTTA TTTTTATTTA*

ATTTTTCTTT TATTTTATTT TTATTTAATT TTTCTTTTAT TTTTATTTAA TTTTTCTTTT GTTTTTATTT TTATTTTGTT TTTTTTTGTC ATATTATTAT CATTTTTAAA

TTCATTTCTA TTTCCCATAA TAAATTATTC TACAAAATAT TCAAACGAAG ATTATTTAGC AAACGAAAAC AATTTAACAT TTATTTAAAA AA**ATG**

2) **3' flanking elements enhance expression of the pgs28 gene in *Plasmodium gallinaceum*.** Linnie M. Golightly, Wilfred Mbacham, Johanna Daily, T.J. Sullivan, and Dyann F. Wirth* Department of Tropical Public Health Harvard School of Public Health, Boston, MA 0215 U.S.A.

We investigated whether the pgs28 3' gene flanking region regulates the developmental expression of the pgs28 gene in the mature zygotes and ookinetes of *Plasmodium gallinaceum*. Sexual stage parasites were transfected with plasmids containing either total or partial deletions of the 3' pgs28 gene flanking region utilizing either luciferase (LUC) or β-glucuronidase (GUS) as markers of transient expression. The parasites were allowed to develop in vitro to the ookinete stage and assayed for enzymatic activity. Gene expression correlated with deletions of the 3' gene flanking region present in the plasmid vector. Analysis of the 3' gene flanking region revealed that the sequence necessary for maximal gene expression contained a T-rich tract.

To determine the utility of GUS as a marker of transient transfection in *P. gallinaceum* we transformed gametes and fertilized zygotes with pgs28.1GUSF plasmid DNA. Mature ookinetes expressed significant GUS activity when compared to background levels (Figure 4). Cells transformed with the pgs28.1GUSR plasmid did not express significant GUS activity (data not shown). A relationship of GUS activity relative to input plasmid DNA for amounts 100 μg and 200 μg was observed. For amounts of plasmid DNA greater than 200 μg (400 μg and 600 μg) there was not a corresponding increase in GUS activity (data not shown). Experiments were performed to determine if the pgs28.1GUSF and pgs28.1LUC plasmids functioned independently when introduced simultaneously into cells by electroporation . In cells simultaneously transfected with both plasmids, their respective enzyme activities could be detected at levels comparable to those found in cells transfected with either plasmid separately (Figure 4). These experiments demonstrated that cells transformed with the pgs28.1GUSF plasmid reliably expressed significant GUS activity over background. The co-transfection of pgs28.1GUSF and pgs28.1LUC resulted in their simultaneous expression in ookinetes without diminution in their individual enzyme activities. We therefore internally controlled our experiments by co-transfecting cells with plasmids containing the full length 3' pgs28 gene flanking region and GUS or LUC with a pgs28 3' gene flanking deletion plasmid containing LUC or GUS respectively.

To determine whether the pgs28 gene 3' flanking region was necessary for gene expression, cells were transformed with plasmids either with or without the 3' gene flanking region. In two experiments, cells were transformed either with plasmids pgs28.1GUS d28 or pgs28.1GUS d12 (which lack the 3' flanking region), or pgs28.1GUS d20 (which contains the full length 3' gene flanking region), and pgs28.1LUC as an internal control (Figure 5). Cells transformed with either pgs28.1GUS d28 or pgs28.1GUS d12 expressed an average of 94% and 97 % less GUS activity respectively than cells transformed with the pgs28.1 d20 plasmid. In addition they did not express significant GUS activity over background. pgs28 gene expression therefore required sequence elements present in the 3' pgs28 gene flanking region of pgs28.1 GUS d20 that were deleted from the pgs28.1GUS d12 and pgs28.1GUS d28 plasmids.

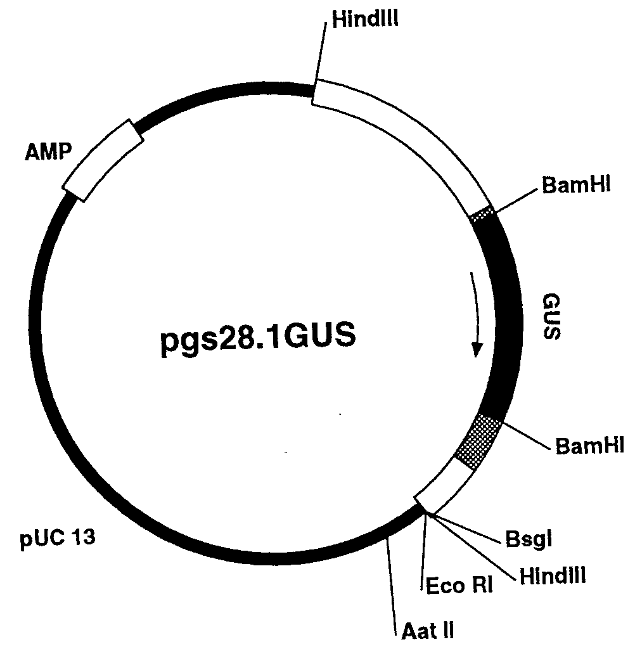
To determine which segments of the 3' gene flanking sequence were necessary for pgs28 gene expression, cells were transformed with plasmids containing partial 3' gene flanking deletions. In two experiments cells were simultaneously transformed with either plasmid pgs28.1 ILUC (which contains the full length 3' gene flanking region), pgs28.1LUC 191 (which contains 534 nucleotides of the 3' gene flanking region), pgs28.1 LUC 110 (which contains 210 nucleotides of the 3' gene flanking region) or pgs28.1LUC 3dt (which does not contain 3' gene flanking sequences) and pgs28.1GUSF as an internal control. Cells transformed with plasmid pgs28.1LUC 191 expressed an average of 38% less LUC activity than those transformed with pgs28.1LUC. In contrast cells transformed with pgs28.1LUC 110 and pgs28.1LUC 3dt expressed an average of 92% and 97% less LUC activity respectively (Figure 6). These results suggested that 3' gene flanking sequences important for optimal pgs28 gene expression were present in the pgs28.1LUC and pgs28.1LUC 191 plasmids but were deleted from the pgs28.1LUC 110 and pgs28.1LUC 3dt plasmids. To determine the specific DNA sequences responsible for the enhancement of pgs28 gene expression, we sequenced the entire 3' pgs28 gene flanking region.

Sequence and analysis of the 3' flanking region of the pgs28 gene. The 3' gene flanking region of the pgs28 gene was determined to be 727 bp (Figure 7). In the region between 210 and 534 nucleotides, which represented sequences deleted from the pgs28.1LUC 110 plasmid but present in the pgs28.1LUC 191 plasmid, a T-rich sequence (82%) was noted. It is comprised of approximately 156 nucleotides and is located between 215 to 370 nucleotides from the stop codon of the pgs28 gene. Six potential eukaryotic polyadenylation consensus sequences (ATTAAA /AATAAA) were identified but no significant homologies to other genes or gene flanking regions were detected by sequence comparison of databases.

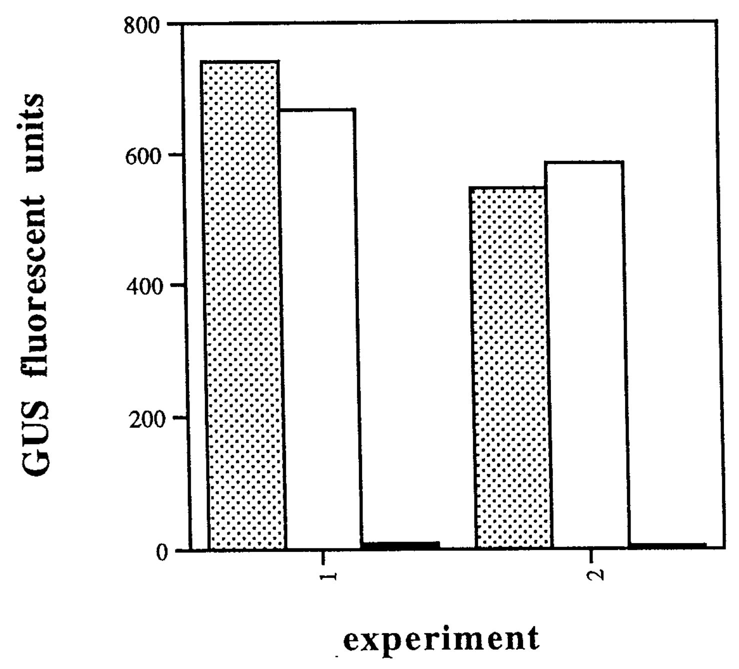
Our experiments, utilizing the *P. gallinaceum* transfection system, suggest that 3' pgs28 gene flanking sequences, which contain a T- rich region, are important in regulating pgs28 gene expression. This has potentially important implications to our understanding of the molecular biology of the malaria parasite.

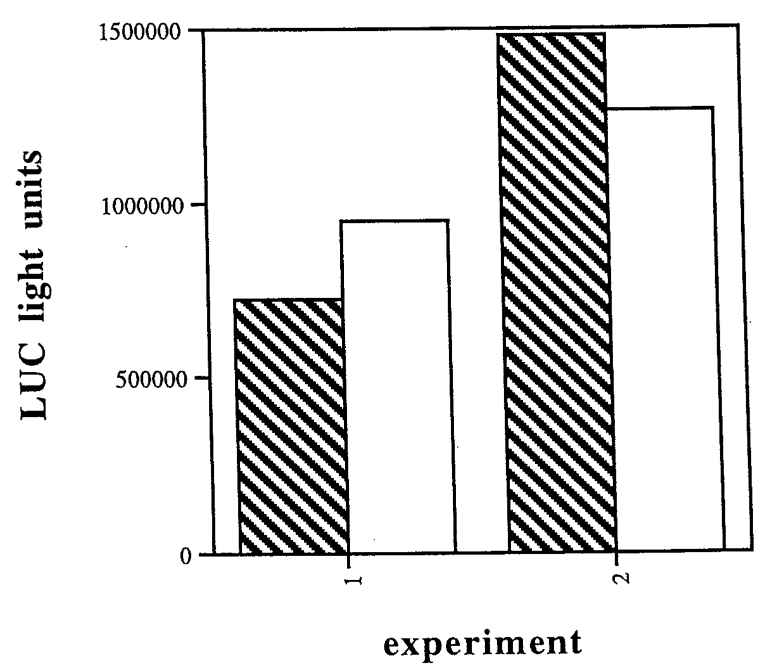
Figure 4. (A) Map of the pgs28.1GUSF plasmid. Amp, ampicillin-resistance gene. Coding regions of the pgs28 gene are shaded. Open boxes represent pgs28 gene flanking regions. (B) As described in the materials and methods $1X10^7$ cells were electroporated with 100 ug of each plasmid as indicated or without DNA. Cell aliquots were assayed for GUS and LUC activity. The results of each assay in 2 experiments are depicted separately.

A



B





pgs28.1GUSF
pgs28.1LUC
pgs28.1GUSF + pgs28.1LUC
without DNA

Fig. 4

Figure 5. Effect of the total deletion of the pgs28 gene 3' flanking region. (A) As described in the materials and methods $1X10^7$ cells were electroporated with 100mg of the indicated plasmid and 50mg or 100mg of the pgs28.1LUC plasmid in experiments 1 and 2 respectively as an internal control. Cell aliquots were assayed for GUS and LUC activity. Normalized GUS values a re plotted. (B) The HindIII fragment of the constructs indicated are diagrammed. Open boxes represent pgs28 gene coding regions.

Fig. 5

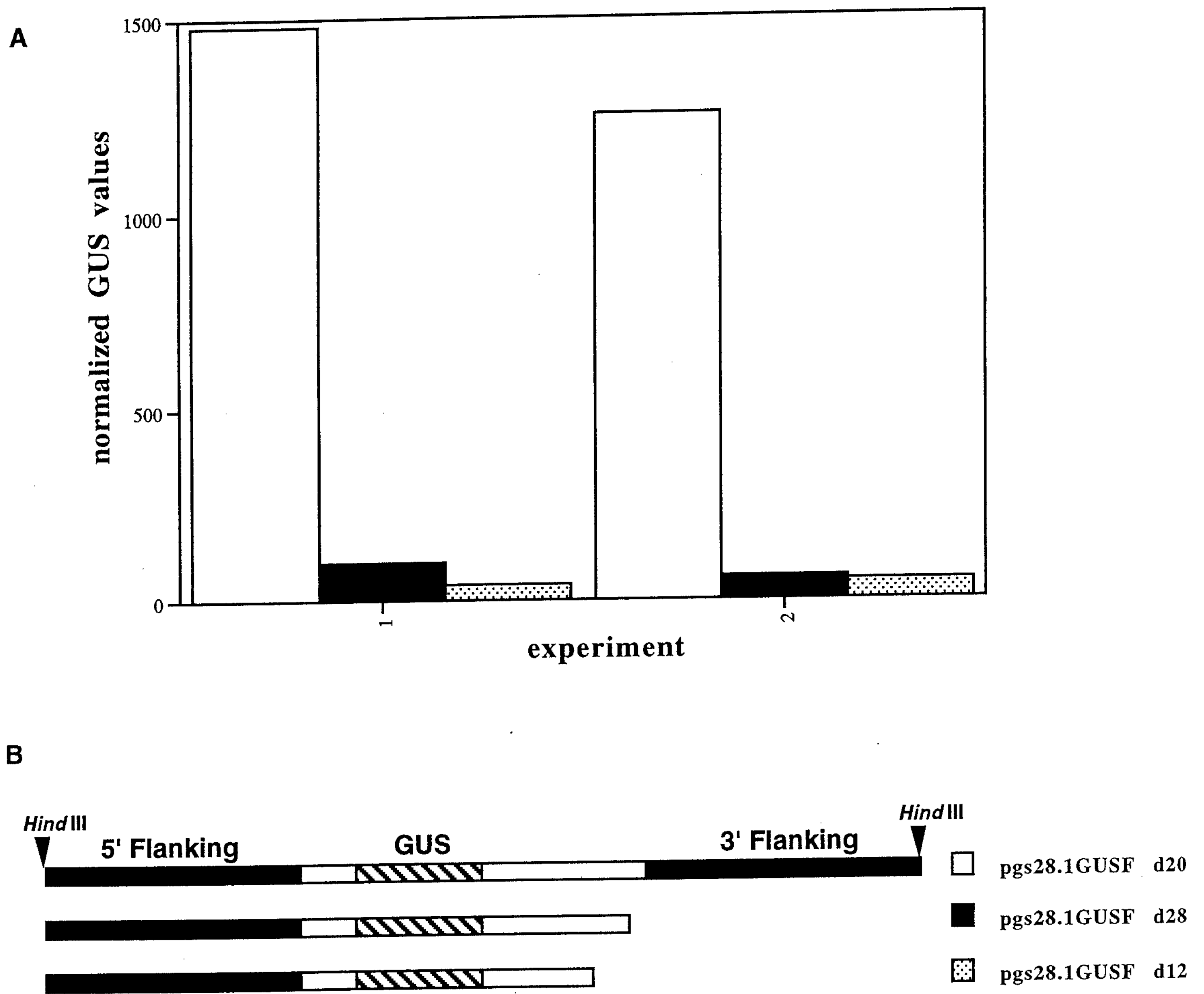
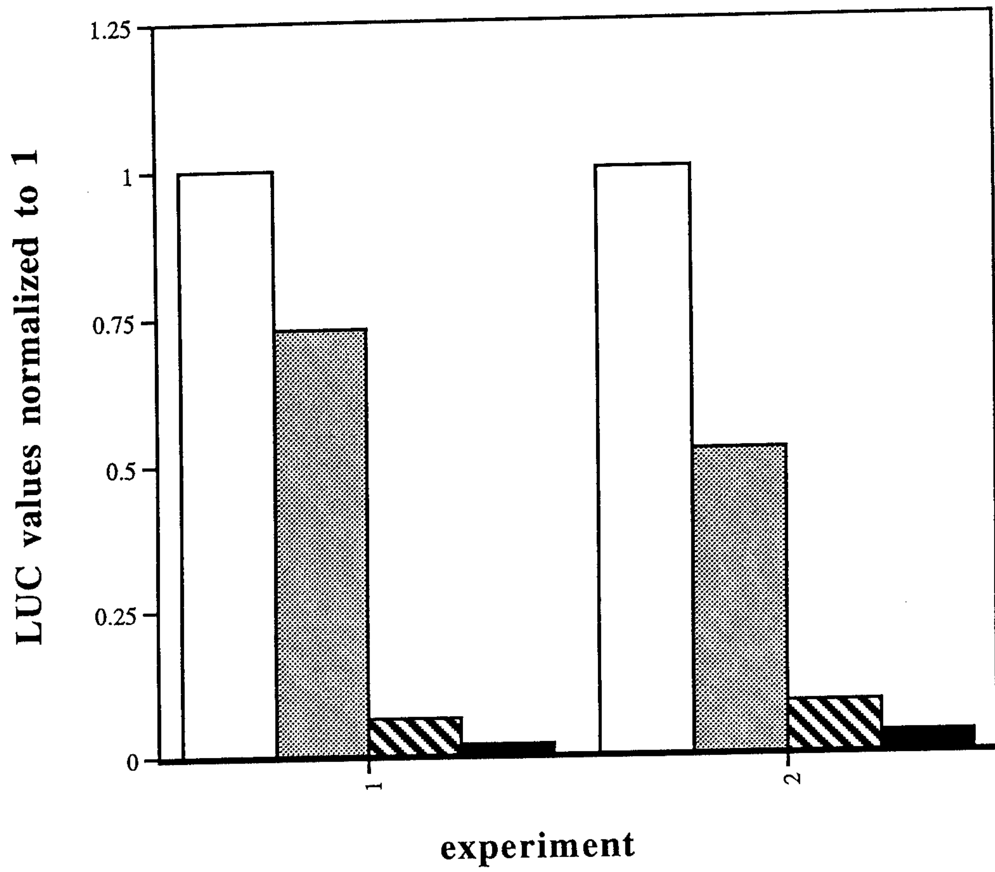


Figure 6. Effect of partial deletion of the pgs28 gene 3' flanking region. (A) As described in the materials and methods $1X10^7$ cells were electroporated with 50mg or 100mg respectively in experiments 1 and 2 of the indicated plasmid with 100mg of the pgs28.1GUSF plasmid as an internal control. Cell aliquots were assayed for GUS and LUC activity. LUC values were normalized to GUS values. Plotted LUC values were weighted to a maximal value of 1. (B) The 5'HindIII - 3'SacI fragment of the constructs indicated are diagrammed. PCR fragments utilized to generate the constructs are depicted with arrowheads representing primers. Open boxes represent pgs28 gene coding regions.

Fig. 6

A



B

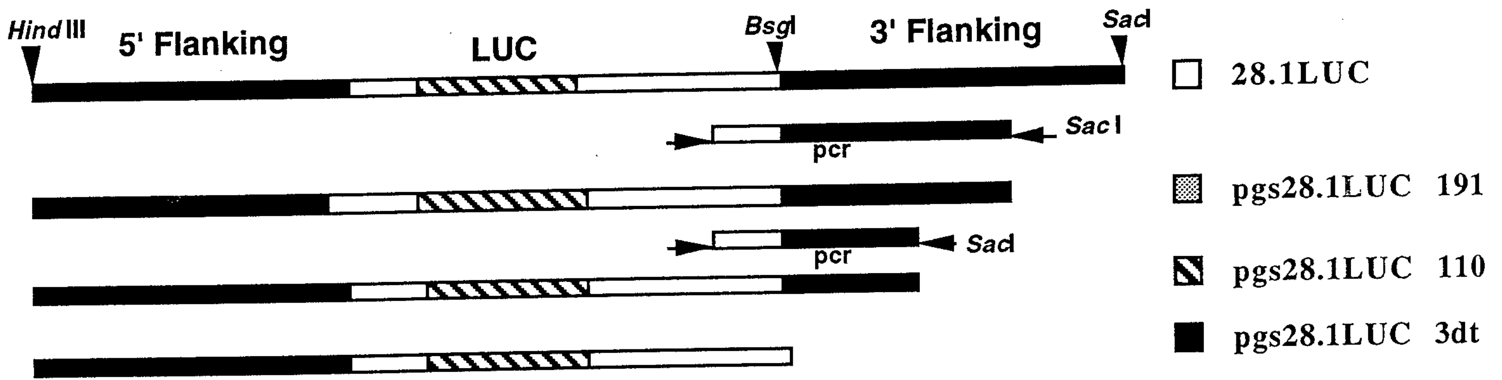


Figure 7. Sequence of the pgs28 gene 3' flanking region. The sequence is numbered from the A of the pgs28 gene start methione (GenBank accession no. M96886). The sequence begins the first nucleotide after the pgs28 stop codon which is indicated by a labeled arrowhead. Eukaryotic polyadenylation signals are bolded. PCR primers 110 and 191, utilized in deletion plasmids pgs28.1LUC 110 and pgs28.1LUC 191 respectively, as described in the materials and methods, are unlined with arrowheads. Arrowheads above the primers indicate the extent of pgs28 3' gene flanking sequence included in the pgs28.110 and pgs28.1LUC 191 plasmids respectively. The poly-T rich tract is boxed

Fig. 7

stop

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792  ATATATGGCT GCACTTAATG AAAGTAATAT AATTACCAGA CCAAATTAAA
842  TCATAATTAT ATGCACTAAT TTGAATAAAA TTTACTTCAG TTTTTCTTTT
892  TTTGTTAACT TTTTAAAGAA AAATATAAAA AAATAAATTT TATTGCATAA
942  AAGAAGAATA AAAAAAATTC ATATAAAAAA ATAATATGAA TGTATAACTA
992  AATGAAAAAG AAAATTTATT TTTTAATATT CTTTTTTCCT CTTTTTTTTT
1042 TTTTCCTTTT TTCTTTTTTT TGATTATTTA TTTTTTTCTT TTTTTTTTTA
1092 TTTTGTATTT TTTTTTTTTT TTTTTTGTTT TTTTTTTTTT TGTTTTGTTT
1142 CTTGTCTTCC TCTTTTTTTA CAAAATTGTT TTAACTAATT TGATCTATAT
1192 AAAATTTAAT ATTGTTAAGG ACTCATTAAA TTCAAATATT TATTTTTTAT
1242 TTATCGTTGA AAAAAAGTAT TTCTTTTAAT TTTTCATTAT AGTTGTAATA
1292 AATTATGTTG TCACTGAGGT TACGTTTGAT TTAATTTATA ACAATTGATA
1342 CTTAGCTCAA AAATGTAAAA GTTTTAAATG CATATGTAAT ACTGTATTTT
1392 TATTAGAAAA TTAAAGGATT TATGAAAAGG AATATCCATA CAATATACAT
1442 AATTTAGGAA GAATTATGAT ATATTATTAT ATATATAAAA AAATATATTT
1492 ATTCAGAAAA AACAAAATTT GAAGCTT
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110

191

3) **Transfection of *P. falciparum* and functional analysis of pfmdr1 gene expression.** Sarah Volkman, Bai Xiong and Dyann Wirth

This work addresses technical objectives 2 and 3. As we have previously described, we were the first to establish transient transfection in a malaria parasite using the *P. gallinaceum* model system. While we were in the process of transferring our technology to the *P. falciparum* system, two other groups successfully developed transfection systems in *P. berghei* and *P. falciparum* and we have chosen to use those vectors developed in the *P. falciparum* system by Dr. Yimin Wu in Dr. Tom Wellems' laboratory, rather than continue to develop our own independent system. This saved considerable time and redundant effort and has allowed us to focus on developing vectors for the functional analysis of the pfmdr1 gene and in developing methods for gene replacement.

The transient transfection technology using a luciferase reporter gene is now well established in our laboratory. The efficiency of transfection, as measured by the amount of luciferase activity detected after transient transfection is low consistent with the original report by Wu and colleagues. Dr. Volkman has also been successful in establishing stable transfection using the DHFR selectable plasmids developed by Wu. This technology required considerable adaptation from the published procedure and was successful only after sixteen attempts. The key observation made by Dr. Volkman was that cutting the plasmid DNA such that there was homologous sequence at each end of the linear vector increased the frequency of site specific integration. Dr. Volkman has now successfully repeated this experiment three times and is in the process of characterizing these transfected cells. In parallel, Dr. Volkman has developed a targeting vector for the disruption of the pfmdr1 gene and has used this vector in a stable transfection experiment designed to disrupt the pfmdr1 gene. She has selected stable transfectants and is now determining if the pfmdr1 gene is disrupted.

4) **Expression of the pfmdr1 gene of Plasmodium falciparum Modulates Drug Sensitivity in Yeast**. Sarah Volkman, Stephen Woodcock and Dyann Wirth

Recently [Volkman, 1995] we have demonstrated that the pfmdr1 gene expressed in a heterologous yeast system complements the ste6 gene function in yeast [Reviewed in Michaelis, 1993], a pheromone transport molecule required for mating. Furthermore, we showed in this same yeast system that expression of the pfmdr1 gene containing two mutations associated with naturally occurring chloroquine resistance [Foote, 1990], failed to complement ste6 gene function and did not restore a mating phenotype. These data argue that Pgh1 is a transport molecule and that mutations associated with chloroquine resistance alter the ability of Pgh1 to function as a transport molecule in this yeast system, perhaps in the malaria parasite itself. This heterologous expression system has been used to show that mdr3, another member of the ABC gene family can also complement ste6 Expression of mdr3 in *Saccharomyces cerevisiae* confers resistance to FK520, a drug which belongs to a class of compounds that function as MDR modulators, some of which are transported by the P-glycoproteins

Therefore, we are interested in using heterologous expression of pfmdr1 in S. cerevisiae as an assay system to isolate the function of Pgh1 and to examine the interaction of this gene product with different drug compounds. In this work we extend our previous analysis to demonstrate that expression of the pfmdr1 gene containing a mutation associated with mefloquine resistance in the wild failed to complement STE6 function in this yeast system. Furthermore, we examined the ability of pfmdr1 expression in this system to modulate drug sensitivity to various drugs, and examined the functional role of the mutations in pfmdr1 associated with either chloroquine or mefloquine resistant parasites on this drug modulation. Our goal is to develop this yeast system as a rapid screen for compounds that may be involved with the transport of drug and the reversal of drug resistance.

Construction of pYpfmdr1F. The pfmdr1 gene, either from the drug sensitive parasite, or from drug resistant parasites, was cloned into the pYES-2 vector and transfected into yeast. Ura+ clones were analyzed by Southern and northern analysis to determine the presence of the coding region and the expression of the gene [Volkman, 1995 or data not shown]. Previously it had been demonstrated that the wild-type pfmdr1 transformant, designated pYpfmdr1, and a mutant form of pfmdr1, designated pYpfmdr1CD, expressed Pgh1, whereas, yeast transformed with the null pfmdr1 gene, designated pYpfmdr1N or yeast transformed with the vector alone, designated pY did not express Pgh1 [Volkman, 1995]. To extend this analysis, the effect of a mutation in pfmdr1 associated with mefloquine resistant malaria [Wilson, 1993], was examined in this yeast system. A plasmid, designated pYpfmdr1F, containing pfmdr1 sequences with this mefloquine-associated mutation was constructed and used for transformation of yeast.

Expression of pYpfmdr1F in Yeast. The expression of Pgh1 in the pYpfmdr1F transformant is demonstrated by an indirect immunofluorescence assay using an a-Pgh1 antiserum which illustrated similar expression to both the wild-type Pgh1, and the Pgh1 molecule containing a cysteine at amino acid position 1034 and aspartic acid in amino acid position 1042, as previously described [Volkman, 1995]. The pYpfmdr1F cells incubated with pre-immune rabbit serum as primary antibody showed no staining. These results demonstrated that the yeast transformed with the pfmdr1F gene construct expresses the Pgh1 protein, and as detected at the level of light microscopy, with a similar distribution of Pgh1 to both the pYpfmdr1 wild-type and the pYpfmdr1CD mutant transformants [Volkman, 1995].

Mating Analysis of pYpfmdr1F Transformant. Mating assays were used to test whether this mutation in pfmdr1, associated with mefloquine resistance could complement the mutation in ste6 when expressed in the DST strain, SM1563. Previously we had demonstrated that number of diploids formed between the MATa mating strain and the pYpfmdr1 transformant was at a level 1000-fold above background [Volkman, 1995]. In a similar experiment using the yeast transformed with either the control plasmid, pY, or the plasmid containing the mutant pfmdr1 gene, pYpfmdr1CD, only background level mating was observed. Here we demonstrate that the pYpfmdr1F mutant fails to restore mating. Failure to detect mating by the pYpfmdr1F transformant is not due to the lack of pfmdr1 expression, since pYpfmdr1, pYpfmdr1CD and pYpfmdr1F expressed pfmdr1 similarly, as demonstrated by IFA analysis.

Drug Assays. We have demonstrated that Pgh1 can complement STE6 function and restore mating [Volkman, 1995], and that specific mutations associated with either chloroquine resistance [Volkman, 1995] or mefloquine resistance can abolish this mating phenotype. Next, we wanted to determine if the expression of pfmdr1 in yeast would have any effect on the sensitivity of the transformants to various chemotherapeutic compounds. Furthermore, we were interested in the effect of the mutations in pfmdr1 associated with drug resistance on this drug sensitivity.

We first asked if drugs known to interact with P-glycoprotein would modulate drug sensitivity in yeast. Yeast are refractory to many of the compounds that are transported by P-glycoprotein, therefore, we decided to examine the survival of pfmdr1 transformants under FK520 pressure, based upon the findings of with mammalian mdr genes. To examine the ability of pfmdr1 expressed in yeast to confer resistance to FK520 in this heterologous system, transformants were exposed to different concentrations of FK520 over the course of 24 hours. As shown in Table 1, the yeast transformants pY and pYpfmdr1N grew to similar levels in the presence or absence of FK520. Yeast strains pYpfmdr1 and pYpfmdr1CD also grow to a similar density after 22 hours in the absence of drug, but this growth is inhibited in FK520, as reflected in a Sensitivity Index (SI) of 3.2 and 3.8 respectively (Table 1). The pfmdr1 mutation associated with mefloquine resistance expressed in yeast, pYpfmdr1F, also modulates the sensitivity of yeast to FK520, but at a level approximately half of the wild-type or pYpfmdr1CD transformant (SI = 2) (Table 1). These data demonstrated that expression of pfmdr1 in yeast makes the yeast more sensitive to FK520, and that mutations associated with chloroquine resistance do not significantly alter this sensitivity, while a mutation associated with mefloquine resistance reduces this sensitivity to approximately half this level.

In similar experiments the growth of yeast expressing pfmdr1 and mutant forms of this gene was tested in the presence of known anti-cancer agents actinomycin D, daunomycin and vinblastine, as well as an MDR-reversing agent, verapamil (Table 1). These are compounds which are known to interact with the P-glycoprotein molecule in mammalian systems and we were interested in whether or not expression of Pgh1 might alter the sensitivity of yeast to these compounds. As seen previously, growth of yeast transformed with vector alone or with plasmids containing deletion mutations was similar in the presence or absence of drug. In contrast, the growth of yeast expressing wild-type pfmdr1 exhibited a SI of 2 and a SI of 1.7 in the presence of 100 mg/ml actinomycin D and 100 mg/ml daunomycin respectively (Table 1), and significantly reduced growth was observed in the presence of verapamil (SI = 3.2). However, no difference was observed in 1 mg/ml vinblastine (Table 1) over a 22 hour period for pYpfmdr1. The pYpfmdr1CD transformant demonstrated different growth patterns under these same conditions with similar growth in the presence or absence of both actinomycin D, and daunomycin (Table 1); however, pYpfmdr1CD had slightly reduced growth in the presence of vinblastine (SI = 1.5), and in the presence of verapamil (SI = 1.8) (Table 1). The pYpfmdr1F transformant exhibited no significant difference in growth in the presence of these compounds, as compared to growth in the absence of drug (Table 1).

To extend this analysis, we decided to use compounds which are common chemotherapeutic agents used for malaria, including chloroquine, mefloquine and quinine.

Chloroquine, to a level 1 mg/ml, had no effect on any of the yeast transformants (Table 1). Differences were observed for mefloquine where pYpfmdr1 had a significant response (SI = 2), while neither mutant displayed a difference in growth in the presence of mefloquine as compared to growth without (Table 1). Both pYpfmdr1 and pYpfmdr1CD showed a significant difference in quinine, with a SI of 3.4 and 1.7, respectively, but only a slight effect was observed for pYpfmdr1F (SI = 1.4) (Table 1).

These data argue that pfmdr1 expressed in yeast modulates sensitivity to various chemotherapeutic compounds. Although all forms of pfmdr1 expressed in this system modulate sensitivity to FK520, variable responses of these transformants in actinomycin D, daunomycin, verapamil, mefloquine and quinine was observed, and no difference of growth in chloroquine was observed for any of these transformants.

Table 1 **Sensitivity Index**

Drug	*pY*	*pYpfmdr1*	*pYpfmdr1CD*	*pYpfmdr1N*	*pYpfmdr1F*
FK520	1	**4.2**	**3.8**	1	**2.3**
Actinomycin D	1	**2**	1.1	1.1	1.2
Daunomycin	1.1	**1.7**	1.4	1.1	1.2
Chloroquine	1.1	1.2	1.1	1.2	1.1
Mefloquine	1	**2**	1.2	1.1	1.3
Vinblastine	0.9	1.1	**1.5**	1.2	1
Verapamil	1.1	**3.2**	**1.8**	1	1.3
Quinine	1	**3.4**	**1.7**	0.9	1.4

Drug Assays. FK520 (L-683,590) was kindly provided by Merck Sharp & Dohme Research Laboratories (Rahway, NJ) and diluted to 10 mg/ml in methanol. Other drugs included actinomycin D (2 mg/ml), daunomycin (5 mg/ml), vinblastine (10 mg/ml) and chloroquine (10 mg/ml) where stock solutions were in water, and verapamil (10 mg/ml), mefloquine (1 mg/ml) and quinine (1 mg/ml) where stock solutions were in ethanol. The concentration of those stock solutions are given in parentheses.

Growth inhibition assays were performed using a microtiter technique in 96-well flat-bottom plates with well containing equal volumes (0.05 ml) of YPD media with or without drug. Dilutions were made 2-fold, with the highest final concentration being 100 mg/ml for FK520, actinomycin D, daunomycin, mefloquine, and quinine and 1 mg/ml for chloroquine and vinblastine. Yeast transformants grown to stationary phase in SD-URA media were rinsed in

YPD media, then diluted to an OD570 of 0.5 in YPD media. Aliquots of these cell dilution (0.05 ml) were added to each well and incubated for 22 hours. Cell growth was measured optically at 570 nm at approximately 2 hour intervals on a microtiter plate reader (Molecular Devices, Inc.) Control growth was measured in the presence of solvent only.

Growth inhibition is expressed as a Sensitivity Index (SI) which is calculated as the optical density of cells grown in the absence of drug relative to the optical density of cells grown in the presence drug, as measured after 22 hours at 30°C. A SI = 2 means that the drug reduced growth to a level 50 percent of no drug control growth. In this assay, a SI of 1.5 or greater was considered significant.

7. CONCLUSIONS

Progress has been made on all of the technical objectives. Cis-acting elements which control gene expression have been identified and characterized in the malaria parasite. This is the first such work in malaria and will contribute to our overall understanding of the fundamental functions of the parasite and should have immediate applications in future vaccine and drug development applications. We have succeeded in transferring the technology to the human malaria parasite, *P. falciparum* and are in the progress of using molecular genetic techniques to functionally test the role of the putative drug transporter gene, pfmdr1. Further progress has been made in the development of the high throughput screening method using the heterologous system of yeast (*Sacchomyces cerivisiae*). This screening system for the function of the pfmdr1 gene is now ready for screening large numbers of new antimalarial drugs targeted to the pfmdr1 gene product.

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Biochemistry

Functional complementation of the *ste6* gene of *Saccharomyces cerevisiae* with the *pfmdr1* gene of *Plasmodium falciparum*

(multiple drug resistance)

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ABSTRACT **The *pfmdr1* gene has been associated with a drug-resistant phenotype in *Plasmodium falciparum*, and overexpression of *pfmdr1* has been associated with mefloquine- and halofantrine-resistant parasites, but little is known about the functional role of *pfmdr1* in this process. Here, we demonstrate that the *pfmdr1* gene expressed in a heterologous yeast system functions as a transport molecule and complements a mutation in *ste6*, a gene which encodes a mating pheromone a-factor export molecule. In addition, the *pfmdr1* gene containing two mutations which are associated with naturally occurring chloroquine resistance abolishes this mating phenotype, suggesting that these genetic polymorphisms alter this transport function. Our results support the functional role of *pfmdr1* as a transport molecule in the mediation of drug resistance and provide an assay system to address the nature of this transport function.**

The emergence of drug-resistant organisms that cause human disease has plagued clinical and epidemiological efforts to combat them. Drug-resistant *Plasmodium falciparum*, which causes malaria in humans, emerged over 30 years ago, but increasing levels of resistance to drugs such as chloroquine and mefloquine, as well as cross-resistance to these and other compounds, such as quinine and halofantrine, has made treatment and prophylaxis of malaria increasingly difficult. Neither the targets of these drugs nor the mechanisms of drug resistance are well understood. A candidate gene, the *P. falciparum* multiple drug resistance gene or *pfmdr1*, proposed to encode a transport molecule involved in drug resistance, has been identified (1, 2).

The *pfmdr1* gene is a member of the ATP-binding cassette gene family (3), whose members encode transport molecules, such as the P-glycoproteins, known to efflux drugs and thereby confer drug resistance in mammalian cells (4). It has been hypothesized that the *pfmdr1* gene has a similar role in drug resistance in *P. falciparum* (5). Although the mechanism of drug resistance in *P. falciparum* is not known, it has been demonstrated that chloroquine-resistant parasites accumulate less chloroquine than chloroquine-sensitive parasites (6). It has been suggested that an efflux mechanism is involved in drug resistance (6) and that agents like verapamil which reverse P-glycoprotein-mediated drug efflux in mammalian cells also reverse chloroquine resistance in *P. falciparum* (5). However, more recent studies have demonstrated rapid efflux of chloroquine in both chloroquine-sensitive and chloroquine-resistant parasites (7), suggesting that decreased chloroquine accumulation in resistant parasites may involve mechanisms other than efflux of drug and, furthermore, that reversing agents, such as verapamil, may function by a mechanism other than by inhibition of drug efflux (8). In addition, a proposed role for *pfmdr1* in drug resistance is controversial since other work involving a genetic cross between a chloroquine-sensitive and a chloroquine-resistant strain of *P. falciparum* has dissociated the *pfmdr1* gene from these phenotypic characteristics of efflux and reversal by verapamil (9). Finally, overexpression of *pfmdr1* is not associated with chloroquine-resistant parasites from the wild (2), and parasites selected *in vitro* for increased chloroquine resistance deamplify the *pfmdr1* gene (10) while increasing their sensitivity to mefloquine.

In contrast with chloroquine resistance, there is consistent evidence for amplification of the *pfmdr1* gene in mefloquine-resistant *P. falciparum*, both in field isolates (11) and in laboratory strains subjected to mefloquine pressure (12, 13). In addition, cross-resistance to compounds such as halofantrine (11–13) and quinine (13) has been associated with mefloquine resistance, suggesting that mechanisms involved in mefloquine resistance are distinct from those involved in chloroquine resistance (ref. 14; reviewed in ref. 15).

The goal of the work presented here was to develop a system for functional analysis of the *pfmdr1* gene and its product, the Pgh1 protein (16), and to resolve its role as a transport molecule in drug resistance. In addition, we wanted to develop a screening system to identify drugs which interact with the Pgh1 protein in this system, as well as modulators which may affect these drug interactions.

The *ste6* gene of *Saccharomyces cerevisiae* is also a member of the ATP-binding cassette gene family (17, 18) whose function is defined. The *ste6* gene encodes a membrane protein that exports **a**-factor mating pheromone, and mutants which are deficient in *ste6* expression are sterile and cannot mate (18). Mating is used as a measure of **a**-factor export in a very sensitive complementation assay, where mating frequencies of 0.01% are considered to reflect significant **a**-factor export (19). Other members of the ATP-binding cassette gene family have been shown to complement *ste6*, including the mouse *mdr3* (*mdr1a*) gene (20) and a chimera of the *cftr* gene (21); however, the human *MDR1* gene failed to complement a mutation in *ste6* (22). The demonstration that some ATP-binding cassette gene family members can complement *ste6* function suggests a specific structural requirement for **a**-factor transport. This assay system may therefore be used to address the functional nature of a transport molecule and to characterize the structural components of this molecule which determine or specify this function.

The purpose of these experiments is to develop a functional assay for the *pfmdr1* gene of *P. falciparum* in *S. cerevisiae* and to determine if expression of *pfmdr1* can functionally complement *ste6* and transport mating pheromone **a**-factor. A *pfmdr1* gene containing genetic polymorphisms associated with chloroquine resistance (23) is also expressed in this yeast system to address the effect of these genetic changes on the function of *pfmdr1* in this complementation analysis.

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MATERIALS AND METHODS

Media and Strains. Yeast strains and transformants were grown at 30°C in liquid culture or on solid agar plates containing yeast/peptone/dextrose (YPD) medium or selective synthetic dextrose-uracil (SD-URACIL) medium, and mating assays were performed on minimal synthetic dextrose (SD) agar plates (24). For growth in galactose, dextrose was replaced in the above media at a final concentration of 2%. Yeast strains (25) (the kind gift of Susan Michaelis, Johns Hopkins University, Baltimore) included SM1068 (MATα *lys1*), an α-tester strain for mating, or SM1086 (MATα *sst2-1 rme his6 met1 can1 cyh2*), an α-tester strain for pheromone assays, and SM1563 (MAT**a** *trp1 leu2 ura3 his4 can1 ste6::LEU3*), a yeast strain with a mutation in *ste6*. Yeast transformations were carried out by the lithium acetate method (26).

Constructs. Shuttle plasmids constructed with the pYES-2 vector (Invitrogen) contained either the wild-type *pfmdr1* gene (pYpfmdr1) or a mutant *pfmdr1* gene with a serine to cysteine change at position 1034 and an asparagine to aspartic acid change at position 1042 (pYpfmdr1CD) (2). As a control, the *ste6* gene was also inserted into this vector (pYste6) by using a *Hin*dIII restriction fragment from the pSM580 vector (Susan Michaelis, personal communication).

Northern Analysis. Yeast RNA was isolated from transformants (20-ml cultures) grown to a density of 2×10^8 cells per ml in SD-URA. Cells were centrifuged (Sorvall 6000T; 3500 rpm; 4°C; 10 min), resuspended in 1 ml of extraction buffer (0.1 M Tris·HCl, pH 7.5/0.1 M LiCl/0.1 M EDTA), added to 0.5 g of acid-washed glass beads (425–600 μm in diameter) in 1 ml of phenol/chloroform (50:50), and vortexed repeatedly with equal, intermittent incubations on ice for 5 min. The volume of each phase was increased to 10 ml and extracted twice with an equal volume of phenol/chloroform (50:50). Both aqueous phases were combined and precipitated with ethanol. Northern analysis was performed (14) by using one-tenth of the total yeast RNA for each sample (2×10^8 cells) with hybridization as described (14). The *pfmdr1* probe was a mixture of PCR-amplified DNA from the regions between nt 510 and 830 and 3609 and 4792 (2).

Quantitative Mating Assay and Quantitative Pheromone Assay. Quantitative mating assays were performed by a plate mating procedure as described (25). Mating frequency was calculated as the number of diploids formed relative to the number of input MAT**a** cells. The results that are presented represent three or more separate experiments and are given with the standard deviation. Quantitative pheromone assays were performed as described (25) and are represented as a 1:2 dilution series of concentrated culture supernatant from each of the transformants. Experiments were performed in medium containing either dextrose or galactose. Mating of both the MAT**a** strain SM1058 (25), which is isogenic to SM1563 but contains a wild-type *ste6* gene, and the pYste6 transformant was inhibited in the presence of galactose. Thus, all the data presented here are from cells grown in dextrose-containing media.

Indirect Immunofluorescence Assay of Yeast. Yeast transformants were subjected to an indirect immunofluorescence assay (27) with a polyclonal rabbit anti-Pgh1 antiserum (α-Pgh1) as the primary antibody and a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin as the secondary antibody. The α-Pgh1 antiserum was raised in a rabbit immunized with a fusion protein generated from a construct containing the *pfmdr1* gene region from nt position 4158 to 4428 (2) in frame with the β-galactosidase sequences of the pUR vector (28) and expressed in *Escherichia coli.* Yeast transformants were visualized at a magnification of 630 and at a wavelength of 495 nm.

Sequence Comparison. Analysis of sequences for the Pgh1 (2), Mdr3 (29), and STE6 (17, 18) protein coding regions was performed by using the software programs from PG/GENE (IntelliGenetics) and the Genetics Computer Group (University of Wisconsin; ref. 30). Data base searching was performed at the National Center of Biotechnology Information by using the BLAST (31) network services with a gap weight of 3.00 and gap-length weight of 0.10.

RESULTS

Expression of the *pfmdr1* Gene in Yeast. Shuttle plasmids containing either the *pfmdr1* coding region (Fig. 1*A*) or the *ste6* coding region (17, 18) were introduced into SM1563 (25), and independent Ura$^+$ colonies were chosen for further analysis. The *pfmdr1* gene expressed in these transformants was either the wild-type sequence (pYpfmdr1) or the mutant sequence (pYpfmdr1CD) containing two naturally occurring mutations in *pfmdr1* associated with chloroquine-resistant *P. falciparum* (23). Ura-selected transformants were analyzed by Southern analysis to demonstrate the presence of predicted plasmids (data not shown), and expression of *pfmdr1* in these yeast transformants was examined by Northern analysis (Fig. 1*B*). This analysis demonstrated that the *pfmdr1* probe hybridized with a transcript of ≈4 kb in RNA harvested from yeast transformed with either the wild-type *pfmdr1* gene or the mutant *pfmdr1* gene but not in RNA from yeast transformed with the control plasmid (pY) alone. This transcript size of 4 kb is consistent with the size of the *pfmdr1* coding region, which was put into the shuttle vector for expression in yeast (Fig. 1*A*). The two *pfmdr1* transcripts from *P. falciparum*, which are shown for comparison, have previously been shown to have sizes of 8.5 kb and 7.5 kb (14) since they include sequence information beyond the *pfmdr1* coding region of 4 kb.

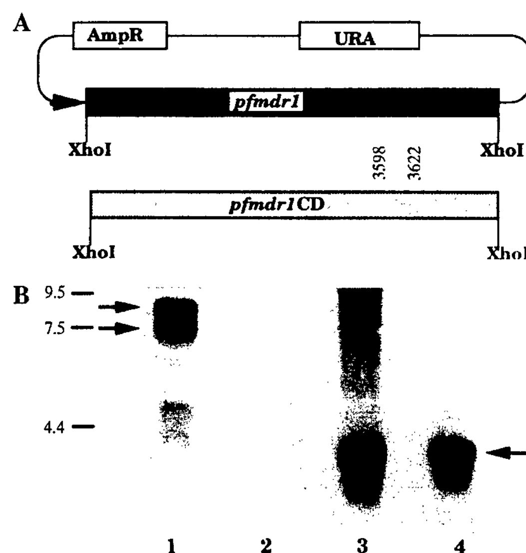

FIG. 1. Construction of expression plasmids and Northern analysis of yeast transformants. (*A*) Shuttle plasmids containing the wild-type *pfmdr1* gene (solid box) or the mutant *pfmdr1* gene (stippled box) containing the C1034 and D1042 mutations were constructed by using the *Xho* I restriction site of the pYES-2 plasmid. The mutations in *pfmdr1*CD are indicated by nt number 3598 (2), resulting in cysteine at position 1034, and by nt number 3622 (2), resulting in an aspartic acid at position 1042 (23). (*B*) Northern analysis of RNA isolated from either the W2mef strain of *P. falciparum* (1), lane 1; or the yeast transformants pY, lane 2; pYpfmdr1, lane 3; or pYpfmdr1CD, lane 4. Size markers for 9.5 kb, 7.5 kb, and 4.4 kb are indicated at the left, along with two arrows next to lane 1 to identify the two *pfmdr1* transcripts of 8.5 kb and 7.5 kb (14) and the single arrow on the right to identify the 4-kb *pfmdr1* transcript in the pYpfmdr1 and pYpfmdr1CD transformants.

To determine if the Pgh1 protein was expressed in yeast transformed with *pfmdr1*, indirect immunofluorescence analysis was performed. Immunofluorescence was detected with α-Pgh1 antiserum in yeast transformed with either the wild-type *pfmdr1* (pYpfmdr1) or the mutant *pfmdr1* (pYpfmdr1CD) gene containing two chloroquine-resistance-associated mutations but not in yeast expressing the control plasmid (pY) only (Fig. 2). The staining pattern in the yeast transformed with either the wild-type or the mutant *pfmdr1* gene was punctate rather than homogeneous, and immunofluorescence was observed as discrete points of signal localized either in the plasma membrane or in internal vesicles (see Fig. 2). These same cells incubated with preimmune rabbit serum as the primary antibody showed no staining (data not shown). These results demonstrated that the yeast transformed with either the wild-type or the mutant *pfmdr1* gene construct express Pgh1 protein and that, as detected at the level of light microscopy, there appeared to be a similar distribution of Pgh1 in each of these transformants.

Phenotypic Analysis of *pfmdr1* Expression. Mating assays were used to test whether *pfmdr1* could complement the mutation in *ste6* when expressed in the Δ*ste6* strain SM1563. Representative plates of these mating assays are shown with the calculated mating frequency in Fig. 3. This analysis demonstrated that the number of diploids formed between the MATα mating strain (25) and the pYpfmdr1 transformant was at a level 1000-fold above background. In a similar experiment using the yeast transformed with either the control plasmid, pY, or the plasmid containing the mutant *pfmdr1* gene, pYpfmdr1CD, only background-level mating was observed. Failure to detect mating by the pYpfmdr1CD transformant is not due to the lack of *pfmdr1* expression since both pYpfmdr1 and pYpfmdr1CD expressed *pfmdr1* similarly (Fig. 2). The level of complementation of the null *ste6* allele by the wild-type *pfmdr1* gene expressed in yeast is similar to the results of Raymond *et al.* (20) for expression of *mdr3* in a similar yeast system, where the *ste6* transformant mated at a frequency of 6.7×10^{-2} and the *mdr3* transformant mated at a frequency of 7.1×10^{-4}.

Quantitative pheromone assays were used to demonstrate the presence of **a**-factor in the culture supernatant of these cells. Results of these experiments (Fig. 4) show that the culture supernatant of pYpfmdr1 contains an **a**-factor titer which inhibited growth of the MATα lawn to a dilution of 1:8, whereas the supernatant from pYste6 transformants inhibited this lawn growth to a dilution of 1:64. Neither pY nor pYpfmdr1CD inhibited the growth of the MATα lawn in this analysis. These data demonstrated that detectable levels of mating factor were present in the supernatant of yeast transformants expressing wild-type *pfmdr1* but not in yeast expressing *pfmdr1* containing the two chloroquine-resistance-associated changes.

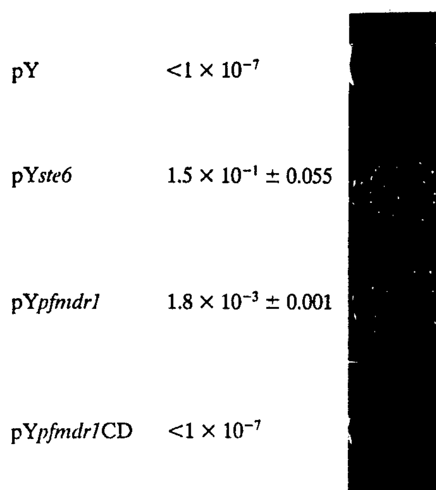

FIG. 3. Mating analysis of yeast transformants. SM1563 yeast transformed with plasmid only (pY), plasmid containing the *ste6* gene (pYste6), plasmid containing the wild-type *pfmdr1* gene (pYpfmdr1), or plasmid containing the mutant *pfmdr1* gene (pYpfmdr1CD) were mixed with MATα cells (SM1068) and grown on minimal medium agar plates to select for diploid yeast. Representative plates are shown with 10^7 transformed MAT**a** (SM1563) cells for each of the transformants except pYste6, which is shown at a 1:10,000 dilution, and pYpfmdr1, which is shown at a 1:100 dilution, to illustrate the similar cell densities observed at these dilutions. Mating frequency was calculated as described in *Material and Methods.*

Location of the Chloroquine Resistance-Associated Mutations. The genetic changes in the *pfmdr1* gene expressed in the pYpfmdr1CD transformant reside in the predicted transmembrane domain 11 of the *pfmdr1* gene, a region which is proposed to form an amphipathic helix (20, 23) and may be involved in substrate recognition and transport by the P-glycoprotein superfamily of transporters. A comparison of these changes in Pgh1 with the STE6 and Mdr3 protein sequences is shown in Fig. 5. The *mdr3* gene which complements ste6 contains a serine residue at position 939, a site proposed to be analogous to the serine residue at position 1034 in *pfmdr1* (20) (Fig. 5), and a change in this serine residue to a phenylalanine residue abolishes the ability of *mdr3* to complement *ste6* (20). These data suggest that the serine at

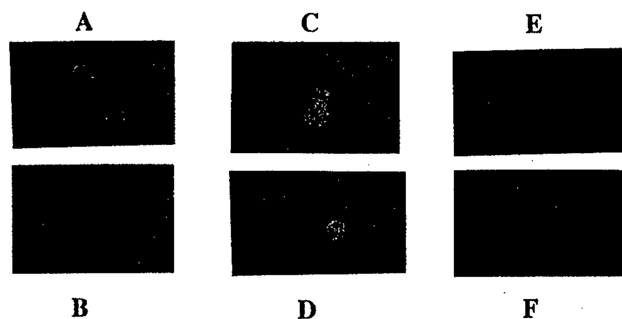

FIG. 2. Identification of Pgh1 in yeast transformants. SM1563 yeast were transformed with plasmid pY only (*A* and *B*), with plasmid pYpfmdr1 containing the wild-type pfmdr1 gene (*C* and *D*), or with plasmid pYpfmdr1CD containing a mutant *pfmdr1* gene (*E* and *F*). Formaldehyde-fixed cells were probed with α-Pgh1 antisera followed by secondary staining with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin and visualized by light microscopy at 495 nm (*B–F*). *A* shows a phase-contrast image of *B*.

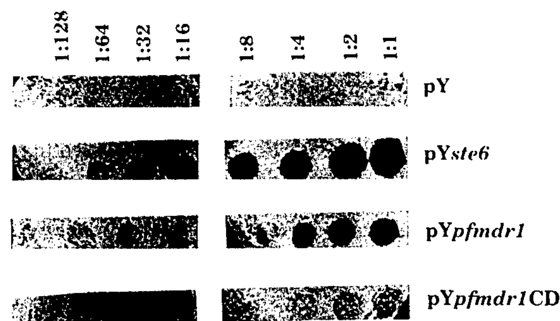

FIG. 4. Pheromone analysis of **a**-factor present in the culture supernatant. SM1563 yeast transformed with plasmid only (pY); plasmid containing the *ste6* gene (pYste6); plasmid containing the wild-type *pfmdr1* gene (pYpfmdr1); or plasmid containing the mutant *pfmdr1* gene (pYpfmdr1CD) were grown as described in *Material and Methods.* A dilution series (1:2) from the fully concentrated culture supernatant (1:1) to a dilution of (1:128) from these transformants was spotted onto a lawn of MATα (SM1086) cells.

Consensus	1081	KRAIV-G---	SFTQ----FI	-A--Y-FG--	LV-----T--	1120
STE6	940	KRAIATGFGI	SMTNMIVMCI	QAIIYYYGLK	LVMIHEYTSK	979
Mdr3	929	KKAHVFGITF	**S**FTQAMMYFS	YAACFRFGAY	LVAQQLMTFE	968
Pgh1	1022	RRIIVNAALW	GFSQSAQLFI	NSFAYWFGSF	LIKRGTILVD	1061
CLQR	1022	**********	**C*******	D*********	**********	1061

FIG. 5. Comparison of the amino acid sequences from the predicted transmembrane domain 11 of the STE6, Mdr3, and Pgh1 proteins. The alignment of the amino acid sequences from Pgh1 (from aa 1022 to aa 1061; ref. 2), Mdr3 (from aa 929 to aa 968; ref. 29), and STE6 (from aa 940 to aa 979; refs. 17 and 18), as well as a consensus sequence from aa 1081 to aa 1120 derived using BLAST as described in *Materials and Methods*, is shown. The chloroquine-resistance-associated Pgh1 sequence (CLQR) is identical to Pgh1, except for the mutations in position 1034 and 1042 of the Pgh1 sequence (23), with the asterisks indicating identity with the Pgh1 sequence above. The proposed transmembrane region of the Pgh1 sequence is underlined (2), and the serine residue indicated in boldface in the Mdr3 sequence corresponds to the amino acid changed to a phenylalanine residue in the work of Raymond *et al.* (20).

position 1034 of Pgh1 may be important for the transport function of this protein.

DISCUSSION

This work demonstrates that the *pfmdr1* gene expressed in a strain of yeast with a mutation in *ste6* complements STE6 function and transports mating pheromone **a**-factor. In addition, our results suggest that the expression of a *pfmdr1* gene with two naturally occurring polymorphisms associated with chloroquine resistance, C1034 and D1042 (23), abolishes this functional complementation of **a**-factor transport, as determined by mating assays, and that **a**-factor is not present in significant amounts in the supernatant of yeast cultures that express this mutant *pfmdr1* gene. The functional role of the *pfmdr1* gene in the mediation of drug resistance in malaria is not known; however, genetic polymorphisms in *pfmdr1* associated with a chloroquine-resistant phenotype (23) abolish this transport function in yeast, suggesting that these changes may be important for accumulation of drug in the parasite itself.

These genetic changes reside in the predicted transmembrane domain 11 of the *pfmdr1* gene, a region proposed to form an amphipathic helix and which may be involved in substrate recognition and transport by the P-glycoprotein superfamily of transporters (20, 23). In addition, the *mdr3* gene that complements *ste6* contains a serine residue at position 939, a site proposed to be analogous to the serine residue at position 1034 in *pfmdr1* (20). Altering this Ser-939 residue similarly abolishes the ability of *mdr3* to complement *ste6*. This change to a phenylalanine residue at position 939 in *mdr3* has been associated with dramatic changes in the substrate specificity of this gene product in mammalian systems (32–34). These data argue that the serine at position 1034 in *pfmdr1* has an important functional role in transport of yeast mating factor and perhaps other substrates.

A similar yeast system has been used to test if another ATP-binding cassette family gene, the human *MDR1* gene, could complement STE6 function when expressed in yeast deficient for *ste6*. Expression of *MDR1*, which is the human homologue of the mouse *mdr3* gene and is known to confer multiple drug resistance in mammalian systems, did not complement *ste6* in this experiment. One possible explanation for these results (19) is the presence of a mutation in the *MDR1* gene which resulted in a change from glycine to valine at amino acid position 185. This mutation, which resides near transmembrane domain three, phenotypically enhances colchicine selectivity in mammalian cells. These data imply that changes at or near transmembrane regions are important for transport in these ATP-binding cassette gene family members and are consistent with findings in mammalian systems where substrate specificity is often determined by transmembrane domain regions (4).

The observation that residues in transmembrane domain regions determine the specificity and level of resistance to different drugs, as well as the hydrophobic nature of the compounds which the *mdr* genes transport, leads to a current model which views drug transport as a biphasic process involving partitioning into the membrane followed by transport across it (35). This model proposes that substrates which can be transported by the P-glycoprotein molecule partition into the lipid bilayer of the membrane because of their hydrophobic nature, then interact with the P-glycoprotein transporter in the membrane at or near the transmembrane regions.

The genetic changes in the mutant *pfmdr1* gene used in the work presented here occur naturally in chloroquine-resistant parasites (23). The functional relevance of these mutations in *pfmdr1* on chloroquine sensitivity was tested in another system. The *pfmdr1* gene expressed in Chinese hamster ovary cells results in an increased sensitivity of these cells to chloroquine, and these same mutations in *pfmdr1* at positions 1034 and 1042 abolish this increase in chloroquine sensitivity in this system (36). These data support the findings in this work which demonstrate that these genetic polymorphisms identified in a naturally occurring, chloroquine-resistant parasite (23) are associated with altered transport of substrate. This argues that expression of the *pfmdr1* gene may play an important role in the mediation of drug transport in *P. falciparum* itself.

Additional experiments have been done with a similar heterologous yeast system expressing *mdr3* to demonstrate that expression of *mdr3* in this yeast system confers drug resistance to the immunosuppressive and antifungal agent FK520 (37). Furthermore, the *mdr3* gene containing a serine to phenylalanine mutation at position 939 expressed in this yeast system failed to confer this resistance phenotype. This suggests that expression of *pfmdr1* in yeast may be similarly tested for a functional role in drug transport and that the effect of the chloroquine-resistance-associated mutations in *pfmdr1* (23) may be addressed.

We have demonstrated that at the level of light microscopy *pfmdr1* is expressed in yeast in both internal vesicles and the plasma membrane. Although higher resolution of this localization is required, this observation is consistent with the finding that Pgh1 is localized to the digestive vacuole in *P. falciparum* (16). These data may seem to contrast with the finding that *pfmdr1* expression in Δ*ste6* yeast allows **a**-factor to be exported from the cell for mating, but recently it has been demonstrated that STE6 is mainly associated with internal membranes and not the cell surface in yeast, suggesting that STE6 secretes **a**-factor into endocytic vesicles and that the pheromone is released extracellularly through exocytosis (38). We propose that expression of *pfmdr1* in yeast allows for the transport of **a**-factor in a manner similar to STE6, and that Pgh1 may provide an analogous drug transport function in the malaria parasite.

Drug resistance in malaria is possibly mediated by several different mechanisms. This work demonstrates that the malaria parasite *pfmdr1* gene can complement the function of the *ste6* gene in yeast cells by transporting the mating pheromone **a**-factor from these cells but that a naturally occurring mutation in *pfmdr1* associated with chloroquine resistance is inactive. This establishes that Pgh1 is likely to function to alter drug accumulation primarily as a drug transporter rather than as an ionophore (39). The accumulation of drugs other than chloroquine, such as mefloquine and halofantrine, may be influenced differently by *pfmdr1* expression in the parasite, and it is possible that some drugs may be transported by proteins other than Pgh1. Further analysis of the role of Pgh1 as a transport molecule and how expression of *pfmdr1* may mediate a mechanism of drug resistance in malaria needs to be carried out. This yeast expression system can be utilized to identify the characteristics of *pfmdr1* which are important for transport

functions possibly relevant to a mechanism of drug resistance in *P. falciparum.*

We have functionally expressed *pfmdr1* in *S. cerevisiae* and have established that the *pfmdr1* gene product functions as a transporter. In this system, *pfmdr1* transports a small hydrophobic peptide, the yeast mating pheromone **a**-factor, easily assessed by mating assays. Furthermore, this yeast expression system can be easily manipulated to address the characteristics of *pfmdr1* that are important for this transport function and which may be relevant to a mechanism of drug resistance in *P. falciparum.*

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