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UNIVERSITY OF ALBERTA

STUDIES ON *Plasmodium falciparum* ANTIGENS OBTAINED BY EUKARYOTIC EXPRESSION CLONING IN COS CELLS

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled STUDIES ON *Plasmodium falciparum* ANTIGENS OBTAINED BY EUKARYOTIC EXPRESSION CLONING IN COS CELLS in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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To Tom and Bonny,
Kevin and Susan,
and especially,
Vaughn

ABSTRACT

Plasmodium falciparum is the causative organism of the most severe form of malaria, which kills millions of people each year. The existence of drug-resistant *P. falciparum* strains and the failure of existing malaria vaccines emphasizes the need for new vaccines incorporating novel parasite antigens.

Parasite proteins associated with cellular membranes are particularly promising as vaccine candidates. An efficient way to isolate genes encoding these antigens is by using eukaryotic expression cloning in COS cells and selection with hyper-immune human serum. In 1989, Elliott and colleagues used this method to clone a completely new cysteine-rich *P. falciparum* antigen called Pf12. The major goal of this thesis was to determine the sub-cellular location and time of expression of Pf12.

In preliminary panning experiments, three genes encoding parasite antigens were identified in addition to Pf12. As these three antigens had been previously characterized, Pf12 was chosen as the sole candidate for further study. Recombinant subfragments of Pf12 were expressed using *E. coli*, baculovirus, and DNA immunization expression systems. Two of these proteins, Pf12ec A2 and Pf12ec B2, were produced in *E. coli* and antiserum specific for each purified subfragment was used to characterize wild-type Pf12.

The anti-Pf12ec B2 antiserum, which had the strongest specificity for Pf12, was used to identify Pf12 as a 37 kDa protein expressed on the surface of mature schizonts within the infected RBC (iRBC), the parasitophorous vacuolar membrane, the merozoite surface, and perhaps the inner leaflet of the iRBC membrane. The anti-Pf12ec A2 and B2 antisera also had significant specificity for human serum albumin (HSA).

The time of expression and localization of Pf12 suggest that it may be involved in release of merozoites from the iRBC, stabilization the iRBC, re-invasion of RBCs, or perhaps evasion of merozoites from the host immune response by mimicking host proteins

such as HSA. These studies suggest that Pf12 would make an excellent asexual stage vaccine candidate.

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3D7	*P. falciparum* strain
Ab	Antibody
AcNPV	*Autographa californica* nuclear polyhedrosis virus, the wild-type baculovirus used to make target protein expressing recombinant viruses.
Ad	Prefix used for recombinant adenovirus constructs
ADCI	Antibody dependent cellular inhibition, a host immune response against malaria involving monocytes and antibodies
anti-B2	Mouse polyclonal antisera specific for Pf12ec B2, the best Pf12 specific antibody reagent generated in these studies.
anti-A2	Rat polyclonal antisera specific for Pf12ec A2, a Pf12 specific antibody reagent.
AT rich	Reference to DNA sequences that have a high proportion of adenine and thymidine residues
BL21DE3	*E. coli* strain containing an integrated λ phage that expresses T7 RNA polymerase; used in the expression of toxic genes.
BL21DE3pLysS	BL21DE3 strain harboring a lysozyme producing plasmid; lysozyme prevents leaky expression of target genes by inactivating constitutively produced T7 RNA polymerase.
BLAST	Basic local alignment search tool, used to search DNA and protein databases for sequence homologies.
bME or 2ME	Beta-mercaptoethanol
BMV	Brome Mosaic Virus
bp	Base pair

BSA	Bovine serum albumin
bv	Baculovirus
CAT	Chloramphenicol acetyl transferase
cDNA	Complementary deoxyribonucleic acid
CLUSTAL W	Computer program for aligning multiple DNA or amino acid sequences
CMV	Cytomegalovirus
COS	African green monkey kidney cell line
C-terminal	Carboxy-terminal
CTL	Cytotoxic T lymphocyte
D10	*P. falciparum* strain
DAF	*Decay accelerating factor* anchor used in the vectors pJFE14DAF, pBJT2, and pPSC2DAF
DAF B+	*P. falciparum* cDNA library
DBP	Duffy binding protein
DH5α	*E. coli strain* used for standard genetic engineering techniques such as mini and maxi preparations of DNA.
DHFR-TS	Dihydrofolate reductase-thymidylate synthase, a selectable marker used in *P. falciparum* transfection experiments
DMEM	Dulbecco's modified eagle's medium

DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence; a sensitive Western blotting detection development system
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
Fa3, 4+	*P. falciparum* genomic library
FBS, FCS	Fetal bovine serum
FITC	Fluorescein Isothiocyanate
GPI	Glycophosphatidyl inositol
HB3	Honduran isolate of *P. falciparum*
HRP	Horseradish peroxidase
HSA	Human serum albumin
IFA	Immunofluorescence assay
IFN	Interferon

Ig	Immunoglobulin
iRBC	Parasite-infected RBC, same as PRBC
kb	Kilobase
kDa	Kilodalton
KIA	Thailand isolate of *P. falciparum*
LMP	Low-melting point
MAb	Monoclonal antibody
MilliQ water	De-ionized water
MRGS-His6	Epitope and purification tag incorporated into recombinant proteins
M-RPMI	Tissue culture media supplemented with hepes, hypoxanthine and 10% human serum, used to grow *P. falciparum* parasites
NEB	New England Biolabs
NO	Nitric oxide
N-terminal	Amino-terminal
OD_{600}	Optical density at 600 nm
Oligo	Oligonucleotide
ORF	Open reading frame

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pf12 A2 bv	Pf12 A2 protein produced by a Pf12 A2 recombinant baculovirus.
Pf12 tag	Pf12 protein lacking the native parasite signal sequence and anchor; these were replaced by the T1/leu-1 signal and DAF anchor of pBJT2.
Pf12ec A1	Deletant Pf12 protein lacking signal and anchor sequences, produded in *E. coli.*
Pf12ec A2	Deletant Pf12 protein with same N-terminus as A1, plus a further deletion at the C-terminus.
Pf12ec B2	Deletant Pf12 protein missing 60 N-terminal amino acid residues and the native parasite anchor; this protein was used to generate the best anti-Pf12 antisera.
Pf12sec	Deletant Pf12 protein lacking the native anchor signal.
Pf12sec bv	Pf12 sec produced in baculovirus.
PI	Phosphatidyl inositol
PNG	Papua New Guniea pooled antisera used for antibody panning
PRBC	Parasitized red blood cell
PVM	Parasitophorous vacuolar membrane
RBC	Red blood cell
RNA	Ribonucleic acid

SDS PAGE	Sodium dodecyl sulfate polyarcylamide gel electrophoresis
Sf9	*Spodoptera frugiperda* insect cell line used in baculovirus expression systems
SGB	Sulfated glycoconjugate binding motif
SV40	Simian virus 40
TCR	T-cell receptor
T1/leu-1 signal	Signal sequence from T1/leu-1 glycoprotein used to make secreted forms of Pf12, and incorporated into the vectors pBJT2 and pPSC2DAF
TNF	Tumor necrosis factor
TVM	Tubovesicular membrane, a network of membranes set up by the parasite within the red blood cell
UV Microsope	Ultra-violet light microscope, used in immunofluorescence experiments
WHO	World Health Organization
WM1100	*E. coli strain* used to amplify Hirt panning clone pool for transfection into COS cells at the beginning of each new panning cycle.

CHAPTER I

BACKGROUND AND INTRODUCTION

A. Biology, pathology, and immunology of malaria infection

Introduction

In the following sections I review the biology of malaria and the most important antigens characterized to date in *P. falciparum*, with special emphasis on the asexual blood stages, which are relevant to the studies described in this thesis. In addition, I have included the most salient features of natural immunity to malaria in man, immune evasion tactics employed by the parasite, and the development of vaccines for human clinical trials. The last section details the use of COS cell panning, the eukaryotic expression cloning technique used to isolate Pf12 (the main parasite protein characterized in this thesis) as well as several new *P. falciparum* antigens described in chapter 5.

Malaria is a tropical disease that kills children, migrant workers, and non-immune travellers

Malaria is a major global public health problem infecting annually up to 500 million people, and killing up to 2.3 million people, most of whom are children under the age of 5, migrant workers, or immune 'naive' travellers to areas of malaria transmission (Sturchler, 1989; W.H.O., 1995). Forty percent of the world's population is at risk of contracting malaria, which is endemic in 91 tropical or subtropical countries, with the majority of cases occurring in tropical Africa, India, Brazil, Afghanistan, Sri Lanka, Thailand, Indonesia, Viet Nam, Cambodia, and China. The WHO sponsored Global Malaria Eradication Program (1955-1969), while eliminating malaria from Southern Europe, the former Soviet Union, and parts of Asia and South America, fell short of its objectives in transmission hotspots such as Africa and India. The urgency for new methods of malaria control is emphasized by the deadly epidemics of 1968 (Sri Lanka), and 1988 (Madagascar), and by

the emergence of drug resistant parasites in almost all areas of malaria transmission except Central America (W.H.O., 1998).

Malaria transmission tends to concentrate in the warmer, humid regions that favor mosquito growth and development. The disease is caused by a protozoan parasite (genus *Plasmodium*) transmitted when female anopheline mosquitoes take a blood meal to nourish their eggs. It is from the still, swampy mosquito breeding grounds that malaria, meaning 'bad' or 'evil' air, gets its name. The use of the term malaria is often attributed to the writer Horace Walpole in 1740, who described the disease as "a horrid thing called mal'aria that comes to Rome every summer and kills one." (Desowitz, 1991)

Plasmodium falciparum is responsible for the most severe form of malaria

Plasmodium species infect a wide variety of different hosts including snakes, birds, rodents, monkeys, and man. Plasmodia belong to the phylum Apicomplexa: the unifying feature of this phylum is the presence of invasion organelles collectively called an apical complex (Perkins, 1992). *Plasmodia* differ from other genera in this phylum in that they have two asexual phases in the vertebrate host, one in the red blood cells and one in the liver (and/or other organs), and a sexual phase which starts in the vertebrate host and ends in the mosquito vector (Garnham, 1988). The four species of *Plasmodium* parasites capable of infecting humans are *P. vivax*, *P. malariae*, *P. ovale*, and *P. falciparum*. Although significant morbidity is caused by each of these different species of parasite, only *P. falciparum* is capable of causing death due to severe disease complications such as anemia, hypoglycemia, and most notably coma due to cerebral sequestration of parasites. This introductory chapter will concentrate mainly on the pathogenesis of *P. falciparum* in man.

The malaria life cycle

The malaria life cycle, described in figure 1.1, consists of a sexual stage of reproduction in the mosquito and two distinct asexual stages in the host. It begins with the injection of sporozoites into the host when a female Anopheles mosquito takes a blood meal to nourish its eggs. The parasites then undergo an asexual exoerythrocytic cycle in the host liver, followed by an asexual erythrocytic cycle in the host red blood cells (RBCs). After a number of rounds of replication, a population of asexual blood stages will differentiate into sexual stages which complete the life cycle when ingested by a mosquito taking a bloodmeal.

During the erythrocytic cycle of growth, *P. falciparum* induces a number of changes in their RBC hosts. The most prominent of these alterations is the formation of knob structures on infected RBCs which concentrate key proteins (see table 1.3) that mediate cytoadherence and consequently the pathology of cerebral malaria (MacPherson et al., 1985; Aikawa et al., 1990; Aikawa et al., 1996). Of central importance to the studies of Pf12 in this thesis is the formation of a network of vesicles within the infected RBC extending from the parasitophorous vacuolar membrane (called the PVM; Trigg, 1988) created when the parasite invades the RBC to the outer membrane of the RBC. This network is called the tubovesicular membrane or TVM, and it figures prominently as a means by which the asexual blood-stage parasite acquires and exports proteins (Grellier et al., 1991; Haldar et al., 1991; Pouvelle et al., 1991; Elmendorf and Haldar, 1994; Elford and Ferguson, 1993).

Stage specific parasite virulence factors: key organelles and proteins

Tables 1.1-1.3 provide a more detailed review of some of the *P. falciparum* organelles and proteins that are important for parasite invasion of host cells and parasite

evasion of specific host immune responses. This review is given in the context of the parasite life cycle from sporozoite invasion of hepatocytes to merozoite invasion of RBCs. Special emphasis is placed upon the kinds of parasite antigens that may elicit a protective immune response; specific antibodies to these antigens are the key selective tool in the COS cell expression and antibody panning experiments upon which this thesis is based.

Genetic manipulation of malaria parasites

In order to determine the function of *Plasmodium* proteins, several groups have successfully introduced and maintained exogenous DNA sequences in *Plasmodium* parasites either episomally or as stably integrated constructs. *Plasmodium* parasites have a very AT-rich genome consisting of approximately 2-4 x 10^7 base pairs contained on 14 chromosomes (Weber, 1988). Parasite genes are relatively GC-rich compared to flanking noncoding sequences and introns, and the changes in AT content at the ends of genes are often so abrupt that this characteristic alone can be used to locate coding regions (Weber, 1988). Unlike other apicomplexan parasites such as *Toxoplasma*, the transfection of malaria parasites is hampered by the intracellular growth of many of the life cycle stages of the parasite, and the slow replication of the parasite. By contrast, the high rate of recombination observed in malaria parasites works in favor of targeted insertion of DNA sequences (Menard and Janse, 1997).

Table 1.4 details the results of transfection experiments conducted on malaria parasites to date. Four different *Plasmodium* species, *P. gallinaceum, P. berghei, P. falciparum,* and *P. knowlesi* have been transfected with DNA constructs by electroporation (Waters et al., 1997). Initially, extracellular sexual stage parasites were transfected (Goonewardene et al., 1993), followed by the extracellular RBC-infective merozoites (van Dijk et al., 1995), infected RBCs (Wu et al., 1995; Wu et al., 1996; Crabb et al., 1997), and sporozoites (Menard et al., 1997).

The constructs used in transfection experiments usually contain a selectable marker or reporter gene flanked by 5' and 3' untranslated *Plasmodium* gene sequences, and a prokaryotic origin of replication for production of large amounts of plasmid DNA in a bacterial host. *Plasmodium* 5' and 3' sequences are important in these constructs because they provide the necessary parasite promoter, untranslated region (UTR) and transcriptional stop signals for controlling expression of the introduced genes (Menard and Janse). These elements are approximately 2-3 Kb in length. The selectable marker used in malaria transfection experiments has been the dihydrofolate reductase-thymidylate synthase gene (DHFR-TS). Two kinds of DHFR-TS gene have been used: the *P. berghei* (*Pb*) and *P. falciparum* (*Pf*) DHFR-TS containing a serine 110 to asparagine mutation conferring resistance to pyrimethamine, or the pyrimethamine-resistant *Toxoplasma gondii* gene, *Tg* DHFR-TS. The latter gene was favored in 'gene knockout' experiments because its lack of homology with the *Plasmodium* DHFR-TS gene decreased the chance of integration of the introduced DNA at the DHFR-TS site as opposed to the desired "knockout" site (Waters et al., 1997). Other selectable and reporter gene constructs are detailed in table 1.4.

Exogenous DNA is introduced into parasites by electroporation either as uncut circular plasmid, or linearized plasmid (Menard and Janse, 1997). Uncut circular plasmid is maintained episomally, and may be lost when selection pressure is removed (van Dijk et al., 1995). By contrast, linearized plasmid can become integrated into the parasite chromosome, although this process is somewhat less efficient in *P. falciparum* than *P. berghei* (Menard and Janse, 1997). Stable recombinants can be obtained in *P. falciparum* using circular DNA, but only after several weeks of selection combined with intermittent periods of removal of selective pressure. Two kinds of linearized constructs have been used to stably transform malaria parasites: insertion and replacement vectors (Menard and Janse, 1997). The former construct disrupts the target locus by inserting the entire plasmid sequence into the chromosome through a single crossover event; the latter replaces the plasmid sequence with the chromosomal target sequence via a double crossover event.

The development of these constructs has led to the targeted disruption of the *P. berghei* circumsporozoite protein, CSP (Menard et al., 1997), the *P. falciparum* knob-associated histidine rich protein, KAHRP (Crabb et al., 1997), and the *P. berghei* thrombospondin-related anonymous protein (Sultan et al., 1997), as shown in table 1.4. The advent of stable transformation of malaria parasites promises to shed new light on the function of key parasite proteins in the process of infection.

Natural immunity to *Plasmodium falciparum*

Parasite burden and disease symptoms decline with age in the face of continuing infection

Natural immunity to *Plasmodium falciparum* in humans can be broken down into three general types, based on the age of the host. In infants, after the protective effects of maternal antibody have waned (Reeder and Brown, 1996), parasite burdens are relatively heavy, and a high mortality and morbidity is observed due to poor immune responses to parasites (Snow et al., 1994). In older children an anti-toxic immunity develops, characterized by high parasite burdens, less severe disease and an increase in antimalarial antibodies (McGregor et al., 1965). This type of immunity is frequently seen in children born in regions with heavy malaria transmission rates (McGregor et al., 1956; Greenwood et al., 1987). From the time of adolescence to adulthood, an antiparasite immunity develops that reduces the density and frequency of parasitemia, and consequently the morbidity and mortality associated with infection (McGregor and Smith, 1952; McGregor et al., 1956; Miller, 1958).

Concomitant immunity develops slowly and wanes quickly in the absence of infection

A strain-transcending 'concomitant' immunity or 'premunition' keeps the parasite load below the 'threshold of pathogenicity', and is commonly seen in adults in areas where

malaria transmission is high, such as sub-Saharan Africa, Papua New Guinea, Thailand, and the Solomon Islands (Druilhe and Perignon, 1994). Although this immunity is believed to develop slowly, and wane quickly if not frequently boosted by natural infections (Day and Marsh, 1991), studies of migrant workers from Java (a region of low malaria transmission) residing in Irian Jaya (i.e. Indonesian New Guinea, an area of high malaria transmission) have shown that natural immunity can develop in adults within 12-24 months (Baird et al., 1991). Frequency and density of parasitemia in adult newcomers from Java was equal to that of lifelong residents of Irian Jaya, suggesting that the immigrant population was able to build up an antitoxic immunity in a brief period of time.

Antibodies mediate immunity to asexual parasite stages

The role of anti-parasite antiserum in malaria infections was first discovered in 1917 with the clinical improvement of malaria patients following injection with sera from naturally immunized donors (Sotiriades, 1917). In 1961, Cohen and colleagues showed that passive immunotherapy using the IgG fraction of pooled West African adult immune serum protected children from disease symptoms (Cohen et al., 1961). This immunity was directed towards late stage schizonts or merozoites, was short-lived, and sometimes was only partially effective, with a secondary rise of parasitemia being noted (Cohen and McGregor, 1963). This recrudescence was interpreted as the emergence of a serologically distinct population of parasites, perhaps a result of antigenic variation, as discussed below. Despite the apparent strain specificity of some immune sera, strain transcending immunity was also noted in experiments where West Africans pooled IgG protected East Africans (McGregor et al., 1963) and Thai malaria patients in a manner similar if not better than that achievable with antimalarial drugs (Sabchareon et al., 1991).

Experimental malaria infection in mice has shed some light on the role of T cells in the human immune response to malaria

The use of murine malaria models to determine the role of T cells in the immune response is a logical choice because of the detailed information available on the mouse immune system and the availability of many different kinds of mouse strains and parasites. Murine malaria parasites can be divided into those causing lethal disease, such as *P. berghei*, *P. vinkei*, *P. yoelli*, and some strains of *P. chabaudi*, and those causing infection which can be resolved such as *P. chabaudi chabaudi*, *P. chabaudi adami*, and *P. vinkei petteri* (Cox, 1988). Non-lethal parasites such as *P. c. chabaudi* serve as excellent models for dissecting the role of T cells in immunity to malaria. This parasite has many features in common with *P. falciparum* in that it infects RBCs and undergoes sequestration, although infected RBCs lack knobs, and sequester primarily in the liver (Cox et al., 1987).

T helper cells are key thymus-derived lymphocytes which respond to antigens presented in the context of the MHC class II molecules on antigen presenting cells by producing distinct cytokines capable of mobilizing antigen-specific cellular (cytotoxic T lymphocytes, CTLs) and humoral (antibody) effector arms of the immune system. The importance of T helper cells was shown in the inability of thymectomized mice to resolve *P. chabaudi* infection (McDonald and Phillips, 1978), the transfer of resistance to infection from immune to lethally irradiated mice via T cell adoptive transfer experiments (McDonald and Phillips, 1980), and the susceptibility of TCR αβ- knock-out mice (lacking the T cell receptor) to infection (Good and Zevering, 1994). Depletion experiments using monoclonal antibodies specific for both CD4+ and CD8+ cells showed that CD4+ cells are critical for acquired immunity in mice (von der Weid and Langhorne, 1993), and that the acquired immunity is driven by CD4+ Th1 (T helper type 1) responses initially, followed by CD4+ Th2 responses (see next section) (Langhorne, 1989). While adoptive transfer of CD4+ T cells from naive or infected mice was shown to prevent death due to infection in

SCID mice, the chronic parasitemia that resulted could only be resolved by the transfer of both B cells and CD4+ T cells (Meding and Langhorne, 1991).

Immunity to *Plasmodium* is a two step process involving an early Th1 response followed by a Th2 response

In the late 1980s, two different T cell responses were defined among a panel of mouse T cell clones that displayed distinct cytokine secretion patterns: one group expressed IL-2 and IFNγ, called Th1, the other expressed IL-4, 5, 6, and 10, and was labelled Th2 (Mosmann et al., 1986; Cherwinski et al., 1987). In general, Th1 immune responses involved activation of antigen specific CTLs that kill cells presenting foreign antigen in the context of MHC class I; Th2 responses involved activation of antigen-specific B cells and production of antibody. Although these specific cytokine profiles may be distinct in T cell clones and observable in inbred mouse strains, a more complex picture of cytokine profiles and immune responses probably exists in natural populations of mice and humans.

The immune response to experimental malaria infections in mice is believed to involve an early Th1 response followed by a Th2 response. The Th1 response is a non-specific and antibody independent process, involving the production of IFNγ and TNFα, which stimulate the production of reactive nitrogen (NO) and oxygen intermediates by macrophages (Galinski and Barnwell, 1996). NO concentrations measured by serum nitrate have been shown to increase to a maximum at peak parasitemia (Taylor-Robinson and Phillips, 1993), and the NO synthase inhibitor L-N-monomethylarginine (L-NMMA) partially inhibits the protective effects of NO. At least two other mechanisms whereby TNFα inhibits the growth of *Plasmodium* blood stages may exist. Work with human neutrophils has shown that activation of these cells by IFNγ and TNFα results in increased phagocytosis and killing of merozoites and parasitized RBCs (Phillips, 1994). Fever caused by the action of TNFα and other cytokines may also inhibit the growth of *P. falciparum* parasites, as the death of trophozoites and schizonts grown in culture at 40°C

was higher than those grown at 37°C (Kwiatkowski, 1989). Thus, the initial Th1 response to parasite blood stages involves a nonspecific inhibition of parasite growth through the action of cytokines such as IFNγ and TNFα.

The release of TNFα is both beneficial and detrimental to the host

The release of proinflammatory cytokines such as TNFα in the early Th1 response to blood stages of *P. falciparum* can create as much harm as it does good. Observation of cancer patients undergoing TNFα trials revealed that the symptoms resulting from TNFα treatments were similar to those of malaria, including fever, rigors, headaches, nausea, vomiting, and thrombocytopenia (Clark and Rockett, 1994). TNFα is a potent pyrogen (Dinarello et al., 1986), and serum TNFα levels have been shown to correlate with disease severity in malaria-infected individuals (Clark et al., 1989; Kwiatkowski et al., 1989). Serum TNFα levels were highest in Gambian children that died from severe cerebral malaria , intermediate in survivors, and lowest in children with uncomplicated malaria (Grau et al., 1989; Kwiatkowski et al., 1990). It was also shown that anti-TNFα monoclonal antibodies reduce TNFα levels, but neither prevent nor reverse cerebral malaria (Kwiatkowski et al., 1993). Studies using the murine malaria parasite *P. berghei* ANKA, a strain that reproduces the pathology of human cerebral malaria in mice, also showed that overproduction of TNFα can inhibit survival (Taverne et al., 1987), and that monoclonal antibodies specific for TNFα can reduce the severity of disease (Grau et al., 1987).

TNFα and cytoadherence are likely responsible for cerebral malaria

The concerted action of TNFα and parasite cytoadherence may be responsible for the uncommon yet frequently fatal condition of cerebral malaria. Cerebral malaria is a syndrome of unrousable coma affecting up to 5 % of infected individuals (mostly children and non-immune 'naive' adults), and killing up to 15% of those affected, even with treatment (Brewster et al., 1990). Factors affecting the chance of progression from mild disease symptoms to cerebral malaria include: 1) parasite strain specific expression of

cytoadherence proteins such as PfEMP1, rosettin, sequestrin, and modified band 3 protein, 2) parasite strain specific expression of antigens that stimulate TNFα release, 3) the genetically determined capacity for TNFα release by the host, and 4) the level of host concomitant immunity (Facer and Tanner, 1997).

Two separate hypotheses as to the cause of cerebral malaria have been postulated: 1) obstruction of the circulation in post capillary venules of the brain due to sequestered parasitized RBCs (MacPherson et al., 1985), or 2) disruption of neurotransmission due to TNFα stimulated NO production in cerebral endothelium (Clark et al., 1991). These mechanisms may actually both be working to promote disease. The expression of ICAM-1 and other cellular adhesion molecules, which are endothelial receptors for the parasite cytoadherence protein PfEMP1, are up-regulated by TNFα (Berendt et al., 1989; Berendt et al., 1992). The local upregulation of cellular adhesion molecules could lead to sequestration of parasitized RBCs, and the action of activated monocytes and other immune effector cells at these lesions may lead to local increases in neurotransmission, which in turn would interfere with more general neurotransmissions (Clark et al., 1991).

Individuals homozygous for TNF2 are more susceptible to the harmful effects of TNFα

The detrimental effects of TNFα release promoted by Th1-type responses may be influenced by the genotype of the host. It has been shown that two different TNFα alleles exist in humans (Wilson et al., 1992), and that the less common allele, TNF2 is associated with greater constitutive and inducible levels of TNFα (Wilson et al., 1997). Gambian children homozygous for this allele have been shown to have four times the risk of progressing to cerebral malaria than those with TNF1 (McGuire et al., 1994). Thus, although the Th1 response may be important in controlling the malaria infection, in certain instances it may lead to overproduction of cytokines with harmful consequences.

Th2 responses promote antibody-mediated protection against severe malaria

Whereas purified mouse splenic CD4+ T cells obtained before parasitemia has reached its peak produce high levels of IFNγ, followed by IL-2, CD4+ T cells obtained after parasitemia declines produce predominantly IL-4 and IL-10 (Langhorne et al., 1989; Taylor-Robinson and Phillips, 1992). This late-phase Th2 response to *P.chabaudi* infections in mice involves specific IgG1 antibodies, and blocking these antibodies diminishes the protection they confer (Taylor-Robinson and Phillips, 1993). The action of two different kinds of antibodies promoted by Th2 type responses in humans helps to overcome the symptoms of *P. falciparum* infections.

Antibodies specific for parasite 'endotoxins' block the harmful effects of TNFα release

One of the key observations from early malaria studies was that the onset of symptoms was linked to the asexual growth cycle of the parasite (Golgi, 1889), with the bursting of billions of synchronously infected RBCs and the release of parasite factors being responsible for induction of the host response. The release of TNFα by murine monocytes in *in vitro* cultures and *in vivo* was shown to be stimulated by *P. berghei* and *P.yoelli* heat stable antigens (Taverne et al., 1990b; Taverne et al., 1990a). Antibodies specific for these antigens block TNFα secretion, and prevent mortality due to high levels of TNFα (Taverne et al., 1990b). In addition, the TNF stimulating activity of these antigens was protease resistant, but sensitive to the activity of phospholipase C and other lipases, suggesting the stimulation was due to a phospholipid structure (Bate et al., 1992c). Antisera raised against *P.yoelli* TNF-stimulating antigens inhibited the TNF inducing activity of antigens of *P. falciparum* and *P. vivax* parasites (Bate et al., 1992a), as did anti-phosphatidyl inositol antibodies, suggesting that the malaria 'endotoxin' was parasite derived PI molecules (Bate et al., 1992b).

Parasite Glycophosphatidyl inositol (GPI) and GPI-linked proteins stimulate TNFα release

Autologous TNFα-stimulating *P. falciparum* antigens that have been identified include: RESA, MSP-1, MSA-2, and Ag7, a complex of heat stable soluble antigens (Kwiatkowski and Bate, 1995). Both MSP-1 and MSA-2 are anchored to the merozoite membrane through glycophosphatidyl inositol (GPI) linkages with C-terminal residues. Schofield and colleagues have shown that the loss of TNFα-inducing activity of MSP-1 and 2 correlates with the loss of GPI by digestion or chemical degradation, and that purified parasite GPI stimulates secretion of TNFα and IL-1 from human macrophages (Schofield and Hackett, 1993; Schofield et al., 1993). Patients infected with either *P. falciparum* or *P. vivax* make antibodies specific for phosphatidyl inositol (Facer and Agiostratidou, 1994), and these antibodies are capable of inhibiting TNFα induction by *P. falciparum* (Bate and Kwiatkowski, 1994). An overall picture of anti-toxic immunity has developed from these studies: namely that this immunity can be mediated by anti-PI antibodies specific for GPI-linked parasite proteins, and the parasite GPI moiety itself.

Parasite burden may be controlled by antibody mediated cellular inhibition (ADCI)

Although purified antibodies from individuals protected from severe malaria (Wahlin et al., 1984; Ahlborg et al., 1993), and antibodies generated against several asexual stage antigens such as MSP-1 (Pirson and Perkins, 1985), AMA-1 (Deans et al., 1984), RAP-1/2 (Schofield et al., 1986), and EBA-175 (Sim et al., 1990a) have been shown to block merozoite invasion of RBCs *in vitro*, direct correlation between the presence of invasion inhibitory antibodies and protection from disease symptoms or heavy parasite burdens has not been established (Druilhe and Perignon, 1994). Phagocytosis of free parasites or parasitized RBCs has been shown to have little influence on parasite growth *in vitro* (Druilhe and Khusmith, 1987), but the levels of antibodies promoting merozoite phagocytosis have been shown to closely correlate with protection (Khusmith

and Druilhe, 1983). These antibodies, when combined with monocytes (but not neutrophils, lymphocytes, or tissue macrophages) from either naive or immune recipients, inhibited the growth of parasites *in vitro*. When added to parasite cultures without monocytes, these antibodies had no effect. This phenomenon was termed antibody dependent cellular inhibition (ADCI) (Khusmith and Druilhe, 1983), and represented the first correlation between antibody mediated protection *in vivo*, and antibody mediated parasite inhibition *in vitro*. The ADCI hypothesis developed by Druilhe and colleagues describes the inhibition of intracellular parasites as a result of the action of monocytes stimulated by cytophilic antibody-merozoite complexes. While ADCI may reduce parasitemia, it is self-limiting in that it is not a long-lived response, and some merozoites are always needed to promote it (Druilhe and Perignon, 1994).

Cytophilic antibodies play a crucial role in ADCI

The role of cytophilic IgG1 and IgG3 molecules is central in the promotion of monocyte ADCI activity. This has been shown in studies describing profound differences in the ratios of cytophilic and non-cytophilic antibodies in protected and susceptible subjects. IgG1/ 3 isotypes were found to predominate in protected individuals, whereas IgG2, IgM isotypes predominated in susceptible subjects. Interestingly, the action of non-cytophilic antibodies from susceptible individuals was shown to interfere with the ADCI effect mediated by cytophilic antibodies (Bouharoun-Tayoun and Druilhe, 1992). Further experiments identified the merozoite surface antigens MSP-1 (Ferrante and Rzepczyk, 1997), MSA-2 (Taylor et al., 1995), MSP-3 (Oeuvray et al., 1994), and the RBC antigen RESA (Beck et al., 1995) as targets of cytophilic antibodies. One study showed that IgG3 isotype antibodies specific for MSA-2 predominated over IgG1 isotypes in protected individuals. It was hypothesized that these specific IgG3 antibodies, which have a relatively short half-life, and therefore decline rapidly if not continuously stimulated (as opposed to the IgG1 isotype, which predominates even after long lapses in the period of

immune stimulation), may be responsible for the short-lived immunity seen in protected individuals.

The role of complement in host defense against *P. falciparum*

The complement system is a highly regulated enzymatic cascade of the humoral immune system generating products that promote an inflammatory response and a lytic effector response against targets via a macromolecular membrane attack complex (Muller-Eberhard, 1988). A number of studies, reviewed in (Phillips, 1994), suggest that the role of complement in phagocytosis of parasitized RBCs by monocytes and neutrophils is unclear, although it may act in concert with antibodies via the classical pathway to stimulate the production of oxygen radicals by neutrophils in response to parasitized RBCs in *in vitro* experiments. Complement-mediated lysis of sexual stage parasites has been documented (Healer, 1997), and transmission-blocking antibodies specific for Pfs 230, a sexual stage vaccine candidate, work via a complement mediated mechanism (Williamson et al., 1995). Although a role for complement in immunity to both asexual and sexual stages has been has been revealed, more studies are needed to establish the specific mechanism of complement-mediated inhibition of asexual parasite growth *in vivo*.

A general scheme of immunity to *P. falciparum* infection

From these experiments, an overall picture emerges of immunity to malaria in mice and men. The main response to the initial assault of sporozoites on the liver probably involves Th1 type antigen-specific CTL responses to intrahepatic parasite peptides displayed by MHC class I proteins, rather than neutralizing antibody to specific sporozoite proteins such as CSP. This initial Th1 type response may serve to limit the number of merozoites that burst from hepatocytes, giving the immune defenses against blood parasites a 'fighting chance'. Presentation of processed blood-stage parasite antigens in the context of MHC class II molecules to CD4+ cells by antigen presenting cells stimulates the production of Th1 cytokines such as IL-12, IFNγ, and IL-2. IFNγ mediated activation of

macrophages and monocytes results in TNFα production, which may inhibit parasites indirectly by elevating body temperature, promote phagocytosis of parasites by neutrophils, and induce the production of reactive oxygen and nitrogen intermediates. Later in infection, as parasite-specific B cells start to expand, these cells efficiently capture the lower amounts of circulating parasite antigen and present it to CD4+ T cells. Here a Th2 response is favored, with the production of cytokines such as IL-4, 5, 6 and 10. This in turn promotes the expansion of antigen specific B cells and the elimination of residual parasitemia via specific anti-disease and anti-parasite antibodies (Taylor-Robinson, 1995).

Parasite strategies for evading the human immune response

Antigenic Diversity

In order to circumvent the immune responses of the host, malaria parasites have developed a number of immune evasion strategies. Different strains of malaria parasites synthesize homologous proteins that have slightly different amino acid sequences. This antigenic diversity has been postulated by some investigators to account for the development of immunity in individuals only after repeated infections over many years (Anders et al., 1993b; Reeder and Brown, 1996). Antigenic diversity may arise through a number of different mechanisms including mutations resulting in single amino acid substitutions (McConkey et al., 1990), and allelic forms of certain antigens that contain variable tandem repeats (Anders et al., 1993b). DNA recombination occurring in parasite sexual stages further promotes antigenic diversity by giving rise to new alleles (Kerr et al., 1994) and new parasite genotypes (Kemp et al., 1990).

Antigens with both minor and major polymorphisms have been documented in *P. falciparum*, and the lack of cross-reactivity between different antigenic types might explain the slow acquisition of immunity in humans. It has been shown that a series of point mutations restricted to two regions of Apical membrane antigen-1 (AMA-1), are responsible for the antigenic polymorphism between different AMA-1 alleles (Thomas et

al., 1990). AMA-1 was described earlier in this chapter as a 60 kDa antigen associated with the electron dense neck of the rhoptries of merozoites, and the surface of more mature merozoites (Peterson et al., 1989; Crewther et al., 1990). This protein consists of two domains separated by a transmembrane anchor: a C-terminal cytoplasmic domain, and an N terminal external domain containing 16 highly conserved cysteine residues that may stabilize the structure of AMA-1 through disulfide bridging. Alignments of five different *P. falciparum* AMA-1 sequences revealed a concentration of mutations in two main regions: in the 'hypervariable loop' between the first two of sixteen conserved cysteine residues, and within a 9 amino acid region in the C-terminal domain.

Two distinct merozoite surface antigens, MSP-1 and MSA-2, exhibit a more extensive form of antigenic polymorphism which includes changes in repetitive sequences. Both MSP-1 and MSA-2 are found on the surface of merozoites, and both are anchored through the attachment of glycophosphatidyl inositol to the C-terminus (Haldar et al., 1985; Smythe et al., 1988). Alignments of several MSP-1 alleles reveals 17 separate blocks of conserved, semi-conserved and variable sequences (Tanabe et al., 1987; Peterson et al., 1988). The variable sequences are dimorphic, and intragenic recombination at the 5' end of MSP-1 has resulted in the exchange of the two forms of variable blocks 2 and 4 between different alleles. The most conserved region of MSP-1 is at the 3' end of the gene which encodes the 19 kDa merozoite surface-anchored fragment containing the EGF repeats described above (Blackman et al., 1991).

MSA-2 is also a dimorphic antigen, but this dimorphism occurs in the large central variable region of the protein (Thomas et al., 1984). This region is composed of repetitive sequences flanked by non-repetitive variable sequences. One form of MSA-2 is characterized by tandem repeats of 12 and/or 32 amino acids, whereas the other has shorter glycine or alanine-rich tandem repeats. Different alleles of the same dimorphic family encode a different number of tandemly arranged repeats, directly contributing to the observed variation in the size of different MSA-2 forms (45-55 kDa). Intragenic

recombination between similar sequences within the repeat regions also confers a further degree of diversity on these alleles (Marshall et al., 1991).

A much greater amount of antigenic diversity has been attributed to S-antigens, soluble proteins synthesized by mature asexual parasites and released upon iRBC rupture (Wilson et al., 1975; Wilson, 1980; Anders et al., 1983). The molecular basis of S-antigen diversity has been reviewed by Anders (Anders et al., 1993a). Comparative sequence analysis of many different S-antigen genes reveals a central region of multiple tandem repeat sequences flanked by conserved regions. The tandem repeats, present in up to 100 copies in some S-antigens, vary in length, sequence, and number, but are strongly conserved within a single antigen. It has been proposed that S-antigen allelic variants have arisen from point mutations, insertions, deletions, and even frameshift mutations (Brown et al., 1987; Saint et al., 1987).

Antigenic variation

In addition to having strain specific variants of homologous proteins, malaria parasites can evade human immune responses by switching antigenic phenotype during the ongoing process of infection. This antigenic variation was first identified in African trypansomes, which switch antigenic forms of variable surface glycoprotein, VSG, through genomic rearrangements of non-expressed VSG genes to active expression sites (Pays and Steinert, 1988). Trypanosome parasites can thus circumvent an immune response generated against one antigenic form of VSG by switching to a novel one. Antigenic variation was first described in experimental *P. knowlesi* infections of rhesus monkeys (Brown, 1965). Monkeys infected with this parasite die within a week of infection, but if drug cured, go on to develop a strain-specific immunity. On reinfection with the same strain, a population of parasites can be recovered that are not recognized by the original strain-specific antiserum, as tested by *in vitro* agglutination experiments. Antigenic variation was shown in *P. falciparum* with the spontaneous emergence of serologically distinct parasites in *in vitro* cultures that are not subject to immune selective

pressures normally present within the host (Biggs et al., 1991; Roberts et al., 1992). Distinct antigenic variants arose at an estimated rate of 2% per generation, and the observed antigenic switching was accompanied by changes in the size of PfEMP1 (Roberts et al., 1992), the high molecular weight protein associated with knob structures and believed to play a role in cytoadherence (Leech et al., 1984). Further studies addressing the co-modulation of antigenic variation and cytoadherence demonstrated that cloned parasite lines with different cytoadherence profiles expressed PfEMP1 proteins of widely variable molecular weights (Biggs et al., 1992).

In 1995 two teams cloned the genes encoding PEMP1 by separate approaches. One group, using antiserum specific for a distinct PfEMP1 protein, cloned two different PfEMP1 genes from cDNA and genomic DNA libraries (Baruch et al., 1995); the other group, while looking for chloroquine resistance genes, unexpectedly discovered a series of genes having homology to EBA-175, the glycophorin A-binding protein expressed on the surface of merozoites (Su et al., 1995; Smith et al., 1995). Through the combined efforts of these groups, the discovery of a large and varied family of sequences, called *var* genes, was fully realized.

The *var* genes are present in 50-150 copies or 2.6% of the entire haploid genome, representing a considerable genetic investment. Although the *var* genes encode antigenically diverse proteins, they share a common general structure. This consists of a highly conserved N-terminal region (DBL1) with homology to the Duffy binding-like domains of *P. falciparum* EBA-175, and *P. vivax* Duffy blood group-binding receptor, followed by a conserved cysteine interdomain region (CIDR), one to four extra DBL (DBL2,3,etc.) domains, a transmembrane region, and a cytoplasmic acidic terminal segment (ATS). It has been suggested that the conserved DBL1/CIDR region may be involved in adherence, whereas relatively conserved central DBL domains play a secondary structural role. Highly variable regions between these DBL domains may mask immune recognition of the conserved structural regions of these proteins (Reeder and Brown,

1996). Although no evidence exists for a specific mechanism of antigenic switching amongst different *var* genes, work with yeast artificial chromosome mapping of *P. falciparum* chromosome 12 shows that *var* genes are present at both central and sub-telomeric sites, and more importantly, are interspersed amongst repetitive elements in recombination 'hotspots' (Rubio et al., 1996; Corcoran et al., 1988).

Cross-reactive immunodominant repeats: The smokescreen effect

Some studies have suggested that cross-reactive tandem repeats within certain parasite proteins may help to divert an active immune response away from sensitive parasite epitopes. Four classes of immune cross-reactivity exist between *P. falciparum* antigens: between different epitopes within one protein, between epitopes of different proteins from the same life cycle stage, between epitopes of different proteins from different life cycle stages, and through the expression of the same antigen during different life cycle stages (Moelans and Schoenmakers, 1992). Examples of these cross reactivities include the circumsporozoite protein CSP and Exp-1 expressed during blood stages (Coppel et al., 1985), RESA and a number of other asexual stage antigens, and S-antigens (Moelans and Schoenmakers, 1992; Schofield, 1991). A hallmark of these tandem repeat structures is their immunodominance: when sera from immune individuals exposed to sporozoites is absorbed with the NANP repeat peptide of the circumsporozoite peptide, all reactivity to sporozoites is lost (Zavala et al., 1985). Similar findings from absorption experiments using both RESA and S-antigen repeat peptides and blood stage parasites confirms the immunodominance of these repeats. The parasite may incorporate tandem repeat structures close to epitopes responsible for host cell invasion or cytoadherence as a means to evade the immune response.

Two separate hypotheses have arisen describing the role of these immunodominant regions: 1) in the prevention of affinity maturation of antibodies specific for key epitopes due to the existence of a large number of repeats that cross react with similar structures on heterologous proteins (Anders, 1986) , or 2) the promotion of an inefficient T-independent

B-cell response to proteins displaying these tandem repeats (Schofield, 1991). While little evidence supporting the first hypothesis has been obtained, work with *P. berghei* sporozoite immunization in mice suggested that parasites do induce a T-cell independent B-cell response to CSP NANP repeats (Schofield and Uadia, 1990). T-cell independent responses occur by cross-linking of B-cell surface Ig by repetitive structures such as polyacrylamide, bacterial carbohydrates and lipopolysaccharide, collagen and flagellin (Schofield, 1991). This cross-linking enables the B-cell to respond to antigen without interaction with T cells through the MHC II/TCR complex, or stimulation from T-cell derived cytokines. This response is often short lived, restricted to mainly to IgM type antibodies, lacks any affinity maturation or memory components, and may lead to generalized polyclonal activation, a hallmark of malaria infection.

The development of malaria vaccines

The development of malaria vaccines has been met with limited success at best, due to some of the factors outlined above including the parasite mechanisms of immune evasion, and the complex nature of the human immune response to infection. The current wisdom on this topic suggests that a successful vaccine must be a multicomponent vaccine, eliciting immune responses against several stages and by several mechanisms, such as: anti-sporozoite antibodies for preventing hepatocyte invasion, CTL responses specific for intrahepatic parasites, anti-merozoite antibodies for preventing RBC invasion, antibodies capable of killing parasitized RBCs and preventing cytoadherence of parasitized RBCs, antitoxic antibodies for preventing harmful host cytokine-mediated disease symptoms, and antibodies capable of preventing sexual stage parasite transmission to the mosquito vector.

Sporozoite vaccines

Immunization of volunteers with irradiated *P. falciparum* or *P. vivax* sporozoites gave the first hope that protective malaria vaccines could be developed (Clyde, 1975). Invasion competent yet replication deficient irradiated sporozoites, when given to human

volunteers conferred a complete strain-specific protection that required repeated boosting in order to remain effective. It was shown that the irradiated sporozoites were capable of invading hepatocytes, but remained as undivided parasites within the hepatocyte (Ramsey et al., 1982). Because of the difficulties in obtaining large amounts of healthy sporozoites for human immunization, interest turned to vaccines containing sporozoite components such as the CS protein and SSP-2/TRAP, discussed earlier in this chapter. While rodent trials showed that antibodies specific for immunodominant CS repeat NANP mediated protection (Nussenzweig and Nussenzweig, 1989a), human trials using this repeat motif as an immunogen were less successful (Fries et al., 1992; Herrington et al., 1987); high antibody titres to repeats and inhibition of hepatocytes in *in vitro* studies did not correlate with protection.

The failure of other synthetic and recombinant NANP vaccine candidates (Ballou et al., 1987) suggested an importance not only in the immunogen, but the adjuvant used in the vaccine formulation. This has been emphasized in recent studies using CS NANP peptide fused to hepatitis B surface antigen (HBsAg), and emulsified in an oil containing the immune stimulants monophosphoryl lipid A and QS21 (Stoute et al., 1997). This vaccine protected six of seven volunteers from challenge with infected mosquitoes, the best efficacy rating of any malaria sub-unit vaccine to date.

Vaccines aimed at inducing CTL responses against sporozoite and liver stage antigens

Early studies with mouse malaria parasites implicated MHC class I restricted CTL activity in protection against sporozoite challenge through destruction of infected hepatocytes (Romero et al., 1989; Hoffman et al., 1989). HLA class I restricted CTL responses to parasite proteins *in vitro* have been identified in Africans, although at a relatively low level (Hill et al., 1991b). CTL epitopes have been identified in four pre-erythrocytic proteins: 1) the CS protein, 2) sporozoite surface protein 2 (SSP-2), a

polymorphic sporozoite surface protein that may function in hepatocyte recognition (Charoenvit et al., 1987), 3) liver stage antigen 1(LSA-1), a protein residing in the parasitophorous vacuole of liver stage parasites (Zhu and Hollingdale, 1991), and 4) STARP, a recently discovered novel liver and sporozoite expressed antigen (Aidoo et al., 1995). Although excitement has been generated over the effectiveness of vaccines stimulating CTL responses, it has been tempered by the finding that polymorphisms within CTL epitopes may lead to loss of CTL recognition due to inefficient loading of peptides onto HLA class I molecules (Lalvani et al., 1997).

Asexual stage vaccines

By far the most popular asexual stage vaccine has been Spf66, a chemically synthesized vaccine comprising peptides from asexual blood and pre-erythrocytic stages of the parasite (Patarroyo et al., 1988). Patarroyo and colleagues performed N-terminal protein sequencing on a large number of *P. falciparum* proteins, synthesized the corresponding peptides, and for each peptide performed separate immunization and challenge studies on *Aotus* monkeys. Those peptides which gave some protection were combined in the final synthetic vaccine. Peptides included in the Spf66 vaccine included the highly conserved region 45-53 derived from the 83 kDa cleavage product of MSP-1, sequences from two anonymous blood stage antigens of 55 and 35 kDa, and two NANP repeat sequences from the CS protein, added in part to create hairpin bends in the chimaeric peptide. Cysteine residues were attached to each end of the chimaeric peptide to effect polymerization through oxidation. The final 50 kDa polymer was adsorbed to aluminum hydroxide adjuvant (Lopez et al., 1994).

Vaccine trials conducted with Spf66 in both South America and Africa have shown that the vaccine reduces the risk of first time episodes of malaria by 27 %, and the total number of malaria episodes by 38 % (Facer and Tanner, 1997). Examination of individual trials reveals a striking difference between protection in individuals in Latin America and Africa. In Colombian trials, an overall protection from first malaria episodes of 38.8% was

achieved, with an observed efficacy of 77.2% in children under the age of 5 (Valero et al., 1993). Although a Tanzanian trial with 586 children showed partial protection (efficacy of 31 %) (Alonso et al., 1994), other subsequent trials with children in the Gambia (D'Alessandro et al., 1995), and Thailand (Nosten et al., 1996) failed to protect children aged 2 to 15 years. Although the reasons for differences between the Colombian and Gambian/Thai trials are undetermined, it has been postulated that differences in transmission rates, parasite populations, vaccinee ages, and pre-existing immunity may be responsible (Facer and Tanner, 1997).

MSP-1, MSA-2 and RESA, all of which show some degree of protection against parasite challenge, have undergone safety and immunogenicity trials in Australia and Papua New Guinea, and are being prepared as a combination vaccine for phase III efficacy trials in children in Papua New Guinea. Immunization with these blood stage antigens has been accomplished in animal studies with encouraging results. The MSP-1 19 kDa merozoite-associated fragment, though immunogenic in mice, failed to protect *Aotus* monkeys from challenge with heterologous or homologous *P. falciparum* strains (Facer and Tanner, 1997). *Aotus* and *Saimiri* monkeys immunized with whole recombinant MSP-1 protein but not peptides from MSP-1 conserved regions were protected from parasite challenge (Deans et al., 1988). Mice immunized with MSA-2 conserved sequences are protected against an otherwise lethal challenge of asexual *P.chabaudi* parasites (Saul et al., 1992). Although monoclonal antibodies specific for RESA inhibit merozoite invasion *in vitro*, monkey vaccination experiments using full-length recombinant RESA protein or RESA subfragments provides only partial protection from challenge (Collins et al., 1991). Other blood stage vaccine candidates include the RAP1/2 protein complex of rhoptries and AMA-1, which protect *Saimiri* monkeys from parasite challenge (Ridley et al., 1990a; Howard and Pasloske, 1993) and EBA-175, which contains regions that may be the target for invasion blocking antibodies (Sim et al., 1990a).

Another vaccine candidate, NyVac7 (Tine et al., 1996), incorporates a number of parasite proteins including CSP, SSP-2, LSA-1, MSP-1, AMA-1, SERA, and Pfs25 into a live attenuated vaccinia vector (Tartaglia et al., 1992). This vaccine is well-tolerated in *Rhesus* monkeys, and antiserum from immunized monkeys recognizes CSP, SSP-2, MSP-1 and Pfs 25. No protection data has been published for this vaccine.

DNA vaccines

Although DNA had been delivered into mammalian somatic tissues by *in vivo* infection using recombinant retroviral vectors (Dubensky et al., 1984), by encapsulation of DNA in liposomes (Kaneda et al., 1989), or by complexing DNA to protein carriers (Wu et al., 1989), the use of naked DNA alone to immunize animals was not developed until 1993 through collaborative work done by Merck Inc. and Vical Inc. scientists. The discovery that injection of naked DNA encoding influenza nucleoprotein into the quadricep muscles of mice elicited both specific CTL and antibody responses to nucleoprotein, and was protective against challenge with influenza A virus (Ulmer et al., 1993), opened up a new field of vaccination called DNA immunization. DNA vaccines satisfy some of the important criteria for a successful malaria vaccine: they can be multi-subunit vaccines consisting of a number of different sequences, injected DNA can persist for long periods of time providing continuous immune stimulation (Wolff et al., 1992), they can elicit both T and B cell responses (Ulmer et al., 1993), they are cheap and easy to make, and they do not require refrigeration to maintain their integrity. The drawbacks of DNA immunization include the potential to elicit harmful anti-DNA antibody responses and the potential for injected DNA to activate oncogenes by integrating into the host chromosome (Robertson, 1994; Griffiths, 1995; Nichols et al., 1995).

DNA vaccine experiments with murine *P.yoelli* homologs of *P. falciparum* genes such as CSP, SSP-2, Exp-1, and MSP-1, have given some insights into the importance of restricted CTL mediated immunity in protecting mice. DNA immunization of mice differing in H-2 haplotypes with CSP, SSP-2 or Exp-1 constructs alone only protected a fraction of

the inbred strains tested, and this protection was due to the effects of CD8+ T cells, interferon gamma, and nitric oxide. Combined immunization with CSP and Exp-1 DNA resulted in protection of a much greater fraction of these strains (Sedegah et al., 1994; Doolan et al., 1996). This data emphasizes the need to include multiple parasite sequences in vaccination of a genetically heterogenous population such as humans. DNA immunization experiments with this construct in monkeys has revealed the induction of high-titre antigen specific antibodies, and "Phase I" clinical trials, designed to test safety and immunogenicity trials are now underway in humans (Facer and Tanner, 1997).

Transmission blocking vaccines elicit parasite-specific antibodies that kill parasites within the mosquito vector

Transmission blocking vaccines provide no protection for the infected host, but are intended instead to curb transmission of the parasites. By contrasting some key parameters of the asexual and sexual stages of replication, it is evident that transmission blocking vaccines capitalize on a number of weaknesses of sexual stage parasites: the number of parasites required to complete the life cycle decreases from billions in the asexual stages, to hundreds in the sexual stages; sexual stage parasites are extracellular for hours as opposed to seconds; and many sexual stage antigens are not expressed in the human host, and thus are not subject to immune selection (Kaslow, 1997). Transmission blocking vaccines are intended to work in conjunction with other preventative measures such as insecticide treated bednets, chemotherapy, and other vaccines, reducing transmission to a rate that would make an otherwise partially effective measure highly effective.

Both pre-fertilization and post-fertilization parasite proteins are being considered as candidates for a transmission blocking vaccine. Pre-fertilization vaccines are aimed at killing gametes in the gut of the mosquito, or preventing them from fusing to form a zygote. The lead pre-fertilization vaccine candidates are Pfs 230 (Williamson et al., 1993), and Pfs 48/45 (Kocken et al., 1993). Pfs 230 is a 310 kDa protein expressed on the

surface of gametes. This protein is processed from its 363 kDa precursor form by cleavage of an N-terminal 50 kDa region containing immunodominant GGNV repeats (Williamson et al., 1996; Riley et al., 1995). Pfs 48/45 is present on the surface of gametes as two GPI anchored proteins of 48 and 45 kDa (Kocken et al., 1993). Although no function has been attributed to either protein, both share a number of cysteine rich domains, which may mediate interaction between the two (Carter et al., 1995; Kaslow, 1997). Antibodies directed against recombinant proteins expressing two of the cysteine containing motifs of Pfs 230 blocked transmission by a complement mediated method (Williamson et al., 1995); monoclonal antibodies specific for B cell epitopes of Pfs 48/45 block fertilization in the absence of complement (Targett et al., 1990). No clinical or pre-clinical vaccine trials have been designed for these antigens yet.

Post-fertilization vaccine candidates prevent the maturation of parasite sexual stages

Two major post-fertilization vaccine candidates include Pfs28 (Duffy et al., 1993), and Pfs25 (Kaslow et al., 1988). These vaccines work by preventing the transformation from the zygote into the motile ookinete form. Higher titres of antibodies are required for this type of vaccine, because proteolysis of the antibodies in the mosquito mid-gut is likely to occur in the time it takes for these later stage antigens to be expressed (Kaslow, 1997). Pfs25 is a 25 kDa GPI-anchored protein expressed in zygote and ookinete sexual stages. It contains four cysteine rich EGF-like domains, the third domain overlapping with part of the major B-cell epitope. Efforts to elicit conformation dependent anti-Pfs25 antibodies that block transmission have failed when prokaryotic systems have been used to express recombinant Pfs 25 (Kaslow and Shiloach, 1994). Pfs28 is a 28 kDa antigen expressed on *P. falciparum* zygotes at a later stage than Pfs25. It was discovered as a homologue of Pgs28, the 28 kDa *P. gallinaceum* protein containing EGF repeats and responsible for inducing strong transmission blocking immunity in chickens (Duffy et al., 1993).

Responses to these two antigens expressed sequentially on zygote stages may result in a much more potent transmission blocking immunity. The transmission-blocking vaccine TBV25H, consisting of a recombinant 6-histidine tagged Pfs25 protein produced in *Saccharomyces cerevisiae*, has been shown to elicit short-lived transmission blocking immunity, and is being prepared for safety and immunogenicity trials in humans (Kaslow, 1997).

Summary of malaria biology, pathology, and immunology

In summary, both the disease process and the human response to malaria infection is complex. While many years of observations regarding naturally acquired immunity in humans reveals that age and frequency of exposure to the parasite are key determining factors in the degree of morbidity and mortality for a given population, many important questions still remain regarding the specific parasite molecules involved in generating a protective immune response, and in promoting the severe cerebral pathology. No specific markers for protection have been discovered. Although vaccine candidates such as Spf66 have shown a moderate degree of protection, many others have failed. The best vaccine for this disease will most likely be a cocktail of a number of different proteins expressed at different life cycle stages, reflecting the complexity of the malaria parasite.

It is also evident that the immune response to malaria parasites has a significant antibody component, specific for an incompletely defined set of parasite proteins. Although the importance of proteins such as MSP-1, AMA-1, MSA-3, PfEMP1, has been revealed through *in vitro* studies and animal immunization, the degree of protection conferred by immune responses to these antigens remains questionable, partly due to immune evasion mechanisms employed by the parasite to circumvent a specific immune response. The fact still remains that individuals can mount a successful anti-parasite and anti-disease immunity, and that this immunity, at least in part, is mediated by antibodies specific for parasite molecules. This provides motivation for attempting to characterize the

major parasite antigens which can be recognized by antibodies from immune individuals, using a variety of expression cloning strategies.

B. Using COS cell expression and antibody panning to clone novel *Plasmodium falciparum* genes

The importance of shape

It is evident that many *P. falciparum* antigens must maintain a native conformation in order to carry out their function. For example: 1) the EGF domain of the 19 kDa merozoite surface-associated MSP-1 fragment is conformation dependent (Spetzler et al., 1994); 2) AMA-1, localized in the neck of the rhoptry as well as the merozoite surface, contains a set of complex disulfide bonded motifs (Hodder et al., 1996), and 3) the Duffy binding-like regions of EBA-175 (Sim et al., 1994) and Pf EMP1 (Baruch et al., 1995) both contain conserved cysteine-rich regions that may be important in RBC binding and cytoadherence respectively. Antibodies from protected individuals probably recognize a number of different proteins, and may be generated predominantly to conformationally constrained regions, at least for some of these proteins.

COS cell expression cloning has two main advantages for cloning *P. falciparum* genes

In the past many important *Plasmodium falciparum* antigens have been characterized and cloned using one or more of four different strategies: 1) direct purification of proteins from parasite lysates (eg. MSP-1) (Siddiqui et al., 1978), 2) screening of parasite DNA expression libraries (plasmid or λ vectors) in *E. coli* using human immune or more specific antiserum (eg. MSP-1(Hall et al., 1984; Holder et al., 1985), CSP (Ellis et al., 1983), and S-antigen (Coppel et al., 1983), 3) probing of gene libraries using oligonucleotides designed from known protein sequence or from segments

of protein sequence known to be conserved between species (e.g. dihydrofolate reductase thymidylate synthase; DHFR (Bzik et al., 1987)), or 4) PCR amplification of genomic DNA using oligonucleotides designed from known protein sequence or from segments of protein sequence known to be conserved between species.

The identification of transformed *E. coli* colonies expressing parasite antigens of interest may be hampered by the fact that the recombinant antigens are unlikely to be folded in the same way as the native parasite antigens. In order to circumvent this problem, Elliott and Howard attempted to clone *P. falciparum* genes by eucaryotic expression cloning using COS cells and antibody panning (Seed and Aruffo, 1987; Aruffo and Seed, 1987; Elliott et al., 1990). This process is summarized in figure 1.1, and explained in more detail below. In comparison to bacterial expression cloning, the use of eukaryotic expression cloning should allow for the production of parasite proteins in a relatively more native state. Thus eukaryotic expression cloning is more likely to be successful in cloning genes that encode parasite antigens with conformationally dependent epitopes, and some of these will be important targets of a protective immune response.

COS cell expression cloning also has a second potential advantage. This relates to the fact that any parasite gene which is selected for must encode a parasite protein or glycoprotein which is attached to the surface of the COS cell (see figure 1.1). In other words, by its very nature COS cell expression cloning and antibody panning automatically selects for genes encoding parasite antigens which are attracted to a membrane. Most typically, such proteins would contain a hydrophobic N-terminal signal sequence (also called leader peptide) which would target the protein into the rough endoplasmic reticulum and golgi, and a more C-terminal hydrophobic transmembrane segment which serves to anchor the protein in the cytoplasmic membrane. Alternately there may be a hydrophobic segment at the extreme C-terminus of the protein, which serves as a signal for cleavage and covalent linkage of the protein to a glycosylphosphatidylinositol lipid membrane anchor, such as is found for the mammalian decay accelerating factor (DAF) protein. Among all

possible parasite antigens, by their nature those parasite proteins or glycoproteins which are attached to membranes are more likely to represent targets of protective antibodies, particularly if they appear on the surface of the merozoite or on the surface of infected erythrocytes.

Rescuing cDNA clones by expression in COS cells and antibody capture

As shown in figure 1.1, COS cell panning typically utilizes transient expression of cDNA libraries in mammalian COS cells (an African green monkey kidney cell line (Gluzman, 1981) and the rescue of specific clones by antibody capture and panning (Simmons, 1993). COS cells transfected with DNA libraries initially express perhaps hundreds of different recombinant proteins, depending on the numbers of different plasmids that are present in each cell. Cells expressing immunogenic molecules anchored to the cell surface can be enriched using monoclonal antibodies, and after a number of rounds of transfection, selection by panning, and amplification of the selected plasmids in *E. coli*, the specific gene (typically a cDNA) expressing the antigen of interest can be obtained. Essentially panning minimizes the work involved in searching for a target cDNA by using an antibody to rescue it. COS cell panning has been used in the past to isolate a number of important cDNA sequences, most of which are reviewed by Simmons (Simmons, 1993). Some of these include: CD2, CD58, CD28, ICAM-1 and 2, ELAM-1, CD22, CD44, CD36, and VCAM-1. In most cases, a monoclonal antibody was available to help pan out the cDNA of interest, although sometimes the molecule's ligand or even cells themselves were used as panning reagents.

Antisera collected from individuals living in malaria endemic regions contain antibodies that are specific for a number of proteins including parasite antigens. In 1989 Elliott and Howard used a pool of hyperimmune serum from 3 individuals living in a malaria endemic region (rural Ghana) to select COS cells which had been transfected with *Plasmodium falciparum* genomic libraries cloned in a novel eukaryotic expression vector called pJFE14. After three rounds of panning two predominant genes were selected for,

which were named Pf7 and Pf12. Upon DNA sequencing, Pf7 turned out to be a strain variant of a known merozoite surface antigen, MSA 2. Pf12, on the other hand, appeared to encode a completely new parasite antigen, which was unlike any protein present in the GenBank database (Elliott et al., 1990).

The nature of Pf12

The Pf12 gene cloned by Elliott contained a 1044 base pair open reading frame which encoded a cysteine-rich 39.4 kDa protein (figure 1.2). The protein sequence displayed an N-terminal hydrophobic leader peptide (likely cleavage after Cys23), and a C-terminal hydrophobic segment which is typical of a signal for cleavage and covalent attachment to a glycosylphosphatidylinositol lipid membrane anchor. The hydropathy plot for Pf12 is shown in figure 1.3. This was all the information about Pf12 that was available when I began my Ph.D. thesis project in 1990.

C. Goals of this thesis project

This thesis project had a specific goal as well as a more general goal. The specific goal was to further characterize the Pf12 antigen which had just been cloned by Dr. Elliott. Initially we wished to know when in the parasite life cycle the Pf12 protein was expressed, and in what sub-cellular location the antigen would be found. Besides satisfying our basic curiosity, characterization of the Pf12 antigen was important to the field of malaria vaccine development because: a) it was a novel membrane-associated antigen that could potentially have utility in a vaccine, and b) subsequent discoveries showed that Pf12 was related, by virtue of its unique disulfide-bonded domain structure, to Pfs 230 and Pfs 48/45, two sexual stage antigens considered to be promising candidates for a malaria transmission-blocking vaccine.

The more general goal of this thesis was to develop a method or methods whereby novel membrane associated *P. falciparum* antigens which were cloned by COS cell

expression and antibody panning could be simply and efficiently characterized. This characterization would be with respect to stage of expression, sub-cellular site of expression, and appropriateness (or not) for inclusion in a potential malaria vaccine. The initial part of this aspect of the project involved using the COS cell expression and antibody panning method to isolate my own novel *P. falciparum* genes.

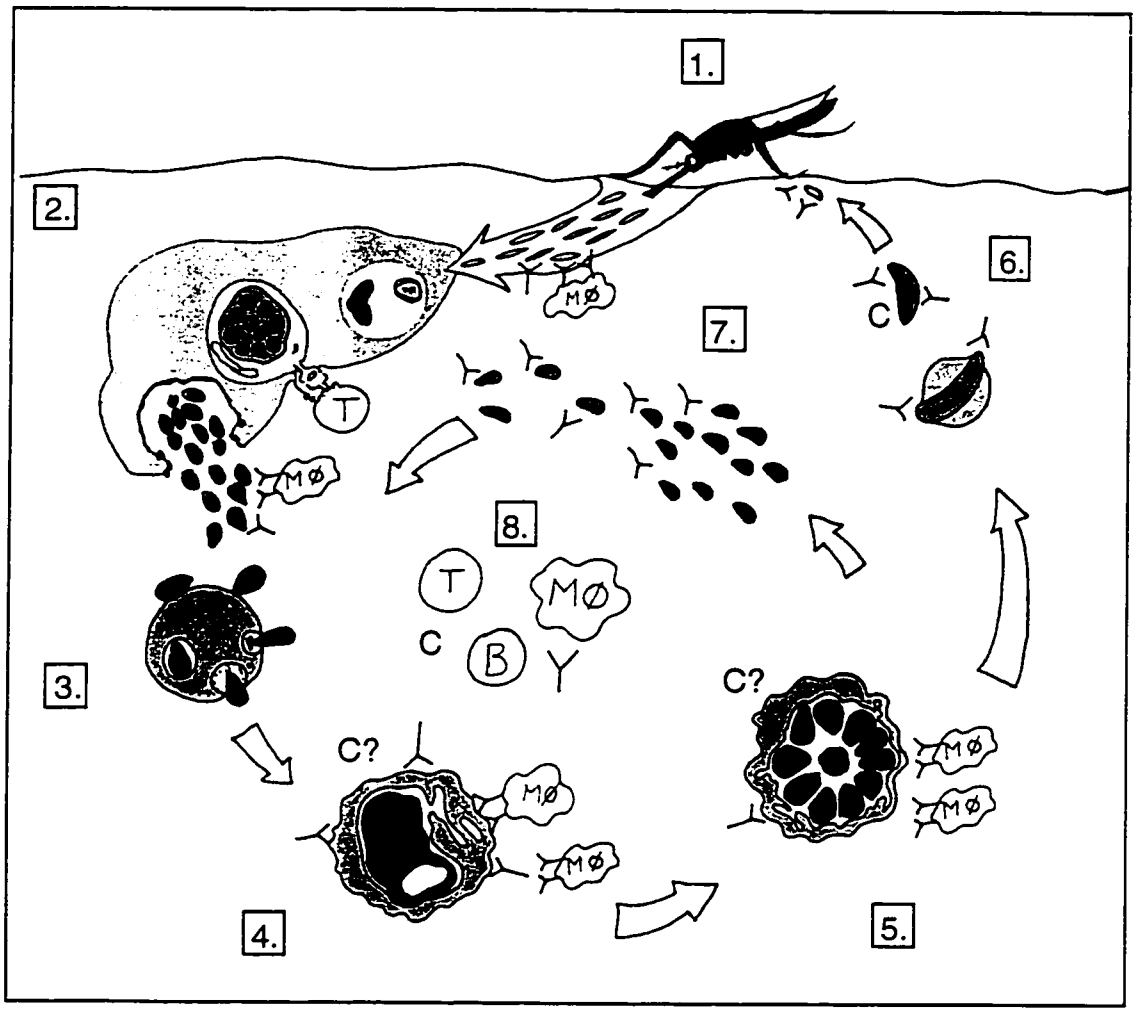

FIGURE 1.1. The life cycle of *Plasmodium falciparum.* (1) Sporozoites are injected into the bloodstream when a female *Anopheles* mosquito takes a bloodmeal. (2) Sporozoites invade liver cells and differentiate into RBC-infective merozoites. (3) Merozoites invade RBCs by binding to the RBC and discharging proteins and lipid complexes from the apical organelles into the RBC membrane. (4) As the parasite develops from the ring stage to the amorphous trophozoite stage, it induces changes within the host RBC such as the formation of the tubovesicular membrane network (TVM) within the RBC cytoplasm, and knob structures on the surface of the RBC. (5) The parasite develops into a schizont containing a number of infective merozoites and a central residual body composed of hemozoin. These infected RBCs (iRBCs) avoid splenic clearance by cytoadhering via knob structures to endothelial cells lining host capillaries. Cytoadherence of iRBCs is associated with the most severe symptom of *P. falciparum* infection, cerebral malaria . When the schizont bursts, new merozoites are released that can infect RBCs and develop into (6) gametocytes, which complete the sexual cycle in the mosquito vector, or (7) new merozoites that continue the asexual cycle in host RBCs. (8) T-cells (T), B-cells (B), monocytes (MØ) and antibodies (Y) are all important in the host immune response to infection. The role of complement in lysing iRBCs is debatable (C?), whereas complement fixing antibodies specific for sexual stage parasites have been characterized (C).

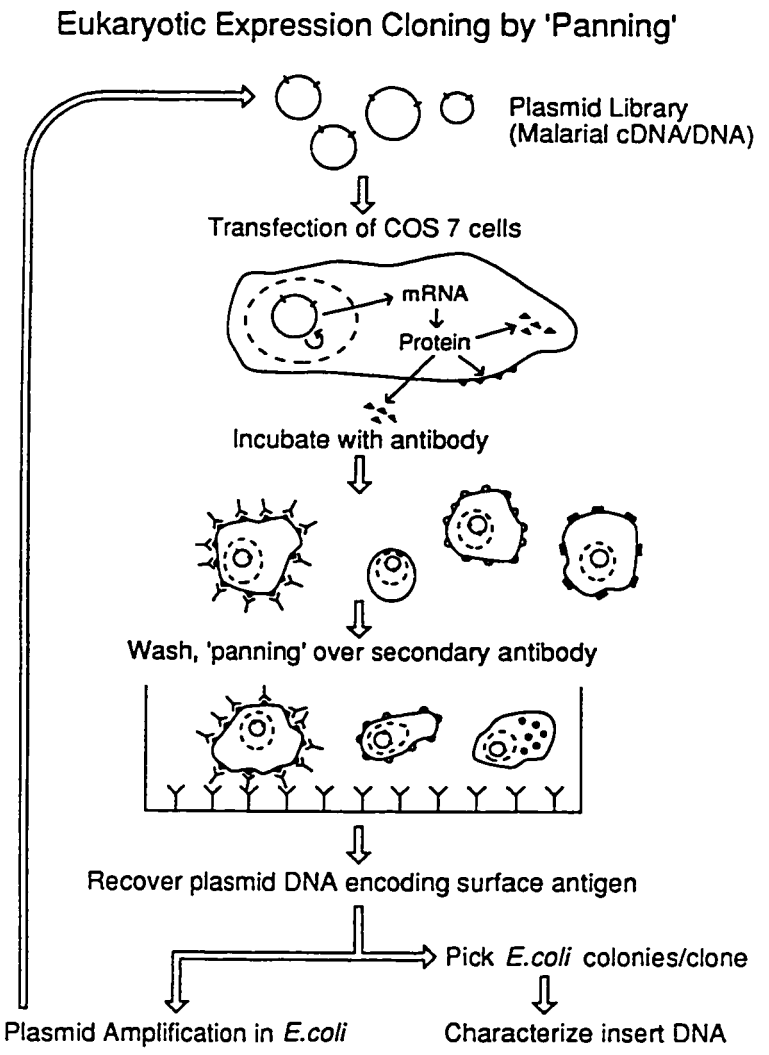

FIGURE 1.2. Overview of the process of eukaryotic expression cloning and selection by antibody panning. See text for details.

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1/1                                                 31/11
ATG ATA AAA TTA AGT AAG AAG TAT TGT TTA GGG ATA TCC TTT GTA TTA TAT ATT TTG TTG
M   I   K   L   S   K   K   Y   C   L   G   I   S   F   V   L   Y   I   L   L
61/21                                               91/31
TCT GTT TGT GAA GGG CAT AAA AAT TTA ACA TGT GAC TTT AAC GAT GTA TAC AAA TTA GAA
S   V   C   E   G   H   K   N   L   T   C   D   F   N   D   V   Y   K   L   E
121/41                                              151/51
TTT CAT CCT AAT CAA CAA ACA AGT GTT ACT AAA TTA TGT AAT GTA ACT CCT AAT GTA TTA
F   H   P   N   Q   Q   T   S   V   T   K   L   C   N   V   T   P   N   V   L
181/61                                              211/71
GAA AAG GTA ACT ATA AAA TGT GGT TCA GAT AAA TTA AAT TAT AAT TTA TAT CCT CCA ACT
E   K   V   T   I   K   C   G   S   D   K   L   N   Y   N   L   Y   P   P   T
241/81                                              271/91
TGT TTT GAA GAG GTA TAT GCA TCT AGG AAT ATG ATG CAT TTA AAA AAA ATA AAA GAG TTT
C   F   E   E   V   Y   A   S   R   N   M   M   H   L   K   K   I   K   E   F
301/101                                             331/111
GTA ATC GGA TCA TCA ATG TTT ATG AGA CGT AGT TTA ACA CCA AAT AAA ATT AAC GAA GTT
V   I   G   S   S   M   F   M   R   R   S   L   T   P   N   K   I   N   E   V
361/121                                             391/131
TCT TTC AGA ATT CCA CCT AAT ATG ATG CCT GAA AAA CCT ATA TAT TGT TTT TGT GAA AAT
S   F   R   I   P   P   N   M   M   P   E   K   P   I   Y   C   F   C   E   N
421/141                                             451/151
AAA AAA ACA ATA ACT ATT AAT GGT TCC AAT GGA AAT CCT TCA AGT AAA AAA GAT ATA ATA
K   K   T   I   T   I   N   G   S   N   G   N   P   S   S   K   K   D   I   I
481/161                                             511/171
AAT AGA GGA ATA GTT GAA ATT ATT ATA CCT TCA TTA AAT GAA AAA GTT AAA GGA TGT GAT
N   R   G   I   V   E   I   I   I   P   S   L   N   E   K   V   K   G   C   D
541/181                                             571/191
TTT ACA ACA AGC GAA TCT ACA ATT TTC TCA AAA GGA TAT AGT ATT AAT GAA ATA TCT AAT
F   T   T   S   E   S   T   I   F   S   K   G   Y   S   I   N   E   I   S   N
601/201                                             631/211
AAA TCA TCA AAT AAC CAA CAA GAT ATT GTA TGT ACA GTT AAG GCA CAT GCT AAT GAT TTA
K   S   S   N   N   Q   Q   D   I   V   C   T   V   K   A   H   A   N   D   L
661/221                                             691/231
ATC GGA TTT AAA TGT CCA AGC AAT TAT TCT GTT GAA CCA CAT GAT TGT TTT GTT AGT GCA
I   G   F   K   C   P   S   N   Y   S   V   E   P   H   D   C   F   V   S   A
721/241                                             751/251
TTC AAT TTA AGT GGG AAA AAT GAA AAC CTA GAA AAT AAA CTT AAA TTA ACA AAT ATA ATT
F   N   L   S   G   K   N   E   N   L   E   N   K   L   K   L   T   N   I   I
781/261                                             811/271
ATG GAT CAT TAT AAT AAT ACT TTC TAT TCA AGA TTA CCA AGT TTA ATT TCT GAT AAT TGG
M   D   H   Y   N   N   T   F   Y   S   R   L   P   S   L   I   S   D   N   W
841/281                                             871/291
AAA TTC TTT TGT GTA TGT TCA AAA GAT AAT GAA AAA AAA TTA GTC TTT ACC GTA GAA GCA
K   F   F   C   V   C   S   K   D   N   E   K   K   L   V   F   T   V   E   A
901/301                                             931/311
AGC ATT TCA TCA AGT AAT ACT AAA CTT GCT TCA AGA TAT AAT ACA TAC CAA GAT TAT ATA
S   I   S   S   S   N   T   K   L   A   S   R   Y   N   T   Y   Q   D   Y   I
961/321                                             991/331
TCC AAC TCT TCT TTC TTA ACC CTT TCA TCA TAT TGT GCC TTC ATC ACT TTT ATT ATC ACA
S   N   S   S   F   L   T   L   S   S   Y   C   A   F   I   T   F   I   I   T
1021/341
TCA TTC TTA TCA TTC ATC TTA TAA
S   F   L   S   F   I   L   *
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FIGURE 1.3 DNA, protein sequence, and hydropathy plot of Pf12. The 1044 bp open reading frame of Pf12, plus the deduced amino acid sequence were determined using the DNA analysis program, DNA Strider 1.2.

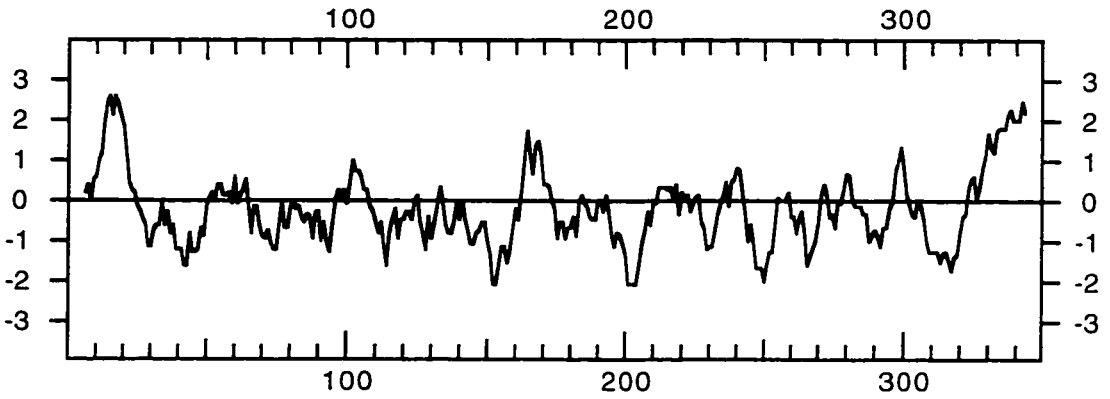


FIGURE 1.4 Hydropathy plot of Pf12. This Kyte-Doolittle hydropathy plot of the amino acid sequence of Pf12 shows N-terminal (signal sequence) and C-terminal (anchor signal) hydrophobic amino acid regions. The numbers at the top of the plot correspond to the amino acid residue number, the numbers indicated to the side correspond to arbitrary values of relative hydrophobicity (positive numbers, more hydrophobic) of amino acid sequences in a given region.

TABLE 1.1. Summary of *Plasmodium falciparum* sporozoite and liver stage proteins important in pathogenesis

Antigen	Size	Life Cycle Stage	Comments	References
Circumsporozoite antigen, CSP	40-60 kDa	Within the oocyst; sporozoite surface	Contains tandem immunodominant repeats, B and T cell epitopes Cysteine rich RII region containing a sulfated glycoconjugate binding (SGB) motif mediates binding to hepatocytes CSP- knock-out sporozoites do not mature, and are no longer infective Sporozoite vaccine candidate	Yoshida et al., 1981; Nussenzweig and Nussenzweig, 1989b; Cerami et al., 1992a; Cerami et al., 1992b; Shakibaei and Frevert, 1996; Menard et al., 1997
Sporozoite surface protein 2/ thrombospondin-related anonymous protein, SSP-2/TRAP	63 kDa	Within sporozoite microneme bodies and on the sporozoite surface	Contains RGD and SGB motifs that may mediate hepatocyte binding TRAP- knock-out sporozoites are no longer infective	Charoenvit et al., 1987; Robson et al., 1988; Rogers et al., 1992; Muller et al., 1993; Sultan et al., 1997
Pfs16	16 kDa	Surface of gametocytes and sporozoites	Pfs16-specific antibodies block sporozoite invasion but not transmission of infective gametocytes	Moelans et al., 1991; Moelans et al., 1995
Liver stage antigen, LSA-1	200 kDa	Parasitophorous vacuole of infected hepatocytes	Contains immunodominant repeats HLA-Bw-53 CTL responses to LSA-1 peptides have been identified Liver stage vaccine candidate	Guerin-Marchand et al., 1987; Hill et al., 1991a; Hill et al., 1992; Nardin and Nussenzweig, 1993; Fidock et al., 1994b

TABLE 1.1. Summary of *Plasmodium falciparum* sporozoite and liver stage proteins important in pathogenesis, continued

Antigen	Size	Life Cycle Stage	Comments	References
Sporozoite threonine and asparagine rich protein, STARP	78 kDa	Sporozoite surface Parasitized hepatocytes Ring infected erythrocytes	Threonine and asparagine rich protein containing tandem repeats	Fidock et al., 1994a
Sporozoite and liver stage antigen, SALSA	70 kDa	Sporozoite surface, parasitophorous vacuole of infected hepatocytes, possibly merozoite surface	Individuals living in regions of low malaria transmission have high antibody titres to the two distinct B-cell epitopes of SALSA	Bottius et al., 1996
Exported protein 1, Exp-1	23 kDa	Parasitophorous vacuole of infected hepatocytes and RBCs	Contains homology with tandem repeats of CSP MAb specific for Exp-1 inhibit sporozoite invasion of hepatocytes	Sanchez et al., 1994

TABLE 1.2. Summary of *Plasmodium falciparum* merozoite surface proteins important in pathogenesis

Antigen	Size	Life Cycle Stage	Comments	References
Merozoite surface protein 1, MSP-1, also called MSA-1	83, 38, 33, 19, kDa	Merozoite surface	190-250 kDa precursor is proteolytically processed to four polypeptides Cysteine-rich EGF modules of the 19 kDa fragment mediate binding to RBC Growth-inhibiting MAbs specific for 19 kDa fragment recognize reduction sensitive epitopes Immunization of mice with renatured but not reduced MSP-1 confers protection against parasite challenge	Freeman and Holder, 1983; Holder et al., 1985, McBride and Heidrich, 1987; Appella et al., 1988; Perkins and Rocco, 1988; Heidrich et al., 1989; Blackman et al., 1990; Cooper et al., Holder et al., 1992a, Holder et al., 1992b; Chappel and Holder, 1993; Daley and Long, 1993; Burghaus and Holder, 1994; Ling et al., 1994; Urquiza et al., 1996
Merozoite surface antigen 2, MSA-2	45-55 kDa	Merozoite surface	Diverse central region of tandem repeats MAbs specific for S-T-D-S peptide of MSA-2 inhibit growth of the parasite *in vitro* Immunization of mice with conserved peptides confers protection against parasite challenge	Thomas et al., 1984; McBride et al., 1985; Clark et al., 1989; Saul et al., 1992
MSP-3	48 kDa	Merozoite surface	Contains conserved repeats MSP-3 specific MAbs plus monocytes inhibit growth of *P. falciparum* in culture.	Ouevray et al., 1994

TABLE 1.2. Summary of *Plasmodium falciparum* merozoite apical organelle proteins important in pathogenesis, continued

Antigen	Size	Life Cycle Stage	Comments	References
Erythrocyte binding antigen, EBA-175	175 kDa	Micronemes of merozoites; apical end of merozoites	Cysteine rich RBC binding domain shares homology with *P. vivax* and *P. knowlesi* Duffy binding proteins RBC binding domain recognizes both sialic acid residues and peptide backbone of glycophorin A	Camus and Hadley, 1985; Orlandi et al., 1990; Sim et al., 1990; Adams et al., 1992; Klotz et al., 1992; Orlandi et al., 1992; Sim et al., 1994
Rhoptry high molecular weight complex, RhopH	140, 110, 130 kDa	Merozoite rhoptries	Binds inner leaflet of the RBC membrane May be involved in facilitating entry of merozoite into RBC by deforming RBC membrane	Brown and Coppel, 1991; Sam-Yellowe, 1992; Sam-Yellowe and Ndengele, 1993; Perkins and Zeifer, 1994
Serine rich antigen, SERA	120 kDa	Merozoite rhoptries, released on rupture of infected RBC	Associates with RhopH and contains an RBC binding domain	Sam-Yellowe, 1992
Rhoptry -associated protein, RAP-1	80 kDa	Rhoptry organelle; surface of the ring-infected RBC	Cysteine rich protein with amphiphilic sequences that may promote association with RBC membrane Forms a complex with RAP-2 protein, called RAP1/2; MAb specific for RAP1/2 complex inhibit parasite growth in culture *Saimiri* monkeys immunized with RAP1/2 were protected against challenge with asexual parasites	Schofield et al., 1986; Clark et al., 1987; Crewther et al., 1990; Ridley et al., 1990a; Ridley et al., 1990b; Ridley et al., 1991; Perkins et al., 1992

TABLE 1.2. Summary of *Plasmodium falciparum* merozoite apical organelle proteins important in pathogenesis, continued

Antigen	Size	Life Cycle Stage	Comments	References
Rhoptry-associated protein 2, RAP-2	42 kDa	Rhoptry organelle; surface of the ring-infected RBC	Cysteine rich protein that associates with RAP-1 to form the RAP 1/2 complex	Crewther et al., 1990; Ridley et al., 1991; Stowers et al., 1995
Apical merozoite antigen 1, AMA-1	83 kDa	Neck of the Rhoptries; merozoite surface	Integral membrane protein with cystine knot disulfide bond structure Proteolytically processed to a 62 kDa fragment prior to invasion *Rhesus* monkeys immunized with *P.knowlesi* AMA-1 were protected against challenge with asexual parasites AMA-1 may be involved in the release of merozoites from the infected RBC	Deans et al., 1988; Peterson et al., 1989; Crewther et al., 1990; Hodder et al., 1996
Ring-infected erythrocyte surface antigen, RESA		Initially in merozoite dense granules; transferred to the PVM on invasion, then to the inner leaflet of the RBC membrane	May stabilize the RBC membrane by associating with spectrin RESA disappears from the membrane as the parasite matures	Aikawa and Miller, 1983; Coppel et al., 1984; Perlmann et al., 1987; Foley et al., 1990; Da Silva et al., 1994; Foley et al., 1994

TABLE 1.3. Summary of *Plasmodium falciparum* proteins produced in parasite-infected RBCs

Antigen	Size	Life Cycle Stage	Comments	References
P. falciparum erythrocyte membrane protein 1, Pf EMP1	200-400 kDa	Knob structures of infected RBCs	Transmembrane protein with N-terminal binding domain and C-terminal cytoplasmic domain containing acidic terminal repeats May contribute to cerebral malaria by mediating adherence of infected RBCs to endothelial cells via CD36, ICAM-1, VCAM-1, ELAM-1 receptors Belongs to a family of proteins containing Duffy-like binding motifs (EBA-175, and *P. vivax* DBP are also members) Undergoes antigenic variation; 50-150 copies of Pf EMP1 are present per haploid genome	Aley et al., 1984; Leech et al., 1984; Howard et al., 1988; Berendt et al., 1989; Oquendo et al., 1989; Ockenhouse et al., 1992; Pasloske et al., 1993; Baruch et al., 1995; Rogerson et al., 1995; Smith et al., 1995; Su et al., 1995
Knob-associated histidine rich protein, KAHRP	80-95 kDa	Cytoplasmic face of the RBC membrane underneath knob structures	Histidine and lysine rich protein Binds the acidic terminal repeats of PfEMP1 Associates with RBC cytoskeletal proteins and Band 4.1	Pologe et al., 1987; Kilejian et al., 1991; Chishti et al., 1995; Deitsch and Wellems, 1996
Mature erythrocyte infected surface antigen, MESA	200 kDa	Cytoplasmic face of the RBC membrane underneath knob structures	Contains a N-terminal repeats Binds Band 4.1 via a regions flanking the N-terminal repeats	Coppel et al., 1986b; Foley and Tilley, 1995

TABLE 1.3. Summary of *Plasmodium falciparum* proteins produced in parasite-infected RBCs, continued

Antigen	Size	Life Cycle Stage	Comments	References
P. falciparum erythrocyte membrane protein 3, Pf EMP3	300 kDa	Outer surface of RBC membrane; concentrated at knob structures	Co-expressed with KAHRP Absent in parasitized RBCs lacking knob structures	Pasloske et al., 1993; Pasloske et al., 1994
Rosettin	28 kDa	Surface of the infected RBC	Binds uninfected RBCs forming clumps of RBCs around an infected RBC called a 'rosette' May contribute to cerebral malaria	Wahlgren et al., 1989; Scholander et al., 1996

TABLE 1.4 Transfection of DNA into malaria parasites

Transfected sequence	Parasite life cycle stage transfected	DNA construct	Result of transfection	Comments	References
Luciferase flanked by *P. gallinaceum* Pgs 28 5' and 3' regulatory regions	*P. gallinaceum* gametes and zygotes	Circular plasmid	Transient expression of luciferase by episomal plasmid	First demonstration of transfection of malaria parasites Revealed the importance of both 5 and 3' flanking sequences	Goonewardene et al., 1993
Pyrimethamine resistant *P. berghei* DHFR-TS gene flanked by 5' and 3' regulatory regions	*P. berghei* merozoites	Linearized and circular plasmid	Generation of pyrimethamine resistance by episomal plasmid	First use of the selectable marker DHFR-TS	van Dijk et al., 1995
CAT genes flanked by *Pf* hsp86, hrp3 5' and hrp2 3' regulatory regions	*P. falciparum* ring, troph and schizont-infected RBCs	Circular plasmid	Transient CAT expression by episomal plasmid	Demonstration that CAT genes with different codon usage were expressed at similar levels	Wu et al., 1995
Tg DHFR-TS flanked by hrp3 5' and hrp2 3' regulatory regions	*P. falciparum* ring, troph and schizont-infected RBCs	Circular plasmid	Integration of DHFR-TS at chromosomal sites	First demonstration of targeted homologous integration of transfected sequences	Wu et al., 1996
Tg DHFR-TS flanked by 5' upstream regions of *Pf* and *Pb* DHFR-TS	*P. falciparum* ring, troph and schizont-infected RBCs	Circular plasmid	Generation of pyrimethamine resistance by episomal and integrated DNA	Identification of promoter elements of Pf and Pb DHFR-TS	Crabb and Cowman, 1996

TABLE 1.4 Transfection of DNA into malaria parasites, continued

Transfected sequence	Parasite life cycle stage transfected	DNA construct	Result of transfection	Comments	References
Pb DHFR-TS flanked by *P. berghei* CSP regulatory regions	*P. berghei* sporozoite	linearized replacement plasmid	Knockout of CSP by stable integration of marker	Delayed development of sporozoites in the oocyst Loss of infectivity of sporozoites	Menard et al., 1997
Tg DHFR-TS flanked by *P. falciparum* KAHRP 5' and 3' regulatory regions	*P. falciparum* ring, troph and schizont-infected RBCs	linearized replacement plasmid	Knockout of the KAHRP gene, loss of knobs on infected RBCs	KAHRP is responsible for the formation of knobs PfEMP1 distribution on infected RBC surface is altered, and cytoadherence decreased	Crabb et al., 1997
P. berghei TRAP gene upstream of DHFR-TS	*P. berghei* merozoites	linearized insertion plasmid	Knockout of TRAP gene	TRAP is not essential for sporozoite development TRAP is essential for sporozoite invasion of mosquito salivary glands, mouse liver cells, and sporozoite gliding motility	Sultan et al., 1997
GFP flanked by hrp3 5' and hrp2 3' regulatory regions	*P. falciparum* ring, troph and schizont-infected RBCs	circular plasmid	Expression of GFP in asexual stage parasites	GFP expressed within parasite cytoplasm First estimate of the efficiency of transfection of *Plasmodium* by electroporation: $1x10^{-2}$	VanWye and Haldar, 1997

CHAPTER II

MATERIALS AND METHODS

General DNA preparation, detection, and cloning techniques

Plasmid DNA preparation by alkaline lysis and CsCl gradient centrifugation.

E. coli strain DH5α transformed with the appropriate plasmid were grown overnight in 500 ml of 2XYTMT (Sambrook et al., 1989) plus 100 μg/ml ampicillin, and harvested by centrifugation. 'Maxiprep' plasmid DNA was obtained using the alkaline lysis method (Sambrook et al., 1989), and purified by CsCl/ ethidium bromide gradient centrifugation (Sambrook et al., 1989). The plasmid DNA was collected from the gradient, extracted several times with n-butanol, precipitated using 0.3 M sodium acetate (pH 5.2) and an equal volume of isopropanol, washed with 70% ethanol, and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) . The precipitated DNA was extracted 1 time with phenol, 2 times with a 1:1 mixture of phenol chloroform, and 1 time with chloroform alone. This extracted DNA was precipitated with 0.3 M sodium acetate and 2.5 volumes of cold 95% ethanol, washed with 70% ethanol, and resuspended in TE.

Plasmid 'minipreps' were obtained using the alkaline lysis miniprep method described in Maniatis (Sambrook et al., 1989) which is essentially the same as the protocol described above without the CsCl gradient and isopropanol precipitation steps.

Restriction endonuclease digestion of DNA

DNA was digested using restriction enzymes (New England Biolabs, Promega, Stratagene, Boehringer Mannhiem) according to the manufacturer's protocol. In general, 4U of enzyme was used to digest 1 μg of DNA, and the amount of enzyme added to digestion reactions did not exceed 10% of the total volume.

Agarose gel electrophoresis

DNA digested by restriction endonucleases was resolved by agarose gel electrophoresis according to the methods described by Sambrook et al., (1989). Agarose (Life Technologies, Grand Island NY) gels ranging in concentration from 0.8-2% in TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA), 100 µg/ml ethidium bromide, were used depending on the size of the DNA fragment being resolved.

DNA ligation and *E. coli* transformation

DNA fragments digested with restriction endonucleases (approximately 0.5 to 2 µg of DNA), and resolved by agarose electrophoresis using low-melting point agarose (FMC BioProducts Rockland ME) gels were commonly used in DNA ligation reactions. DNA bands were excised from agarose gels, mixed with an equal volume of MilliQ water, melted at 70° C, then cooled at room temperature before adding to the ligation mix. A typical ligation involving DNA fragments with cohesive-end termini (e.g. Xba I/ Not I digested gene fragment plus Xba I/ Not I digested vector) was carried out in a 40 µl total volume containing the DNA fragments to be ligated (5-100 ng of DNA from melted gel slices, 5-15 µl) 50 mM Tris-HCl pH 8, 10 mM $MgCl_2$ 10 mM DTT, 1 mM ATP, 200 U of T4 ligase. After incubating this reaction for 1-18 hours at room temperature, 10 µl was removed and mixed gently with 200 µl of *E. coli* DH5α competent cells. This mixture of cells and DNA was incubated at 42° C for 90 seconds, grown in 1 ml of 2XYTMT for 30 minutes at 37° C, plated out on 2XYTMT/ 100 µg/ml ampicillin plates, and incubated overnight at 37° C. Transformant colonies were picked from this plate, and transformant DNA was harvested by the alkaline lysis miniprep method.

Vector dephosphorylation

In order to ligate DNA fragments digested with one restriction endonuclease (e.g. a DNA fragment with Xho I sites at both ends) into a vector digested with the same endonuclease, it was necessary to dephosphorylate the 5' phosphoryl termini of the vector to favor the formation of vectors with DNA inserts over recircularized vector (background). This was achieved by incubating the digested vector in a reaction containing calf intestinal phosphatase according to the manufacturer's instructions (NEB). The reaction was incubated at 37° C for 30 minutes, and heat inactivated at 75° C for 20 minutes. The DNA was resolved by low melting point agarose gel electophoresis, excised, and freed from the gel using beta-agarase (NEB) according to the manufacturer's instructions. The DNA was then extracted 2 times with phenol/chloroform, 2 times with chloroform, and ethanol precipitated with 0.3 M sodium acetate. This DNA was used in standard ligation reactions.

Polymerase Chain Reaction

Amplification of DNA fragments was achieved by polymerase chain reaction (PCR) using Thermophilus aquaticus (Taq; made in our lab by A. Atrazhev) or Pyrococcus furiosis (Pfu) (Stratagene, La Jolla CA) DNA polymerase. PCR was used mainly to add restriction sites or other sequences to 5' and 3' ends of DNA. A typical reaction contained 1-10 ng of DNA template, 0.5 μM primers, 0.2 mM dNTPs, 50 mM KCl, 10 mM Tris-HCl pH 9, 1.5 mM $MgCl_2$, and 0.01% w/v gelatin, made up to 50 μl in MilliQ water. PCR reactions were conducted in a PTC-100 Programmable Thermal Controller (MJ Research, Watertown MA) using the conditions: 94° C for 50 seconds, 50-60° C for 50 seconds, 72° C for 3 minutes, for 25 cycles, followed by a 5 min extension at 72° C. One tenth of the PCR reaction was examined by 0.8% agarose gel electrophoresis, and the remainder was separated from primers by a microcon-100 filter unit (Amicon, Beverly MA), extracted 2 times with a 1:1 mixture of phenol/ chloroform, 2 times with chloroform,

and precipitated with 2.5 volumes of 95% ethanol and 0.3 M sodium acetate. This DNA was then digested with restriction enzymes and ligated into the appropriately digested vector. Table 2.1 describes the primers used to create Pf12 subfragments. These primers were made on a PCR-Mate model 391 DNA Synthesizer (ABI, Foster city CA).

Random oligonucleotide labelling of DNA probes

Clone#2 and #7 fragments from chapter 5, were made into ^{32}P-labelled probes for Southern blotting using the random oligonucleotide priming method of Feinberg and Vogelstein (Feinberg and Vogelstein, 1983). Xho I digested CsCl-purified maxiprep DNA was separated by 0.8% LMP agarose electrophoresis, the desired DNA fragments excised (avioding the vector backbone), mixed with an equal volume of MilliQ water, boiled 7 minutes to denature the DNA, and warmed to 37° C. 25 µl (approximately 1 µg) of this DNA was added to 2 µl of 10 mg/ml BSA, 5 µl of [α-^{32}P]-dCTP (50 µCi, NEN, >3000Ci/mmol), 10 µl of OLB mix (100 µM dA,G, and TTP, 250 mM Tris-HCl pH 8, 25mM $MgCl_2$, 1.0 mM Hepes pH 6.6, 45 mM 2ME, 540 µg/ml random hexadeoxyribonucleotides-Pharmacia #2166), 1 µl of Klenow (2 U/µl, Boehringer Mannheim) and 9 µl of MilliQ water. The reaction was incubated at 37° C for 1 hour, then stopped by addition of 5 µl of 10% SDS /0.5 M EDTA pH 8. [α-^{32}P]-dCTP-labelled DNA (approximately 1-2 x 10^8 dpm/ µg) was separated from unincorporated counts by size exclusion chromatograpy using Sephadex G-50F poured into a 1.5ml pasteur pipette containing a small glass wool plug. Labelled probe was eluted in TE pH 8, and the peak fractions were identified with a hand-held radiation monitor. Fractions from the first peak (labelled probe) were pooled, fractions from the second peak (unincorporated [α-^{32}P]-dCTP) were discarded.

Southern blotting

DNA was digested with restriction endonucleases, resolved by agarose electrophoresis, denatured by gently shaking the gel in 0.5M NaOH, 1.5 M NaCl for 10 minutes, and neutralized by shaking in 1M Tris-HCl pH8, 1.5 M NaCl. The neutralized gel was placed on a glass plate and Hybond Super C (Amersham) cut to the size of the gel, wet first in MilliQ water, then 20X SSPE, was layered onto the gel (avoiding air bubbles). This was followed by three layers of filter paper soaked in 20X SSPE, and three inches of dry paper towels, all cut to the size of the gel. Next, the stack was flipped over, the glass plate removed, and the same procedure of layering nitrocellulose, filter paper and paper towels was completed. A plastic sheet was placed on the top of this double blot stack, and a weight placed upon this to facilitate the transfer process. The double blot setup was incubated overnight at room temperature. By setting up two different stacks of nitrocellulose, filter paper, and paper towels, two identical blots can be generated from one gel. It is important to make sure that the filter paper and paper towels from the two different stacks do not touch each other; this would 'short circuit' the wicking action that pulls the DNA from the gel to the nitrocellulose membranes. After transfer, DNA was fixed to the nitrocellulose membranes by baking in a vacuum oven for 2 hours at 80° C.

Nitrocellulose membranes containing DNA were washed in 4X SSC placed DNA side down in a Tupperware dish and prehybridized in 50 % formamide, 5X SSPE, 5X Denhardt's solution, 0.1% SDS, and 200 µg/ml salmon sperm DNA , for 30 minutes at 37° C. The hybridization was set up by denaturing the labelled DNA probe in 25 ml of formamide, and then mixing this with 25 ml of 10X SSPE, 10X Denhardt's solution, 0.2% SDS, and 400 µg/ml salmon sperm DNA, resulting in a hybridization mixture of the exact same composition as the pre-hybridization mixture. The pre-hybridized DNA was placed in a Tupperware container with the probe-hybridization solution, and shaken gently

overnight, 37° C. The blots were then washed three times at 65° C with 2X SSPE, 0.1% SDS, followed by two stringent washes at 65° C with 0.2X SSPE, 0.1% SDS. At this point the blots were checked for radioactivity with the hand-held radiation monitor, and washed further if necessary. The blots were allowed to dry, were taped to filter paper and wrapped in saran wrap, and then exposed to X-ray film (Kodak) for 2 hours.

DNA sequencing

DNA sequencing was performed by the method of Sanger (Sanger et al., 1977). Aliquots from DNA sequencing reactions were resolved on 6% acrylamide TBE gels containing 8.3 M urea and analyzed on a ABI 373 automated DNA sequencer (ABI). Double stranded DNA templates were sequenced using the methods described in the ABI Taq "dyedeoxy" terminator cycle sequencing kit (ABI). Clones 2, 7, and 18 from chapter 5 were sequenced initially by this method using the primer 5'pJFE14 (see table 2.1). Single-stranded DNA templates were generated from fragments subcloned into pBluescript SK- and KS- as described in Maniatis (Sambrook et al., 1989) and sequenced using T3 or T7 dye-labelled primers (constructed in our lab; see table 2.1 for details) and USB Sequenase by the manufacturer's instructions (USB). Single stranded DNA templates were also sequenced using T3 and T7 primers and the Amersham Thermosequenase dye-terminator cycle sequencing protocol.

SDS-PAGE analysis of proteins

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of protein samples was carried out according to the method of Laemmli (Laemmli, 1970). To make a 12% acrylamide separating gel, 6 ml of 30% acrylamide and 0.8% N,N'-methylene bisacrylamide (called reagent A; both products from Bio-Rad Laboratories Hercules CA), 3.75 ml of 1.5 M Tris-HCl/ 0.4% SDS (pH 8.8), 5.25 ml of MilliQ water, 50 µl of 10% ammonium persulfate (APS; Life Technologies, Grand Island NY), and 10 µl of TEMED

(Life Technologies, Grand Island NY) were mixed, poured into a BioRad MiniProtean II module, and overlayed with 95% ethanol until polymerization was complete. A 4 % A stacking gel consisting of 650 µl of 30% A, 1.25 ml of 0.5 M Tris-HCl/ 0.4% SDS (pH 6.8), 3.05 ml of MilliQ water, 25 µl of 10% APS, and 5 µl of TEMED, was poured over the separating gel, and an 8 or 15 well comb was inserted.

Protein samples were prepared in SDS lysis buffer containing 62.5 mM Tris-HCl, (pH 6.8), 2% SDS, 25% glycerol, 100 mM dithiothreitol (DTT), and 0.01% bromphenol blue. These samples were boiled for 7 minutes, insoluble material was pelleted by centrifugation at 16 000 xg, and the supernatant was loaded onto the gel. Polyacrylamide gels were run 1 hour at 20 mA/ gel (BioRad Power Pac 200, constant current) in electrophoresis buffer containing 192 mM glycine, 25 mM Tris-HCl, and 0.1% SDS. After electrophoresis the gels were fixed in 10% acetic acid and 10 % methanol (fixation), proteins were visualized by staining the gel in 0.25% Coomassie Brilliant Blue R250, 50% methanol and 10 % acetic acid, and the gel was destained using the fixation solution.

Immunological Techniques

Western blotting

Unfixed, unstained polyacrylamide gels from SDS-PAGE experiments were washed for 10 minutes in 25 mM Tris-HCl, 192 mM glycine, and 10% methanol (transfer buffer), then transferred to an open gel holder cassette containing a fibre pad and three sheets of filter paper moistened with transfer buffer. A nitrocellulose membrane, pre-wetted in transfer buffer, was layered onto the gel (avoiding bubbles), followed by three additional pre-wetted filter papers and the other fibre pad, forming a 'sandwich'. The gel holder cassette was closed and placed into the BioRad Mini Trans-Blot Cell. The cell was run at 200 mA (BioRad Power Pac 200), for 1 hour in transfer buffer. After protein transfer the nitrocellulose membrane was stained in 0.5% Ponceau S, 1% glacial acetic

acid for two minutes, and washed with water for two minutes to visualize the proteins. The nitrocellulose filter was blocked with either 10% milk/ 0.5% Tween-20/ PBS, or 5% BSA/ 0.5% Tween-20/ PBS at 4° C for 30 minutes. Both primary and secondary antibody incubations were carried out by diluting antibody in 1-2 % BSA/ 0.5% Tween-20/ PBS, and adding it to the blot for 1 hour at 4° C. Primary and secondary antibody concentrations in these experiments depended on the method of detection: using the Amersham ECL kit, a 1:10000 dilution of polyclonal antiserum such as rat anti-A2 was used, and a 1:10000 dilution of goat anti-rat HRP (Amersham). For diaminobenzidine (Sigma) development, 1:1000 dilutions of both primary and secondary antibodies were used. The blots were washed at least five times between each antibody incubation with 0.5% Tween-20/ PBS, and developed according to the manufacturer's instructions.

Enzyme-Linked Immunosorbent Assay

Linbro EIA II Plus 96 well plates coated (4° C, 18 hours) with 50-100 ng of Pf12 recombinant protein /PBS (100 µl) per well were blocked with 3% BSA/PBS for 30 minutes at 37° C. After washing the plates twice with PBS, 50 µl antiserum diluted in 1% BSA/ PBS/ 0.05% Tween-20 was incubated in each well for 30 minutes at 37° C. Plates were then washed several times with PBS/ 0.05% Tween-20, and 50 µl of goat anti-mouse or goat anti-rat horseradish peroxidase secondary antibody (Jackson) diluted 1:1000 in 1% BSA/ PBS/ 0.05% Tween-20 was added to each well. After incubating and washing samples as described above, 100 µl of substrate solution consisting of 10 mg/ml ABTS (Sigma), 90 mM Na_2HPO_4, 30 mM citric acid, 0.01% v/v H_2O_2, was added to each well. As soon as noticeable color development occurred (15-20 minutes at room temperature, with mixing), the reaction was stopped with 100 µl of 60 mM citric acid per well, and measured at 405 nm.

Immunofluorescence

Immunofluorescence experiments were conducted with either COS cells in solution, or *P. falciparum* parasites fixed to glass slides. COS cells ($5x10^5$) lifted from tissue culture plates (Falcon) with PBS/0.5mM EDTA pH 8, centrifuged at 200 xg, and resuspended in primary antibody diluted in 5% FBS/ PBS/ 0.5 mM EDTA pH 8. These cells were incubated at 4° C for 20 minutes, overlayed onto an FBS cushion, and centrifuged at 200 xg. The cells were then resuspended in fluorescein isothiocyanate (FITC) or rhodamine-conjugated goat anti-rat, goat anti-mouse, or goat anti-human antibody. After centrifugation through an FBS cushion as before, the cells were resuspended in PBS/ EDTA and examined using a Leitz UV microscope at 700X magnification.

RBCs directly from parasite cultures or from percoll gradient purifications were washed 2 times in RPMI, and air-dried onto slides. These slides were fixed in either (1) 100% methanol (-20° C, 2 minutes), or (2) 100% acetone (-20° C, 1 hour), followed by 95% ethanol (room temperature, 2 minutes). The slides were stained with primary and secondary antibodies diluted in 2% BSA/ PBS for 20 minutes at room temperature, with three successive three minute PBS wash steps after each antibody incubation. The slides were mounted with coverslips, sealed with nail polish, and viewed using a Leitz UV microscope at 700X magnification, or a Leica Confocal Laser Scanning Microscope (University of Alberta Anatomy and Cell Biology) at 2000X magnification.

Production and purification of recombinant proteins in *E. coli* expression systems

Detection of recombinant proteins produced by *E. coli* transformed with Pf12 subfragments in pT7-7His6 and pBT1TØ

A fresh colony of Pf12/pT7-7His6 or Pf12/pBT1TØ-transformed *E. coli* BL21DE3 grown on a 2XYTMT/ 100 µg/ml ampicillin (2XYTMT/AMP) plate was inoculated into 2.5 ml of 2XYTMT/AMP liquid broth and grown on a roller wheel at 37° C until the culture density reached O.D. 0.6. The culture was then split into (2) 1 ml samples: an 'induced' sample (IPTG added to a final concentration of 0.4 mM), and a non-induced sample (no IPTG added). Approximately 0.5 ml of remaining culture was grown for an additional 4 hours in 1 ml 2XYTMT/ AMP/ 10% glycerol and frozen at -80° C. These non-induced and induced samples were grown for 5 hours on a roller wheel, harvested by centrifugation at 16 000 xg, and resuspended in 100 µl of MilliQ water. 10 µl of each sample was solubilized in SDS lysis buffer, resolved by SDS-PAGE, and in some cases, Western blotted with specific antibodies.

A modification of this protocol was developed for the expression of recombinant Pf12ec B2 in chapter 4: isolated *E. coli* colonies were streaked to confluence on new 2XYTMT/AMP plates to give a larger initial inoculum of bacteria in the expression experiment. Bacteria from these plates were used to inoculate starter cultures to an O.D. of 0.3; these cultures consequently reached O.D. of 0.6 quickly, decreasing the incidence of plasmid loss before induction. The secretion of beta-lactamase from the bacteria as they enter the log phase of growth is enough to inactivate the ampicillin used in the growth medium in a very short period of time (30 minutes to an hour). Thus, the option of growing the culture to a sufficiently high density before induction (such as O.D. 0.6, for a high yield of recombinant protein), must be weighed against the possibility of plasmid loss due to inactivation during this pre-induction growth phase. Starting the culture with a

larger initial inoculum from plates (where the selection pressure is maintained longer because the beta-lactamase from colonies does not diffuse as fast on plates as it does in liquid growth medium), minimized this pre-induction phase.

Large-scale recombinant protein production was performed by inoculating 1 L of 2XYTMT/AMP media with a starter culture grown from a fresh plasmid-transformed *E. coli* colony or glycerol stock of a confirmed protein 'producer'. The large-scale culture was induced under the same conditions as the small-scale cultures described above. 1 ml of non-induced and induced cultures were grown separately from the induced large-scale culture and used to confirm the presence of recombinant protein by SDS-PAGE or Western blotting before proceeding with the purification of protein from the large scale culture.

French press lysis of Pf12ec A2 expressing *E. coli* and solubilization Pf12ec A2 inclusion bodies in urea buffer.

The 1 L culture of *E. coli* BL21 DE3 that had been shown to express Pf12ec A2 was harvested by centrifugation at 3000 xg and resuspended in 25 ml of 0.1M NaH_2PO_4/ 0.01M Tris pH 8 containing 2 µg/ml RNase, 4µg/ml DNase, 2 mM $MgCl_2$. This pellet was mixed thoroughly by pipetting then lysed in a French Press three consecutive times at a pressure of 1000 psi . The lysate was centrifuged at 6000 xg in a Beckman centrifuge, and the pellet and supernatant were kept for SDS-PAGE analysis. The supernatant from the 6000 xg spin was centrifuged at 17 000 xg and the pellet and supernatant from this centrifugation step were also kept. In the end, three portions of the original bacterial lysate were kept for analysis: a 6000 xg pellet, a 17 000 xg pellet and a 17 000 xg supernatant. SDS-PAGE analysis of these fractions confirmed that Pf12ec A2 was present in inclusion bodies in the 6000 xg pellet. These inclusion bodies were solubilized by resuspending the pellet in 10 ml of 8M urea, 0.1M NaH_2PO_4, 0.01M Tris pH 8, called urea buffer. The reducing agent betamercaptoethanol (2ME) was added to this buffer to a final concentration

of 10%. This mixture was incubated overnight at 4° C to ensure complete solubilization of the protein.

Purification of Pf12ec A2 by nickel chelate affinity chromatography, preparative SDS-PAGE, and electroelution

Both Pf12ec A2 and B2 were purified by nickel chelate chromatography (Hochuli et al., 1987). Solubilized Pf12ec A2 was loaded onto a 12.5 ml Ni-NTA column that had been previously equilibrated with urea buffer (pH 8). The column was washed with ten column volumes each of urea buffers (plus 1% 2ME) at pH 8, 6.3, 5.9, 4.5, and 3.3 in order to elute pure Pf12ec A2. Fractions (5.5 ml) were collected and monitored by a UV spectrometer. Fractions corresponding to peaks recorded by the detector were analyzed by SDS-PAGE; those containing Pf12ec A2 were pooled and lyophilized in order to reach a total volume appropriate for loading onto a preparative SDS-PAGE gel. During this process some urea came out of solution . 15% polyacrylamide preparative gels were made using 20 cm X 20 cm large plates and 3mm spacers. A large well for the eluted protein was made using extra spacers and a smaller well was made for protein size makers. Fractions containing eluted Pf12 were solubilized in SDS-lysis buffer and loaded onto two preparative gels. After electrophoresis (80 mA constant current, 4-6 hours) was complete, the gel was washed with 3 changes of MilliQ water, and stained for 10 minutes at room temperature with 0.05% Coomassie blue in MilliQ water. The gel was then washed with MilliQ until a distinct band could be seen. This band was excised with a razor blade, and minced into very small cubes. The minced gel containing Pf12 protein was placed in an electroelution chamber (Schliecher and Schuell) containing 192 mM glycine, 25 mM Tris-HCl buffer, pH 8, and electroeluted at 100V overnight. This resulted in the migration of the protein out of the gel, through a permeable membrane, and into a 'trap'. The electroeluted protein was analyzed by SDS-PAGE, and quantitated using the Bicinchoninic acid (BCA) assay (Pierce). In this assay proteins react with copper ions (Cu^{+2}) under

alkaline conditions, giving rise to Cu^{+1}. These ions react with two BCA molecules to form a purple product which exhibits a strong absorbance at 562 nm. The protein was then lyophilized in 160µg aliquots.

Hypotonic lysis of Pf12ec B2 expressing *E. coli* and solubilization of Pf12ec B2 inclusion bodies in GuHCl buffer.

Pf12ec B2-producing *E. coli* BL21DE3 pLysS were solubilized by the method of K.H. Ling and S.Y. Cheng (Lin and Cheng, 1991), taking advantage of the overproduction of lysozyme in this strain (included primarily as an inhibitor of leaky T7 RNA polymerase expression under non-inducing conditions). Briefly, a 1 L pellet of transformed *E. coli* BL21DE3 pLysS was resuspended in a 50 ml solution of 20 mM Tris-HCl pH 7.5, 20% sucrose, 1 mM EDTA, incubated on ice for 10 minutes at 4° C, centrifuged at 4000 xg, and resuspended in cold water to promote outer cell wall lysis and spheroplast formation. Lysed outer cell wall components were separated from the spheroplasts by an 8000 xg centrifugation, and the pelleted spheroplasts were resuspended in 10 ml of sonication buffer containing PBS/ 5 mM EDTA, 1 µg/ml leupeptin, 20 µg/ml aprotinin, 0.5mM PMSF, 40 µg/ml DNase I, and 10 µg/ml RNase A (both from Boehringer-Mannheim). The cells were lysed by pulsing with a sonicator (Misonix, Farmingdale NY; 30 seconds/ pulse, 30 second pause between each pulse; 50 W setting) 5 times at 4° C. The sonicated suspension was incubated at room temperature for 1 hour, diluted to 38 ml in sonication buffer, and centrifuged at 13000 xg . The pellet was resuspended in 38 ml washing buffer containing PBS/ 25% sucrose, 5 mM EDTA, 1% Triton X-100, incubated on ice for 10 minutes, and centrifuged at 25 000 xg. The pellet was washed two more times in this manner, and resuspended in 6M GuHCl, 0.1M NaH_2PO_4, 0.01M Tris pH 8 overnight at 4° C.

Purification and renaturation of Pf12ec B2 protein

Pf12ec B2 was eluted from nickel columns using increasing concentrations of imidazole in urea buffer as opposed to the decreasing pH method described above. Pf12ec B2 was loaded onto a nickel column as above, but eluted with 200 mM imidazole in urea buffer, pH 7.5 (Hoffman and Roeder, 1991). Eluted fractions containing Pf12ec B2 (as judged by SDS-PAGE) were pooled and diluted 1:10 in 3M urea buffer containing 1% SDS, 1mM DTT. A 40 ml aliquot of this protein was dialyzed overnight (using Spectra/Por 12 000-14 000 molecular weight cutoff dialysis membrane; Spectrum Los Angeles CA) at room temperature against 4 L of Laemmli buffer containing 1%. This process was repeated two more times, first against 'modified Laemmli buffer' containing 0.1% SDS, then against 'modified Laemmli buffer' containing 0.01% SDS. Finally, the protein aliquot was dialyzed against 4 mM Hepes buffer (pH 7.5) containing 2 mM oxidized glutathione and 0.02 mM reduced glutathione (Sigma). This protein was concentrated, analyzed by SDS-PAGE, then prepared for immunization.

Immunization of rats with recombinant Pf12 proteins

Lyophilized Pf12ec A2 protein was resuspended in PBS, and mixed with an equal volume of Freund's complete adjuvant (FCA). Sprague Dawley rats were immunized intraperitoneally with 50 μg of Pf12ec A2 initially, then boosted three more times with the same amount of Pf12 in Freund's incomplete adjuvant. Blood was obtained by rat jugular vein puncture, centrifuged for 2 minutes at 3000 xg in serum separation tubes (Becton Dickinson), and rat serum was collected for further studies. Purified and renatured Pf12ec B2 was prepared in the same manner as indicated above and injected by the same route into four Balb/c, A/J, and Biozzi mice (25 μg DNA per injection). Ten to fourteen days after the fourth injection, mouse serum was collected by tail vein puncture for further studies. The anti-A2 and anti-B2 antiserum in these experiments is polyclonal; the isotype of the anti-A2

and B2 antiserum was not determined, and antibodies were not purified from the original polyclonal antiserum stocks.

In vitro transcription and translation of recombinant Pf12 in a wheat germ lysate

Pf12 was modified by PCR using the primers Pf12 5'-Nco I and Pf12 3' 1A (see table 2.1) to generate a sequence identical to Pf12ec A1 plus a methionine start codon. This sequence was subcloned into Sma I-digested pBluescript SK- by blunt end ligation. Five micrograms of the Pf12/SK- construct was digested with Not I to linearize the DNA, extracted 2 times with chloroform, and sodium acetate precipitated. This DNA was incubated for 2 hours at 37° C in a 50 µl transcription reaction containing 10 mM DTT, 125 µg/ml BSA, 250 µM rATP, rCTP, and rUTP, 25 µM rGTP, 250 µM $m^7G(5')ppp(5')G$ (Boehringer-Mannheim), 5 µl of 5X transcription buffer (Promega), 40 units of Rnasin (Promega), and 20 units of T7 RNA polymerase (Promega). After confirming the presence of transcribed RNA by agarose gel electrophoresis, 5 U of RQ1 RNase-Free DNase (Promega) was added to the reaction which was incubated at 37° C for 15 minutes. The RNA was then extracted 2 times with chloroform and ethanol precipitated with 0.3 M sodium acetate. From 0.5-4 µg of RNA was translated into ^{35}S methionine-labelled protein (20 µCi of 10 µCi/µl stock; Dupont NEN) using the Promega wheat germ extract system according to the manufacturer's protocols. This labelled protein was used in the immunoprecipitation experiments described in chapter 3.

Production and purification of protein from baculovirus expression systems

Sf9 cell culture

Spodoptera frugiperda Sf9 cell lines (Pharmingen) capable of supporting *Autographa californica* nuclear polyhedrosis virus (AcNPV, baculovirus) infection and replication were grown in EX-CELL 401 media (JRH Biosciences) plus 10% FBS

(Hyclone), 2.5 µg/ml fungizone (Life Technologies), 50 µg/ml gentamicin (Life Technologies). This medium was called Excell-complete. These cells were grown at 27° C as monolayers on tissue culture plates (Falcon) or as suspension cultures in spinner flasks (Bellco Biotech). Monolayer cultures were split by tituration and transfer of cells three times per week; suspension cultures were seeded at $0.5x10^6$ cells/ml and split at culture densities of $2X10^6$ cells/ml. Pluronic acid (Life Technologies) was added to suspension cultures to minimize shear stress on the cells.

Transfection of Sf9 cells using lipofectin, and detection of recombinant baculoviruses by plaque assay

A mixture of 0.5 µg of AcNPV DNA, 1 µg of plasmid DNA containing the target gene, and 10 µl of lipofectin reagent (BRL), and 150 µl of Grace's Supplemented Insect Medium (Grace's medium; Life Technologies) were incubated for 15 minutes at room temperature. This mixture was added dropwise to one well of a 6 well dish (Nunc) containing $2X10^6$ Sf9 cells previously washed and overlaid with 150 µl of Grace's medium. This was incubated for 4 hours at 27° C, then brought up to a total volume of 3 ml with Excell-complete. After five days of incubation, the supernatant was removed, diluted in Grace's medium in decrements of 10^{-1} to 10^{-6}, and 2 ml of each dilution was added to $1.4X10^6$ Sf9 cells in the corresponding well of a 6 well dish. After 1 hour of incubation at 27° C with gentle rocking, the supernatant was aspirated from the monolayers and overlaid with 1% Seaplaque low-melting-point agarose (FMC) in Excell-complete (cooled to just below 42° C before adding). The overlays were placed on a level, vibration-free surface to solidify, then placed at 27° C in a humid incubator until plaques developed (5-7 days). If the target gene was on a plasmid containing the beta-galactosidase gene (such as the baculovirus expression vector pETL-JFE, figure A1.1B), recombinant baculoviruses could be detected as blue plaques by adding Bluo-gal (BRL) to the overlay mixture. Otherwise, recombinant plaques were detected using a dissecting microscope and

a strong light source hitting the surface of the plate at a 30° angle. In this case recombinant plaques were visualized as faint opaque spots with a translucent center on a translucent background of Sf9 cells. They were differentiated from wild-type AcNPV plaques by their lack of highly refractile occlusion bodies.

Recombinant plaques that were sufficiently isolated from AcNPV plaques were picked with a pasteur pipette and incubated in 1 ml of Excell-complete at 37° C for 4 hours to elute the virus from the gel plug. Dilutions of virus (10^{-1} and 10^{-2}) were made in Graces medium, and these were applied to new monolayers in 6 well dishes, which were in turn overlaid with agarose as before. This process of picking plaques and replating eluted virus was repeated two more times in order to isolate pure recombinant virus. Pure recombinant viruses were amplified to titres of $1x10^7$-$1x10^8$ pfu/ml by adding 200 µl of virus to $2x10^7$ Sf9 cells in Excell-complete and growing for 3 days.

Transfection of Sf9 cells using the Pharmingen Baculogold kit

The Pharmingen Baculogold kit was also used for generating recombinant baculoviruses, according to the manufacturer's instructions (Pharmingen). This method uses calcium phosphate transfection to introduce replication-defective linearized baculovirus and plasmid carrying the target gene into Sf9 cells. Recombination events occurring between the plasmid and the linearized virus generate recombinant replication competent viruses. The level of wild-type virus (or recombinant viruses without the target DNA insertion) generated from this recombination process is very low, making isolation of pure recombinant viruses by plaque assay much easier. Recombinant virus obtained from this method were subject to the same amplification process as above.

Detection of recombinant baculoviruses by dot blotting

Recombinant baculoviruses were identified by plaque assay, picked, and eluted from the gel plug into Excell-complete media. Two hundred microliters of this stock was used to infect a monolayer of $6x10^5$ Sf9 cells in Excell-complete medium for 2 days, then the supernatant was removed, and the cells were lysed in fresh 0.5 N NaOH for 15 minutes. The lysate was neutralized by addition of 20 µl of 10 M ammonium acetate, and then transferred to one well of the BioRad dot blot apparatus (BioRad) containing a nitrocellulose membrane prewetted in MilliQ water, followed by 1 M ammonium acetate, 0.02 N NaOH. After the lysate had been immobilized onto the nitrocellulose, it was washed once with 1 M ammonium acetate, 0.02 N NaOH. The membrane was then removed from the apparatus, rinsed one time in 4X SSC, and allowed to dry. DNA was fixed to the membrane by heating in a vacuum oven, and DNA hybridization was carried out with a Pf12-specific probe as described previously in this section.

Harvesting proteins produced by Sf9 cells infected with recombinant baculoviruses

A time course of recombinant virus growth and secreted protein production was conducted over 5 days. Fifty µl of plaque-purified recombinant baculovirus (Pf12sec bv #3 or #4) was added to five separate monolayer cultures of $6x10^5$ Sf9 cells grown in Excell (no FBS). Each supernatant was collected at different time points (0, 1,3,4,5 days), and resolved by 12% SDS-PAGE as described earlier. 18 µl of supernatant plus 6 µl of SDS lysis buffer was loaded per well.

Recombinant virus (Pf12bv sec #1,13,14,17, Pf12bv A2 #1, 2, 3), was similarly incubated with Sf9 cells for five days in Excell-complete medium. At the end of this period, both infected cells and supernatants were kept for further SDS-PAGE, Western blotting, and protein solubility analysis. Sf9 cells were lysed directly in 50 µl of SDS lysis

buffer and the cellular DNA was sheared by passing the lysate through a 26 gauge needle on a 1 ml syringe 10 times. 10 µl of this sheared lysate was resolved by SDS-PAGE. Supernatants from these long-term growth experiments were also kept for SDS-PAGE analysis. 10 µl of supernatant was also added to 10 µl of SDS-lysis buffer, and resolved by SDS-PAGE.

Determining the solubility of Pf12bv A2 protein by incubation in various denaturants and detergents

Two microliters of Sf9 cells ($6x10^5$) infected with recombinant viruses were incubated with 18 µl of either 1% SDS, 8M urea, 1% NP-40, or RIPA buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 0.1% SDS, 1% sodium deoxycholate, 1% TritonX-100) at 4° C for 1 hour, microfuged at 16000 xg, added to 4 µl of SDS lysis buffer, and resolved by 15% SDS-PAGE.

DNA Immunization methods

Quantitation of CAT activity in mice injected intramuscularly with three different CAT expression plasmids

Milligram quantities of CAT and control pPSC1 plasmid DNA was obtained using Qiagen tip 500 columns (Qiagen) according to the manufacturer's protocol. The DNA was resuspended to a final concentration of 2 µg/µl in 10% India Ink/ PBS. Each plasmid was injected into a separate group of Balb/c mice (n=3). Each mouse quadriceps muscle was immunized with 20 µl of DNA solution (40 µg) using a 26 g needle. The quadriceps muscles were harvested after 4 days, monitored for the presence of ink, and then homogenized in 800 µl of 0.25 M Tris-HCl pH 7.8. 10 µl of a 1:20 dilution of the homogenate was used in the Pierce BCA assay to quantitate the amount of protein in each homogenate. Each sample was normalized to 100 µg of protein in a total volume of 132 µl. This volume of quad homogenate was added to 15 µl of 2M Tris pH 7.8, 2 µl of 40

mg/ml acetyl CoA (Sigma), and 1 µl of ^{14}C-Chloramphenicol (0.25 µCi/µl, Amersham). The reaction was incubated at 37° C for 1 hour and extracted with 1 ml of ethyl acetate. The upper layer was removed to a new tube, evaporated in a speed vacuum apparatus, and resuspended in 30 µl of ethyl acetate. This aliquot was resolved by thin layer chromatography using a 250 µm layer silica gel plate (Whatman) and a 190:10 mixture of chloroform:methanol. Monoacetylated ^{14}C chloramphenicol was visualized using a phosphorimager.

DNA Immunization of Mice and Rats

Plasmid DNA for immunization experiments in rats and mice was obtained using Qiagen columns according to the manufacturer's protocol (Qiagen). The first rat DNA immunization used DNA that was resuspended in TE at a final concentration of 1µg/µl; this protocol was changed for all remaining rat immunizations to the use of a 1µg/µl solution of DNA in PBS. Each rat quadricep was injected with 100 µg of DNA in a 100 µl volume using a 21 gauge needle. For mouse DNA immunizations a 2µg/µl DNA solution in 20% sucrose/PBS was used, following the method of Wolff and colleagues (Wolff et al., 1990). Each mouse quadricep was injected with 40 µg of DNA in a 20 µl volume using a 26 gauge needle. After the initial immunization, these animals were boosted three times at 4 weeks intervals, and bled by either the jugular (rat), or tail (mouse) vein to test for Pf12 specific antibodies.

Immunoprecipitation of biotinylated COS cell surface proteins using antiserum from DNA immunized mice

COS cell surface proteins were biotinylated using NHS-LC-Biotin (Pierce) and the method of Lantz (Lantz and Holmes, 1995). $5x10^7$ COS cells were transfected with Pf12/pPSC1 using the DEAE method, harvested post-transfection using PBS/ 0.5 mM EDTA, centrifuged at 500 xg, and resuspended in 1 ml of PBS containing 1 mM NHS-LC-

Biotin. This mixture was incubated for 25 minutes at room temperature, then washed three times with PBS. The biotinylated cells were then lysed by resuspension in a 1 ml solution of 20 mM Tris-HCl pH 7, 150 mM NaCl, 0.5% NP-40, 0.5% Tween-20, 0.5% BSA, 5 mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, 10 µg/ml aprotinin (TN lysis buffer). After incubating the lysate for 1 hour at 4° C, it was centrifuged at 3000 xg to pellet cell debris and nuclei. The supernatant was kept for immunoprecipitation studies.

Supernatants from biotinylation experiments were precleared by adding 10 µl of a 1:1 slurry of Protein G sepharose (Pharmacia) in TN lysis buffer and incubating overnight at 4° C. This preclearing step was undertaken to remove any specificity proteins in the supernatant may have for Protein G sepharose. The precleared lysate was then centrifuged at 3000 xg and the supernatant was kept for further studies. 1 µl of test antibody, 20 µl of a 1:1 slurry of Protein G sepharose in TN lysis buffer, and 600 µl of TN lysis buffer was added to 200 µl of the precleared supernatant. This was incubated at 4° C for 1.5 hours, then washed 2 times with TN lysis buffer containing 600 mM NaCl, 2 times with TN buffer containing 300 mM NaCl, and 2 times with TN buffer. With each wash, almost all of the wash solution was aspirated, and after the final wash the Protein G sepharose pellet was resuspended in 40 µl of SDS lysis buffer. This sample was then resolved by SDS-PAGE and Western blotted with 1 µl of Streptavidin-HRP (Pierce) in 10 ml of 5% BSA/ PBS+0.3% Tween-20 at 4° C for 1 hour. The blot was washed 3 times in PBS+0.3% Tween-20, followed by 3 times in PBS+0.1% Tween-20. The blot was then developed using the Amersham ECL kit, according to the manufacturer's instructions.

Plasmodium falciparum culture in human red blood cells

Several *P. falciparum* strains were used in experiments in chapter 3 and 6. D10 is a chloroquine sensitive clone of the FC27 isolate (Culvenor, 1987); 3D7 is a chloroquine sensitive clone of the NF54 isolate (Chawira, 1987); K1A is a multi-drug resistant clone of

the Thailand K1 strain (Chawira, 1987), and HB3 is a chloroquine sensitive clone of the Honduras I/CDC strain (Bhasin, 1984). All parasite strains were stored as glycerolyte-preserved fractions in liquid nitrogen. To make initial frozen parasite stocks 0.3 packed cell volumes (V) of glycerolyte preservative (Baxter) was added to a 200-500 µl pellet of ring-infected RBCs, incubated at room temperature for 5 minutes, then an additional 1.33 V was added to the mixture. These stocks were frozen for 18 hours at -70° C, then transferred to liquid nitrogen. Frozen parasite stocks were prepared for culture by (1) thawing quickly and slowly adding 0.2 volumes of 12% NaCl, (2) incubating the mixture for 2 minutes at room temperature, (3) adding 2 volumes of 1.6% NaCl, and incubating as in (2), (4) centrifuging the mixture at 200 xg, (5) adding 2 volumes of 0.9% NaCl, 0.2% dextrose to the RBC pellet, (6) centrifuging the mixture as in (4), and using the RBC pellet as a seed stock for a new parasite culture. These stocks were cultured with human type AB RBCs at a 5% hematocrit concentration (a very kind gift from Dr. Elliott, originally from the laboratory of Dr. Russell Howard) in M-RPMI containing: RPMI 1640 (Life Technologies) supplemented with 25 mM hepes, 0.4 mM hypoxanthine, 0.2 % sodium bicarbonate (pH 7.2) and 10% human AB serum (provided for initial growth of the parasites by Dr. Elliott and for continued parasite growth as pooled serum from The Canadian Red Cross blood donor clinic). This parasite growth media was called M-RPMI. Blood used for malaria cultures was separated from the serum component by centrifugation at 1500 xg for 20 minutes, the lymphocyte 'buffy coat' was removed by aspiration, the RBCs were washed 2 times in RPMI, then resuspended in RPMI to a hematocrit of 50%. These washed RBCs were stored at 4° C for up to one month. The parasites were grown in a 3% CO_2/ 1% O_2 (balance N_2) atmosphere at 37° C. The medium was carefully aspirated and replaced each day (and RBCs every second day), and the cultures were diluted 1:10 into new media containing RBCs when 10% of the RBCs became infected with parasites. The cultures were monitored by making a culture blood smear on a glass slide, air drying the slide, fixing the slide for 2 minutes at room temperature with 100%

methanol, then staining the slide with Giemsa stain (BDH) for 5 minutes at room temperature and viewed with an Olympic light microscope at a 1000X magnification.

Sorbitol synchronization of *P. falciparum* cultures

This treatment enriches for ring stage parasites by selectively lysing trophozoites and schizonts. Cultures with a high percentage of RBCs infected with ring stage parasites were harvested by centrifugation at 700 xg, and 1 packed cell volume of RBCs was added to 4 volumes of 5% w/v Sorbitol (Sigma) prewarmed to 37° C. The RBCs were incubated at 37° C for 10 minutes, centrifuged as before, and washed 2 times with RPMI before resuspending in M-RMPI.

Separation of ring, trophozoite, and schizont stage parasites using percoll gradients.

Percoll gradients were set up in 15 ml conical tubes (Falcon) by first adding 2ml of 90% v/v percoll/ RPMI (Stock: 9 ml Percoll, 1 ml 10x RPMI), then layering 80%, 70%, 60%, and 40% percoll/ RPMI on top of this. 2 ml of a standard parasite culture at 10% parasitemia was carefully layered on top of this and centrifuged at 2000 xg for 20 minutes at room temperature. Parasites gathered at the interface of each layer were aspirated using a pasteur pipette, and washed 2 times in RPMI. These parasites could then be counted by Giemsa staining, introduced into new cultures, fixed to slides for immunofluorescence, or prepared for immunoelectron microscopy.

In order to harvest proteins from the various parasite life cycle stages, the same protocol was used with minor modifications. 5% sorbitol was added to the percoll/ RPMI solutions in order to lyse the schizont and trophozoite infected-RBCs right on the gradient. Protease inhibitors were also added to the percoll/RPMI solutions: 50 µg/ml leupeptin, 2 mM PMSF, 100µg/ml aprotinin, and 10 mM EDTA. The lysed schizonts and trophozoites could be harvested quickly from the gradient, washed twice in cold PBS + protease

inhibitors, and immediately solubilized in 50-100 µl of SDS lysis buffer. By contrast, ring-infected RBCs had to be lysed in PBS/ 0.15% saponin after separation on the gradient. The amount of parasite proteins resolved by SDS-PAGE was normalized using the BCA assay (Pierce) to 0.1-0.2 µg per sample.

Metabolic labelling of parasite cultures and Immunoprecipitation of ^{35}S-methionine-labelled parasite proteins.

A total volume of 5 ml of parasite culture at 20% parasitemia was centrifuged at 700 xg, and the pelleted RBCs were washed 2 times with RPMI (no methionine, no human serum). These cells were resuspended in 2 ml of M-RPMI (no methionine), and 100 µl of ^{35}S methionine (Amersham) was added. This culture was incubated under standard conditions for 18 hours, then RBCs and culture supernatants were harvested for immunoprecipitation studies.

After ^{35}S-methionine labelling of parasite proteins, the RBC pellet and supernatant were solubilized, precleared with Protein A, and incubated with antiserum + Protein A to immunoprecipitate parasite proteins. The RBC pellet was solubilized for 1 hour at room temperature by adding 200 µl of 2% SDS in NETT buffer containing 2 mM PMSF, 100 µg/ml leupeptin, 100 µg/ml aprotinin, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.5, 0.5% Triton X-100. 400 µl of NETT buffer was added to 100 µl of the culture supernatant and incubated under the same conditions as the RBC pellet. The solubilized RBCs were centrifuged at 16000 xg, and the supernatant was collected. The RBC lysate and culture supernatants were incubated for 18 hours at 4° C with 1/5 volumes of a 1:1 mixture of Protein A:NETT buffer. The supernatants were collected after centrifugation at 16000 xg, mouse polyclonal antiserum was added at a 1:100 dilution, and the samples were incubated for 1 hour at 4° C. Next, a 1/5 volume of 1:1 mixture of Protein A:NETT buffer was added to the samples, and the incubation was continued for another hour.

Finally these samples were centrifuged at 16000 xg, washed five times with NETT buffer, and the Protein A pellets were resuspended in SDS lysis buffer.

Invasion inhibition experiments using anti-Pf12 antiserum and *P. falciparum in vitro* cultures

These assays were done using small scale 3D7 and D10 parasite cultures, anti-A2 and anti-B2 antiserum. Twenty µl of parasitized RBCs synchronized to the schizont stages were added to 160 µl of M-RPMI, 20 µl of fresh RBCs, and 2 µl of anti-Pf12 or pre-immune control antibody (final dilution of 1:100). This mixture was incubated for 12-18 hours under standard culture conditions. Giemsa stained smears were made of the parasites cultures, and newly infected RBCs were enumerated. Approximately ten fields or 1000 RBCs were counted for experiment, and the percent of newly invaded RBCs was calculated as the number of newly invaded RBCs divided by the total number of uninfected RBCs x 100. This value was used to compare the effect of the anti-Pf12 and pre-immune serum on the invasion of RBCs by merozoites.

Preparation of *P. falciparum* infected RBCs for immunoelectron microscopy

Fixation of specimens prior to antibody staining

Parasitized RBCs harvested from both asynchronous and synchronous cultures were centrifuged at 1500 xg, washed once in RPMI and then resuspended in different concentrations of glutaraldehyde or paraformaldehyde in 50 mM phosphate buffer, pH 7.4 (PB) for 1-24 hours at 4° C. The cells were then washed 2 times in PB, and dehydrated through a graded series of alcohols (30/ 50/ 75/ 95/ 100% at -20° C) The specimens were embedded in K4M-Lowicryl (Marivac), sectioned (Reichert-Jung Ultracut, Austria), and mounted on 300 mesh copper grids. The grids were blocked in 1% BSA/ PB for 10 minutes, incubated with primary antibody (1:100 dilution in 1% BSA/ PB) for 30 minutes at room temperature, and similarly with secondary 10 nm gold-conjugated goat anti-mouse or goat anti-rat antibody (EY Laboratories, San Mateo CA; 1:100 dilution in 1% BSA/ PB)

with a 1% BSA/ PB wash between each incubation. These grids were incubated in distilled water for 10 minutes, and then examined in a Phillips model 410 transmission electron microscope.

Fixation of specimens after RBC permeabilization and antibody staining

Parasitized RBCs from cultures were washed with RPMI, and lysed with 20 packed cell volumes of 0.15% Saponin (Sigma)/ PBS at 4° C for 2 minutes. The lysed cells were centifuged at 1500 xg and washed 2 times with PBS before antibody incubation. Parasitized RBCs were also permeabilized by resuspending 100 µl of cells in 500 µl of PBS containing 10 µg/ml of Streptolysin O (Sigma). After incubating the cells at 37° C for 5 minutes, the cells were centrifuged at 1500 xg, and washed 2 times in PBS. These cells were then reacted with various dilutions of primary antibody and secondary 10 nM gold-conjugated goat anti-mouse antibody with 2 PBS washes after each antibody incubation.

Permeablilized, parasitized RBCs incubated with primary and secondary antibody as described above were fixed overnight in 2% glutaraldehyde/ PB. These cells were washed 3 times with PB, then stained with 1% OsO_4/ PBS for 1 hour at room temperature. The specimen was then incubated at room temperature in a graded series of alcohols (25%, 50%, 75%, 90%, and 100% ethanol, 1 hour each at room temperature), followed by propylene oxide, 1:1 propylene oxide: TAAB 812 resin (Marivac) (overnight at room temperature), and TAAB resin alone (overnight at 65° C). The cured resin was then sectioned and examined by transmission electron microscopy.

COS cell expression methods

DEAE dextran transfection COS cells

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DME; Life Technologies) + 10% fetal bovine serum (FBS; Hyclone) as monolayers in a 5% CO_2

incubator at 37° C. These cells were grown on 10 cm tissue culture plates to approximately 80% confluence, washed once with DME, then overlaid with 10 ml of a mixture of 1-10 μg of DNA in DME containing 500 μg/ml of DEAE dextran (Sigma), 100 μM chloroquine (Sigma). The cells were incubated with the DNA/DEAE mixture for 2-4 hours in 5% CO_2/ 95% air, at 37° C. The supernatant was aspirated completely, and 2 ml of PBS + 10% DMSO was added to the cells for 2 minutes at room temperature and then quickly aspirated. Finally, 20 ml of DME+10% FBS was added, and the cells were incubated overnight in 5% CO_2/ 95% air, at 37° C. The following day, these cells were lifted from the culture plates using trypsin/EDTA (Life Technologies), and seeded onto new plates. Three days after the transfection the cells were harvested by removing the DME+10%FBS, and incubating the cells with PBS + 0.5 mM EDTA in 5% CO_2/ 95% air at 37° C for 10 minutes. These cells could then be used for immunofluorescence, Western blotting, or biotinylation experiments.

Isolation of cDNA and genomic clones by COS cell panning.

COS cell panning experiments followed essentially the same protocol described by Elliott (Elliott et al., 1990). *P. falciparum* genomic libraries (Fa3+, Fa4+ in pJFE14; 40 μg) were mixed 6.7×10^7 COS cells, and cDNA libraries (DAF B+, in pJFE14 DAF; 160 μg), mixed with 1.3×10^8 COS cells in Dulbecco's Modified Eagle's Medium (DME, Life Technologies). These cells were transferred to 4 mm gap BioRad electroporation cuvettes and electroporated at 250 V, 960 μF using a BioRad gene pulser. The cells were recovered in DME/10 % Fetal Bovine Serum (FBS) and grown on tissue culture plates at 37°C, 5% CO_2/ 95% air for 72 hours to allow for maximum expression of surface proteins.

COS cells were detached using PBS/ 0.5mM EDTA, centrifuged at 200 xg, and resuspended in PBS/0.5mM EDTA/ 5% FBS plus Papua New Guinea Pooled antiserum (PNG, 1:40 dilution). After 1 hour incubation at 4°C, the unbound antibody was separated

from the COS cells by pelleting through an FBS cushion. The COS cells were resuspended in PBS/ 0.5mM EDTA, passed through a 100 μm nylon mesh (Tetko) and added to 10 cm bacteriological petri dishes (Fisher Scientific) that had been previously coated at room temperature with 10μg/ml goat anti-human IgG (Cappel Laboratories) in 50 mM Tris pH 9.5. The panning process was conducted by incubating the COS cells on these plates for three hours at room temperature with occasional swirling. Unbound COS cells were washed away with PBS/EDTA, and bound COS cells were lysed and recovered in Hirt lysis solution (0.6% SDS/ 10mM EDTA).

Cellular DNA was precipitated from this fraction by adding 1:5 volume of 5M NaCl and incubating overnight at 4°C. Soluble plasmid DNA was separated from cellular DNA by centifugation (9000 xg, 30 minutes, 2°C). The supernatant was collected, and plasmid DNA was precipitated from this fraction by adding 10 μg of 10 mg/ml tRNA, 1/10 volume of 3M sodium acetate, an equal volume of isopropanol, and freezing on dry ice. After thawing, plasmid DNA was centrifuged at 10000 xg, 4°C, washed in 75% ethanol, resuspended in TE buffer, and extracted three times with phenol/chloroform followed by chloroform. The extracted Hirt DNA was precipitated in 0.3M sodium acetate and 95% ethanol, washed in 75% ethanol, and resuspended in 10 μl of TE.

Electroporation of Hirt DNA into *E. coli* WM1100

Two microliters of Hirt DNA was mixed with 40 μl of electocompetent WM100 in a cold 2 mm gap BioRad cuvette and pulsed with the BioRad gene pulser at 2.5 kV, 25 μF, 200 Ω. The electroporated bacteria were immediately resuspended in 2 ml of SOC media (2% Tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose), grown for 90 minutes at 37° C, inoculated into 500 ml 2YTMT media (1.6% Tryptone, 1% yeast extract, 10mM NaCl, 2mM $MgCl_2$, 10 mM Tris pH 7.5)

plus 100 μg/ml Ampicillin (Sigma), and grown overnight. The harvest bacterial pellet was used for spheroplast fusion.

Spheroplast Fusion

This process involved making spheroplasts out of the Hirt-transformed WM110 bacteria described above, and fusing these to COS cells. Using this less efficient method of transfection and larger amounts of DNA (from the large-scale growth of electroporated WM1100) increases the chances of having individual COS cells containing plasmids with distinct parasite DNA inserts, which minimizes the rescue of 'passenger' plasmids. WM1100 bacteria containing Hirt plasmid DNA were made into spheroplasts by successive additions of 35 ml of cold 20% sucrose/Tris-HCl 50 mM pH8, 1 ml of 5 mg/ml Lysozyme (Sigma), 2 ml of cold 0.25 M EDTA pH 8, and 2 ml 50 mM Tris-HCl, 37°C. 20 ml of cold DME/ 10% sucrose/ 10 mM $MgCl_2$ was slowly added to the spheroplasts, and this suspension was pipetted onto COS cell monolayers. COS cells were centrifuged at 1000g for 10 minutes at room temperature, the supernatant was removed, and spheroplasts were fused to these COS cells by the addition of 50% PEG/DME for 2 minutes. After washing the PEG-treated monolayers with DME, the cells were grown for another 2 days in DME/ 10% FBS, and prepared for another round of panning.

The panning process was continued two more times, for a total of three panning experiments, the first using COS cells electroporated with the initial libraries, the second and third using COS cells transfected with the Hirt plasmid DNA pools by spheroplast fusion. The final plasmid pool, Hirt#3, was electroporated into WM1100, and a large scale alkaline lysis prep (with CsCl purification) of the plasmid DNA was made. This DNA was transformed into *E. coli* strain DH5α, and individual colonies were obtained for further study.

TABLE 2.1. Sequences of primers used in the creation of Pf12 deletants, sequencing of panning clones, and the creation of new expression vectors.

Primer	Sequence
Pf12 A series	
Pf12 5' 1A	CTCTCTGGATCCGAAGGGCATAAAAATTTAACATGT
Pf12 5' 2A	CTCTCTGGATCCATGTTTATGAGACGTAGTTTA
Pf12 3' 1A	TCTCTCCTGCAGCTAGTATGTATTATATCTTGAAGC
Pf12 3' 2A	TCTCTCCTGCAGCTAGATTAAATCATTAGCATGTGC
Pf12 B Series	
Pf12 5' 1B	ATCGATCTAGAACTATTAATGGTTCCAATGGA
Pf12 5' 4B	CGGGATCCTCTAGATGTTTAGGGATATCCTTTG
Pf12 5' 5B	CGGGATCCTCTAGATTAGAAAAGGTAACTATA
Pf12 5' 6B	CGGGATCCTCTAGAACAATAACTATTAATGGTTCC
Pf12 5' 7B	GGATCCTCTAGAGGATTTAAATGTCCAAGCA
Pf12 5' 8B	GGATCCTCTAGAAATGGAAATCCTTCAAGTAA
Pf12 3' 1B	TATAATACATACCAAGATTAAGCGGCCGCATGAC
Pf12 3' 2B	CTATTAATGGTTCCAATGGAACGCGTTAAGCGGCCGCACGTCA
Pf12 3' 3B	GTCAGTGCGGCCGCTTATCCGGATTCACAAAAACAATATATAGG
Pf12 3' 4B	GTCAGTGCGGCCGCTTATCCGGATCCATTGGAACCATTAATAG
Pf12 3' 5B	GTCAGTGCGGCCGCTTATCCGGAATCATGTGGTTCAACAGAA
Pf12 3' 6B	GTCAGTGCGGCCGCTTATCCGGAGTTGGATATATAATCTTGGT
Baculovirus constructs	
BxBv5'	TACGGATCCTCTAGAGATCCCTCGACCTCG
Pf12 Bv 3'	GTTGCGGCCGCTTAGTTGGATATATAATCTTGGT
ETL-JFE up	CTAGCACCCGGGACTGCAGAGCGGCCGCGGAGGCCTG
ETL-JFE dn	GATCCAGGCCTCCGCGGCCGCTCTGCAGTCCCGGGTG
In vitro Transcripton/ Translation	
Pf12 5' Nco I	CTCTCTCCATGGAAGGGCATAAAAATTTAACATGT
Sequencing	
5'pJFE14	ACTTCTAGGCCTGTACGGA
T3	ATTAACCCTCACTAAAG
T7	AATACGACTCACTATAG

TABLE 2.1. (continued)

Primer	Sequence
pBT1TØ	
Tag His6 dn	GGAATTCGGATCCTCTAGAGTGATGGTGATGGTGATGCGAGCCT
Tag His6 up	GACTAGTCATATGAGAGGCTCGCATCACCATCACCATCACTCTA
BT1 poly dn	GGAATTCACGCGTTGCGGCCGCGCTAGCACTAGTG
BT1 poly up	GATCCACTAGTGCTAGCGCGGCCGCAACGCGTGAATTCCTGCA
Tø5' II	ATATATCTGCAGGCGGCCGCAAAGCTTGGCTGCTAACAAAGCCCG
Tø3'	ATATATCTGCAGGCGGCCGCAAAGCTTGGCTGCTAACAAAGCCCG
pPSC1	
SV40 ori 5'	AGCTAGAAGCTTGTCTTACTCCGCCCATCCC
SV40 ori 3'	TAGGCTTTTGCAAAAAGCTAAAGATCTTCGATC
CMV 5'	AGCTAGAGATCTTTGGTTATATAGCATAAATCAAT
CMV 3'	GCAGTCACCGTCCTTGACTCTAGAAGCTAG
Sig1 5'	CTCTCTACTAGTCCACCATGCCCATGGGGTCTC
Sig2 5'	CTCTCTTCTAGAACCACCATGCCCATGGGGTCTC
Sig1 3'	CTCTCTGAATTCTCTAGAGACTTCTCCGAGGCAGGAAGC
CAT	
CAT 5' 2	CTCTCTTCTAGAACCACCATGGAGAAAAAAATCACTGGATAT
CAT 3'	CTCTCTGCGGCCGCTTACGCCCCGCCCTGCCACTC

CHAPTER III

GENERATION OF ANTI-Pf12 ANTISERUM USING A Pf12 SUBFRAGMENT (Pf12ec A2) EXPRESSED IN *E. coli*

A. Introduction and background

The main goal of this project was to determine when and where Pf12 was expressed during the life cycle of *Plasmodium falciparum*. In order to accomplish this, it was necessary to raise antiserum specific for Pf12, and to use this antiserum as a reagent for detecting the parasite protein. Generation of antiserum in turn required the immunization of rats or mice with either the purified native protein, a recombinant version of the native protein, or a synthetic peptide made from the protein sequence. I considered using recombinant Pf12 to generate Pf12 specific antibodies for two reasons: early experiments with Pf12 peptides failed to produce specific antibodies after rabbit immunization experiments, and there was no obvious source of pure, native Pf12 protein.

Usually one of the first steps in characterizing a newly discovered antigen for which the amino acid sequence is known is to synthesize a peptide fragment of the protein and use the peptide to raise specific antibodies. This method is relatively expensive but fast. However, it may fail if the chosen peptide is not a good B-cell epitope, or if the region within the native protein corresponding to the peptide is inaccessible to antibodies due to its hydrophobicity, charge, or presence in regions of secondary and tertiary structural domains. In my preliminary experiments, Pf12-peptide specific antibodies generated by Elliott and Howard failed to stain Pf12-transfected COS cells in experiments prior to this thesis work, indicating that the peptide either did not elicit a robust anti-Pf12 response in test animals, or that the epitope was not accessible to antibodies in the larger context of the native protein.

In addition, there was no obvious source of pure, native Pf12. Because Pf12 was newly discovered and had no significant homology to other proteins in the database, devising a strategy for purifying Pf12 from parasites based on the protein sequence alone

would have been difficult. Growing sufficient parasite material would have also been a daunting task. For all of these reasons I decided to make recombinant Pf12 using an established prokaryotic expression system.

This chapter describes the production of recombinant Pf12 in *E. coli*, and use of this material to generate specific antibodies to localize Pf12 in the parasite. Even though recombinant Pf12 made in this system would probably not be folded in a native conformation, I hypothesized that a strong polyclonal response against a denatured protein might generate highly specific antibodies for epitopes which would be present on both the native as well as the denatured protein.

B. Results

Cloning of Pf12 subfragment DNA sequences into the prokaryotic expression vector, pT7-7His6

Using wild-type Pf12 DNA (see chapter 1) as a template, I used *Taq* DNA polymerase and PCR to create four Pf12 subfragments (figure 3.2; see table 2.1 for PCR primers), each with a 5' Bam HI and a 3' Pst I restriction site. These were inserted into the prokaryotic expression vector pT7-7His6 (figure 3.1), a modified version of the Tabor and Richardson vector pT7-7 (Mizushima and Nagata, 1990). By using this vector, recombinant proteins could be synthesized and purified in large quantities quickly and easily, and the combination of the pT7-7His6 vector and *E. coli* host strain BL21 DE3 were created specifically to minimize (but unfortunately not totally eliminate) leaky expression of the target gene, thus minimizing the effect of recombinant protein toxicity on the host cells. BL21 DE3 is a lysogen harboring a lambda derivative containing the T7 RNA polymerase gene under the control of the lac UV5 promoter. T7 RNA polymerase and consequently the target gene expression can be induced by the addition of IPTG to the culture medium (Perlmann et al., 1987).

The Pf12 A1 protein (lacking native signal and anchor sequences) failed to be expressed in *E. coli*

The first subfragment was designed to contain as much of the wild-type Pf12 sequence as possible: only the signal sequence and the putative anchor signal were deleted (figure 3.2). *E. coli* BL21 DE3 competent cells were transformed with the A1 containing plasmid, and two separate clones were chosen for further study. These were grown to an OD_{600} of 0.6, then induced with IPTG. As shown in figure 3.3, I was unable to detect any recombinant protein in lysates from non-induced or induced cultures (clones 1 or 2) resolved by 12% SDS-PAGE and stained with Coomassie blue. It should be noted that both clones synthesized less total protein after IPTG induction. The intensity of staining of many proteins including the 45 and 37 kDa species decreased dramatically, suggesting that induction of the Pf12 protein in *E. coli* may have affected the growth of the bacteria and/or the overall production of bacterial proteins.

Testing for expression of other Pf12 subfragments; Pf12 A2, A3, and A4

Three other subfragments of Pf12 were constructed as described above. Although the A1 protein was not made at detectable levels in the previous experiments, it was possible that Pf12 subfragments would be expressed at detectable levels. These were named A2, A3, and A4, representing the N-terminal two-thirds, middle third, and C-terminal two-thirds of the protein, respectively (figure 3.2). The region each subfragment covers was selected randomly, with the intent to divide Pf12 into three different sub-regions, with some overlap between sub-regions.

Of the three subfragments, A2 was expressed at the highest level on IPTG induction, as shown in figure 3.4. In this case a ≈27-28 kDa protein was synthesized on induction of A2-transformed *E. coli*, whereas no induced proteins were detected in *E. coli* transformed with the A3 or A4 plasmids. Three additional A2 transformed colonies were

analyzed to confirm the initial result (figure 3.5). Given that all three of the colonies produced A2 protein, it was decided to proceed with large-scale protein production.

Large scale production of Pf12ec A2

Large scale production of Pf12ec A2 followed the protocol given in chapter 2. Briefly, an A2-transformed colony of *E. coli* BL21 DE3 was inoculated into 1L of 2YTMT medium, grown at 37 °C until the culture reached an OD_{600} of 0.6, and then induced with IPTG and grown for an additional 5 hours. The harvested bacteria were lysed in a French Pressure cell, and the resulting washed pellet (consisting of A2 protein as inclusion bodies) was solubilized in 8 M urea. Boiling the pelleted inclusion bodies in the presence of 1% SDS sample buffer and 10% beta-mercaptoethanol for 15 minutes was not enough to completely solubilize the A2 protein for SDS-PAGE analysis. Rather, the inclusion bodies had to be fully denatured in 8M urea for at least 30 minutes prior to incubation with SDS-PAGE sample buffer in order to be resolved adequately on protein gels. Figure 3.6 details an experiment which demonstrates this fact. No recombinant protein was detected on the gel when the pellet is solubilized solely in SDS (figure 3.6). In this experiment insoluble aggregates of protein present in the SDS extract are most likely not loaded onto the gel because they are lost in the final centrifugation step intended to remove insoluble debris still present after sample buffer treatment. Previous analysis of small-scale cultures, such as those shown in figure 3.4, did reveal some recombinant protein because such a small amount of insoluble debris was present that these 'preclearing' spins (immediately prior to gel loading) were deemed unnecessary.

Two-step purification of Pf12ec A2 using nickel affinity chromatography and preparative SDS-PAGE

Nickel chelating columns were prepared as described in chapter 2, equilibrated with 8M urea pH 8, and the urea-solubilized Pf12 A2 pellet was loaded onto the column. After

washing the column extensively and applying a series of elution buffers of decreasing pH, A2 was eluted at pH 4.3, and 3.3 (figure 3.7). A number of fractions from the column were resolved by 12% SDS-PAGE (figure 3.8). The predominant protein species in fractions 17-23 has an observed molecular weight of 27-28 kDa, close to that estimated for Pf12ec A2. In addition there are several other proteins present, both of higher and lower molecular weights. While the low molecular weight species is most likely a proteolytic product of Pf12ec A2, the higher molecular weight species is probably a contaminating *E. coli* protein that co-eluted with A2.

Because of the presence of possible contaminating proteins, the Pf12ec A2 protein eluted from the nickel column was further purified by preparative SDS-PAGE and electroelution. One small well was created for protein markers, along with a single large central well in a large (20 x 20 x 0.3 cm) 12% polyacrylamide gel. The nickel column fractions containing Pf12ec A2 were loaded into the central well and resolved by SDS-PAGE. After separation, the gel was lightly stained in a Coomassie/water to visualize the protein without fixing it in the gel. A large diffuse band migrating at approximately 20 kDa was observed. The stained region plus a few millimeters on either side was excised in order to obtain a maximum yield of protein. The gel slice was then finely diced with a scalpel and added to the electroelution chamber of an Elutrap apparatus. Pf12ec A2 was electoeluted from the slice fragments, and the resulting soluble protein was resolved by 12% SDS-PAGE. A single protein of the expected size was observed (figure 3.9) . This electroeluted protein was pooled, quantitated using a BCA assay, and used for rat immunizations. The final yield of protein from these experiments was approximately 0.5 mg recombinant protein/L of original culture.

Generation of rat anti-A2 antiserum

Once purified Pf12ec A2 protein was obtained, two rats were immunized by IP injection of 50 µl of protein (1 mg/ml) emulsified in an equal volume of Complete Freund's Adjuvant (CFA). The animals were boosted three more times at three to four week

intervals, using the same dose of antigen in IFA. Two weeks after the final boost, antisera were collected and assayed for Pf12ec A2 specificity by ELISA. Both of the immunized rats showed high titres (>1:10,000) compared to no signal in the pre-immune sera or in rats who received the adjuvant alone (data not shown). These Pf12-specific antibodies were characterized further in Western blotting, immunoprecipitation, and immunfluorescence experiments.

Rat polyclonal anti-A2 antiserum gave a weak but detectable and specific signal on Western blots of *E. coli* but not COS cell generated protein

As shown in figure 3.10, the rat anti-A2 antiserum recognized Pf12ecA2 from three different steps in the purification process: 1) the inclusion body pellet obtained immediately after French press lysis (6K), 2) a fraction eluted from the nickel column (F21), and 3) Pf12ec A2 further purified by preparative SDS-PAGE and electroelution (A2). In addition to the 25 kDa A2 protein, rat anti-A2 antiserum stains a 14 kDa protein in the crude lysate (6K). This proteolytic product of A2 was observed previously in figures 3.6 and 3.8.

By contrast, rat anti-A2 antiserum failed to recognize Pf12 produced by Pf12/pJFE14-transfected COS cells and analyzed by Western blotting (data not shown). COS cells were transfected with pJFE14 or Pf12/pJFE14 and harvested 72 hours later. A small portion of the transfected cells were retained for surface staining (see below for results of IFA), and the remainder were lysed and used to generate Western blots. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and stained using rat pre-immune or anti-A2 antiserum. No difference in staining was observed between the two types of antisera for vector or for Pf12-transfected COS cells.

Rat anti-A2 antiserum gave a weak but detectable signal on Western blots of *P. falciparum* parasites

Western blotting of protein extracts obtained from *in vitro* parasite cultures also revealed the specificity, albeit weak reactivity, of the rat anti-A2 antiserum for parasite Pf12 (figure 3.11). Infected RBCs containing parasites from three different strains were lysed with 0.15% Saponin detergent, which disrupts the RBC membrane without affecting the parasite membrane itself. In this way a significant proportion of the RBC membranes could be separated from the parasites by low-speed centrifugation and washing steps. The washed parasites were then lysed in SDS lysis buffer, resolved by SDS-PAGE, and Western blotted with rat anti-A2 antiserum or with pre-immune control antiserum. Initial experiments revealed the anti-A2 antiserum reacted with protein bands of different sizes, depending on the strain; a 35 kDa band was seen in the K1A strain parasites, whereas a 32-34 kD doublet was seen in the 3D7 parasites. No signal in this part of the gel was seen in the pre-immune serum, and the signal was also absent from the D10 strain in the post-immune serum. In all instances where signal was obtained, the affinity of the anti-A2 antiserum for these lower molecular weight proteins was apparently low, since only a weak signal was obtained. Control experiments done with uninfected RBCs (RBC lane in the 3D7 blots) revealed an additional cross-reactive band at 65 kDa detected by the post-immune serum, which was also seen in D10 parasites, as well as in the molecular weight marker (corresponding to BSA; the latter not shown). The conclusion from these experiments was that the rat anti-A2 antiserum functioned, but just barely, as a reagent for detecting the full-length Pf12 protein generated by parasites.

Immunoprecipitation studies with rat anti-A2 antiserum

To further characterize the rat anti-A2 antiserum another approach was taken, as shown in figure 3.12. In this case radiolabelled Pf12 A1 protein (Pf12 lacking both anchor

and signal sequences, see figure 3.2) was made by *in vitro* transcription and translation, and the anti-A2 antiserum was tested for its capacity to specifically immunoprecipitate the *in vitro* transcribed and translated polypeptide. Briefly, A1 DNA was inserted into the plasmid vector pBluescript SK- and RNA was made using the upstream T7 RNA polymerase promoter and T7 RNA polymerase. A1 RNA was added to a wheat germ translation system containing ^{35}S-methionine for metabolic labelling of any translated proteins. The labelled protein (figure 3.12A) was then incubated with either rat control pre-immune or rat anti-A2 antiserum. Immunoprecipitated protein was harvested, resolved by SDS-PAGE and visualized by autoradiography. Results of this experiment showed that the rat anti-A2 antiserum immunoprecipitated a 34 kDa protein, whereas the control pre-immune serum did not (figure 3.12B). Brome Mosaic Virus (BMV) RNA was included in these experiments as a positive control for RNA translation, and the ^{35}S-methionine-labelled BMV proteins were included in the immunoprecipitation experiment as a specificity control. In Figure 3.12B, some nonspecific immunoprecipitation of BMV high molecular weight proteins was noted for both the pre-immune and the post-immune antiserum. This highly sensitive *in vitro* transcription and translation assay revealed that the anti-A2 antiserum was specific for the Pf12 protein, but again it also suggested that the reactivity was very weak.

Immunofluorescence experiments using rat anti-A2 antiserum

Rat anti-A2 antiserum showed a stronger specificity for Pf12 protein in some immunofluorescence experiments. COS cells transfected with wild-type Pf12 in pJFE14 were stained brightly by the anti-A2 antiserum, whereas pJFE14 -transfected cells (i.e. empty vector) were not stained by the anti-A2 antiserum. Moreover, Pf12/pJFE14 transfected cells were not stained by the pre-immune serum. Representative fluorescence staining experiments on transfected COS cells are shown in figure 3.13, and these results are summarized in table 3.1.

In contrast to Pf12 transfected COS cells, malaria parasites from *in vitro* asexual stage cultures did not stain as brightly with the rat anti-A2 antiserum. This antiserum failed to stain unfixed malaria-infected intact RBCs from *in vitro* cultures, or saponin-lysed RBCs containing schizonts, rings or merozoites (table 3.2). However, rat anti-A2 antiserum did stain methanol-fixed smears of unsynchronized parasite-infected RBCs, with staining localizing to either the periphery of the RBC, or to the parasite within the RBC (figure 3.14). Using ethidium bromide staining as an indicator for the presence of parasite DNA within the RBC, it was observed that approximately ten percent of ethidium-stained RBCs in a smear were also stained with the rat anti-A2 antiserum.

Immunoelectron microscopy experiments using rat anti-A2 antiserum

ImmunoEM experiments using sections of glutaraldehyde and paraformaldehyde-fixed parasite-infected RBCs (see materials and methods) were conducted in an attempt to further pinpoint the subcellular location of Pf12. The anti-A2 antiserum stained neither parasites nor parasitized RBCs any stronger than did the control pre-immune serum in these experiments (data not shown). In addition, uninfected RBC components and regions outside of RBCs were stained nonspecifically by the anti-A2 antiserum (data not shown).

Invasion inhibition experiments using anti-A2 antiserum and *P. falciparum in vitro* cultures

Preliminary invasion inhibition experiments were conducted to determine if anti-A2 antiserum could inhibit the invasion of new RBCs by merozoites in tissue culture. These experiments were conducted as described in chapter 2, using 3D7 and D10 strain parasites. Control and anti-A2 antiserum was added to the cultures at a dilution of 1:100. The results from these experiments indicated that the percent invasion obtained in the presence of anti-Pf12, 7%, was not significantly different from that obtained in the presence of the control

antiserum, 10%. More extensive studies using larger numbers of parasites were not undertaken.

C. Discussion

The goal of these studies was to produce enough recombinant Pf12 to generate Pf12-specific antibodies, and to use these antibodies as a tool for localizing the protein in the parasite. The previous failure of Pf12 peptides to generate antiserum that was specific for the protein suggested that it may have a distinctive conformation, and that generating antibodies to epitopes of the protein that were accessible at the surface was of key importance. Consequently, in addition to the creation of the recombinant Pf12ec A2 protein, much effort was devoted to discerning the nature of the anti-A2 Ab specificity.

Full length Pf12 protein was not expressed at detectable levels in initial *E. coli* expression experiments described in this chapter. Pf12 may be toxic when overproduced in heterologous expression systems, although no direct evidence proving this has been presented. It may be that other factors such as the high cysteine content of Pf12 inhibit the production of large amounts of the protein in *E. coli*. It is interesting to note that Pf12 is not toxic to COS cells: wild-type Pf12, containing its own native signal and anchor sequences, can be expressed on the surface of COS cells at levels comparable to positive controls such as T8/DAF. However, the amount of protein made by this system was not enough for generating anti-Pf12 antiserum. Thus I turned to the use of *E. coli* expression systems to produce recombinant protein for raising Pf12-specific antibodies.

The first subfragment I created, Pf12ec-A1, which lacks only the hydrophobic signal and anchor sequences, could not be expressed in *E. coli* at levels detectable by Coomassie staining of SDS-PAGE gels. I had created this mutant to encompass as much of the wild-type sequence as possible, excluding the amino and carboxy terminus hydrophobic regions, which were most likely signal and anchor sequences that were cleaved in eukaryotic cells, but would not be cleaved by *E. coli*. Moreover, there is

evidence that hydrophobicity, along with a number of other parameters such as charge average, turn-forming residue fraction, cysteine fraction, and total number of residues correlate positively with inclusion body formation (Wilkinson, 1991). The inclusion of these hydrophobic sequences that are most likely not part of the mature native protein would either hamper the production of significant quantities of soluble recombinant protein, or result in a recombinant protein with different properties from the native protein.

After testing a number of *E. coli* clones transformed with A1/pT7-7His6 for the presence of A1 protein, it became evident that this protein was not expressed at detectable levels in *E. coli*. I therefore went on to make three new subfragments encompassing different portions of the A1 sequence. The reasoning behind creating these subfragments was simply to avoid regions that may hamper the production of the recombinant protein. The number of cysteines present in Pf12 led me to suspect that disulfide bridged domains may be important in the native conformation of the molecule, but I had no hypothesis to predict which disulfide bridges were important.

Of the three new subfragments that were created, A2 was the only one to be expressed at levels detectable by Coomassie blue staining of protein extracts resolved by SDS-PAGE. This protein encompassed the N-terminal two-thirds of the A1 sequence, and had an observed molecular weight close to the predicted 24.9 kDa. The other constructs, A3 and A4, encompassed the middle third and back two-thirds of A1 respectively. The observation that A4 was not produced in detectable amounts suggested that the C-terminal one-third of the protein was directly involved in the poor expression of this protein, because both A2 and A4 shared the middle portion. The observation that A3, which encompassed the middle portion of the protein, was also not produced in detectable amounts, suggested that the poor expression levels of Pf12 may have less to do with a specific 'toxic' amino acid sequence, and more to do with correct folding of certain domains which exist within the protein.

When expressed in *E. coli*, A2 protein was present as insoluble protein in inclusion bodies. After centrifuging a French press lysate from a large scale A2-transformed *E. coli* culture, I observed that the A2 protein remained associated with the pellet, and had to be solubilized with 8M urea or 6M guanidine HCl in order to be resolved on SDS-PAGE. This protein may have aggregated for a number of reasons, including the presence of parasite "preferred" codons in the Pf12 sequence that are not abundantly recognized by *E. coli* tRNAs AGA, AAG (Chen and Inouye, 1994), or the presence of numerous disulfide bridges both of which have been proposed to stall polypeptide elongation and result in misfolding and degradation of the nascent polypeptide chain (Moran et al., 1991).

A2 protein was successfully purified from A2 inclusion bodies using a nickel chelating column, preparative gel electrophoresis, and electroelution. Rats immunized with this purified Pf12ec A2 protein produced antiserum that was more specific for denatured, reduced forms of recombinant Pf12 made in *E. coli* than it was for Pf12 produced by transfected COS cells or by *P. falciparum* parasites themselves. Initial Western blot experiments showed that anti-A2 antiserum recognized the original A2 protein expressed in *E. coli* and purified by nickel chromatography, preparative gel electrophoresis and electroelution. This protein was fully denatured in SDS lysis buffer and reduced with DTT before being resolved by SDS-PAGE and blotted to nitrocellulose. Furthermore, anti-A2 antiserum also was specific for only the 27-28 and the 14 kDa proteins in the pelleted crude lysate from large scale cultures. This strongly suggests that the 14 kDa protein is a proteolytic fragment of the 27-28 kDa A2 protein, as only the full-length purified A2 was used to generate the anti-A2 antiserum.

In Western blot experiments using lysates from three different strains of *P. falciparum*, rat-anti-A2 antiserum weakly recognizes proteins of 32 (strain K1A), 60 (D10), or 31 and 65 (3D7) kDa. Initially it was surprising that the sizes of the D10 strain proteins recognized by the rat anti-A2 antiserum (60 kDa) were so much greater than those of the K1A and 3D7 strains (32-35 kDa). I hypothesized that this could be due to different

alleles of Pf12 expressed by different strains, insufficiently denatured and reduced dimers of Pf12 (which might aggregate to form higher molecular weight complexes), post-translationally modified Pf12, or a protein with cross-reactive epitopes. I favored the cross-reactive protein hypothesis after including an RBC control in the third Western blotting experiment with 3D7 parasite proteins (figure 3.11C). In this experiment, anti-A2 Ab recognized a 65 kDa protein in both parasite and RBC control lanes. Although *P. falciparum* parasites, which are routinely cultured in RPMI containing 10% human serum, were washed before lysis and resolution by SDS-PAGE, some human serum albumin (HSA) could have adsorbed onto lysed RBC membranes that invariably remain associated with the parasites even after saponin lysis and washing steps. This protein could also be present in the RBC controls, which are washed uninfected RBCs, prepared in the same manner as the infected RBCs. Although it is unlikely that HSA was present as a contaminant in the protein preparation used to generate the anti-Pf12 antiserum, it is possible that the antiserum still binds to HSA on the Western blots through specific recognition or non-specific binding.

These Western blotting experiments began to reveal the nature of the specificity of anti-A2 antiserum. It seemed to recognize denatured epitopes strongly, such as those of the prokaryotic Pf12ec A2, which needed to be denatured and reduced before resolution on SDS-PAGE gels. Smaller amounts of protein produced under more native conditions such as in COS cells or in the *P. falciparum* parasites themselves, were only weakly recognized by the antiserum at best. These proteins, potentially having post-translational modifications and more native folding patterns may have been difficult to completely denature, thus masking some or perhaps many of the epitopes recognized by the anti-A2 antiserum. This explanation of the specificity of A2 is neither complete, nor satisfying, as later immunoprecipitation and immunofluorescence experiments suggest that the anti-A2 Ab also recognizes 'native' Pf12.

The Pf12 immunoprecipitation experiments were chosen to study the specificity of the anti-Pf12 antiserum because a highly labelled and thus easily detectable amount of relatively native Pf12 could be used as the target protein. The experiments described in figure 3.12 show that anti-A2 antiserum specifically immunoprecipitates a 34 kDa ^{35}S-labelled Pf12ec A1 made by *in vitro* transcription/translation. This protein encompasses the entire wild-type coding sequence less the signal and anchor regions. It was neither denatured nor reduced before incubating with anti-A2 antiserum in immunoprecipitation experiments, but it is questionable as to whether this protein was folded into a native state in the wheat germ lysate used for protein translation and labelling.

Immunofluorescence experiments with Pf12-transfected COS cells further illustrate the ability of the rat anti-A2 antiserum to recognize relatively native Pf12 protein. Anti-A2 antiserum brightly stains the surface of unfixed Pf12/pJFE14-transfected COS cells compared to controls. Pf12 in this environment is more native than both Pf12 A2 protein made in *E. coli*, and that made by *in vitro* transcription/translation, because COS cells can provide elements such as chaperones to assist in proper protein folding and enzymes that catalyze post-translational modifications such as glycosylation.

By contrast, anti-A2 antiserum did not stain the surface of unfixed parasites and parasite-infected RBCs, and only weakly stained methanol-fixed parasite-infected RBCs. These observations suggested that Pf12 protein was not present on the surface of the infected RBC like RESA or PfEMP1 proteins, but either on the cytoplasmic face of the RBC, within the parasitophorous vacuole, or on the surface of the parasite within the RBC. Because previous experiments with Pf12-transfected COS cells showed that the anti-A2 antiserum recognized Pf12 on the surface of COS cells, I expected that any Pf12 on the surface of parasite-infected RBCs should be recognized. The observation that staining of the parasite only occurred after permeabilization of the infected RBCs by air-drying and methanol fixation suggested that Pf12 was targeted to a site inside the infected erythrocyte. In these experiments the antibody staining seemed associated with a membranous portion

of early stage schizonts, separated from the nucleus which was stained by EtBr. It was difficult to discern whether this membrane belonged to the parasite or the infected RBC, as in some instances it seemed compact and irregularly arranged around the parasite nucleus, whereas in others it seemed to bulge out to the size of a normal RBC membrane.

Immunoelectron microscopy studies were conducted in order to unequivocally localize Pf12 protein in *P. falciparum* parasites. These experiments failed to reveal any new information about the time of expression or the location of Pf12. This is probably due to the effects of glutaraldehyde and paraformaldehyde fixation on parasite Pf12 protein which may have destroyed key epitopes recognized by the anti-A2 antiserum. Although this antibody did strongly recognize denatured A2 protein made in *E. coli*, it only weakly recognized parasite proteins in immunofluorescence and Western blotting experiments. The addition of harsh fixatives such as glutaraldehyde, which have been shown to mask or destroy epitopes of *P. falciparum* and other proteins, may have had an adverse effect on Pf12 which was only weakly recognized by anti-A2 antiserum in the first place. Table 3.2 details the effects of a number of different fixation conditions on the ability of anti-A2 antiserum to stain *P. falciparum* parasites. Of all methods tested, methanol fixation by itself appeared to be the only one that does not abrogate staining. Fixation with a number of different concentrations of paraformaldehyde and glutaraldehyde (down to 0.24% glutaraldehyde) abolished staining completely. Although stronger staining on immuno-EM was evident for anti-A2 antiserum than for pre-immune serum, the staining in general was both weak and non-specific, labelling the parasite, infected and uninfected RBCs, and some of the extracellular spaces.

Finally, preliminary experiments were undertaken in order to determine whether the anti-A2 antiserum could inhibit the invasion RBCs by merozoites in parasite cultures. While these experiments did not involve the enumeration of large number of RBCs, the results did suggest that anti-A2 did not inhibit invasion of RBCs when compared to pre-immune control antiserum (both at a 1:100 dilution).

In conclusion, the experiments described in this chapter served to generate antiserum which in theory could be used to characterize the newly discovered Pf12 parasite protein with respect to subcellular location and time of expression. It was shown that wild-type Pf12 could not be expressed in significant amounts in *E. coli*, and that the one subfragment that could be expressed, Pf12ec A2, was present as an insoluble protein in inclusion bodies. This denatured protein was purified by a combination of nickel affinity chromatography and preparative SDS-PAGE, used to generate Pf12ec A2 antiserum. This antiserum was strongly specific for denatured and reduced Pf12ec A2, weakly specific for relatively native Pf12. Although it stained the surface of Pf12-transfected COS cells, it only weakly stained methanol-fixed parasite-infected RBCs, suggesting the subcellular location of Pf12 to be either the inner RBC membrane, the parasitophorous vacuolar membrane, or the surface of the parasite itself. No further information was obtained from immunoEM experiments, probably due to the effect of glutaraldehyde/paraformaldehyde fixation on key Pf12 protein epitopes, or the relatively weak specificity of the anti-A2 antiserum for reduced or denature parasite Pf12, as indicated in the Western blotting experiments in figure 3.11.

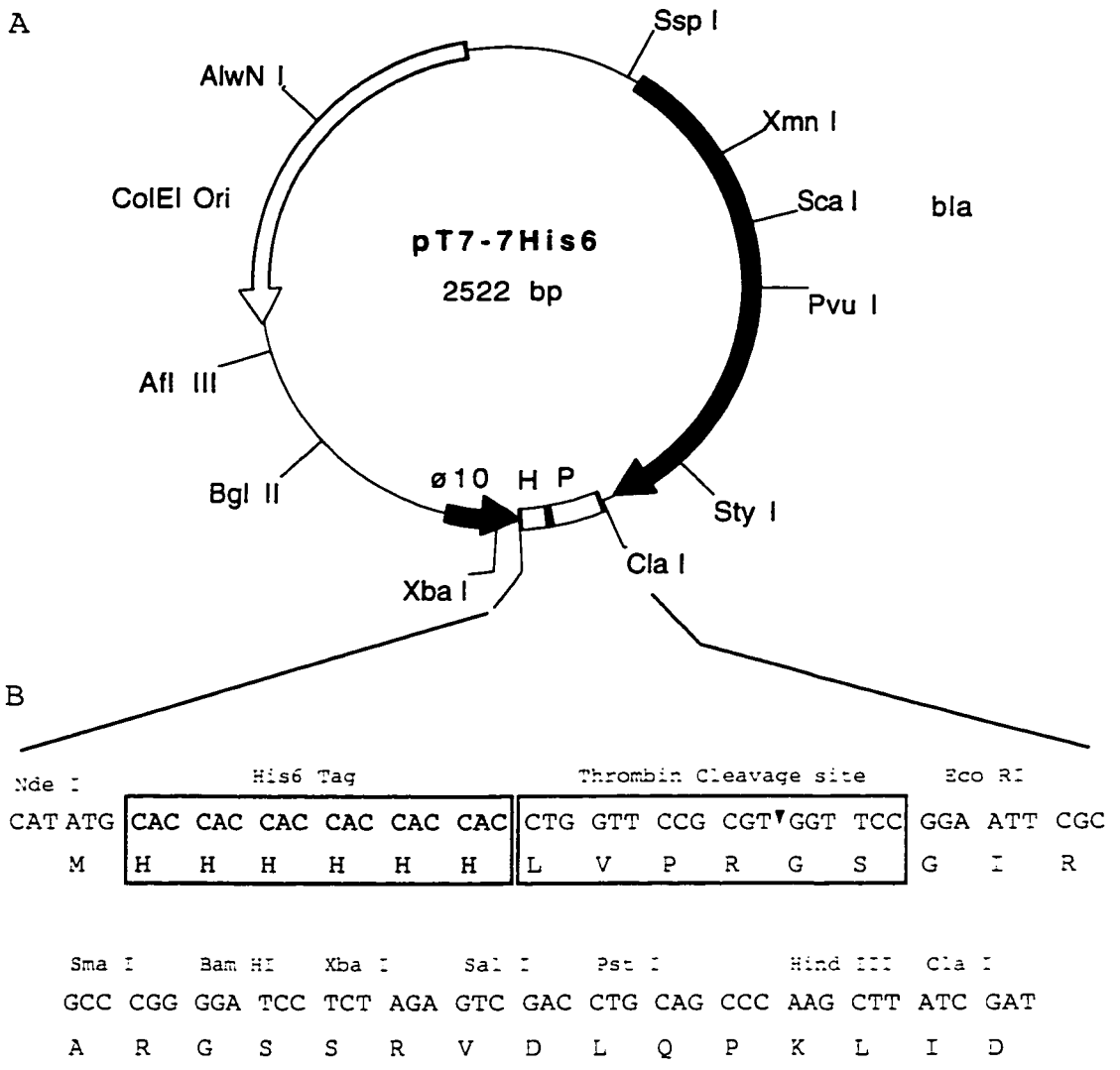


FIGURE 3.1. pT7-7His6, a prokaryotic expression vector for production of recombinant proteins containing an N-terminal hexahistidine tag. **A.** This vector is a modified version of the prokaryotic expression vector pT7-7. Features include: T7 gene 10 RNA polymerase promoter, ø10; His6 tag for purification by nickel affinity chromatography, H;polylinker, P; beta-lactamase, bla; Col EI origin. **B.** Expanded view of the DNA (above) and protein sequence (below) from the translation start site to the end of the polylinker. Shaded box, His6 tag; open box, thrombin cleavage signal for His6 tag removal and site (arrowhead) of cleavage. Restriction enzyme sites are labelled above the DNA sequence. Expression of any gene inserted at the polylinker is inducible by IPTG in *E.coli* BL21 DE3, a lysogen harboring a λ derivative containing the T7 RNA polymerase gene under control of the lac UV5 promoter.

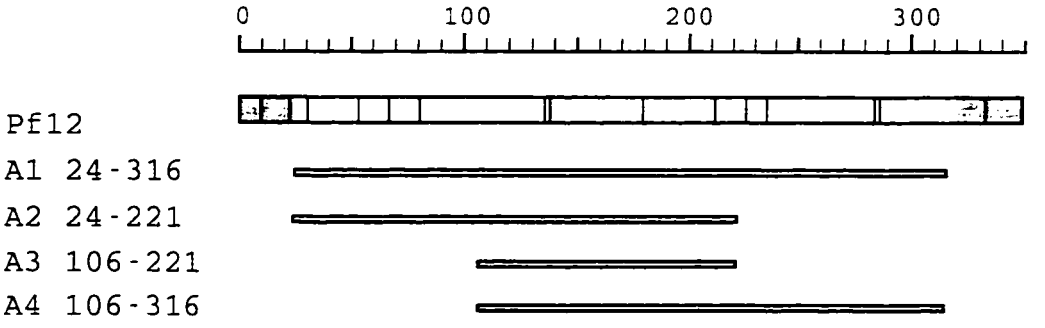


FIGURE 3.2. Pf12ec 'A series' subfragments. All subfragments were engineered by PCR using primers shown in table 2.1. The wild-type sequence, Pf12, is shown as a wide bar at the top. Shaded box (left), N-terminal signal sequence; shaded box (right), C-terminal anchor signal; vertical bars: cysteine residues. Subfragments are numbered A1-4, followed by the range of wild-type Pf12 amino acids each spans. The thin horizontal bars corresponding to each deletant approximate the region of wild-type Pf12 contained in each recombinant protein. The predicted molecular weights of each is as follows: A1, 37.2 kDa; A2, 24.9 kDa; A3, 15.5 kDa; A4, 26.4 kDa. These subfragments were cloned into the prokaryotic expression vector pT7-7H6 using 5' Bam HI and 3' Pst I sites (see Fig. 3.1).

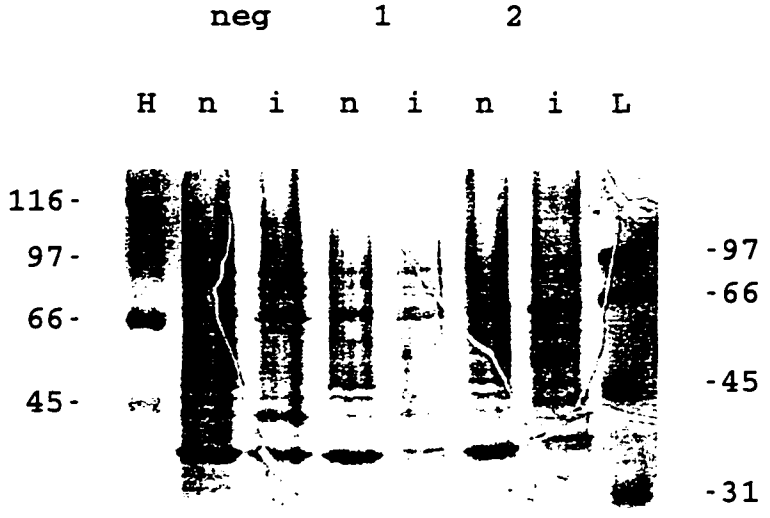


FIGURE 3.3. SDS-PAGE analysis of lysates from BL21 DE3 cells transformed with Pf12ec A1 in pT7-7His6. Two separate A1-transformed colonies (1,2) were picked and grown under noninducing(n) and inducing(i) conditions. Lysates from these cultures and cultures of BL21 DE3 transformed with empty vector alone were resolved by 12% SDS-PAGE and stained with Coomassie blue. H, high-range protein marker; L, low-range protein marker; sizes corresponding to the marker bands are shown beside the marker lanes. The mobilities of the high molecular weight markers are slightly distorted due to the presence of the 116 kDa protein.

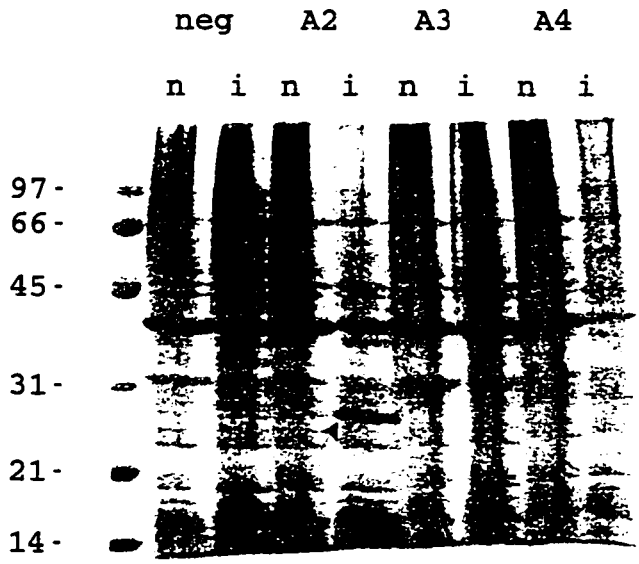


FIGURE 3.4. SDS-PAGE analysis of Pf12ec A series deletants. Lysates from non-induced (n), and induced (i) cultures of A2-A4 constructs or empty pT7-7His6 plasmid alone (neg) were resolved by 12% SDS-PAGE and stained with Coomassie blue. The arrow indicates expressed A2 protein, with a predicted molecular weight of 24.9 kDa. Expected sizes for A3 and A4 are 15.5 kDa and 26.9 kDa respectively.

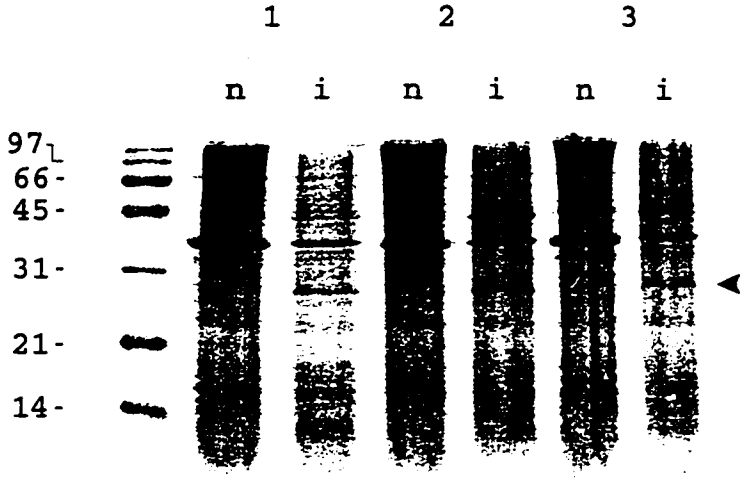


FIGURE 3.5. SDS-PAGE analysis of protein expression from three independent BL21 DE3 colonies (1, 2, 3) transformed with the Pf12ec A2 construct in pT7-7His6. Lysates of non-induced (n) and induced (i) cultures were resolved by 12.5% SDS-PAGE and stained with Coomassie Blue. Arrow indicates the A2 protein present only in the induced cultures, with an observed molecular weight of 25.0 kDa.

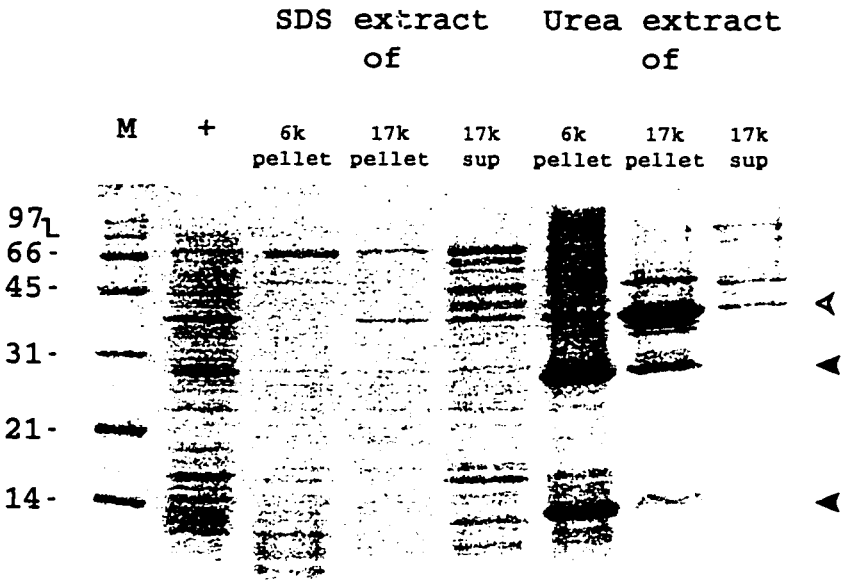


FIGURE 3.6. SDS-PAGE analysis of solubilized A2 protein from large-scale *E.coli* cultures. Cells from a 1 L culture of BL21 DE3 expressing A2 protein were harvested and lysed using a French Press. To determine the solubility of A2, the lysate was centrifuged at two different speeds, and the pellets and supernatants from each spin were examined. The lysate was first centrifuged at 6000 xg, split into pellet (6k pellet) and supernatant, then the 6k supernatant was centrifuged at 17,000 xg, and subsequently split into 17k pellet and 17k sup. Each sample was solubilized in either 1% SDS or 8 M urea, and microfuged. Supernatants from the solubilization process were resolved by 12% SDS-PAGE and stained with Coomassie Blue. Closed arrow, Pf12ec A2 protein migrating at 25.0 kDa, along with a putative A2 proteolytic fragment migrating at 14.0 kDa; open arrow, contaminating *E.coli* protein; M, Low-range protein markers; +, lysate from small-scale culture known to express A2.

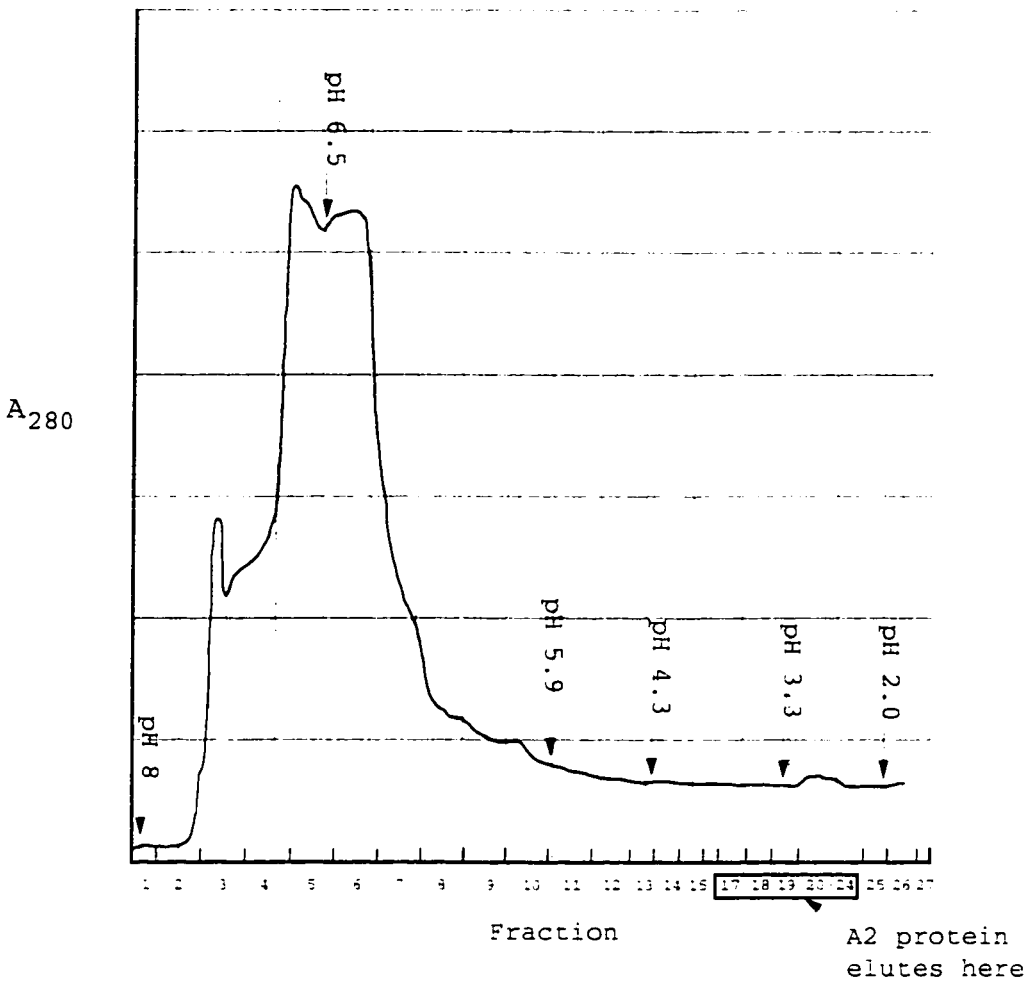


FIGURE 3.7. Purification of recombinant A2 protein using nickel chelating resin and affinity chromatography. Urea-solubilized A2 protein was loaded onto a nickel column. The column was then washed with 8M urea/tris buffers at the various pH values shown. Fractions were numbered and assayed for the presence of eluted A2 protein by SDS-PAGE. The open box indicates the fractions containing A2 protein. Y-axis values are arbitrary absorbance units measured for fractions eluted from the column.

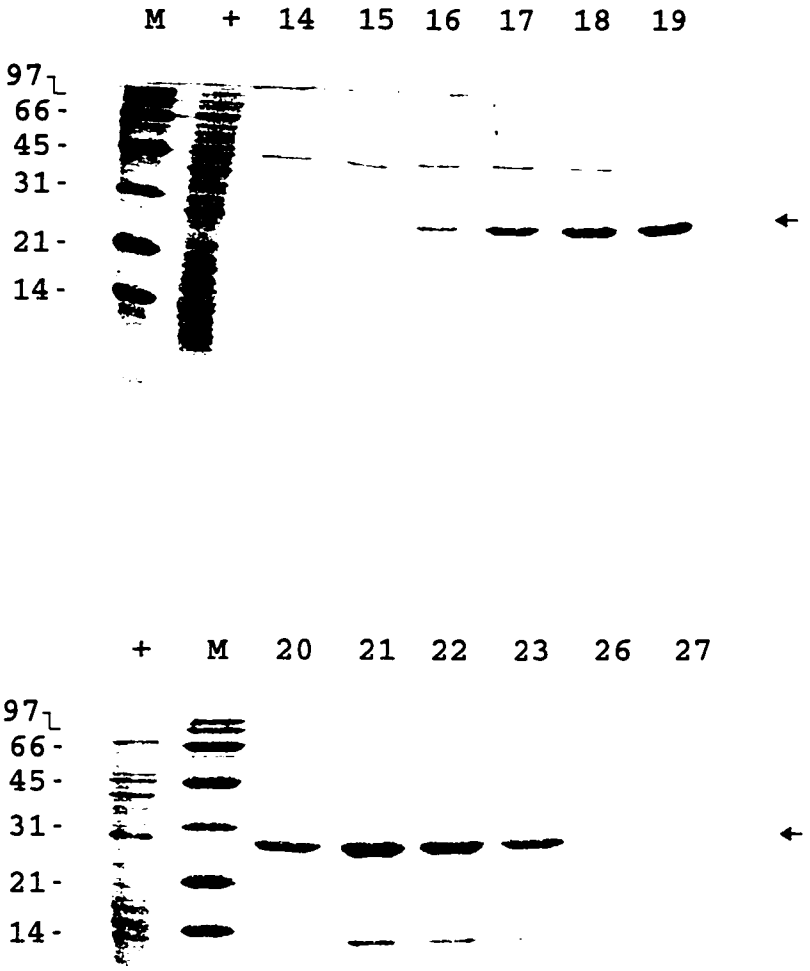


FIGURE 3.8. SDS-PAGE analysis of fractions eluted from the nickel column in figure 3.7. Aliquots from various fractions eluted from the nickel column were diluted in SDS sample buffer, resolved by 15% SDS-PAGE, and stained with Coomassie blue. M, low-range protein markers; +, lysate from a culture expressing A2 (arrow indicates A2 band); 14-27, column fractions.

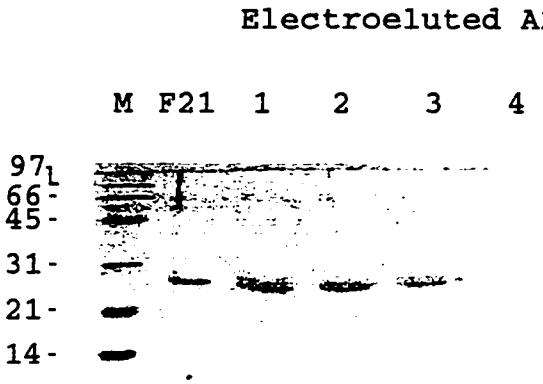


FIGURE 3.9. SDS-PAGE analysis of A2 protein purified by preparative SDS-PAGE and electroelution. Nickel column fractions containing A2 protein were purified further by 12.5% preparative SDS-PAGE and A2 protein was electroeluted from excised gel slices in a Tris-HCl/glycine buffer. Eluted protein was analysed by 12% SDS-PAGE. M, Low-range protein markers; F21, A2 protein fraction from nickel column; 1-4, four different A2 protein fractions electroeluted from 12.5% preparative SDS-PAGE. These were subsequently pooled and used for immunization of rats.

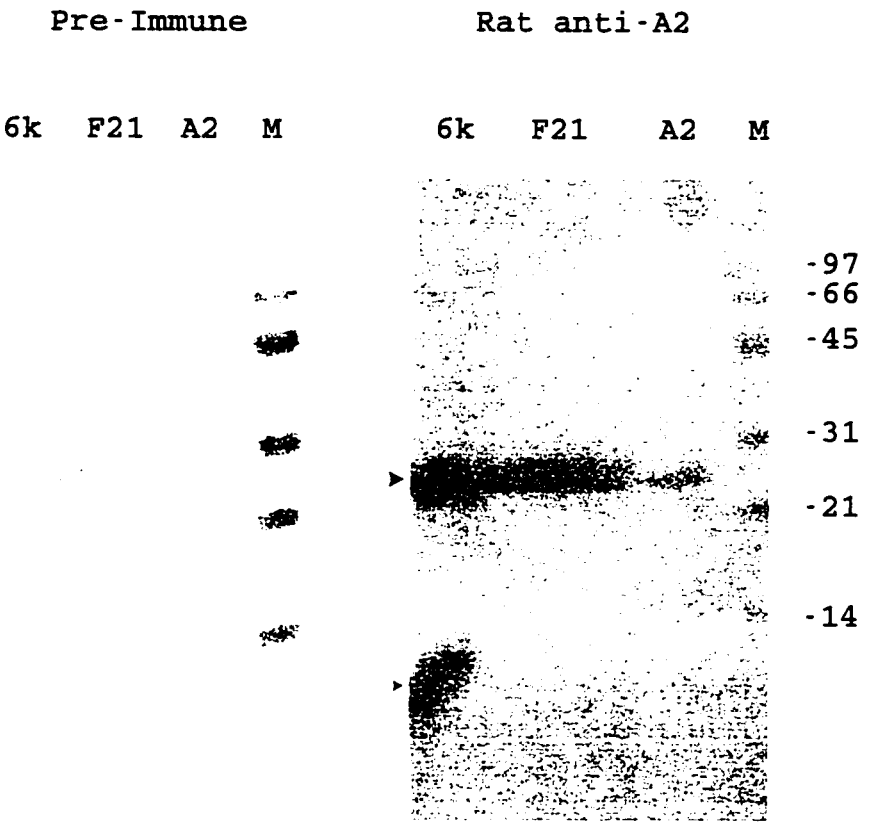


FIGURE 3.10. Western blot analysis of A2 proteins using rat anti-A2 antiserum or rat pre-immune serum. A2 protein obtained from three different steps in the purification process was resolved by 12% SDS-PAGE and Western blotted with either rat pre-immune or rat anti-A2 post-immune antiserum (1:500). Secondary antibody was horseradish peroxidase conjugated goat anti-rat (1:500). Antibodies were visualized by diaminobenzidine staining. 6k, urea-solublized A2 protein from 6k pellet in figure 3.6, closed arrows indicate A2 protein and 13 kDa proteolytic A2 fragment; F21, A2 protein fraction 21 eluted from the nickel column in figure 3.7; A2, electroeluted A2 protein from figure 3.9.

FIGURE 3.11. Parasite proteins detected by Western blot analysis using anti-A2 antiserum. Infected RBCs containing *P. falciparum* parasites from three independent strains (K1A, D10, 3D7), were lysed with 0.15% saponin/ PBS, and parasites were pelleted, washed, and solubilized in SDS lysis buffer. The lysates were resolved by 5-20% SDS-PAGE (K1A, D10), or 12% SDS-PAGE (3D7), and Western blotted with either rat pre-immune or rat anti-A2 antiserum. Closed arrows denote Pf12 protein, open arrows denote a cross-reacting protein, most likely human serum albumin. The Pf12 protein was not detectable on the original D10 Western.

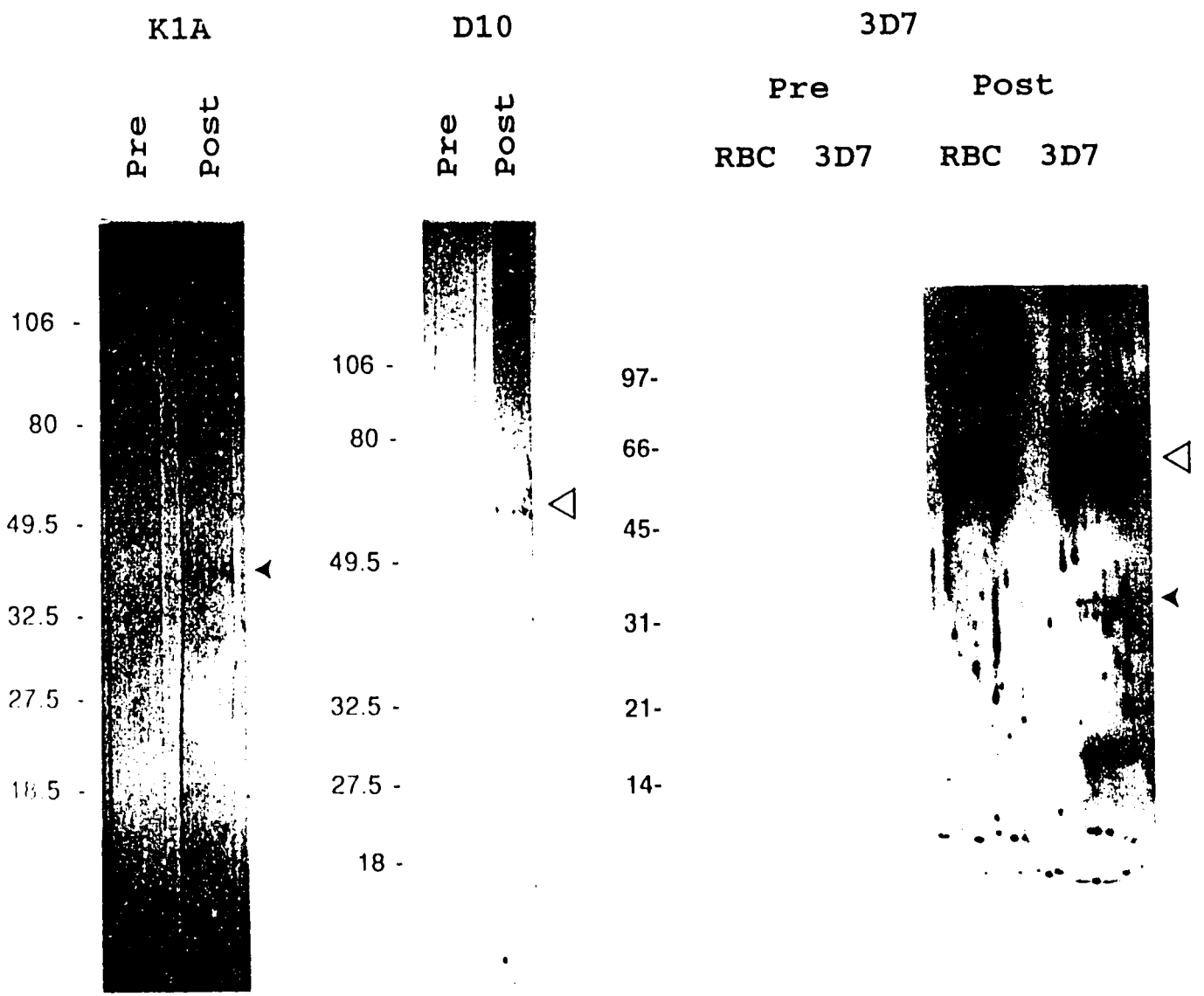

K1A
Pre
Post
106 -
80 -
49.5 -
32.5 -
27.5 -
18.5 -
D10
Pre
Post
106 -
80 -
49.5 -
32.5 -
27.5 -
18 -
3D7
Pre
Post
RBC 3D7
RBC 3D7
97-
66-
45-
31-
21-
14-

FIGURE 3.12. SDS-PAGE analysis of in vitro transcribed and translated 35S-methionine labelled Pf12 and immunoprecipitation of the labelled protein. A. A deletant of Pf12 identical in sequence to Pf12ec A1 (created with primers Pf12 5' Nco I, Pf12 3' 1A; see table 2.1) was cloned as a blunt-ended fragment into Sma I-digested pBluescript SK- (Stratagene). This construct was linearized downstream of the Pf12 insert with Not I, and transcribed using T7 RNA polymerase. Pf12 RNA was translated to generate 35S-methionine-labelled Pf12 protein in a wheat germ lysate system. This protein was resolved by 15% SDS-PAGE, the gel was dried, and labelled protein was visualized by autoradiography. Different inputs of Pf12 RNA(0.5, 1, 2, and 4 μg) were used, and compared to brome mosaic virus (BMV) RNA control. B. The BMV and Pf12 labelled proteins from (A) were immunoprecipitated by rat pre-immune or anti-A2 antiserum, resolved by 12.5% SDS-PAGE, and visualized by autoradiography. The closed arrow indicates immunoprecipitated Pf12 protein with an observed molecular weight of 34 kDa, close to the predicted molecular weight of 37.2 kDa. Note that this is longer than the A2 protein which arises from a deletant of the original full length Pf12 clone.

A


Pf12 RNA (ug)
BMV .5 1 2 4
97
66
45
31
21
14

B

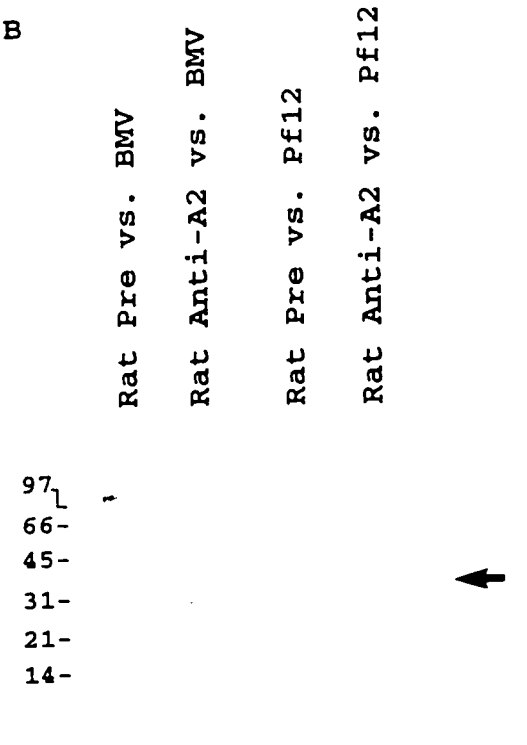

Rat Pre vs. BMV
Rat Anti-A2 vs. BMV
Rat Pre vs. Pf12
Rat Anti-A2 vs. Pf12
97
66
45
31
21
14

TABLE 3.1. Rat anti-A2 antiserum stains the surface of Pf12/ pJFE14-transfected COS cells, but not control transfected cells.

	COS cells transfected with*		
Antiserum	pJFE14	CD8/pJFE14	Pf12/pJFE14
anti-CD8	-	++++	-
rat pre-immune	-	-	+/-
rat anti-A2	-	-	+++

*COS cells were transfected with either pJFE14 (empty vector, negative control), CD8/pJFE14 (positive control), or wild-type Pf12/pJFE14 using the DEAE dextran method. The cells were harvested after two days, and incubated with either anti-CD8 monoclonal antibody, rat pre-immune serum, or rat anti-A2 antiserum (1:50 dilution) followed by fluorescein-conjugated secondary antibody (1:200 dilution). Surface staining was scored as either negative (-), or positive (from weak, +, to strong, ++++) using a Leitz UV microscope (400X magnification).

TABLE 3.2. Immunofluorescence experiments using different fixation conditions on parasites and staining with rat anti-A2 antiserum.*

Fixation Conditions	Rat Pre-Immune	Rat anti-A2
Unfixed mixed culture (Strains K1A, HB3, 3D7)	-	+/-
Unfixed Schizont-infected RBC (K1A, D10)	-	-
100% MeOH fixed mixed culture (K1A, HB3, 3D7)	-	++
100% MeOH fixed Schizont-infected RBC (D10)	-	-
100% Acetone fixed Schizont-infected RBC (3D7)	-	-
2%P/2%G fixed Schizont-infected RBC (K1A,D10)	-	-
1%P/1%G fixed Schizont-infected RBC (K1A,D10)	-	-
1%P/.5%G fixed Schizont-infected RBC (K1A,D10)	-	-
.25%G fixed mixed culture, (K1A, HB3, 3D7)	-	-

(G) Glutaraldehyde
(P) Paraformaldehyde
Parasite strains in brackets

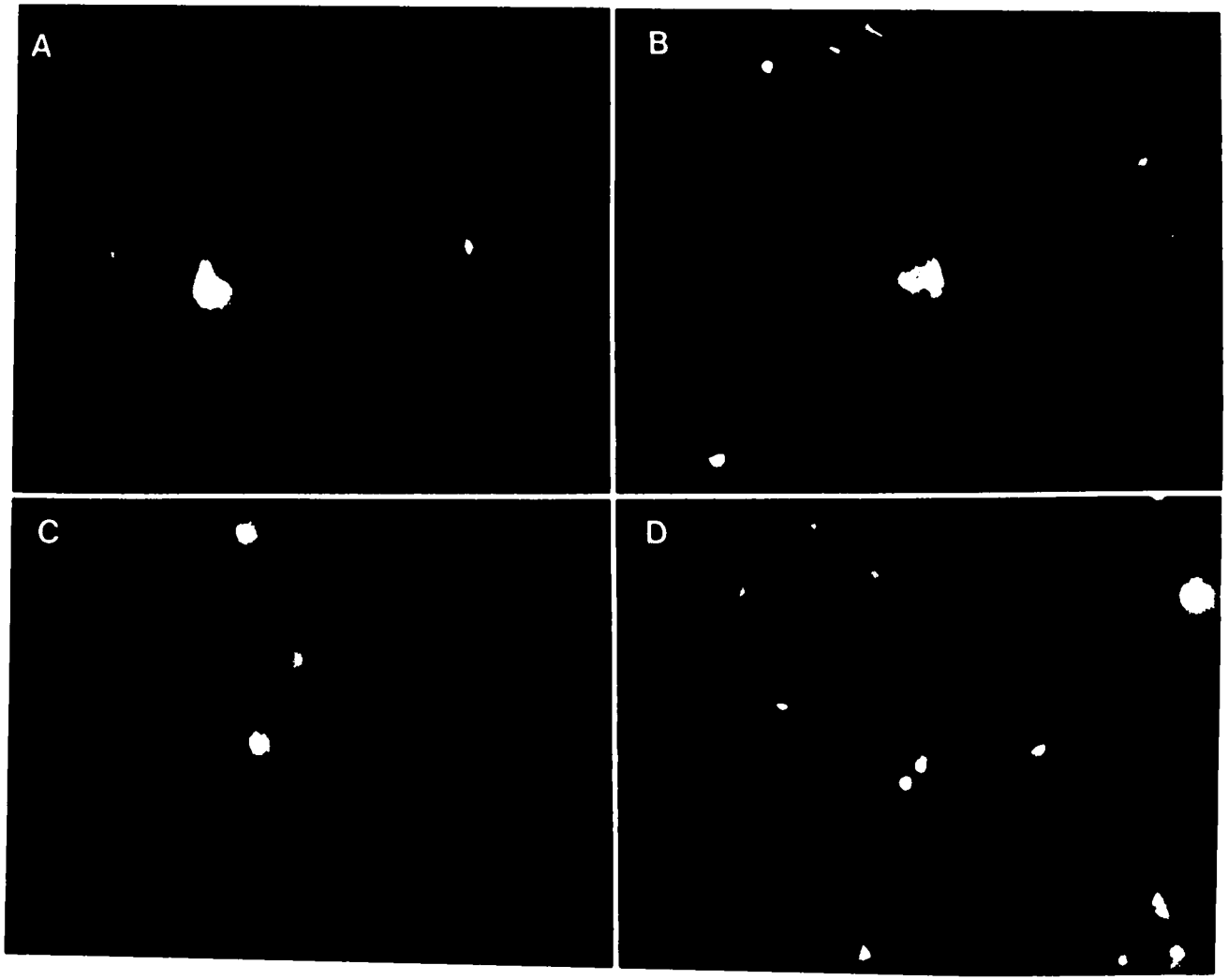
++++ Strong staining
- No staining

**P. falciparum* parasites grown *in vitro* were stained with rat anti-A2 antiserum and pre-immune control serum under a number of different fixation conditions. Unfixed parasites were incubated with primary antibodies (1:200) followed by fluorescein conjugated goat anti-mouse secondary antibodies (1:200), and examined as wet mounts. For methanol, acetone and paraformaldehyde/glutaraldehyde (P/G) fixation, air-dried thin blood smears of mixed cultures or percoll-separated schizonts were used, with the same staining protocol as above. All parasite specimens were viewed at using a Leitz UV microscope (1000X magnification). Staining was quantitated as in figure 3.1.

FIGURE 3.13. Detection of Pf12 protein on the surface of Pf12/pJFE14 transfected but not control transfected COS cells by immunofluorescence using rat anti-A2 antiserum. Representative photographs of the stained cells described in table 3.1 are shown (400X magnification). A and C, light-field microscopy of COS cells; B and D, dark-field microscopy of COS cells under UV illumination. A and B, pJFE14 control transfected COS cells reacted with rat anti-A2 antiserum, C and D, Pf12/pJFE14 transfected COS cells reacted with rat anti-A2 antiserum.

C
B
D

FIGURE 3.14. Immunofluorescence analysis of methanol-fixed smears of *P. falciparum* HB3 parasites using rat anti-A2 antiserum. *P. falciparum* HB3 parasites from *in vitro* asexual stage cultures were smeared onto glass slides, air-dried, and fixed with 100% Methanol at -20°C for 2 min. Fixed smears were incubated with either rat pre-immune or anti-A2 antiserum (both at a 1:100 dilution), labelled with FITC conjugated goat anti-rat antibody (1:200). Slides were counter-stained with 5 μg/mL ethidium bromide (EtBr; stains DNA which is only present in parasitized cells), and visualized using a Leitz UV microscope (1000X magnification). **A.** Parasite-infected RBCs incubated with rat pre-immune antiserum. Parasites are revealed by the round regions of EtBr fluorescence. **B,C, and D**. HB3 Parasite-infected RBCs incubated with rat anti-A2 antiserum. EtBr fluorescence can be seen as rounded regions where parasite nuclear DNA resides. In some parasites regions of fluorescence are present around the nuclei (halo effect) suggestive of the parasite membrane or infected RBC membrane.


A
B
C
D

CHAPTER IV

E. coli EXPRESSION REVISITED: GENERATION OF NEW Pf12 SUBFRAGMENTS TAKING INTO ACCOUNT A MODEL DISULFIDE-BONDED DOMAIN STRUCTURE

A. Introduction and Background

After attempting to produce relatively native Pf12 protein using the baculovirus expression system and DNA immunization, (see Appendix A1 and A2), I decided to make the Pf12 recombinant protein in *E. coli* once again, this time taking into account some new knowledge and a hypothesis Richard Carter had developed about the way the disulfide bridges in Pf12 form to give the protein a distinct shape (Carter et al., 1995). A hint that Pf12 had an ordered disulfide bond structure came from Carter's initial visual inspection of a sexual stage antigen, Pfs 230. The placement of cysteine residues in this protein followed a distinct and simple pattern. This pattern can be seen also in Pf12 (see figure 3.2), the only protein in the database with similarity to Pfs 230. After conducting motif searches by computer, a more detailed hypothesis of the disulfide bond structure of Pfs 230 and Pf12 (figure 4.1) was developed. If I could make a recombinant subfragment that maintained some of these potential disulfide domains, the antiserum specific for this protein might reveal more information about the cellular location of Pf12.

I hypothesized that subfragments that maintained the native disulfide bridging pattern may be more likely to fold properly in *E. coli*. This could mean that the protein would not be present in inclusion bodies on induction, but might be soluble, or at least, expressed at a higher level. Also, if I had to denature these new subfragments to solubilize and purify them, subsequent renaturing might be easier, because all the requisite cysteine residues were present to form a disulfide bonded domain.

Rationale for the experiments of Chapter 4

In this chapter a number of prokaryotic expression, and protein characterization studies were undertaken that were similar to those of chapter 3, although different in a number of key areas. I knew that Pf12 was a difficult protein to express in a native form, and that antibodies specific for denatured and reduced forms of Pf12, such as Pf12ec A2, may not be good reagents for detecting the native protein. I also suspected that the fixation process used in immunoEM experiments may destroy certain key epitopes of Pf12. I addressed these pitfalls in the following ways. A new vector was created, pBT1TØ, that could potentially produce higher levels of recombinant proteins in *E. coli* . This vector introduced an epitope tag onto the N-terminus of the recombinant protein so that protein production could be monitored by Western blotting. A new strain of *E. coli* that reduced the leaky expression of recombinant proteins, BL21 DE3 pLysS, was also used. A number of Pf12 subfragments were created that maintained the disulfide bonded domains proposed by Carter (1995), with the intent of identifying a subfragment that was tolerated by *E. coli* , encompassed most of the wild-type protein sequence, and was expressed in a soluble form. The Nickel column purification of the His6-tagged recombinant proteins was improved upon by using imidazole and the purified recombinant Pf12 protein was partially renatured before being used to generate Pf12-specific antibodies. Western blotting of proteins from a number of different sources, including purified asexual stages of *P. falciparum* parasites was undertaken to determine the time of expression of Pf12. Finally, attempts were made to optimize the permeabilization and fixation of parasites for both immunofluorescence and immunoEM experiments conducted to determine the subcellular location of Pf12.

B. Results

New Pf12 'B' series subfragments cloned into the prokaryotic expression vector pBT1TØ.

In designing the new Pf12 subfragments, I created primers that would amplify Pf12 DNA sequences encoding regions of the protein where the hypothetical disulfide bridges and antigenic loops were maintained (figure 4.1). This new group of Pf12 constructs, designated the 'B' series, were created using Pfu DNA polymerase and wild-type Pf12 sequence as a template, and cloned into the vector pBT1TØ (figure 4.2). This vector has all the features of pT7-7H6 plus two important additions: an MRGS-His6 sequence and the T7 gene 10 transcriptional terminator (TØ) downstream from the multiple cloning site. The former sequence replaces the His6 tag of the pT7-7His6 vector, adding an epitope tag in addition to the His6 affinity handle onto the amino terminus of the target protein (Adapted from a Qiagen vector sequence). Expression of smaller quantities of protein can be monitored by Western blotting using a commercial antibody specific for the epitope tag, and expression conditions can subsequently be optimized for poorly expressed proteins that may otherwise not have been detected by less sensitive methods such as Coomassie staining. The latter sequence encodes a stem-loop structure in the nascent RNA, preventing readthrough transcription. The TØ element was included in this vector because it has been shown that transcriptional terminators can enhance mRNA stability, and can substantially increase target protein production (Hayashi and Hayashi, 1985).

Subfragments cloned into pBT1TØ were transformed into a BL21 DE3 host which harbors another plasmid useful in the prevention of 'leaky' expression, pLysS. This plasmid consists of the T7 lysosyme gene cloned into pACYC184 BamHI site (Chang and Cohen, 1978). T7 lysosyme produced by pLysS under the control of the T7 Ø3.8 promoter can bind and inhibit T7 RNA polymerase produced under non-inducing conditions by the integrated lambda derivative DE3 (Moffatt and Studier, 1987). This low

concentration of lysosyme is usually enough to prevent leaky expression of the target gene, but does not adversely affect target gene expression under inducing conditions, where much more T7 RNA polymerase is present than lysozyme. In addition, T7 lysozyme will not severely weaken *E. coli* bacteria when produced in this manner because it cannot pass through the inner membrane to reach the peptidoglycan layer, where it normally acts by cleaving the 1,4-ß linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine of peptidoglycan. In summary, the combination of the new vector pBT1TØ and a modified host system was implemented in order to increase the amount of protein made by stabilizing target mRNA and decreasing leaky expression, and increase the sensitivity of detection of target proteins using a new epitope tag.

Western blotting analysis of B series subfragments expressed in *E. coli*.

Pf12 'B series' subfragments 1-12 (figure 4.3) were cloned into pBT1TØ, transformed into *E. coli* BL21 DE3 pLysS, and target protein production was induced by addition of IPTG once cultures had reached OD 0.6. Lysates from both non-induced control and induced cultures were resolved by SDS-PAGE (figure 4.4), Western-blotted with anti-MRGS His6 commercial antibody (Qiagen), and visualized using anti-mouse HRP and chemiluminescence (figure 4.5). The Pf12ec B2 mutant was expressed at higher levels than most other 'B' series proteins. It was the second largest of the B mutants, and encompassed (Dr. Carter's terminology) domain 1 and part of domain 2-- the domain 2 loop 'a' being deleted (figure 4.1). Although some of the shorter B subfragments were also expressed at levels equal to B2 (B4, B5, B6, B9), I chose B2 for further study because it encompassed most of the wild-type Pf12 protein sequence.

Large scale purification of and renaturation of Pf12ec B2

This protein was purified on a large scale using nickel affinity chromatography (Hochuli et al., 1987) and denaturing conditions (6M GuHCl) to solubilize the inclusion

body pellet obtained after the Pf12 expressing bacteria were lysed. In this case I decided to use a different method of eluting bound protein from the nickel column than the pH-based method used in Chapter 3. I chose to use increasing concentrations of imidazole instead of decreasing pH so that I might elute Pf12ec B2 more efficiently at a neutral pH. Under these conditions the protein would be less susceptible to pH induced damage and might be easier to renature. Before eluting B2 from a preparative column, I determined the optimum imidazole concentration to be used in washing and elution steps. I did this by loading a commercial nickel 'spin' column (Qiagen) with urea-denatured B2 protein from a crude French Press lysate, washing the column with urea buffers containing increasing concentrations of imidazole, and then monitoring the amounts of B2 that was eluted from the column in each step (figure 4.6). The results indicated that imidazole concentrations as low as 60 mM were sufficient to elute B2 protein from the nickel column, and that 20 mM imidazole did not elute significant amounts of B2 protein from the column. With this information, I continued with the large-scale purification of Pf12ec B2 protein. B2 protein denatured with 6M GuHCl was loaded onto a preparative nickel column that was equilibrated with 8M urea and 20 mM imidazole, pH 7.5 (in a Tris/ phosphate buffer). The column was then washed and eluted using 200 mM imidazole, pH 7.5 (at least a three-fold excess over the minimum concentration of imidazole needed)(figure 4.7). By loading the protein onto the column in the presence of imidazole, and washing extensively, I was able to improve on the purity of the eluted protein from the initial 'A' series experiments. The eluted protein was analyzed by SDS-PAGE (figure 4.7) and pooled for renaturation experiments.

Partial Renaturation of Pf12ec B2 by Dialysis

At this point I wanted to renature the protein to some extent, hopefully reforming some of the disulfide bridges (that were reduced by addition of 2-mercaptoethanol) that I believed to be crucial in maintaining a Pf12 in a native conformation. My attempts at the

stepwise removal of urea from the protein solution using dialysis and buffer replacement invariably resulted in the precipitation of Pf12 once the urea concentration in the protein aliquot went below 3 M. Consequently, I decided to solubilize my combined fractions of eluted protein in 1% SDS/ 3 M urea, and then slowly remove the urea by dialysis using a buffer containing SDS, followed by the stepwise removal of SDS using a 4 mM HEPES buffer containing the conjugate thiol:disulfide pairs oxidized and reduced glutathione as disulfide bond exchangers (Saxena and Wetlaufer, 1970) . I was able to completely remove the urea, and partially remove the SDS, without significant precipitation of B2 protein. I was unsure of the extent of SDS removal after dialyzing the 1% SDS-solubilized protein in 0.1% SDS, 0.01% SDS, and finally the HEPES buffer. Although the final dialysis step was quite extensive (2 days), it has been suggested that the complete removal of SDS by dialysis can be quite difficult as this detergent can bind strongly to certain proteins (Tanford, 1968). After dialysis, B2 protein was concentrated using a centriprep 10 concentration unit (Amicon), quantitated by BCA assay, analyzed by SDS-PAGE (figure 4.8) and stored at 4C for mouse immunizations. No protein precipitation was noted on centriprep concentration, but after four months storage, a fine precipitate was detected lining the sides of the tube. The initial yield of protein after column purification was estimated to be 10-20 mg/L culture. After a number of attempts at dialyzing and refolding the protein, the final yield was 3 mg/L culture.

Generation of polyclonal Pf12ec B2 specific Antiserum

Five Balb/c, five A/J and three Biozzi mice were each immunized with 20 µg of B1 protein in Freund's Complete Adjuvant, and boosted three more times with 20 µg of B2 in Incomplete Adjuvant at three week intervals. Preimmune serum was taken as a control. After the final boost, mouse anti-B2 antiserum was obtained and tested for specificity to Pf12. In ELISA experiments the average titre of anti-Pf12ec B2 antibody from Balb/c, A/J, and Biozzi mice was approximately $6X10^5$ vs. denatured B2 and $7X10^5$ vs. renatured

B2 protein (figure 4.9). All three strains of mice had approximately the same titres of anti-B2 antibodies, so the antiserum was pooled and used to further characterize Pf12.

Western Blotting of Lysates from Pf12-transfected COS cells with pooled polyclonal anti-B2 antiserum.

In these experiments, two different forms of Pf12 were used, one lacking the native signal and anchor sequences (Pf12tag) which were replaced by the T1/leu-1 signal and DAF anchor of pBJT2 eukaryotic expression vector (figure 4.11), the other the wild-type Pf12 in pPSC1. Vector pBJT2 was created as a eukaryotic expression vector 'companion' to the prokaryotic expression vector pBT1TØ described earlier in this chapter (figure 4.2). Both secreted and anchored forms of the target protein could be expressed in pBJT2, and each of these contain an N-terminal MRGS-His6 epitope/affinity purification tag. In the Western Blotting experiments, I first transfected COS cells with either Pf12tag/pBJT2, Pf12/pPSC1 or pBJT2 control. I wanted to determine whether the anti-B2 antiserum could recognize Pf12 produced in COS cells, and I wanted to be able to compare it with the tagged form of Pf12 in pBJT2, which I could detect with the anti-MRGS commercial monoclonal antibody. I reasoned that the wild-type Pf12 and Pf12 tag subfragment should be approximately the same size because the signal and anchor positions for both proteins were the same.

Lysates from COS cells transfected with these Pf12 constructs (figure 4.10) were resolved by SDS-PAGE and Western-blotted with mouse pre-immune, anti-B2, and anti-MRGS antibody, labelled with goat anti-mouse HRP, and visualized by chemiluminescence. The anti-B2 antiserum recognized a smear of proteins from the Pf12/pPSC1 lysate in the range of 40-60 kDa, whereas negative control antiserum and vector-transfected COS cell lysates failed to produce a signal. Most predominant in this series of bands was a protein with an observed molecular weight of approximately 39 kDa. The anti-B2 antiserum also recognized a similar smear of proteins ranging from 40-66kDa

in the Pf12 A1/pBJT2 lysate, although the staining was not as strong as in the wild-type Pf12 lysate. In addition, anti-MRGS antibody recognized an identical group of proteins the Pf12 A1/pBJT2 lysate. These staining profiles suggested that the anti-B2 antiserum was indeed specific for Pf12 produced in COS cells as it recognized a range of proteins similar to that recognized by the anti-MRGS His6 antibody, which was specific only for MRGS His6 tagged Pf12 A1. The smear of proteins recognized by both antibodies could have been a number of differentially glycosylated or folded forms of Pf12, the unmodified 39 kDa form of the protein being the most predominant.

Western Blot experiments using asexual *P. falciparum* parasites and mouse anti-B2 antiserum

Initial western blot experiments using extracts from *P. falciparum in vitro* cultures suggested that the mouse anti-B2 antiserum recognized a protein of molecular weight similar to Pf12, and showed some cross-reactivity with either BSA or other RBC components (figure 4.12). In these experiments, RBCs containing asexual stage schizonts were harvested using 60% percoll separation (see materials and methods section), lysed using 0.15% saponin/PBS, washed, resolved by SDS-PAGE, and Western-blotted with anti-B2 antiserum. The saponin lysis step serves to disrupt the RBC but not the parasite, leaving an intact free parasite with some remnant RBC membrane fragments attached to it. Although some parasite proteins will be lost in this step, namely secreted proteins and to some extent parasite RBC membrane, PVM and TVM proteins, it is essential to release contaminating RBC proteins that otherwise distort SDS-PAGE gels and contribute to background (e.g. hemoglobin) in Western blotting experiments. The freed parasites were washed at 4°C lysed immediately in SDS lysis buffer, resolved by SDS-PAGE, and Western-blotted with anti-B2 antiserum. For the strains tested, HB3, K1A, D10, 3D7, the anti-B2 antiserum recognized a 35 kDa protein and in some cases additional high molecular weight proteins (including 65 kDa band in the D10 strain). The anti-B2 antiserum also

recognized a 65 kDa protein in K1A, D10 parasites, and non-infected RBCs in western blot experiments using acetone precipitated parasite and RBC extracts (figure 4.13). The reactivity of the anti-B2 antiserum to the 65 kDa band in RBC lysates was abolished by extensive washing of the saponin lysed cells in PBS, but remained present to some extent in the parasite lysate.

The cross-reactive epitope recognized by the anti-B2 antiserum could either be an RBC component that remained after washing of lysed parasites, or components of human serum, such as serum albumins that remain bound to the proteins in the parasite extract. Western blots of recombinant B2 protein, BSA, and human serum proteins were incubated under a number of different blocking conditions to determine if the cross-reactivity of the antiserum could be abolished. In the first series of experiments, blots containing the three proteins were blocked with 0.5% Tween 20/PBS and incubated with anti-B2 antiserum that had itself been incubated for 30 minutes at room temperature with either 0.5% Tween-20/PBS alone, 2% BSA/PBS, or 2% BSA and 2% Human serum/PBS. This step was intended to block any antibodies that recognized BSA or human serum proteins before the antiserum was applied to the blot. In the second set of experiments, blots were blocked first with 5%BSA+0.5% Tween-20/PBS, then incubated with antibody that was itself incubated under the three different conditions.

The results from this experiment (figure 4.14) suggested that the HSA reactivity of anti-B2 could be almost completely abolished, whereas the BSA reactivity could be partially abolished, when the blots were initially blocked with 5%BSA+0.5% Tween-20/PBS, and the anti-B2 was preincubated with 2%BSA+2% human serum/PBS. The preblocking step with BSA and human serum seemed to be significantly better at abolishing cross-reactivity than BSA or Tween-20 alone, although reactivity to Pf12 protein was also lessened to some extent. It should also be noted that the B2 protein used in this experiment was the same lot of pure protein used for mouse immunization, but that it had been incompletely reduced. Thus when resolved on SDS-PAGE and blotted to nitrocellulose, a

number of bands were seen in addition to the predicted 31kDa band, namely a 60 kDa band, and other low molecular weight bands which were most likely proteolytic products.

To further test this preblocking protocol, Western Blots containing saponin-lysed HB3 strain schizonts, RBCs, or human control antiserum were blocked with 5% BSA/PBS and incubated with anti-B2 antiserum preincubated in either 0.5% Tween-20/PBS, or 2% human serum /2% BSA /PBS. Again, the preincubation step almost completely abolished the HSA and BSA reactivity of the anti-B2 antiserum, while having no effect on its specificity for Pf12, seen as a 37.8 kDa band (figure 4.15).

After creating a blocking protocol that would diminish the reactivity of anti-B2 to BSA and HSA, new parasite Western blot experiments were conducted to determine the time of expression of Pf12 during the parasite life cycle (figure 4.16). 3D7 strain parasites from *in vitro* cultures were chosen for these studies because they could be easily grown compared to some of the other strains used, and because both asexual (merozoites through to segmenters) and sexual (gametocytes) could be tested. Each different life cycle stage was separated from asynchronous parasite cultures using stepwise percoll gradients. RBCs from cultures of 5-10% parasitemia (percentage of RBCs infected with parasites as determined by microscopic inspection of Giemsa stained culture smears) were centrifuged through gradients consisting of successive layers of 40, 60, 70, 80 and 90% percent percoll plus 4% sorbitol. RBCs infected with distinct life cycle stages were separated by density, collecting at various interfaces between different percoll layers. After centrifugation, parasites at each interface were aspirated with a pasteur pipette into separate tubes. Smears of parasites from each layer were stained with Giemsa examined to ensure that a relatively pure population of each life cycle stage had been isolated. The 4% sorbitol was included in the gradient layers to lyse the trophozoite, schizont and segmenter-infected RBCs giving a cleaner separation, whereas the smaller ring-infected RBCs, which are resistant to sorbitol, were lysed with saponin after being collected from the gradient. The separated parasites were washed extensively in PBS, and prepared for SDS-PAGE

separation and Western blotting. All parasite lysates were quantitated by BCA assay and normalized before loading. In later experiments (figure 4.16 A), protease inhibitors PMSF, aprotinin, and leupeptin were used, and all lysis steps and washes were completed swiftly at 4°C. The integrity of these parasite protein preparations is much better than that of earlier experiments (figure 4.16 B).

The anti-B2 antiserum used in Western blotting experiments in figures 4.16 A and B recognizes a number of proteins in the schizont and to some extent trophozoite, but not RBC control, ring, gametocyte or lysed schizont (top layer of the percoll gradient) protein preparations. The recognition of trophozoite proteins by anti-B2 antiserum could be due to the presence of small amounts of schizonts in the trophozoite preparation. In figure 4.16 A, anti-B2 recognizes 37 and 34 and 19 kDa proteins, whereas in figure 4.16 B the antiserum recognizes 37, 31, and 17 kDa proteins. The 19 and 17 kDa proteins of these figures are most likely proteolytic products of Pf12 resulting from the release of parasite proteases during the process of preparing parasite proteins for Western blotting. This proteolytic pattern is reminiscent of that seen in chapter 3 with Pf12ec A2 produced in *E. coli*, and earlier in this chapter with the Western Blot analysis of the B series subfragments in produced in *E. coli*. Figure 4.16 B demonstrates how the use of protease inhibitors, reduced temperature washes and swift lysate preparation minimized this proteolysis. From these experiments it is evident that Pf12 is expressed during the schizont stage of the asexual life cycle as a protein of an observed molecular weight of 37 kDa.

Immunoprecipitation of ^{35}S-methionine-labelled parasite Pf12 protein using anti-B2 antiserum.

In order to confirm the previous Western Blotting results, and to determine if Pf12 was secreted into the parasite culture media, immunoprecipitation experiments were conducted using anti-B2 antiserum. I chose this method in order to avoid problems with background staining of RBC or HSA proteins as only parasite protein would be labelled. Asynchronous HB3 strain *in vitro* cultures were washed and incubated with ^{35}S-

Methionine in methionine-free media. 18 hours after addition of the labelling media, the parasitized RBCs and culture supernatants were collected, the RBCs lysed and washed in PBS, the resulting preparations precleared with protein G sepharose, and finally incubated with anti-B2 antiserum. Antibody-protein complexes were then precipitated using protein G-sepharose, washed extensively, resolved by SDS-PAGE, and analyzed using a phosphorimager. The results of this experiment are detailed in figure 4.17. The anti-B2, but not pre-immune antiserum recognizes a protein of approximately 34 kDa that associates with the parasitized RBC. Although some high molecular weight proteins have been immunoprecipitated from both culture supernatant and parasite lysates, no proteins are specifically immunoprecipitated from the supernatant by the anti-B2 antiserum. This experiment confirms that the anti-B2 antiserum specifically binds parasite-synthesized Pf12, and that this protein stays associated with the parasite or infected RBC.

Immunofluorescence experiments using Pf12-transfected COS cells and anti-B2 antiserum.

Pf12/pPSC1-transfected COS cells were stained brightly by the anti-B2 antiserum compared to controls (table 4.1). In these experiments, COS cells were transfected (DEAE dextran) with either pPSC1 or Pf12/pPSC1 DNA, incubated with a mouse pre-immune or post-immune anti-B2 antiserum and labelled with goat anti-mouse FITC-conjugated antibody. Antiserum from the three different strains of mice injected with recombinant B2 were assayed separately, and all found to stain the surface of Pf12-transfected COS cells, whereas the controls did not.

Immunofluorescence experiments using *P. falciparum* parasites and anti-B2 antiserum.

In parasite immunofluorescence experiments, the choice of fixation protocol had a significant impact on the ability of anti-B2 antiserum to stain parasites (table 4.2). 3D7

strain schizonts and segmenters from sorbitol-synchronized cultures were used for these experiments based on the observations from the Western blotting experiments that Pf12 was synthesized predominantly during the these stages. Anti-B2 antiserum did not stain the surface of unfixed schizont-infected RBCs, suggesting that the protein was present in the interior of the parasitized RBC, or present on the surface of merozoites which were often lost during the washing steps of this experiment. Methanol fixed parasitized RBCs were also stained with anti-B2 antiserum, following the protocols used in chapter 3 with the anti-A2 antiserum. Unfortunately, anti-B2 antiserum and pre-immune control staining of these methanol-fixed smears was not significantly different.

Immunofluorescence experiments using acetone-fixed parasites and anti-B2 antiserum showed that Pf12 is present during the late schizont and merozoite stages (figure 4.18-4.21). In schizonts, but not trophozoites, Pf12 can be detected in a diffuse punctate distribution throughout the entire infected RBC (figure 4.19 B, D), and it also seems to "shower forth" from the infected RBC, in some cases (figure 4.19 B). When immunofluorescence studies were conducted with an additional ethidium bromide incubation step to stain the parasite nuclei, it was evident that Pf12 was associated with merozoites bursting from the infected RBC (figure 4.20 A and B). Pf12 was both associated with the merozoites, and present in vesicle-like packets that did not associate directly with the merozoites. Using confocal microscopy and two different antibodies, one to detect Pf12 (anti-B2) and one to detect e Ag on the surface of red cells, it was shown that Pf12 localizes to schizont-infected erythrocytes (figure 4.21 A, white arrowhead) and free merozoites (figure 4.21 A and B black arrowheads). After prolonged intervals of washing the acetone fixed parasites used in the immunofluorescence experiments, it was observed that Pf12 signal is diminished if not lost entirely.

Immunoelectron microscopy experiments using *P. falciparum* parasites and anti-B2 antiserum

The anti-B2 antiserum has failed to localize Pf12 in immunoelectron microscopy experiments. These experiments were considered to be crucial in assigning a subcellular location to Pf12. Although a number of different protocols were tested, all failed to add any further information about the location of Pf12 in the parasite. The first set of protocols used involved the fixation of washed HB3 or 3D7 parasites with glutaraldehyde and paraformaldehyde, dehydration of the parasites through stepwise incubations in ethanol of increasing concentrations, immobilization of parasites in embedding media, sectioning and staining of the embedded parasites with mouse preimmune and anti-B2 antiserum. A number of different fixative concentrations were used, as detailed in table 4.3. In these instances, virtually no staining of parasites was noted with either preimmune or anti-B2 antiserum. In addition, this antiserum was sent to collaborators (Dr. M. Aikawa, Case Western University) where similar experiments were conducted. Sections of 0.1% glutaraldehyde and 1% paraformaldehyde fixed parasites were incubated with both preimmune and anti-B2 antiserum (figure 4.23). While the anti-B2 antiserum seemed to stain the schizont and trophozoite stronger than the pre-immune antiserum, the staining was still generally weak and non-specific. Anti-B2 also stained nearby uninfected RBCs with approximately the same intensity as the parasite infected RBCs. Together, data from this set of experiments suggested that the anti-B2 antiserum had no specificity for parasite protein. It was hypothesized that the fixation of parasites prior to antibody staining was responsible for the loss of signal, and that either epitopes of parasite synthesized Pf12 were masked or by fixation, or the protein itself was lost during this process.

A second set of experiments was conducted to circumvent the potential fixative sensitivity of Pf12. The main idea behind these experiments was to incubate parasites with preimmune or anti-B2 first, then proceed through the fixation, dehydration, embedding, and sectioning steps. Thus anti-B2 antibodies could interact with Pf12 in its native

conformation, before being altered by fixatives. The main obstacle to overcome was to make parasite Pf12 accessible to the antibody. Previous immunofluorescence experiments had shown that Pf12 resided within schizont and segmenter-infected RBCs, not on the surface. The challenge was to permeabilize the infected RBC and maintain its general integrity (without fixation), allowing the anti-B2 to diffuse in and bind Pf12. The observation from the immunofluorescence experiments that Pf12 tended to shower forth from segmenters and merozoites bursting from RBCs caused some concern, as permeabilization of infected RBCs may result in a loss of Pf12.

Two methods of permeabilization were used in these experiments: saponin lysis, a relatively harsh detergent that completely lyses the infected RBC, leaving only the parasite and remnant RBC membrane components associated with it, or streptolysin O, a bacterial pore-forming toxin that gently permeabilizes the RBC membrane without completely lysing it, maintaining to some extent the network of protein trafficking vesicles established by the parasite, the parasitophorous vacuolar membrane/ tubovesicular membrane (PVM/TVM). Immunofluorescence experiments (figures 4.19, 4.20, 4.22) had suggested that Pf12 was on the surface of the schizont or segmenter, on the surface of merozoites, and perhaps on the inner leaflet of the RBC membrane. This suggested that it may have access to the PVM/TVM network used by the parasite for trafficking and targeting of parasite proteins. By both harsh and gentle permeabilizing agents, I hoped to be able to localize Pf12 to one or more of these regions.

Unfortunately, neither of these experiments revealed any new information about the location of Pf12. Both anti-B2 and preimmune antiserum stained permeabilized schizont infected RBCs poorly (table 4.3). No subcellular organelles were labelled specifically, and in the case of the saponin-lysed parasites, it was difficult to identify parasites and RBCs. Staining of streptolysin O permeabilized RBCs was also poor, although parasites and RBCs were still intact.

Invasion inhibition experiments using anti-B2 antiserum and *P. falciparum in vitro* cultures

These experiments were conducted as described in chapter 3 and in the materials and methods section using 3D7 parasites and anti-B2 antiserum at a dilution of 1:100. These experiments revealed that anti-B2 does not inhibit invasion of RBCs compared to pre-immune control serum. The percent invasion calculated for cultures incubated with control or anti-B2 antiserum was approximately 10%. As with the invasion inhibition experiments completed with the anti-A2 antiserum, these experiments need to be confirmed by enumerating greater quantities of parasites, and different strains of parasite.

C. Discussion

The goal of experiments described in this chapter, like chapter 3, was to generate a Pf12 subfragment that could be produced in *E. coli* in relatively large amounts, purified, and used to generate Pf12 specific antiserum for localizing the protein in malaria parasites. The studies conducted in this chapter differ from chapter 3 in that a more extensive set of subfragments was created based on the hypothetical disulfide bonding pattern described by Elliott and Carter (Carter et al., 1995), a new vector and modified host strain of *E. coli* was used to produce and purify recombinant Pf12 protein, an effort was made to renature the purified protein, and a more extensive optimization of parasite preparation for immunofluorescence and immunoEM was conducted. Antibodies generated against the new recombinant Pf12 subfragment, Pf12ec B2, were used to localize Pf12 to the surface of schizonts and segmenters in infected RBCs, the inner leaflet of the infected RBC membrane, and free merozoites. Although this anti-B2 antiserum was superior to the anti-A2 antiserum described in chapter 3, both anti-A2 and anti-B2 antiserum failed to localize Pf12 definitively in immunoEM experiments. Thus, the exact location of Pf12 is still not known; though immunofluorescence and Western blotting experiments can localize it to the

schizont iRBC and merozoite stages of the asexual cycle, its precise association with specific membranes and subcellular organelles remains undetermined.

In the first part of this chapter, an attempt was made to first identify a portion of Pf12 that could be produced at higher levels in *E. coli* than the previous subfragment (Pf12ec A2) described in chapter 3, purify the recombinant protein, and renature it to a conformation resembling that of the native parasite protein. I attempted to design subfragments that would maintain the hypothetical folding pattern because I believed that this might prevent aggregation of recombinant proteins within *E. coli* via intramolecular disulfide bridges. Unfortunately this had no effect whatsoever on the formation of substantial Pf12ec B2 protein inclusion bodies in large-scale *E. coli* cultures, and most likely the same is true for the other subfragments expressed at high levels (Pf12ec B4-6, B9-12). Even though key cysteine residues were present to form the native disulfide bridges of the protein, the presence of relatively large amounts of recombinant B2 protein in the oxidizing environment of the interior of *E. coli*, and other factors such as codon bias may have conspired to promote inclusion body formation. Future directions for the expression of disulfide rich proteins should include the use of *E. coli* strains such as AD494 (Derman et al., 1993) a thioredoxin reductase mutant that allows disulfide bond formation in the *E. coli* cytoplasm, and the co-expression of chaperone proteins that can assist in the formation of disulfide bridges.

The 'B series' subfragments are interesting themselves in their proteolytic cleavage and self-association patterns, as observed in (figure 4.5). The incorporation of the MRGS-His6 epitope tag on each of these proteins was a key improvement on the methods used in chapter 3, allowing for their detection and visualization by a commercial antibody and Western blotting, as opposed to Coomassie staining of polyacrylamide gels. As a result, not only can the level of expression of each mutant be determined, but also other interesting features of the recombinant proteins can be studied.

The subfragments that were expressed at relatively high levels were: B9, B6, B2, while B4, B10-12 were expressed at moderate levels. B2 was chosen because it was expressed at relatively high levels, and it encompassed most of the wild-type protein sequence. B2 protein is present in figure 4.5 as a number of different species, the predominant one with a calculated molecular weight of 30.5 kDa (compared to a predicted 31.9 kDa), with other minor species of 35.2, 24.6, 14.4, 13.0, and 10.3 kDa. The lower molecular weight species are also present to some extent in B4, B6, B9-12, suggesting the action of a protease on specific sites in the full-length protein to cleave it to these smaller forms. Although the specific sites remain undetermined, it is possible to speculate, based on the size of the proteolytic fragments, that proteases may cleave Pf12 to give loop fragments contained in subfragments B9, B10, B11, and B12.

The presence of larger molecular weight species in the B2 lane of figure 4.5 is interesting, and could be due to the presence of acidic residues in the protein that may affect its overall mobility. Another more intriguing explanation is that the presence of high molecular weight species is due to the tight association of B2 and proteolytic fragments of B2, even under the denaturing and reducing conditions of the experiment. While this may seem unlikely, an examination of the B6 (predicted molecular weight of 21.5 kDa) and B9 (predicted molecular weight of 13.7 kDa) lanes reveals two high molecular weight proteins of 74.2 and 27.5 (B6 lane) and 33.4 (B9 lane). An example of how this might occur is as follows: the association of the predominant 15.2 kDa protein in the B9 lane with itself to form a 30.1 kDa protein, which is close to the size of the 33.4 kDa protein seen in B9. It is interesting to note that the B9 'loop' is present in B6, B5, and B2, all of which show some potential to self-associate even under the denaturing and reducing conditions of this experiment. This, coupled with the hypothesis by Elliott and Carter that disulfide-bonded domains of Pf12, Pfs 48/45, and Pfs 230 may form interlocking 'cystine knots' suggests that Pf12 is indeed a protein with a unique and unusual native conformation.

Comparison of the 'A series' subfragments of the chapter 3 and the newer 'B series' subfragments revealed that the closest 'relative' to the A2 protein in the 'B series', B3, was produced at undetectable levels in *E. coli*. This was unexpected in that A2 was produced at a levels detectable by Coomassie staining. Closer examination of B3 protein revealed that an N-terminal region of the protein encompassing some of the amino acid residues of the signal sequence was not present in A2. In other subfragments containing this region, B1, B7, B8, recombinant protein production was also nearly undetectable, making it a good candidate for a 'toxic' region, although B1, the largest of the subfragments, was still expressed at a detectable level despite the presence of this sequence. The expression profile of these subfragments suggests that the low level of production of various subfragments in *E. coli* may be due to a number of factors, including the presence of rare codons, the misfolding of certain regions that may only be exposed in certain subfragments, and the aggregation or degradation of proteins early in the experiment. The presence of one toxic region of the protein does not provide a complete explanation for the results obtained.

Further comparison of the A series and B series subfragments revealed that the method of detection was crucial in identifying these proteins. The closest 'A series' relative to B5, A4, contains two unpaired cysteines, and part of loop b (see figure 4.1 for loop designation), which may explain why it was produced at undetectable levels in chapter 3. A simpler explanation is that the method of detection (Coomassie staining) was not sensitive enough. Although B5 was produced at detectable levels using Western blotting, it was only barely detectable by Coomassie staining. This is also evident when comparing A3 and its B series relative B10. While B10 is readily detectable by Western blotting techniques, it is not visible in Coomassie stained polyacrylamide gels. The observations from these experiments strongly suggest that sensitive methods such as Western blotting should always be used in the detection of newly produced recombinant proteins. Low

levels of protein expression, which might otherwise be increased by careful optimization of growth conditions, may not be detected by SDS-PAGE analysis and Coomassie staining.

After identifying Pf12ec B2 as the best subfragment to pursue further, based on its size and expression level, I proceeded to purify it on a large scale and renature it. While the purification process using nickel affinity chromatography involved only one new aspect (the use of imidazole for protein elution, as opposed to pH), the renaturation process was completely new and thus required more attention. The use of imidazole for elution of B2 protein from the nickel column was chosen to minimize any detrimental effects pH may have on the recombinant protein itself, and to have the recombinant protein in a neutral pH environment for the renaturation process. While the total protein obtained from the preparative column was much higher than that of chapter 3, a significant portion was utilized in many different renaturation experiments. Using dialysis to effect the stepwise removal of 8M urea denaturant from the B2 protein eluate, I was able to keep B2 protein soluble to a denaturant concentration of 3 M urea. As I removed more denaturant from the protein it began to precipitate, whether I removed it in a stepwise fashion, or by gradual dilution of the dialysis buffer using a gradient maker. The addition of 1% SDS to the denatured protein eluate solved this problem. By maintaining the concentration of SDS while I removed the urea I was able to keep B2 solubilized, and it did not precipitate when SDS was removed in a stepwise fashion in the presence of Hepes buffer and reduced and oxidized glutathione. I decided to add the last two components to facilitate the formation of B2 disulfide bridges. Only a very small amount of protein precipitate was noted after the final concentration steps. I used this protein for mouse immunizations assuming that it had a partially native conformation based solely on its solubility properties. My main assumption was that any precipitate formed would most likely be due to disulfide bridges formed between improperly folded B2 proteins.

The anti-B2 antiserum obtained from mice immunized with B2 protein was a much better reagent for detecting Pf12 than the rat anti-A2 antiserum from chapter 3. The

antiserum had higher titres of antibodies specific for both relatively native and denatured B2 as assayed by ELISA. Three different strains of mice were immunized to test the possibility that one might produce higher titres of anti-B2 antibodies than the others, but ELISA results suggest that this was not the case. Consequently, all antiserum from all B2 immunized mice was pooled and used for further characterization of Pf12.

This antiserum, called anti-B2, showed specificity for a group of proteins ranging in size from 39-60 kDa made in Pf12-transfected but not control COS cells in Western blotting experiments. The smear of bands recognized suggested that the protein was glycosylated. As a control, protein preparations from COS cells transfected with the Pf12tag sequence were Western-blotted with commercial antibody specific for the tag. The proteins recognized by this antibody were similar in size to those recognized by the anti-B2 antibody. Both anti-B2 and anti-MRGS-His6 antibodies recognized a predominant protein of 39 kDa in addition to the higher molecular weight smear, confirming that the anti-B2 antiserum was indeed specific for Pf12. Anti-B2 did not recognize the epitope-tagged Pf12 protein as strongly as anti-MRGS-His6 did. This may reflect the different titres of the two antibodies (anti-B2 is mouse polyclonal antiserum; anti-MRGS-His6 a mouse monoclonal) or the different epitopes they recognize. Interestingly, anti-B2 showed a stronger specificity for Pf12 than the MRGS-His6 tagged protein. This might be due to the presence of lower amounts of MRGS-His6 Pf12 produced by COS cells than wild-type Pf12. Conversely the epitopes recognized by anti-B2 may be dependent on a conformation (that is maintained even in the denaturing and reducing conditions of the experiment) that is partially disrupted by the presence of the epitope tag at the N-terminus of the protein. The results from these preliminary experiments using COS cell-produced Pf12 confirmed that anti-B2 was specific for this protein, and that Pf12 was glycosylated in COS cells.

Western blotting experiments using this anti-B2 antiserum identified a 35-37 kDa protein in a number of different *P. falciparum* strains (figure 4.12). The protein itself had a greater observed molecular weight when reduced with DTT than the non-reduced form.

This may be due to a compactness imparted on the protein when it is in a native conformation. It was interesting to note that for one strain of parasite, D10, a 66 kDa protein was also seen in addition to the 37 kDa protein. This species was present in the reduced protein preparation, but not the non-reduced one, which is counterintuitive considering the potential for protein association under non-reducing conditions. I considered that this protein might be contaminating human serum albumin, which is present in the parasite *in vitro* culture (10% human serum is needed to culture parasites, along with human red blood cells), the source of parasite protein in these studies. Inadequate washing of this particular preparation (which was made separately from the others) may have resulted in the retention of some HSA, which could non-specifically bind to the anti-B2 antiserum used in the Western blot experiments.

I continued to study this possibility by preparing new parasite protein from K1A and D10 strain parasite infected RBCs and normal uninfected RBCs, acetone precipitating this protein, and conducting new Western blot experiments with anti-B2 antiserum (figure 4.13). The results from these experiments confirmed my hypothesis, showing that anti-B2 did indeed have specificity for a 66 kDa protein in both parasite strains, and the RBC control in addition to its specificity for a 37 kDa protein in the parasite strains alone. All three protein preparations had human RBC proteins in them, and any other proteins that were present in the culture media that bound to these proteins. Although I washed each preparation after saponin lysis of RBCs, RBC membrane components are still present in the RBC control and parasite protein preps. I was unsure about the identity of this 66 kDa protein, but the fact that the anti-B2 antiserum was also specific for a number of proteins in the molecular weight markers used in these Western blot experiments (especially BSA) suggested that BSA or HSA were good candidates. There were two other possibilities: anti-B2 was specific for an RBC protein of similar molecular weight, or that anti-B2 was recognizing a larger form of Pf12, present only in the D10 strain where it was produced at much higher levels than in the other strains.

I attempted to block the specificity of the anti-B2 antiserum for the 66 kDa protein by pre-incubating it in buffer containing either 0.5% Tween-20 detergent, 2% BSA, or 2% human serum before applying this solution to the blot (figure 4.14). The purpose of the pre-incubation was to adsorb the anti-66 kDa protein antibodies from the anti-B2 antiserum, before it was applied to the blot. I was interested in showing that the anti-B2 specificity for the 37 kDa protein (in parasite-infected RBCs only) was separate from the 66 kDa specificity (in parasite- infected and uninfected RBCs). The 37 kDa protein recognized by anti-B2 in parasite-infected RBCs could have simply been a proteolytic product of 66 kDa protein recognized in uninfected RBCs, possibly cleaved as a result of proteins produced by parasite infection, as opposed to a protein synthesized by the parasite itself. The Western blots in figure 4.14 show that pre-incubation of anti-B2 with human serum does reduce the binding of anti-B2 to HSA and BSA proteins, without reducing the reactivity of the antibody to the renatured B2 protein discussed earlier in this chapter. This renatured protein was incompletely denatured and reduced before resolution on SDS-PAGE gels and Western blotting. As a result, B2 protein most likely self-associated to form multimer aggregates, all of which are recognized by the anti-B2 antiserum. While the anti-B2 reactivity to some of the multimers has been reduced as a result of Human serum pre-incubation, it is not as marked as the reduction in reactivity to BSA and HSA on the blot, and anti-reactivity to the main 31 kDa species remains unchanged. It is interesting to note that even in the renatured purified B2 protein lane, anti-B2 antiserum shows significant recognition of a 60 kDa protein, migrating at the same mobility as HSA.

The use of a 2% human serum pre-incubation step was continued in Western blotting studies with HB3 strain parasite protein (figure 4.15). In these studies it was shown that the pre-incubation step eliminated anti-HSA reactivity without reducing anti-Pf12 reactivity. Furthermore no anti-66kDa protein reactivity was noted in protein preparations from the parasitized RBCs. The RBC control used in this step, while containing some higher molecular weight proteins that were recognized by anti-B2

antiserum, did not contain the 66 kDa that was recognized in RBC controls by anti-B2 previously. As these were new preparations that had been washed more extensively than the previous ones, it was evident that the 66kDa activity was most likely an RBC or media protein that adhered to both the parasites and control RBCs after the saponin lysis step, probably HSA, as evidenced by anti-B2 reactivity to BSA present in the molecular weight marker. Although no BSA was present in the parasite culture media, the saponin lysis, or PBS wash buffers, HSA was present in the original culture media, and could have remained bound to the parasites or lysed portions of the RBC membranes after these preparation steps.

These studies did not address the possibility that another phenomenon may also be occurring, namely that Pf12 may be the 'cross-reactive' protein recognized by the anti-B2 antiserum. The presence of the cross-reactive band in figure 4.12 could be explained by an increased expression of Pf12 by D10 above that of the other strains tested. If this hypothesis was true, Pf12 would have to be present as a dimer (approximately 70 kDa), and have some cross-reactivity with a 66 kDa RBC-associated protein (HSA, as hypothesized above), as shown in figure 4.13. This is a remote possibility, as the cross-reactivity of anti-B2 can be significantly blocked by HSA, with little reduction in reactivity for the monomeric form of HB3 strain Pf12 (figure 4.15). Further studies using D10 strain Pf12, where the strongest cross-reactivity was originally noted, would have to be undertaken to confirm this hypothesis.

Pf12 was localized to the late trophozoite, and schizont stages using percoll gradient separation of parasites and Western blotting. The percoll gradient separation method has been used quite frequently for separating different parasite stages, based on the densities of the infected RBCs. Ring-infected RBCs, which contain fine ring-like parasites and maintain their overall shape, can be collected from a different part of the gradient than trophozoite-infected RBCs which contain a much larger parasite, and schizont-infected RBCs, which also contain larger parasites, and have lost their characteristic shape. At the

top of the gradient, lysed RBCs, parasite membranes, and merozoites can be obtained. A stepwise percoll gradient can be used to collect samples from each stage of the asexual parasite life-cycle, with minimal cross-contamination between stages. The percentage of each life cycle stages purified from the various interfaces of the gradient were determined by Giemsa staining, and only samples with less than 5% cross-contamination were used. Sorbitol was also included in these gradients because it lyses the deformed schizont and trophozoite-infected RBC, leaving intact parasites, which separate better on the gradient.

Of the stages purified, anti-B2 antiserum recognized 34 and 37 kDa proteins in schizont-infected RBCs (figure 4.16). This size corresponds well with the size of protein recognized in Western blots using Pf12-transfected COS cells, and with the calculated molecular weight of the protein, 39.4 kDa. In these experiments smaller proteolytic protein bands were noticed, a motif seen in a number of instances previously discussed, and this proteolysis was minimized by the inclusion of protease inhibitors and swift preparation of parasite proteins at 4°C. In addition, it is also evident that Pf12 is not heavily glycosylated, or else a smeared ladder of bands representing a number of protein species with differing degrees of glycosylation would be evident, as noted previously in the Westerns conducted using protein from Pf12-transfected COS cells (figure 4.10).

I conducted an immunoprecipitation study as a means of confirming the Western blot results, and to determine if Pf12 was secreted in addition to being associated with the parasite. As the native Pf12 sequence includes a PI membrane anchor signal, and proteins with these kinds of signals are sometimes produced in both anchored and secreted forms, I thought it would be worthwhile to see if an anchored form of Pf12 existed. My main concern was that if Pf12 was secreted, it would most likely be lost in the saponin lysis and washing steps in the protocol for preparing parasite protein for Western blotting.

Western blot experiments described earlier could not be used to identify secreted Pf12 for a number of reasons. I would be sampling both supernatants and parasites to detect Pf12, and the presence of HSA and other proteins in the parasite growth medium

would not only distort the polyacrylamide gels when these proteins were resolved, but serve as a major contributor to nonspecific binding in Western blot experiments. Western blot experiments where the source of protein was parasitized RBCs and culture media directly lysed in SDS sample buffer always resulted in protein profiles distorted by HSA, hemoglobin, and other proteins. Different proteins recognized by control and anti-B2 antiserum were hard to determine because of the distortion in these blots. By separating parasites from culture media and using saponin lysis to disrupt the parasitized RBC without lysing the parasite, I was able to resolve these problems, the trade-off being a loss of any parasite protein present in the RBC cytoplasm or secreted into the culture supernatant.

By labelling parasite proteins (not RBC proteins, which have already been made earlier in the maturation process of the RBC), with ^{35}S-methionine, and immunoprecipitating these proteins with control and anti-B2 antiserum, I was able to circumvent the problems mentioned above. Figure 4.17 shows that anti-B2 antiserum immunoprecipitated a protein of approximately 34 kDa, in agreement with the size of Pf12 recognized in previous Western blotting experiments. This protein is precipitated only from parasite lysates, not from culture supernatants. This does not rule out the possibility for Pf12 secretion for a number of reasons. First, these experiments were not as sensitive as the Western blotting experiments. Because unsynchronized HB3 cultures were used, only a fraction of the parasites were at the schizont stage of growth, when Pf12 is synthesized. Synchronizing cultures, introducing label prior to the schizont stage, and harvesting parasite protein soon thereafter would have definitely improved on the amount of Pf12 immunoprecipitated. Also, Pf12 may be secreted, but still associate with RBC and parasite membranes based on hydrophobicity. Although the most hydrophobic sequences of Pf12 were detected in the signal and anchor sequence regions, other regions of significant hydrophobicity may be brought together as a result of the folding or unfolding of the native parasite protein. A number of other high molecular weight proteins were detected by both the control and anti-B2 antiserum. These are most likely due to non-

specific binding of parasite proteins to the antibodies or the protein G sepharose used to precipitate the antibody-protein complexes.

While the Western Blotting and immunoprecipitation studies established Pf12 protein was present in a number of different isolates as a 37 kDa schizont protein, immunofluorescence experiments revealed the location of Pf12 in schizont-infected RBCs, and a new aspect of Pf12 not realized in previous experiments: it was also present on merozoites. The success of these experiments hinged on the type of fixation used prior to the staining process. Whereas parasites fixed with methanol were stained to some degree by anti-A2 as described in chapter 3, methanol staining seemed to abolish the ability of anti-B2 to stain parasites. An acetone-based fixation method was developed for washed, air-dried parasitized RBCs from *in vitro* culture. Anti-B2 antiserum, but not control (or anti-A2 antiserum from the previous chapter) strongly stained schizont-infected RBCs obtained from synchronized *in vitro* cultures. I used synchronized cultures because I knew from previous experiments when Pf12 was expressed, and I wanted all the parasites harvested to be at that stage when I stained them. By monitoring the growth of the parasites in culture I was able to predict when schizonts and merozoites would be the predominant forms in culture, confirm this by inspection of Giemsa-stained smears, and then harvest these parasites for immunofluorescence experiments.

These immunofluorescence experiments reveal that Pf12 is targeted to a number of regions of the mature asexual stage parasite before and after merozoites break free of the infected RBC. Staining of unfixed, unpermeabilized infected RBCs shows that Pf12 is not present on the surface of these cells. Conversely, staining of permeabilized (by air-drying), acetone-fixed, infected RBCs reveals the presence of Pf12 on the surface of the schizont within the infected RBC, possibly on the inner leaflet of the infected RBC membrane, and on the surface of merozoites as they escape the RBC. The observation that no stained merozoites are visible in immunofluorescence experiments with unfixed, unpermeabilized parasites does not necessarily mean that Pf12 is not present on the surface

of the merozoite; often it was difficult to observe merozoites under these conditions, and many merozoites are lost during the washing stages of the experiment. The use of air-dried, fixed smears on the other hand, immobilized all parasite forms to the slide and effectively prevented parasite loss in washing steps.

Two-color immunofluorescence experiments using normal UV microscopy and confocal microscopy confirmed that Pf12 was present in schizonts, and more importantly, free merozoites. The use of FITC as a secondary reagent to detect Pf12 and ethidium bromide to label parasite DNA showed discrete merozoite nuclei surrounded by Pf12 protein, while in other regions Pf12 swirled around the entire grouping of merozoites as they burst from the infected RBC (figure 4.20). In some instances Pf12 staining did not coincide with the staining of parasite DNA (figure 4.20). This may have been due to the weaker intensity of the EtBr staining compared to the FITC staining, or due to Pf12 containing membrane fragments remaining after the merozoites were released from the infected RBC. Figure 4.20 D shows two such Pf12 stained regions seemingly free of EtBr staining. In addition, the Pf12 staining around the merozoite DNA is suggestive of vesicle-like packets separate from the merozoites.

Confocal microscopy experiments using two-color immunofluorescence were conducted to address the question of whether or not Pf12 staining was present on the surface of the parasite inside the RBC, or the surface of the parasitized RBC itself (figures 4.21 and 4.22). This was a concern because during the end of the asexual stages of the parasite life cycle, the infected RBC membrane loses its characteristic shape, becomes difficult to see using light microscopy of unstained or Giemsa stained parasites and eventually breaks down. It was difficult to tell if the strong Pf12 surface staining was on a crumpled RBC membrane that looked like a parasite because of its shape, or the parasite membrane itself. Using a second antibody to the RBC eAg on RBC membranes, it was possible to distinguish Pf12 and RBC membrane staining. Although these experiments are not conclusive, they suggest that Pf12 is associated with the membrane of the mature

schizont as it divides into 8-32 merozoites and begins to occupy most of the RBC cytoplasm. It was possible to detect bright Pf12 staining with small amounts of RBC membrane staining suggesting that this protein is present when the RBC membrane is breaking down around the maturing parasite. The green FITC staining of the RBC membrane and the red staining of Pf12 did not colocalize to form a yellow signal, but instead remained as discrete regions of red staining with some punctate green staining outside of it. This suggests that Pf12 is present on the surface of the parasite before it bursts, and as the parasite grows in size, Pf12 may localize to the inner surface of the RBC membrane.

In addition, these experiments strongly suggested that merozoites contain Pf12 on their surfaces. In figures 4.22 A and B, open arrowheads denote rounded or oval-shaped regions of strong Pf12-staining, suggestive of merozoites by their size (approximately 2 μM), shape, and lack of anti-RBC e antigen staining. Moreover, in Figure 4.22 B, these oval bodies are seen bound to RBCs in a manner reminiscent of merozoite attachment. Finally, the presence of flattened regions of staining on RBC surfaces (figure 4.22 B), suggests that Pf12 protein may be sloughed from the merozoite membrane and remain on the iRBC surface after invasion. From these experiments it was evident that in addition to schizonts, merozoites contained Pf12 as well.

These experiments also reveal the importance of immunoelectron microscopy as a gold standard in confirming the presence and location of antigens. The immuno-fluorescence studies strongly suggested where Pf12 was located and when it was synthesized, but immunoEM studies would have confirmed these observations. The reason for failure of the immunoEM experiments is most likely due to the susceptibility of parasite-synthesized Pf12 to fixatives such as glutaraldehyde and paraformaldehyde, and the difficulty (in the absence of any fixation steps) of permeabilizing parasites and making Pf12 accessible to antibodies without losing the protein to washing steps or proteolysis. Experiments using rat anti-A2 antiserum in chapter 3 had suggested that Pf12 was

susceptible to glutaraldehyde and paraformaldehyde fixation (as are many antigens); and both fixatives were also shown to affect anti-B2 staining of parasite smears in immunofluorescence experiments (table 5.2). Thus even though an attempt was made to generate antiserum specific for a more native form of Pf12, this was not sufficient to overcome the effects of glutaraldehyde or paraformaldehyde fixation of Pf12 in parasite specimens.

A final set of RBC invasion inhibition experiments suggests that Pf12 may not be involved in mediating invasion of RBCs by merozoites. This is supported by similar experiments conducted in chapter 3, although more of these experiments need to be completed in order to confirm these initial observations. A titration of both anti-A2 and anti-B2 antiserum should be undertaken, and the experiments themselves should be redesigned to include the enumeration of more RBCs or microscope fields. Also, the initial percent parasitemia before the addition of antiserum could be increased to 3-5% as opposed to 1% used in these studies, promoting healthier parasite growth.

A model for Pf12 localization

A model for the sub-cellular localization of Pf12 can be proposed based on the results described above. This model still needs to be confirmed by immuno EM experiments. Pf12 expression probably begins in the late trophozoite stage, where it is transported to the outer membrane of the parasite (anchored by a PI linkage), and most likely the PVM/ TVM. Pf12 accumulates in the TVM of mature schizonts but does not penetrate into the individual membranes of the developing merozoites. The ability of Pf12 to self-aggregate suggests that it may form some sort of matrix or meshwork of associated proteins within the TVM. When the iRBC bursts, some Pf12 may be proteolytically cleaved from a 37 kDa fragment to a 19 kDa fragment, the smaller soluble protein associating with free merozoites, the larger anchored protein remaining associated with remnants of the PVM/TVM. By contrast, anchored Pf12 may transfer from the TVM to the

merozoite membrane intact. Pf12 may also associate with the inner leaflet of the RBC membrane before the burst of merozoites, but wet immunofluorescence experiments discussed above suggest that it is not exposed on the outer surface of the iRBC. Pf12 stays associated with the outer surface of free merozoites, and upon merozoite invasion of RBCs, Pf12 may be transferred to the iRBC membrane.

This model of Pf12 localization and expression accounts for all the observations recorded in chapter 3 and 4 except one: the fact that iRBCs incubated with anti-A2 or B2 antiserum do not enter the iRBC via the TVM and label Pf12. This can be explained by the fact that these 'wet' immunofluorescence experiments were all conducted at 4° C. At this temperature the RBC membrane may not be fluid enough to transport antibodies into the TVM. One crucial experiment that should be conducted in the future is the incubation of iRBCs with anti-B2 at 37° C, which may result in the transport of the antibodies into the TVM. Also, the isotype of anti-B2 or A2 (both polyclonal antiserum), was not determined. This could be important, because if this antibody is primarily IgM, it may be present in a pentameric form and thus not easily traverse the 'parasitophorous duct' into the TVM. The determination of the isotype of the anti-A2 and anti-B2 antiserum, along with the purification of these antibodies may help to further localize Pf12.

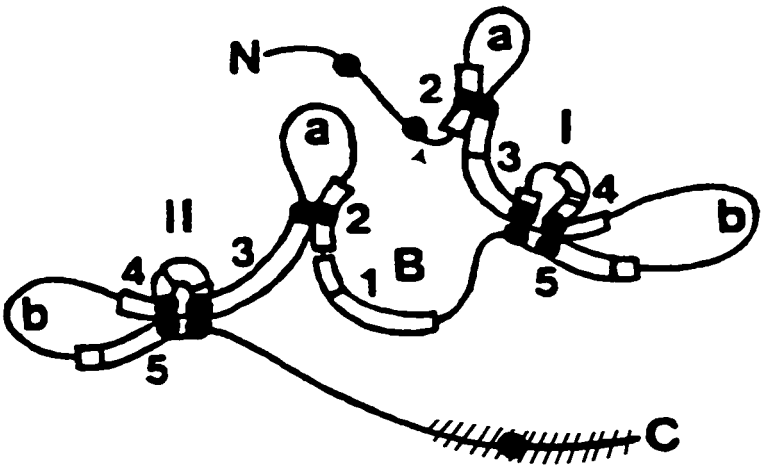
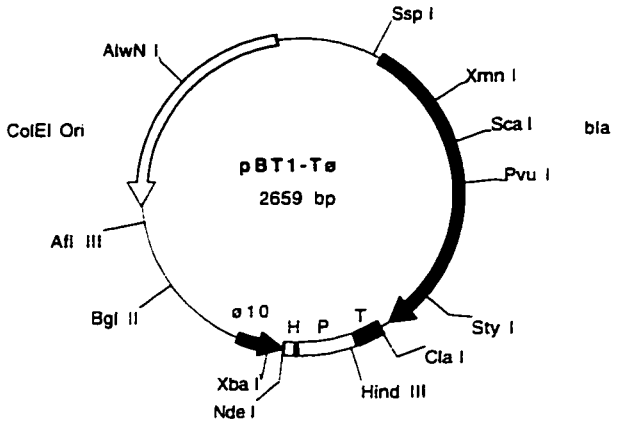


FIGURE 4.1. Schematic diagram of a possible model for the disulfide linkages in Pf12 developed by R. Carter and J. Elliott. Two hypothetical disulfide-bonded domains, which are structurally related to each other arise from this model and are labelled I and II. These are joined by a bridge segment B. Each domain consists of linked cysteine residues (dark circles) with intervening loops "a" and "b". Blocks of sequence which contain particular repeated sequence motifs (numbered 1-5), are indicated by open double lines (domain 1 does not contain motif 1). Strokes across the open double lines indicate proline residues, and the arrow indicates the cleavage site of the N-terminal signal peptide. The C-terminal hydrophobic segment, which is likely cleaved during linkage to a phoshoinositol anchor, is indicated by the hatched segment.

A.



B.

```
                                                                  XbaI        BamHI
CAT ATG AGA GGC TCG CAT CAC CAT CAC CAT CAC TCT AGA GGA
    M   R   G   S   H   H   H   H   H   H   S   R   G

        SpeI    NheI    NotI            MluI    EcoRI   PstI
TCC ACT AGT GCT AGC GCG GCC GCA ACG CGT GAA TTC CTG CAG
T   S   A   S   A   A   A   T   R   E   F   L   Q
```

C.

```
Pst I  Not I     Hind III
ctgcaggcggccgcaaagcttggctgctaacaaaGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA

                                                                    Cla I
ACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGgaggaactatatccgatcgat
```

FIGURE 4.2. pBT1TØ, a prokaryotic expression vector for production of recombinant protein containing an N-terminal MRGS-His6 epitope/purification tag. **A.** This vector is a modified version of the prokaryotic expression vector pT7-7 (figure 3.1). Features of this plasmid include: T7 gene 10 promoter, ø10; MRGS-His6 tag, H; Poly-linker, P; T7 transcription terminator, T; ColEI origin; betalactamase selective marker, bla. **B.** Expanded view of the DNA and protein sequence of the MRGS-His6 tag and polylinker regions. Restriction sites are printed above the DNA sequence.Open box: amino acid sequence recognized by anti-MRGS-His6 monoclonal antibody (Qiagen). Shaded box: amino acid sequence required for protein purification using nickel affinity chromatography. Expression of inserted genes can be confirmed by Western blotting using anti-MRGS-His6 antibody prior to large scale expression and purification. **C.** DNA sequence of the T7 transcriptional terminator generated by PCR using the primers Tø5'II and Tø3' (see table 2.1) and the prokaryotic expression vector pET11a (Novagen) as a template. This segment would be expected to promote higher expression levels than pT7-7 (figure 3.1).

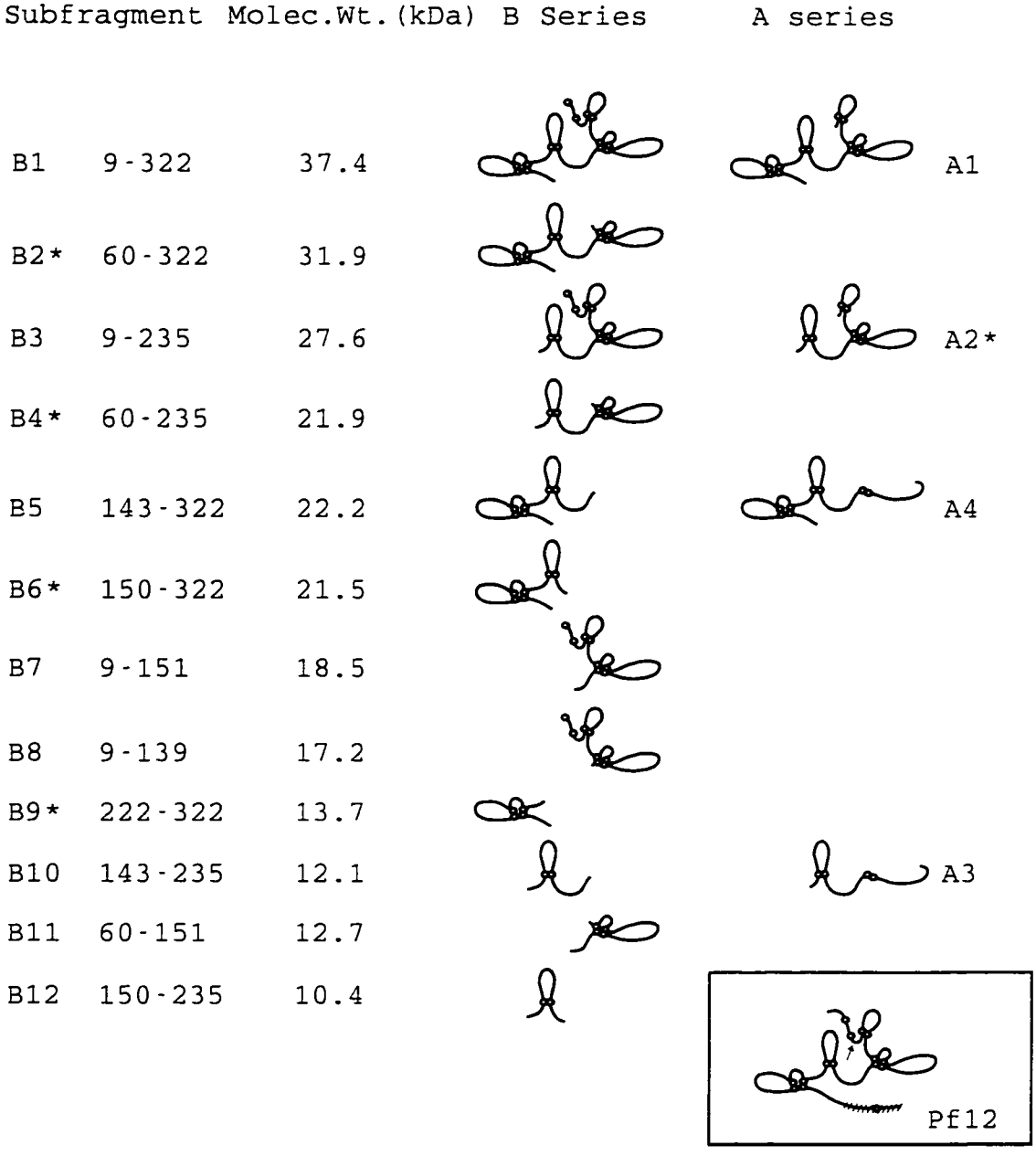


FIGURE 4.3. Pf12ec B series subfragments, compared to the A series subfragments. The boundaries, predicted molecular weight, and model disulfide structure for deletants B1-12 are shown on the left. Model disulfide structure for the previously constructed deletants A1-4 (from figure 3.2) are shown on the right. directly beside the B deletant they most closely resemble. The line diagram of Pf12 (open box) matches the one in figure 4.1, with the open circles representing the cysteine residues. B series constructs were generated via PCR using primers shown in table 2.1, and cloning into the Bam HI and Not I sites of the vector pBT1Tø. All constructs were transfected into *E.coli* BL21 pLysS for expression studies. (*) indicates recombinant proteins whose expression could be detected directly on Coomassie blue stained gels (see figure 3.4 and 4.4).

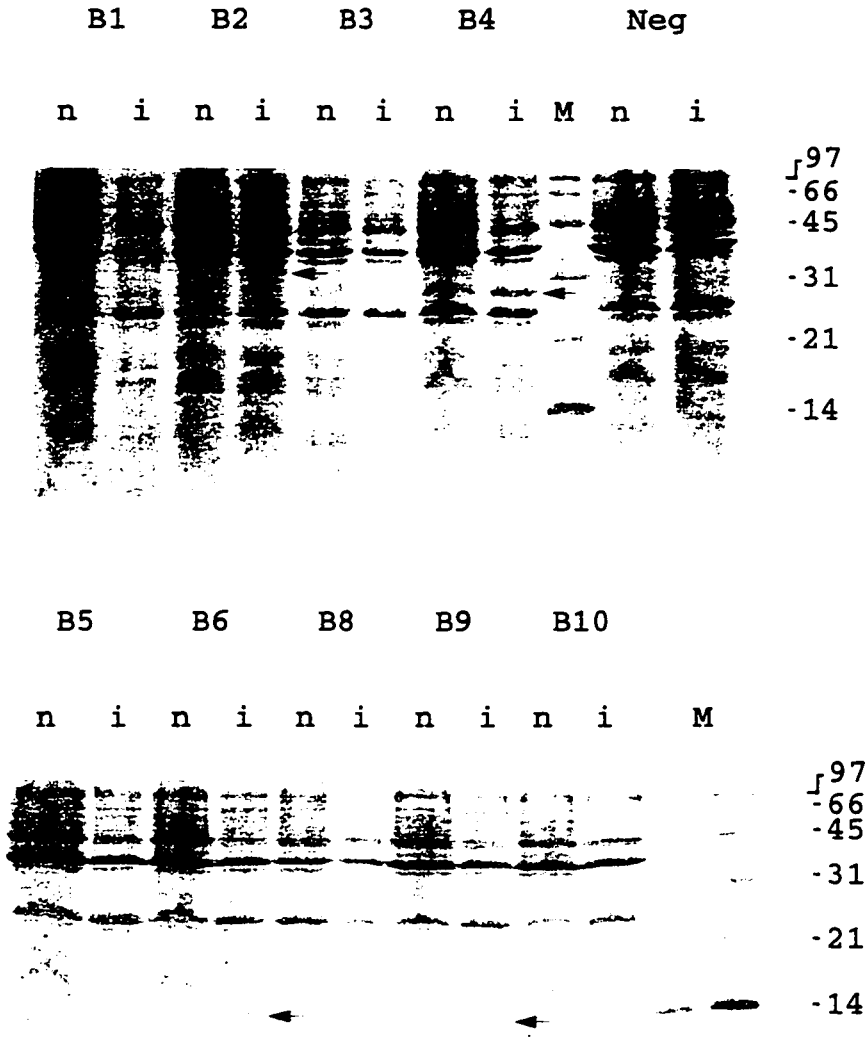


FIGURE 4.4. SDS-PAGE analysis of protein expression from Pf12B series deletants in *E.coli*. Lysates from non-induced (n) and induced (i) BL21 DE3 pLysS bacteria transformed with Pf12 B series deletants were resolved by 12% SDS-PAGE (using 100 mM DTT). Gels were stained with Coomassie blue and examined for the presence of induced proteins, which are indicated by arrows for deletants B2, B4, B6, and B9. M: Low-range protein marker, with marker sizes shown on the right.

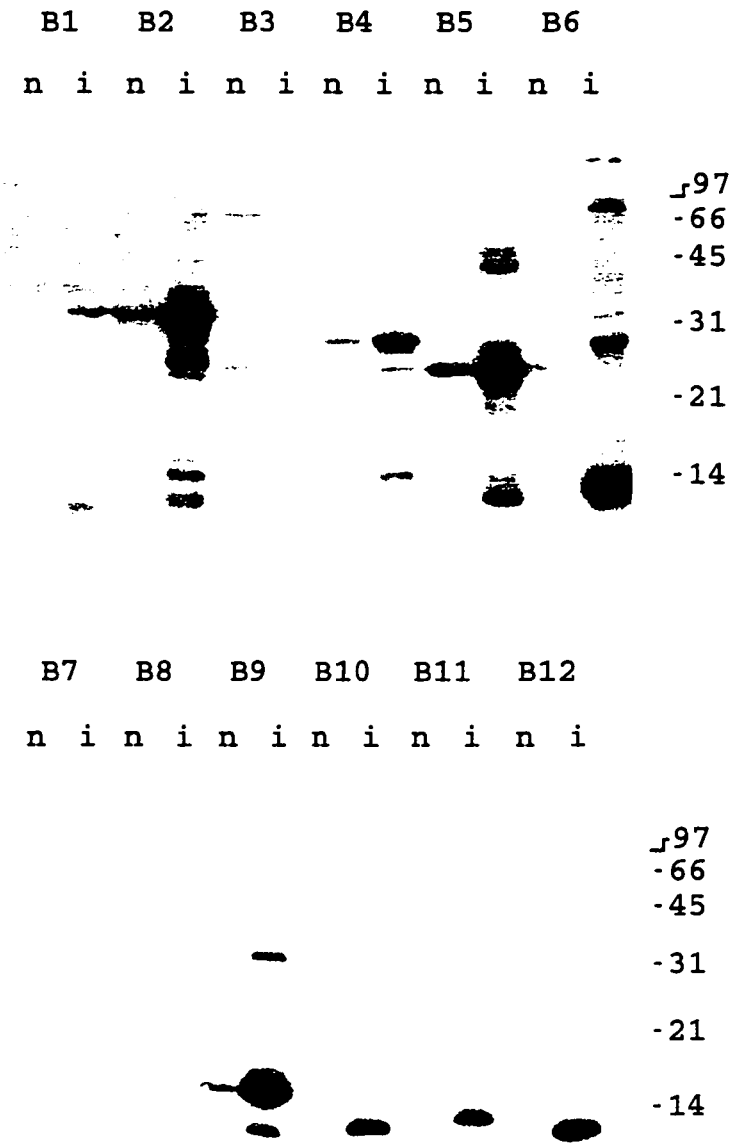


FIGURE 4.5. Western blot analysis of protein from B series deletants expressed in *E.coli*. Cell pellets were resolved by 12% SDS-PAGE as in figure 4.4, and blotted onto nitrocellulose. Proteins were detected using a monoclonal antibody specific for the MRGS-His6 tag which was incorporated at the N-terminus of each recombinant protein. Low-range molecular marker sizes are shown to the right; n, noninduced cultures; i, induced cultures.

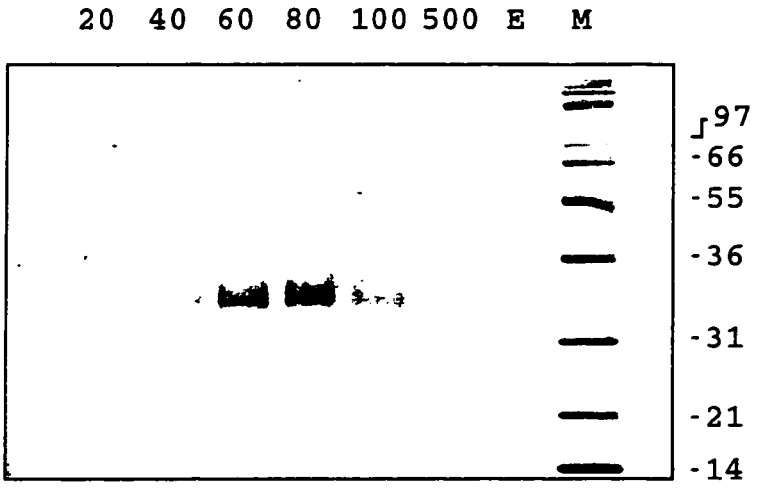


FIGURE 4.6. Pilot nickel column purification of recombinant Pf12ec B2, showing SDS-PAGE analysis of column fractions eluted by various concentrations of imidazole. B2 protein (from inclusion bodies; solubilized in 6M GuHCl) was loaded onto a nickel chelating column, and eluted using increasing concentrations of imidazole in 8M urea 0.01 M Tris-HCl pH 7.5, 0.1 M Na_2HPO_4 (urea buffer). The column was washed with 20mM imidazole urea buffer until no further protein was eluted as judged by Bradford assay. This was repeated for 40, 60, 80, 100, and 500mM imidazole urea buffer, and with 100mM EDTA urea buffer (E). Each sample was resolved by 12% SDS-PAGE and stained with Coomassie blue. Imidazole concentrations used to elute each fraction are given at the top of the gel. Broad range marker (M) sizes are indicated to the right of the gel.

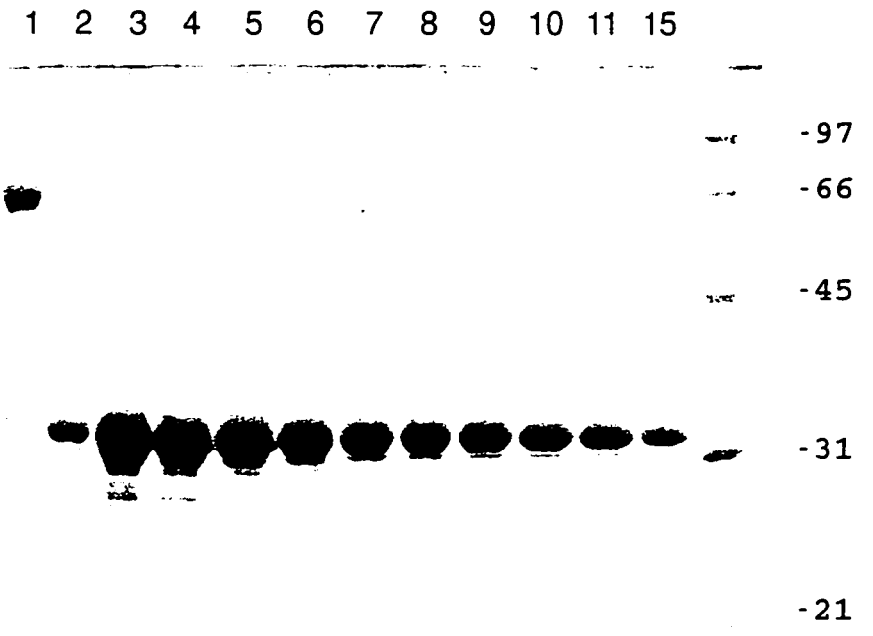


FIGURE 4.7. SDS-PAGE analysis of nickel affinity column fractions containing Pf12ec B2 protein. Solubilized protein from an induced culture of B2-transformed *E.coli* BL21 DE3 pLysS was applied to a 12.5 ml nickel chelating column that had been equilibrated with 20 mM imidazole urea buffer (see figure 4.6). The column was washed with 20 volumes of 20 mM imidazole urea buffer, and protein content of these wash fractions was monitored by Bradford assay (protein concentration reached baseline levels after 10-15 volumes of wash buffer had been eluted from the column). Pf12ec B2 protein was eluted using 100 mM imidazole in urea buffer. Eluted fractions (1-11, 15; indicated at the top of the gel) were resolved by 12% SDS-PAGE and stained with Coomassie blue. Column fractions 3-15 were pooled for renaturation. Fraction 1 contained a protein much larger than the expected molecular weight of Pf12ec B2; 1 and 2 were therefore excluded from the pool in order to avoid contamination of Pf12ec 2 with other proteins.

FIGURE 4.8. SDS-PAGE analysis of purified, renatured, Pf12ec B2 protein. Pf12ec B2-containing fractions eluted from a single large nickel chelating column run was pooled, and separate 40 ml aliquots (B2-1, 2, 3, 4) and diluted 1:10 in 3M urea buffer (see figure 4.7) containing 1% SDS, 1mM DTT. Each aliquot was dialyzed overnight at room temperature against 4 L of Laemlli buffer. This process was repeated using modified Laemmli buffer containing 0.1%, 0.01% SDS, and then finally in 4 mM Hepes buffer containing 2 mM oxidized glutathione and 0.02 mM reduced glutathione. The protein was concentrated, resolved by 15% SDS-PAGE, and stained with Coomassie blue. Low range protein markers (M) sizes are indicated to the left. protein from aliquots B2-1, B2-2, and B2-4 were combined and used to immunize mice; protein was apparently lost from the B2-3 aliquot.

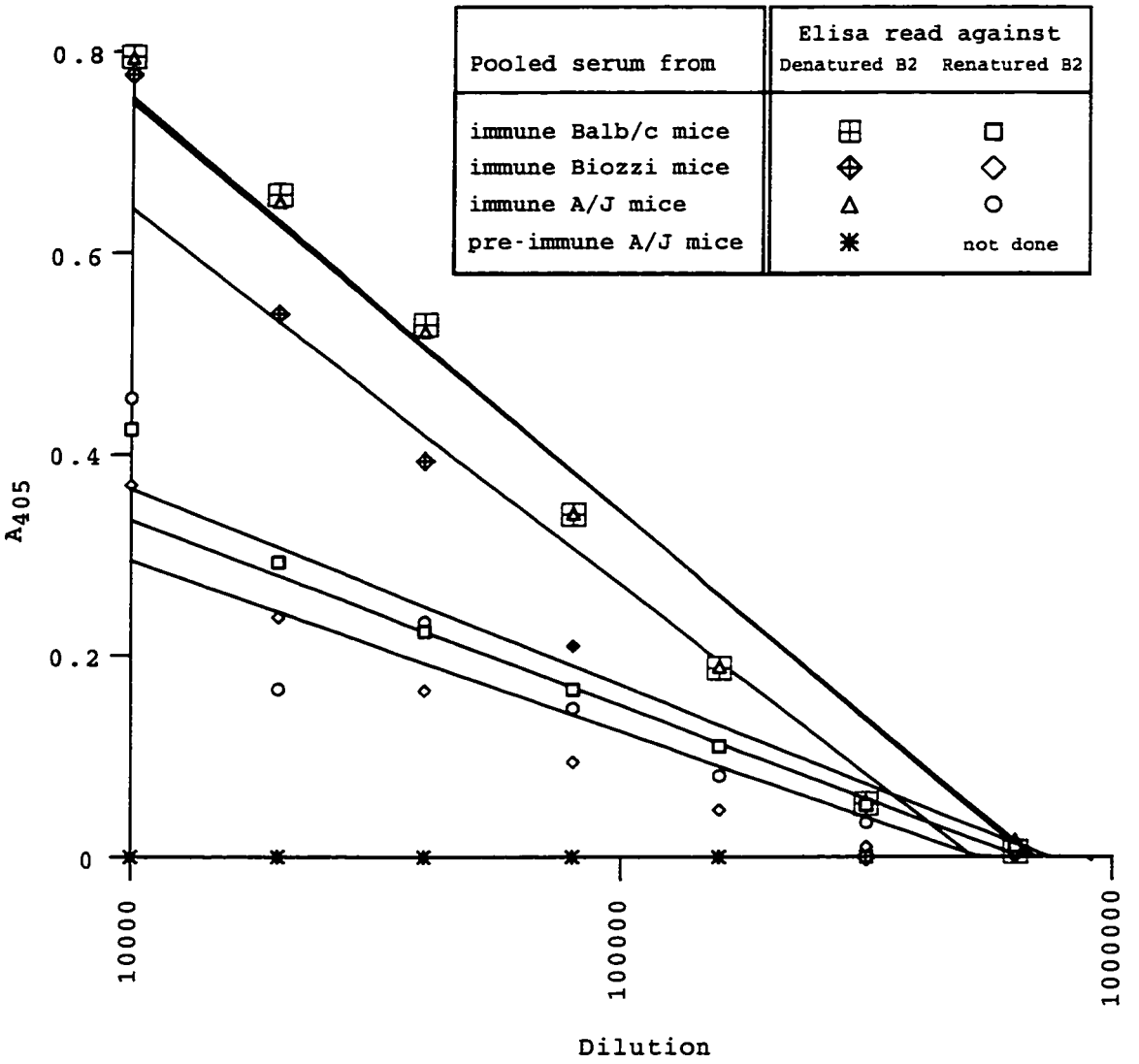


FIGURE 4.9. Anti-Pf12ec B2 antibody titres in three different strains of mice immunized with the 'renatured' B2 protein. Balb/c, Biozzi, and A/J mice (n=5 per group) were pre-bled and then immunized with 'renatured' Pf12ec B2 protein shown in figure 4.8. Mice received 200 µl of protein (0.1 mg/ml in PBS), emulsified with an equal volume of CFA (first injection) or IFA (subsequent two injections), with injections given at four week intervals. Fourteen days after the last injection mice were bled via the tail vein and serum from each group was pooled and analyzed by solid phase ELISA. Titres against the denatured/ reduced B2 protein (denatured in 1% SDS/ 50 mM DTT, then bound to the ELISA plate) and the 'renatured' protein (see legend figure 4.8) were compared.

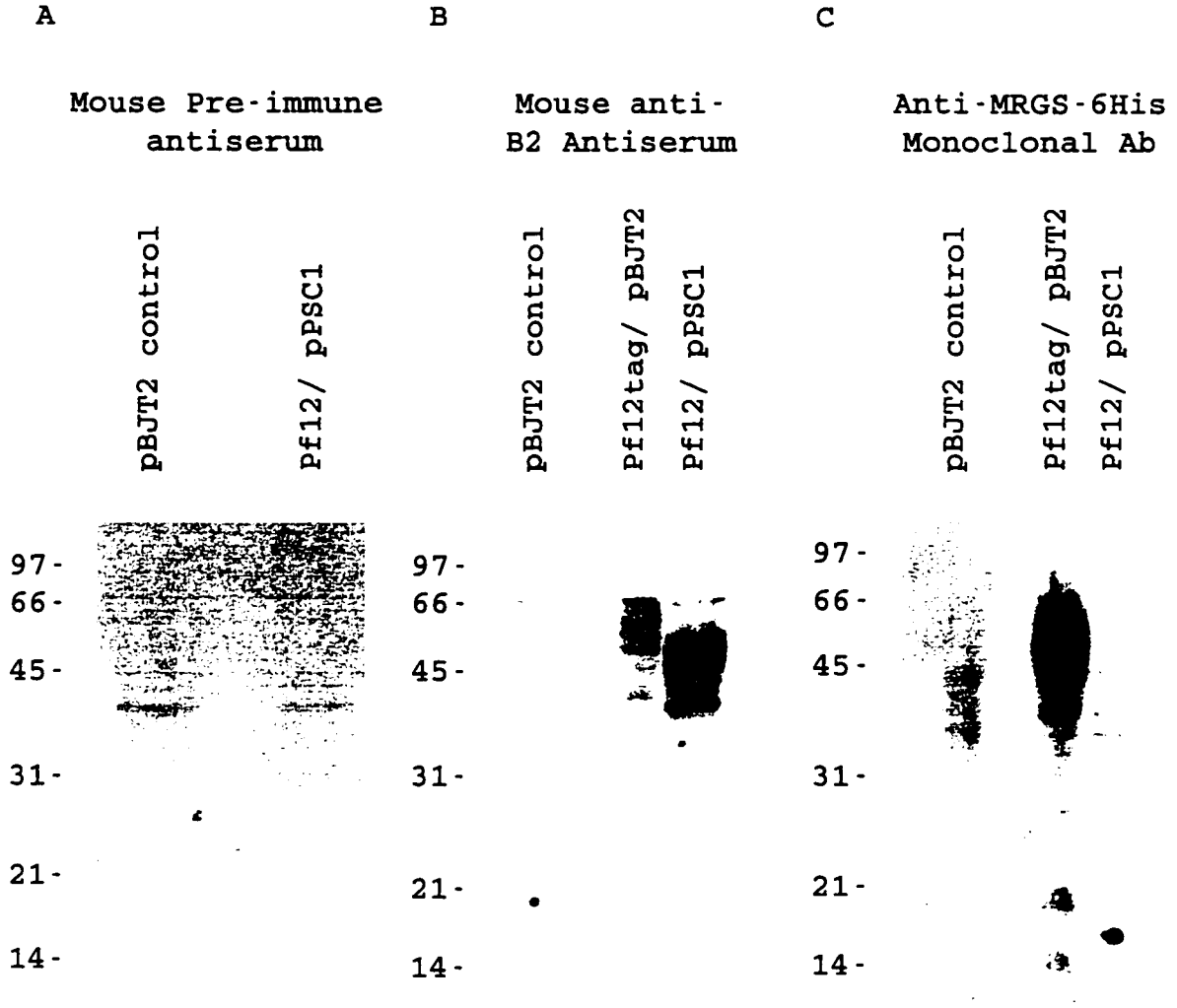
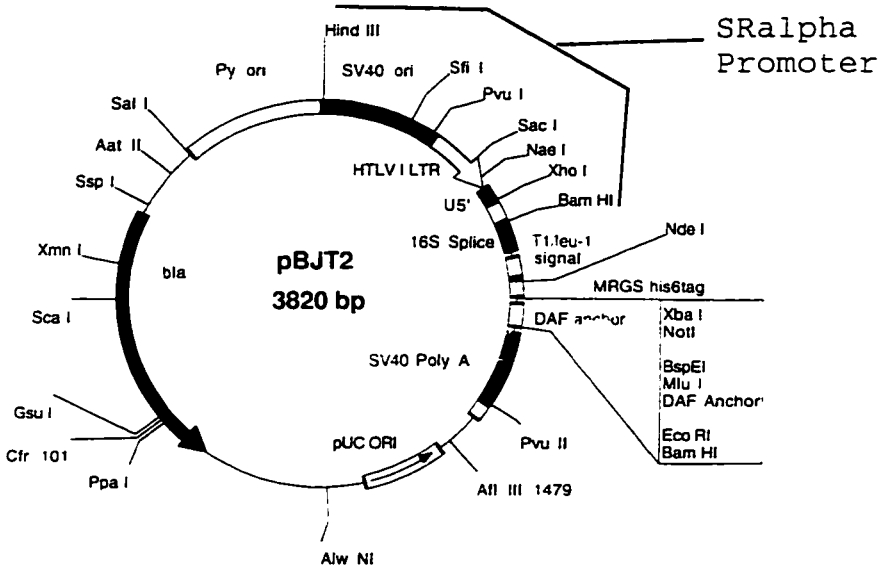


FIGURE 4.10. Western blot analysis of lysates from Pf12-transfected COS cells with mouse anti-B2 antiserum. COS cells transfected with Pf12/pPSC1 (gives wild type protein; see figure A2.5) or Pf12tag /pBJT2 were harvested 72 hours post transfection. Lysates (approximately $1x10^5$ cell equivalents/lane) were resolved by 12% SDS-PAGE, blotted to nitrocellulose (blocked with 10% milk/ PBS/ 0.5% Tween-20) and probed with (A) mouse pre-immune control antiserum, (B) mouse anti-B2 antiserum, or (C) MRGS-His6 specific monoclonal antibody (all at 1:10,000 dilution), followed by goat anti-mouse HRP-conjugated secondary antibody (1:10,000). (Pf12tag lacks the anchor and signal sequences of the native protein; it was created with primers Pf12 5' 1A and Pf12 3' 6B, (table 2.1). The PCR amplified fragment was cloned into pBJT2, which adds an N-terminal signal sequence, MRGS-His6, and C-terminal DAF anchor to target sequences (see figure 4.11). The blots demonstrate that although the B2 protein (i.e. original immunogen) contains the MRGS-His6 motif, the mouse anti-B2 antisera strongly recognizes the wild type Pf12 protein (at least in a denatured/reduced form), and with about the same affinity as it recognizes a version of Pf12 containing an MRGS-His6 tag.

A.



B.

```
                T1/leu-1 signal sequence begins
tctagtccacc ATG CCC ATG GGG TCT CTG CAA CCG CTG GCC ACC TTG TAC CTG CTG GGG
            M   P   M   G   S   L   Q   P   L   A   T   L   Y   L   L   G
ATG CTG GTC GCT TCC TGC CTC GGA GAA GTC TCT AGT CAT ATG AGA GGC TCG CAT CAC
M   L   V   A   S   C   L   G ▲ E   V   S   H   H   M   R   G   S   H   H

                    Polylinker                  DAF Anchor begins

                    Xba I    Not I Sac II       Bsp EI
CAT CAC CAT CAC tct aga gcg gcc gcg g  TCC GGA ACT ACG CGT CTT  TCT GGG CAC
H   H   H   H   S   R   A   A   A      S   G   T   T   R   L    S   G   H

                Mlu I
ACG TGT  TTC ACG TTG ACA GGT TTG CTT GGG ACG CTA GTA ACC ATG GGC TTG CTG ACT
T   C    F   T   L   T   G   L   L   G   T   L   V   T   M   G   L   L   T

  Eco RI
TAg  aa ttc
Z
```

FIGURE 4.11. Map of pBJT2, a eukaryotic expression vector for producing N-terminal MRGS-His6-tagged surface or secreted proteins in COS cells. **A.** Target open reading frames which are cloned into the pBJT2 vector at the multiple cloning site are fused to upstream T1-leu signal (for secretion) and an MRGS-His6 tag which is retained in the mature protein for identification and/or purification. If a membrane anchor is not already present in the ORF, the downstream DAF anchor allows for phosphatidyl inositol-mediated linkage of recombinant proteins to the cell surface, if desired. This plasmid is composed of elements from pJFE14DAF (SRalpha promoter; figure 5.1), YPO4 (T1/leu-1 signal sequence; Dr. Brian Seed), pBT1Tø (MRGS-His6; see figure 4.2), pSRalpha DAF 319 (polylinker and DAF anchor shown above; a gift from Dr. D. Denney), and pPSC1 (vector backbone; figure A2.1). **B.** DNA sequence of the T1/leu-1 signal (arrow indicates signal cleavage site), MRGS-His6 tag (underlined), and DAF anchor of pBJT2. Polylinker, 5' and 3' untranslated plasmid sequences are in lower case. Z= stop codon.

TABLE 4.1 Immunofluorescence analysis of Pf12-transfected COS cells incubated with anti-B2 antisera.

	COS cells transfected with:	
Antisera	Pf12/pPSC1	pPSC1
A/J Pre	-	-
A/J Post	+++	-
Balb/c Pre	-	-
Balb/c Post	+++	-
Biozzi Pre	-	-
Biozzi Post	+++	-

COS cells were transfected with either Pf12/pPSC1, or pPSC1 using the DEAE dextran method. Transfected cells were harvested after two days and incubated with pooled mouse pre-immune or anti-B2 immune antisera (1:200 dilution), and then an FITC-conjugated goat anti-mouse secondary antibody (1:500). Surface staining was scored as negative (-), or positive (from weak,+, to strong, ++++) using a Leitz UV microscope (400X magnification). The immune antisera from the A/J, Balb/c, and Biozzi mice were pooled to give the 'mouse anti-B2 antiserum' used in the remainder of the staining and Western blot experiments shown in this chapter. Note that in this experiment the transfected COS cells were not fixed prior to staining.

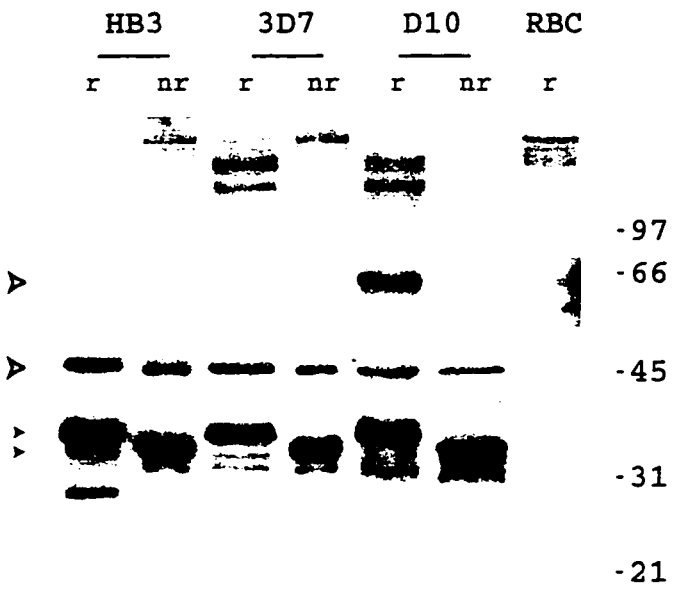


FIGURE 4.12. Detection of Pf12 protein in three strains of *Plasmodium falciparum* by Western blotting and mouse anti-B2 antiserum. Parasites from asexual stage cultures were enriched for schizonts by passage over a 60% percoll gradient and lysed with 0.15% saponin /PBS. Parasite lysates were prepared under reducing conditions (r) with 50mM DTT, and under non-reducing conditions (nr). The lysates (and a control uninfected red cell lysate) were normalized for protein by Bradford assay, resolved by 12% SDS-PAGE (100 ng protein per lane), and blotted onto nitrocellulose. The membranes were blocked with 10%Milk/ PBS/ 0.5% Tween, incubated with mouse anti-B2 antiserum (1:10,000 dilution), washed thoroughly in PBS/ 0.5% Tween, and then probed with HRP conjugated goat anti-mouse secondary antibody (1:10,000). Protein bands were visualized by chemiluminescence (Amersham ECL kit). M, low range protein markers; closed arrows indicate Pf12 protein, open arrows indicate proteins containing cross-reactive epitopes.

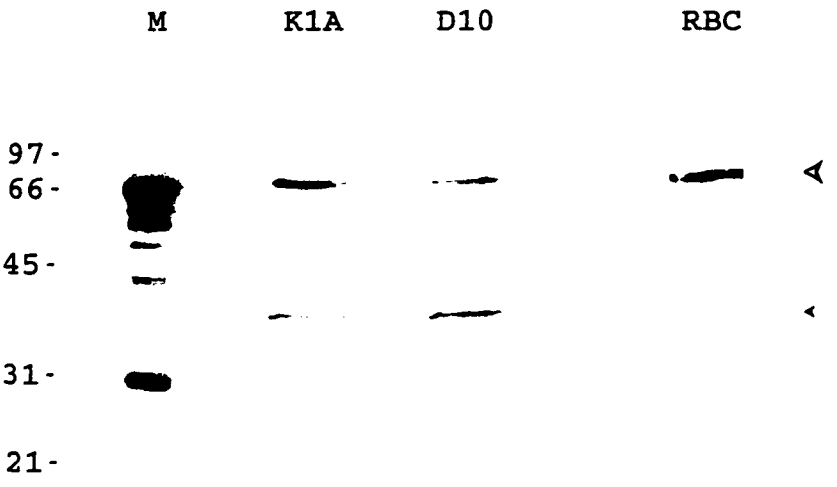
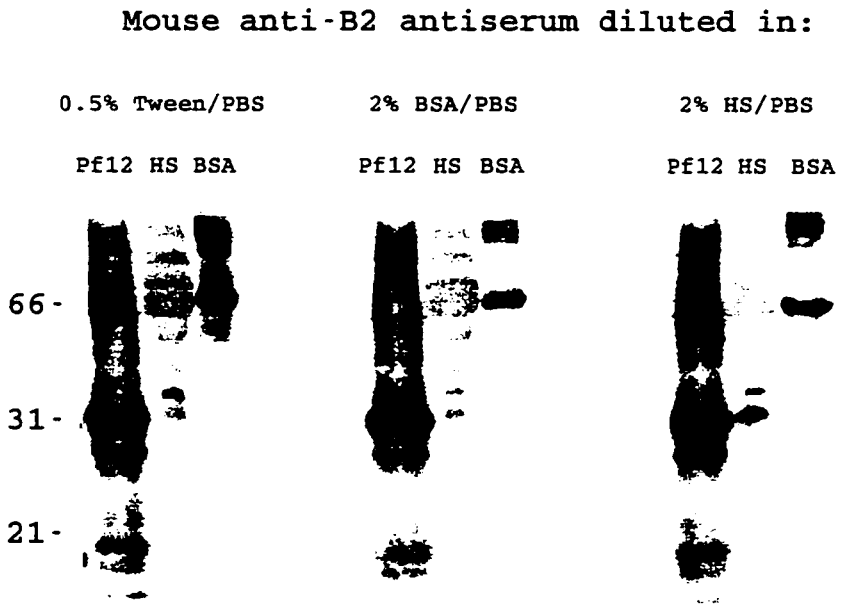


FIGURE 4.13. Detection of a cross-reative 66kDa protein band in Western blots of uninfected RBCs probed with mouse anti-B2 antiserum. Lysates from asynchronous parasite cultures (K1A and D10 strains; lysed in 0.5% saponin/ PBS) were precipitated with 100% acetone (4°C, 2 hours), pelleted in a microfuge, resolved by 12% SDS-PAGE (0.1 ug/ lane), Western blotted to nitrocellulose, and probed with mouse anti-B2 antiserum (as in figure 4.12). An equivalent quantity of uninfected red blood cell lysate, treated in the same manner as the parasite lysates, was included in the right lane (RBC). Closed arrow indicates the native Pf12 protein, migrating at 37 kDa, open arrowhead indicates the 66 kDa cross-reactive band which is present in both uninfected and infected culture.

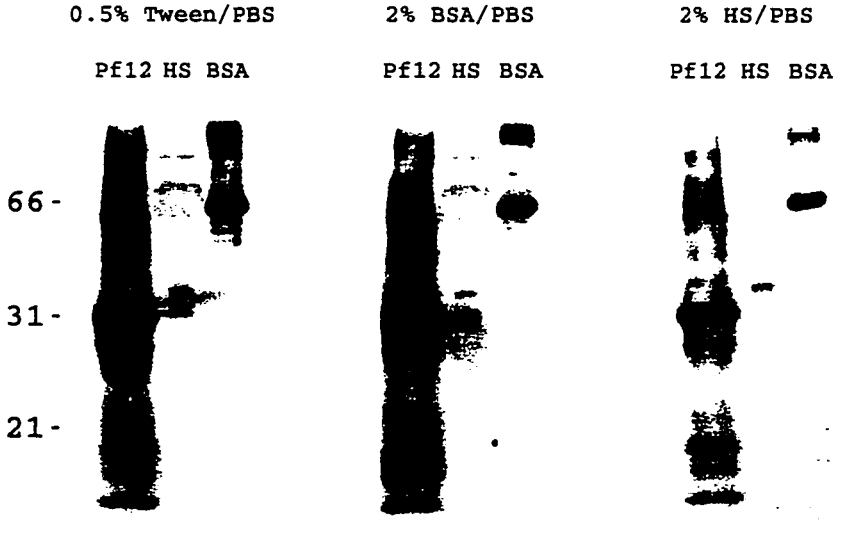
FIGURE 4.14. Determination of blocking conditions that minimize binding of mouse anti-B2 antiserum to BSA and human serum proteins. Purified recombinant Pf12ec B2 protein, BSA, and human serum proteins (HS) were resolved by 12% SDS-PAGE (2 μg/lane), blotted to nitrocellulose and blocked with: (A) 0.5% Tween-20/PBS or (B) 5% BSA/ PBS/ 0.5% Tween-20. The anti-B2 antiserum was diluted (1:10,000) in either 0.5% Tween-20/PBS, 2% BSA/PBS , or 2% human serum/ PBS, and incubated at room temperature for 30 minutes before being added to blots blocked under conditions (A) or (B). The blot was washed, probed with secondary antibody, and developed as in figure 4.12. In this experiment purified Pf12ec B2 was incompletely reduced, thus it appears as one major protein at 31 kDa, but also appears in a number of minor bands representing aggregates (> 31 kDa), or degradation products (<31 kDa). The best protocol for minimizing the reactivity of anti-B2 antiserum to BSA was (B) an initial blocking step with 5%BSA/ PBS/ 0.5% Tween-20, combined with the incubation of anti-B2 in 2% human serum/PBS before addition to the blot.

A



Nitrocellulose blocked with PBS/0.5% Tween-20

B

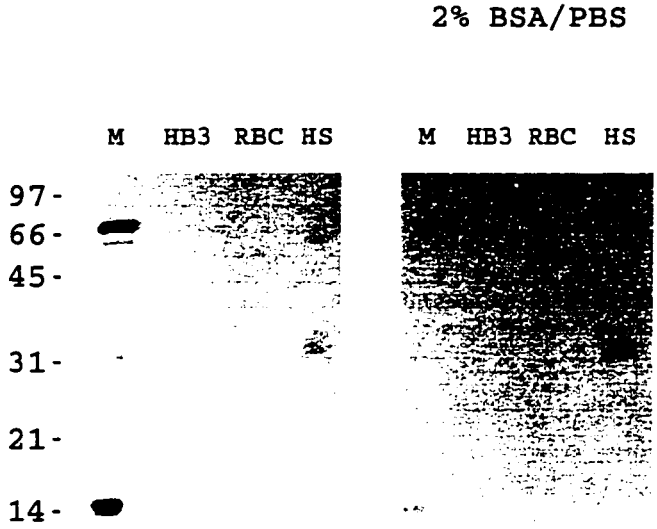


Nitrocellulose blocked with 5% BSA/PBS/0.5%Tween-20

FIGURE 4.15. Western blot analysis of HB3 parasite proteins using mouse anti-B2 antiserum diluted in PBS/ 0.5% Tween-20 or 2%BSA/2% human serum/PBS. Parasite-infected and fresh uninfected RBCs were lysed with 0.15% saponin/PBS (parasites remain intact; RBCs are lysed), washed 2 times with PBS by centrifuging at 1500 xg, and resuspended in SDS lysis buffer. The parasite (HB3) and red cell lysates (RBC), along with Human serum (Hu), were resolved by 12% SDS-PAGE (0.1 ng/lane), blotted to nitrocellulose, and blocked with 5% BSA/PBS. Mouse pre-immune and mouse anti-B2 antiserum were diluted 1:10,000 in either PBS/0.5% Tween-20, or 2% BSA/2% Human serum/ PBS/0.5% Tween-20 as indicated, and incubated for 30 minutes before being added to the nitrocellulose blots. Incubating the anti-B2 antiserum with BSA and human serum before applying it to the blot did not appear to the diminish binding to a 37.8 kDa protein in the parasite lysate, while the reactivity to other proteins (e.g. HSA in the human serum, BSA in the markers) is reduced.

A

Mouse pre-immune antiserum preincubated in:



B

Mouse anti-B2 antiserum preincubated in:

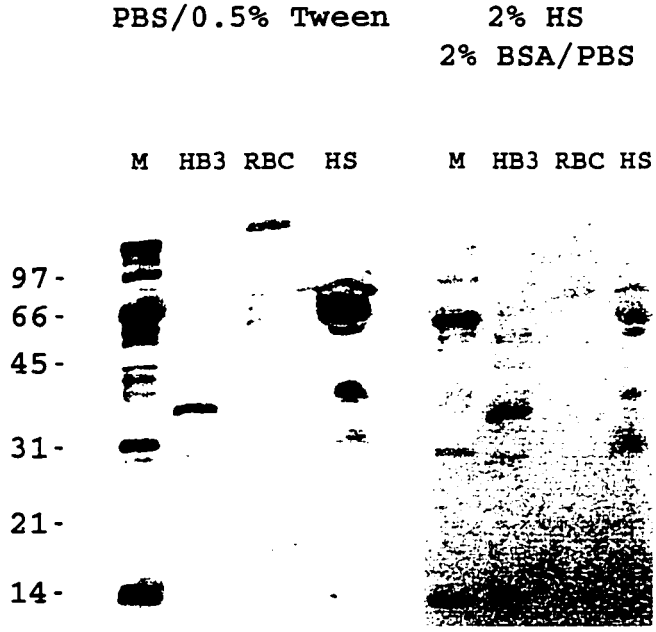
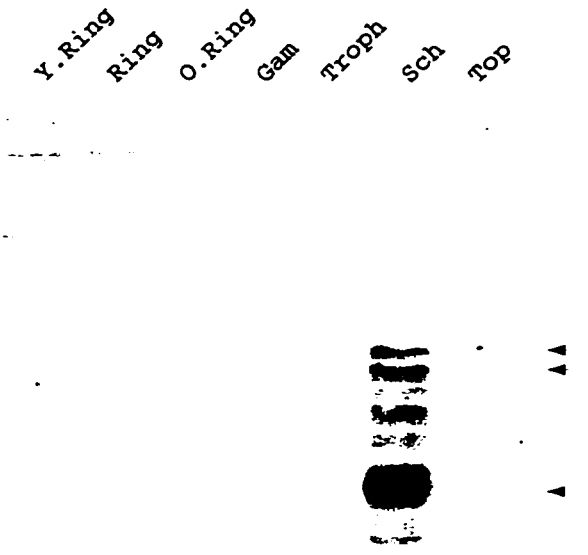


FIGURE 4.16. Western blot analysis of *P.falciparum* 3D7 proteins from different life cycle stages probed with mouse anti-B2 antiserum. Parasitized RBCs were separated on a 40/60/70/80/90% per-coll+4% sorbitol stepwise gradient as described in chapter 2. As determined by Giemsa staining, parasites from different life cycle stages formed discrete bands on the gradient: Y.Rings, young rings at the 80/90% interface; Rings, rings at the 70/80% interface; O.Rings, older rings within the 70% layer; Troph, trophozoites at the 60/70% interface; Gam, gametocytes within the 60% layer; Sch, schizonts at the 40/60% interface; Top, cellular debris at the top of the gradient. Also included in (B) is an uninfected RBC contol (RBC). Parasitized RBCs collected from different segments of the gradient were lysed in 0.15% saponin /PBS, washed in PBS, and pelleted (1500 xg, 4° C). Parasite pellets were lysed in SDS lysis buffer, proteins resolved by 12% SDS-PAGE, and Western blotted with mouse anti-B2 antiserum. Arrows indicate Pf12 protein migrating at 37 and 34 kDa with a major proteolytic band at 19 kDa. In (A), the proteolytic degradation of Pf12 is much more extensive than that seen in (B), where PMSF, leupeptin, aprotinin protease inhibitors were included, and harvesting, resolving, and blotting of parasite proteins was completed as quickly as possible (i.e. within approximately 6-8 hours).

A.


Y.Ring
Ring
O.Ring
Gam
Troph
Sch
Top
97-
66-
45-
31-
21-
14-

B.

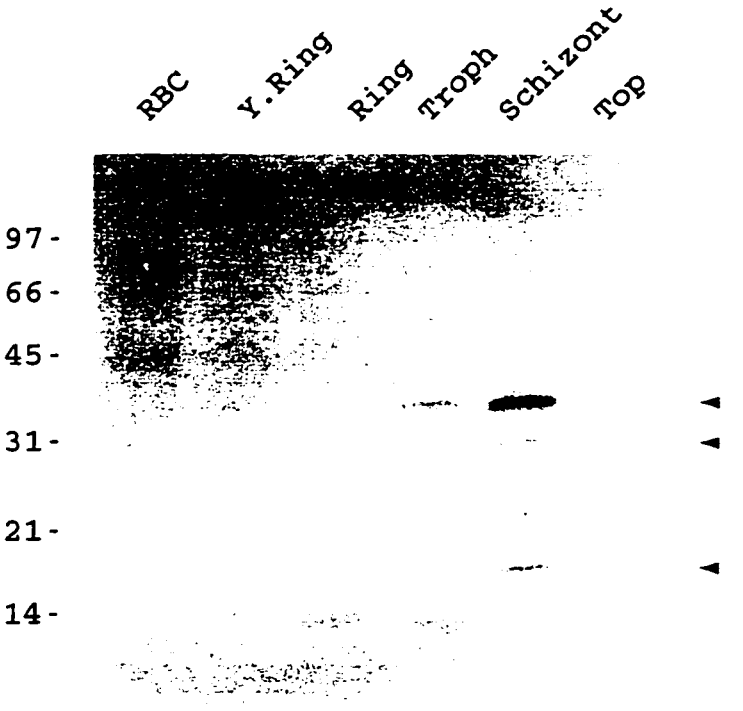

RBC
Y.Ring
Ring
Troph
Schizont
Top
97-
66-
45-
31-
21-
14-

FIGURE 4.17. Autoradiograph of immunoprecipitated metabolically labelled Pf12 protein from parasite lysates. P.falciparum HB3 parasites were grown for 18 hours in methionine-free RPMI supplementedwith 50 µCi of ^{35}S-methionine. Following incubation the parasites (P) and culture supernatant (S) were separated, solubilized, pre-cleared with Protein G-sepharose and incubated with either the pre-immune or anti-B2 antiserum. Antigen-antibody complexes were captured onto Protein G-sepharose beads, which were washed, and boiled in SDS lysis buffer prior to loading onto a 12% polyacrylamide gel. The gel was fixed (10% methanol, 10% acetic acid), dried down, and exposed to a phosphorimager plate. Autoradiographs were captured on a Fuji phosphorimager. Immunoprecipitated Pf12 is indicated by the arrow.

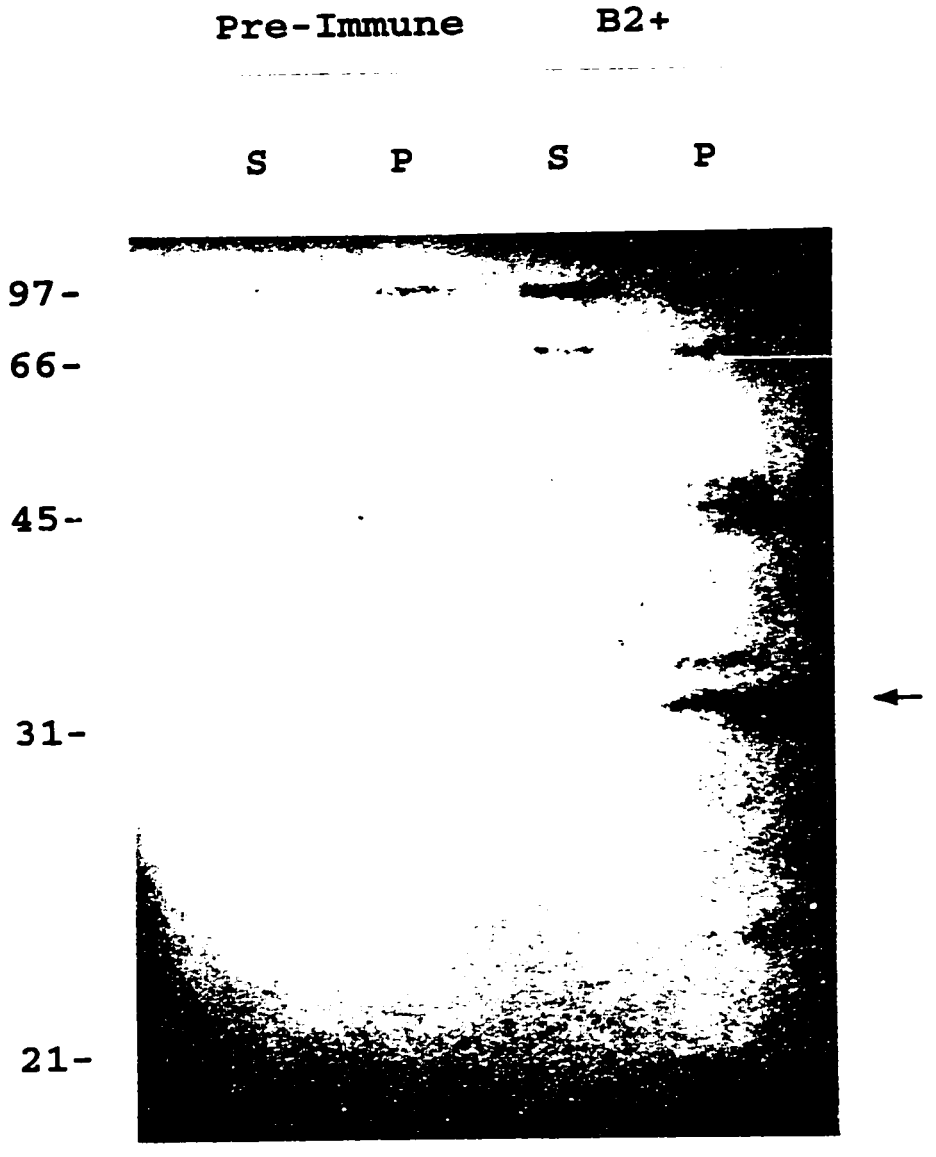

Pre-Immune
B2+
S
P
S
P
97-
66-
45-
31-
21-

FIGURE 4.18. Immunofluoresence staining of *P. falciparum* strain 3D7 schizonts with pre-immune mouse sera, and normal RBCs with mouse anti-B2 antisera. These representative experiments provide negative controls for all subsequent staining experiments. Human RBCs infected with *P. falciparum* strain 3D7 parasites were synchronized at the ring stage using percoll gradient centrifugation followed by sorbitol synchronization. Synchronous parasite cultures at the late schizont stage were smeared onto slides, air-dried, fixed in 100% acetone followed by 100% ethanol, and then stained with mouse pre-immune sera (1:100), and goat anti-mouse FITC Ab (1:200). Slides were visualized using a Leitz UV microscope (700X) under light (A) and fluorescence (B) conditions. Uninfected RBCs were fixed and stained in a similar fashion with mouse anti-B2 antisera, and viewed under light (C), and fluorescence (D) conditions.

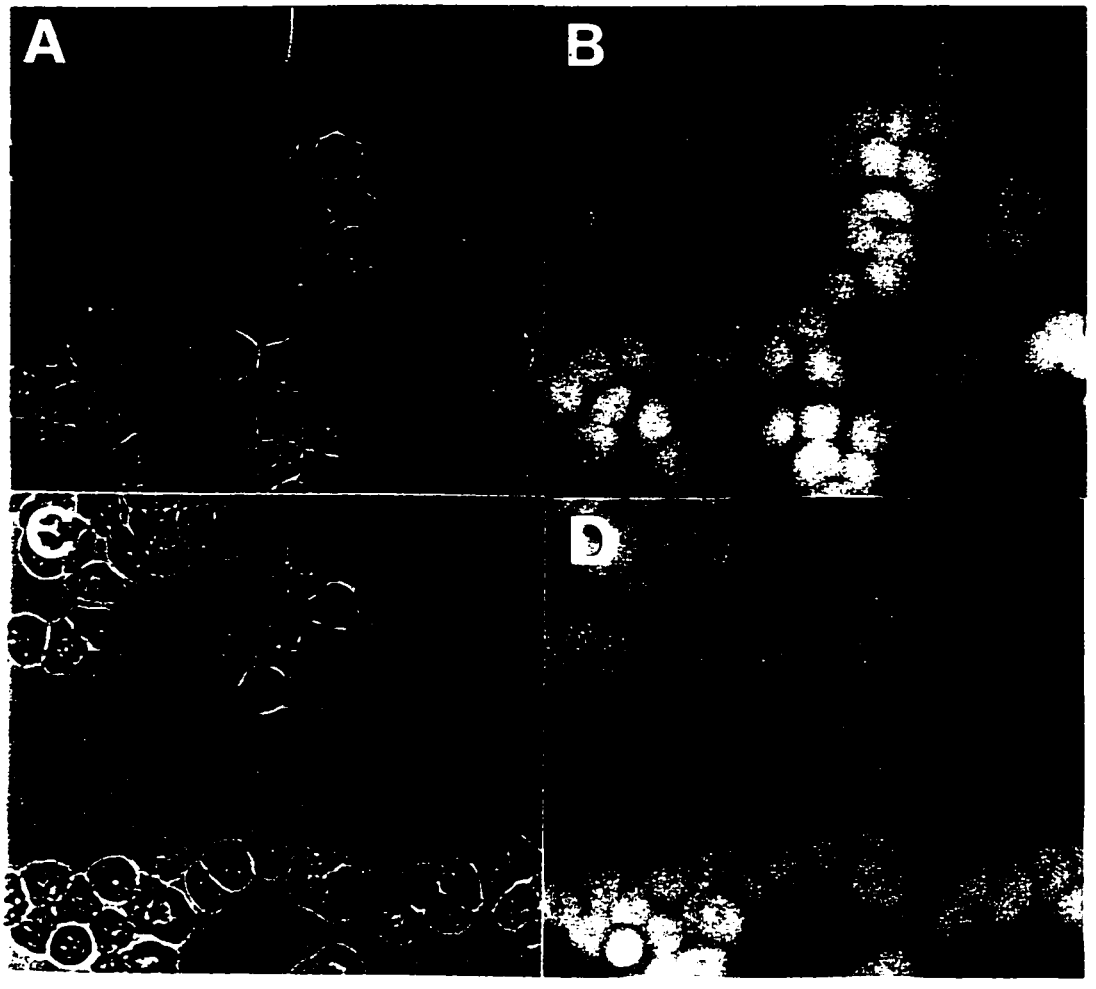

A
B
C
D

FIGURE 4.19. Immunofluorescence staining of schizont stage parasites with mouse anti-B2 antisera. Schizont stage 3D7 parasites were generated and stained with anti-B2 antiserum as described in figure 4.18. A,C,E; Light field conditions: B,D,F; UV illumination. Parasites can be identified under light field conditions by the presence of patches of membrane surrounding a dark circular residual body. Corresponding regions viewed under UV illumination appear bright green due to the specificity of the mouse anti-B2/FITC-Ab complex for the parasite (700X magnification). RBCs themselves show a degree of yellow autofluorescence due to hemoglobin. This was not washed away during antibody staining due to the short incubation and washing times used.

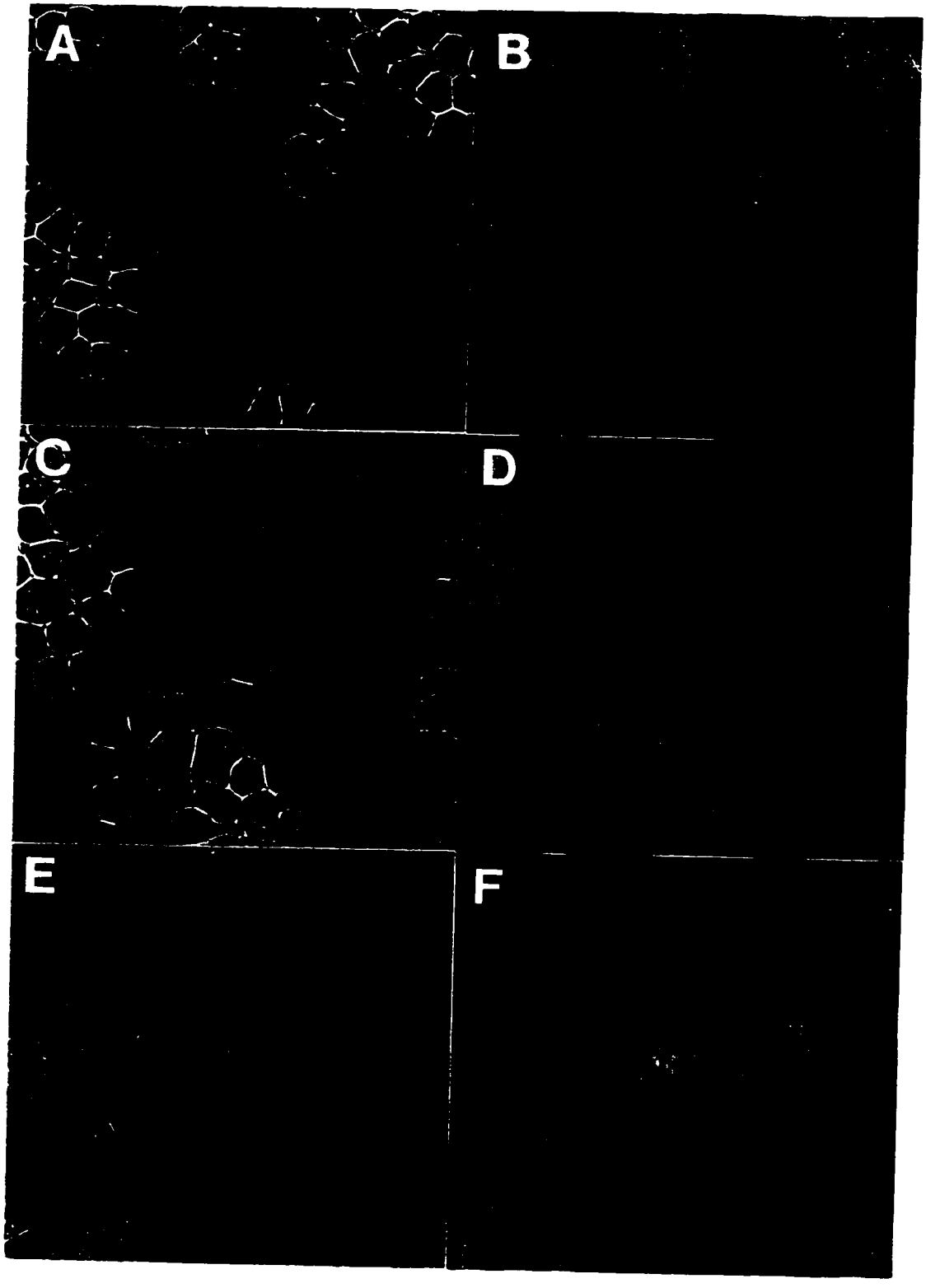

A
B
C
D
E
F

FIGURE 4.20. Colocalization of Pf12 with merozoites is observed by double staining with mouse anti-B2 antiserum and ethidium bromide. Schizont stage 3D7 parasites were generated and stained with anti-B2 antiserum as described in figure 4.18, with an additional ethidium bromide incubation (5 μg/ml) in order to stain parasite nuclei. A,C; Light field conditions: B, D; UV illumination. Parasites can be identified by the presence of residual pigment bodies under light field illumination, and as individual merozoite nuclei by speckled red fluorescence under UV illumination (700X magnification). Pf12 appears as green fluorescence in patches surrounding the merozoite nuclei (B and D). A single merozoite is attached to the surface in (D).

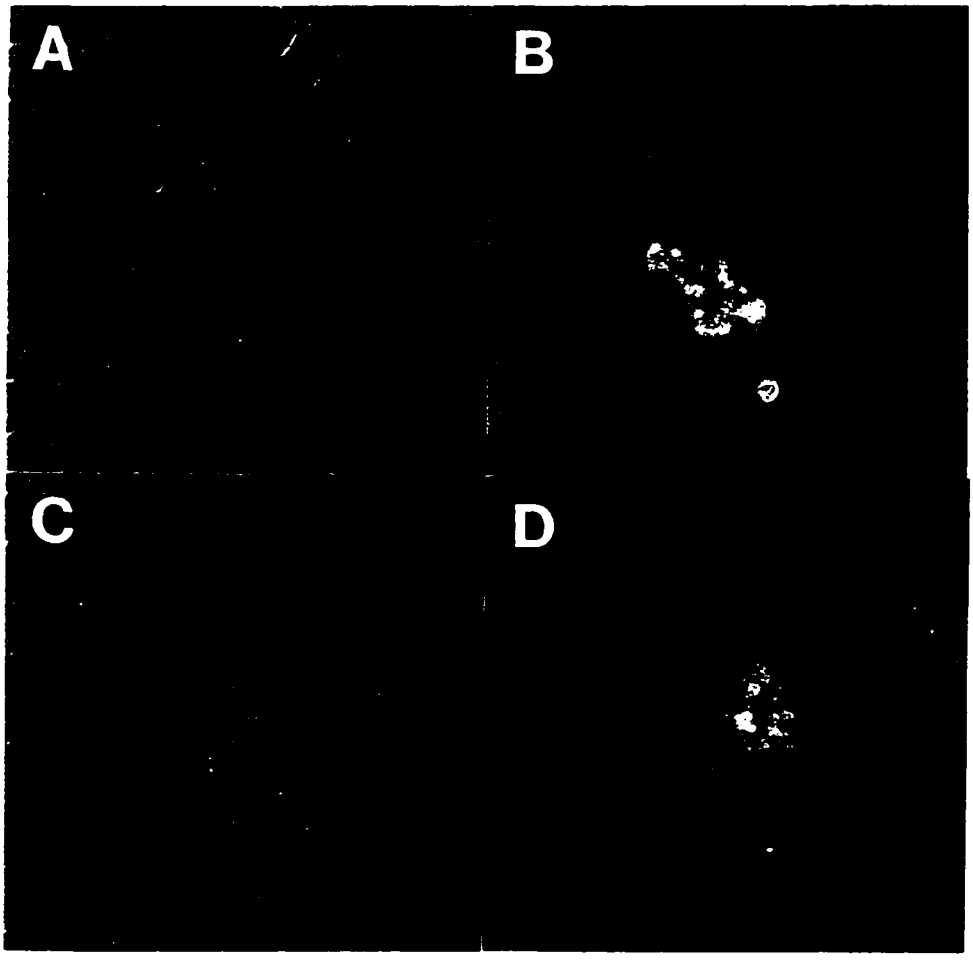
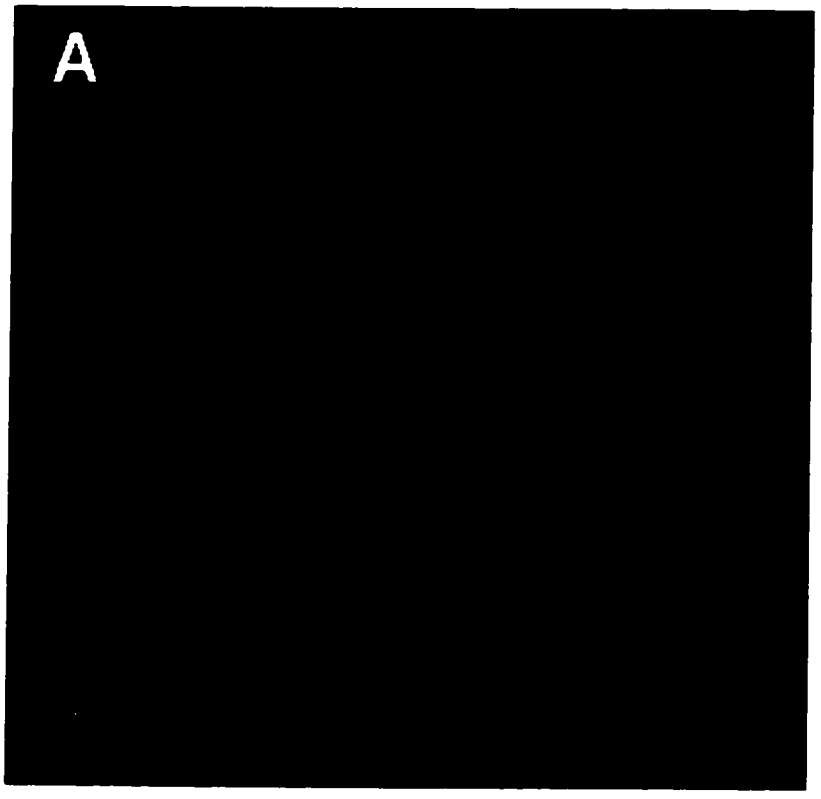

A
B
C
D

FIGURE 4.21. Two-color confocal microscopy control experiments using uninfected (A) and infected (B) RBCs. These experiments were conducted as controls for the confocal experiments in figure 4.22, comparable to the control experiments in figure 4.18. Uninfected and infected RBC smears were prepared, fixed and stained as described in figure 4.18 and chapter 2. **A.** Uninfected RBCs incubated with (1) RBC anti- 'e Ag' antiserum plus FITC-conjugated goat anti-human IgG (labels the RBC surface green) and (2) anti-B2 antiserum plus rhodamine-conjugated goat anti-mouse antibodies, as a control to measure the reactivity of anti-B2 for RBCs (bright red staining; no signal in this panel indicates no background reactivity of the antiserum). **B.** Control experiment similar to (A) using anti-'e Ag' to stain RBCs (green staining) and mouse pre-immune antiserum (bright red staining; no signal in this panel indicates no background reactivity of this serum). All antisera were used at a 1:100 dilution for these experiments. The faint reddish fluorescence seen in the interior of some RBCs is due to hemoglobin which has not been completely washed away from the specimen. RBCs were visualized using a Leica confocal microscope (2000X magnification).


A

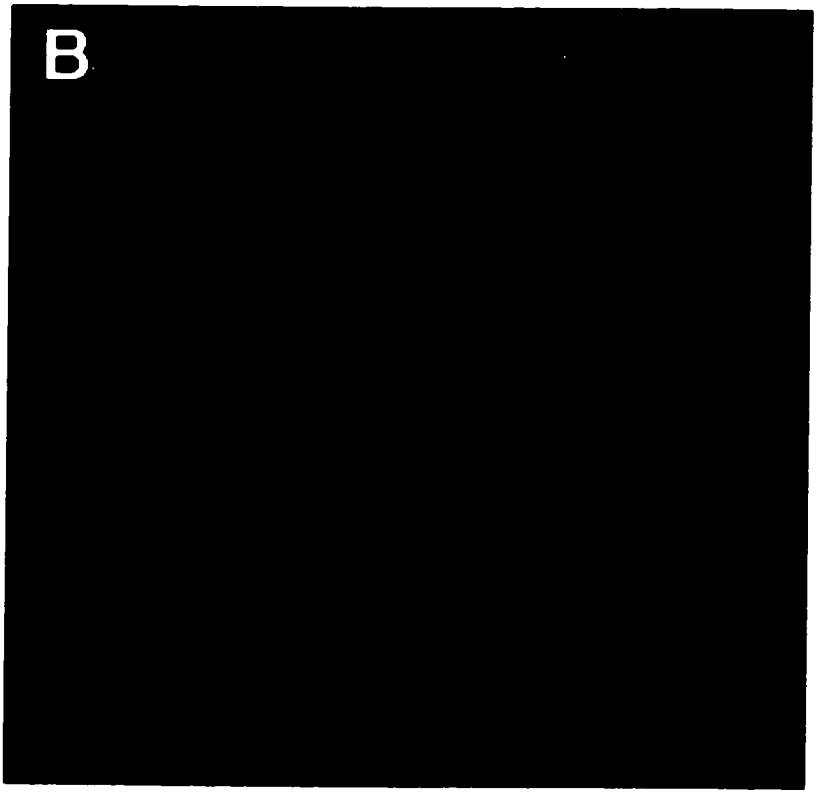
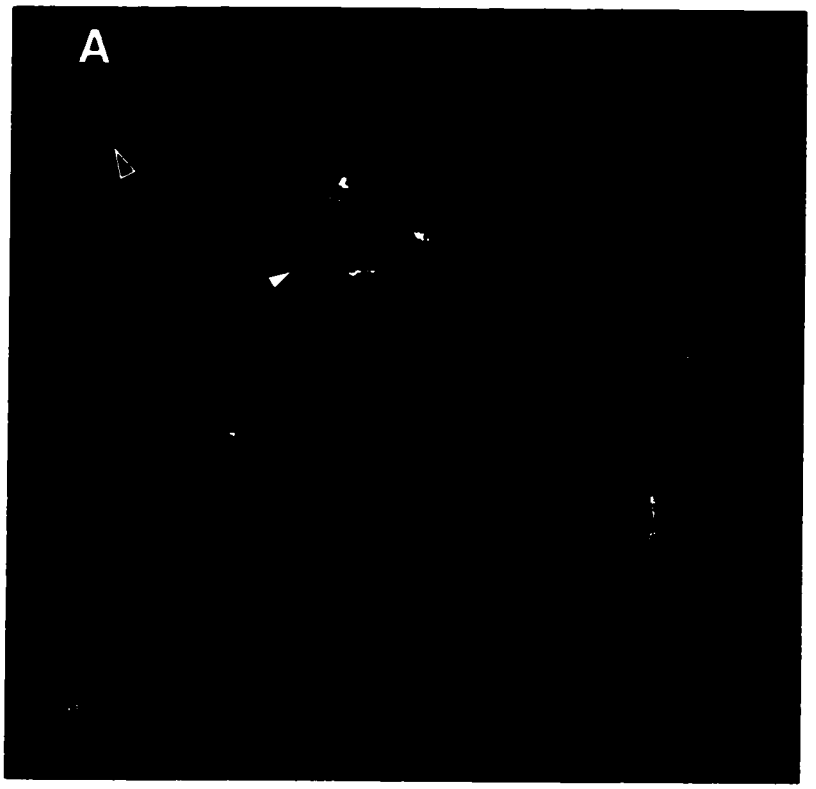

B

FIGURE 4.22. Two-color confocal microscopy shows that Pf12 is localized to the erythrocyte membrane in schizont infected RBCs as well as to the outer surface of the merozoite. Parasite cultures were synchronized, and schizont infected RBCs were harvested, fixed and stained using the methods described figure 4.18, except that Pf12 was visualized as red fluorescence by using mouse anti-B2 antisera in conjunction with rhodamine-conjugated goat anti-mouse IgG, and the RBC surface membrane was visualized as green fluorescence by using human anti-e Ag antisera in conjunction with FITC-conjugated goat anti-human IgG (see figure 4.21). Parasite specimens were viewed with a Leica confocal laser scanning microscope. **A.** The outer surface of both normal and infected RBCs appears as a somewhat granular thin green line, except where cells are in direct contact. Faint red hemoglobin autofluorescence is seen within the uninfected cells, whereas this is completely absent in the single schizont infected cells, (white arrowhead). Pf12 staining appears as bright red fluorescence following the surface contours of the infected cell, which is also outlined by the green staining. Pf12 surface staining is also seen for two free parasites (upper left corner, black arrowhead), most likely merozoites as judged by their size and shape. Except for one small remnant of erythrocyte membrane, little or no FITC staining co-localizes with Pf12 staining in this case. **B.** Pf12 staining (bright red) is seen on the surface of, and extending some distance from the surface of free merozoites (black arrowheads). Pf12 staining is also seen on the surface of the RBC (bottom left corner), perhaps a result of merozoite membrane 'sloughing' after invasion. No intact schizonts are seen in this field. These specimens were viewed with a Leica confocal microscope as in figure 4.21 (2000X magnification).


A

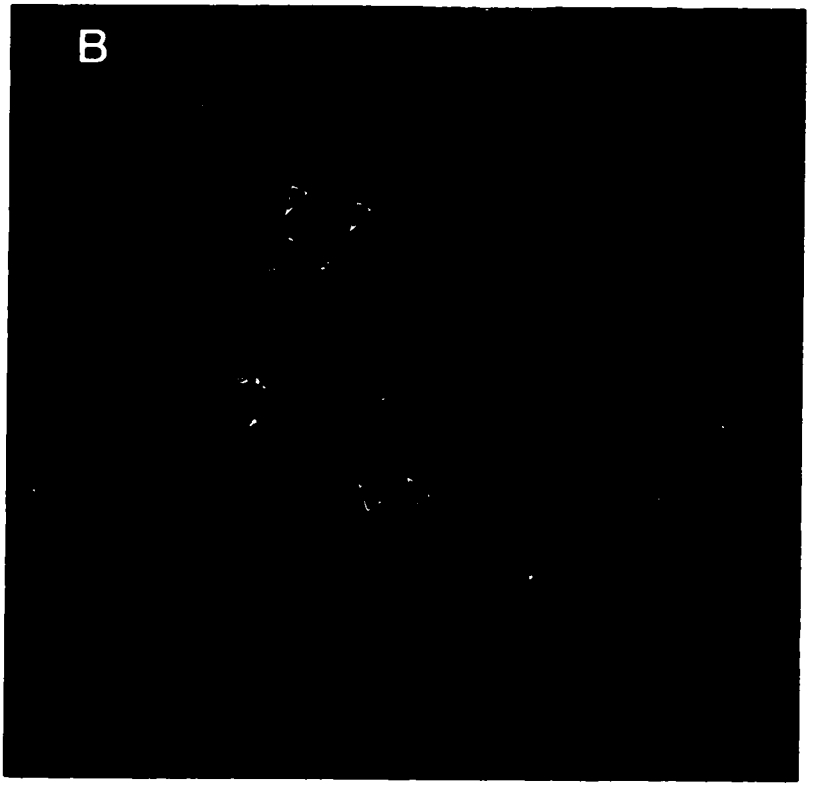
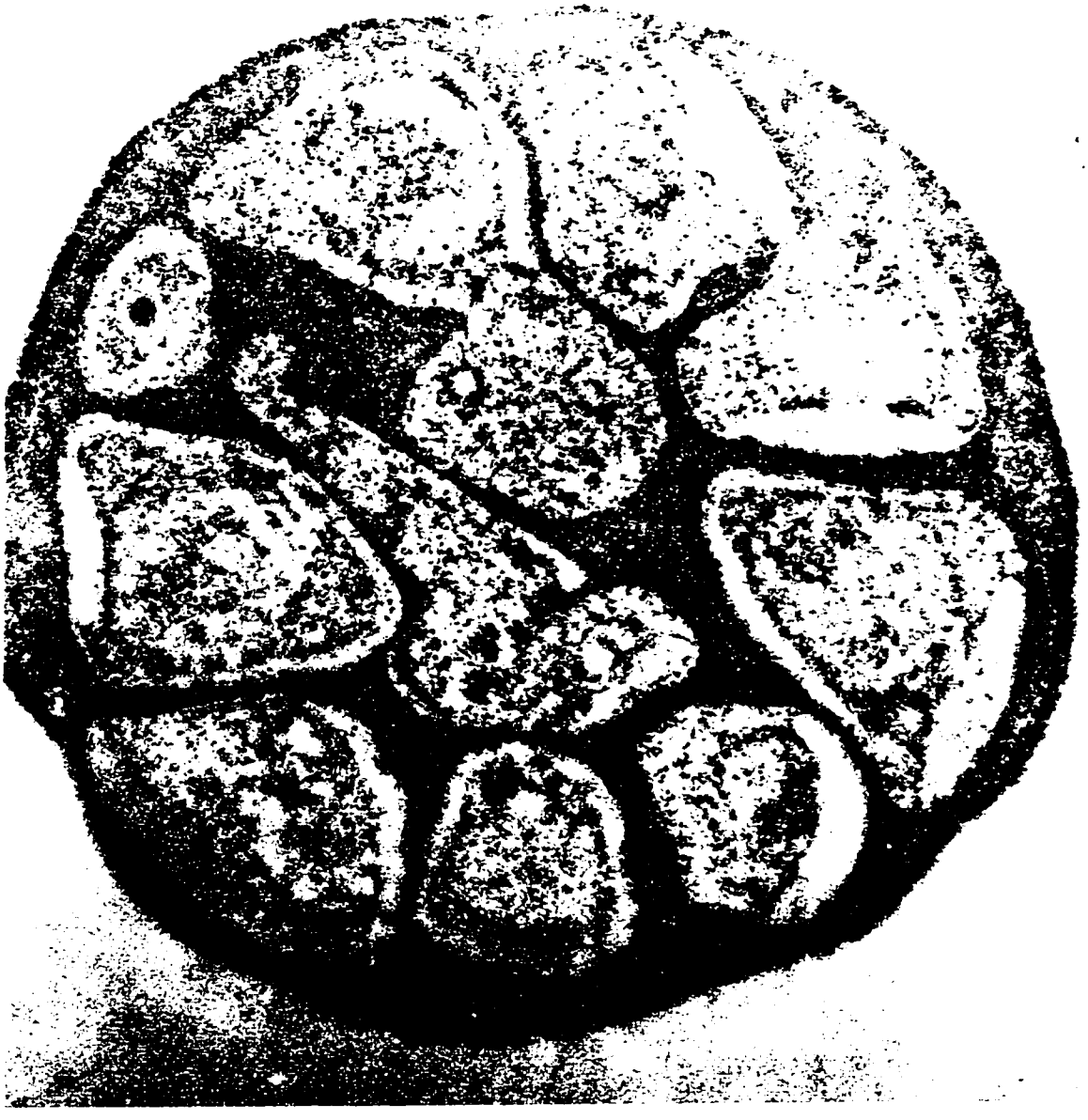

B

FIGURE 4.23. Immuno-electronmicroscopic analysis of *P.falciparum* strain 3D7 parasites using mouse pre-immune and anti-B2 antiserum. Sections of asexual and sexual stage parasites were prepared by Dr. M. Aikawa as described in chapter 2, and incubated with either mouse pre-immune or anti-B2 antiserum (1:100), followed by goat anti-mouse 10nm colloidal gold-conjugated Ab (1:100). **A.** Segmenter-stages stained with mouse anti-B2 antiserum. This is a mature asexual stage parasite which has undergone nuclear and cytoplasmic division to form a number of merozoites. The parasites label poorly with the anti-B2 antiserum, and no specific regions of labelling, either within the parasites or the parasitized RBC cytoplasm can be detected. (7000X magnification) **B.** Gametocyte-infected RBC and uninfected RBC stained with mouse anti-B2 antiserum. Although the sexual stage parasite is labelled by the anti-B2 antiserum, so is the uninfected RBC beside it. This reactivity of the anti-B2 antiserum with normal RBC components is reminiscent of Western blotting results described previously that reveal a reactivity towards a 65 kDa antigen of uninfected RBCs, possibly HSA. (12000X magnification) **C.** Gametes and uninfected RBCs stained with mouse pre-immune antiserum. As with previous Western blotting and immuno-fluorescence experiments, some weak reactivity is seen towards normal RBCs as well as to parasite components (12000X magnification).



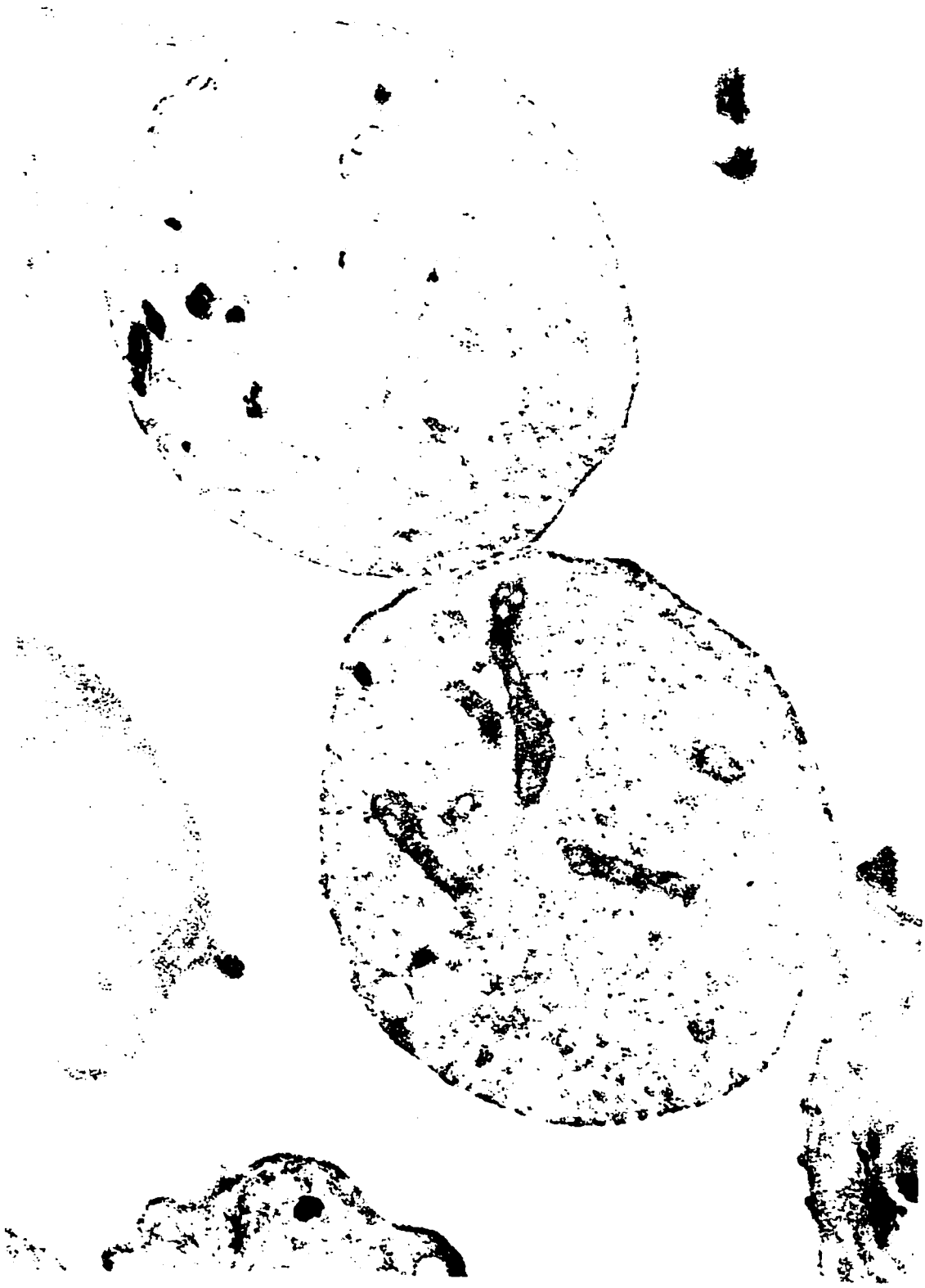


TABLE 4.2. Summary of immunofluorescence results obtained using mouse anti-B2 antiserum and various fixation methods on two different strains of *P.falciparum* *

Fixation Conditions	Mouse Pre-Immune	Mouse Anti-B2
No fixation mixed culture# (strains HB3,3D7)	-	-
100% methanol fixation§ mixed culture (HB3,3D7)	-	-
100% methanol + 1%P/0.2%G§ fixation mixed culture (HB3)	-	-
100% acetone, then 100% EtOH fixation§		
Mixed culture (HB3,3D7)	+	++++
Rings	+	+
Trophs	+	++
Schizonts (and released merozoites)	+	++++
Gametocytes	+	+
Schizonts@	-	++

P: Paraformaldehyde
G: Glutaraldehyde

+ Weak staining, ++++ Strong Staining
- No Staining

*Parasites from *in vitro* cultures were stained with mouse anti-B2 antiserum or pre-immune control sera under a number of different fixation conditions. All parasite specimens were viewed using a Leitz UV microscope (700X magnification). #Unfixed parasites were incubated with primary antibodies (1:200), followed by fluorescein conjugated goat anti-mouse secondary antibodies (1:200), and examined as wet mounts. §For methanol, paraformaldehyde/ glutaraldehyde (P/G) fixation and acetone/ ethanol fixation, air-dried thin blood smears of mixed cultures or percoll-separated schizonts, trophs, rings, and gametocytes were used, with the same staining protocol as above. @In this experiment only, both antisera were preabsorbed with normal RBC prior to IFA staining of parasites.

TABLE 4.3. Summary of the different fixation conditions tested in an attempt to visualize Pf12 by immuno-electronmicroscopy*

Fixation Conditions	Mouse Pre-Immune	Mouse Anti-B2
HB3, 3D7 Mixed Culture		
4%P/1%G	-	-
1%P/.5%G	-	-
HB3 Schizonts		
4%(P)/1%(G)	-	-
2%(P)/2%G)	-	-
HB3 Rings		
4%(P)/1%(G)	-	-
2%(P)/2%(G)	-	-
HB3 Schizonts Saponin lysed, fixed with.5%(P), incubated with Ab first, then fixed with 2%P/2%G	+/-	+/-
HB3 Schizonts Saponin lysed, incubated with Ab first, then fixed with 2%(P)/2%(G)	+/-	+/-
3D7 Schizonts Streptolysin O lysed, incubated with Ab, then fixed with 2.5%G	+/-	+/-
3D7 Schizonts Acetone fixed, incubated with Ab, then fixed with 2.5%G	+/-	+/-

P Paraformaldehyde ++++ Strong staining
G Glutaraldehyde - No Staining

*Mixed cultures were grown until 10% of RBCs were infected with parasites and then harvested, fixed and processed. Schizonts and ring stages were isolated on percoll gradients. The primary antibodies used in this experiment were mouse pre-immune control or mouse anti-B2 antiserum (1:200); secondary antibodies used were 10 or 20 nm gold-conjugated goat anti-mouse antibody (1:200).

CHAPTER V

ISOLATION OF ADDITIONAL NOVEL *P. falciparum* GENES BY COS CELL EXPRESSION CLONING AND ANTIBODY PANNING

A. Introduction and background

This chapter describes the final part of my thesis involving another logical avenue of pursuit stemming from the studies of Elliott and colleagues: the identification of new *P. falciparum* genes, and consequently new antigens using COS cell expression of *P. falciparum* cDNA and genomic libraries and antibody panning.

Two eukaryotic expression vectors, pJFE14 (Elliott et al., 1990) and pJFE14DAF, were constructed by Dr. J. F. Elliott specifically for the creation of *P. falciparum* genomic and cDNA libraries to be used in COS cell panning (Simmons, 1993). The vector pJFE14 was designed for the expression of parasite cDNA or genomic sequences encoding antigens containing native signal and anchor sequences. These parasite sequences seem to function properly in COS cells, as Pf12, which contains its own native 'parasite' signal and anchor sequences, it expressed at the surface of the cell. By contrast, proteins encoded by longer cDNAs cloned into pJFE14DAF are expressed on the surface of COS cells even though they may not have a native anchor sequence. Because many parasite genes are quite large, it is difficult to construct libraries containing complete gene sequences. Partial cDNA sequences that encode protein regions that are immunogenic can be cloned using pJFE14DAF, because these partial protein sequences will be anchored to the surface of COS cells as DAF fusion proteins. The promoter and vector backbone of pJFE14DAF (figure 5.1), are essentially the same as pJFE14 (see figure 5.2).

The key elements of pJFE14DAF include the SRalpha promoter responsible for the high levels of expression of target protein, and the DAF (Kinoshita et al., 1986) signal, which anchors the target protein on the surface of the COS cell. SRalpha, a fusion of the SV40 early promoter-enhancer with the R-U5 segment of the HTLV-1 LTR, gives a 100-

fold increase the level of target gene expression above that of the original promoter (Takebe et al., 1988). The expression of large amounts of target protein on the surface of COS cells is essential in promoting strong avidity interactions between the cells and the panning antiserum, effectively separating the COS cells expressing immunogenic target proteins from the rest of the population. The choice of the COS cell line itself is also essential in expressing large amounts of target protein. COS cells are an African Green monkey kidney cell line transformed with a replication defective SV40 virus (Gluzman, 1981). These cells produce large T antigen which promotes the replication of large amounts of plasmid containing an SV40 origin, resulting in a significant increase in the amount of target protein produced.

The DAF anchor is an essential part of this vector because when the upstream target DNA coding sequence is in frame with the vector sequence encoding the last 28 amino acids of DAF (and a target signal sequence is present), the target protein will be directed to the surface of COS cells. In order for this system to work, the parasite cDNAs must be fused in frame without the interruption of the cDNA stop codon. This vector is intended for cloning parasite DNA molecules that may only encode portions of genes. DAF protein itself is a glycophosphatidyl inositol-linked membrane protein that inhibits the complement cascade by binding activated complement fragments C3b and C4b (Kinoshita et al., 1986). Sequences fused in frame with the DAF anchor encode proteins that are directed to the endoplasmic reticulum where they undergo proteolytic cleavage of the anchor signal, attachment of GPI to the first residue of the anchor signal (serine 319 of DAF), and targeting to the cell surface. Because cDNA sequences can be in three different reading frames and two different orientations when inserted into the Bst XI cloning site of the vector, only one in six of these target DNAs will be fused in frame with the DAF anchor. The existence of parasite anchors as part of the inserted DNA fragment increases the chance of expressing target proteins on the surface of COS cells. Also it should be noted that this

vector allows for the cloning of immunologically relevant secreted proteins by anchoring them to the surface of COS cells with the vector-encoded DAF anchor.

P. falciparum genomic and cDNA libraries were used in the COS cell panning experiment. Genomic Fa3 and Fa4 libraries (cloned into pJFE14) consisting of large DNA fragments, not all of which may be parasite genes (i.e.. intron sequences may be present), were included because they had been successful in the first study by Elliott and colleagues. The expected sequences cloned from panning experiments using these libraries would be complete genes containing native signal and anchor regions. Although the presence of introns may prevent the expression of whole genes by this method, the low intron frequency in *P. falciparum* made the use of genomic libraries feasible. DAF B+ cDNA libraries (cloned into pJFE14DAF) prepared by Dr. Elliott were used because they contain only gene sequences that are expressed by the parasite. Sequences cloned from these libraries could be expected to encode either secreted or anchored proteins, or portions of those proteins. These sequences required only a secretion signal to direct the target protein to the endoplasmic reticulum, the DAF signal ensuring the protein was anchored on the surface of the COS cell. Thus immunogenic domains of proteins encoded by very large genes could be recovered using this library even though the complete cDNAs might not be present. After identifying regions of genes that were enriched by panning, it would be possible to go back to the original cDNA or even genomic library to recover the complete sequence using a labelled probe and hybridization techniques. The use of the two different kinds of DNA libraries ensured that both partial and complete parasite genes could be recovered that express either naturally anchored or secreted immunogenic proteins.

The COS cell panning process is described in figure 1.1., and in section B of the introduction. It involves an initial electroporation of COS cells with *P. falciparum* genomic and cDNA libraries followed by panning COS cells over pooled immune serum from individuals frequently infected with malaria. After three rounds of panning transfected COS cells, the pool of remaining cDNAs is highly enriched for those encoding parasite

antigens. This pool could consist of the antigens necessary to stimulate protective responses in a vaccine.

B. Results

Transfection of *P. falciparum* genomic and cDNA libraries into COS cells by electroporation

The first step in the panning experiment was the electroporation of COS cells with the *P. falciparum* DAF B+ cDNA library cloned into vector pJFE14DAF, and the Fa3+/ Fa4+ genomic libraries cloned into vector pJFE14 created by Dr. J.F. Elliott (Elliott et al., 1990). The DAF B+ cDNA library (160 µg) and the Fa3+/Fa4+ genomic libraries (40 µg) were transfected into $6.7x10^7$ and $1.3x10^8$ COS cells respectively. These cells were grown at 37°C for three days before incubation with Papua New-Guinean pooled human antiserum (PNG) and panning over goat anti-human antibody coated plates.

Restriction endonuclease analysis of separate cDNA and genomic clones from the final panning pool.

After three successive rounds of panning, the final pool of cDNA and genomic clones, called the 'Hirt', was transformed into *E. coli*, and 17 separate Hirt clones were examined by restriction analysis and agarose gel electrophoresis (figure 5.3). Clones that were relatively large and equal in size were favored as these were most likely to be the panning-enriched cDNAs encompassing the entire coding sequence of a gene. Of the 17 clones studied five groups of interest emerged (table 5.1): (clone 2, 17; 1.4 Kb), (clone 4, 13; 0.7 Kb), (clone 6,11; 1.3 Kb), (clone 7, 10; 2.6 Kb), and (12, 16; 0.9 Kb). The 5 Kb bands of clones 1, 2, 8, 17 were shown by further restriction analysis to be due to incomplete digestion. In addition, Clone 8 was shown to be a mixed population of different clones, probably generated by cross-contaminating two colonies from the original

transformation outgrowth plate. Clones 2, 4, 6, 7, 8, 12, and 18 were selected for further study.

Southern Blotting analysis of clones 2 and 7

Xho I-digested DNA fragments from clones 2 and 7 were used as probes in Southern blotting experiments to determine if any of the other clones contained similar DNA sequences. In the first experiment, the clone 7 probe cross-hybridized strongly with clones 7 and 10, noticeably with clones 8 and 17, and weakly with clone 2 (figure 5.4). The identical hybridization band profiles and signal intensities of clones 7 and 10 suggested that these were identical cDNA clones, whereas the profiles of clones 2, 8, and 17 suggested a significant, but lesser degree of amino acid homology to clone 7. Southern blotting experiments using clone 2 as a probe confirmed the presence of shared sequences by clone 2 and 17, although no cross-hybridization was seen with clones 7, 8 or 10. In this experiment the clone 2 probe did not hybridize as strongly with clone 17 as it did with itself suggesting that although these clones were of similar size and shared a significant sequence homology, they were probably not identical cDNAs.

Analysis of Hirt Clones by Immunofluorescence

The Hirt clones 2, 4, 6, 7, 8, 12 and 18 were also analyzed by transfection into COS cells and immunofluorescence using the panning antiserum. The panning antiserum stained COS cells transfected with clones 7, 8 and 18 strongly, and clone 2 to a lesser degree (figures 7.5 and 7.6). COS cells transfected with clones 4, 6, and 12 stained weakly. Based on staining with panning antiserum, size, and frequency of representation in the studied pool of 18 clones (size matching and cross-hybridization), clones 2, 7, and 18 were chosen as the main candidates for DNA sequencing and database comparisons.

Clone 2 DNA sequence has significant homology to the heat shock protein hsp90

Clone 2 was sequenced using dye-labelled dideoxy terminators or primers by the standard Sanger method adapted for the ABI 370A automated DNA sequencer (described in chapter 2). Clone 2 was sequenced directly, or subcloned by digesting with Xho I (which cuts outside of the insert sequence, in the pJFE14DAF polylinker region) and ligating into similarly cut pBluescript SK- and KS-. Altogether, approximately 460 bases of 5' and 960 bases of 3' DNA sequence were obtained (figure 5.7). An ORF was identified in the 3' sequence of clone 2 which was shown to be in frame with the downstream DAF anchor sequence, confirming that the protein was indeed anchored to the surface of COS cells via fusion to this region (figure 5.8). The clone 2 open reading frame has a strong homology to hsp 90 proteins such as *P. falciparum* Pf hsp86 (Su and Wellems, 1994), Pf hsp90 (Bonnefoy et al., 1994), human hsp90α (Hickey et al., 1989) and hsp90β (Pebbe et al., 1987), and *C.familiaris* grp94 (Cala and Jones, 1994) (an endoplasmic reticulum targeted hsp homolog). This homology is shown in a multiple alignment with these sequences using the CLUSTALW tool (figure 5.8) (Higgins et al., 1998). The sequence homology to Pf Hsp 86 is 60%, it extends over a large region of the aligned sequences, and it is comparable to homologies of the various hsp90 genes of other species. This clone was not pursued any further, because it had already been characterized to some extent by other investigators (Bonnefoy et al., 1994).

Clone 7 DNA has significant homology to the *P. falciparum* sporozoite and liver stage antigen, SALSA

Clone 7 was also sequenced in the same fashion as clone 2, using both the original clone and the subcloned cDNA (Xho I digested) in pBluescript SK- and KS- as a template. Approximately 1150 bases of 5' DNA and 670 bases of 3' cDNA sequence were obtained (figure 5.6, and figure 5.9 for sequence). The 5' sequence has a high frequency of A and

T residues, and contains many stop codons. From position 634 to 994 a open reading frame (no methionine codon is present) was discovered that has highly significant homology to SALSA, the *P. falciparum* sporozoite and liver stage antigen (Sanchez et al., 1994) (see figure 5.10). The 3' sequence is also very AT rich, and basic local alignment search tool (BLAST) database searches revealed no significant homologies to any proteins sequences (Altschul et al., 1998). The sequence of the central region of clone 7 (approximately 800 bases) remains to be sequenced.

Clone 18 DNA sequence has significant homology to MESA, the mature erythrocyte-infected surface antigen

Clone 18 was sequenced in the same manner as the previous two clones. Approximately 480 bases of 5' sequence, and 500 bases of 3' sequence was obtained for this clone (figures 7.6). An region (position 828-1194; see figure 5.11) was identified in the 3'sequence that had a strong homology to one of the amino acid repeat motifs of MESA, or the mature erythrocyte-infected surface antigen (Coppel et al., 1986a). An alignment between the clone 18 ORF and MESA, shown in figure 5.12., reveals a homology of approximately 60% for these two sequences, indicating that clone 18 was most likely Pf MESA. This clone was not pursued any further because it had been characterized previously.

C. Discussion

Using COS cell expression of *P. falciparum* cDNA and genomic libraries, and antibody panning, I successfully isolated separate *P. falciparum* DNA sequences encoding proteins with homology to three previously characterized *P. falciparum* antigens: Pf hsp 90, SALSA, and MESA. Pf hsp 90 and MESA had already been cloned and the antigens characterized; the SALSA gene has not been fully characterized.

Clones 2, 7, and 18 were picked as promising candidates from a pool of 17 clones because they were relatively large (thus capable of containing an entire gene), and because the PNG panning antiserum brightly stained COS cells transfected with these clones, suggesting that the inserts encoded parasite proteins recognized by the antiserum. Clones 7, and 18 gave the best surface immunofluorescence profile, and clone 2 gave a weaker, diffuse yet significant signal.

I conducted the Southern blotting experiment to determine whether clones 2 and 7 had been enriched by the panning process. The insert profile in figure 5.3 suggested that these clones (and a number of other clones, see table 5.1), had been enriched because one other clone of similar size (for both 2 and 7) was present in the pool. The Southern blotting experiment was a fast way of determining if the inserts of similar size were actually homologous (faster than sequencing all of the inserts). The clone 7 probe hybridized with the clone 7 and 10 DNA on the blot, as expected. In addition, the clone 7 probe hybridized to a lesser degree with a DNA fragment of equal size in clone 8 (possibly clone 7 was one of the contaminating plasmids in the clone 8 mixed population), and the 2.0 and 5.0 Kb fragments of clones 2 and 17. The high degree of AT repeats in clone 7, described in the results section, may be responsible for the cross-hybridization with clones 2 and 17. Polyadenine molecules were not included in this experiment, which would have minimized the amount of hybridization between parasite AT-rich sequences. The ^{32}P-labelled clone 2 DNA probe hybridized with the 5.0 and 2.0 Kb fragments of the clone 2 partial digest on the blot, and only faintly with the clone 17 DNA, suggesting that although the two inserts were of similar size, they were not homologous. Interestingly, the clone 2 probe did not hybridize with the clone 7 DNA, even though the clone 7 probe had weakly hybridized with the clone 2 DNA. This may be due to the increased amount of AT-rich regions in the clone 7 probe, but not the clone 2 probe.

The partial sequences obtained for clones 2, 7, and 18 were analyzed using DNA Strider, and compared to Genbank database entries using BLAST (Altschul et al., 1998).

Although complete sequences for each clone were not obtained, enough information was generated to determine if each clone warranted further study. I developed an overall picture of the orientation of the sequences in pJFE14 or pJFE14DAF, and then I searched each sequence for an open reading frame in the proper orientation (downstream from the SRalpha promoter, upstream of the SV40 Poly A sequence). I used this sequence to conduct a BLAST database search for proteins with significant amino acid homology.

An ORF found in the clone 2 sequence showed significant homology to *P. falciparum* hsp90 heat shock protein. Two groups have identified nearly identical *P. falciparum* hsp90 genes, called hsp86 and hsp90 (Su and Wellems, 1994; Bonnefoy et al., 1994). The hsp90 family is a group of phylogenetically conserved stress proteins that function as molecular chaperones (Wiech et al., 1993), possess ATPase activity (Nadeau et al., 1993), associate with protein kinases (Brugge et al., 1981), and associate with cytoskeletal proteins such as tubulin and actin (Lindquist and Craig, 1988; Koyasu et al., 1986). In parasites with hosts that have different core temperatures (such as mosquitos and man), heat-shock proteins that have specific functions may be induced as they are transferred from one host environment to another. In the case of *P. falciparum*, which induces fever and the production of potentially toxic molecules by host immune cells (such as reactive oxygen and nitrogen intermediates), heat shock proteins may be important in prolonging parasite survival. After identifying a number of hsp90 proteins having homology with clone 2 by BLAST database searches, I used the CLUSTALW program (http: //www2.ebi.ac.uk/ clustalw/, European Bioinformatics Institute) to perform a multiple alignment of these sequences. The results of this alignment show a number of regions where the clone 2 sequence the five hsp90 proteins have both strong homology (KLYVRRV and GVVDS D/E DLPLN V/I SRE), and no homology such as the 30 residue insertion present in clone 2, but absent in the other proteins. The homology between clone 2 and Pf hsp90 was the strongest seen for all the searches completed in this chapter; consequently no further studies on clone 2 were undertaken.

It is interesting that the hsp90 sequence was cloned by panning, considering that this protein does not contain an N-terminal hydrophobic signal sequence (Bonnefoy et al., 1994). It still seems to be directed from the interior of the parasite to the parasitophorous vacuole, thus this protein may contain a novel parasite specific targeting signal. This parasite signal does not seem to function as efficiently in COS cells (shown in figure 5.5); the clone 2-transfected cells are stained diffusely with the PNG sera, as opposed to the distinct punctate surface staining seen with COS cells transfected with clone 7.

Antibody responses to Pf hsp90 in monkeys vaccinated with the protein have been correlated with protection from *P. falciparum* challenge. It is possible that this protein is accessible to the immune system either by pre-mature lysis of the parasites, release of the protein on merozoite burst, or via the parasitophorous duct (Pouvelle et al., 1991). The precise mechanism of the protective response to heat shock proteins is not known, but it may be that neutralization of these proteins may make the parasite more vulnerable to host defense mechanisms, or may disable chaperone functions of the protein that help fold and target other parasite proteins to specific cellular locations.

The sequence obtained for clone 7 was very AT rich, and contained numerous stop codons. The longest open reading frame detected in the 5' sequence of clone 7 (base 634-747), encodes a protein that is nearly identical in sequence to the *P. falciparum* sporozoite and liver stage antigen, SALSA (Sanchez et al., 1994). A portion of SALSA gene was cloned by screening *P. falciparum* genomic libraries in λgt11 with human polyclonal antiserum having strong specificity for sporozoites on 'wet' immunofluorescence assay, but weak reactivity to previously characterized antigens such as CSP, the major sporozoite surface protein, and LSA-1, a liver stage protein. Only a portion of this sequence has been published, the exact sequence shown in figure 5.11, plus an N-terminal arginine, which is not present in clone 7. SALSA has been identified by Western blotting and immuno-electron microscopy experiments (using human affinity purified antibodies, and antiserum raised in chimpanzees against the λgt11 protein) as a 70 kDa protein expressed on the

surface of sporozoites, and in the parasitophorous vacuolar space of mature liver stage parasites, possibly on the merozoites. The work of Bottius and colleagues (Sanchez et al., 1994) showed that SALSA has at least two distinct B-cell epitopes (i.e. not cross-reactive epitopes, a phenomenon seen in many other malaria antigens), and that a high proportion of individuals living in regions of low malaria transmission (Senegal) have antibodies that recognize these epitopes. This also seems to be true of the PNG sera used in the panning experiments in this chapter. The homology between clone 7 and SALSA was discovered only recently. No methionine start codon was identified for clone 7 (or for SALSA, either). This could be explained if clone 7 is a genomic DNA sequence inserted in pJFE14 (the identity of the vector and hence the nature of the insert, cDNA or genomic DNA, has not been identified), and the open reading frame I have identified is actually the second exon of the protein. Analysis of upstream sequence in all three frames did not immediately reveal strong candidates for exon 1 of this protein. Exon 1 may still reside in the region of the DNA remains to be sequenced. In the 670 bp of sequence obtained from the 3' end of clone 7 (also very AT rich, and containing many stop codons) no ORFs were detected on analysis with DNA Strider, and no sequences showing homology to SALSA or other proteins on BLAST database search.

With the clone 18 alignment I was more successful in finding an open reading frame and homologous protein sequences in the database. The ORF in the 3' DNA sequence showed distinct homology to *P. falciparum* mature-parasite-infected erythrocyte surface antigen, MESA (Coppel et al., 1986a), also known as Pf EMP2 (Howard et al., 1987). MESA is an antigenically diverse, 200 kDa polymorphic protein that associates with the RBC membrane skeleton band 4.1 protein (Lustigman et al., 1990). MESA is not required for knob formation or cytoadherence of infected RBCs to C32 melanoma cells in *in vitro* sequestration studies (Petersen et al., 1989), but cloned parasite lines that do not express MESA cytoadhere to a lesser extent than MESA positive parasite lines (Magowan et al., 1988). MESA may play a role in stabilizing the RBC membrane or parasite protein

complexes within the membrane. Although MESA is not exposed on the surface of parasitized RBCs, it does contain a number of repeat motifs, which may elicit non-protective antibody responses (i.e. the smokescreen effect, described in chapter 1) if the protein is exposed to the immune system (after merozoite burst).

Clone 18 has significant homology to one of the repeat regions of MESA, the ESKETG sequences. COS cells transfected with clone 18 were stained brightly with the PNG antiserum, indicating that this protein was indeed immunogenic. Because MESA had been cloned and characterized previously, it was not studied further.

The main difficulty that arose from the COS cell panning experiments of this chapter was not in isolating enriched cDNAs, but in finding novel ones within this pool. The ideal candidate sequence would have been an open reading frame having no strong homology to *P. falciparum* sequences (ie. sequences had already been cloned), or even any other DNA sequences in the database, for that matter. Significant homologies with proteins from other species would have suggested a possible function for the novel parasite protein, but this information was unnecessary.

A better chance of success might have been achieved by screening the reactivity of PNG antiserum to a larger number of individual panning clones transfected into COS cells using immunofluorescence (eg. 96 vs. 17 studied in this chapter). This makes sense because many clones that are selected may not only have been previously discovered, but of the novel clones perhaps only a fraction might encode proteins that elicit effective immune responses to infection. If the 'smokescreen' hypothesis of immune evasion is correct, many antibody responses to parasite proteins may be directed to irrelevant 'decoy' proteins, which could be isolated along with 'relevant' antigens in panning experiments (although the PNG sera, a pool of serum from immune adults, should have a relatively low titer of antibodies with cross-reactive specificities).

In addition to surveying more clones, panning experiments might be improved by using the 'differential' antiserum, such as the kind used to isolate SALSA described earlier.

These antiserum was selected because of its high specificity for sporozoites, but not previously cloned antigens such as CSP and LSA-1. A similar type of experiment could be conducted using antiserum showing merozoite surface staining, or contributing to ADCI activity (described by in the introduction, (Lunel and Druilhe, 1989), but not showing reactivity to previously cloned antigens. If this differential antiserum could not be found in natural populations, it could be created by removing antibodies specific for a number of previously cloned antigens. This may be a somewhat laborious task, but I believe the key to continued success with COS cell expression and antibody panning, short of increasing the number of clones studied, is to use a more refined tool for rescuing the cDNA clones, such as a pool of antiserum with restricted specificity.

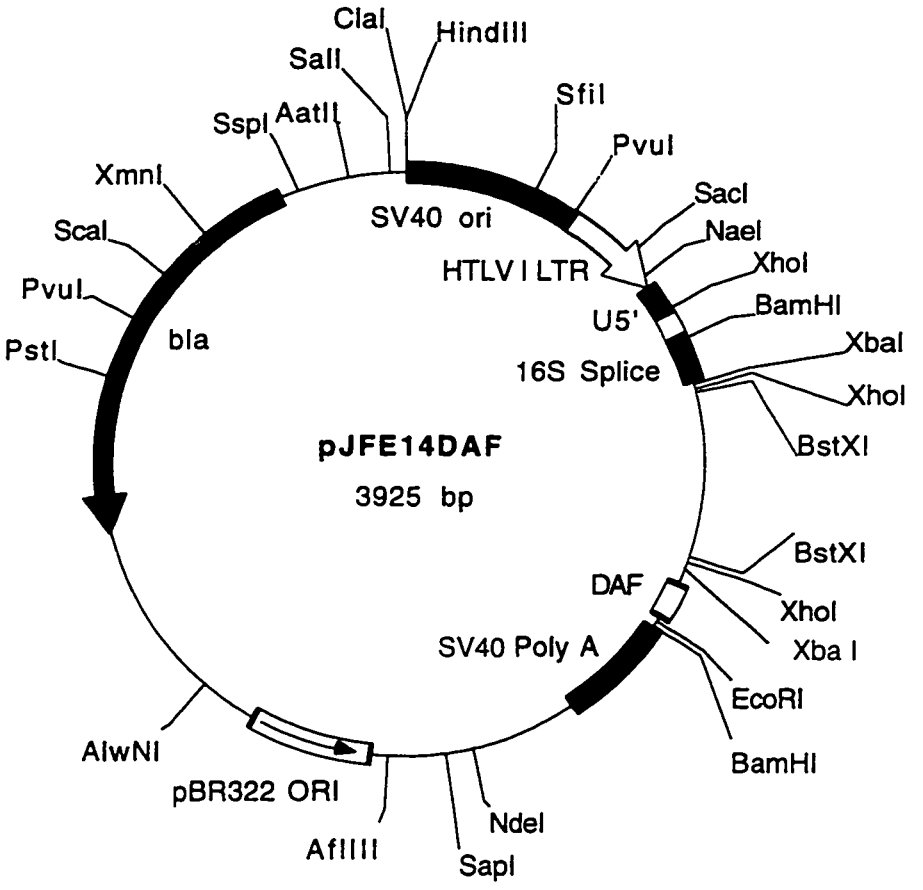


FIGURE 5.1. Map of pJFE14DAF, a plasmid vector for use in COS cell expression cloning. Features of this vector include: an SRalpha promoter containing an SV40 early gene promoter/enhancer unit, partial R-U5' sequence from the HTLV-1 LTR, and SV40 16S late-gene splice donor and acceptor sites; Bst XI sites for efficient cloning of cDNA or genomic fragments; the decay accelerating factor (DAF) anchor signal for expression of any potential in-frame secreted coding sequence as an anchored protein on the surface of COS cells; an SV40 polyadenylation signal; pBR322 origin and beta-lactamase gene. pJFE14 (not shown) is similar except that the DAF anchor is absent.

A. DNA sequence of the polylinker from pJFE14

```
5'TCTAGAGATCCCTCGACCTCGAGATCCATTGTGCTGGC--------------360bp'stuffer'-------------
GCCAGCACAATGGATCTCGAGGGATCTTCCATACCTACCAGTTCTGCGCCTGCAGGTCGCGGCCGCGGGAGGCCGAATTC3'
```

B. DNA sequence of the polylinker from pJFE14DAF

```
5'TCTAGAGATCCCTCGACCTCGAGATCCATTGTGCTGGC-------------360bp'stuffer'---------------
GCCAGCACAATGGATCTCGAGGTCTAGAGCCAAATAAAGGAAGTGGAACCACTTCAGGTACTACCCGTCTTCTATCTGGGCA
CACGTGTTTCACGTTGACAGGTTTGCTTGGGACGCTAGTAACCATGGGCTTGCTGACTTAGGCATCGAATTC3'
```

C. C-terminal sequence of any DAF-fusion protein encoded by pJFE14DAF

?LEHNGSRGLE PNKGSGTTSGTTRLLSGHTCFTLTGLLGTLVTMGLLT
(amino acids encoded by the BstXI linker and by restriction sites of the polylinker are underlined; the remaining amino acids are encoded by the DAF 'anchor' sequence)

With respect to DNA, the sequence would read:

```
-----------------------------sequence of the DNA insert--------------------------------, then
CC CTA GAG CAC AAT GGA TCT CGA GGT CTA GAG CCA AAT AAA GGA AGT GGA ACC ACT TCA GGT ACT ACC CGT
 ?   L   E   H   N   G   S   R   G   L   E   P   N   K   G   S   G   T   T   S   G   T   T   R
CTT CTA TCT GGG CAC ACG TGT TTC ACG TTG ACA GGT TTG CTT GGG ACG CTA GTA ACC ATG GGC TTG CTG ACT
 L   L   S   G   H   T   C   F   T   L   T   G   L   L   G   T   L   V   T   M   G   L   L   T
TAG GCATCGAATTC (in this case only the DNA sequence contributed by the 12 bp BstXI linker is underlined)
```

FIGURE 5.2. Polylinker sequences of pJFE14 and pJFE14DAF.

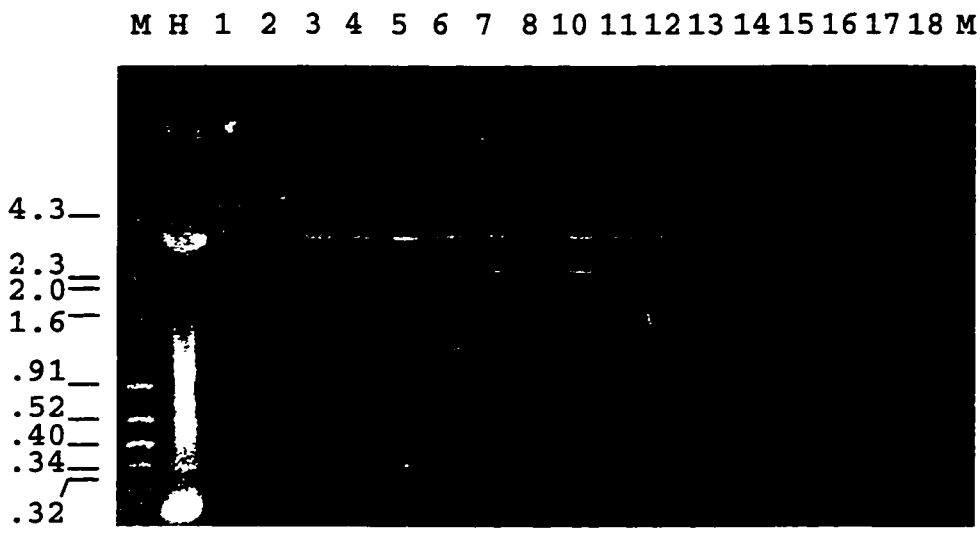


FIGURE 5.3. Restriction analysis of 17 selected clones. Hirt#3 DNA was prepared by large-scale alkaline lysis and CsCl gradient purification and transformed into *E.coli* DH5α. Seventeen independent colonies were picked at random, plasmid DNA was isolated by mini-prep alkaline lysis, digested with Xho I, resolved by 0.8% agarose gel electrophoresis, and inserts visualized by ethidium bromide staining. Each clone is designated by a number at the top of the gel. M, marker; H, Xho I digestion of the large-scale Hirt#3 DNA prep.

TABLE 5.1 Summary of clones obtained from COS cell expression and antibody panning

Clone	XhoI Fragment Size(Kb)	Comments
1	1.4	Incomplete digest*
2#,17	2.0	Incomplete digest*
3	.52	
4#,13	.70	
5	.32	
6#,11	1.3	
7#,10	2.6	
8	2.8, 2.6, 1.8	Likely mixed clones
12#,16	.9	
14		empty clone
18	1.4	

#clones in bold type were pursued further by separate transformation into COS cells, and immunofluorescence staining, and by DNA sequencing.

*confirmed by extended Xho I digestion

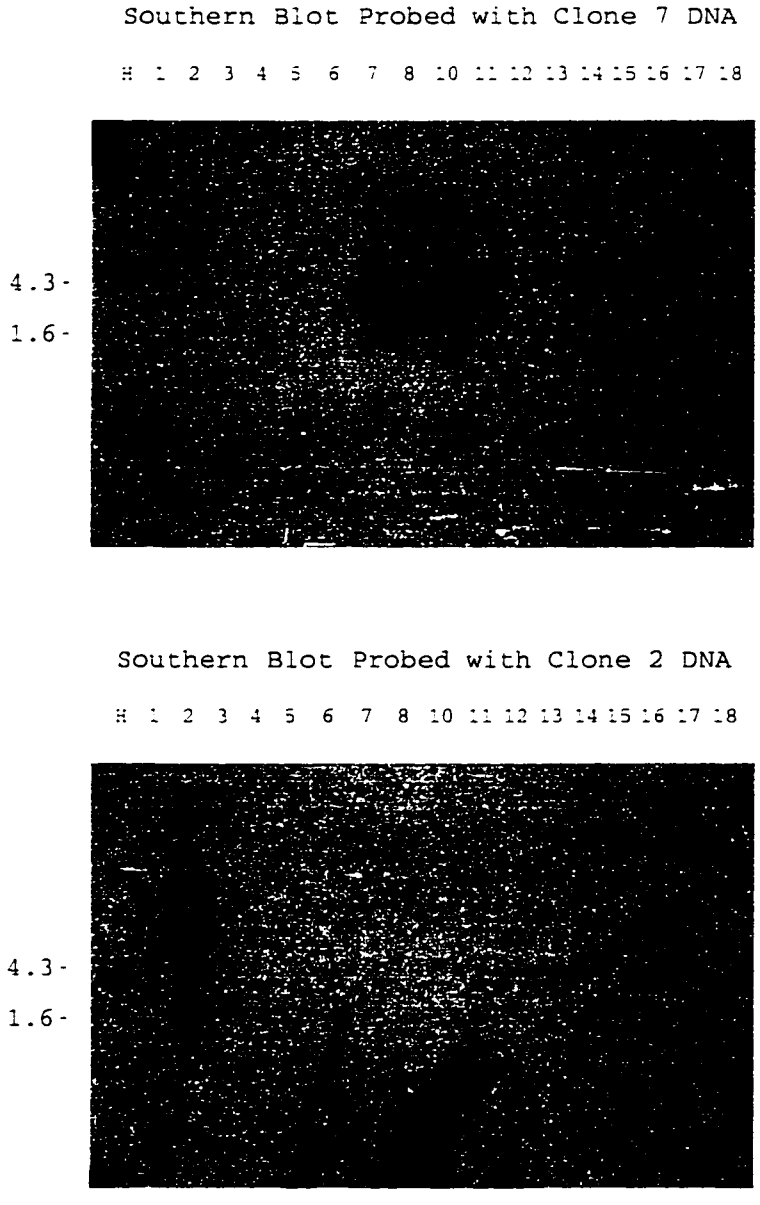


FIGURE 5.4. Southern blot analysis of the 17 selected clones using DNA inserts from clones 2 and 7 as probes. Representative clones from Hirt#3 were digested with Xho I, resolved by 0.8% Agarose electrophoresis, blotted to nitrocellulose, and probed with either clone 2 or 7 DNA that had been ^{32}P labelled. Each clone is designated by its number at the top of the gel, and DNA size markers (in Kb) are indicated at the side. Clones are the same as those shown in figure 5.3.

FIGURE 5.5. IFA of COS cells transfected with selected clones 2 or 7 and stained using PNG panning antisera. Hirt#3 clones 2 (A,B) and 7 (C,D) were transfected into COS cells using the DEAE dextran method. Transfected cells were lifted from culture vessels 72 hours later using PBS/ 0.5 mM EDTA, stained with either normal human serum (1:500; A,C), or pooled Papua-New Guinean (PNG) immune serum (1:500; B,D). Cells were washed, stained with goat anti-human FITC-Ab (1:200), and visualized with a Leitz UV microscope at 250X magnification.

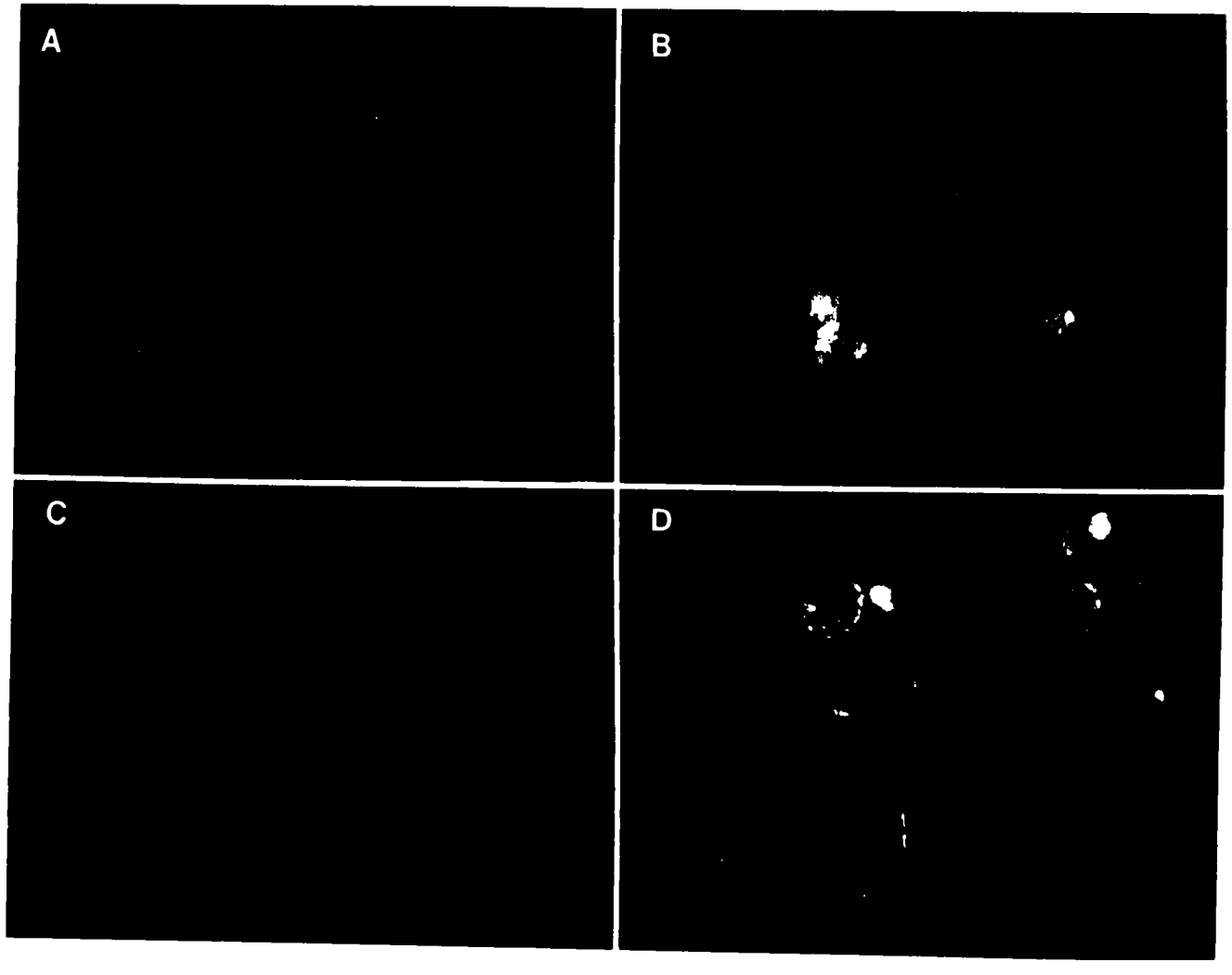
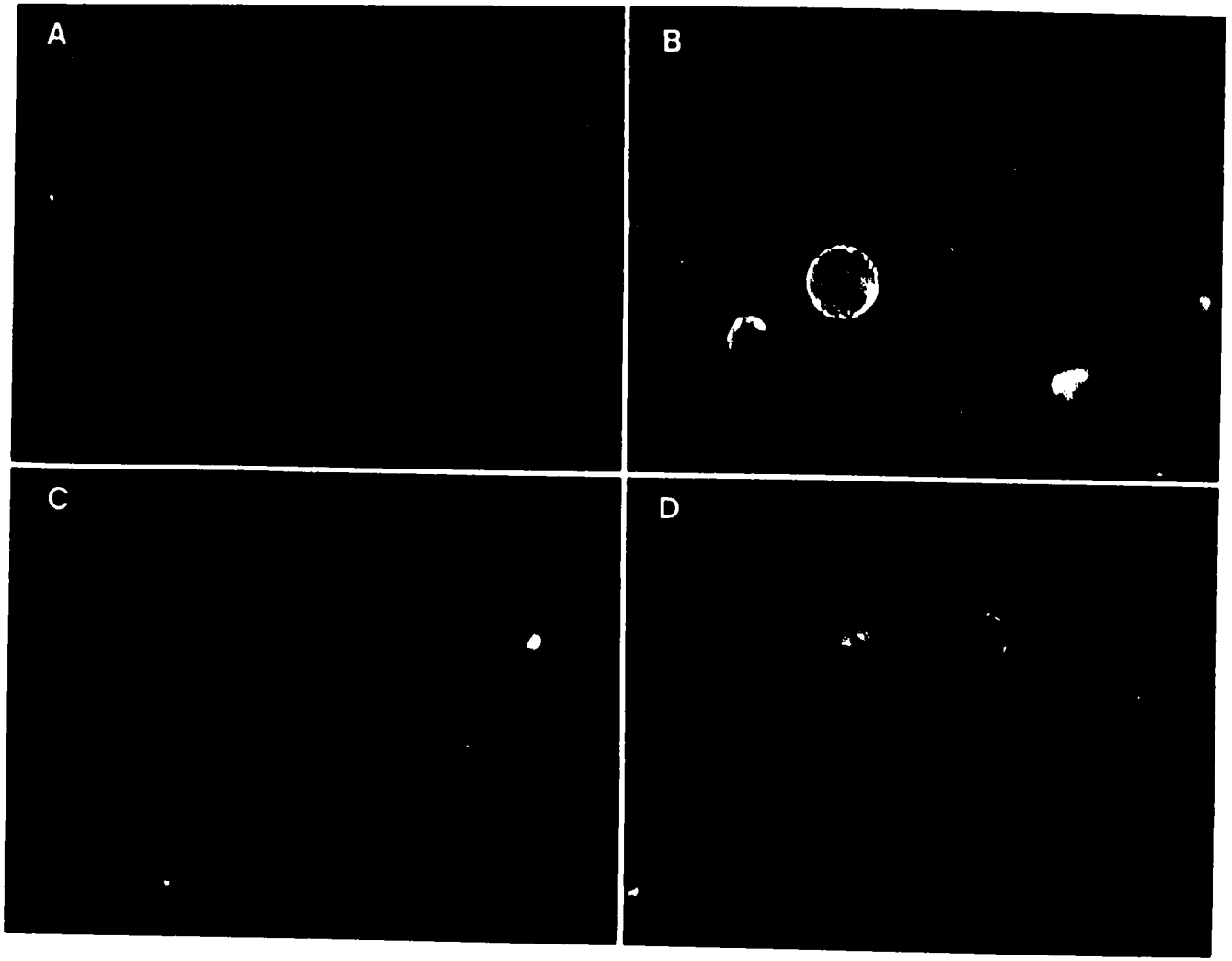

A
B
C
D

FIGURE 5.6. IFA of COS cells transfected with selected clones 8 or 18 and stained with PNG antisera. COS cells were transfected with Hirt#3 clones 8 (A,B) or 18 (C,D), stained, and visualized as described in the previous figure (250X magnification).


A
B
C
D

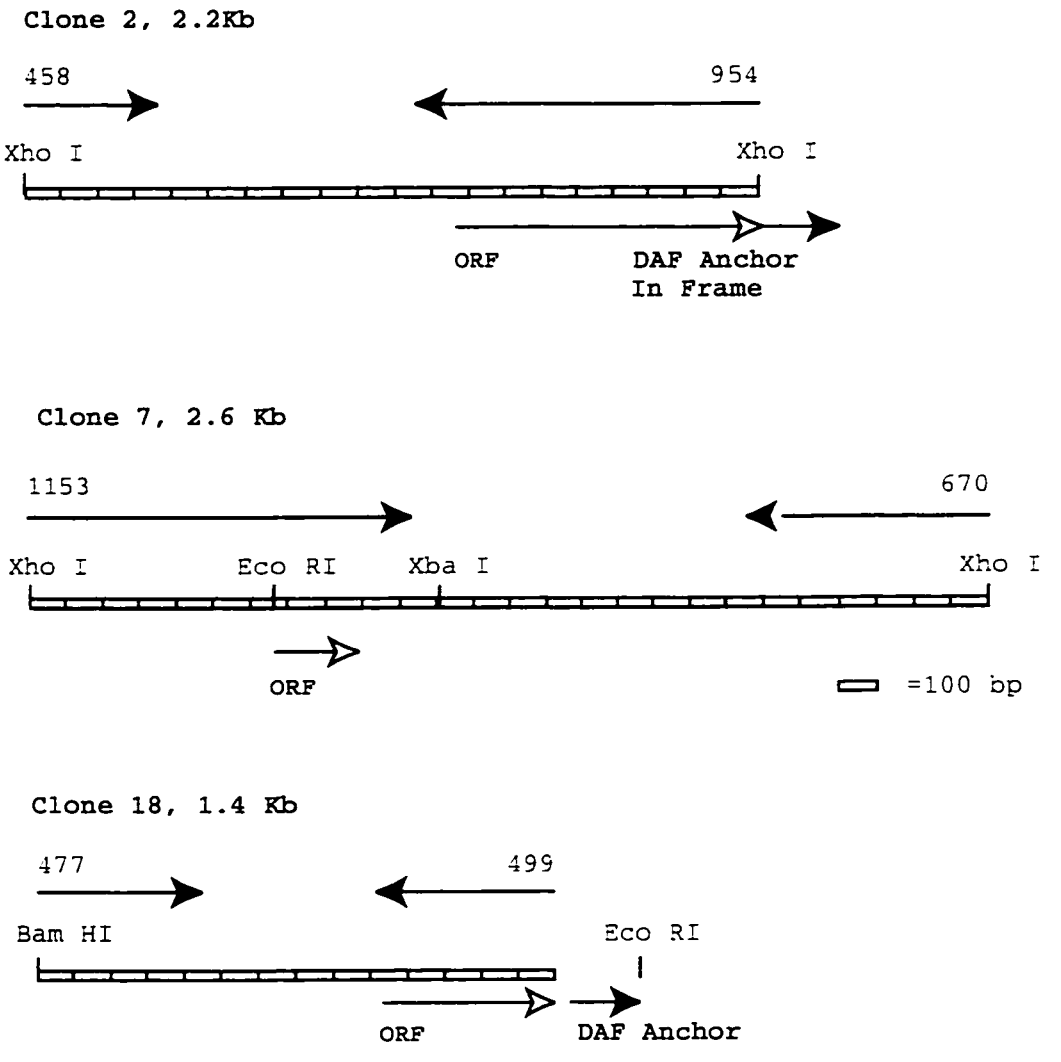


FIGURE 5.7. Summary of DNA sequences obtained for clones selected by COS expression and antibody panning. Clones were sequenced using dye-labelled dideoxynucleotide terminators, or dye-labelled primers, double-stranded and single-stranded DNA templates. Regions of each cDNA clone sequenced are spanned by closed arrows with the number of bases sequenced directly above. Open reading frames are indicated as ORF, and one continuous ORF between the clone 2 sequence and the DAF anchor of pJFE14DAF is shown. Clones 7 and 18 ORFs have no initiating methionine codons detected thus far.

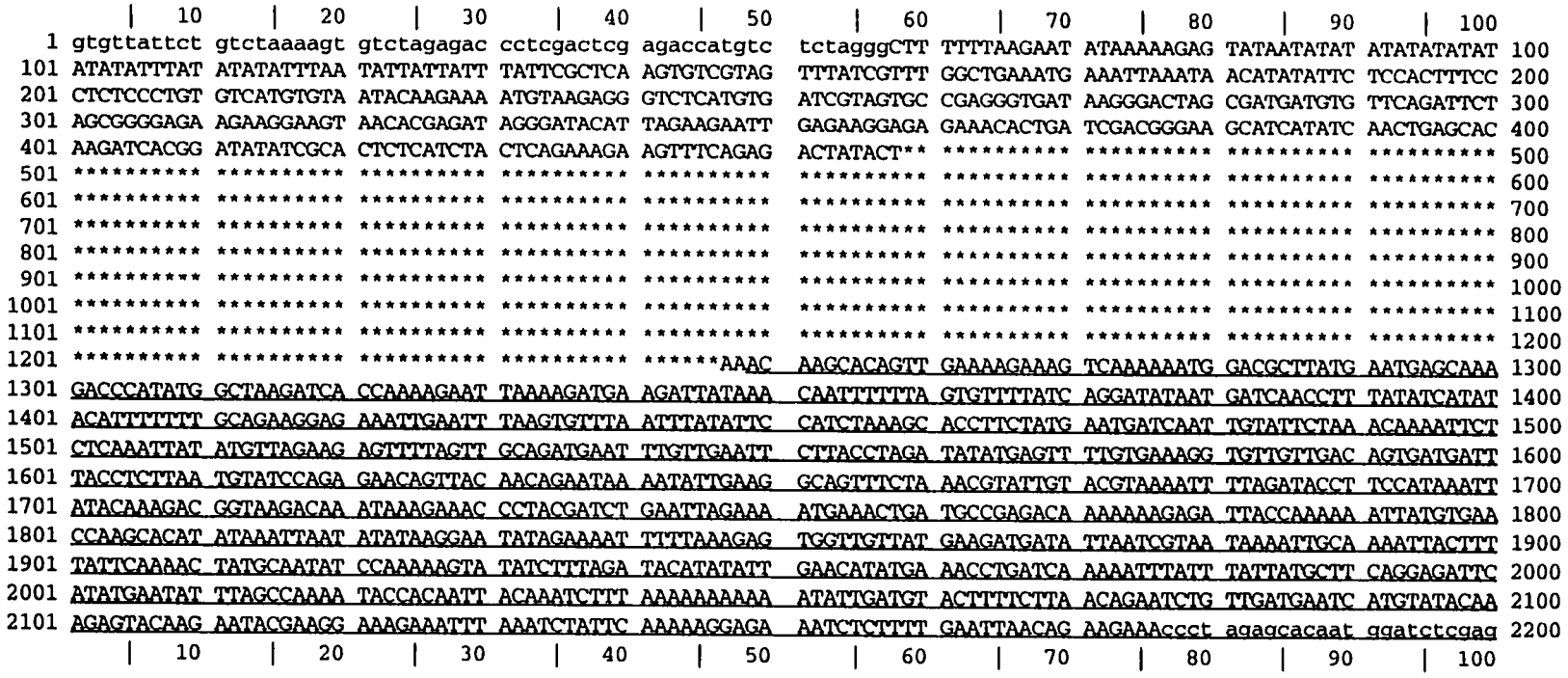


FIGURE 5.8. Clone 2 DNA sequence. Clone 2 5' DNA sequence (1-458) was obtained using the original clone 2 and the primer 5'pJFE14 (Table 2.1), which anneals 62 bases upstream of the Xba I site in the pJFE14 vector. Clone 2 3' DNA sequence (1256-2200) was determined using clone 2 DNA digested with Xho I/ Eco RI and subcloned into pBluescript KS- and SK-. (*) tract represents an approximation of the region of clone 2 that remains to be sequenced. pJFE14DAF sequences are shown in lower case. The underlined sequence indicates an open reading frame (the upstream ATG remains to be determined by sequencing) extending to the Xho I site of pJFE14DAF, in frame with the DAF anchor. This sequence encodes a protein that has homology to *P.falciparum* heat shock protein hsp90.

```
Hu hsp90a ---------DQEELNKTKPIWTRNPDDITNEEYGEFYKSLTNDWEDHLAVKHFSVEGQLE 337
Hu hsp90b ---------DQEELNKTKPIWTRNPDDITQEEYGEFYKSLTNDWEDHLAVKHFSVEGQLE 337
Pf hsp86  ---------EWEELNKQKPLWMRKPEEVTNEEYASFYKSLTNDWEDHLAVKHFSVEGQLE 337
Pf hsp90  ---------EWEELNKQKPLWMRKPEEVTNEEYASFYKSLTNDWEDHLAVKHFSVEGQLE 337
Cf grp94  TKKVEKTVWDWELMNDIKPIWQRPSKEVEDDEYKAFYKSFSKESDDPMAYIHFTAEGEVT 382
Clone 2   TSTVEKKVKKWTLMNEQRPIWLRSPKELKDEDYKQFFSVLSGYNDQPLYHIHFFAEGEIE 60
                 .  .*. .*.* * .... ...*  *.. ..   .. .   ** .**..

Hu hsp90a FRALLFVPRRAPFDLFENR--KKKNNIKLYVRRVFIMDNCEELIPEYLNFIRGVVDSEDL 395
Hu hsp90b FRALLFIPRRAPFDLFENK--KKKNNIKLYVRRVFIMDSCDELIPEYLNFIRGVVDSEDL 395
Pf hsp86  FKALLFIPKRAPFDMFENR--KKRNNIKLYVRRVFIMDDCEEIIPEWLNFVKGVVDSEDL 395
Pf hsp90  FKALLFIPKRAPFDMFENR--KKRNNIKLYVRRVFIMDDCEEIIPEWLNFVKGVVDSEDL 395
Cf grp94  FKSILFVPTSAPRGLFDEYGSKKSDYIKLYVRRVFITDDFHDMMPKYLNFVKGVVDSDDL 442
Clone 2   FKCLIYIPSKAPSMNDQLYS--KQNSLKLYVRRVLVADEFVEFLPRYMSFVKGVVDSDDL 105
          *......*  **     .     * . .******.. *.  ...*....*..*****.**

Hu hsp90a PLNISREMLQQSKILKVIRKNLVKKCLELFTELAED-KEN------------------- 434
Hu hsp90b PLNISREMLQQSKILKVIRKNIVKKCLELFSELAED-KEN------------------- 434
Pf hsp86  PLNISRESLQQNKILKVIKKNLIKKCLDMFSELAEN-KEN------------------- 434
Pf hsp90  PLNISRESLQQNKILKVIKKNLIKKCLDMFSELAEN-KEN------------------- 434
Cf grp94  PLNVSRETLQQHKLLKVIRKKLVRKTLDMIKKIADE-KYN------------------- 481
Clone 2   PLNVSREQLQQNKILKAVSKRIVRKILDTFHKLYKDGKTNKETLRSELENETDAETKKEI 165
          ***.*** *** *.**.. *....* *. . .. .. * *

Hu hsp90a ---------YKKFYEQFSKNIKLGIHEDSQNRKKLSELLRYYTSASGDEMVSLKDYCTRM 485
Hu hsp90b ---------YKKFYEAFSKNLKLGIHEDSTNRRRLSELLRYHTSQSGDEMTSLSEYVSRM 485
Pf hsp86  ---------YKKFYEQFSKNLKLGIHEDNANRTKITELLRFQTSKSGDEMIGLKEYVDRM 485
Pf hsp90  ---------YKKFYEQFSKNLKLGIHEDNANRTKITELLRFQTSKSGDEMIGLKEYVDRM 485
Cf grp94  ----------DTFWKEFGTNIKLGVIEDHSNRTRLAKLLRFQSSHHPSDITSLDQYVERM 531
Clone 2   TKKLCEPSTYKLIYKEYRKFLKSGCYEDDINRNKIAKLLLFKTMQYP-KSISLDTYIEHM 224
                . ... . . .* *  **  ** ....** . .    . .*. *  .*

Hu hsp90a KENQKHIYYITGETKDQVANSAFVERLRKHGLEVIYMIEPIDEYCVQQLKEFEGKTLVSV 545
Hu hsp90b KETQKSIYYITGESKEQVANSAFVERVRKRGFEVVYMTEPIDEYCVQQLKEFDGKSLVSV 545
Pf hsp86  KENQKDIYYITGESINAVSNSPFLEALTKKGFEVIYMVDPIDEYAVQQLKDFDGKKLKCC 545
Pf hsp90  KENQKDIYYITGESINAVSNSPFLEALTKKGFEVIYMVDPIDEYAVQQLKDFDGKKLKCC 545
Cf grp94  KEKQDKIYFMAGSSRKEAESSPFVERLLKKGYEVIYLTEPVDEYCIQALPEFDGKRFQNV 591
Clone 2   KPDQKFIYYASGDSYEYLAKIPQLQIFKKKNIDVLFLTESVDESCIQRVQEYEGKKFKSI 284
          *  *. **. .*.. .   . . .. . *.. .*... ...** ..* . ...** .

Hu hsp90a TKEGLELPEDEEEKKKQEEKKTKFENLCKIMKDILEKKVEKVVVSNRLVTSPCCIVTSTY 605
Hu hsp90b TKEGLELPEDEEEKKKMEESKAKFENLCKLMKEILDKKVEKVTISNRLVSSPCCIVTSTY 605
Pf hsp86  TKEGLDIDDSEEAKKDFETLKAEYEGLCKVIKDVLHEKVEKVVVGQRITDSPCVLVTSEF 605
Pf hsp90  TKEGLDIDDSEEAKKDFETLKAEYEGLCKVIKDVLHEKVEKVVVGQRITDSPCVLVTSEF 605
Cf grp94  AKEGVKFDESEKTKE---------------------------------------------- 606
Clone 2   QKGEISFELTEETLEHNG------------------------------------------- 302
           *  ...   *.  .
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FIGURE 5.9. Alignment of clone 2 protein sequence with five different hsp90 sequences.

C. familiaris grp94, human hsp90a,b, *P. falciparum* hsp86 and hsp90 were identified as sequences with significant homology to clone 2 by BLASTX 'nr' database search (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST). These sequences were aligned using the CLUSTALW tool. The locus name for each sequence is given to the left of the sequence, and the amino acid number of the last residue in each line is shown to the right. The clone 2 ORF is indicated at position 1248 in the previous figure (see clone 2 DNA sequence, figure 5.8). Amino acid homology is indicated by (*), identity by (.), and gaps introduced into the sequence by (-). Clone 2 has approximately 60% homology to the hsp90 sequences analysed.

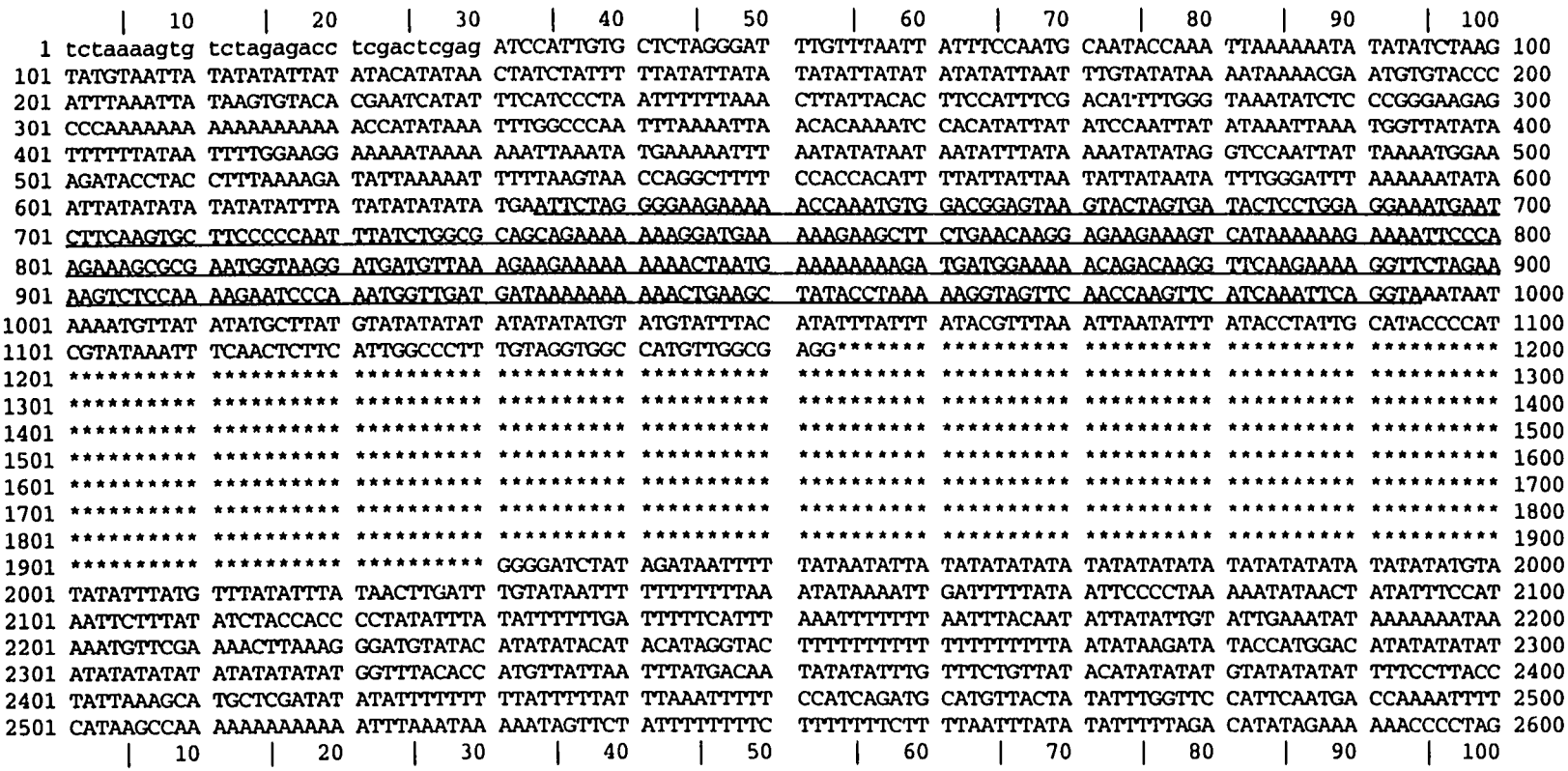

FIGURE 5.10. Clone 7 DNA sequence. Clone 7 5' DNA sequence (1-1153) was obtained by dideoxy sequencing using dye-labelled dideoxy terminators. The initial 5' sequence was determined using the original clone 7 and the 5'pJFE14 primer. Further sequence analysis was completed using clone 7 DNA digested with Xho I, Xho I/ Eco RI and Xho I/ Xba I (see figure 7.7) and subcloned into pBluescript SK- and KS-. Clone 7 3' sequence (1931-2600) was determined by the same method as above, using clone 7 DNA digested with Xho I and subcloned into pBluescript KS-. (*) tract represents an approximation of the region of clone 7 that remains to be sequenced. pJFE14 sequences are shown in lower case. The underlined sequence indicates an uninterrupted reading frame encoding a protein that has significant homology to the *P.falciparum* sporozoite and liver stage antigen, SALSA.

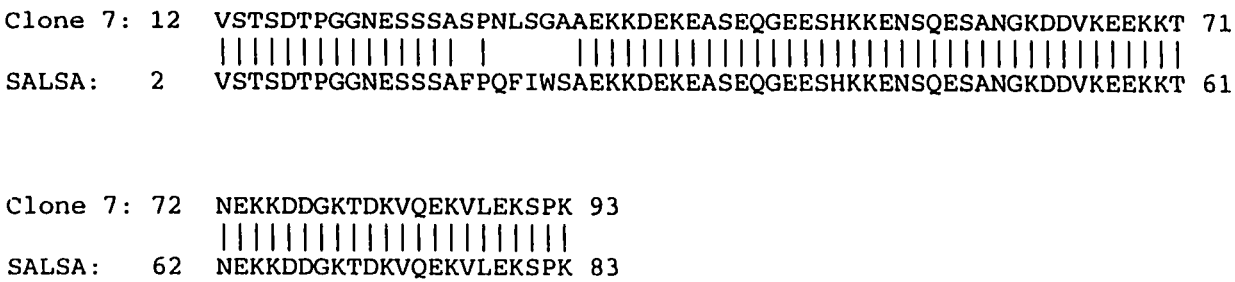

FIGURE 5.11. Clone 7 protein sequence alignment using BLAST2. The Clone 7 5' DNA sequence was used as a query in a BLASTX search of the non-redundant 'nr' database (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST). This search translates the DNA sequence into all 6 possible reading frames and compares it to the entries in the database. The sporozoite and liver stage antigen, SALSA showed significant homology to a significant portion of an uninterrupted reading frame (base 634-994 in figure 1.9, translated into a 121 amino acid sequence lacking a methionine start codon) of the clone 7 5' DNA sequence. Numbers to either side of the sequence indicate amino acid residue number in the translated protein sequence. Sequence homology is indicated by a line between the two sequences.

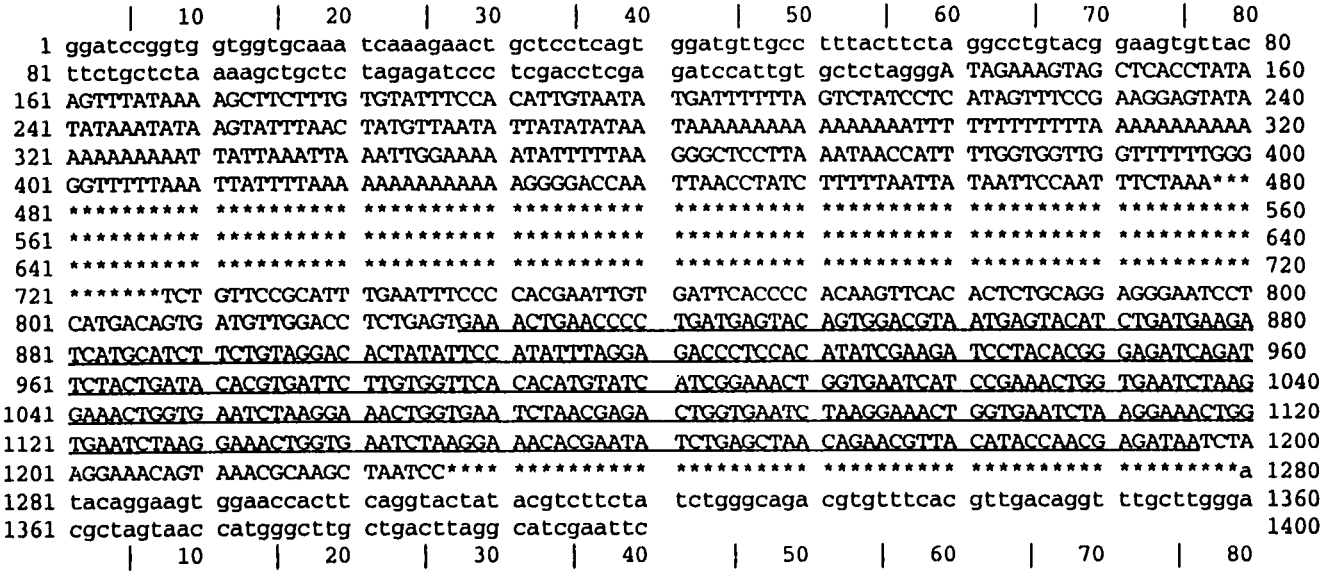

FIGURE 5.12. Clone 18 DNA sequence. Initial clone 18 5' (1-480) DNA sequence was obtained using clone 18 DNA digested with Bam HI/ Eco RI and subcloned into pBluescript SK-. Further 5' sequences (728-1226) were determined (by internal primers) using clone 18 DNA digested with Xho I and subcloned into pBluescript SK- and KS-. (*) tract represents an approximation of the regions of clone 18 that remain to be sequenced. Lower case letters denote both 5' and 3'pJFE14DAF sequences. The underlined sequence indicates a reading frame that encodes a protein with homology to the *P.falciparum* mature erythrocyte-infected antigen, MESA.

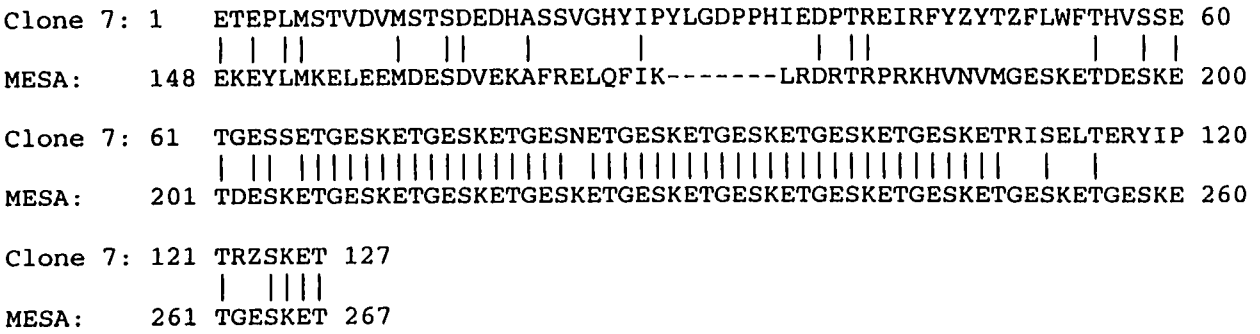

FIGURE 5.13. Clone 18 protein sequence alignment using BLAST. The clone 18 3' DNA sequence was used as a query in a BLASTX search of the non-redundant 'nr' database, as with clones 2 and 7. Protein translated from a reading frame (clone 18 bases 828-1194, see figure 1.11) interrupted by 3 stop codons (Z) showed significant homology to mature erythrocyte infected surface antigen, MESA. Position 1 of the clone 18 protein sequence corresponds to base 828 of clone 18 (see figure 1.11). Numbers to either side of the sequence indicate amino acid residue number in the translated sequence. Gaps are indicated by (-). Sequence homology is indicated by a line between the residues.

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

The cloning of Pf12 by COS cell expression and antibody panning was the beginning point for my thesis. By showing that an established eukaryotic expression cloning technique could be used to isolate genes encoding immunologically relevant parasite antigens, Elliott and colleagues not only expanded upon the existing methodologies for discovering new parasite antigens but offered an improvement: in choosing a eukaryotic system to express parasite cDNAs, they ensured that the proteins encoded by these cDNAs were in a more native conformation than existing prokaryotic expression systems. This advance was central to the problem of cloning parasite genes that expressed proteins with conformationally dependent epitopes. Studies with merozoite antigens such as MSP-1 (Spetzler et al., 1994) and AMA-1 (Hodder et al., 1996) showed that conformation was important in not only the function, but also the immune recognition of these proteins. It was certainly possible that many genes encoding immunogenic proteins could not be cloned by screening lambda expression libraries with immune sera because many parasite antigens required disulfide bridges to maintain native conformations, and the conditions needed for the formation of native disulfide bridged domains were not present in *E. coli* (Moran et al., 1991). The use of COS cell expression of *P. falciparum* cDNA and genomic libraries (COS cells could support the folding of parasite proteins into relatively native conformations) and antibody panning as a complimentary method for cloning new parasite genes became a worthwhile avenue of pursuit.

I began my project with the study of Pf12, a parasite gene cloned by Elliott and colleagues (Elliott et al., 1990) using COS cell expression and antibody panning. This gene encoded a potentially GPI-linked protein with a predicted molecular weight of 39.4 kDa. Initial sequence analysis revealed that this protein contained putative secretion and GPI anchor signals, suggesting that it was present on the surface of either the parasite, the

parasite-infected RBC, the parasitophorous vacuolar membrane (PVM), or the network of vesicles that extend from the intracellular parasite to the RBC membrane, the tubovesicular membrane (TVM). Pf12 had a number of potential glycosylation sites (although N-linked glycosylation has not been described in erythrocytic stages of the parasite), and a relatively high cysteine content. BLAST searches revealed little significant homology with database proteins.

My goal to characterize Pf12 was dependent upon raising Pf12 specific antiserum to use as a diagnostic tool for localizing the protein in the parasite. In order to accomplish this, I attempted to synthesize Pf12 protein using eukaryotic and prokaryotic expression systems, and use this recombinant product to raise Pf12 specific antiserum in test animals.

My first attempt to express Pf12 protein using a prokaryotic system was successful, but I was unable to generate antiserum that could specifically localize Pf12, or reveal the time of expression of Pf12 during its life cycle. My first attempts at making full-length Pf12 less the anchor and signal sequences failed, but subsequent attempts to make subfragments of Pf12 were more successful. Initially, I used PCR to generate subfragments encompassing the N-terminal two-thirds, the middle third, and the C-terminal two-thirds of Pf12, and cloned this into an expression vector that added a His6 tag to the target protein for later purification by nickel affinity chromatography. I discovered that the N-terminal two thirds of the protein, called Pf12ec A2, could be expressed at low levels in inclusion bodies in *E. coli*, whereas the remaining two proteins were not expressed at detectable levels. I purified this protein under denaturing conditions and used it to generate anti-Pf12 immune serum in rats. This rat anti-A2 antiserum was specific for the denatured Pf12ecA2, and for wild-type Pf12 expressed on the surface of Pf12-transfected COS cells. On the other hand, the anti-A2 antiserum had only a weak specificity for methanol fixed parasite infected RBCs in immunofluorescence experiments, and no specificity for iRBCs in immunoEM experiments. While the immunofluorescence experiments suggested that Pf12 was present within the iRBC, perhaps at the surface of the

parasites or the inner leaflet of the RBC membrane, no definitive localization of Pf12 was possible.

I chose to produce recombinant Pf12 in a eukaryotic expression system with the hopes that the protein would be folded in a more native conformation and that antibodies specific for this protein would be more useful in characterizing it than the rat anti-A2 antibodies generated previously. Although Pf12 could be made in COS cells transiently (it was cloned by COS cell panning), obtaining enough protein from transient or permanently transfected eukaryotic cells to raise specific antiserum would have been difficult. I chose to generate a recombinant baculovirus expressing this protein, as this system had been shown to produce milligram amounts of recombinant protein in insect cells. Unfortunately, I was unable to make secreted versions of the Pf12 using by infecting Sf9 cells with a Pf12 recombinant baculovirus. Using a recombinant virus containing a truncated form of Pf12 identical to the Pf12ec A2 sequence, I was able to make a small amount of insoluble protein in this system. I decided to abandon these experiments because my original aim had been to generate relatively native secreted protein that could be easily harvested and purified.

Another method I chose for generating antiserum specific for native Pf12 was DNA immunization. This method circumvented the need for producing and purifying recombinant protein for immunization because the animal muscle cells themselves would take up purified injected DNA, produce the protein encoded by the DNA, and then the animal would mount an immune response to this protein. Though this method was successful in eliciting a humoral response to mouse IL-10, which was used as a positive control, no specific anti-Pf12 response was observed in the mouse and rat DNA immunization experiments I conducted. Possible reasons for this may include the toxicity of Pf12 in muscle cells, the aggregation or improper folding of Pf12, or that Pf12 itself is not a very immunogenic protein. One difficulty with these experiments was that no good source of native Pf12 was available to test the specificity of the anti-Pf12 antiserum. Initially denatured, truncated prokaryotic Pf12 (described later in this section), was used in

ELISA experiments to detect Pf12-specific antiserum, but if antibodies were generated specifically to conformation dependent epitopes, these may not have been detected. Later experiments involved the use of Pf12 on the surface of Pf12-transfected COS cells to identify anti-Pf12 antibodies first by immunofluoresence, then later using immunoprecipitation of biotinylated COS derived Pf12 for increased sensitivity. Neither of these methods could detect anti-Pf12 antiserum in DNA immunized animals. Finally, adenoviruses expressing IL4 were co-injected with Pf12 DNA, to provide an adjuvant effect, promoting a humoral response in the microenvironment where Pf12 protein was being produced. Although this method did elicit a nonspecific antibody response to COS cell proteins, no anti-Pf12 activity was detectable. In summary, DNA immunization was an attractive method because the potential existed to generate specific antibodies to a target protein without having to produce large amounts of the protein. This method probably failed because Pf12 was not made by the muscle cells after DNA immunization.

After failing to produce native Pf12 in eukaryotic systems, I decided explore prokaryotic expression systems once again, with the intent to carefully renature solubilized recombinant Pf12 protein and use it to elicit specific antiserum in mice. I generated a series of new Pf12 subfragments, based on a hypothetical disulfide-bonded domain structure for Pf12 and two other related sexual stage antigens, Pfs 230 and Pfs 48/45 (Carter et al., 1995). Using pattern searches, Richard Carter and colleagues had discovered that Pfs 230 protein contained an ordered number of repetitive cysteine-containing sequences with the potential to form disulfide bonded domains. I created new Pf12 subfragments with this hypothetical model in mind: I hoped the proper matching of cysteine residues would facilitate formation of disulfide bridges and avoid premature degradation or formation of inclusion bodies in *E. coli* transformed with the sequences encoding these proteins. Although I was able to generate a number of subfragments that expressed at moderate levels in *E. coli*, the subfragment I chose to study further, Pf12ec B2, was present in insoluble inclusion bodies. I chose this particular subfragment because it encompassed

most of the Pf12 sequence. Before immunizing mice with this protein I attempted to renature it with some success. Pf12ec B2 specific antibodies obtained from these immunized mice recognized both denatured Pf12ec B2, and relatively native Pf12 expressed on the surface of Pf12-transfected COS cells. Unlike the rat anti-A2 antiserum, this mouse anti-B2 antiserum recognized Pf12 as a 36-38 kDa protein in parasite Western blots, and could be used to localize the protein to the schizont stages of the erythrocytic cycle of parasite growth. Immunofluorescence studies using acetone fixed iRBCs suggested that Pf12 was present either within the parasitophorous vacuole surrounding mature schizonts, or at the inner leaflet of the RBC prior to merozoite burst, and on the surface of merozoites after they have broken free of the RBC. As noted for the rat anti-A2 antiserum, the anti-B2 antiserum was unable to localize Pf12 to a specific parasite membrane or organelle. This may be due to the destruction of critical Pf12 epitopes by fixatives such as glutaraldehyde and paraformaldehyde. Further studies conducted to address this point by incubating iRBCs with anti-B2 prior to fixation may have failed because the antigen was inaccessible, or the permeabilization process was did not make the antigen readily available to the antibody.

In addition to the time of expression of Pf12, I had hoped to determine its exact subcellular location in order to develop a better understanding of the function of Pf12. The information I have obtained begins to shed some light on the role of Pf12. Pf12 is a 37 kDa protein expressed during the late trophozoite, schizont and merozoite stages. It is most likely transported to the PVM/ TVM, where it remains until the merozoites burst free of the iRBC. At this time it is transferred to the surface of the merozoites. It is cleaved by proteolytic enzymes both in *E. coli* and in *P. falciparum* to generate one large fragment (approximately 30 kDa) and one smaller (14-20 kDa) fragment. Recombinant Pf12 subfragments (and proteolytic forms of these subfragments) expressed in *E. coli* may self-associate. Preliminary evidence for this possibility was presented in the original work by

Elliott and colleagues that showed Pf12 expressed in COS cells could form dimers and trimers (Elliott et al., 1990).

Pf12 seems to be distributed around the developing merozoites within the schizont. Immunofluorescence experiments show that this staining encompasses the entire 'packet' of developing merozoites, but does not stain each one individually. As the merozoites grow and take up a significant portion of the RBC cytoplasm, the Pf12 staining seems to localize to the inner leaflet of the RBC membrane. Instead of an actual translocation of the protein to this site, the staining profile may simply be reflective of the growth of the merozoites to fill the entire RBC (see figure 4.21 A). The staining profile of Pf12 in figure 4.20 reveals that Pf12 associates loosely with the merozoites as they are released from the RBC, but is also present in vesicle-like packets separate from the merozoites. Finally, figures 4.21 A and B suggest that Pf12 remains associated with free invasive merozoites, and may be shed onto the membrane of newly invaded RBCs.

Pf12 closely resembles MSP-1, a major asexual vaccine candidate, in that both antigens are expressed on the surface of merozoites, both antigens undergo proteolytic cleavage to give similar sized fragments (MSP-1 undergoes a number of proteolytic cleavages, including a cleavage of its 42 kDa fragment to give merozoite associated 19 and soluble 33 kDa fragments), and both antigens have cysteine residues which play key roles in their conformations. Unlike MSP-1, Pf12 does not seem to be readily accessible to antibodies on the merozoite surface. Unfixed schizonts and merozoites were not stained by the mouse anti-B2 antiserum; only after acetone fixation was this staining visualized. This suggests that Pf12 is either made accessible to the antibody by acetone fixation, or that the protein is modified to reveal epitopes recognized by the antibody after the acetone fixation. If Pf12 is initially made in the parasitophorous vacuolar membrane and later transferred to the surface of the merozoites (or released in membranous vesicles), it is difficult to envisage how it would translocate to a region of the merozoite (e.g. on the inner surface of the merozoite membrane) where it is inaccessible to antibodies. By contrast, Pf12 could

contain post-translational modifications (such as acylation, glycosylation; see 'smear' of Pf12 proteins made in COS cells, figure 4.10; addition of GPI anchor) or specific conformations (cysteine knot motifs, see ; self association, see results of Pf12ec B2 expression in *E. coli* figure 4.5), that mask epitopes which are revealed by the effects of acetone fixation (partial denaturation, or more importantly extraction of hydrophobic molecules such as lipids). The extraction of lipid or 'relaxation' of the conformation of Pf12, may allow antibodies to bind to otherwise inaccessible epitopes. Thus Pf12 may be present on the surface of merozoites just as MSP-1 is, except that it is present in a conformation that is not recognized by the anti-Pf12 antibodies generated in chapter 4.

Pf12 also shares common features with another asexual stage vaccine candidate, apical membrane antigen 1, or AMA-1 (see table 1.2). This antigen is first expressed in the neck of the rhoptries before merozoite release, then proteolytically processed and transferred onto the surface of the merozoite after the iRBC bursts. AMA-1, like Pf12, is a cysteine-rich protein with a disulfide bond structure reminiscent of the cystine knot motif proposed for Pf12 and Pfs 230 (Hodder et al., 1996; Carter et al., 1995).

The localization and properties of Pf12 suggest a number of potential functions for this protein. If it is in a compact conformation as the studies in this thesis and work described in (Carter et al., 1995) suggest, it may play a role in maintaining the structure of the iRBC until mature, infective merozoites have formed. Premature lysis of these iRBCs would mean the release of uninfective merozoites, derailing the infection process, and potentially priming the host immune system to attack infective merozoites. By contrast Pf12 may facilitate the timely release of merozoites from the iRBC. The presence of Pf12 on the surface of merozoites also suggests that this protein is involved in merozoite invasion of host RBCs. The failure of anti-A2 and B2 antiserum to inhibit merozoite invasion in preliminary *in vitro* experiments suggests that Pf12 may not be directly involved in this process. Finally, the recurring cross-reactive specificity of both the anti-A2 and anti-B2 antiserum for HSA suggests a role for Pf12 in helping merozoites to evade

the host immune response by mimicking a host protein. Merozoites coated with Pf12 resembling portions of HSA may avoid host antibody responses, which are usually not generated towards proteins that resemble host molecules.

The final portion of my thesis involved a new round of COS cell panning experiments. By combining both genomic and cDNA libraries in these panning experiments, I hoped to isolate parasite clones expressing both intact proteins or portions of proteins recognized by human immune serum. This exercise was successful in that the DNA sequences I did isolate, Pf hsp90, SALSA, and MESA, all play important roles in the parasite life cycle, and both Pf hsp90 and SALSA have been considered as vaccine candidates. With the exception of SALSA, these genes had already been fully sequenced and the corresponding proteins characterized. The entire SALSA sequence has not been published; determining the rest of the sequence of clone 2 may be a worthwhile pursuit.

Future Directions

This thesis demonstrates that although COS cell panning is a reliable method for isolating parasite genes, it is only the first step in a long process of characterizing the encoded protein and assessing its importance in the pathogenic process. An additional question remains once the protein has been characterized: will it be a good vaccine candidate? In the case of Pf12, the presence of the protein on the surface of the blood stage infective form, the merozoite, coupled with the fact that the PNG panning antiserum contains high titres of antibodies specific for Pf12 suggests that it would be a good vaccine candidate. A much more detailed analysis of Pf12 is still needed to support this recommendation. It is unfortunate that a more definitive method of assessing whether novel parasite proteins will actually make good vaccine candidates does not exist. The most common studies for determining whether a protein will make a good vaccine candidate include sequencing a number of genes from different parasite strains to determine the potential for polymorphism in the parasite protein, *in vitro* studies that determine

whether a given protein is important for merozoite invasion or parasite growth, and vaccine studies in animals such as mice and monkeys. These methods do not offer definitive proof that a protein will be an effective vaccine candidate; antibodies that inhibit parasite growth *in vitro* may have no effect on parasite growth within the human host, and the malaria parasites of mice and monkeys are sufficiently different from *P. falciparum* that it is difficult to make predictions about the efficacy of a protein as a vaccine candidate based on its performance in an animal study. While future studies to determine the function of Pf12 in the parasite are important, a more definitive method for determining the potential of candidate proteins to provide protection from malaria infection is still desperately needed.

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APPENDIX A1

EXPRESSION OF RECOMBINANT Pf12 IN BACULOVIRUS

A. Introduction and background

This chapter describes attempts to make Pf12 using the baculovirus eukaryotic expression system. Producing Pf12 in a eukaryotic system is a good idea because *Plasmodium falciparum* itself is a eukaryote: therefore all the necessary machinery for ensuring that the disulfide-bridged domains of Pf12 are properly assembled and post-translational modifications are made would most likely be present. Although the yield of protein from these systems is usually quite low compared to prokayotic systems, and more time is required to express substantial amounts of recombinant protein, it may be necessary to accept these disadvantages in order to achieve the goal of producing protein in a native conformation. With increasing knowledge about the importance of Pf12 secondary and tertiary structure, I was convinced that polyclonal antiserum generated against Pf12 in a native conformation would be the best reagent both for localizing Pf12 and studying its structure.

In order to accomplish this goal I used separate approaches described in the following two chapters: the creation of a recombinant baculovirus expressing Pf12 protein, and the immunization of mice and rats with eukaryotic expression plasmids containing Pf12 DNA sequences. Baculoviruses infect a number of different moth species (Cory and Entwistle, 1990), and can be cultured in cell lines derived from the fall armyworm, *Spodoptera frugiperda* (Sf9) cells (O'Reilly et al., 1992). During infection two forms of viral progeny are produced: early phase extracellular virus for infection of new target cells, and late phase virus embedded in a protective proteinaceous "polyhedrin", made up of a 29 kDa protein (Volkman et al., 1976; Summers and Smith, 1978). This non-essential protein can accumulate at levels of up to 50% of the total protein produced by infected Sf9 cells, and is easily visualized as rounded highly refractile bodies within the cell cytoplasm.

Recombinant baculovirus expression systems aim to replace the viral polyhedrin gene by co-transfecting wild-type or linearized baculoviruses with plasmids containing target genes flanked by polyhedrin sequences (O'Reilly et al., 1992). While the consecution of a recombinant virus may be time consuming and require some skill, Sf9 cells infected with recombinant baculoviruses produce milligram quantities of target protein, often in the correct conformation and enzymatically active. In addition, these cells are capable of adding post-translational modifications to recombinant proteins, and secreting them into a protein free culture medium if the proper secretion signals are provided (Vlak, 1990). The technique of generating recombinant viruses was considered difficult at the time, because the Sf9 cells themselves are not a hardy cell line, like COS cells, are prone to fungal, bacterial, and low-level wild-type baculovirus contaminations, and must be kept under optimal growth conditions in order to survive transfection of target DNA. Furthermore, because the generation of a recombinant baculovirus (between co-transfected plasmid carrying target gene and wild-type baculovirus DNA) occurs infrequently and involves the disruption of the wild-type polyhedrin gene, recombinant viral plaques on an Sf9 cell monolayer are difficult to identify, especially in an overwhelming background of wild-type viral plaques. The use of beta-galactosidase expressing plasmids for co-transfection and replication-defective linearized baculoviruses decreased the difficulty of this process in that recombinant baculoviruses provided with X-gal substrate were blue, and only the recombinant baculoviruses were capable of replication; linearized virus could not replicate in Sf9 cells unless it recombined with the co-transfected vector containing the target gene.

B. Results

Identification of Recombinant Baculovirus containing Pf12 sequences using Dot-Blot Hybridization

Three different Pf12 constructs were used to generate recombinant baculoviruses. The first construct, Pf12sec (encompassing amino acids 1-322 of Pf12; see figure A1.2 A), was inserted into the baculovirus expression vector pETL-JFE, provided by Dr. Chris Richardson, and modified by the addition of a new polylinker (figure A1.1 A). This construct, along with wild-type baculovirus AcNPV, was transfected into Sf9 cells using lipofectin, and recombinants containing Pf12sec DNA sequences were screened by plaque assay. Eighteen blue recombinant plaques (producing Lac Z, a marker for the generation of recombinant baculoviruses) were picked and screened for the presence of Pf12sec DNA sequences using a ^{32}P-labelled probe and dot-blot hybridization (figure A1.3). Of these, six strong positives, four weak positives, and eight negatives were observed.

SDS-PAGE Analysis of Pf12 Recombinant Baculoviruses

Several dot-blot positive and negative baculoviruses were chosen for further study. Initially a time course study of secretion of Pf12 into Pf12sec bv-infected Sf9 cell culture supernatants indicated that neither dot-blot positive or negative recombinant baculoviruses were secreting Pf12 (figure A1.4). After collecting culture supernatants daily over a course of five days, resolving these samples by 15% SDS-PAGE and staining with Coomassie Blue, no significant difference could be seen in the protein band profiles of either the dot-blot positive or negative supernatant samples. A new set of experiments was conducted with the supernatants and cell pellets of Sf9 cell cultures infected with Pf12sec bv 1, 14, 17

(dot-blot positive), and 13 (dot-blot weak positive). In both infected cells and supernatants from infected cultures, no recombinant Pf12 protein was identified (figure A1.5).

Western Blot Analysis of Pf12bv sec #14, #17

Western blot analysis of proteins synthesized by dot-blot positive baculoviruses was conducted because of the increased sensitivity this assay provides. Cellular and supernatant proteins from Sf9 cultures infected with either of these viruses, or wild-type baculovirus, were resolved by SDS-PAGE and Western-blotted with rat anti-A2 antiserum (figure A1.6). The antiserum was not specific for any proteins present in the recombinant or wild-type baculovirus lanes, although it did bind strongly to the Pf12ec A2 positive control. These experiments suggested that while the recombinant baculoviruses had incorporated a portion of the Pf12 sequence as shown by dot-blot hybridization, they were unable to synthesize detectable amounts of Pf12 protein in infected Sf9 cells.

Construction of a Recombinant Baculovirus Containing a Pf12 subfragment sequence.

Information from experiments conducted in prokaryotic expression systems suggested that Pf12 subfragments such as Pf12ec A2 were expressed at higher levels than the wild-type Pf12 sequence. Therefore, I inserted the A2 sequence described in Chapter 1 into the baculovirus expression vector pAcGP67 (figure A1.1 B and 4.2 B). This vector contained its own start codon and signal sequence from the baculovirus GP67 glycoprotein upstream from the target gene insertion site. Inserted target genes were then fused with the vector ATG and signal sequences. I used this vector to create recombinant baculoviruses in conjunction with the Baculogold transfection kit (Pharmingen) described in more detail in the materials and methods section. Briefly, this system uses linearized baculovirus, which is co-transfected into Sf9 cells with the pAcGP67 expression plasmid. A recombination event between the plasmid and the linearized virus is required to generate an infective

virion. After the initial transfection, these recombinant viruses can be amplified and then plaque-purified. Plaque-purification of relatively slow-growing recombinant viruses is easier with this system, because there are no wild-type viruses present to contaminate picked plaques.

After transfecting the A2 baculovirus expression plasmid and amplifying recombinant baculoviruses, I attempted to plaque-purify recombinants for further study. I observed many A2 plaques at 1/10000 dilution of amplified transfection supernatant. I picked several of these plaques for further study.

SDS-PAGE and Western blot analysis of proteins synthesized by A2 recombinant baculoviruses

Supernatant and cell proteins from recombinant baculovirus-infected Sf9 cells cultures were resolved by 12% SDS-PAGE and Western-blotted with rat-anti-A2 antiserum or control antiserum. In the first SDS-PAGE experiment (figure A1.7), two proteins having calculated molecular weights of 27 and 30 kDa were observed in moderate quantities in the cellular fraction of Sf9 infected with Pf12 A2 bv that were not present in the non-infected or wild-type baculovirus-infected cells. No differences were noted in the protein profiles of the supernatants from each test sample. In Western blot experiments the rat anti-A2 antiserum recognized proteins having molecular weights of 25, 31, and 32 kDa in the cell pellets of Sf9 infected with Pf12 A2 bv#1 (figure A1.8). These protein bands were not observed on the blot for any of the other samples tested, including Pf12 A2 bv#1, isolated from a different plaque than Pf12 A2 bv#1.

Pf12bv A2 is present as insoluble protein in A2bv-infected Sf9 cells

The main goal of this study was to synthesize large amounts of native Pf12 for mouse immunization. Observations from the SDS-PAGE analysis of Pf12 A2 bv produced in insect cells suggested that a moderate amount of the protein was being made, but

unfortunately, it was not secreted into the protein-free supernatant media as hoped, but instead was present in insoluble granules similar to the inclusion bodies formed in *E. coli*. To complete this study, I assessed the solubility of Pf12 A2 bv, finding that it was indeed comparable to that of Pf12ec A2 described in Chapter 3.

Cell pellets from Pf12 A2 bv-infected Sf9 cells were washed and solubilized in a number of different agents including: 1% SDS, 1% NP40, 8M urea, and 1% 'RIPA' buffer (see chapter 2) containing a number of agents and denaturants including 1% sodium deoxycholate. The detergent extracts were then resolved by 12% SDS-PAGE and stained with Coomassie Blue. The Pf12 A2 bv protein doublet (figure A1.8) was solubilized by 8 M urea and sodium deoxycholate only (figure A1.9), indicating that this protein is present in Sf9 cells in a non-secreted form, probably as insoluble protein aggregates, not unlike inclusion bodies seen in *E. coli*.

C. Discussion

In this chapter I have described experiments where I attempted to express Pf12 in the baculovirus eukaryotic protein expression system. Although I was unable to make a recombinant virus expressing full-length Pf12 in this system, I was successful in generating a Pf12 A2 expressing recombinant baculovirus. This baculovirus recombinant had been generated using a transfection vector (pAcGP67) containing the Pf12 subfragment A2 sequence, identical to the one best expressed in *E. coli* in chapter 3, and the linearized replication-defective baculovirus from the Baculogold kit. I had much more success using this kit than other methods of generating recombinant baculoviruses including use of a transfection vector containing beta-galactosidase (beta-Gal) (pETL-JFE), because co-transfection of the vector containing the target gene with the linearized virus of the kit almost never results in generation of a wild-type replication-competent virus, making subsequent recombinant plaque identification and purification easy.

The first set of experiments involved the generation of a recombinant baculovirus containing full-length Pf12 less its anchor signal. This sequence, called Pf12sec, was intended to encode a secreted protein that could be recovered in the protein-free baculovirus medium supernatant, and used immediately for immunization. I was able to visualize recombinant plaques approximately seven days post-transfection as a few blue plaques in amongst a background of white wild-type baculovirus plaques. Under a microscope, wild-type viral plaques could be easily identified by the highly refractile occlusion bodies formed inside virally infected Sf9 cells by the viral polyhedrin protein. Recombinant plaques, conversely, contained cells infected with viruses where the polyhedrin sequence had been replaced with Pf12sec/beta-galactosidase by a recombination event between the transfection vector and the wild-type virus. The presence of beta-galactosidase in the recombinant virus plus the use of Bluo-gal chromogenic substrate in the agarose overlay, made identification of the blue recombinant plaques relatively easy. Plaques were chosen that were distinct and physically separated from wild-type baculoviral ones, picked with a pasteur pipette, and used for two subsequent plaque purifications. The recombinant virus obtained from the final round was considered pure, and used for further studies.

Although the recombination event occurring between the virus and the transfection vector should transfer both beta-galactosidase and the Pf12sec sequence, there was also a chance the beta-Gal sequence was transferred without the target gene sequence. I wanted to make sure that the Pf12 sequence was present in the recombinant viruses I had purified, so I probed the DNA from Sf9 cells infected with my recombinant viruses using a ^{32}P-labelled Pf12-specific probe in a dot-blot experiment (figure A1.3). This study revealed that 6 of the 18 recombinants had a strong Pf12-specific signal, and 4 of 18 had a weak signal, as visualized by autoradiography. Together, 10 of 18 recombinants had most likely incorporated the Pf12 DNA into their genomes, whereas all had incorporated the beta-Gal sequence by virtue of their blue color in plaque assays. I conducted this particular experiment because I had anticipated that detecting recombinant Pf12 in itself might be

difficult, so I wanted to be sure to choose the viruses with the strongest Pf12 signal for protein expression experiments.

In the next set of experiments I attempted to determine if the dot-blot positive recombinants did indeed produce Pf12 by Coomassie staining lysates from virus-infected Sf9 cells resolved by SDS-PAGE, and later, Western blotting using anti-A2 antiserum. Unfortunately, I could not detect any Pf12 protein by the first method described, using a dot-blot negative virus as a control (figure A1.4). This time course experiment showed no apparent production of proteins in the culture supernatants of Sf9 cells infected with either dot-blot positive or negative virus, up to five days after initial infection of the cells with the viruses. I pursued this further by attempting to detect Pf12 protein by the same method in more concentrated supernatants and cell pellets from cultures containing each of the strong dot blot positive recombinant baculoviruses (figure A1.5). Like the earlier experiments, I was unable to detect the production of Pf12 by any of the recombinant viruses. Using the Pf12 specific rat-anti-A2 antibody, I was able to determine if any Pf12 protein had been made at all by these recombinant baculoviruses (figure A1.6). As described in the results section, no Pf12 protein could be detected by Western blotting cellular and supernatant proteins from Sf9 cells infected with dot-blot positive recombinant baculoviruses.

In the second set of baculovirus experiments, conducted using the transfection vector pAcGP67, the Pf12 A2 subfragment, and the linearized, replication-defective baculovirus of the Baculogold kit, I was able to obtain a recombinant baculovirus that produced Pf12 A2 protein. These new experiments I conducted involved the use of a transfection vector that provided a secretion signal from the baculoviral GP67 protein, known for its efficient secretion. This signal was fused to the A2 sequence used in chapter 3, and the resulting vector sequence was cotransfected with the linearized virus to generate a recombinant. The vector used in this case did not have a beta-Gal gene to render recombinant plaques blue in the presence of Bluo-gal/agarose overlays, but detecting a recombinant plaque was not difficult due to the absence of any contaminating wild-type

virus. Recombinant plaques were purified as before, and SDS-PAGE and Western blotting experiments, similar to the ones described previously, were conducted.

SDS-PAGE analysis of Sf9 cell lysates and supernatants from cultures infected with recombinant viruses revealed the expression of a 27/30 kDa doublet in the cell lysate test but not cell lysate controls or any of the supernatant samples (figure A1.7). This doublet is larger than the predicted 24.9 kDa, perhaps due to glycosylation of the protein by the Sf9 cellular machinery, or the effect of acidic residues in the protein. The reason this protein is present as a doublet is also undetermined. The 27 kDa species may be a proteolytic product of the 31 kDa species as a distinct low molecular weight protein was detected in the Pf12 A2 bv lane that could be the other polypeptide generated by the proteolysis. A2 protein was not produced in large amounts, and was not secreted into the supernatant as hoped, suggesting that some aspect of this protein, perhaps the complex nature of the disulfide bridges, was contributing to its retention and low level of expression within the virus-infected Sf9 cells.

I confirmed that the protein expressed in this experiment was Pf12 by Western blotting these proteins from lysates of recombinant virus-infected Sf9 cells with rat anti-A2 antiserum (figure A1.8). This experiment revealed that the anti-A2 antiserum did indeed recognize a group of proteins of similar size to the 27/30 kDa doublet. These proteins formed a ladder of bands ranging from 25-35 kDa, with the most prominent proteins being 25, 31, and 32 kDa species. This smeared ladder of bands was suggestive of differentially glycosylated proteins.

After confirming that the Pf12 A2 recombinant viruses produced A2 protein, but only within Sf9 cells, and not as secreted proteins, I wanted to determine the solubility of A2 within the cells. If this protein was soluble, but not secreted, it would be a relatively simple matter to lyse the cells and obtain the protein. Even though the amount of protein produced was probably not comparable to amount of secreted protein that could have been made, I was still interested in obtaining soluble protein from any source. Unfortunately,

after lysing infected cells and trying to solubilize Pf12 A2 with NP40, SDS, sodium deoxycholate, and urea, I discovered that A2 protein was present in insoluble granules similar to those present in the *E. coli* experiments described in chapters 3 and 4. The strength of the polyhedrin promoter coupled with the complex disulfide bonding pattern of A2 may have contributed to the stalling of protein folding processes in the endoplasmic reticulum, ultimately resulting in the retention and degradation of Pf12 A2 within the Sf9 cells.

In summary, the experiments conducted in this chapter were undertaken for the purpose of expressing secreted Pf12 for rat and mouse immunization and generation of anti-Pf12 antiserum. Sf9 cells infected with Pf12 dot-blot positive recombinant baculoviruses did not produce secreted Pf12, nor were they able to produce the truncated A2 subfragment in a soluble form. Future improvements for expression of toxic parasite proteins in baculovirus should include some aspects mentioned earlier for prokaryotic expression, namely: the inclusion of an epitope and affinity tag in the target protein sequence for easy identification and purification of recombinant proteins and inclusion of chaperone sequences to assist in proper protein folding.

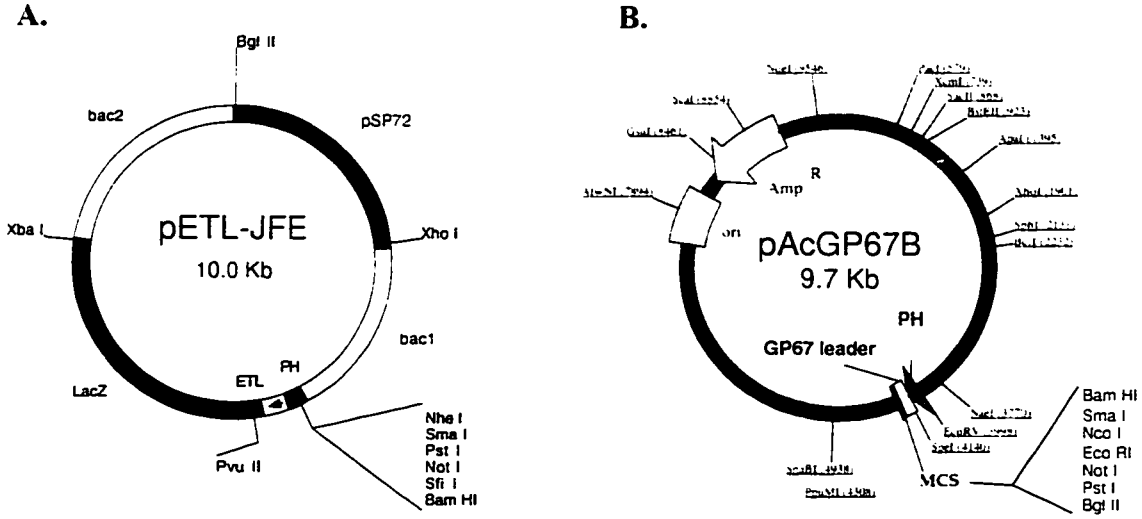


FIGURE A1.1. Maps of the baculovirus expression plasmids pETL-JFE, and pAcGP67B. These vectors, when cotransfected into Sf9 cells with wild-type baculovirus DNA, undergo homologous recombination with the viral DNA (via bac1 and bac2 sequences in pETL-JFE, for example), replacing the polyhedrin gene with the vector and target gene sequences. **A.** The vector pETL-JFE was designed for the production of recombinant proteins containing native secretion signals (or no signal). It is identical to the baculovirus expression vector pETL (a gift from Dr. C.D. Richardson), with the addition of the polylinker sequence shown to the left between the Nhe I and Bam HI sites (made with the primers ETL-JFE up and ETL-JFE dn; see table 2.1. for details). Sequences inserted into the polylinker are expressed under the control of the polyhedrin promoter (PH). The ETL (early to late) promoter drives the production of betagalactosidase in recombinant virus. Recombinant viruses are identified by plaque assay (as blue plaques using Bluo-gal agarose overlays). Bacterial plasmid pSP72 (Promega) sequences in pETL-JFE are for propagation of the plasmid in *E.coli* under ampicillin selective conditions. **B.** The vector pAcGP67B (Pharmingen) is designed for production of secreted proteins. The target gene inserted into the multiple cloning site (MCS) is expressed as a gp67 signal peptide fusion protein under the control of the polyhedrin promoter. This vector lacks a beta-galactosidase reporter gene; recombinant viruses are detected as occlusion body negative (polyhedrin negative) plaques using a phase contrast microscope and a strong light source.

A.

Xba I
TCTAGAGATCCCTCGACCTCGAGATCCATTGTGCTCTAGGGAAAGT

ATG ATA AAA TTA AGT AAG AAG TAT TGT TTA GGG ATA TCC TTT GTA TTA
M I K L S K K Y C L G I S F V L

Not I
TAT ATT TTG TTG TCT GTT TG T GAA GGG CAT... TCC AAC TAA GCG GCC GC
Y I L L S V C ▲ E G H ...S N Z

B.

ATG CTA CTA GTA AAT CAG TCA CAC CAA GGC TTC AAT AAG GAA CAC
M L L V N Q S H Q K P N K E H

ACA AGC AAG ATG GTA AGC GCT ATT GTT TTA TAT GTG CTT TTG GCG
T S K M V S A I V L Y V L L A

Bam HI
GCG GCG GCG CAT TCT GCC TTT GCG GCG GAT CTT GGA TCC **GAA GGG**
A A A H S A P A ▲ A D L G S E G

Pst I Bgl II
CAT AAA AAT TTA ... CAT GCT AAT GAT TTA ATC TAG CTG CAG ATC T
H K N L ...H A N D L I Z

FIGURE A1.2. Cloning strategies for Pf12sec and Pf12 A2 in pETL-JFE and pAcGP67B respectively. **A.** Pf12sec bv was engineered by PCR (using the primers BxBv5' and Pf12 bv 3' in table 2.1, and Pf12/pJFE14 as a template) to contain 5' Xba I and 3' Not I sites, and to exclude the native GPI anchor. This fragment was inserted into Nhe I/ Not I digested pETL-JFE, to generate Pf12sec bv/pETL-JFE. Note that Nhe I and Xba I ends are compatible. **B.** The Pf12 A2 coding segment from chapter 3 (see figure 3.2) was digested with Bam HI/ Pst I and ligated into Bam HI/ Pst I digested pAcGP67B to generate Pf12 A2 bv/pAcGP67B. Native Pf12 DNA and protein sequences are shown in bold, and vector sequences in plain text. Restriction endonuclease sites are shown directly above the DNA sequence, and the signal cleavage sites are indicated by arrows. Only the 5' and 3' ends of each construct are shown, separated by dots. Z indicates a stop codon.

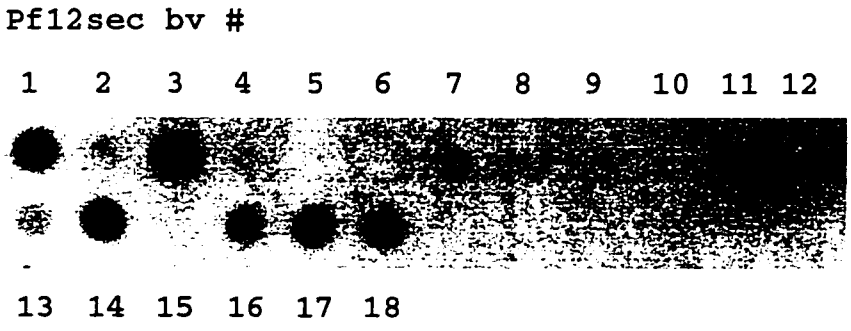


FIGURE A1.3. Identification of recombinant Pf12 baculoviruses using a radiolabelled Pf12 DNA probe and dot-blot hybridization. Sf9 cells infected with plaque-purified potentially recombinant baculoviruses (created with Pf12sec bv/pETL-JFE) were lysed and immobilized on nitrocellulose using a dot-blot vacuum manifold. Recombinants containing Pf12 DNA were identified using a ^{32}P-labelled Pf12-specific probe. Autoradiography showed strong positives (Pf12sec bv #1,3,11,14,17,18) and weak positives (#7,12,13,16), as well as negative plaques.

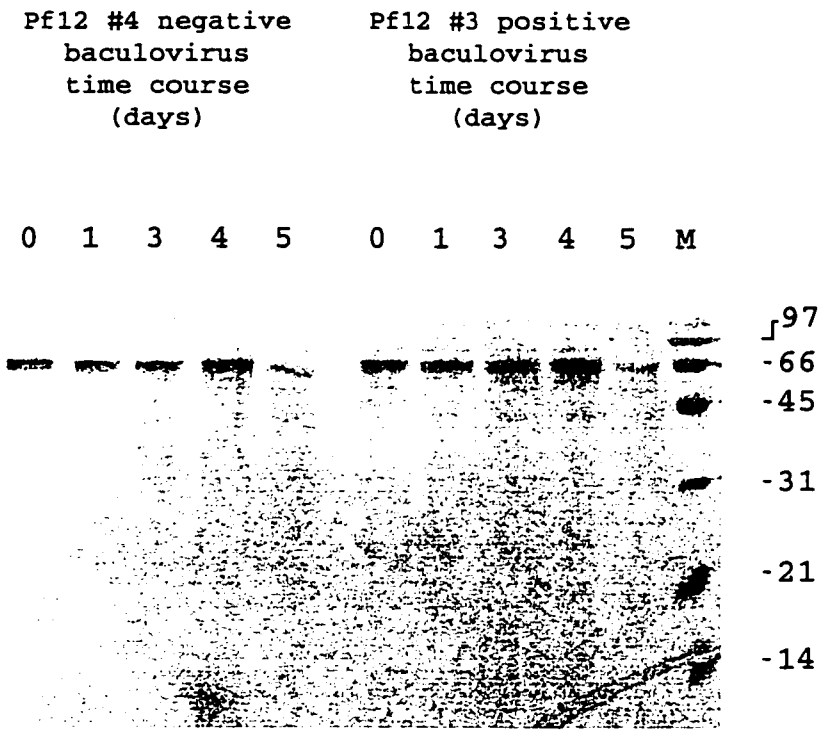
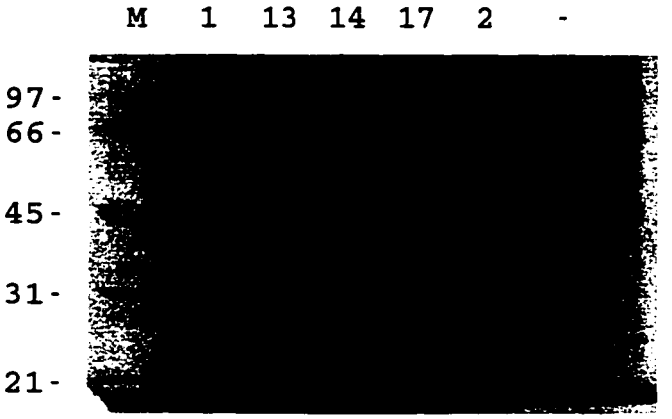


FIGURE A1.4. SDS-PAGE analysis of secreted proteins made by a recombinant baculovirus containing Pf12 DNA. In this experiment 150 µl of day 3 supernatant from Sf9 monolayers infected with either plaque-purified Pf12 dot-blot positive baculovirus (Pf12sec bv #3) or Pf12 dot-blot negative baculovirus (Pf12sec bv #4) was incubated with $6x10^5$ Sf9 cells. Supernatants (18 µl of 1ml total culture supernatant) from this infection were harvested at 0, 1, 2, 3,4, and 5 days and resolved by 12% SDS-PAGE. Proteins were visualized using Coomassie-blue staining. No bands corresponding to secreted Pf12 protein were seen in either supernatant. The position of low-range protein markers is indicated to the right.

A.



B.

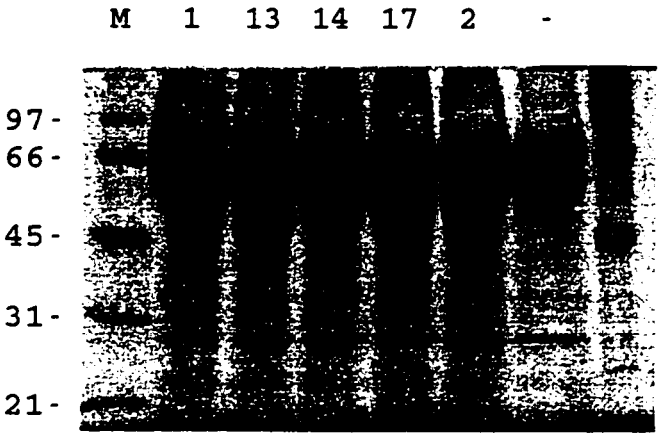


FIGURE A1.5. SDS-PAGE analysis of Sf9 cultures infected with Pf12 DNA positive recombinant baculoviruses. Sf9 cells (A; $1x10^5$ cell equivalents/ lane) and supernatants (B; 10 µl of 1ml culture supernatant/ lane) from cultures infected with recombinant Pf12sec DNA positive baculoviruses (Pf12 #1,3,14,17; from figure A1.3), recombinant Pf12sec DNA negative baculovirus (Pf12sec #2, figure A1.3) or no baculovirus (-) were harvested after 5 days of culture, solubilized in reducing SDS lysis buffer, resolved on 12% polyacrylamide gels, and visualized with Coomassie blue staining. Low range protein markers, M, are indicated to the left.

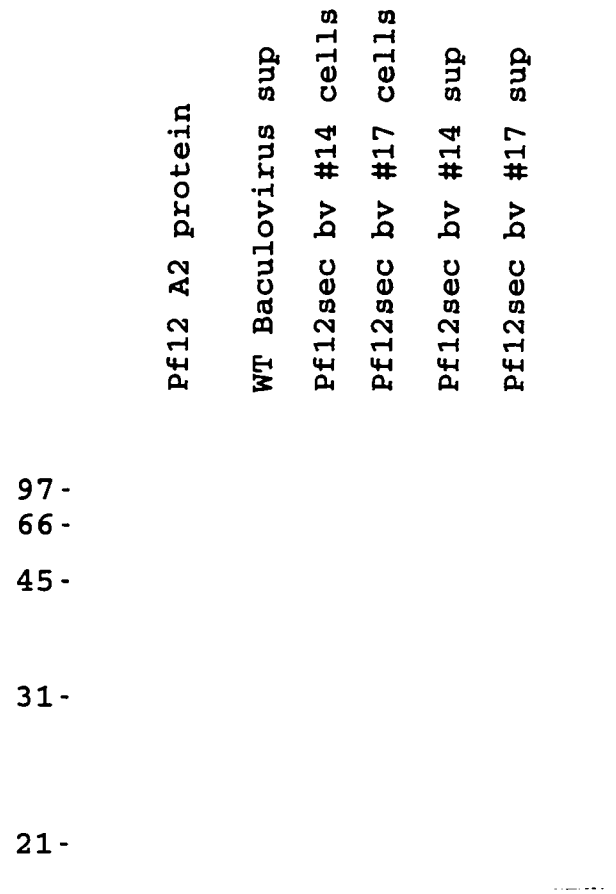


FIGURE A1.6. Western Blot analysis of proteins synthesized by recombinant baculoviruses Pf12sec #14 and #17 assayed using anti-A2 rat antiserum. Sf9 cellular and supernatant proteins from cultures infected with Pf12sec bv #14 and #17 recombinant baculoviruses were solubilized in SDS lysis buffer, resolved by 12% SDS-PAGE, and Western blotted with rat anti-A2 antiserum (1:400). Recombinant A2 protein from *E.coli* (chapter 3) and supernatant from baculovirus-infected Sf9 cells were included as positive and negative controls respectively. The secondary antibody used was a goat anti-rat alkaline phosphatase (1:1000), and protein bands were visualized using BCIP/NBT chromogenic reagents. The position of low-range protein markers is indicated to the left.

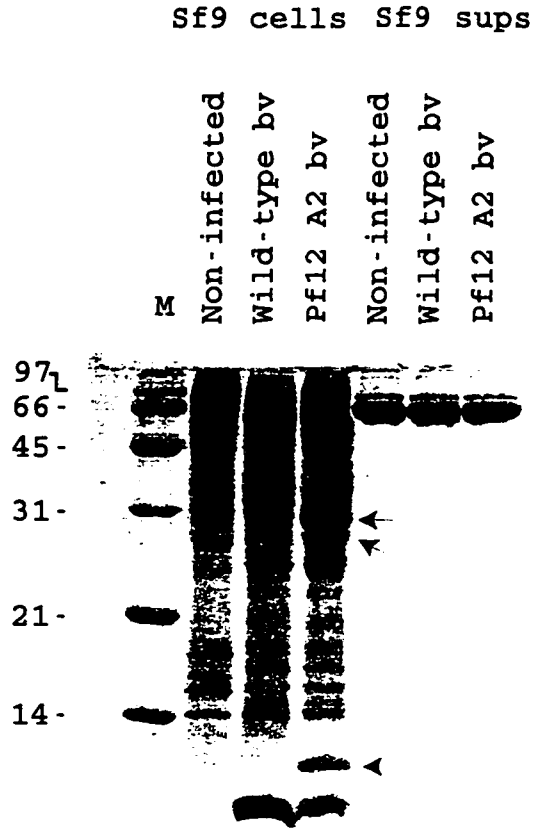


FIGURE A1.7. SDS-PAGE analysis of Sf9 cells infected with a recombinant baculovirus encoding the Pf12 A2 protein. Sf9 cellular ($1x10^5$ cell equivalents/lane) and supernatant (10 µl of 10 ml total culture supernatant) proteins from cultures infected with a Pf12 A2 recombinant bv were solubilized in SDS lysis buffer, resolved by 15% SDS-PAGE, and visualized with Coomassie blue staining. Negative controls included supernatants and cells from uninfected and wild-type bv-infected cultures. Arrows indicate recombinant Pf12 A2 proteins: 6 kDa (most likely a proteolytic fragment), 27 kDa, and 30 kDa. Low-range protein markers are indicated to the left.

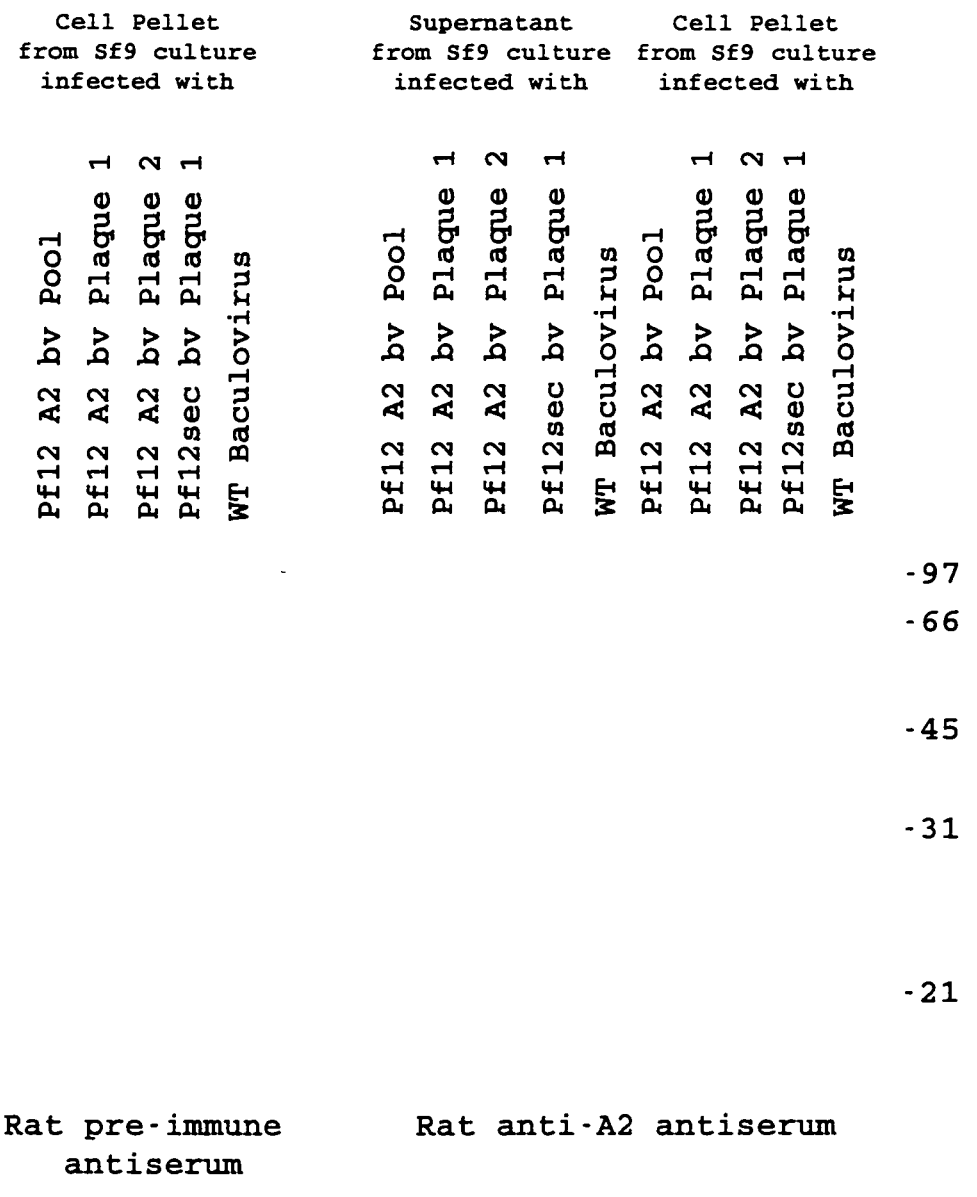


FIGURE A1.8. Western blot analysis of proteins synthesized by Pf12sec bv and Pf12 A2 bv recombinant baculoviruses probed with rat anti-A2 antiserum. Sf9 cells and supernatants from cultures infected with wild-type baculovirus, Pf12sec bv #1 (see figure A2.3), Pf12 A2 bv #1 or #2 (the latter two plaques chosen arbitrarily) were solubilized in SDS lysis buffer, resolved by 12% SDS-PAGE, blotted to nitrocellulose, and probed with either rat pre-immune or rat anti-A2 antiserum (1:1000 dilution). Only the Pf12 A2 bv #1 lane from the cell pellet shows a signal.

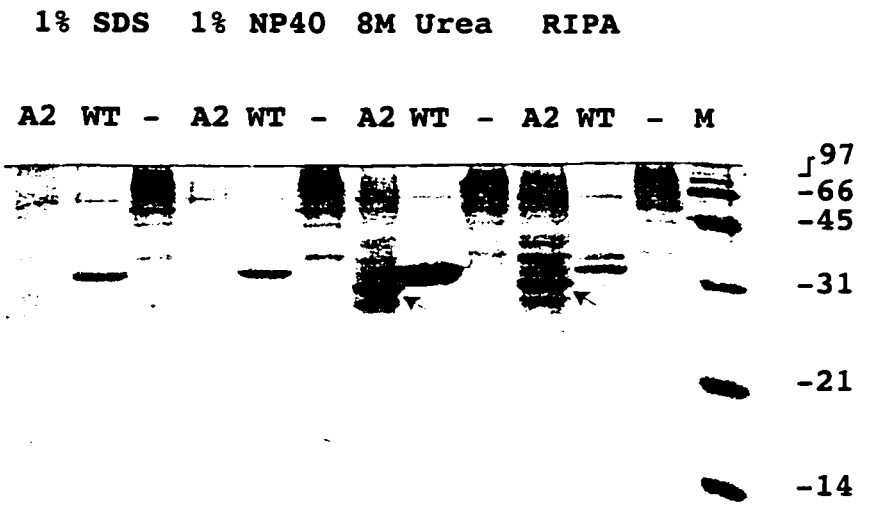


FIGURE A1.9. SDS-PAGE analysis of Pf12 A2 bv protein, testing its solubility in various denaturants and detergents. Sf9 cellular proteins from uninfected (-), Pf12 A2 bv-infected (A2), or wild-type bv-infected (WT) cultures were harvested and extracted with either 1% SDS, 1% NP40, 8M Urea, or RIPA buffer (contains deoxycholate; see chapter 2) at 4°C for 1 hour. Extracted proteins were separated from cells by centrifugation in microfuge, the supernatant added to SDS lysis buffer, resolved by 15% SDS-PAGE, and visualized by Coomassie blue staining. Arrows indicate the Pf12 A2 protein doublet. Low-range protein markers are indicated to the right.

APPENDIX A2

ATTEMPTS TO GENERATE ANTI-Pf12 ANTISERUM BY DNA IMMUNIZATION IN MICE AND RATS

A. Introduction and background

After encountering difficulties expressing wild-type and subfragments of secreted Pf12 in baculovirus, I concentrated on another method to generate antiserum specific for Pf12 protein that was made in a eukaryotic environment: DNA Immunization. This method was attractive because the process of producing, purifying, and renaturing a recombinant protein could be completely circumvented. Instead, generating antiserum specific for a protein could be accomplished by immunizing the test animal with a eukaryotic expression plasmid containing the gene encoding the target protein, relying on the mouse cells to produce this foreign protein themselves, and then the mouse immune system to mount a response to it. In other words, the main problem I had been encountering, namely production of recombinant Pf12 in a native form, could be circumvented by using the mouse cells to make the protein themselves *in vivo*. Even though Pf12 was not expressed at detectable levels in baculovirus and *E. coli* expression systems, I still had reason to believe that these experiments would work because the original Pf12 gene had been cloned by COS cell panning, a process that relied heavily on the production of native parasite proteins by COS cells, and the recognition of this protein by antiserum from individuals continually exposed to malaria parasites.

Intramuscular injection of DNA in animal models results in the uptake of DNA into muscle cells and the expression of the protein encoded by the DNA . These kinds of experiments were first described by J.B. Ulmer and colleagues (Ulmer et al., 1993) at Merck Research Laboratories, who immunized Balb/c mice with plasmid DNA encoding influenza A nucleoprotein, generating both humoral and cellular responses to the nucleoprotein. DNA-immunized mice were subsequently protected from challenge with a

heterologous strain of influenza. The implications of these experiments were far-reaching. From an entirely practical viewpoint, DNA immunization could be used to generate antiserum specific for any protein encoded on an injected target gene. Although some years have passed since the original DNA immunization experiments were conducted, the complete story of the uptake and intracellular fate of injected DNA has not been elucidated. Even less knowledge existed about the best promoters for DNA immunization, so I began these experiments by creating my own vector for DNA immunization, and then using an established method to immunize rats and mice with DNA.

B. Results

pPSC1, DNA immunization vector containing a CMV IE1 promoter

I created vector pPSC1 (containing polyoma origin, SV40 origin, and the CMV IE1 enhancer/promoter) for DNA immunization, and for high levels of gene expression in cells transformed with SV40 or Polyoma viruses. The CMV IE1 enhancer/promoter is one of the strongest eukaryotic promoters known, being active in a broad range of host cells (Dubensky et al., 1984). In addition, other regions upstream of the IE1 gene contain transcriptional signals that may favor gene expression in muscle cells (Dubensky et al., 1984), the common target tissue for DNA immunization. Finally, the first intron of the IE1 gene, intron A, has been shown to have a positive effect on gene expression. Using primers specific for the upstream regions of the CMV IE1 gene, I amplified a 1678 bp fragment (figure A2.1B) containing the elements described above and inserted it into a vector backbone containing a polylinker for insertion of target genes, an SV40 polyadenylation signal, a pUC origin and beta-lactamase gene for growth in bacteria under ampicillin selective conditions, and polyoma and SV40 origins for plasmid amplification in polyoma and SV40-transformed cell lines. In later work I created another DNA immunization vector, pPSC2DAF, that can be used to produce both anchored and secreted

forms of the target protein (described in figure B.2, Appendix B). Even though this vector was intended for DNA immunization, it is also a excellent general eukaryotic expression vector, with a promoter strong enough to produce significant amounts of target protein in many different kinds of cells.

Initially I compared three vectors, pPSC1/CAT, pPyEF-1α/CAT, and pRSV/CAT (see figure B.1, Appendix B), for their ability to express chloramphenicol acetyl transferase (CAT) when injected into mouse quadricep muscles. The CAT reporter gene from the plasmid CAT 3MS (a gift from Dr. L-J. Chang) was modified by PCR to contain 5' Xba I and 3'Not I sites, inserted into pPSC1, and tested to ensure that CAT activity had been maintained. The CMV promoter was then removed from this vector by Sal I/Xba I digestion, and a new promoter, the human polypeptide chain elongation factor 1 α (EF-1α), originally from the vector pEF-BOS (Mizushima and Nagata, 1990), was inserted in its place to create pPyEF-1α/CAT. pRSV/CAT was is described in (Gorman, 1985).

I used these three plasmids to determine which promoter gave the strongest expression of CAT after DNA immunization (table A2.1). I immunized the quadriceps of Balb/c mice (n=5) with 40 μg of either pPSC1/CAT, pPyEF-1α/CAT, or pRSV/CAT plasmids mixed in a 10% Ink/saline solution. This solution allowed me to ensure that I had properly injected the DNA into the quadricep as opposed to subcutaneously, and to roughly monitor the amount of solution that reached the quadricep later on in the experiment. Four days after injection, the mice from each group were euthanized and I removed the mouse quadriceps for further study. I examined the degree and pattern of ink staining, and then homogenized each quadricep, normalized each homogenate for protein content, and assayed each homogenate for CAT activity, using a standard protocol described in the materials and methods section. CAT activity, as quantitated using a Fuji phosphorimager and Mac-BAS software, was revealed to be highest on average for quadriceps injected with pPSC1/CAT. This experiment suggested that the pPSC1 was the best plasmid for expression of genes via DNA immunization (table A2.1).

Pf12 DNA immunization in rats

The first Pf12 DNA immunization experiments in rats were conducted using the wild-type Pf12 gene inserted into the expression vector described above, pPSC1 (figure A2.2). A murine IL-10/pPSC1 construct was used as a positive control in these experiments. Because it was low molecular weight secreted protein sufficiently different from rat IL-10, it was hypothesized that this protein would make a good immunogen. In addition, the cytokine properties of mIL-10 in a rat microenvironment might promote a TH2 response, consequently stimulating production of specific antibodies. pPSC1 vector alone was used as a negative control. In Sprague Dawley rats immunized with the mIL-10/pPSC1, but not the Pf12/pPSC1 or pPSC1 construct alone, a specific antibody response was detected. Specificity of rat antiserum collected after four DNA immunizations for either Pf12ec A2 or recombinant mIL10 protein was measured by ELISA. Titres of rat antiserum specific for mIL-10 were observed to be significantly higher than preimmune and pPSC1 controls. By contrast, titres of Pf12ec A2-specific antiserum from rats immunized with Pf12/pPSC1 or pPSC1 alone were not significantly different from each other or pre-immune controls. In Western blotting experiments, antiserum collected from mIL10/pPSC1 immunized rats recognized an 18 kDa protein, comparable to the predicted molecular weight of mIL10 (figure A2.4). Antiserum from rats immunized with Pf12/pPSC1 did not recognize Pf12 A2 protein in Western blotting experiments (figure A2.3). In summary, this first attempt to elicit Pf12 specific antibodies through DNA immunization had failed, but the protocol itself was effective for generating antibodies specific to mIL10.

Immunization of rats with Pf12sec, and Pf12 A2 DNA sequences

A second set of DNA immunization experiments in rats was conducted using new PCR-engineered constructs described in figure A2.5. The wild-type Pf12 sequence lacking

an its native anchor signal, Pf12sec, and the A2 sequence of Pf12 (see figures 3.2 and 4.2 A) containing an N-terminal T1/leu-1 secretion signal, were inserted into pPSC1. These new Pf12 constructs were chosen because the secreted versions of the proteins may be more accessible to immune surveillance than the anchored versions, and because the A2 sequence had been identified as a portion of Pf12 that could be expressed at detectable levels in *E. coli* and baculovirus expression systems.

In this new experiment, 16 Sprague Dawley rats were immunized in the same way as the previous experiment. After four immunizations, antiserum was collected from each rat and assayed for specific binding to either Pf12ec A2 recombinant protein in ELISA experiments (figure A2.6), or native Pf12 expressed on the surface of pPSC1-transfected COS cells (table A2.2).

Unfortunately, this experiment failed to generate Pf12 specific rat antiserum. No significant difference was seen in ELISA titres of antiserum from rats immunized with Pf12sec or A2/pPSC1 constructs, pPSC1 vector controls, or pre-immune control antiserum. Immunofluorescence experiments using Pf12sec/pPSC1 and pPSC1-transfected COS cells revealed no significant differences in staining profiles between the control and test experiments. In a further attempt to determine if any of the rats produced Pf12 specific antibodies, lysates from COS cells transfected with Pf12/pPSC1 or pPSC1 vector alone were resolved by SDS-PAGE and western blotted with antiserum collected from each of the 16 rats immunized. Again, no significant differences were seen between control and test antiserum (data not shown).

Injection of mice with a mixture of Pf12 DNA and a recombinant adenovirus expressing IL4

New DNA immunization experiments were designed using Balb/c mice as test animals, and recombinant IL-4 adenoviruses to provide an adjuvant effect. IL4 is a potent cytokine that promotes the development of Th2 (T helper 2) subsets (Mosmann et al.,

1986; Cherwinski et al., 1987). Th cells activated *in vitro* by antigen such as ovalbumin in the presence of IL-4 will develop into Th2 cells, secreting IL-3,4,5,10, and 13, and providing help for total antibody production. Recombinant IL-4 producing adenoviruses coinjected with DNA, would be taken up by the same cells in the microenvironment of the muscle, and newly produced IL-4 could exert its effects close to where the DNA was expressed and therefore affect the local immune response to the newly expressed protein. If the other DNA immunization experiments had failed because Pf12 was only being expressed in small amounts, or was not very immunogenic, it was hypothesized that the presence of the IL-4 secreting adenoviruses might help to overcome this lack of reactivity.

Balb/c mice (n=5 for each different plasmid) were immunized intramuscularly (quadriceps) with 40 μg of each of the following constructs pPSC1, Pf12/pPSC1, Pf12sec/pPSC1, A2sec/pPSC1 in 16% Sucrose /PBS. In parallel experiments, mice were immunized similarly with the above constructs and approximately $7x10^7$ PFU of IL-4-producing adenoviruses (Ad 5 IL4). In addition, one group of mice was immunized with $7x10^6$ PFU of recombinant beta-gal producing adenovirus (Ad 5 beta-gal) and pPSC1 vector. After three immunizations spaced approximately four weeks apart, antiserum was harvested from each group and tested for specificity for Pf12 protein.

Testing for production of anti-Pf12 antibodies using biotinylated Pf12 expressed in COS cells

A new method for testing the specificity of the antiserum for biotinylated Pf12 expressed by Pf12/pPSC1-transfected COS cells was developed. COS cells expressing Pf12 were biotinylated using a commercial reagent, NHS-LC-Biotin, selective for surface proteins of intact cells. Some biotinylation of cytoplasmic proteins may also occur if cells have lysed before exposure to NHS-LC-Biotin, or due to minor amounts of diffusion of the reagent across the cell membrane. Biotinylated cells were solubilized in a 1% NP40 lysis buffer, and recovered biotinylated membrane proteins were immunoprecipitated by the

mouse antiserum. This immunoprecipitated protein was resolved by SDS-PAGE, Western blotted with streptavidin-horseradish peroxidase, (SA-HRP), and visualized by chemiluminescence. I believed this method was superior to ELISA and Western blotting methods used previously because the biotinylated Pf12 was probably more native than the Pf12ec A2 denatured protein used in previous experiments, while the sensitivity of the assay was not compromised. I was concerned that any Pf12 protein produced as a result of DNA immunization might be present in a native conformation, and consequently any antibody response generated would be to "native" epitopes. These antibodies therefore may not recognize the truncated, denatured Pf12ec A2 used in previous ELISA experiments, or the denatured Pf12 used in Western blots. It was sensitive enough, by virtue of the strong bond formed between the biotinylated protein and the SA-HRP indicator that minor but significant signal that might not normally be noticed in immunofluorescence experiments might be detected by blotting with SA-HRP and visualizing by chemiluminescence.

The results of both the new DNA immunizations (figure A2.7) and DNA/adenovirus co-immunizations (figure A2.8) showed that antibodies specific for Pf12 were not generated. The specificity of antiserum from Pf12 DNA-immunized mice (Pf12/pPSC1, Pf12sec/pPSC1, and A2/pPSC1) for a number of proteins ranging from 49.5 kDa to 116.5 kDa, was not significantly different from the specificity of post-immune antiserum from pPSC1-immunized mice. Two positive controls were included in this experiment: (a) the antiserum used to clone Pf12 originally, PNG antiserum, immunoprecipitated a number of proteins ranging from 27.5 kDa to 205 kDa in a profile similar to that described in the original experiments by Elliott and colleagues (Elliott et al., 1990); (b) anti-CD36 monoclonal antibodies were shown to immunoprecipitate CD36 from lysates of biotinylated CD36-transfected COS cells (figure A2.7). In addition to the immunoprecipitation experiments, no specific staining of Pf12-transfected COS cells with

antiserum from DNA-immunized mice was seen in immunofluorescence experiments (table A2.2).

The results from the DNA/adenovirus co-immunizations (figure A2.8) show a similar lack of specific antibody response to biotinylated Pf12. In these experiments the post-immune response to biotinylated COS proteins seems to be weaker, although one 90 kDa protein is consistently immunoprecipitated by the post-immune antiserum of mice immunized with pPSC1/IL4-adenovirus, Pf12sec/pPSC1/IL-adenovirus, and A2 /pPSC1/IL4-adenovirus. This protein is not immunoprecipitated by the antiserum of mice immunized with pPSC1/beta-Gal-adenovirus or Pf12/pPSC1IL4- adenovirus. Immunofluorescence experiments with the antiserum from DNA/adenovirus coimmunized mice did not show any specific staining of Pf12-transfected COS cells (table A2.2).

C. Discussion

In this chapter DNA immunization was used in an attempt to generate antibodies specific for Pf12. This method was attractive because antibodies could be obtained without having to produce and purify recombinant protein for immunization, a problem I had been struggling with. Although initial pilot experiments with CAT plasmids and positive controls confirmed that the DNA injection technique worked, I was unable to generate Pf12-specific antibodies by DNA immunization in rats or mice.

Initially I created a vector especially for the purpose of DNA immunization, called pPSC1. This vector contained the CMV IE1 promoter which directs the expression of proteins at high levels in a number of different cell types, and has regulatory elements that may specifically upregulate protein expression in muscle cells, which were the target cells for my experiments. I conducted experiments measuring the amount of CAT made under the control of three different promoters, each of which had been described in the DNA immunization literature. Comparisons between the RSV, EF-1alpha and CMV promoters indicated that in mouse muscle cells injected with CAT plasmid DNA, CAT expression

under the control of the CMV promoter was significantly higher than expression from the other promoters.

The first DNA immunization experiments in rats showed that no specific response to Pf12/pPSC1 was generated, even after four immunizations. I had chosen the wild-type Pf12 DNA for these experiments, along with mouse IL10 DNA as a positive control. I assayed for anti-Pf12 and anti-mIL10 antibody production by ELISA and Western Blotting using Pf12ec A2 and recombinant IL10 as the binding targets for antibodies generated by DNA immunization. Antibodies specific for mIL10, but not Pf12ec A2 were detected. The specificity of these antibodies was confirmed by Western blotting. In the case of Pf12, I was concerned that if antibodies were being generated to properly folded Pf12 protein, they may not recognize the reduced, denatured Pf12ec A2 used in the above experiments. I therefore chose to use Pf12-transfected COS cells to assay for Pf12-specific antibodies in immunofluorescence experiments. These cells were not stained significantly by the DNA immunization antiserum.

New DNA immunization experiments were conducted with Pf12sec/pPSC1 and Pf12 A2/ pPSC1, the secreted version of A2 described in figure A2.5. Secreted versions of Pf12 were chosen for this experiment because these proteins may be made in larger quantities, and more accessible to the immune system because they are secreted from the cell. A2 protein was also chosen because it had been expressed successfully in both *E. coli* and baculovirus where attempts to express the full length version of the protein had failed. These experiments however, like the previous ones, failed to generate Pf12-specific antibodies as measured by ELISA and immunofluorescence. Some steps in the process where problems might have occurred include: the inability of rat muscle cells to produce Pf12, or the death of cells that did express the protein, the retention and degradation of Pf12 in muscle cells, preventing the protein from stimulating an immune response, or the stimulation of a cellular as opposed to a humoral immune response to Pf12. It is possible

that the aggregation or improper folding of Pf12 in both prokaryotic and eukaryotic cells was a major factor in the failure of the DNA immunization experiments.

I decided to conduct one final experiment based on the assumption that the amount of Pf12 produced by the muscle cells was not enough to stimulate an immune response (either because only a small amount of Pf12 was produced before the cells died, or because this protein was degraded rapidly). Using Balb/c mice for immunization, I co-administered pPSC1 plasmid DNA encoding either Pf12, Pf12sec, or Pf12 A2 and recombinant adenoviruses that constitutively expressed IL4. I included the recombinant adenovirus to provide an adjuvant effect: by targeting the same cells as the Pf12 plasmids and secreting IL4 in the same microenvironment as Pf12 protein, this virus could theoretically promote a humoral or Th2 response against Pf12. In previous experiments enough Pf12 may not have been present to stimulate this response; I hoped the co-injection of adenoviruses expressing a Th2 cytokine would help 'tip the balance' in favor of the generation of antibodies with specificity for Pf12.

To increase the sensitivity of detection of anti-Pf12 antibodies, I used biotinylated Pf12 protein in immunoprecipitation experiments instead of denatured Pf12ec A2 protein in ELISA experiments. This involved transfecting COS cells with Pf12/pPSC1, biotinylating the intact cells with NHS-LC-Biotin, solubilizing the membranes, and using the labelled membrane proteins for immunoprecipitations. Fortunately, only a small fraction of cytoplasmic proteins are labelled by this reagent, which does not readily traverse intact membranes. This meant that Pf12 protein would constitute a greater fraction of the total labelled protein. Antiserum from DNA/adenovirus-immunized mice was used to immunoprecipitate these biotinylated proteins, which were then resolved by SDS-PAGE and Western Blotted with Streptavidin-HRP. The strength of the Streptavidin-biotin bond, coupled with the native conditions of the immunprecipitation, greatly increased the sensitivity of this assay for anti-Pf12 antibodies over ELISAs, Western blots, and even immunofluorescence. One concern with previous experiments had been that denatured Pf12

was used in ELISA and Western blot experiments to detect an antibody that may be specific for native epitopes. Moreover, the use of Pf12-transfected COS cells to detect these antibodies by immunofluorescence may have failed because of the lower sensitivity of these assays. The biotinylation -immunoprecipitation method united the best aspects of both Western blot and immunofluorescence assays: the sensitivity of chemiluminescent detection and the use of native Pf12 protein as a target.

The results from these experiments indicated that detectable anti-Pf12 antibodies were not produced in DNA-immunized or DNA/adenovirus IL4 co-immunized mice. If anti-Pf12 antibodies had been produced, biotinylated Pf12 should have been immunoprecipitated by postimmune antiserum from the DNA-immunized but not pre nor postimmune antiserum from the control-immunized mice. In figure A2.7, the postimmune antiserum from both the control and DNA-immunized mice immunoprecipitates a number of proteins not recognized by the pre-immune controls. Although a general increase in antibody specificity for biotinylated COS proteins is observed in this experiment, the post-immune pooled antiserum from mice immunized with the three different Pf12 constructs does not specifically immunoprecipitate a protein of the expected size, nor is the specificity of this antibody sufficiently different from the postimmune control. Also included in this experiment are immunoprecipitations of Pf12-transfected COS cells with PNG antiserum used originally to clone Pf12 by panning, and immunoprecipitations of CD36-transfected COS cells by a commercial monoclonal antibody. The PNG immunoprecipitation was intended as a positive control for the presence of biotinylated Pf12, and the CD36 immunoprecipitation a positive control for the biotinylation process itself. The PNG pooled antiserum, but not control human antiserum, immunoprecipitated a number of proteins similar in size to those seen in immunoprecipitations of ^{125}I-labelled Pf12 from Pf12-transfected COS cells (Elliott et al., 1990). This immunoprecipitation was not as clean as the one described in (Elliott et al., 1990), and it was difficult to a identify specific Pf12 bands. The results from the CD36 immunoprecipitation indicated that an 80 kDa protein

was immunoprecipitated, along with 35 and 42 kDa proteolytic products. These positive control experiments indicate that it is possible to use this biotinylation/immunoprecipitation method to detect antibodies specific for a particular protein, but whether the experiment worked in this case remains questionable due to the absence of a good positive control for Pf12 expression and immunoprecipitation.

The DNA/ adenovirus co-administration experiments also failed to generate Pf12-specific mouse antiserum. Although no anti-Pf12 antiserum was generated, results from this experiment (figure A2.8) suggest that the presence of IL4-secreting adenovirus did in some cases stimulate antibody production in these mice, and that the presence of wild-type Pf12 may have lessened this effect. Antiserum from mice immunized with pPSC1 vector and IL4- expressing adenovirus recognize a 90 kDa protein produced by Pf12-transfected COS cells, whereas mice immunized with a control adenovirus expressing betagalactosidase and pPSC1 do not. Although the identity of this protein remains unknown, it is similar in size to a major protein recognized by the antiserum from DNA-immunized mice in the previous experiment (figure A2.7). Co-immunization of the IL4 adenovirus with wild-type Pf12 DNA in mice does not generate antibodies specific for this protein, whereas co-immunization of IL4 adenovirus with Pf12sec or Pf12 A2sec does, but to a lesser extent than the control. Pf12 protein could either kill cells that express it, and decrease the amount of IL4 expressed if these cells also harbored the IL4 adenovirus, or it could have an immunosuppressive effect that counteracts IL4.

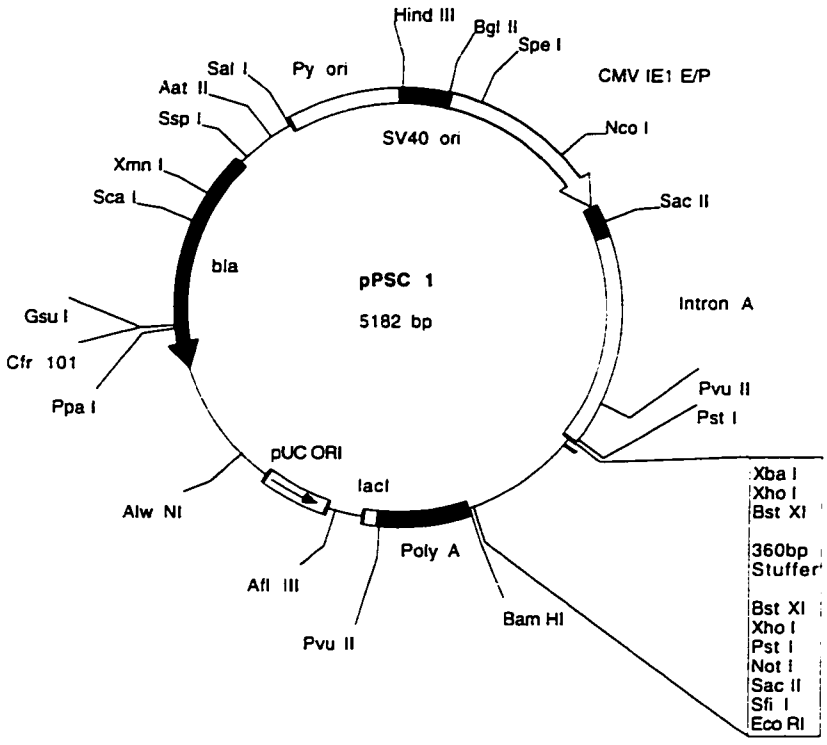
This particular method of detecting Pf12 has several drawbacks, the most important being the impurity of the biotinylated protein used to detect the antibodies. Because all of the surface proteins of COS cells transfected with Pf12 were biotinylated and harvested for immunoprecipitations, the presence of antibodies specific for COS cell proteins could obscure the interpretation of the results. In the first immunoprecipitation experiment (figure A2.7), a relatively large amount of protein was immunoprecipitated by both pre- and post-immune mouse antiserum. The intensity and variety of different proteins recognized by the

pre-immune antiserum suggests that these preparations could have been washed more stringently at the protein G-sepharose stage, before resolving by SDS-PAGE. The second experiment using DNA/adenovirus co-immunization is much cleaner in this respect. An obvious improvement on these experiments would have been the purification of the biotinylated Pf12 produced by COS cells. Although MRGS-His6-tagged Pf12 was not available at the time, it would have been ideal for transfection into COS cells, biotinylation by NHS-LC-Biotin, and purification using nickel affinity chromatography. Even small amounts of labelled, purified Pf12 would have been enough for these experiments. The streptavidin-biotin bond is one of the strongest molecular associations known, and, coupled with chemiluminescent detection, adds a level of sensitivity to these immunoprecipitations similar to that achievable using 125I-labelled protein. Thus, although the use of biotinylated proteins offered good sensitivity in detecting anti-Pf12 antibodies, the need for pure biotinylated Pf12 was underscored by the high background observed in these studies.

Of the experiments conducted in this chapter (and the rest of my thesis), the 'gold-standard' for detecting anti-Pf12 was immunofluorescence. The PNG antiserum used to clone Pf12 by panning, the anti-A2 and anti-B2 antiserum all strongly label Pf12-transfected COS cells. Although this method is not as sensitive as FACS analysis, it is easy to perform and interpret. The biotinylation/ immunoprecipitation experiment was a compliment to the immunofluorescence experiment. It is perhaps a more sensitive means of detecting anti-Pf12 antibodies, but if I could not detect labelling of Pf12-transfected COS cells by mouse antiserum initially, the titre of antibodies present was probably quite low. I had hoped that the biotinylation/ immunoprecipitation experiments would give me more information: instead of observing a positive or negative signal I could detect which proteins were immunoprecipitated, and if they were of the expected size. Regardless of the experiment, I included an immunfluorescence assay for confirmation. This data is presented in table A2.2, and it confirms that all DNA immunization experiments failed to

generate detectable amounts of antiserum that specifically stained Pf12-transfected COS cells with intensity comparable to rat-anti-A2 antiserum.

A.



B.

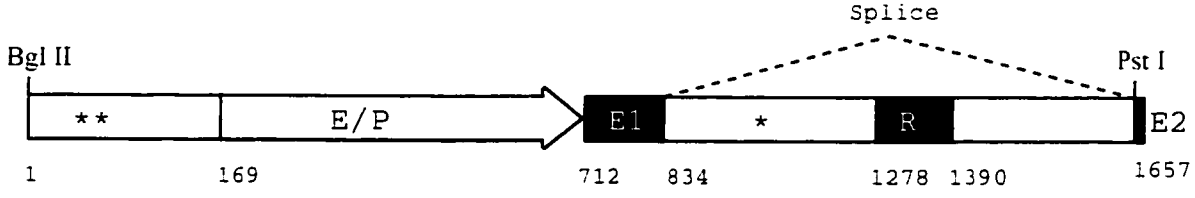


FIGURE A2.1. Map of pPSC1, a eukaryotic expression vector for DNA immunization. **A.** The pPSC1 eukaryotic expression vector consists of the following elements: a CMV IE1 enhancer/promoter including intron A (CMV IE1 E/P+Intron A, engineered using the primers CMV 5', CMV 3' detailed in table 2.1 and the Towne strain of CMV as a template) for high expression of insert DNA in many different cell types, SV40 and Polyoma origins (SV40 ori, engineered from pcDNA-Amp using primers SV40 ori 5' and 3' detailed in table 2.1; Py ori digested from pJFE14-Py) for maintenance of high plasmid copy number in cell lines transformed by replication defective SV40 or Polyoma viruses respectively, the pJFE14 polylinker, SV40 late region polyadenylation signal (Poly A, from pCSD6, a gift from Dr. D. Denney), pUC-derived vector sequences including the beta-lactamase (bla; from pCSD6) gene and the pUC origin of replication (pUC ORI; from pCSD6). **B.** The structure of the CMV IE1 enhancer/promoter/IntronA segment (bases 1-1678) is as follows: Positons 32, 86, and 1051, Nuclear factor 1 (NF1) binding sites (*); 169-650, complex enhancer/promoter region (E/P) containing many clustered Sp1, CRE/ATF, and NF-kB transcription factor binding sites; 712-834, IE1 Exon 1 (E1); 835-1657, IE1 Intron A; 1657-1678, IE1 Exon 2 (E2); 1278-1390, region of homology to regulatory elements contained in troponin I muscle specific enhancer (R).

TABLE A2.1. CAT activity in homogenates of quadricep muscles from mice immunized with three different CAT expression vectors.

Plasmid injected[#]	Promoter	Number of mouse quadriceps success -fully injected[‡]/ total quads injected	CAT activity[§] obtained from independent quad homogenates	CAT activity[†] Mean ± S.E.
pPSC1 negative control*	CMV	6/6	0.2, 0.27, 0.31 0.35, 0.45, 0.48	0.34 ± 0.04
pPSC1/ CAT*	CMV	6/6	1.01, 1.76, 1.96 3.07, 4.67, 7.23	3.28 ± .945
pPyEF/ CAT*	EF1alpha	5/6	0.19, 0.28, 0.35, 0.67, 1.47	0.592 ± 0.20
pRSV/ CAT*	RSV	5/6	0.08, 0.15,0.13, 0.17,0.24	0.154 ± 0.03

[#]Plasmid DNA was prepared using Qiagen columns. Both quadricep muscles on each mouse were injected with 40 µg of DNA in a 40 µl volume of 10% India ink/PBS. Four days later, mice were sacrificed and quadriceps harvested.

[‡]Quadriceps that were stained with India ink were considered to be successfully injected with the plasmid. Only 2 of the 24 quadriceps showed no India ink staining (in the pPyEf/CAT and pRSVcat groups). The CAT values from these quadricep homogenates were not used in this study.

[§]CAT activity was expressed as percent conversion to acetylated form, calculated using the formula: CAT activity = [acetylated chloramphenicol counts/ acetylated + nonacetylated chloramphenicol counts)] x 100. Acetylated and nonacetylated chloramphenicol counts from each reaction were quantitated by running TLC and using a Fuji phosphorimager. The protein concentration of each quadricep homogenate was determined by BCA assay and 100 ug of protein was used in each CAT assay.

*The CAT coding segment from CAT 3MS was engineered by PCR (see vector primers table 2.1) to have a 5' Xba I site and a 3' Not I site, and this was cloned into the corresponding sites in the vectors (described figures A2.1 and B.1). Note that this CAT construct has a eukaryotic consensus sequence for initiation. The pRSVcat vector was not changed from its original form.

[†]Mean and standard error values for each data set were calculated using Statview 512+ (Brain Power Inc, Calabasos CA). CAT activity values obtained for pPSC1/ CAT were significantly different from pPSC1, pPyEf/ CAT, and pRSVCAT ($P<0.01$; Scheffe F-test).

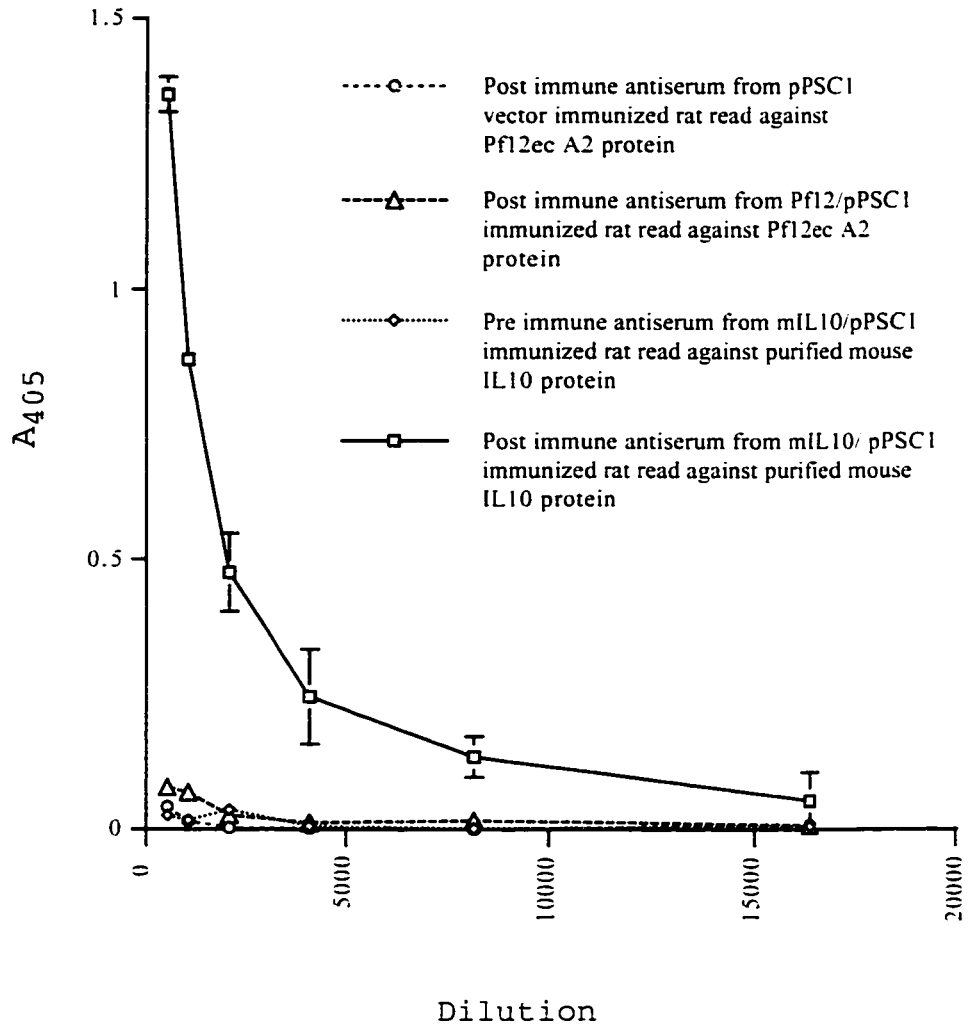


FIGURE A2.2. Antibody titres against Pf12ec A2 or against mIL10 in rats undergoing DNA immunization with either Pf12/pPSC1, or mIL10/pPSC1. Sprague Dawley rats were injected in both quadricep muscles each time, every 4-6 weeks using 100 μg per quadriceps muscle of either Pf12/pPSC1, mIL10/pPSC1, or pPSC1 vector control (1 mg/ml in PBS). Two weeks after the fourth inoculation antisera was collected and tested for recognition of either recombinant purified recombinant Pf12ec A2, or purified recombinant mIL10 using ELISA. Both pre and post-immune serum in pPSC1 immunized rats gave negligible signal against either Pf12ec A2 or mIL10, and only one of these titration curves is shown in the figure. Both pre and post-immune serum in Pf12/pPSC1 immunized animals gave negligible signal, thus only the post-immune result is shown above. There was also no signal against mIL10 in the Pf12/pPSC1 immunized animals, nor was there signal against Pf12ec A2 in the pPSC1/mIL10 immunized animals.

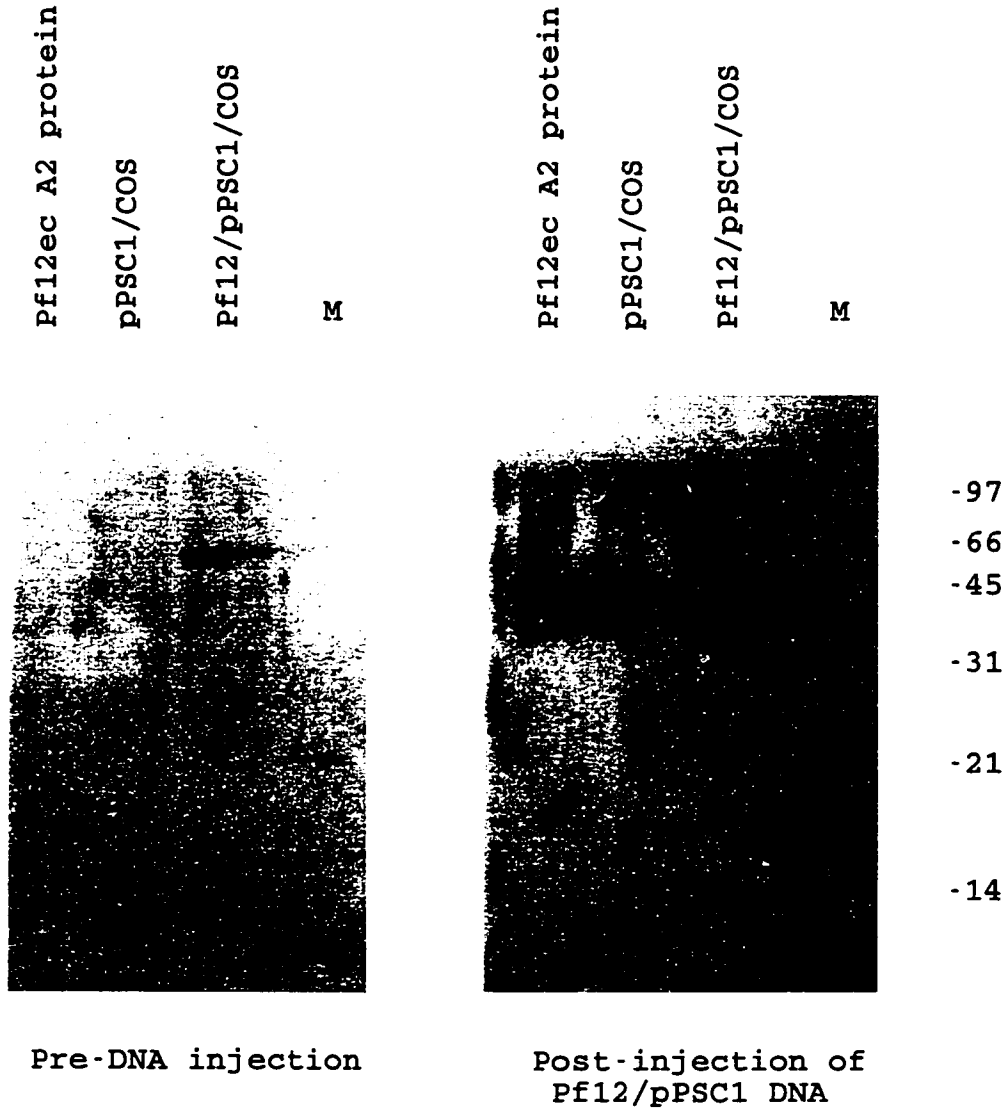


FIGURE A2.3. Western blot analysis of antiserum from Pf12/ pPSC1 DNA-injected rats (same post-immune serum as figure A2.2). Pf12ec A2 protein (500 ng/lane), lysates from pPSC1 and Pf12/ pPSC1-transfected COS cells (protein from approximately $1X10^5$ cells, 72 hours post-transfection) were resolved by 12% SDS-PAGE and Western blotted with pre and post-immune antiserum from Pf12/pPSC1 DNA-immunised rats. No specific signal was observed in these experiments. M, low-range protein marker lane; protein marker sizes are indicated to the right.

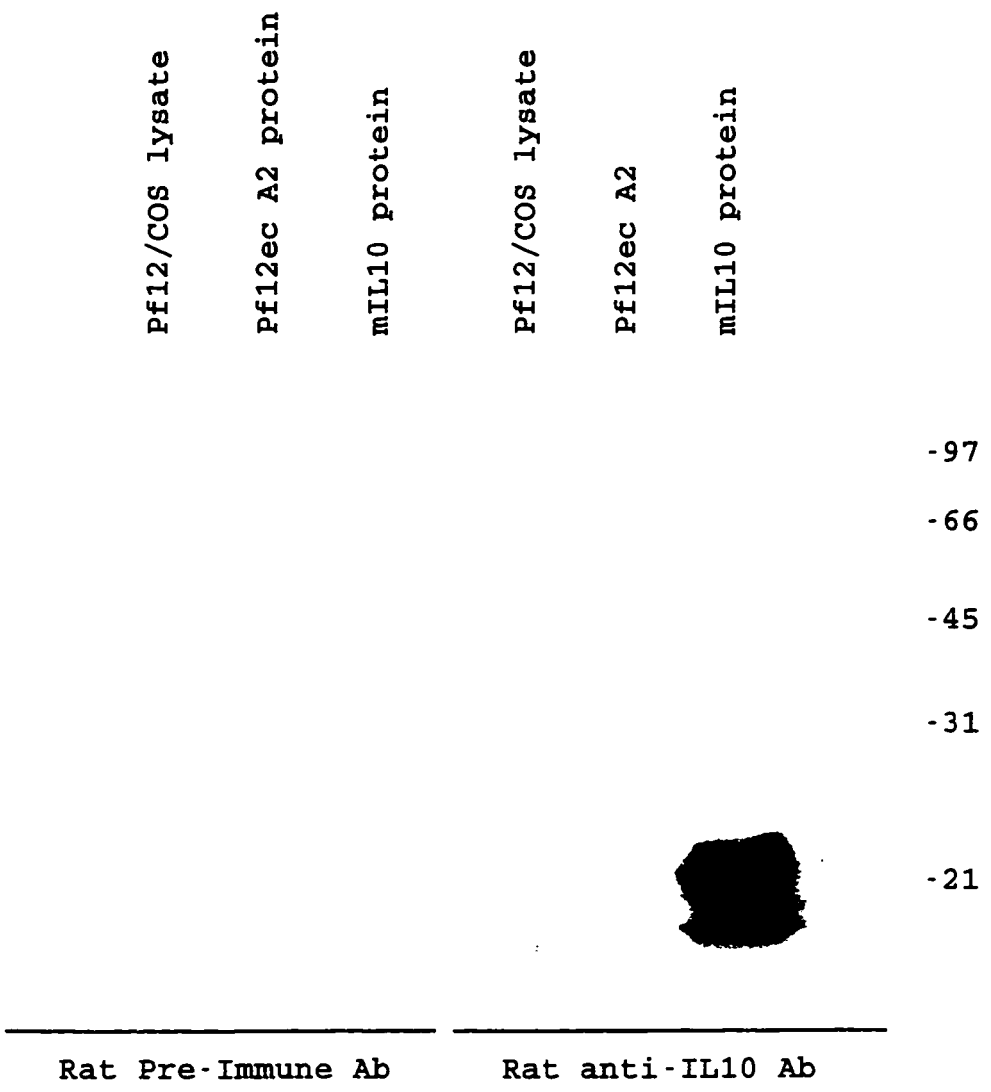
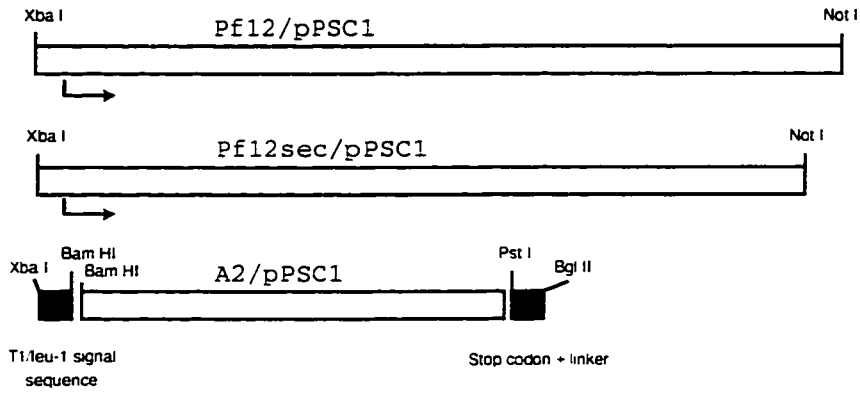


FIGURE A2.4. Western blot analysis of antiserum from mIL10/ pPSC1 DNA-injected rats (same post-immune IL10 serum as in figure A2.2). Pf12/pPSC1 transfected COS cells ($1x10^5$; 72 hours post transfection), Pf12ec A2 purified protein (700 ng/lane), or purified mouse IL10 protein (700 ng/lane) were resolved by 12% SDS-PAGE, Western blotted with either rat pre-immune or rat anti-mIL10 post-immune antisera (1:10,000), and then HRP conjugated goat anti-rat antibody (1:10 000). The blots were developed using the Amersham ECL kit. Low-range molecular weight marker sizes are indicated to the right.

A.



B.

Xba I

TCTAGA CCACC ATG CCC ATG GGG TCT CTG CAA CCG CTG GCC ACC TTG TAC CTG CTG

M P M G S L Q P L A T L Y L L

Bam HI

GGG ATG CTG GTC GCT TCC TGC CTC GGA GAA GTC GGA TCC **GAA GGG CAT AAA . . .**

G M L V A S C L G ↑ E V G S **E G H K**

FIGURE A2.5. Pf12 DNA constructs used in DNA immunization experiments. **A.** Pf12, and Pf12sec were cloned into pPSC1 digested with Xba I/Not I to generate Pf12/pPSC1 and Pf12sec/pPSC1 respectively. Pf12 encodes the wild-type protein with native signal and anchor sequences. Pf12sec encodes a secreted version of the wild-type protein (modified by PCR using BxBv5' and Pf12 Bv 3' primers described in table 2.1, and figure A1.2). Pf12 A2 encodes a secreted version of the A2 protein described in chapters 3 and Appendix 1 (figure 3.2 and A1.2 A). Pf12 A2/pPSC1 was created by a three piece ligation of the fragments: Xba I/ Bam HI cut T1/leu-1 signal (shaded box; T1/leu-1 signal was engineered by PCR using 5'Sig2, 3'Sig1 primers, and the plasmid YPO4 as a template), Bam HI/ Bgl II cut A2 fragment from Pf12 A2 bv/ pAcGP67 (figure A1.2) and Xba I/ Bam HI digested pPSC1 (Bgl II and Bam HI have complementary cohesive ends; both sites are destroyed on ligation). The ligation of the fragments in the proper orientation was confirmed by PCR using the primers Sig2 5' and Pf12 3' 2A (see table 2.1). The pAcGP67 polylinker sequences that were transferred to pPSC1 are indicated by the closed box. **B.** Junction between the T1/leu-1 signal sequence and A2 sequence. The DNA sequence of this region is shown with the encoded protein sequence below. The A2 (i.e. Pf12) sequence is indicated in bold; the signal cleavage site is indicated by an arrow.

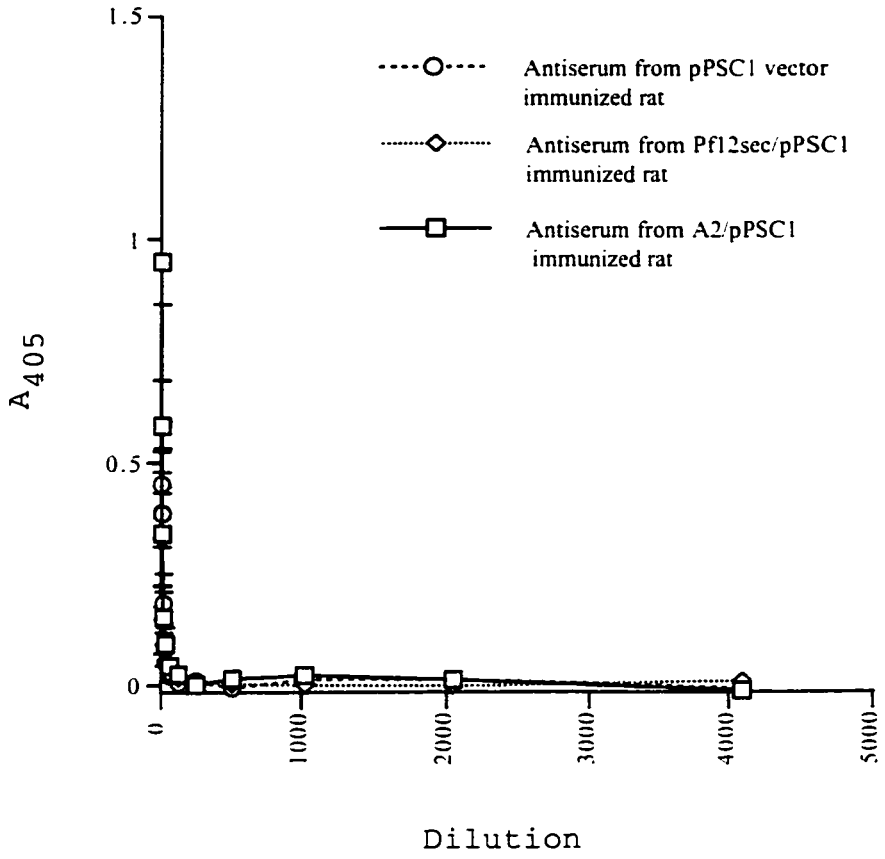


FIGURE A2.6. Antibody titres against Pf12ec A2 protein in rats injected with DNA constructs expressing secreted or truncated forms of Pf12. In this experiment, two different Pf12 sequences were cloned into pPSC1 for DNA immunization: A2/pPSC1 containing the sequence for the truncated version of Pf12, A2, which could be produced in *E.coli* and baculovirus with relatively low toxicity, and Pf12sec/ pPSC1, containing the entire Pf12 sequence less the membrane anchor region (see figure A2.5). Each injection consisted of 100 µg of DNA (1mg/ml in PBS) in each quadricep muscle, and injections were repeated every two to four weeks. Antiserum was collected 10-14 days after the fourth DNA injection, and tested by ELISA for binding to purified Pf12ec A2.

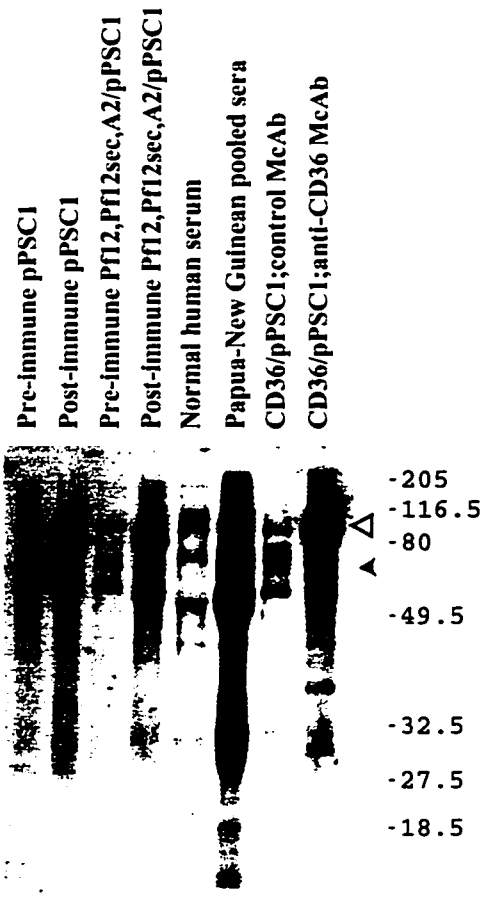


FIGURE A2.7. Immmunoprecipitation of surface biotinylated proteins from Pf12-transfected COS cells using a pool of antisera from mice immunized with either Pf12, Pf12sec, or Pf12 A2/pPSC1 constructs. Mice were immunized by injecting 40 μg of DNA (2 mg/ml in 20% sucrose/PBS) in each quadricep. Two weeks after the fourth injection, antiserum was harvested by tail bleed. For each immunoprecipitation, $1x10^8$ COS cells were transfected using DEAE dextran with either 100 μg of Pf12 /pPSC1 or CD36/pPSC1. Transfected cells were grown for three days, then surface-biotinylated with NHS-LC-Biotin and extracted with 0.1% NP-40. Extracts of these cells were used in immunoprecipitation experiments. After preclearing the extracts with pansorbin, they were incubated with pooled antisera (1:1000) from mice (5 per group) immunized with either one of the three different Pf12/pPSC1 constructs, or with control antisera from mice immunized with pPSC1 alone. As a positive control, extracts from CD36/pPSC1-transfected COS cells were incubated with either anti-CD36 monoclonal antibody (CLB-CD36, 1:1000), or anti-GAD control monoclonal antibody (GAD-6, 1:1000). Protein-antibody complexes were immunoprecipitated with Protein G-Sepharose, resolved by 12% SDS-PAGE, Western-blotted with streptavidin-horseradish peroxidase, and bands were visualized by chemiluminescence (ECL). Arrow indicates a glycosylated form of Pf12 brought down by human immune sera, similar to that obtained previously by Elliott et al., using surface iodination. Open arrowhead indicates CD36, an 88 kDa glycoprotein.

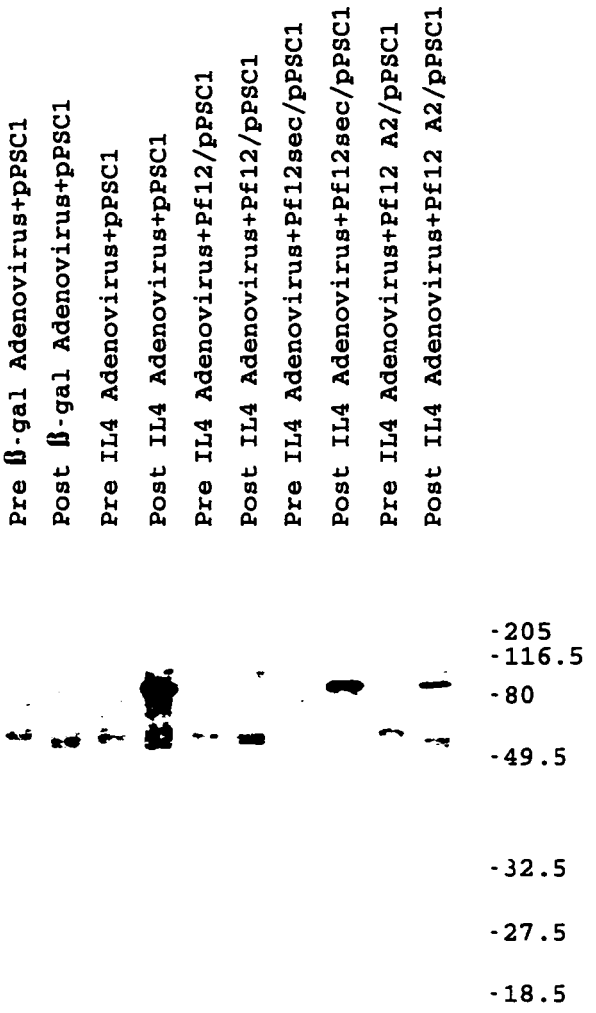


FIGURE A2.8. Immmunoprecipitation of biotinylated proteins from Pf12-transfected COS cells using antisera from mice immunized with a mixture of DNA plus recombinant adenovirus expressing beta-galactosidase or murine IL4. Mice were injected in each quadriceps muscle with 20 µl of a pre-mixed stock of DNA and virus (2 mg/ml DNA, $1x10^9$ Pfu/ml adenovirus in 20% sucrose/PBS) on the first immunization only. Thereafter only the DNA was injected every 2-4 weeks in both quadriceps muscles. Two weeks following the third injection, serum was harvested by tail bleed. Each pre- and post-immune serum is pooled from a group of 4-5 mice. Two recombinant adenoviruses were used in these experiments, one expressing a nuclear localizing form of beta-galactosidase (Ad 5 beta-gal), the other expressing high levels of mouse IL4 (Ad 5 IL4, both gifts from Dean Smith). COS cells were transfected with Pf12/ pPSC1, surface biotinylated, immunoprecipitated, and Western blotted as described in figure A2.7.

TABLE A2.2. Summary of immunofluorescence results obtained using Pf12-transfected COS cells and staining with antisera from rats and mice immunized with various DNA constructs +/- recombinant adenovirus expressing IL4.

Antiserum		Staining of Pf12-transfected COS cells*
DNA Immunization Experiment #1 rats (n=2/group)		
pPSC1	Pre-immune pool	+/-
	Post-immune pool	+/-
Pf12/pPSC1	Pre-immune pool	+/-
	Post-immune pool	+/-
rat anti-A2	Post-immune	+++
DNA Immunization Experiment #2 rats (n=4/group)		
pPSC1	Post-immune pool	+/-
Pf12 A2/pPSC1	Post-immune pool	+/-
Pf12sec /pPSC1	Post-immune pool	+/-
rat anti-A2	Post-immune	+++
DNA Immunization Experiment #3 mice (n=5/group)		
pPSC1	Post-immune pool	+
Pf12/pPSC1	Post-immune pool	+
Pf12sec /pPSC1	Post-immune pool	+
pPSC1/IL4 Ad	Post-immune pool	+
Pf12/pPSC1/IL4 Ad	Post-immune pool	+
Pf12sec /pPSC1/IL4 Ad	Post-immune pool	+
Pf12 A2/pPSC1/IL4 Ad	Post-immune pool	+
rat anti-A2	Post-immune	+++

Strong Ab staining ++++
No Ab staining -

*Immunofluorescence assay was conducted as described in chapter 2. Transfected cells were stained with a 1:200 dilution of the various antisera, washed in PBS/ EDTA, and then labelled with FITC conjugated goat anti-mouse or goat anti-rat antibody at a 1:200 dilution. Cells were washed again, mounted onto slides and observed using a Leitz UV microscope (400X magnification). The rat anti-A2 antisera is from chapter 3.

APPENDIX B

REPORTER CONSTRUCTS AND EXPRESSION VECTORS

The chloramphenicol acetyl transferase (CAT) reporter constructs used in the Appendix A2 (table 5.1) and expression vectors pPSC2DAF (related to pPSC1, described in figure A2.1), pBT1TØ (figure 4.2) and pBJT2 (figure 4.11) are described in further detail in this Appendix. The CAT constructs were created for detemine which of three different promoters directed the highest levels of gene expression in DNA immunization experiments. The expression vectors were created especially for the characterization of parasite proteins using prokaryotic expression in *E. coli* (pBT1TØ), or eukaryotic expression in COS cells, stably transfected cell lines, and DNA immunization immunization experiments (pPSC2DAF, pBJT2).

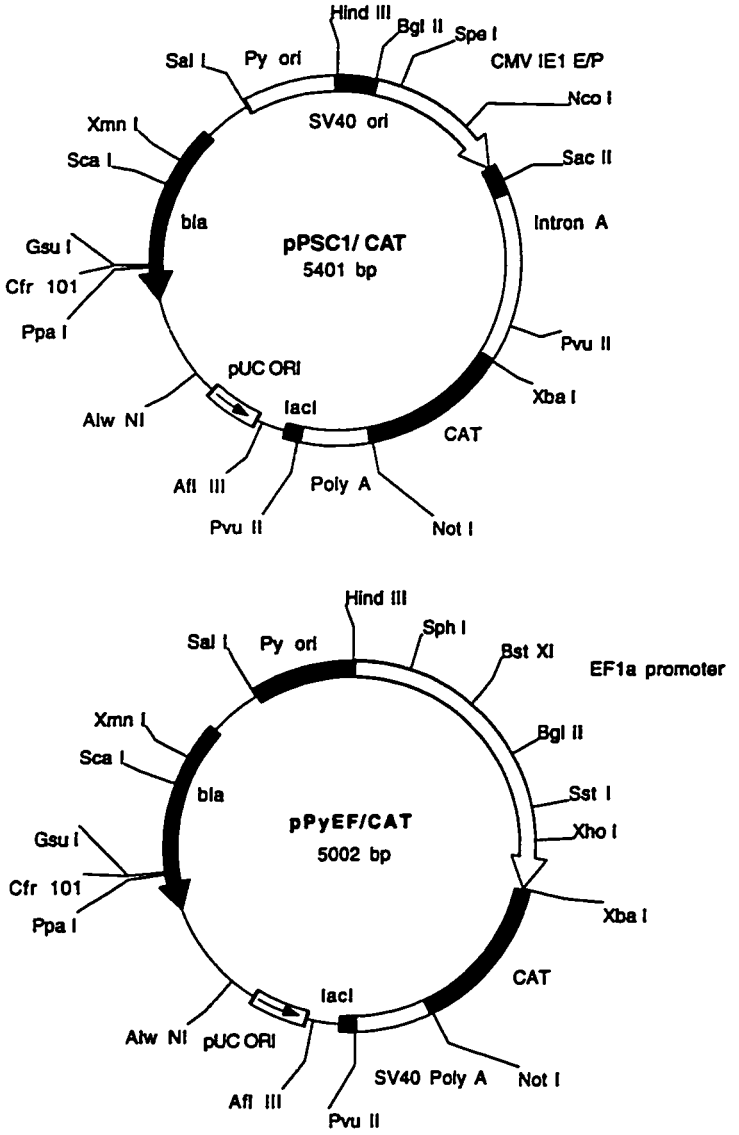
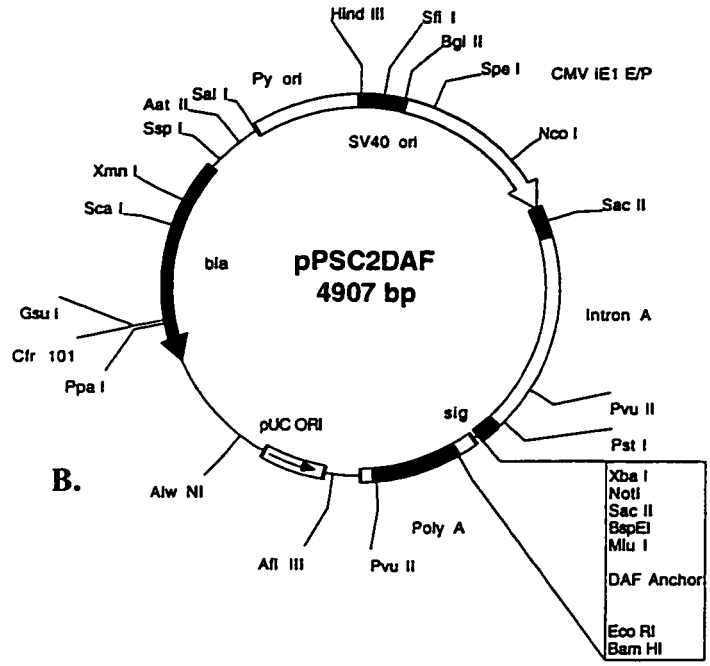


FIGURE B.1. Maps of CAT expression vectors used in Appendix A2. The chloramphenicol acetyltransferase gene (CAT) was amplified from the plasmid CAT 3MS (a gift from Dr. L-J. Chang) by PCR using the primers CAT 5' 2 and CAT 3'(see table 2.1.). These primers add an Xba I site and a Kozak ribosome binding site (CCACC)to the 5' end of the CAT gene, and a Not I site to the 3' end. This CAT fragment was ligated into Xba I/ Not I cut pPSC1, and the resulting CAT vector was called pPSC1/CAT. Another CAT vector was constructed by ligating the Sal I/ XbaI fragment containing the the EF1a promoter from pJFE14-PyEF(constructed by Sunita Bhatti and Dr. John Elliott), into the Sal I/ Xba I cut pPSC1/CAT vector backbone, effectively replacing the CMV promoter of pPSC1/CAT with the EF1a promoter. Finally, a commercial vector containing the RSV promoter driving the expression of CAT (pRSV/CAT) was chosen for comparison with these newly constructed vectors.




```
          T1/leu-1 signal sequence begins
tctagtccaccATG CCC ATG GGG TCT CTG CAA CCG CTG GCC ACC TTG TAC CTG CTG
           M   P   M   G   S   L   Q   P   L   A   T   L   Y   L   L

                                                  Polylinker
                                              Xba      Not  Sac II
GGG ATG CTG GTC GCT TCC TGC CTC GGA GAA GTC tct aga gcg gcc gcg g
G   M   L   V   A   S   C   L   G ▲ E   V   S   R   A   A   A

DAF anchor begins
Bsp EI          Mlu I
TCC GGA ACT ACG CGT CTT TCT GGG CAC ACG TGT TTC ACG TTG ACA GGT
S   G   T   T   R   L   S   G   H   T   C   F   T   L   T   G

                                                      Eco RI
TTG CTT GGG ACG CTA GTA ACC ATG GGC TTG CTG ACT TAg aat tc
L   L   G   T   L   V   T   M   G   L   L   T   Z
```

FIGURE B.2. Map of pPSC2DAF, a DNA immunization vector. **A.** This vector is identical to pPSC1 (see figure A2.1) except it contains the T1/leu-1 signal sequence, polylinker and DAF anchor of pBJT2 (see figure 4.11; the MRGS-His6 tag has been deleted). Target open reading frames which are cloned into the pPSC2DAF vector at the multiple cloning site are fused to upstream T1/leu-1 signal (for secretion). If a membrane anchor is not already present in the ORF, the downstream DAF anchor allows for phosphatidyl inositol-mediated linkage of recombinant proteins to the cell surface, if desired. Polylinker, 5' and 3' untranslated sequences are in lower case, the arrow indicates the signal cleavage site.

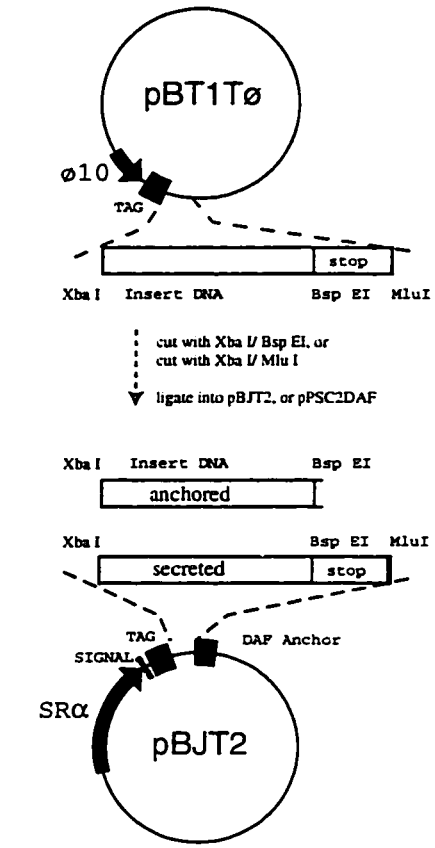


Diagram of pBT1Tø, a prokaryotic expression vector for producing parasite proteins for animal immunization.

Features of this vector include: IPTG-inducible production of high levels of MRGS-His6 tagged proteins, easily detectable by commercially available anti-MRGS-His6 antibody, recombinant protein purification on nickel chelating columns. This vector was designed for producing parasite proteins for animal immunization.

Insert DNA is modified by PCR to contain 5' Xba I sites (or Bam HI, Spe I, or Nhe I, see figure 6.1), and 3' Bsp EI-stop codon (in frame with Bsp EI site)-Mlu I. This DNA can be digested from pBT1Tø and ligated into pBJT2 or pPSC2DAF to encode a secreted protein (Xba I/ MluI; stop codon prevents DAF anchor translation), or an anchored protein (Xba I/ Bsp EI; insert reading frame is fused to downstream DAF reading frame, because Bsp EI-stop-Mlu I sequence is not retained).

Diagram of pBJT2, a eukaryotic expression vector designed for producing secreted or anchored MRGS-His6 tagged proteins.

This vector (see figure 6.11) is intended as a eukaryotic 'companion' vector to pBT1Tø. Native protein can be expressed and purified from COS cells transiently transfected with pBJT2 expressing secreted parasite proteins or BW5147 cells permanently transfected with the construct. COS cells transfected with pBJT2 expressing anchored parasite proteins can be used in immunofluorescence assays.

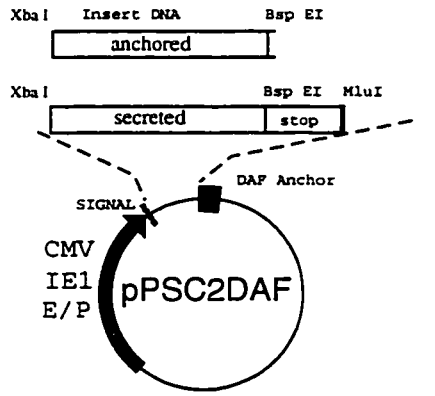


Diagram of pPSC2DAF, a DNA immunization vector designed for production of secreted or anchored proteins *in vivo*.

This vector (see figure A.2) is intended for DNA immunization, using parasite genes shuttled from pBT1Tø. DNA inserted into this vector can be engineered to encode either secreted or anchored proteins (without the MRGS-His6 tag, in this instance).

Figure B.3. Three vectors developed for the characterization of novel *Plasmodium falciparum* antigens.

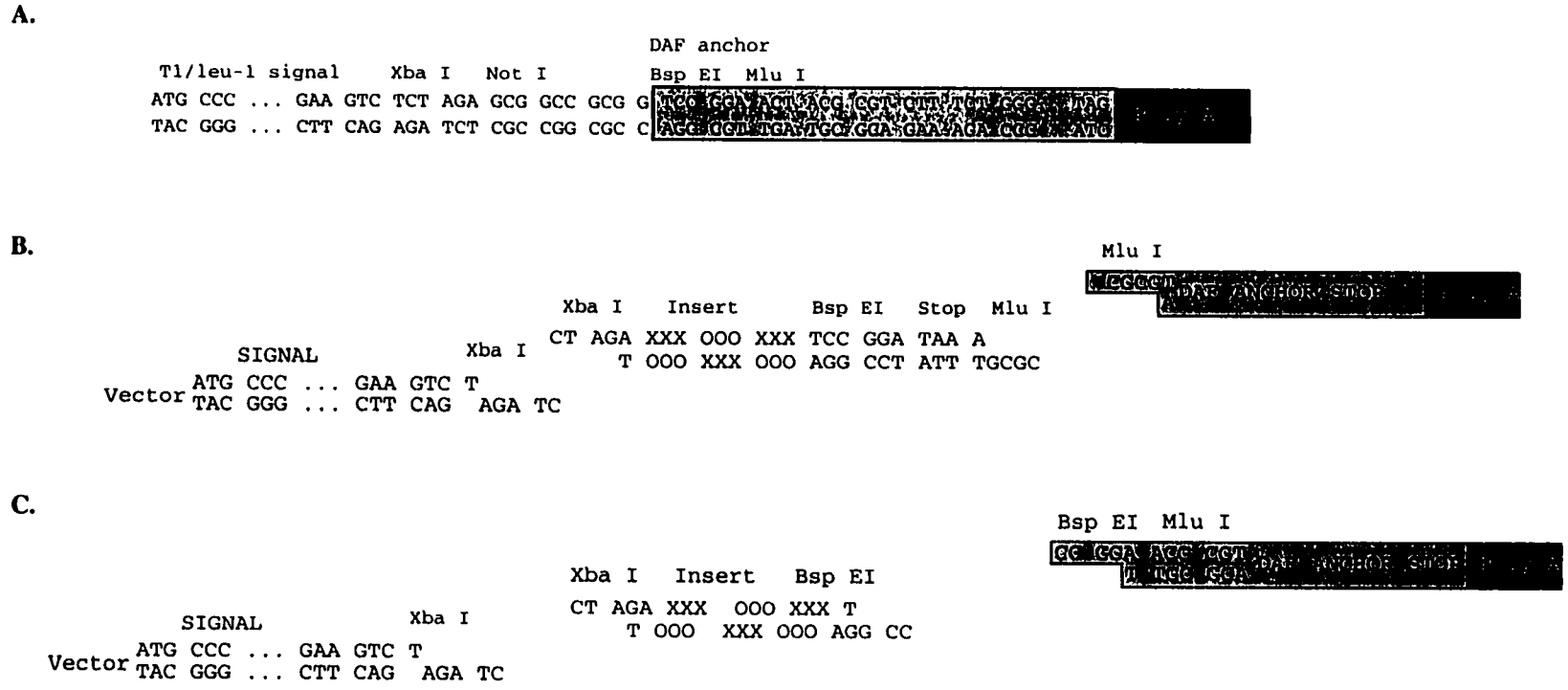


FIGURE A.4. Description of strategy for cloning secreted and anchored proteins encoded by a target sequence by cloning into pPSC2DAF. **A.** Double-stranded sequence of the pPSC2DAF signal, polylinker, and anchor regions. Inserts cloned into this vector must be in frame with the signal sequence (i.e. in frame with the Xba I site). The 5' sites for cloning include Xba I and Not I, and the 3' sites include Bsp EI (secreted protein), and Mlu I (anchored protein). The target insert must be engineered by PCR to have either an 5' Xba I site (or Nhe I, Spe I sites, which have compatible cohesive ends), or Not I site, and a 3' BspEI site followed immediately by a stop codon (in frame with the Bsp EI and insert coding segment), and an Mlu I site (also in frame with the coding segment). B. I order to generate a secreted protein, the PCR modified insert sequence is cut with Xba I and Mlu I and ligated into Xba I/ Mlu I cut vector. The stop codon after the Bsp EI site interrupts the translation of the DAF anchor, generating a secreted protein. C. In order to generate an anchored protein, the insert is cut with Xba I/ Bsp EI and ligated into similarly cut vector. In this case the stop codon has been cut away from the insert, and theinsert segment and DAF anchor coding regions are fused.

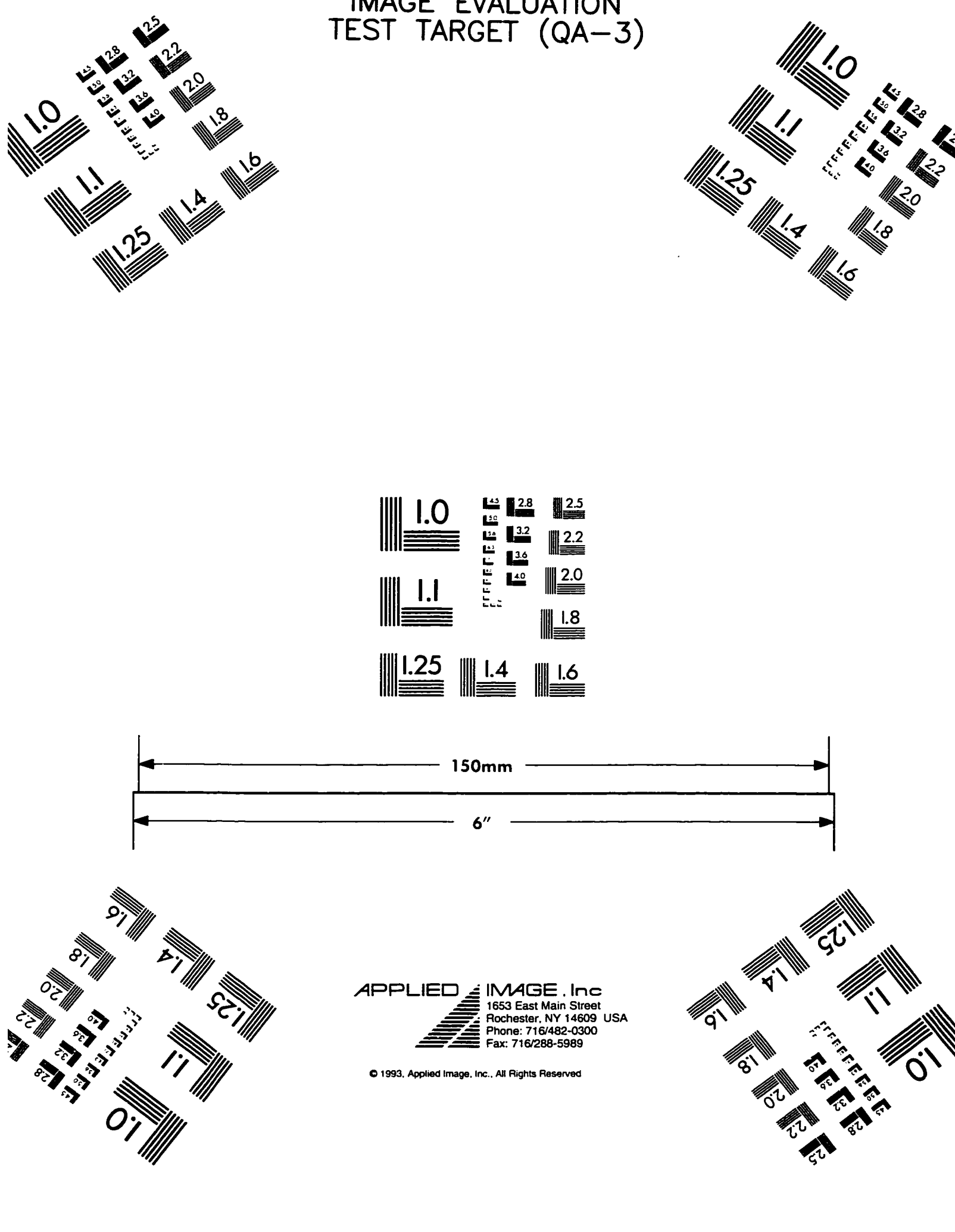


IMAGE EVALUATION
TEST TARGET (QA-3)
1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
150mm
6"
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