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Saccharomyces Cerevisiae-Produced Recombinant Pfs230: A Malaria Transmission-Blocking Vaccine Candidate

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Saccharomyces cerevisiae-PRODUCED RECOMBINANT Pfs230: A MALARIA TRANSMISSION-BLOCKING VACCINE CANDIDATE

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for my family,
especially LCDG

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LIST OF ABBREVIATIONS

ADH	alcohol dehydrogenase promoter
BCIP	5-bromo-4-chloro-3-indolylphosphate p-toludine salt
CFU	colony forming units
dd	distilled/deionized
ddNTP	dideoxyribonucleoside triphosphate
DIG	digoxigenin
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
FCA	Freund's complete adjuvant
HEPES	4-(-2-hydroxylethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
LB	luria broth
MBP	maltose binding protein
MCS	multiple cloning site
MeOH	methanol
mYEp	modified Yeast N-Terminal FLAG expression vector
NBT	nitroblue tetrazodium
Ni^{2+}-NTA	nickel nitrilo-tri-acetic acid
OD	optical density

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isolelectric point
PNK	polynucleotide kinase
SDS	sodium dodecyl sulfate
SCM	synthetic complete medium
SRP	signal recognition particle
TAE	Tris-acetate/EDTA
TBS	Tris-buffered saline
TBE	Tris-borate/EDTA
TCA	tricholoroacetic acid
TE	Tris/EDTA
TRP	tryptophan
YEp	Yeast N-Terminal expression vector
YPD	basic yeast growth media
YPHSM	YP4 high stability expression media

CHAPTER I

INTRODUCTION

Purpose

In an effort to control the morbidity and mortality associated with human malaria, transmission-blocking vaccines have been given careful consideration as a means of halting the spread of the disease (Mendis *et al.,* 1990). The main objective of a transmission-blocking vaccine is to prevent mosquitoes from becoming carriers of the parasite which causes malaria. Parasites in the form of sporozoites are deposited into the bloodstream of a human when a mosquito takes a bloodmeal. This is the beginning of a cycle of infection that proceeds in the human host culminating in the production of both asexually and sexually reproducing parasites. When a mosquito takes an infectious bloodmeal, the parasite undergoes further maturation in the mosquito midgut enabling it to be transmitted to another human. Parasite maturation and subsequent transmission can be blocked if antibodies which recognize parasite surface proteins are also present in the bloodmeal. The focus of this project is on the production of a recombinant protein that can be tested for its ability to induce malaria transmission-blocking immunity. This target antigen is Pfs230 which is found on the surface of the sexual stages of *Plasmodium falciparum* (*P. falciparum*), the parasite responsible for causing the most virulent form of human malaria.

Pfs230 was first identified as a malaria transmission-blocking vaccine candidate by Rener *et al.* who found the protein to be the target of monoclonal antibodies that reduce parasite infectivity in the mosquito midgut (Rener *et al.,* 1983). Recently, six regions of

Pfs230 were expressed as fusions with maltose-binding protein (MBP) in *Escherichia coli (E. coli)* (Williamson *et al.,* 1995). Antisera against one of those six regions, r230.MBP.C, which encodes amino acids 443 to 1132 of Pfs230, was found to generate antibodies that significantly reduce parasite infectivity in the mosquito midgut (between 71.2-89.8%) (Williamson *et al.,* 1995). This region of Pfs230 contains twelve cysteines (Williamson *et al.,* 1993) that in parasite-produced Pfs230 probably form distinct disulfide bonds which help define the conformation of the protein. Pfs230-specific monoclonal antibodies that have transmission-blocking activity only recognize non-reduced parasite-produced Pfs230 (Quakyi *et al.,* 1987) suggesting that the conformation of the monoclonal antibody epitopes in the native protein depends on disulfide bonds. Because recombinant Pfs230 was synthesized in the reduced cytosolic environment of *E. coli,* proper formation of these critical disulfide bonds may have been inhibited. This may explain why antisera against *E. coli*-produced Pfs230 region C (r230.MBP.C) did not completely block parasite infectivity.

The purpose of this study was to express and characterize a region of Pfs230 (C-Nterm) in *Saccharomyces cerevisiae (S. cerevisiae).* A yeast expression system has been previously shown to accurately recreate the conformation in another *P. falciparum* protein, Pfs25 (Kaslow and Shiloach, 1994). C-Nterm (amino acid 447 to amino acid 584 of Pfs230) encodes the amino terminus of Pfs230 region C which may the include the region of Pfs230 that is processed as the parasite emerges from the red blood cell. Additionally, C-Nterm is a small enough region of Pfs230.C to be expressed efficiently in a yeast expression system.

Literature Review

It is estimated that almost one half of the world's population is threatened by malaria (Saul, 1992; WHO, 1993). Present in over 100 countries, the disease, caused by

mosquito-borne parasites, is responsible for 300-500 million clinical cases annually (WHO, 1993). Approximately one to three million people die each year from malaria related illnesses and most of those are children (Hoffman and Miller, 1996). Malaria continues to be a serious problem in the tropics because measures devised to control it, such as the use of anti-malarial drugs, larvicides, and insecticides have proven insufficient in eliminating the parasite and preventing its transmission to the mosquitoes (Hoffman and Miller, 1996).

Human malaria is caused by four species of protozoan parasites of the genus *Plasmodium: P. falciparum, P. vivax, P. ovale, P. malariae* (Oaks, 1991). Although *P. vivax* is responsible for causing the most infections worldwide, the most virulent of those parasites is *P. falciparum* (van Dijck *et al.,* 1995). The parasites are transmitted to humans by a sub-group of 50-60 species of mosquitoes in the genus *Anopheles* (Oaks, 1991).

Malarial parasites were first seen in humans in 1880 (Review by Markell, 1992). Their development both in the anopheline mosquito and in the human bloodstream had been understood and the life cycle elucidated by the early 20th century (Review by Markell, 1992). Upon being bitten by an infected anopheline mosquito, the parasite is introduced into humans in the form of sporozoites (Oaks, 1991). Within forty minutes following injection into the human host sporozoites invade hepatocytes (Markell, 1992). During the following five to fifteen days the sporozoites develop into schizonts within the liver cells (Oaks, 1991). Each of these schizonts goes on to develop into 10,000 to 30,000 merozoites (Oaks, 1991). After rupturing the infected hepatocytes, merozoites are released into the bloodstream where they are able to invade erythrocytes. Once in the erythrocyte merozoites begin to asexually reproduce. Within 48-72 hours each merozoite matures into a schizont containing eight to thirty-two new merozoites (Oaks, 1991). Eventually the infected red blood cells rupture releasing merozoites which are then free to infect other erythrocytes (Oaks, 1991). Rupturing of these red blood cells releases

parasite metabolites which are toxic and may contribute to the symptoms associated with malaria, such as fever (Markell, 1992).

Some time after asexual parasites first appear in the bloodstream a subpopulation of merozoites differentiates into either male or female gametocytes. This form of the parasite grows within red blood cells but does not divide and matures to become the infectious form of the parasite. The trigger for the differentiation between the asexual and sexual life cycle is unknown (Markell, 1992). When an anopheline mosquito draws blood from an infected individual, red blood cells containing parasites (including gametocytes), as well as other serum components such as antibodies and complement, are taken up by the insect. The sexual stage of parasite development then proceeds in the midgut of the mosquito.

Under the influence of a change in temperature and/or chemical environment, intraerythrocytic gametocytes taken up in a bloodmeal emerge from the red blood cells in the mosquito midgut minutes after ingestion (Carter, 1988a). Presumably due to some substance released by the gametocytes themselves, the host red blood cell membranes are disrupted and gametocytes become extracellular (Carter, 1988a). Shortly after emergence, male gametocytes differentiate into eight highly motile microgametes, a process known as exflagellation (Carter, 1988a). These microgametes then proceed to fertilize female gametes derived from corresponding gametocytes (Carter, 1988a). Fertilization is complete less than 30 minutes after the parasite is ingested by the mosquito (Carter, 1988a). From the point of red blood cell emergence onward the parasite is extracellular and is therefore exposed to host components present in the bloodmeal such as complement, cytokines, and antibodies (Carter, 1988a).

In the 12-48 hours following fertilization, a zygote transforms into an ookinete which penetrates the mosquito midgut wall to become an oocyst (Oaks, 1991). The oocyst continues to develop on the serosal surface of the midgut adjacent to the haemocoel (Carter, 1988a). This oocyst enlarges over time forming greater than 10,000 sporozoites.

After the oocyst ruptures, sporozoites are released and migrate to the salivary glands of the insect where they are ready to infect the next human, thus perpetuating the cycle of infection (Oaks, 1991).

Because alternative measures to control or eradicate malaria have met with only limited success, the past two decades have focused on the development of antimalarial vaccines in attempt to control the disease worldwide. There are four major categories of vaccines classified according to the part of the parasite life cycle that they are designed to attack: 1) Pre-erythrocytic vaccines are aimed at preventing sporozoite invasion of liver cells and/or destroying parasite infectivity of hepatocytes; 2) Blood stage vaccines try to limit replication of erythrocytic stage parasites; 3) Anti-disease vaccines attempt to alleviate anemia, fevers, and other clinical illnesses associated with malaria; 4) Transmission-blocking vaccines are designed to prevent infected individuals from transmitting the parasite back to the mosquito thus halting the spread of the disease (Kaslow, 1993). Transmission-blocking vaccines, aimed at the sexual stage of parasite development, target either intraerythrocytic gametocytes in the vertebrate host or extracellular gametes that emerge in the insect (Kaslow, 1993). This type of vaccine will not necessarily protect already-infected individuals, but it may reduce the spread of malaria by slowing or halting the transmission of the parasite from the vector to the host (Oaks, 1991). Most likely, transmission-blocking vaccines would be administered in conjunction with pre-erythrocytic and blood stage vaccines in attempt to combat malaria related illnesses (Oaks, 1991). A combination vaccine targeting more than one stage of parasite development would likely make it more difficult for the escape parasites to mutate in an attempt to evade immune responses.

The earliest demonstration of transmission-blocking activity was in 1958 when Huff *et al.* immunized chickens with the avian parasite *P. gallinaceum* and noted that host factors (possibly antibodies) could decrease the relative infectiousness of gametocytes (Huff *et al.,* 1958). Chickens were immunized with *P. gallinaceum*-infected, formalin

treated erythrocytes and two to three days later challenged with an infective inoculation of live gametocytes (Huff *et al.*, 1958). Experiments revealed that when mosquitoes took a bloodmeal from these immunized chickens there was a significant decrease in the ability of gametocytes to produce oocysts in the mosquito midgut (Huff *et al.*, 1958). This evidence of transmission-blocking immunity was further supported in 1976 when Gwadz immunized chickens with an inactivated preparation of *P. gallinaceum*-infected red blood cells (Gwadz, 1976). Immunized chickens were infected and then *Aedes aegypti* mosquitoes were allowed to take a bloodmeal. *P. gallinaceum* infectivity in the mosquito was measured by counting the number of oocysts per midgut (Gwadz, 1976). In mosquitoes that ingested gametocytes from twice (or more) inoculated chickens infectivity was reduced 95-98% (Gwadz, 1976). It was noted however that by immunizing chickens there was little effect on asexual parasitemia and that immunization did not affect gametocyte production in the host (Gwadz, 1976). In follow-up studies, Carter and Chen used partially purified gametes to immunize chickens and found infectivity was further reduced to 99.9-100% (Carter and Chen, 1976; Carter *et al.*, 1979). Again, asexual parasitemias in experimental animals correlated with the asexual parasitemias found in the controls (Carter and Chen, 1976; Carter *et al.*, 1979). The study by Carter and Chen further noted that immunizations with partially purified preparations of both male and female gametes were more effective in reducing parasite infectivity to the mosquito than were single sex gamete preparations (Carter and Chen, 1976).

While studies had shown that immunizing chickens with gametes resulted in a decrease in oocyst development in the mosquito (Gwadz, 1976; Carter and Chen, 1976; Carter *et al.*, 1979) those same studies failed to indicate that any protection was being offered against the asexual stages of parasite infection. Subsequent studies with monkey and rodent malarias did show transmission-blocking immunity as well as protection against the asexual parasites (Gwadz and Green, 1978; Mendis and Targett, 1979). In a study by Gwadz and Green, rhesus monkeys were immunized with an antigen preparation

of asexual and sexual stage *P. knowlesi* parasites emulsified in Freund's Complete Adjuvant (FCA) (Gwadz and Green, 1978). After challenge with *P. knowlesi*, monkeys immunized with the antigen preparation and FCA were not infectious to feeding mosquitoes (Gwadz and Green, 1978). The authors of this study hypothesized that anti-gamete antibodies produced in the monkeys could have been responsible for blocking the sexual development of *P. knowlesi* in the mosquito (Gwadz and Green, 1978). This was illustrated by the fact that antisera from immunized monkeys showed complete transmission-blocking activity and had microgamete immobilizing activity (Gwadz and Green, 1978). Neither activity was evident in the controls (Gwadz and Green, 1978). In addition, immunized monkeys seemed to be protected against asexual parasites and had significantly reduced parasitemias and consequent pathology (Gwadz and Green, 1978). It was purported that suppression of asexual parasitemias could have been due to the asexual parasites used in the antigen preparation or due to antigens common to both asexual and sexual stages of the parasite (Gwadz and Green, 1978). In a similar study, Mendis and Targett showed that mice vaccinated with a crude preparation of *P. yoelli*-formalin fixed gametes showed significantly reduced infectivity to mosquitoes and well as a pronounced (though not complete) immunity to the erythrocyte stage parasites (Mendis and Targett, 1979). Their study also showed that an effective block on transmission was possible without the use of an adjuvant.

The first study to demonstrate a correlation between transmission-blocking immunity and anti-gamete antibodies in immune serum was performed by Gwadz *et al.* (Gwadz *et al.*, 1979). For this study chickens and monkeys were used as the experimental models. Animals were immunized with preparations containing gametes of their respective malaria parasites, *P. gallinaceum* and *P. knowlesi* (Gwadz *et al.*, 1979). Mosquito feeds and subsequent mosquito midgut oocyst counts showed that transmission-blocking immunity was induced in the animals (Gwadz *et al.*, 1979). In addition, it was shown that when parasitized blood from an immunized chicken or monkey was washed

free of its plasma and then resuspended in non-immunized serum the gametocytes recovered full infectivity to mosquitoes (Gwadz *et al.*, 1979). Furthermore, when parasitized blood from non-immunized chickens was fed to mosquitoes in the presence of serum from immunized birds, infectivity to mosquitoes was completely suppressed (Gwadz *et al.*, 1979). These results suggest that it is some serum component *(i.e.* antibodies) that mediates transmission-blocking immunity. Agglutination reactions and fluorescent antibody tests did indicate anti-gamete antibodies were present in the chicken and monkey serum (Gwadz *et al.*, 1979). The presence of anti-gamete antibodies suggests that reduction in parasite infectivity was mediated by anti-gamete antibodies which were ingested in the mosquito bloodmeal (Gwadz *et al.,* 1979). These antibodies have been shown to interact with extracellular gametes as they emerge from the red blood cells thereby preventing fertilization in the mosquito midgut (Gwadz *et al.,* 1979).

In transmission-blocking immunity, similar to vaccines against the asexual stage of parasite development, surface proteins are targeted. Most transmission-blocking research has focused on antigens present on the surface of parasites during the extracellular, sexual stage of development: namely, proteins on gametes, zygotes, and ookinetes (Oaks, 1991). Three vaccine candidates that appear on the surface of gametes have been identified: Pfs230, Pfs48/45, and Pfs40/10. In addition, Pfs25, which is expressed at the late gamete/early zygote stage of development, and Pfs28, present on the ookinete surface, have also been identified.

The focus of this research project was on Pfs230. Rener *et al.* were the first to indicate that Pfs230 was one of the target antigens for transmission-blocking immunity (Rener *et al.,* 1983). The Rener *et al.* study showed that two monoclonal antibodies (monoclonal antibody IA3-B8 and monoclonal antibody IIC5-B10) acted synergistically to target the surface of *P. falciparum* strain 7G8 (a Brazilian isolate) (Rener *et al.,* 1983). Together, but in the absence of complement, these two monoclonal antibodies mediated almost total suppression of infectivity to mosquitoes (Rener *et al.,* 1983). In addition,

monoclonal antibody IA3-B8 alone, in the presence of activated complement, suppressed infectivity to mosquitoes by 90% (Rener *et al.*, 1983). In the absence of complement either monoclonal antibody had slight effect in reducing infectivity, suggesting that the effects of monoclonal antibody IA3-B8 alone were complement mediated (Rener *et al.*, 1983).

In contrast to the studies by Rener *et al.*, a later study tested the ability of several monoclonal antibodies to block malaria transmission and found that only one of fifteen monoclonals developed, monoclonal antibody 28F1, was monospecific for Pfs230 (Vermeulen *et al.*, 1985). Yet monoclonal antibody 28F1 failed to exhibit transmission-blocking immunity (Vermeulen *et al.*, 1985). Three other monoclonal antibodies which precipitated Pfs230, monoclonal antibody 29F432, monoclonal antibody 7F3, monoclonal antibody 32F1 and also antibody 1771 (rabbit polyclonal antibodies), always did so in conjuction with Pfs48/45 (Vermeulen *et al.*, 1985). The fact that Pfs230 and Pfs48/45 coprecipitated might suggest that these proteins are interrelated and possibly possess a common antigenic site (Vermeulen *et al.*, 1985). Alternative possibilities may be that Pfs230 and Pfs48/45 interact by coprecipitating nonspecifically or that they form a complex on the surface of the parasite. The monoclonal antibodies that coprecipitate Pfs230 (monoclonal antibody 29F432, monoclonal antibody 7F3 and monoclonal antibody 32F1) always bound significantly more Pfs48/45 (Vermeulen *et al.*, 1985). Additionally, a positive reaction to Pfs230 could never be detected with monoclonal antibody 29F432, monoclonal antibody 7F3, monoclonal antibody 32F1, or rabbit polyclonal antibodies (Vermeulen *et al.*, 1985). This may lend support to the theory that Pfs230 coprecipitates with Pfs48/45 nonspecifically or forms a complex with Pfs48/45. The Vermeulen *et al.* study further showed that monoclonal antibody 32F3 and monoclonal antibody 32F5, which specifically precipitate Pfs48/45, most effectively blocked oocyst formation in the mosquito. The authors therefore concluded that Pfs48/45 was a good transmission-blocking vaccine candidate. The authors further suggested that

Pfs230 merely coprecipitated with Pfs48/45 and probably was not a transmission-blocking vaccine candidate antigen (Vermeulen *et al.*, 1985).

A study by Quakyi *et al.* however reestablished Pfs230 as a transmission-blocking vaccine candidate showing that it alone was the target of two transmission-blocking monoclonal antibodies, 1B3 and 2B4 (Quakyi *et al.*, 1987). Pfs230 was also immunoprecipitated by monoclonal antibody IIC5-B10, rabbit serum raised against whole gametes, and rabbit serum raised against purified Pfs230 (Quakyi *et al.*, 1987). In the presence of complement, monoclonal antibody 1B3 and monoclonal antibody 2B4 suppressed infectivity of *P. falciparum* 3D7 (an Amsterdam isolate) to mosquitoes by 95-100% (Quakyi *et al.*, 1987). Neither monoclonal antibody suppressed infectivity when complement was heat inactivated, suggesting that monoclonal antibody 1B3 and monoclonal antibody 2B4 were complement dependent (Quakyi *et al.*, 1987). In addition, monoclonal antibody 1B3 and monoclonal antibody 2B4 only reacted with Pfs230 under reducing conditions suggesting that the conformation of the epitopes recognized by monoclonal antibodies were defined by disulfide bonds (Quakyi *et al.*, 1987). A follow-up study reported that the epitopes defined by monoclonal antibody 1B3 and monoclonal antibody 2B4 were generally well conserved among 45 isolates examined by immunofluorescence assay (Foo *et al.*, 1991). This relative absence of antigenic diversity of Pfs230 among different strains of *P. falciparum* is promising for transmission-blocking vaccine development as the vaccine would probably be effective against most isolates.

Field studies have indicated that Pfs230 is a good transmission-blocking vaccine candidate and is naturally immunogenic (Graves *et al.*, 1988; Carter *et al.*, 1988b; Premawansa *et al.*, 1994; Riley *et al.*, 1994). Trials done in Papua New Guinea evaluated serum from adults living in this malaria endemic area and found that antibodies against Pfs230 correlated with the ability of the serum to block malaria transmission (Graves *et al.*, 1988). Individuals in the study had all been exposed to multiple reinfections (Graves *et al.*, 1988). Naturally occurring antibodies to Pfs48/45 were found in the Papua New

Guinea sera but they did not directly correlate with a decrease in parasite infectivity of the mosquito midgut (Graves *et al.*, 1988). In contrast to the Graves *et al.* study, a recent study in Sri Lanka, an area of lower malaria transmission, has shown that following an acute primary infection, antibodies against Pfs230 did not correlate with anti-parasite activity (Premawansa *et al.*, 1994). Considering the results of both studies it is possible that perhaps only repeated challenges with malarial infection lead to a maturation of transmission-blocking immune response (Premawansa *et al.*, 1994).

In addition to the aforementioned studies Read *et al.* have identified sixteen monoclonal antibodies which specifically recognize Pfs230 under non-reducing conditions and define nine distinct epitopes of the protein (Read *et al.*, 1994). These same sixteen monoclonal antibodies failed to react with Pfs230 when the antigens were treated with β-mercaptoethanol, suggesting that Pfs230 monoclonal antibodies were conformation dependent (Read *et al.*, 1994). This evidence of reduction sensitive antibodies upheld a previous study by Quakyi *et al.* Eight of the monoclonal antibodies in Read's study of the IgG_{2a} isotype mediated complement dependent lysis of gametes (Read *et al.*, 1994). Seven other monoclonal antibodies of murine immunoglobulin isotype IgG_1 (which do not fix complement) failed to lyse gametes. This suggested, as Quakyi *et al.* did before, that anti-Pfs230 antibodies are complement dependent (Quakyi *et al.*, 1987; Read *et al.*, 1994).

Because Pfs230 can elicit an immune response during a natural infection, it may be that an immune response to a subunit vaccine could be boosted during a natural infection in humans (Hoffman and Miller, 1996). This maybe important for inducing long lasting immunity. This could also give a Pfs230 vaccine an advantage over other transmission-blocking candidate antigens (*i.e.* Pfs25) which do not elicit immune responses upon natural infection (Kaslow, 1993).

Pfs230 was cloned and sequenced from both the 3D7 (derived from NF54 Amsterdam Airport isolate) and the 7G8 (derived from a Brazilian isolate) *P. falciparum* strains (Williamson *et al.*, 1993). The gene encodes a 363 kDa protein. There are a

number of distinct physical properties associated with the protein. Pfs230 has three regions of highly net negative charge across the protein which includes the 25 contiguous glutamine (E) residues and sixteen tandem E-E/G-V/E-G repeats located at the amino terminus of the molecule (Figure 1). The charged regions of the surface protein are speculated to act as a shield for the parasite by protecting it with an electrostatic field thereby preventing aggregation by repulsion (Williamson *et al.,* 1993). The remainder of the molecule contains seven cysteine motifs which are presumably involved in the tertiary structure of Pfs230 (Figure 1) (Williamson *et al.,* 1993; Carter *et al.*, 1995).

Pfs230 is present on gametocytes prior to gamete development in the mosquito midgut and is therefore present in the human host (Kumar and Carter, 1984). It was found that Pfs230 protein expression is localized to the parasite plasma membrane in both gametocytes and gametes (Williamson *et al.,* 1996). The protein is synthesized on day two of gametocytogenesis (Vermeulen *et al.,* 1986). Recently it was demonstrated that the 360 kDa form of the molecule is processed to a smaller, 310 kDa protein at a time which corresponds to gamete emergence from the red blood cells (Williamson *et al.,* 1996).

The physical properties of Pfs230 may be linked to immune evasion strategies by the parasite (Williamson *et al.,* 1996). Recall that in humans Pfs230 is expressed by intraerythrocytic gametocytes as a 360 kDa protein containing numerous E-E/G-V/E-G repeats (Figure 1). It is often the case that repeat regions such as these are immunodominant because many copies of the same sequence often increase the avidity for antibodies (Kemp *et al.*, 1987). Although circulating parasites are hidden in host red blood cells there is evidence that a host immune response can be generated to Pfs230 upon a natural infection (Graves *et al.*, 1988; Carter *et al.*, 1988b). This is probably due to parasitized red blood cells that are cleared by the spleen and then presented to the immune system (Carter, 1988a; Carter, 1988b). Because much of the antibody response generated in malaria infections is directed toward repeat epitopes (Kemp *et al.*, 1987), an immune response would probably be directed towards the repeat epitopes in the amino terminal 50

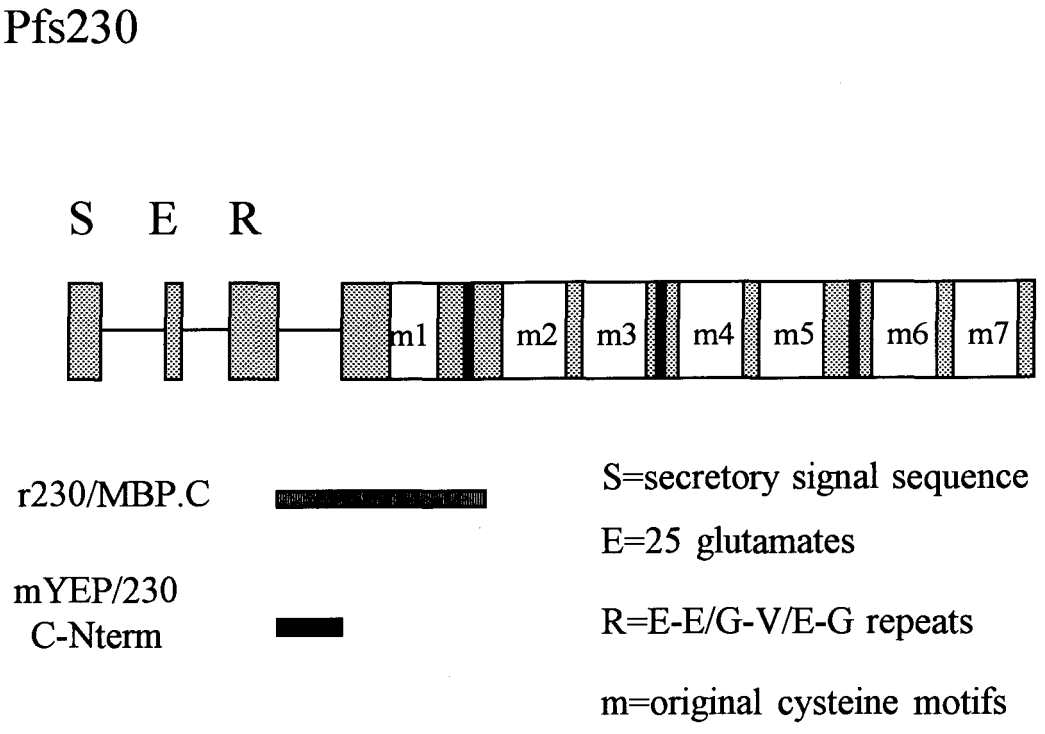

Figure 1. Schematic of Pfs230. The unique physical properties of Pfs230 include the stretch of glutamates and the E-E/G-V/E-G repeats near the amino terminus of the full length 360 kDa protein. In addition, the molecule contains seven cysteine motifs which are likely involved in the tertiary structure of the protein.

kDa of Pfs230, the region which is shed upon parasite emergence from the red blood cell. If the first 50 kDa were targeted by host antibodies, these antibodies would be present in a bloodmeal and the parasite would become exposed to the antibodies in the mosquito midgut. By shedding the amino terminal 50 kDa of the protein, parasites evade an immune response and cannot be recognized by antibodies directed towards the repeat region (Williamson *et al.,* 1995). This strategy-of-evasion theory is supported by studies that suggest that the most immunoreactive regions of recombinant Pfs230 proteins are amino acids which encode the $(E\text{-}E/G\text{-}E/V\text{-}G)_n$ repeats within the first 50 kDa of Pfs230 (Riley *et al.,* 1995).

A recent study reported the production of six recombinant Pfs230 proteins which were produced in *E. coli* as fusions with MBP (Williamson *et al.,* 1995). These recombinants, which encoded 83% of Pfs230, were labeled r230.MBP.A-r230.MBP.F. The immunogenicity of these recombinants was tested in mice (Williamson *et al.,* 1995). One region, r230.MBP.C (Figure 1), induced antibodies that significantly reduced parasite infectivity (71.2-89.8%) in the mosquito midgut (Williamson *et al.,* 1995). r230.MBP.C encodes amino acid 443 to amino acid 1132 of Pfs230. This is the region immediately following the E-E/G-E/V-G repeats and includes the first cysteine motif. The initial recombinants were produced in the reduced cytosolic environment of *E. coli*, not the secretory pathway of a eukaryote, and thus disulfide bonds may have formed randomly during the preparation or purification of Pfs230 region C as an antigen. If Pfs230 region C was expressed in heterogeneous conformations in *E. coli*, crucial epitopes within that region may or may not have been formed as they are in parasite-produced Pfs230. This would result in some antisera that recognizes parasite-produced Pfs230 and some that does not. Accordingly, only a subpopulation of the antibodies produced may effectively block transmission. Thus to further evaluate the ability of region C to induce transmission-blocking immunity it is necessary to express this region in other systems such as yeast or

mammalian cells which target the recombinants to a secretory pathway and permit correct disulfide bond formation.

The objective of this project was to express region C-Nterm of Pfs230 (amino acid 447 to amino acid 584) in yeast. This portion of the molecule is important for two reasons. C-Nterm is a small part of Pfs230 region C, a region which has been previously shown to generate antibodies that reduce *P. falciparum* transmission to mosquitoes. In addition, C-Nterm is a small enough region to be expressed efficiently in yeast. The yeast expressed C-Nterm protein could later be tested for its ability to induce antibodies that decrease parasite infectivity in the mosquito midgut. If yeast expressed C-Nterm is a more potent immunogen than *E. coli*-produced r230.MBP.C, vaccine efforts may be focused on yeast produced C-Nterm. Furthermore, future experiments could be done with C-Nterm protein to determine if it encodes the precise region where stage-specific processing of Pfs230 occurs. This may lead to further insight into parasite immune evasion strategies.

S. cerevisiae, or baker's yeast, has been the first choice of many investigators to supplement prokaryotes in the production of recombinant proteins. The major reasons a yeast expression system was chosen for this project is because it offers high protein yield as well as the possibility of inexpensively scaling up production of protein for a vaccine. In addition, yeast have been shown to produce other recombinant *P. falciparum* proteins, such as Pfs25 and MSA1, in high yield (Kaslow and Shiloach, 1994; Hui *et al.*, 1994). Also, the extensive secretory pathway yeast proteins undergo will likely yield a more properly conformed protein than *E. coli* did (Gellissen, 1992). This may be of particular importance for a Pfs230 vaccine since it has been shown that transmission-blocking monoclonal antibodies which recognize the molecule are conformation dependent (Quakyi *et al.*, 1987; Read *et al.*, 1994).

The secretory pathway in eukaryotes has been firmly established. Polypeptides with hydrophobic amino terminal regions, like the α signal sequence in *S. cerevisiae*, can be targeted for secretion through the rough endoplasmic reticulum (Alberts *et al.*, 1994).

The signal sequence targets the protein for production/translation in the rough endoplasmic reticulum by binding to the signal-recognition particle (SRP) complex (Alberts *et al.*, 1994). One end of the SRP complex binds to the signal sequence while the other end binds to the ribosome (Alberts *et al.*, 1994). This step causes a temporary pause in translation to prevent the protein from being released into the cytosol (Alberts *et al.*, 1994). Once formed, the SRP ribosome complex then binds to the SRP receptor in the rough endoplasmic reticulum membrane and translation continues (Alberts *et al.*, 1994). The polypeptide is then translocated across the rough endoplasmic reticulum where the signal sequence is cleaved by a membrane bound protease (Tuite and Oliver, 1991). Once in the rough endoplasmic reticulum, resident chaperones and isomerases may bind to the nascent polypeptide chain and regulate folding, disulfide bond formation, and posttranslational modification. After translation, the protein is delivered to the Golgi for further processing and then the protein can be shuttled via secretory vesicles to the extracellular space.

Posttranslational modification of yeast proteins begins in the rough endoplasmic reticulum. While N-linked glycosylation, a frequent form of posttranslational modification in yeast, is initiated in the rough endoplasmic reticulum, the extension of oligosaccharide side chains occurs in the Golgi apparatus. Fully glycosylated proteins are then transported to the plasma membrane and the contents are discharged (Tuite and Oliver, 1991). Glycosylation can occur in one of two ways: N-linked glycosylation occurs at asparagine residues in the sequence asn-X-ser/thr where X is any amino acid except proline; O-linked glycosylation occurs at serine or threonine residues. While C-Nterm lacks the requisite sequence for N-linked glycosylation, there are thirteen serines and seven threonines in Pfs230.C-Nterm, leaving C-Nterm sensitive to O-linked glycosylation. In yeast, O-linked glycosylation exclusively involves the addition of mannoses directly to serines or threonines usually in chains of 5 or less (Tanner and Lehle, 1987).

Another posttranslational modification carried out by yeast which can alter protein structure includes phosphorylation, which involves the addition of phosphates to the hydroxyl groups of serine, threonine or tyrosine residues (Alberts *et al.*, 1994). Sulfation, also a posttranslational modification, occurs primarily at tyrosine residues in secretory proteins (Han and Martinage, 1992). In addition to the many serines and threonines in C-Nterm, this region of Pfs230 also contains eight tyrosines. One other type of posttranslational modification is fatty acylation (the addition of hydrophobic acid groups to proteins). Isoprenylation is the addition of a 15 carbon fatty acid (farnesylation) or 20 carbon fatty acid (geranylgeranylation) to the carboxy terminus of proteins (Caldwell *et al.*, 1995). Palmitoylation is also a type of fatty acylation which involves the addition of a 16 carbon chain to cysteine residues throughout the length of polypeptides. Myristoylation, which occurs cotranslationally, is the attachment of a 14 carbon chain to the amino terminus of a protein (Schafer and Rine, 1992). Most reports have these fatty acid additions occurring in the cytosol (Alberts *et al.*, 1994) and are therefore unlikely to appear on a yeast secreted recombinant protein.

CHAPTER II

MATERIALS AND METHODS

Experimental Design

A previous study showed that antisera to an *E. coli*-produced recombinant of Pfs230, r230.MBP.C (encoding amino acid 443 to amino acid 1132), decreased parasite infectivity in the mosquito midgut by 71.2-89.8% (Williamson *et al.*, 1995). This project concentrated on generating *S. cerevisiae*-produced recombinant Pfs230 which can be used in future studies to test for its ability to induce complete malaria transmission-blocking immunity. To do this, mice will be vaccinated with yeast produced Pfs230.C-Nterm. The antisera generated will then be used to test for decreased parasite infectivity in the mosquito midgut.

The expression vector used in this study was the Yeast N-Terminal Flag Expression Vector (YEp) (Eastman Kodak Company, New Haven, CT). YEp is a 7205 base pair plasmid with a multiple cloning site (MCS) region. An f1 origin of replication allows this vector to replicate in *E. coli* and a selectable marker is provided by the ampicillin resistance gene. A two micron circle element and a tryptophan (TRP) selectable marker are encoded in the vector for growth and selection in yeast. The vector was modified to encode a six histidine tag at the 3' end of the multiple cloning site to provide a means of purifying the recombinant protein. To express the amino terminus of Pfs230 region C (amino acid 447 to amino acid 584) the nucleotide sequence was amplified via the polymerase chain reaction (PCR). The resultant PCR product and the modified yeast

expression vector (mYEp) were digested with compatible restriction endonucleases and ligated together. *E. coli* was transfected with this plasmid construct (C-Nterm/mYEp) via electroporation. Transfected bacterial colonies were grown on solid media, selected, amplified in liquid media, harvested, and the construct recovered. The isolated plasmid construct was transfected into yeast, *S. cerevisiae.* Yeast colonies that grew in the absence of tryptophan (TRP) were selected and then grown in a medium conducive to protein expression. After the protein was expressed it was purified and characterized to determine if it was recombinant Pfs230.C-Nterm.

Reagents

Unless otherwise indicated, chemicals were purchased from Sigma Chemical Company, St. Louis, MO and restriction endonucleases and DNA-modifying enzymes were purchased from Life Technologies, Gaithersburg, MD.

Transformation of *E. coli* with YEp Plasmid

Luria-Bertani (LB) medium [1% (w/v) Bacto-tryptone (Difco Laboratories, Detroit, MI); 0.5% (w/v) Bacto-yeast extract (Difco Laboratories, Detroit, MI); 1% (w/v) NaCl (Brown, 1991)] was inoculated with JM109 *E. coli* cells from a glycerol stock (Promega Corporation, Madison, WI) and then incubated at 37^{0}C, 250 rpm (Lab-Line Instruments, Melrose Park, IL) for 48 hours. A 300 µl aliquot of the saturated culture was added to 50 ml fresh LB and grown at 35.5^{0}C, 240 rpm to an OD_{600} of 0.58.

The cells were harvested (2420 x g, five minutes, 4^{0}C) and the supernatant was quickly decanted. The cell pellet was resuspended in ice cold sterile 20 mM NaCl and centrifuged (2420 x g, five minutes, 4^{0}C). The supernatant was removed and cells were resuspended in 0.4M $CaCl_2$ and centrifuged (2420 x g, five minutes, 4^{0}C). After quickly

decanting the supernatant, cells were resuspended in the following solution which was made and filter-sterilized immediately prior to use: 0.056 M $MnCl_2$, 0.04 M $CaCl_2$, 0.032 M ammonium acetate. The resulting calcium competent JM109 *E. coli* was then incubated on ice for 20 minutes.

Five ng of Yeast N-Terminal Flag Expression Vector (YEp) was added to 200 µl of calcium competent JM109 cells. To another 200 µl of the calcium competent JM109 cells 50 ng of YEp was added. Both mixtures were incubated on ice for one hour. The cells were then heat shocked in a 42°C water bath for two minutes (to allow incorporation of the YEp vector into cells) and placed on ice for two minutes. To the cells, 800 µl of SOC medium [2% Bacto-tryptone; 0.5% Bacto-yeast extract; 0.05% NaCl; 20 mM glucose (Sambrook *et al.,* 1989)] was added. Cells were incubated at 37°C for 30 minutes at 250 rpm and then streaked onto LB/agar plates containing 100 µg/ml of the antibiotic ampicillin. Only cells transformed with the YEp vector (which contains the ampicillin resistance gene) can survive in this medium. After incubating upright at room temperature for five minutes, the plates were inverted and placed at 37°C overnight.

The next day, six tubes each containing 5 ml of liquid LB/ampicillin (100 µg/ml) were inoculated separately with six bacteria colonies from the plates. The liquid cultures were grown in a 37°C shaking incubator at 250 rpm overnight. The following day, cultures were removed from the shaker and placed at 4°C for seven hours. An 800 µl aliquot of cells was removed from each culture then gently vortexed with 200 µl of glycerol and stored at -70°C as a renewable glycerol stock of the YEp vector. The remaining cells were harvested by centrifugation (16,000 x g, two minutes) and the supernatants were decanted. The pellets (to be used later for isolating plasmid) were stored at -20°C.

YEp Plasmid Preparation (Minipreps)

To recover the YEp plasmid from the transfected JM109 *E. coli*, Wizard™ Minipreps (Promega Corporation, Madison, WI) were performed. Briefly, bacterial pellets from the six overnight cultures were lysed under alkaline conditions. The lysates were then neutralized causing the *E. coli* plasma membrane and attached genomic DNA to precipitate. The precipitated material was pelleted and the supernatants containing the plasmid DNA were recovered. To isolate the plasmid DNA from the supernatants, a silica-based resin was added. After the resins were washed in high salt to remove unbound contaminants, bound DNA was eluted in low salt Tris/EDTA (TE) buffer [10 mM Tris-HCl; 1 mM EDTA, pH 8.0 (Brown, 1991)] and was stored at -20°C. Reference the 1994 Promega Technical Bulletin, Wizard™ Minipreps DNA Purification Systems for the detailed protocol (Promega Corporation, Madison, WI).

Restriction Endonuclease Digestion of YEp Plasmid

To confirm that plasmid had been isolated from the bacteria, an aliquot of the purified plasmid from each of the six YEp minipreps was digested with restriction endonucleases and visualized on an agarose/Tris-acetate/EDTA (TAE) [40 mM Tris-acetate; 1 mM EDTA (Brown, 1991)] gel. Approximately 100 ng of purified YEp vector was digested with 5 units Kpn I in Buffer A [C_F= 6 mM Tris-HCl, pH 7.5; 6 mM $MgCl_2$; 6 mM NaCl; 1 mM DTT (Promega Corporation, Madison, WI)] for a final reaction volume of 20 µl. After a one hour incubation at 37°C concentrated (6X) DNA loading buffer was added to each sample for a final concentration of 1X [0.042% bromophenol blue; 0.042% xylene cyanol FF; 6.67% (w/v) sucrose in water (Sambrook *et al.*, 1989)]. The digests were evaluated by size fractionation via electrophoresis on a 0.8%

agarose/TAE gel containing 0.04 μg/ml of ethidium bromide. The gel was run at 100 V for 60 minutes and the DNA was visualized using an ultraviolet transilluminator.

YEp Plasmid Modification

1. Oligonucleotide (YEpH6SII/YEpH6AII) Design and Kinasing Reactions. To provide an efficient means of protein purification, an oligonucleotide encoding a six histidine tag was designed to be incorporated into YEp. The sequence of the oligonucleotides (which also encoded an Nhe I restriction site) follows:

Sense strand (YEpH6SII):

5' C/GTC/GAC/CAC/CAC/CAC/CAC/CAC/CAC/TAG/GCT/AGC/CCG/C 3'

Antisense strand (YEpH6AII):

5' GG/GCT/AGC/CTA/GTG/GTG/GTG/GTG/GTG/GTG/GTC/GAC/GGG/CC 3'

To prepare the oligonucleotides for a ligation reaction, a phosphate was added to the 5'-hydroxyl terminus of each oligonucleotide using T4 Polynucleotide Kinase (T4 PNK). The reaction conditions follow:

Kinasing reaction

3 μg YEpH6SII or YEpH6AII
30 Units T4 PNK
70 mM Tris-HCl, pH 7.6
10 mM $MgCl_2$
5 mM DTT
1.67 mM γ ^{32}P-ATP

This reaction was incubated at 37^{0}C for 30 minutes followed by a 10 minute incubation at 65^{0}C to inactivate the T4 PNK. The two phosphorylated oligonucleotides were subsequently annealed by the following reaction:

Annealing oligonucleotides

200 ng phosphorylated YEpH6SII
200 ng phosphorylated YEpH6AII
40 mM Tris-HCl, pH 7.5
20 mM $MgCl_2$
50 mM NaCl

This mixture was first incubated in a 65°C heat block for two minutes then cooled slowly to 37°C (to allow annealing) by placing the heat block on the lab bench.

2. Restriction Endonuclease Digestion of YEp Plasmid. To prepare the purified YEp plasmid for ligation, 800 ng of the purified DNA was digested with 10 units of Apa I in Buffer A (Promega Corporation, Madison, WI) for a total reaction volume of 40 µl. After incubating for 1 hour at 37°C in a heat block 10 units of Sst II were added to the digestion and the reaction continued at 37°C for an additional two and one half hours. To stop the reaction concentrated (6X) DNA loading buffer was added to the sample for a final concentration of 1X. The digest was evaluated by size fractionation via electrophoresis on a 0.8% agarose/TAE gel containing 0.04 µg/ml of ethidium bromide. The gel was run at 100 V for 60 minutes and the DNA was visualized using an ultraviolet transilluminator.

3. Purification of Digested YEp Plasmid from Agarose (Geneclean™). The double digested YEp band was excised from the agarose/TAE gel and the DNA was recovered using the Geneclean™ Kit (Bio101, LaJolla, CA). Briefly, 800 µl of 6 M NaI was added to the excised band and the mixture was incubated at 37°C for five minutes to dissolve the agarose. GLASSMILK™ was added to the tube and after five minutes, the GLASSMILK™/DNA complex was pelleted by centrifugation (16,000 x g, five seconds). The supernatant was decanted and the pellet was washed three times with ice cold NEW WASH™(Bio101, LaJolla, CA). The pellet was desiccated for ten minutes and the DNA

was eluted twice using 12.5 µl of TE buffer per elution. The genecleaned product was stored at -20°C.

4. Ligation YEpH6II/YEp. The purified, double digested YEp plasmid was centrifuged (16,000 x g, 30 seconds) to pellet any residual GLASSMILK™. A 12.5 µl aliquot was taken from the top of the tube and added to the ligation reaction (described below) containing the annealed YEpH6II oligonucleotide.

Ligation reaction

~200 ng genecleaned, Apa I/Sst II digested YEp
~100 ng annealed YEpH6II oligonucleotide
62.5 mM Tris-HCl, pH 7.6
12.5 mM $MgCl_2$
1.25 mM ATP
1.25 mM DTT
25% (w/v) polyethylene glycol-8000
1 unit Ligase

This reaction proceeded for four hours at room temperature.

5. Transformation of *E. coli* with YEpH6II/YEp (mYEp). ElectroMAX DH10B™ *E. coli* cells (Life Technologies, Gaithersburg, MD) were transfected with the YEpH6II/YEp ligation mix via electroporation. Electroporation is a technique which transiently permeabilizes bacteria cell membranes allowing foreign DNA to enter the cells.

The ligation reaction was diluted 1:5 in distilled/deionized (dd) H_2O and 1 µl (3 ng) of the diluted YEpH6II/YEp ligation reaction was added to 40 µl of thawed DH10B™ cells. As a positive control, 1 µl (0.01 ng) of pUC19 DNA (Life Technologies, Gaithersburg, MD) was placed in a tube with 40 µl of cells. The ligation reaction/cell mixture was added to chilled 1 mm gap micro-electroporation cuvettes (BTX Inc., San Diego, CA) and electroporated at 1.8 kV/cm with a time constant of 4.1 msec in the Gene Pulser II electroporator (Bio-Rad Laboratories, Hercules, CA). One ml of SOC medium was immediately added to the cells and the culture incubated in a 37°C shaking incubator

for one hour at 250 rpm. The cells were distributed onto two LB/ampicillin agar plates (500 µl per plate) and after one hour at room temperature the plates were inverted and incubated overnight at 37°C. The same procedure was repeated for the positive control pUC19 DNA.

Six colonies were selected from the YEpH6SII/YEp plates and were grown in LB/ampicillin liquid media at 37°C, 250 rpm overnight. Plasmid DNA was isolated from the overnight cultures (Wizard™ Miniprep) and screened for the presence of the YEpH6II oligonucleotide by restriction analysis with Nhe I. Only the vectors which were modified with the YEpH6II oligonucleotide (mYEp) could be linearized by a Nhe I digest, because the oligonucleotide (and not the original YEp vector) encodes the Nhe I restriction site. Approximately 200 ng of one of the Wizard™ purified plasmids was digested at 37°C for one hour with 7.5 units of Nhe I in React 4 buffer [C_F= 2 mM Tris-HCl, pH 7.4; 0.5 mM $MgCl_2$; 5 mM KCl]. The YEpH6SII/YEp digest was electrophoresed on a 0.8% agarose/TAE gel containing 0.01 µg/ml ethidium bromide and visualized on an ultraviolet transilluminator. As a negative control, approximately 200 ng of YEp vector was digested with Nhe I as per the above reaction conditions and was electrophoresed on the 0.8% agarose/TAE gel with the YEpH6SII/YEp digest. As additional controls, approximately 200 ng of undigested YEp and YEpH6SII/YEp were subjected to the above reaction conditions (with the exception of Nhe I) and size fractionated on the same gel.

Colonies that contained the YEpH6II oligonucleotide ligated into YEp (mYEp) were amplified in LB/ampicillin liquid media at 37°C, 250 rpm overnight. Following the overnight incubation glycerol stocks were made for storage at -70°C. The remaining culture was used to isolate the plasmid.

Construction of Pfs230.C-Nterm Expression Vector

1. DNA Amplification Pfs230.C-Nterm. The polymerase chain reaction (PCR) was used to amplify Pfs230 region C-Nterm base pair #1339 to base pair #1752 (which encodes amino acid 447 to amino acid 584) from *P. falciparum* genomic DNA (3D7 strain). Synthetic oligonucleotides that corresponded to the following base pairs in Pfs230 were used as primers in the reaction: sense oligonucleotide - base pair #1339 to base pair #1351; antisense oligonucleotide - base pair #1719 to base pair #1752. The 5' end of the sense oligonucleotide primer contained the sequence for the restriction enzyme Xho I, while the 5' end of the antisense oligonucleotide primer encoded the sequence for the restriction enzyme Apa I. These restriction enzyme sites were included to facilitate ligation into the multiple cloning site of mYEp.

For PCR amplification, HotStart 50 tubes (Molecular-Bio Products Inc., San Diego, CA) containing wax beads were used. The wax served to separate upper and lower layer components until the reaction had begun and to prevent evaporation during the reaction cycles. Lower layer components of the reaction were added to the tube in the following proportions :

Lower layer components

0.4 mM dNTP
2 mM $MgCl_2$
50 mM KCl
10 mM Tris-HCl, pH 9.0 (at 25^{0}C)
1% Triton X-100
600 ng sense primer
600 ng antisense primer

Reaction tubes were incubated in a thermocycler (MJ Research Inc., Watertown, MA) at 90^{0}C for 30 seconds then cooled to 4^{0}C for five minutes to melt the wax bead over the lower layer. Upper layer components were then added to the reaction tube as follows:

Upper layer components

~1 µg 3D7 *P. falciparum* genomic DNA
2 mM $MgCl_2$
50 mM KCl
10 mM Tris-HCl, pH 9.0 (at 25°C)
1% Triton X-100
5 units Taq polymerase

Initially, the reaction tubes were sequentially incubated (94°C for one minute; 38°C for 30 seconds; 72°C for two minutes) to initiate the mixing of the reaction components. The PCR was then begun using the following program:

PCR Reaction

(A) INITIAL DENATURATION -- 94°C for 30 seconds
(B) ANNEALING OF PRIMERS -- 38°C for 30 seconds
(C) EXTENSION OF PRIMERS -- 72°C for 2 minutes
(D) REPEAT STEPS A-C -- 30 cycles
(E) FINAL EXTENSION OF PCR PRODUCTS -- 72°C for 6 minutes

The size of the PCR amplified DNA was evaluated via electrophoresis on a 1.2% agarose/TAE gel containing 0.07 µg/ml ethidium bromide. DNA loading buffer was added to 2 µl samples of the PCR reaction. The gel was electrophoresed at 100 V for 60 minutes and DNA was visualized using an ultraviolet transilluminator.

The remaining PCR product was concentrated by ethanol (EtOH) precipitation. Briefly, 5 M ammonium acetate was added to the PCR sample to a final concentration of 2.5 M. Ninety-five percent EtOH was added to the PCR amplified DNA to a final concentration of 63.33%. The sample was then incubated at 4°C for 48 hours, then centrifuged (16,000 x g, 20 minutes) and the supernatant decanted. The pellet was then dried and washed with 100 µl of 95% EtOH and centrifuged (16,000 x g) for an additional ten minutes. The supernatant was removed and the C-Nterm pellet was vacuum dried for three minutes and stored at -20°C until use.

2. Restriction Endonuclease Digestion of C-Nterm and mYEp. The amplified C-Nterm pellet was digested with restriction endonucleases to generate compatible ends for

ligation into mYEp. The estimated 5 µg C-Nterm pellet was resuspended in React 2 buffer [C_F= 5 mM Tris-HCl, pH 8.0; 1 mM $MgCl_2$; 5 mM NaCl] and 25 units of Xho I (Promega Corporation, Madison, WI) were added to start the reaction. The reaction was incubated at 37°C for one hour and 15 minutes then EtOH precipitated (as described previously) using 5 M ammonium acetate and 95% EtOH. The Xho I-digested C-Nterm pellet was then resuspended in React 4 buffer and 25 units of Apa I were added to begin the second digestion. This reaction was incubated at 37°C for one hour and DNA loading buffer was added to stop the reaction.

mYEp was isolated from an overnight culture of bacterial cells transfected with the mYEp plasmid using the QIAprep Spin Plasmid Miniprep Kit (50) (Qiagen Inc., Chatsworth, CA). Briefly, bacterial cells were lysed in NaOH/sodium dodecyl sulfate (SDS) in the presence of RNase. The salt concentration of the cell lysate was then increased, causing chromosomal DNA to precipitate. Genomic DNA was removed by centrifugation and the cleared supernatant was loaded onto a QIAprep spin column which has a silica based membrane that selectively adsorbs DNA. After washing the QIAprep spin column, the mYEp DNA was eluted with 50 µl TE buffer.

Qiagen purified mYEp (~500 ng) was digested with 10 units of Xho I in React 2 buffer for one hour at 37°C. The DNA was then EtOH precipitated using 5 M ammonium acetate and 95% EtOH. The resultant Xho I-digested mYEp pellet was resuspended in React 4 buffer and 10 units of Apa I and then incubated for one hour and 20 minutes at 37°C. The reaction was stopped by the addition of DNA loading buffer to the sample. The C-Nterm and mYEp samples were electrophoresed on a 1.2% agarose/TAE gel containing 0.04 µg/ml ethidium bromide for one hour at 114 V. The gel was visualized on an ultraviolet transilluminator for a very short time and the bands representing digested C-Nterm and digested mYEp were excised. The digested DNA was extracted from the agarose and purified using the Geneclean™ Kit (Bio101, LaJolla, CA) as described in the

YEp plasmid modification section of this chapter. The genecleaned products were stored at -20°C.

3. Ligation C-Nterm/mYEp. Because both C-Nterm and mYEp were digested with the same restriction endonucleases, it was possible to perform a compatible end ligation reaction. The reaction conditions follow:

Ligation reaction

~180 ng mYEp
~48 ng C-Nterm
62.5 mM Tris-HCl, pH 7.6
12.5 mM $MgCl_2$
1.25 mM ATP
1.25 DTT
25% (w/v) polyethylene glycol-8000
1 unit Ligase

This reaction incubated at room temperature for four and one half hours. The reaction was then EtOH precipitated using 5 M ammonium acetate and 95% EtOH. The ligation reaction pellet was resuspended in 10 µl ddH_2O and used to transfect *E. coli.*

4. Transformation of *E. coli* with C-Nterm/mYEp construct. One µl (20 ng) of the precipitated C-Nterm/mYEp ligation reaction was added to 20 µl of ElectroMAX DH10B™ *E. coli* cells (Life Technologies, Gaithersburg, MD) and the mixture was placed in a chilled 1 mm gap disposable micro-electroporation cuvette (BTX, San Diego, CA). One µl (0.01 ng) pUC19 DNA (Life Technologies, Gaithersburg, MD) in 20 µl of DH10B™ cells served as a positive control. A 1.7 kV charge was administered using the TransPorator™ Plus (BTX, San Diego, CA). The C-Nterm/mYEp transfected *E. coli* from two electroporations were combined in 1 ml of SOC medium. The cells were incubated for one hour at 37°C, in a 250 rpm shaker and then distributed equally onto two LB/ampicillin agar plates. After a five minutes incubation upright, the plates were inverted and incubated at 37°C overnight. The following day, resultant colonies were selected

from the plates and amplified in LB/ampicillin liquid media overnight at 37°C in a 250 rpm shaking incubator. The same procedure was followed for the cells transfected with the positive control pUC19 DNA.

Cells were harvested from the amplified colonies and plasmid was isolated using the QIAprep Spin Plasmid Miniprep Kit. The isolated plasmids were digested with Kpn I and Spe I which are 5' and 3' to the multiple cloning site in mYEp, respectively. Approximately 100 ng of isolated plasmid (C-Nterm/mYEp) was digested with 10 units of Kpn I and 10 units of Spe I in React 4 at 37°C for one hour. DNA loading buffer was added to stop the reaction. The digestion reaction was electrophoresed on a 1.0% agarose/TAE gel containing 0.01 μg/ml ethidium bromide at 100 V for one hour and the DNA was visualized on an ultraviolet transilluminator.

Colonies that contained the C-Nterm/mYEp construct were amplified in LB/ampicillin liquid media overnight at 37°C, 250 rpm and were then stored as glycerol stocks at -70°C.

Manual DNA Sequencing

To confirm the proper reading frame of the C-Nterm/mYEp construct manual sequencing was done using the Sequenase™ Version 2.0 DNA Sequencing Kit (Amersham, Cleveland, OH). Unless otherwise indicated, all enzymes and buffers used for the sequencing reactions were sold with that kit. One μg of C-Nterm/mYEp was resuspended in 18 μl ddH_2O and 2 μl NaOH, incubated at 37°C for five minutes, then EtOH precipitated. Following incubation on dry ice for 10 minutes, the precipitate was pelleted by centrifugation (16,000 x g, 20 minutes) and the supernatant was decanted. The C-Nterm/mYEp template pellet was washed with 95% EtOH, centrifuged (16,000 x g, 10 minutes) and the supernatant discarded. The template was resuspended in 2.0 μl of

Sequenase Reaction Buffer and 3.5 pmol of YαN-21 primer (Eastman Kodak Company, New Haven, CT), heated for two minutes at 65°C in a heat block, then cooled slowly to 37°C by placing the heat block on the lab bench.

To the cooled template/primer mixture, the following were added: 1 µl DTT (100 mM); 2.0 µl of 1:10 diluted Labeling Mix (1 µl Labeling Mix in 10 µl ddH_20). The reaction was started by the addition of 20 µCi [^{35}S] dATP and 2 µl of 1:8 diluted Sequenase/buffer solution (1 µl (13 units) Sequenase in 8 µl of Enzyme Dilution Buffer). After three and one half minutes at room temperature, a 3 µl aliquot of the reaction mixture was added to four tubes each containing 2.5 µl (0.8 µM) of a distinct ddNTP. The tubes were incubated for seven minutes at 37°C after which time 4 µl of Stop Solution was added. The reactions were stored at -20°C until the following day.

Premixed 6% polyacrylamide gel casting solution (Life Technologies, Gaithersburg, MD) was used to pour the sequencing gel. Eight hundred µl of 10% ammonium persulfate was added to the 75 ml gel mix solution immediately prior to pouring the gel. The sequencing gel polymerized overnight at room temperature.

The following day, the polymerized gel was mounted on the running apparatus and 1X Tris-borate/EDTA (TBE) [0.09 M Tris-borate; 0.002 M EDTA (Brown, 1991)] was added as a running buffer. The power supply was set at 1220 V, 55 mA, 69 W to warm the gel before the samples were loaded. Just before loading, the samples were heated for three minutes at 75°C and placed immediately on ice. Three µl from each ddNTP/reaction tube was added to a separate well. A long sequencing run (4 hours) was done for optimal resolution of larger bands and a short sequencing run (2 hours) was done for optimal resolution of smaller bands. The gel was electrophoresed at 1220 V, 55, 71 W.

At the end of the run, the gel was removed from the apparatus and fixed in 10% methanol (MeOH), 10% glacial acetic acid for one hour at room temperature. The gel was then dried for one and one half hours at 80°C and used to expose X-ray film. The autoradiogram was developed and the sequence of C-Nterm/mYEp was read.

Transformation of *S. cerevisiae* with C-Nterm/mYEp Construct

Liquid YPD growth media [1% Bacto-yeast Extract; 2% Bacto-peptone; 2% Bacto dextrose (Eastman Kodak Company, 1994)] was inoculated with *S. cerevisiae* (2095-6 strain) and incubated at 30°C for 72 hours at 250 rpm. When the OD_{600} of the culture was 1.3, cells were harvested by centrifugation (5,000 x g, five minutes, room temperature). The supernatant was decanted and cells were resuspended in 10 ml YPD and 200 µl 1 M HEPES, pH 8.0. Sterile, cold ddH_20 was added to the resuspended cells to increase the volume to 25 ml. While swirling gently, 275 µl of 0.9 M cold DTT was added. The cells were pelleted by centrifugation (5,000 x g, five minutes, 4°C) and washed sequentially in 30 ml sterile, cold ddH_2O; 25 ml sterile, cold ddH_2O; 2 ml sterile, cold 1 M sorbitol. Following the washes, the cells were pelleted by centrifugation (5,000 x g, five minutes, 4°C) then vigorously resuspended in 50 µl sterile, cold 1 M sorbitol.

Twenty five ng of the C-Nterm/mYEp construct was added to 40 µl of the washed *S. cerevisiae* cells and the mixture was placed in a sterile, chilled 2 mm gap electroporation cuvette. Using the TransPorator™ Plus (BTX, San Diego, CA), the yeast was electroporated at 1.5 kV then immediately diluted with 1 ml cold 1 M sorbitol and gently resuspended. Five hundred µl of cells were plated onto two synthetic complete medium (SCM) minus tryptophan (TRP) plates (Bio101, La Jolla, CA) and incubated at 30°C for 48 hours, inverted. Only yeast transfected with the mYEp expression vector can survive

on TRP minus media because mYEp encodes a TRP selectable marker. Thus, colonies that grew on SCM minus TRP plates should contain mYEP.

Colonies were selected from the plates and grown in liquid SCM minus TRP for 72 hours at 30°C, 250 rpm to an OD_{600} of $\geq$ 8.0. Glycerol stocks [333 µl 60% glycerol; 667 µl yeast culture] were made and stored at -70°C. An aliquot was also taken to inoculate a culture that would be used for protein expression work.

Inducing Protein Expression in *S. cerevisiae* Transformants

A 2.5 µl aliquot of the SCM minus TRP culture described above was diluted 1:20 in each of three tubes containing 47.5 ml YP4 High Stability Expression Media (YPHSM) [1% dextrose; 3% glycerol; 1% Bacto-yeast extract; 8% Bacto-peptone; 20 mM $CaCl_2$; 0.05% adenine; 0.05% uracil (Eastman Kodak Company, 1994)] and were incubated at 30°C for 72 hours at 250 rpm. The cultures were harvested and the cells pelleted by centrifugation (16,000 x g, 20 minutes, 4°C). The supernatants were saved and later evaluated for the presence of secreted recombinant protein.

Trichloroacetic Acid (TCA) Precipitation and SDS-Polyacrylamide Gel Electrophoresis (PAGE)

To better visualize proteins secreted by yeast, 500 µl of each of the three yeast expression culture supernatants were concentrated by precipitation with 125 µl 100% TCA. After centrifugation (16,000 x g, 20 minutes) the supernatants were decanted and the pelleted proteins were washed three times in 500 µl acetone and again centrifuged

(16,000 x g, five minutes, 4°C). The pellets were resuspended in 50 µl 1X sample buffer [0.134 M Tris-base, pH 6.8; 1% SDS (w/v); 10% glycerol; 0.08% bromophenol blue (w/v) (Harlow and Lane, 1988)], 5% β-mercaptoethanol and size fractionated on a denaturing 12% polyacrylamide separating gel, 3.75% polyacrylamide stacking gel using electrophoresis buffer [25 mM Tris-base; 200 mM glycine; 0.1% SDS (Sambrook *et al.*, 1989)]. The gel was electrophoresed at 176 V, 36 mA for two hours and stained in Coomassie brilliant blue [10% glacial acetic acid; 45% MeOH; 0.1% Coomassie brilliant blue (w/v)(Sigma Chemical Company, St. Louis, MO)] overnight while shaking. The gel was destained [5% MeOH, 7.5% glacial acetic acid] on a shaking plate and protein was visualized over a light source.

Ni^{2+}-NTA Purification of Recombinant C-Nterm Protein

An Ni^{2+}-NTA affinity column was used to purify recombinant C-Nterm from the yeast expression media supernatant. Ni^{2+}-NTA resin (Qiagen Inc., Chatsworth, CA) binds the six histidine tagged C-Nterm due to a high affinity interaction between the six consecutive histidines and the Ni^{2+}-NTA complex. Find a detailed reference for the procedure explained below in The QIAexpressionist, Summer, 1992 manual (Qiagen Inc., Chatsworth, CA).

Fifty ml of supernatant from one yeast expression culture was dialyzed against 2 liters of 1X phosphate-buffered saline (PBS) [8.0 g/L NaCl; 0.34 g/L KH_2PO_4; 1.21 g/L K_2HPO_4, pH 7.3 (Brown, 1991)] in Spectra/Por 4 dialysis membrane tubing (molecular weight cut off: 12-14,000)(Baxter Diagnostics Inc., McGaw Park, IL) for four hours at 4°C. The tubing which contained the supernatant was then transferred to 2 liters of fresh 1X PBS and was incubated at 4°C overnight. The following day, 1.5 ml of the Ni^{2+}-NTA resin equilibrated with Column buffer [50 mM Na-PO_4, pH 8.0; 300 mM NaCl (Qiagen

Inc., 1992)] and was added to the dialyzed yeast supernatant. The supernatant/resin slurry was incubated on ice and placed on a shaking plate for one hour. The slurry was subsequently loaded onto two poly-prep chromatography columns (Bio-Rad Laboratories, Hercules, CA) and the material which did not bind to the columns was collected. (After this step, all fractions were collected in 1.0 ml amounts, with the exception of the first fraction from each separate wash which was collected in 200-300 µl fractions). The resin was washed with 5 ml Wash buffer [50 mM Na-PO_4; 30 mM NaCl; 10% glycerol, pH 7.0 (Qiagen Inc., 1992)] then 5 ml Wash buffer containing 0.5 M imidazole, pH 7.0 was applied to elute C-Nterm. Subsequent washes with 5 ml Wash buffer, pH 6.0 and then 5 ml Wash buffer, pH 4.5 were performed to be sure protein had been eluted from the column. Eluants were stored at 4^0C.

To determine which fraction contained the eluted C-Nterm protein, aliquots from the eluted fractions were analyzed on denaturing 12% polyacrylamide separating gels, 3.75% polyacrylamide stacking gels. The gels ran at 176 V, 36 mA, 92 minutes then 120 V, 92 mA for an additional 28 minutes and proteins were visualized by Coomassie blue staining.

Western Blot Analysis of Ni^{2+}-NTA Purified Protein

To determine if protein eluted from the Ni^{2+}-NTA affinity columns reacted with antibodies generated against Pfs230, it was analyzed by western blot. Proteins size fractionated by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane for one hour at 100 V using chilled transfer buffer [25 mM Tris-base; 200 mM glycine; 20% MeOH (Harlow and Lane, 1988)]. Following the transfer, the nitrocellulose filter was blocked in 5% dry milk/1X PBS for 15 minutes at room temperature to prevent non-specific antibody binding. The filter was then incubated overnight at 4^0C with the indicated primary antibodies diluted 1:2000 in 1X PBS. The following day, the

membranes were washed three times with 1X PBS and then incubated for 45 minutes at room temperature with the secondary antibodies, Goat-Anti-Mouse IgG alkaline phosphatase conjugate (Sigma Chemical Company, St. Louis, MO) diluted 1:2000 in 1X PBS. The filter was then washed twice with 1X PBS and once with alkaline phosphatase buffer [100 mM NaCl; 5 mM $MgCl_2$; 100 mM Tris-base, pH 9.5 (Harlow and Lane, 1988)]. The membrane was subsequently incubated with 16.5 µl 5-bromo-4-chloro-3-indolylphosphate p-toludine salt (BCIP) (Life Technologies, Gaithersburg, MD), 22 µl Nitroblue tetrazolium chloride (NBT) (Life Technologies, Gaithersburg, MD) in 5 ml alkaline phosphatase buffer. Bound antibody was visualized by the formation of a blue precipitate on the nitrocellulose.

Glycoprotein Detection Assay

In contrast to *P. falciparum,* proteins secreted by yeast are often post-translationally modified by the addition of large branches of carbohydrates (*i.e.* mannoses) to a secreted protein. This process is known as glycosylation. To determine if recombinant C-Nterm was glycosylated, the DIG Glycan Detection Kit (Boehringer Mannheim, Indianapolis, IN) was used. Unless otherwise referenced, chemicals used for this experiment were supplied with the kit.

The samples were first electrophoresed on a denaturing 12% polyacrylamide separating gel, 3.75% polyacrylamide stacking gel for 20 minutes, 150 V, 36 mA and continued for 70 minutes, 174 V, 36 mA. Samples were then electrophoretically transferred to nitrocellulose for one hour at 100 V in chilled transfer buffer. Following the transfer, the nitrocellulose was washed two times in 1X PBS, pH 6.8. To oxidize the glycoproteins, the filter was subsequently incubated in sodium metaperiodate for 20 minutes while shaking at room temperature. After washing the filter three times for 10

minutes each in 1X PBS, the membrane was incubated in 1 µl DIG-0-3-succinyl-ε–aminocaproic acid hydrazide dissolved in 5 ml sodium acetate buffer, pH 5.5, for one hour, shaking at room temperature. Proteins were then stained with ponceau S for five minutes (while shaking) to determine if the transfer was successful and to identify the major protein bands present. Following this, the nitrocellulose was incubated in Blocking Reagent for one hour at room temperature and then the filter was washed three times for 10 minutes each with 1X Tris-buffered saline (TBS) [0.05 M Tris-HCl; 0.15 M NaCl, pH 7.5 (Boehringer Mannheim, 1995)] while shaking. The nitrocellulose was then probed with 10 µl alkaline phosphatase labeled Anti-digoxigenin antibodies in 10 ml 1X TBS overnight at 4^0C. The following day, the filter was washed three times for 10 minutes each with 1X TBS. Staining solution [10 ml alkaline phosphatase buffer, pH 9.5; 37.5 µl X-phosphate; 50 µl NBT (Boehringer Mannheim, 1995)] made immediately prior to use, was added to the filter to visualize reactivity with glycosylated proteins which was observed by the presence of bands on the nitrocellulose.

C-Nterm Protein Sequencing

To directly determine the identity of the highest molecular weight band produced by C-Nterm/mYEp transfected yeast, the amino acid sequence of that protein was evaluated. Twenty-four ml (~2 µg) of supernatant from yeast transfected with C-Nterm/mYEp construct was concentrated by adding it to SpectraPor dialysis tubing then placing the tubing in one and one half cups of chilled, ground sucrose. As the volume of the supernatant progressively decreased, knots were tied to keep the tubing tight around the supernatant. After nine hours the volume of the supernatant was approximately 5 ml. Three ml of concentrated C-Nterm/mYEp yeast transfected supernatant was sent to Dr.

Bassam Wakim, PhD at Loyola University Medical Center in Maywood, IL. Dr. Wakim purified the highest molecular weight band of the recombinant protein by High Performance Liquid Chromatography (HPLC) using a C4 reversed phase column. A fraction of the purified protein was subjected to *Staphylococcus aureus* (V8) protease digestion and peptides were then purified by HPLC using a C4 reversed phase column. The amino acid sequence of selected peptides was obtained.

Yeast Spheroplast Preparation/DNA Extraction

To determine if the C-Nterm/mYEp construct had been altered after transfection into the yeast, the plasmid was recovered from yeast and transfected into *E. coli*. The plasmid was isolated from the *E. coli*, purified, and then sent for automated DNA sequencing.

More specifically, to do this, sterile YPD media was inoculated with the C-Nterm/mYEp yeast glycerol stock and incubated at 225 rpm, overnight in a 30°C shaking incubator. The following day, when the OD_{600} was 1.3, the culture was centrifuged (2,000 x g, 15 minutes, room temperature) and the supernatant was decanted. The cell pellet was resuspended in 20 ml of 1 M sorbitol, 20 mM Tris-base, pH 8.0 and then centrifuged (2,000 x g, 20 minutes, room temperature). After the supernatant was decanted, the cell pellet was resuspended in 2 ml of 20 mM sodium citrate, pH 5.8; 1 M sorbitol; 2 mg lyticase (Boehringer Mannheim, Indianapolis, IN) and incubated at 37°C for 30 minutes. This mixture was then centrifuged (2040 x g, five minutes, room temperature), the supernatant decanted and the cells resuspended in 1 ml of 0.1 M EDTA, pH 8.0; 10 mM Tris-base; 1% sarkosyl; 2 mg proteinase K. The resuspended cells were placed at 30°C for 30 minutes then 65°C for 15 minutes. The mixture was brought up to a final volume of 10 ml by adding ddH_2O and centrifuged (23,420 x g, 25 minutes, 4° C). To the resultant supernatant, 1/10 the volume of 5 M ammonium acetate and one volume

of isopropanol were added. After the supernatant incubated for 10 minutes at -20°C, the mixture was centrifuged (26,890 x g, 15 minutes, 4°C). The supernatant was decanted and the resultant pellet was resuspended in 500 µl ddH_2O. Fifty µg RNase was added to the tube and the mixture incubated at 37°C for 30 minutes. To remove proteins, a phenol extraction was done by adding 500 µl of Tris-saturated phenol, pH 7.9 and centrifuging the mixture (16,000 x g, 15 minutes, room temperature). The top layer containing the DNA was recovered, transferred to a new tube and the above step was repeated twice more. To the recovered DNA, 200 µl chloroform was added and the mixture was centrifuged at 16,000 x g for 15 minutes. The DNA (top layer) was recovered and precipitated overnight at -20°C with one half the volume of 5 M ammonium acetate and two times the total volume of 95% EtOH. The following day, the DNA was centrifuged (16,000 x g, 20 minutes). The supernatant was decanted and the pellet was resuspended in 100 µl 95% EtOH, centrifuged (16,000 x g, 10 minutes) and the resultant supernatant was decanted. The pellet was resuspended in 20 µl of filter-sterilized TE buffer, pH 8.0.

Transformation of *E. coli* with yC-Nterm/mYEp Construct Recovered from *S. cerevisiae*

The DNA obtained from yeast as described above was electroporated into *E. coli*. One µl of DNA was added to 20 µl ElectroMAX *E. coli* DH10B™ cells (Life Technologies, Gaithersburg, MD) and electroporated at 1.7 kV. This step was repeated and both sets of electroporated cells were immediately combined into 1 ml SOC media. After incubating the culture at 37°C, 225 rpm for one hour, the cells were distributed onto LB/ampicillin agar plates and incubated overnight at 37°C. Only the colonies containing the construct recovered from the yeast (yC-Nterm/mYEp) can survive on this medium due to the presence of the ampicillin resistance gene in the mYEp vector. The following day,

colonies were selected from the plates and grown in LB/ampicillin liquid media overnight at 37°C, 250 rpm.

Plasmid was isolated from the overnight culture by the Qiagen method and was evaluated by restriction analysis. Approximately 100 ng of Qiagen purified yC-Nterm/mYEp was digested with five units of Kpn I and five units of Spe I in 1X React 4 buffer. The reaction was incubated at 37°C for one hour and 6X DNA loading buffer was added to stop the reaction. The digestion reaction was electrophoresed on a 1.0% agarose/TAE gel, 0.02 μg/ml ethidium bromide at 100 V for 70 minutes. The gel was destained in ddH_2O at 4°C for three hours and the DNA was visualized on an ultraviolet transilluminator.

Automated DNA Sequencing yC-Nterm/mYEp Construct

To confirm that the construct recovered from the yeast (yC-Nterm/mYEp) was in the proper reading frame and to determine if the sequence had been altered by the yeast, 1.2 μg of the Qiagen purified yC-Nterm/mYEp was sent for automated sequencing at the University of Chicago (University of Chicago Sequencing Facility, Chicago, IL). The sequence obtained was analyzed using EditView ABI Automated DNA Sequence Viewer, Version 1.0 (Perkin Elmer Corporation, 1996).

CHAPTER III

RESULTS

Vector Modification

To generate *S. cerevisiae*-produced Pfs230 the first step taken was to produce ample quantities of the Yeast N-Terminal Flag Expression Vector (YEp) purchased from Eastman Kodak. Towards that end, *E. coli* strain JM109 was transfected with the YEp vector. Because YEp encodes a gene which confers ampicillin resistance, transfected bacteria was grown and selected for on LB/ampicillin agar plates. Twenty three colonies grew on the plate which contained bacteria transfected with 5 ng of YEp. Twenty colonies grew on the plate which contained bacteria transfected with 50 ng of YEp. Of these, six colonies were selected (three from each plate) and grown separately in LB/ampicillin liquid to generate a clonal population of each colony. Bacteria harvested from the six cultures were lysed and the YEp vector was purified and digested with Kpn I. The digests were electrophoresed on an agarose/TAE gel to verify that YEp vector was purified (Figure 2). Bands from five of the six digests were found to be 7.37 kB as interpolated by DNA size standards. The actual size of YEp is 7.2 kB. No plasmid was recovered from the sixth colony. Purified vector obtained from one of the transfected colonies was used for subsequent modification.

The purified YEp was modified to encode a six histidine tag and a Nhe I restriction site at the 3' end of the multiple cloning site. The six histidines encoded by the modified YEp vector (mYEp) were to be expressed on the carboxy terminus of

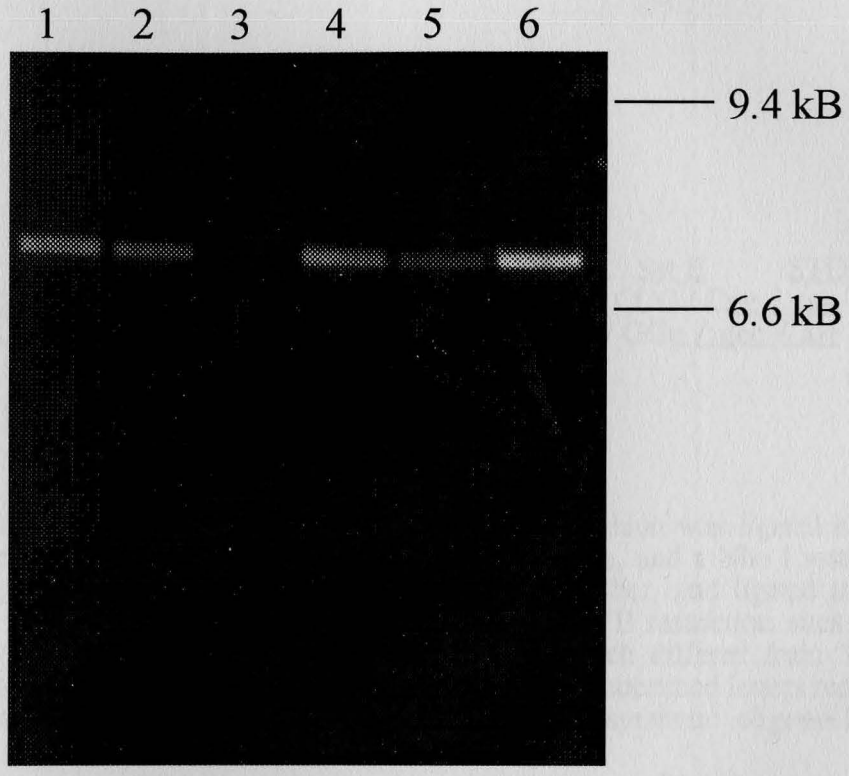

Figure 2. Kpn I digests of YEp vector. Plasmid was Qiagen purified from six colonies of JM109 *E. coli* transfected with the YEp vector. The purified material was digested with Kpn I and then size fractionated on a 0.8% agarose/TAE gel. Lanes 1-6 contain Kpn I digested material purified from bacterial colonies 1-6, respectively. DNA size standards are indicated on the right.

C-Nterm protein thus enabling the recombinant protein to be purified using a Ni^{2+}-NTA affinity column. Briefly, two synthetic complementary oligonucleotides (sense and antisense) which encoded for a six histidine tag and a Nhe I restriction site were phosphorylated and annealed together (Figure 3). The annealed oligonucleotide (YEpH6II) was then ligated into the YEp plasmid which had also been previously digested with Apa I and Sst II.

Annealed oligonucleotide YEpH6II

Apa I	Six His	STOP	Nhe I	Sst II	STOP
ggg/ ccC / GTC / GAC / 6 x CAC / TAG / GCT / AGC / CCG / C**gg / taa**					
cCC/ GGG / CAG / CTG / 6 x GTG / ATC / CGA/ TCG / GG**c / gcc / att**					

Figure 3. This schematic shows the annealed oligonucleotide which was ligated into the YEp vector to encode for six consecutive histidines, a stop codon, and a Nhe I restriction site. The oligonucleotides were phosphorylated, annealed together, and ligated into the YEp vector via a compatible end ligation into the Apa I and Sst II restriction sites. The YEpH6II/YEp ligation generated a modified (mYEp) vector which differed from YEp at the 3' end of the multiple cloning site. Lower case, bolded and underlined letters represent the vector sequence and the upper case letters represent the synthetic oligonucleotide sequence.

E. coli was transfected with the YEpH6II/YEp ligation mix and plated out onto LB/ampicillin agar plates. Resultant colonies were counted to determine the transformation efficiency. This number was compared to the transformation efficiency of *E. coli* that was transfected with the positive control DNA, pUC19 monomer. The equation used to determine the transformation efficiency follows:

$$\text{Colony forming units (CFU)}/\mu g = \frac{\text{CFU in pUC19 plate}}{\text{pg pUC19 used}} * \frac{1 \times 10^6 \text{ pg}}{\mu g} * \text{dilution factor}$$

The transformation efficiency calculated for the control, pUC19, plate follows:

$$\text{CFU}/\mu g = \frac{150 \text{ CFU}}{10 \text{ pg}} * \frac{1 \times 10^6 \text{ pg}}{\mu g} * 100 = 1.5 \times 10^9$$

The transformation efficiency calculated for the mYEp construct follows:

$$\text{CFU}/\mu g = \frac{32 \text{ CFU}}{2.5 \times 10^3 \text{ pg}} * \frac{1 \times 10^6 \text{ pg}}{\mu g} * 2 = 2.56 \times 10^4$$

Six mYEp colonies were selected and grown up overnight in liquid culture. Plasmid was isolated and analyzed for the presence of the synthetic oligonucleotide by restriction analysis using Nhe I. Because only the plasmid containing YEpH6II (mYEp) contains a Nhe I restriction site (and unmodified YEp does not) mYEp plasmids can be linearized by a Nhe I digestion while YEp plasmids cannot. Linearized vector would be represented by a band migrating at approximately 7.2 kB. Undigested vector (which should be seen in the negative control YEp Nhe I digest) would be seen as two bands: one just above 7.2 kB representing relaxed plasmid; one just below 7.2 kB representing supercoiled plasmid. The mYEp and YEp Nhe I digests were electrophoresed on a 0.8% agarose/TAE gel containing 0.01 μg/ml ethidium bromide and were visualized using an ultraviolet transilluminator. Plasmid from a mYEp colony migrated as one 10.68 kB band (as interpolated by size standards using linear regression) following the Nhe I digestion suggesting that this plasmid contained the synthetic oligonucleotide (Figure 4). The actual size of mYEp is 7.206 kB. The control YEp plasmid Nhe I digest migrated as two bands, one interpolated at 11.5 kB and one interpolated at 6.29 kB, consistent with the pattern of relaxed and supercoiled plasmid (Figure 4). Both uncut YEp and uncut mYEp which were run as controls, also migrated as two bands at 11.5 kB and 6.29 kB.

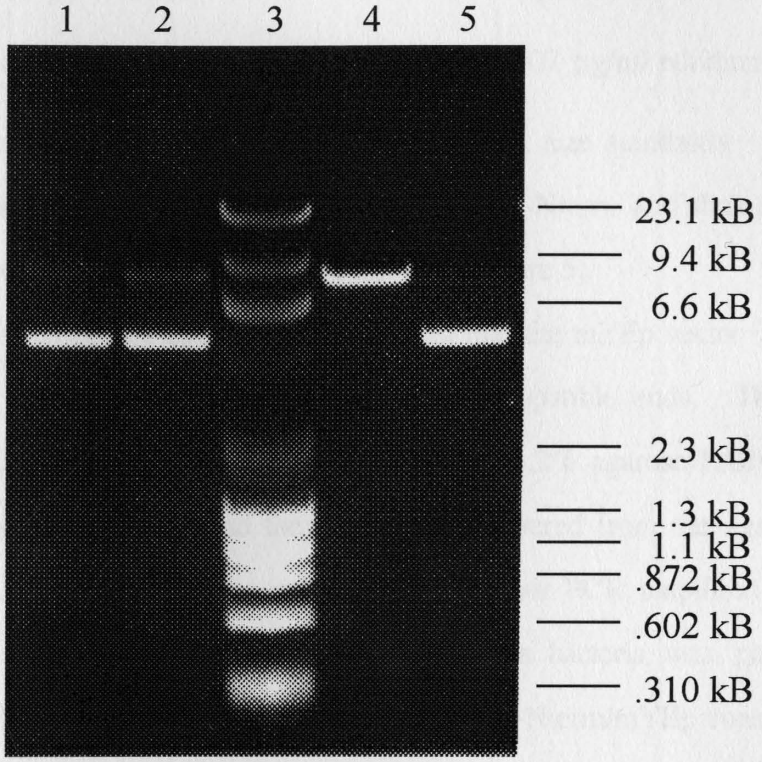

Figure 4. Size fractionation of YEp and mYEp Nhe I digests. Reactions were size fractionated on a 0.8% agarose/TAE gel, 0.01 mg/µl ethidium bromide. Undigested YEp and mYEp were run as controls. Lane 1 contains 5 µl undigested YEp vector . Lane 2 contains 5 µl of YEp vector digested with Nhe I. DNA standards are seen in lane 3 and the corresponding sizes are indicated on the right. Lane 4 contains 5 µl mYEp vector digested with Nhe I. Lane 5 contains 5 µl of undigested mYEp.

Pfs230 Expression Vector

Using mYEp, a Pfs230 expression vector was constructed. To do this, DNA encoding region C-Nterm (base pair #1339 to base pair #1752 of Pfs230) was amplified by PCR. Sense and antisense primers which encoded Xho I and Apa I, respectively, at the 5' end were used in the reaction. The size of the PCR amplified DNA was evaluated via electrophoresis on a 1.2% agarose/TAE gel containing 0.007 μg/ml ethidium bromide and was found to be 473 base pairs by interpolation against size standards. This was consistent with the predicted 414 base pair size of Pfs230.C-Nterm and thus suggested that the appropriate region of Pfs230 had been amplified (Figure 5).

To facilitate insertion of PCR amplified C-Nterm into the mYEp vector (Figure 6) both were digested with Xho I and Apa I generating compatible ends. The double digested PCR product and vector were size fractionated on a 1.2% agarose/TAE gel. The corresponding bands were isolated and the DNA was recovered from the agarose and genecleaned. The 7.2 kB mYEp vector and the 414 base pair PCR amplified C-Nterm were ligated together and electroporated into *E. coli.* The bacteria was plated onto LB/ampicillin agar and the transformation efficiency for the C-Nterm/mYEp construct was calculated as follows:

$$\text{CFU}/\mu\text{g} = \frac{200\ \text{CFU}}{4.56 \times 10^4\ \text{pg}} * \frac{1 \times 10^6}{\mu\text{g}} * 2 = 8.7 \times 10^3$$

The transformation efficiency for the positive control pUC19 was calculated as follows:

$$\text{CFU}/\mu\text{g} = \frac{200\ \text{CFU}}{10\ \text{pg}} * \frac{1 \times 10^6}{\mu\text{g}} * 100 = 2 \times 10^9$$

Three ampicillin resistant colonies were selected, grown up, and the plasmid was isolated. Plasmid was digested with Kpn I and Spe I and size fractionated on a 1.0% agarose/TAE gel. The cleavage site for Kpn I in mYEp is base pair #1457. The cleavage

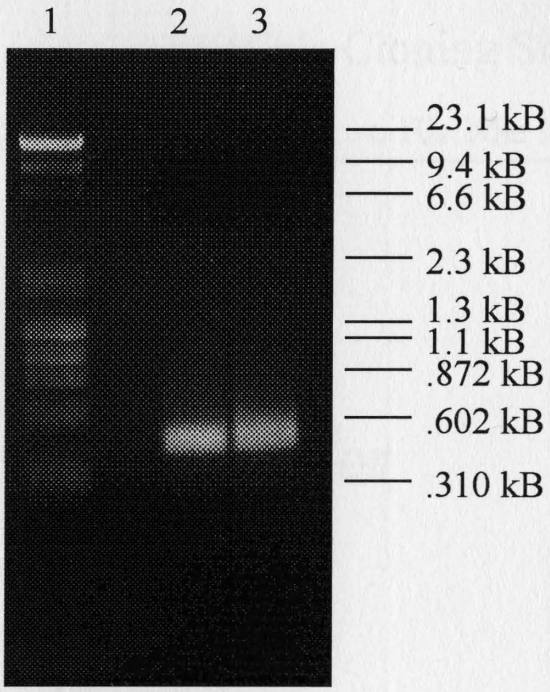

Figure 5. Size fractionation of PCR amplified Pfs230.C-Nterm. Primers designed to flank the sequence of Pfs230.C-Nterm were used to amplify this region via the polymerase chain reaction and the products obtained were size fractionated on a 1.2% agarose/TAE gel. DNA size standards appear in lane 1 and the corresponding sizes are indicated on the right. Lanes 2 and 3 each contain 2 µl of the PCR amplified product.

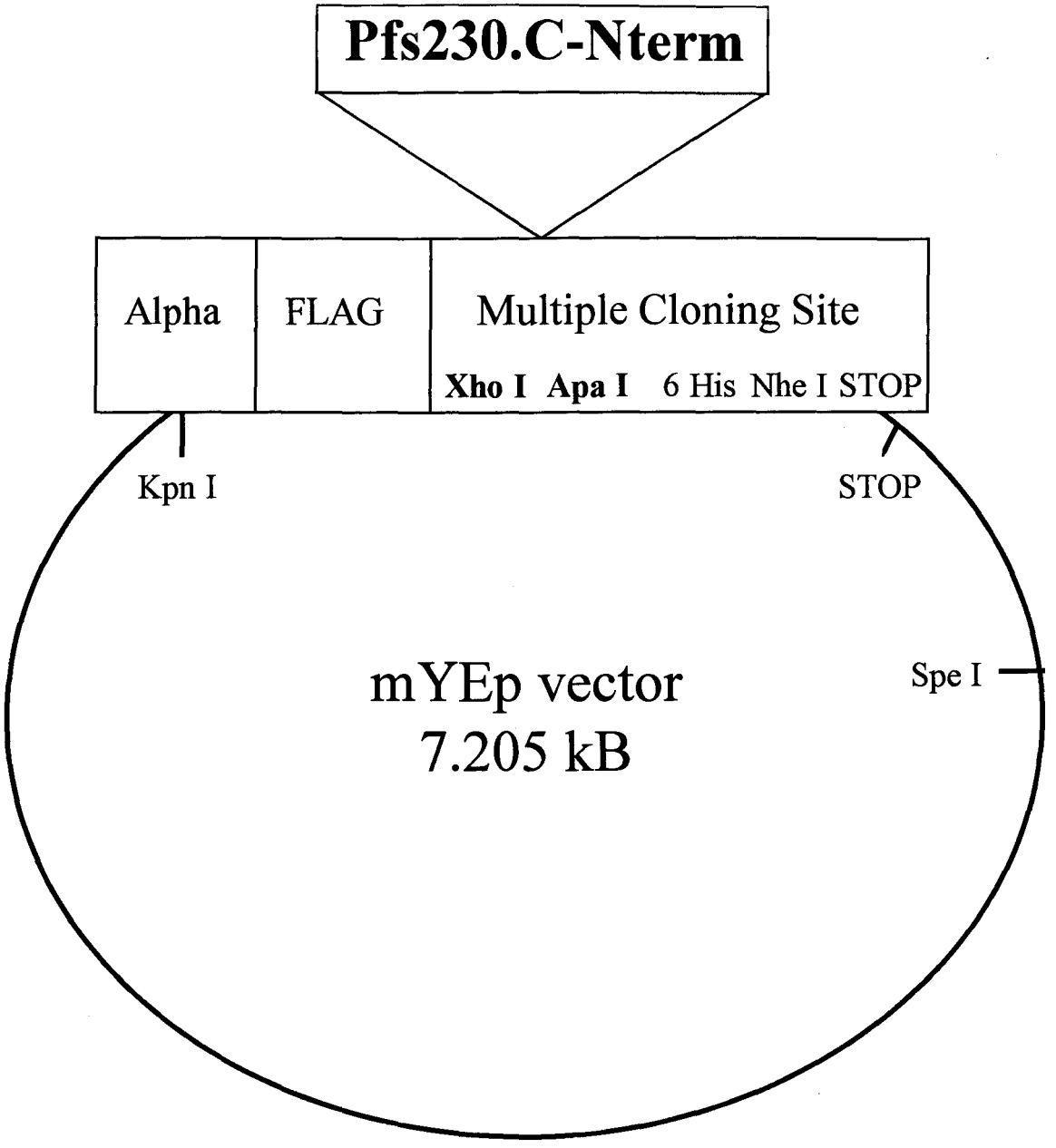

Figure 6. Schematic representing the mYEp vector. The position of the restriction sites that were used in this project are indicated. The relative location of the six histidine tag, the FLAG peptide and stop codons are also indicated.

site for Spe I in mYEp is base pair #2031. Thus if a mYEp vector without C-Nterm insert was digested with these enzymes, two fragments would result: one at 6.626 base pairs representing the vector; one at 574 base pairs representing the area of the vector between Kpn I and Spe I. If mYEp contained the C-Nterm insert, a digest would result in two fragments on an agarose/TAE gel: mYEp at 6.626 kB; C-Nterm at approximately 988 base pairs (the 414 base pair C-Nterm fragment plus the 574 bases between Kpn I and Spe I). Two out of three colonies selected were digested with Kpn I and Spe I. Plasmid from one of these colonies is seen in Figure 7 and migrated as two fragments on the agarose/TAE gel: one interpolated against size standards to be 7.95 kB; one interpolated against size standards to be 931 base pairs (Figure 7). These results suggest that the PCR product had been ligated into mYEp. As a control, a sample of the original mYEp which had not been ligated with C-Nterm insert, was also digested with Kpn I and Spe I. In contrast to the digest of the plasmid purified from C-Nterm/mYEp transfected bacteria, the mYEp digest resulted in a band extrapolated against size standards to be 7.95 kB and a band extrapolated against size standards to be 555 base pairs.

Manual DNA Sequencing

To confirm that the C-Nterm/mYEp construct had been ligated together in the proper reading frame, the Qiagen purified C-Nterm/mYEp DNA was manually sequenced. Sequencing reactions were performed and then electrophoresed on a 6% polyacrylamide gel. An autoradiograph of the sequencing gel was read from base pair #1493 to base pair #1507 in the vector and base pair #1 to base pair #263 in C-Nterm (Figure 8). This was compared with the reported sequence of C-Nterm/mYEp (Figure 8) to verify the proper reading frame. The vector sequence that was read from the autoradiograph was identical to the reported sequence of mYEp base pair #1493 to base pair #1507. However, one discrepancy was identified between the manual sequence of Pfs230.C-Nterm and the

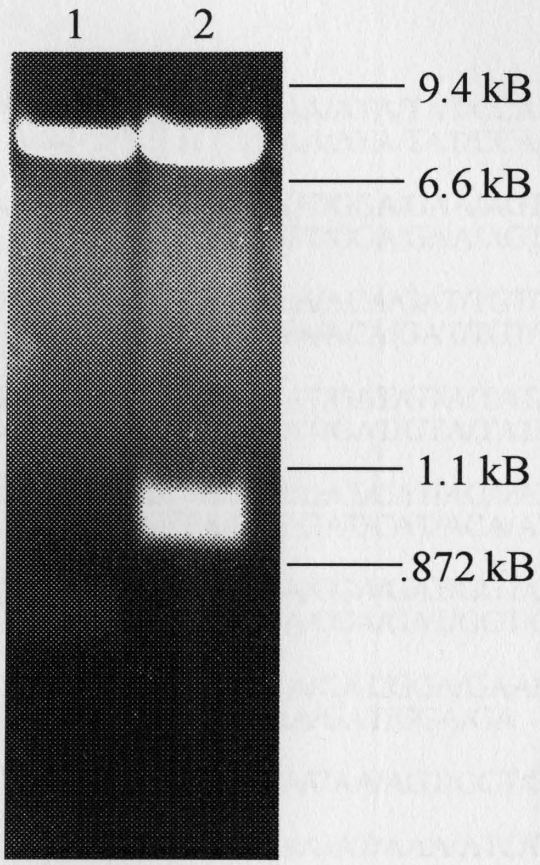

Figure 7. Kpn I and Spe I digests of mYEp and C-Nterm/mYEp. The constructs were digested and size fractionated on a 1% agarose/TAE gel. Lane 1 contains 10 µl of mYEp cut with Kpn I and Spe I. Lane 2 contains 10 µl C-Nterm/mYEp cut with Kpn I and Spe I. The weights of DNA molecular size standards are indicated on the right.

Comparison of reported sequence of Pfs230
and manual sequencing data

Reported	GAA/AAA/GAA/AGG/CAA/GGT/GAA/ATA/TAT/CCA/TTT/GGT/GAT
Manual	GAA/AAA/GAA/AGG/CAA/GGT/GAA/ATA/TAT/CCA/TTT/GGT/GAT
Reported	GAA/GAA/GAA/AAA/GAT/GAA/GGT/GGA/GAA/AGT/TTT/ACC/TAT
Manual	GAA/GAA/GAA/AAA/GAT/GAA/GGT/GGA/GAA/AGT/TTT/ACC/TAT
Reported	GAA/AAG/AGC/GAG/GTT/GAT/AAA/ACA/GAT/TGT/TTT/AAA/TTT
Manual	GAA/AAG/AGC/GAG/GTT/GAT/AAA/ACA/GAT/TGT/TTT/AAA/TTT
Reported	ATA/GAA/GGG/GGT/GAA/GGA/GAT/GAT/GTA/TAT/AAA/GTG/GAT
Manual	ATA/GAA/GGG/GGT/GAA/GGA/GAT/GAT/GTA/TAT/A_A/GTG/GAT
Reported	GGT/TCC/AAA/GTT/TTA/TTA/GAT/GAT/CAT/ACA/ATT/AGT/AGA
Manual	GGT/TCC/AAA/GTT/TTA/TTA/GAT/GAT/CAT/ACA/ATT/AGT/AGA
Reported	GTA/TCT/AAA/AAA/CAT/ACT/GCA/CGA/GAT/GGT/GAA/TAT/GGT
Manual	GTA/TCT/AAA/AAA/CAT/ACT/GCA/CGA/GAT/GGT/GAA/TAT/GGT
Reported	GAA/TAT/GGT/GAA/GCT/GTC/GAA/GAT/GGA/GAA/AAT/GTT/ATA
Manual	GAA/TAT/GGT/GAA/GCT/GTC/GAA/GAT/GGA/GA
Reported	AAA/ATA/ATT/AGA/AGT/GTG/TTA/CAA/AGT/GGT/GCA/TTA/CCA
Reported	AGT/GTA/GGT/GTT/GAT/GAG/TTA/GAT/AAA/ATC/GAT/TTG/TCA
Reported	TAT/GAA/ACA/ACA/GAA/AGT/GGA/GAT/ACT/GCT/GTA/TCC/GAA
Reported	GAT/TCA/TAT/GAT/AAA/TAT/GCA/TCT

Figure 8. The nucleic acid sequence of Pfs230.C-Nterm was determined and compared with the reported sequence of Pfs230. The top line represents the nucleic acid sequence of Pfs230 and the bottom line represents the data obtained from the manual sequencing of C-Nterm/mYEp. The underlined nucleotide represents the base not seen on the autoradiograph obtained by manual sequencing.

reported sequence. Base pair #149 is one of three sequential **A**'s as reported in Pfs230. Only two **A**'s were visible on the autoradiograph in that region. There is, however, clearly an empty space adjacent to those two **A**'s.

S. cerevisiae Transformation

The first step in obtaining *S. cerevisiae* that expresses C-Nterm was to transfect *S. cerevisiae* with the C-Nterm/mYEp construct. *S. cerevisiae* strain 2095-6 was grown in YPD media, harvested, washed, and transfected with the Qiagen purified C-Nterm/mYEp construct as described in Materials and Methods. Electroporated yeast was grown on SCM minus TRP plates. After three days three colonies were selected and grown in SCM minus TRP liquid media. This culture was used to inoculate YPHSM media which induces protein expression. Protein expression was evaluated 72 hours post inoculation. Yeast expression cultures were fractionated by centrifugation (5,000 x g) and the supernatants were harvested. Five hundred µl of supernatant was TCA precipitated and resuspended in 50 µl 1X sample buffer, 5% β-mercaptoethanol. Twenty-five µl samples of the yeast expression media supernatant were electrophoresed on a 12% polyacrylamide separating gel, 3.75% polyacrylamide stacking gel and protein was detected by Coomassie blue staining (Figure 9).

On the Coomassie stained gel seen in Figure 9, there were a number of distinct low molecular weight protein bands (seen in lanes 2-4) associated with C-Nterm/mYEp transfected yeast supernatant that were not seen in the yeast transfected with YEp alone (lane 1). When interpolated by molecular weight standards (Amersham, Cleveland, OH) using linear regression, the unique protein bands on this gel appear to be the following sizes (major bands are in bold print and underlined):

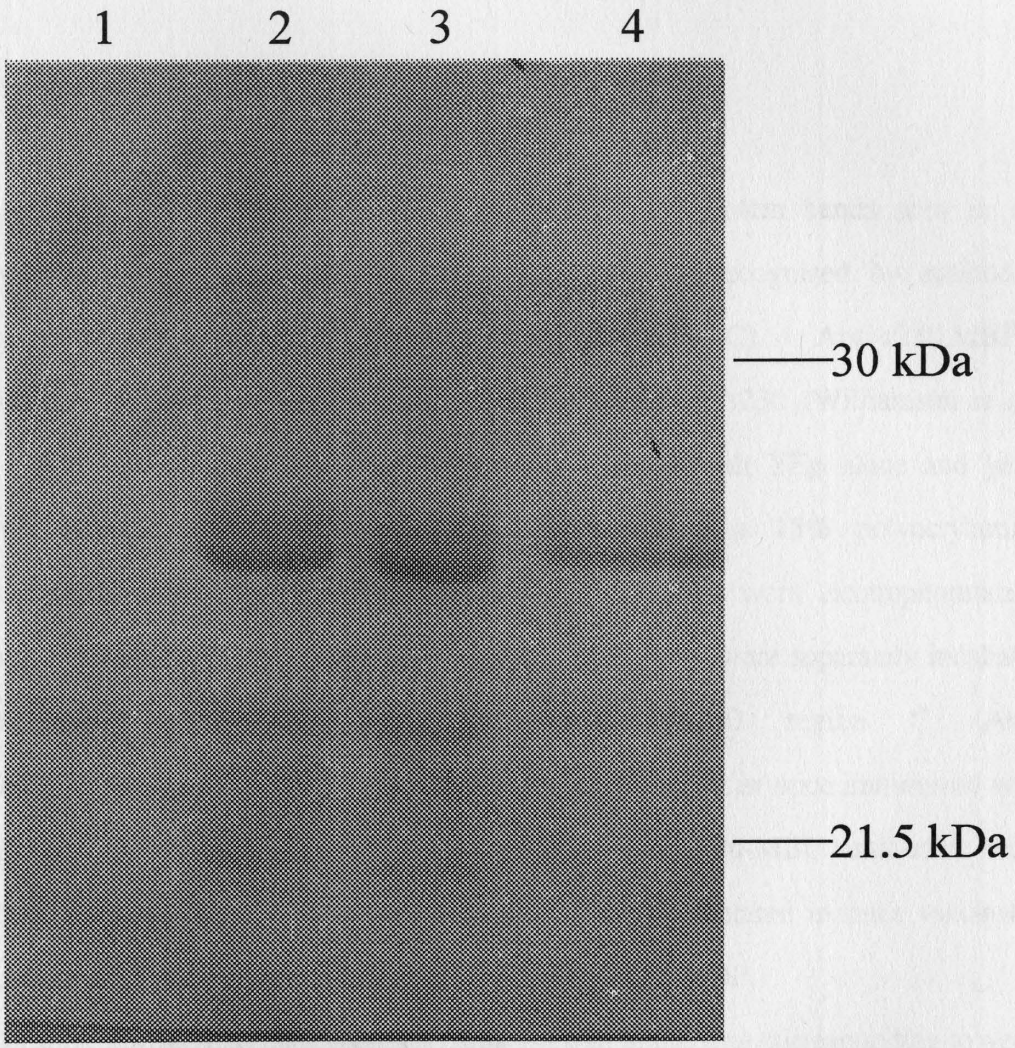

Figure 9. TCA precipitated supernatant of yeast transfected with C-Nterm/mYEp. Protein expression was evaluated on a 12% SDS-polyacrylamide gel. Lane 1 contains the equivalent of 250 µl YEp transfected yeast supernatant. Lanes 2-4 contain the equivalent of 250 µl C-Nterm/mYEp transfected yeast supernatant. Amersham molecular weight size standards are indicated on the right.

Lane 2: 30.58 kDa, **28.48** kDa, 21.04 kDa, 18.58 kDa, 16.11 kDa;

Lane 3: 30.04 kDa, **27.98** kDa, 20.31 kDa, 18.25 kDa, 15.83 kDa

Lane 4: ---- **28.48** kDa. ---- ---- ----

The predicted size of recombinant C-Nterm was 16,270 Da.

Western Blot Analysis of Proteins in Yeast Supernatant

A western blot was performed to determine if the protein bands seen in the supernatant of yeast transfected with C-Nterm/mYEp were recognized by antibodies generated to *E. coli* produced r230.MBP.C (Anti-r230.MBP.C). Anti-r230.MBP.C antibodies have been shown to recognize parasite-produced Pfs230 (Williamson *et al.*, 1995). TCA precipitated supernatant of yeast transfected with YEp alone and yeast transfected with C-Nterm/mYEp were size fractionated on a 15% polyacrylamide separating gel, 3.75% polyacrylamide stacking gel. Proteins were electrophoretically transferred to two nitrocellulose membranes and the membranes were separately incubated with antibodies that were generated against Pfs230 region C (Anti-r230.MBP.C)(Williamson *et al.*, 1995) and antibodies generated in mice immunized with MBP alone (Anti-MBP)(Williamson *et al.*, 1995). The Anti-MBP antibodies were included as a control because antibodies to region C were generated in mice vaccinated with recombinant Pfs230 produced in *E. coli* as fusions with MBP.

Results showed that there were no bands present in the lane corresponding to yeast transfected with YEp on the Anti-r230.MBP.C blot. There was reactivity, however, between the C-Nterm/mYEp yeast transfected supernatant and Anti-r230.MBP.C (Data not shown). The C-Nterm/mYEp protein bands on this blot appeared to migrate in the same molecular weight range as the C-Nterm/mYEp transfected yeast supernatant proteins in the original Coomassie stained gel (Figure 9). No reactivity was seen with YEp transfected yeast supernatant or C-Nterm/mYEp transfected yeast supernatant on the Anti-MBP blot

(Data not shown). This suggested that there was no immuno-crossreactivity between Anti-MBP and the proteins produced in YEp transfected yeast or C-Nterm/mYEp transfected yeast.

Ni^{2+}-NTA Purification of Recombinant C-Nterm Protein

A Ni^{2+}-NTA affinity column, which selectively binds six histidine tagged proteins, was used to purify recombinant C-Nterm from the C-Nterm/mYEp transfected *S. cerevisiae* culture supernatant. C-Nterm/mYEp transfected yeast supernatant was dialyzed against 1X PBS. Ni^{2+}-NTA resin equilibrated with Column buffer was added to the dialyzed yeast supernatant and the mixture incubated for one hour on ice. The supernatant/resin slurry was loaded onto two columns to increase the flow rate through the columns. Fractions eluted from the columns were then evaluated for protein by SDS-PAGE and western blot analyses.

Aliquots taken throughout the purification were size fractionated on a SDS-PAGE gel (Figure 10). Protein bands were interpolated against Sigma molecular weight standards (Sigma Chemical Company, St. Louis, MO). Both high and low molecular weight bands were present in the 1X PBS-dialyzed C-Nterm/mYEp transfected yeast supernatant and in the material which was applied to but did not bind to the Ni^{2+}-NTA affinity column (lanes 1 and 2, respectively). The bands in lanes 1 and 2 were found to migrate in a nearly identical pattern from 75.15 kDa-18.20 kDa except for the band in the 25 kDa range which was fainter in lane 2. Lanes 3-5 which contained fractions from the pH 7.0 wash contained no bands. Only one eluant (lane 8) from the pH 7.0 Wash Buffer/imidazole wash contained protein (Figure 10). The bands in lane 8 were interpolated against Sigma molecular weight standards by linear regression and were found to migrate at: **25.61** kDa, 22.39 kDa, 21.70 kDa, 20.61 kDa, **18.78** kDa. The

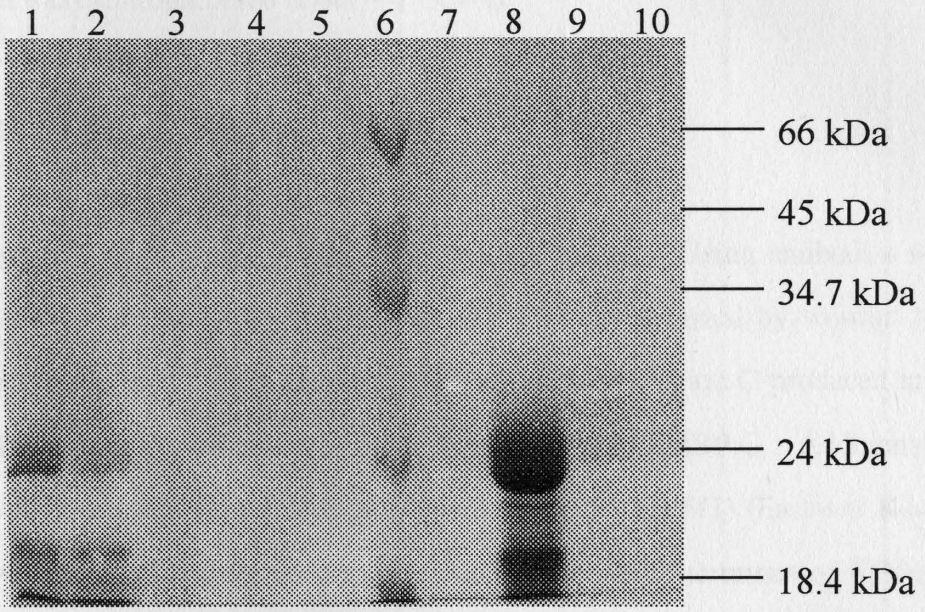

Figure 10. Ni^{2+}-NTA purification of protein from C-Nterm/mYEp transfected yeast. Material eluted from the affinity column was size fractionated on a 12% SDS-polyacrylamide gel and stained in Coomassie blue. Lane 1 contains a 20 µl sample of 1X PBS-dialyzed C-Nterm/mYEp transfected yeast supernatant prior to adding the Ni^{2+}-NTA resin. Lane 2 contains 20 µl of material which was applied to but did not bind to the Ni^{2+}-NTA affinity column. Lanes 3-5 contain 20 µl of material recovered after the first three sequential pH 7.0 washes. Lane 6 contains 10 µl Sigma molecular weight standards and the corresponding sizes are indicated on the right. Lanes 7-10 contain 20 µl fractions of material eluted from the column after applying pH 7.0 Wash buffer/0.5 M imidazole.

bands seen in lane 8 were similar in size to the low molecular weight bands seen in the original C-Nterm/mYEp transfected yeast dialysate (lane 1) and in the material which did not bind to the affinity column (lane 2). However the higher molecular weight bands seen in the dialyzed, unpurified yeast supernatant (lane 1) and in the material which flowed through the affinity column (lane 2) were not present in lane 8. An elution pattern similar to the one in Figure 10 was seen on the gel in which protein eluted from the second Ni^{2+} affinity column was electrophoresed (Data not shown).

Characterization of Purified C-Nterm

1. Western Blot Analyses of Ni^{2+}-NTA Purified Protein. Using antibodies with different specificities, the Ni^{2+}-NTA purified protein was characterized by western blot analyses. To determine the immuno-crossreactivity with Pfs230 region C produced in *E. coli*, protein eluted from the column was probed with Anti-r230.MBP.C. Additionally, monoclonal antibodies against the FLAG polypeptide (Anti-FLAG M1) (Eastman Kodak Company, New Haven, CT) were used to determine if the amino terminus of C-Nterm was present. The DNA sequence which encodes the FLAG peptide is located at the 5' end of the MCS in mYEp but is downstream of the α signal secretory sequence. As the protein moves through the secretory pathway, the α signal sequence is removed, leaving the FLAG peptide at the amino terminus of C-Nterm. Anti-FLAG M1 only reacts with the FLAG peptide when it is on the exposed amino terminus of a recombinant protein. Lastly, protein purified from the Ni^{2+} column was also probed with antibodies to MBP (Anti-MBP) to be sure that reactivity with Anti-r230.MBP.C was specific for r230.C and not its fusion partner, MBP. Reactivity of purified C-Nterm with Anti-MBP would call into question any reactivity seen with Anti-r230.MBP.C.

As a negative control, the protein pattern and immunoreactivity of supernatant from yeast transfected with YEp alone was compared with the immunoreactivity of selected fractions of supernatant from yeast transfected with C-Nterm/mYEp. A Coomassie stained gel of this material (Figure 11.A) revealed a number of higher molecular weight proteins in the YEp transfected yeast that were not visible in the yeast supernatant transfected with the C-Nterm/mYEp construct. The equivalent of 250 µl TCA precipitated yeast transfected with YEp alone was loaded in lane 1. Bands were interpolated against Sigma molecular weight standards by linear regression and were found to migrate between 75.58 kDa and 49.66 kDa. This correlates very well with the pattern of YEp transfected yeast proteins also seen in Figure 10. There were also less intense bands present in the 41.62 kDa to 24.48 kDa range. In lane 2, only 20 µl of 1X PBS-dialyzed supernatant from yeast transfected with C-Nterm/mYEp was loaded. Lane 3 contained 20 µl of C-Nterm/mYEp transfected yeast supernatant which was applied to but did not bind to the affinity column. Lane 4 contained 5 µl of material eluted from the affinity column after applying imidazole. The amount of protein seen in lane 2, which represents material which did not bind to the affinity column, was significantly lower than the amount of protein in lane 4 (Figure 11.A).

Only lanes 2-4 which correspond to C-Nterm/mYEp transfected yeast showed reactivity with Anti-r230.MBP.C (Figure 11.B). No bands were seen in the negative control lane (lane 1) which corresponds to the equivalent of 250 µl TCA precipitated yeast transfected with YEp alone. Reactivity was seen in lane 2 where 20 µl of 1X PBS-dialyzed supernatant from yeast transfected with C-Nterm/mYEp was loaded. Reactivity was also seen in lane 3 which contained 20 µl of C-Nterm/mYEp transfected yeast supernatant that was applied to but did not bind to the affinity column. Additionally,

reactivity was observed in lane 4 which contained 5 µl of material eluted from the affinity column. The protein bands seen in lanes 2-4 migrated in a nearly identical fashion to the bands in the Coomassie stained gel, Figure 11.A. These data suggested that Anti-r230.MBP.C was specific for protein that was only present in yeast transfected with the C-Nterm/mYEp and that the reacting protein was probably Pfs230.C-Nterm. It also suggested that there was no crossreactivity between the Anti-r230.MBP.C antibodies and secreted yeast proteins.

Similar to the previous blot, only lanes 2-4 which correspond to C-Nterm/mYEp transfected yeast showed reactivity with Anti-FLAG M1 (Figure 11.C). The immunostaining pattern was nearly identical to the pattern seen in the Anti-r230.MBP.C blot (Figure 11.B) and Coomassie stained gel (Figure 11.A) . No bands were seen in the negative control lane (lane 1) which corresponds to the equivalent of 250 µl TCA precipitated supernatant from yeast transfected with YEp alone. Because Anti-FLAG M1 only reacts with the FLAG peptide when it is exposed at the amino terminus of a protein, this suggested that the FLAG peptide was present on the amino terminus of recombinant Pfs230.C-Nterm. Additionally, all of the bands that reacted with Anti-r230.MBP.C reacted with Anti-FLAG M1 (Figures 11.B and 11.C). The protein bands in the Coomassie stained gel as well as the two immunoblots seen in Figure 11.A,B,C were found to migrate (by extrapolation using linear regression) between 30.83 kDa to 16.09 kDa.

To assay reactivity of purified C-Nterm with Anti-MBP, protein eluted from the Ni^{2+} affinity column was electrophoresed on a 15% polyacrylamide separating gel, 3.75% polyacrylamide stacking gel. A 15% gel was used in order to better visualize the lower molecular weight bands associated with C-Nterm and determine how they correlate with bands on a 12% denaturing gel. Part of a 15% gel was Coomassie stained while the other

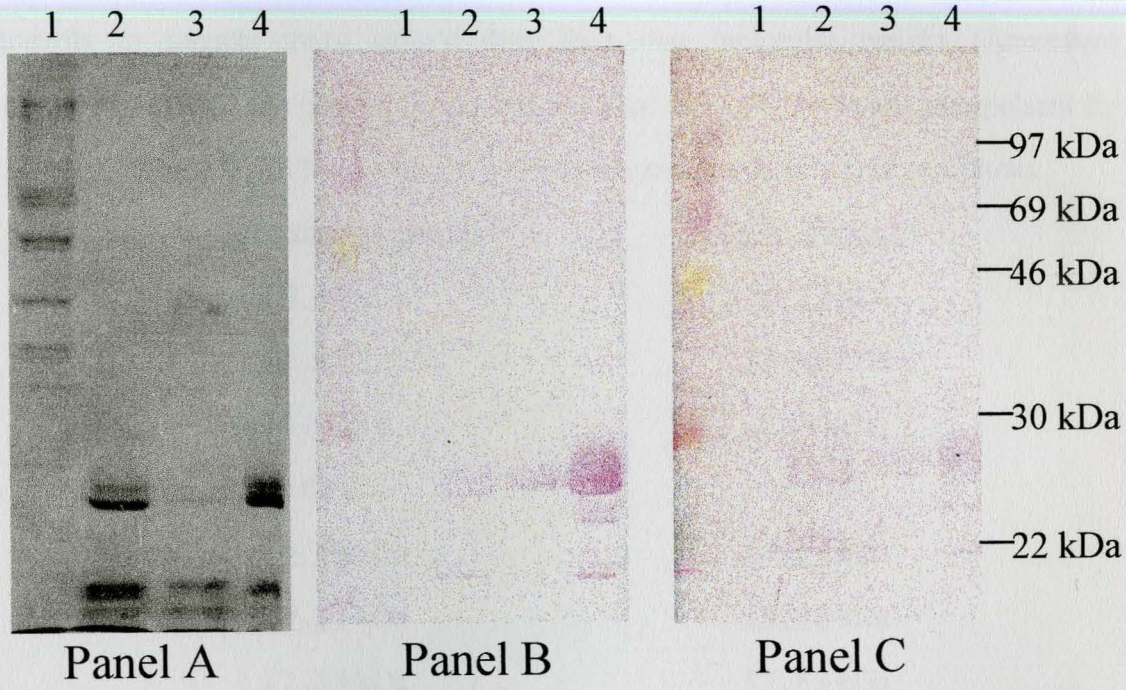

Figure 11.A,B,C. Coomassie staining and immunoblot analyses of purified and unpurified proteins. On a 12% SDS-polyacrylamide gel, samples of YEp transfected yeast supernatant as well as unpurified and purified samples from C-Nterm/mYEp transfected yeast supernatant were electrophoresed. Proteins in panel A were stained by Coomassie blue. Proteins in panel B were electrophoretically transferred to nitrocellulose and probed with Anti-r230.MBP.C. Proteins in panel C were electrophoretically transferred to nitrocellulose and probed with Anti-FLAG M1. Lane 1 in all three panels contained the equivalent of 250 µl YEp transfected yeast supernatant. Lane 2 in all three panels contained 20 µl of 1X PBS dialyzed C-Nterm/mYEp transfected yeast supernatant before it was purified. Lane 3 in all three panels contained 20 µl material which was applied to but did not bind to the affinity column. Lane 4 in all three panels contains 5 µl of protein eluted from the Ni^{2+}-NTA affinity column. Molecular weight standards are seen on the left in panels B and C and the corresponding sizes for all panels are indicated on the far right.

part was electrophoretically transferred to nitrocellulose and probed with Anti-MBP. The Coomassie stained gel showed several bands associated with the C-Nterm eluant and those bands were interpolated against Amersham molecular weight standards as well as Sigma molecular weight standards (Figure 12). In contrast to the Sigma standards, Amersham standards are pre-stained and are therefore a less accurate reflection of size than are the Sigma standards. The covalent attachment of dyes to the proteins causes the Amersham standards to migrate more slowly than their true molecular weight (Amersham Corporation, 1996). As seen in Figure 12, the sizes of C-Nterm bands interpolated by Amersham standards and Sigma standards using linear regression ranged as follows:

Amersham standards	Sigma standards
28.46 kDa	**26.22** kDa
26.94 kDa	**25.06** kDa
24.13 kDa	22.88 kDa
21.62 kDa	20.90 kDa
19.91 kDa	**19.52** kDa
19.36 kDa	**19.08** kDa
17.83 kDa	**17.83** kDa
16.42 kDa	**16.65** kDa
15.12 kDa	**15.56** kDa

No reactivity was seen with C-Nterm on the Anti-MBP western blot (Data not shown) suggesting that Anti-r230.MBP.C is truly recognizing C-Nterm and not MBP.

2. Glycoprotein Detection. Because Pfs230.C-Nterm appeared to be a higher molecular weight than the calculated 16,270 kDa, it was necessary to consider if the protein had been posttranslationally modified. A common posttranslational modification of proteins expressed and secreted by yeast is glycosylation. To test for the presence of carbohydrates on C-Nterm, protein samples were electrophoretically transferred to

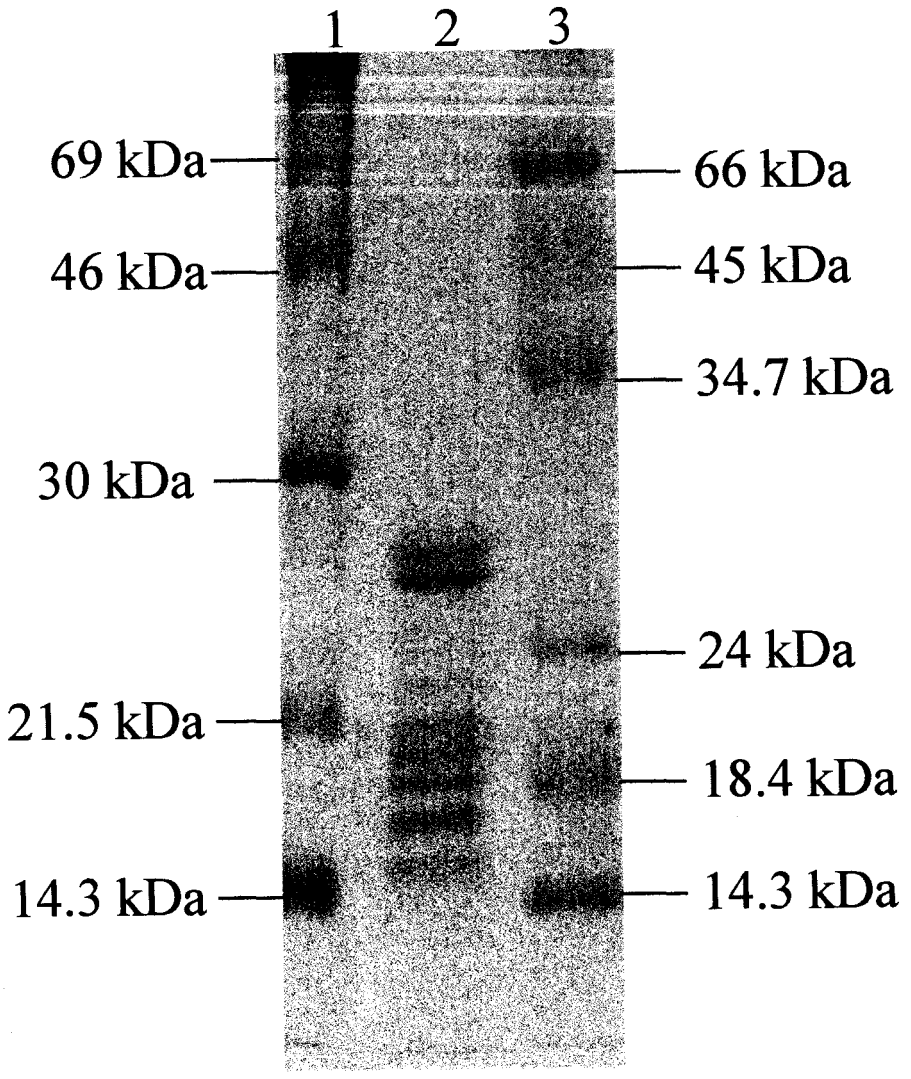

Figure 12. Electrophoretic analysis of purified C-Nterm on a 15% SDS-polyacrylamide gel. Purified C-Nterm was size fractionated on a 15% SDS-polyacrylamide gel and its size interpolated against Amersham and Sigma molecular weight markers. Lane 1 contains Amersham molecular weight standards and the sizes are indicated on the left. Lane 2 contains 3 µl of Ni^{2+} purified C-Nterm. Lane 3 contains Sigma molecular weight standards and the corresponding sizes are indicated on the right.

nitrocellulose. For comparison, 1X PBS-dialyzed yeast supernatant from yeast transfected with C-Nterm/mYEp was loaded in lane 1 while an aliquot of material which did not bind to the Ni^{2+}-NTA affinity column was loaded in lane 3. Glycoproteins were detected using the Glycan Detection Kit (Boehringer Mannheim, Indianapolis, IN) described in the Materials and Methods. As seen in Figure 13 there are several high molecular weight bands which showed a very similar pattern of reactivity in both lanes. The bands in lane 1 were interpolated against Amersham molecular weight standards by linear regression and were found to migrate between 97.51 kDa and 41.35 kDa (Figure 13). The bands in lane 3 migrated between 97.51 kDa and 42.73 kDa (Figure 13). No bands in the 28 kDa-16 kDa range (apparent weight of C-Nterm as interpolated by Amersham standards) showed reactivity (Figure 13).

C-Nterm Protein Sequencing

Because the eluted protein was estimated to be 10 kDa larger than the theoretical weight, the possibility that the amino acid sequence of the protein had been altered, perhaps by a mutation or rearrangement in the DNA, was considered. The major (highest molecular weight) C-Nterm band was purified using HPLC and was then subjected to *Staphylococcus aureus* (V8) protease digestion. Peptides were purified by HPLC using a C4 reversed phase column (Dr. Bassam Wakim, PhD). A peptide 10 amino acids in length and a peptide 17 amino acids in length were sequenced (Figure 14) and found to be identical with the reported sequence of Pfs230.C-Nterm (amino acid 8 to amino acid 17 and amino acid 42 to amino acid 58, respectively)(Figure 14).

C-Nterm DNA Sequencing

Although the amino acid sequence of the peptides obtained from C-Nterm were

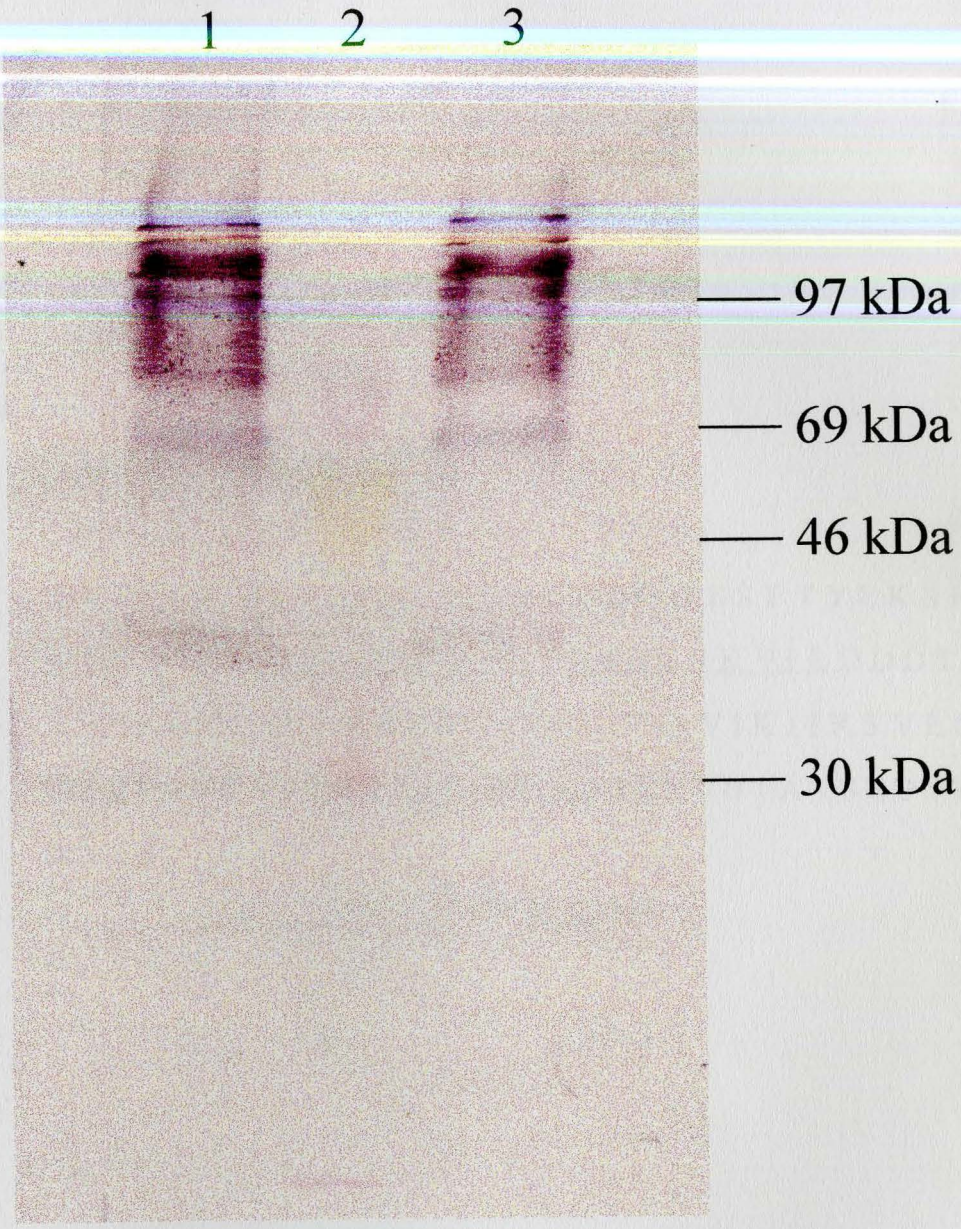

Figure 13. Glycoprotein analysis of C-Nterm/mYEp transfected yeast supernatant. Proteins were size fractionated on a 12% SDS-polyacrylamide gel, electrophoretically transferred to a nitrocellulose membrane, and assayed for the presence of carbohydrate moieties. Lane 1 contains 20 µl of 1X PBS dialyzed C-Nterm/mYEp transfected yeast supernatant. Lane 2 contains Amersham molecular weight standards and corresponding sizes are indicated on the right. Lane 3 contains 20 µl of C-Nterm/mYEp transfected yeast material that was applied but did not bind to the Ni^{2+}-NTA affinity column.

Amino acid sequence of
Pfs230.C-Nterm

Reported E K E R Q G E I Y P F G D E E E K D E G G E S F T Y E K S E V

Reported D K T D L F K F I E G G E G D D V Y K V D G S K V I L D D D T I S

Reported R V S K K H T A R D G E Y G E A V E D G E N V I K I I R S V L Q S

Reported G A L P S V G V D E L D K I D L S Y E T T E S G D T A V S E D S Y D

Reported K Y A S

Figure 14. Peptide sequencing data of yeast expressed Pfs230.C-Nterm. This schematic illustrates the reported amino acid sequence of Pfs230.C-Nterm and the two peptides obtained from protein sequencing are underlined. The bolded, lower case amino acid (#57) obtained in the peptide sequence represents the questionable region of substitution in yC-Nterm/mYEp DNA sequencing data (see Figure 16).

correct, there was still the possibility that the DNA sequence of C-Nterm had been altered downstream of amino acid 58 and had disrupted the stop codons, resulting in a larger protein. This could have been caused by a mutation in the stop codons themselves or a change in the reading frame upstream from the stop codons. To determine if yeast had rearranged the plasmid, the C-Nterm/mYEp construct was recovered. The first step in harvesting the construct from yeast involved spheroplasting the yeast using lyticase. Two tests were used to determine if the spheroplasting was successful: 1) precipitate formation in 1 M sorbitol 2) lysis in 2% SDS. These experiments suggested that the yeast cell wall had been removed making the yeast sensitive to detergent lysis. Proteinase K was then added to the spheroplasts and a phenol extraction was performed to remove protein. The DNA was further purified by a chloroform extraction and was used to transfect *E. coli.* To select for ampicillin resistance, transfected bacteria was plated onto LB/agar plates containing ampicillin. Only colonies which contained mYEp vector should be resistant to ampicillin and therefore able to survive. Both the pUC19 control plate and the experimental plates were confluent therefore transformation efficiency was not determined.

Plasmid purified from amplified yC-Nterm/mYEp colonies was compared with the original construct C-Nterm/mYEp (construct before it was transfected into yeast) by restriction analysis using Kpn I and Spe I. A Kpn I site is 44 base pairs upstream of the C-Nterm insert in the mYEp vector. A Spe I site is 494 base pairs downstream of the C-Nterm insert. A digest with Kpn I and Spe I of both C-Nterm/mYEp and yC-Nterm/mYEp resulted in two fragments on a 1.0% agarose/TAE gel (Figure 15). The sizes of these bands were interpolated against size standards by linear regression. The bands for yC-Nterm/mYEp were found to migrate at: a band at 7.95 kB base pairs (mYEp); a second band at 813 base pairs (the approximate size of the insert (414 base pairs) plus the additional mYEp vector sequence (574 base pairs)). The sizes of the C-Nterm/mYEp bands interpolated as follows: a band at 8.10 kB and a band at 813 base pairs. Thus the size of the mYEp and C-Nterm fragments were the same both before and

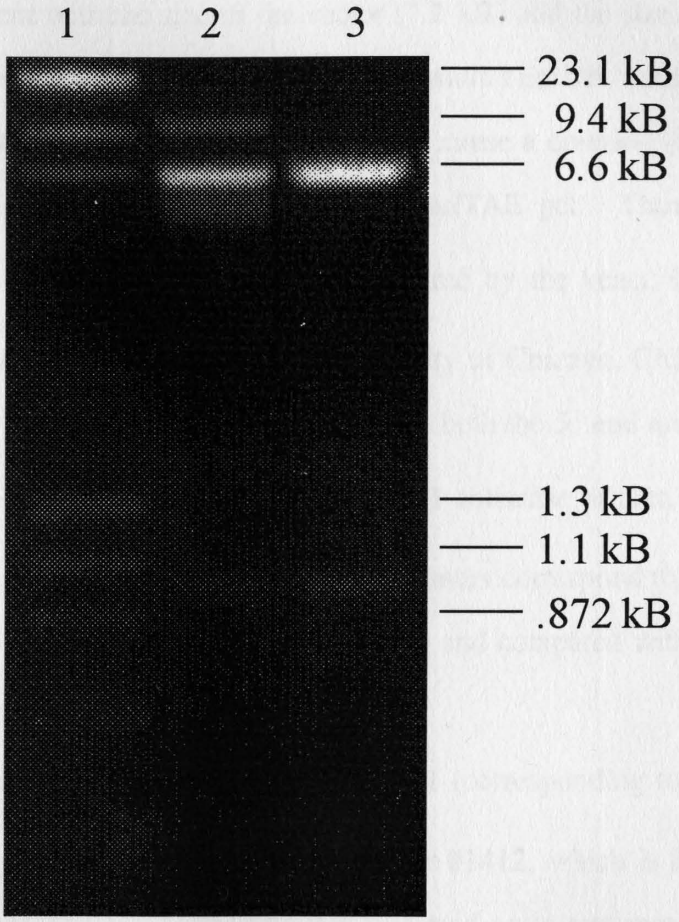

Figure 15. Restriction digests of yC-Nterm/mYEp and C-Nterm/mYEp. To compare to size of the inserts, yC-Nterm/mYEp and C-Nterm/mYEp were digested with Kpn I and Spe I (enzymes flanking the C-Nterm insert). These digests were size fractionated on a 1% agarose/TAE gel. Lane 1 contains DNA size standards with the corresponding sizes indicated on the right. Lane 2 contains 25 µl of the yC-Nterm/mYEp digest. Lane 3 contains 10 µl of the C-Nterm/mYEp digest.

after transfection into yeast. This suggests that the yeast made no major rearrangements that altered the size of the plasmid.

Automated DNA Sequencing

The results of the restriction endonuclease digestion of yC-Nterm/mYEp showed a pattern of bands consistent with the size of the vector (7.2 kB) and the size of the insert (414 base pairs). However, the reading frame of yC-Nterm/mYEp DNA still could have altered by a single base deletion or insertion that would not cause a dramatic change in the size of the mYEp and C-Nterm fragments on an agarose/TAE gel. Thus, to further determine if the yC-Nterm/mYEp construct had been altered by the yeast, 0.3 μg/μl of Qiagen purified yC-Nterm/mYEp was sent to the University of Chicago, Chicago, IL for automated sequencing. The construct was sequenced from both the 5' end and the 3' end using 0.8 pmol/μl of the YαN-21 sense primer and YcC-21 antisense primer, respectively (Eastman Kodak Company, New Haven, CT). These primers correspond to sequence in mYEp. The resulting chromatogram was read (Figure 16) and compared with the known sequence Pfs230.C-Nterm (Figure 16).

The chromatogram generated using primer YαN-21 (corresponding to vector base pairs #1388-#1408) was read beginning at vector base pair #1412, which is in the FLAG sequence of mYEp (Figure 16). The chromatogram was read up to and including vector base pair #1567, which is 3' to the six histidine tag and the two stop codons in the mYEp vector. Pfs230.C-Nterm was inserted into the vector in between base pair #1502 and base pair #1537.

The chromatogram generated using primer YcC-21 (corresponding to vector base pairs #1633-#1613) was read beginning at vector base pair #1582, which is in the CYC1 part of the mYEp vector (Figure 16). The chromatogram was read up to and including

Sequencing Pfs230.C-Nterm

Reported tt/ata/aat/act/act/att/gcc/agc/att/gct/gct/aaa/gaa/gaa/ggg/gta/cct/ttg/gat/aaa/aga/gac/tac/aag
Automated tt/ata/aat/act/act/att/gcc/agc/att/gct/gct/aaa/gaa/gaa/ggg/gta/cct/ttg/gat/aaa/aga/gac/tac/aag
Manual
Protein

Reported gat/gac/gat/gac/aag/gaa/ttc/ctc/gag/GAA/AAA/GAA/AGG/CAA/GGT/GAA/ATA/TAT
Automated gat/gac/gat/gac/aag/gaa/ttc/ctc/gag/GAA/AAA/GAA/AGG/CAA/GGT/GAA/ATA/TAT
Manual GAA/AAA/GAA/AGG/CAA/GGT/GAA/ATA/TAT
Protein E K E R Q G E I Y

Reported CCA/TTT/GGT/GAT/GAA/GAA/GAA/AAA/GAT/GAA/GGT/GGA/GAA/AGT/TTT
Automated CCA/TTT/GGT/GAT/GAA/GAA/GAA/AAA/GAT/GAA/GGT/GGA/GAA/AGT/TTT
Manual CCA/TTT/GGT/GAT/GAA/GAA/GAA/AAA/GAT/GAA/GGT/GGA/GAA/AGT/TTT
Protein P F G D E E E K D E G G E S F

Reported ACC/TAT/GAA/AAG/AGC/GAG/GTT/GAT/AAA/ACA/GAT/TTG/TTT/AAA/TTT
Automated ACC/TAT/GAA/AAG/AGC/GAG/GTT/GAT/AAA/ACA/GAT/TTG/TTT/AAA/TTT
Manual ACC/TAT/GAA/AAG/AGC/GAG/GTT/GAT/AAA/ACA/GAT/TTG/TTT/AAA/TTT
Protein T Y E K S E V D K T D L F K F

Reported ATA/GAA/GGG/GGT/GAA/GGA/GAT/GAT/GTA/TAT/AAA/GTG/GAT/GGT/TCC
Automated ATA/GAA/GGG/GGT/GAA/GGA/GAT/GAT/GTA/TAT/AAA/GTG/GAT/GGT/TCC
Manual ATA/GAA/GGG/GGT/GAA/GGA/GAT/GAT/GTA/TAT/A_A/GTG/GAT/GGT/TCC
Protein I E G G E G D D V Y K V D G S

Reported AAA/GTT/TTA/TTA/GAT/GAT/GAT/ACA/ATT/AGT/AGA/GTA/TCT/AAA/AAA
Automated AAA/GTT/TCA/TTA/GAT/GAT/GAT/ACA/ATT/AGT/AGA/GTA/TCT/AAA/AAA
Manual AAA/GTT/TTA/TTA/GAT/GAT/GAT/ACA/ATT/AGT/AGA/GTA/TCT/AAA/AAA
Protein K V L L D D D T I S R V S K K

Reported CAT/ACT/GCA/CGA/GAT/GGT/GAA/TAT/GGT/GAA/TAT/GGT/GAA/GCT/GTC
Automated CAT/ACT/GCA/CGA/GAT/GGT/GAA/TAT/GGT/GAA/TAT/GGT/GAA/GCT/GTC
Manual CAT/ACT/GCA/CGA/GAT/GGT/GAA/TAT/GGT/GAA/TAT/GGT/GAA/GCT/GTC
Protein H T A R D G E Y G E Y G E A V

Figure 16. DNA sequence of yC-Nterm/mYEp. The top line of the schematic represents the reported sequence of Pfs230 and mYEp. The second line reports the data obtained from automated sequencing of yC-Nterm/mYEp. The third line shows the sequence read from the autoradiograph of manually sequenced C-Nterm/mYEp. Vector sequence is in lower case letters and Pfs230 sequence in upper case. Underlined, bolded nucleotides indicate discrepancies between that sequence and the reported sequence of Pfs230. Stop codons in the vector as well as the six histidine tag are noted. Figure continues on page 70.

Sequencing Pfs230.C-Nterm

Reported GAA/GAT/GGA/GAA/AAT/GTT/ATA/AAA/ATA/ATT/AGA/AGT/GTG/TTA/CAA
Automated GAA/GAT/GGA/GAA/AAT/GTT/ATA/AAA/ATA/ATT/AGA/AGT/GTG/TTA/CAA
Manual GAA/GAT/GGA/GA
Protein E D G E N V I K I I R S V L Q

Reported AGT/GGT/GCA/TTA/CCA/AGT/GTA/GGT/GTT/GAT/GAG/TTA/GAT/AAA/ATC
Automated AGT/GGT/GCA/TTA/CCA/AGT/GTA/GGT/GTT/GAT/G**G**G/TTA/GAT/AAA/ATC
Manual
Protein S G A L P S V G V D E L D K I

Reported GAT/TTG/TCA/TAT/GAA/ACA/ACA/GAA/AGT/GGA/GAT/ACT/GCT/GTA/TCC
Automated GAT/TTG/TCA/TAT/GAA/ACA/ACA/GAA/AGT/GGA/GAT/ACT/GCT/GTA/TCC
Manual
Protein D L S Y E T T E S G D T A V S

6 his tag

Reported GAA/GAT/TCA/TAT/GAT/AAA/TAT/GCA/TCT/ggg/ccc/gtc/gac/cac/cac/cac/cac/cac
Automated GAA/G**G**T/TCA/TAT/GAT/AAA/TAT/GCA/TCT/ggg/ccc/gtc/gac/cac/cac/cac/cac/cac
Manual
Protein E D S Y D K Y A S

Stop **Stop**

Reported cac/tag/gct/agc/ccg/taa/gcg/gcc/gct/gat/ccg
Automated cac/tag/gct/agc/ccg/taa/gcg/gcc/gct/gat/ccg
Manual
Protein

Figure 16 (continued from page 69). DNA sequence of yC-Nterm/mYEp. The top line of the schematic represents the reported sequence of Pfs230 and mYEp. The second line reports the data obtained from automated sequencing of yC-Nterm/mYEp. The third line shows the sequence read from the autoradiograph of manually sequenced C-Nterm/mYEp. Vector sequence is in lower case letters and Pfs230 sequence in upper case. Underlined, bolded nucleotides indicate discrepancies between that sequence and the reported sequence of Pfs230. Stop codons in the vector as well as the six histidine tag are noted.

vector base pair #1343, which is in the α signal secretory sequence of the mYEp vector (Figure 16).

Thus the entire Pfs230.C-Nterm insert sequence and several base pairs before and after were sequenced in both directions. Three discrepancies were found between the chromatogram for these runs and the reported sequence of the Pfs230. The first discrepancy was noted at insert base pair #170, where there was a **C** instead of the predicted base pair, **T** (Figure 16). In contrast, data for the protein sequence of C-Nterm, corresponding to base pair #170 of C-Nterm, read as predicted by the reported sequence of Pfs230 (Figure 16). The second discrepancy was found at insert base pair #329, where there was a **G** instead of the predicted base pair, **A** (Figure 16). Lastly, insert base pair #392 should have been an **A** instead of the **G** seen in the chromatogram (Figure 16). Base pair #392 was actually part of the synthetic primer used in the PCR.

To summarize, the sequencing runs using the YαN-21 and the YcC-21 primers overlapped for much of the mYEp vector and for all of the C-Nterm insert. Sequencing from both directions indicated differences between the automated sequence and the reported sequence of Pfs230 in the same three base pairs. No deletions or insertions were identified in the stop codons suggesting that the reading frame had not been altered.

CHAPTER IV

DISCUSSION

In previous studies Pfs230 was expressed as six separate MBP fusion proteins in *E. coli* (Williamson *et al.*, 1995). Results from mice vaccinations with one of those six recombinants, r230/MBP.C, suggested this region may be a possible transmission-blocking vaccine candidate. In mosquitoes fed on antisera generated to r230/MBP.C parasite infectivity was reduced 71.2-89.8% (Williamson *et al.*, 1995). While 71.2-89.8% reduction in parasite infectivity is impressive, an effective transmission-blocking vaccine must reduce infectivity completely. One possible explanation why r230/MBP.C failed to reduce parasite infectivity in the mosquito midgut entirely may be that the recombinant protein did not fold properly in the *E. coli* cytosol, a reduced environment. Previous studies have determined that Pfs230-specific monoclonal antibodies require proper protein conformation in order to recognize antigens and that these monoclonal antibodies are reduction sensitive (Read *et al.*, 1994).

Possibly the most important aspect in developing a vaccine is that a synthetic antigen accurately mimics the native antigen structure. A previous study with *P. falciparum* protein Pfs25 showed that a yeast expression system produced a potent antigen which induced transmission-blocking while *E. coli* did not (Kaslow and Shiloach, 1994). Being a eukaryote, proteins expressed in yeast can be targeted to an extensive secretory pathway which allows the protein to be generated in a non-reduced environment. A crucial element in this secretory pathway is the rough endoplasmic reticulum (absent in *E. coli*) which contains proteins specifically involved in the regulation of folding (*i.e.* chaperones, isomerases). Therefore synthesis of recombinant polypeptides via a yeast

expression system may generate a more accurate representation of parasite-produced Pfs230 than an *E. coli* expression system.

The objective of this study was to produce a small part of Pfs230 region C, C-Nterm (amino acid 447 to amino acid 584), using a yeast expression system. Yeast N-terminal Flag Expression System (YEp) was chosen as the vector. YEp encodes a two micron circle and a tryptophan selectable marker for growth and selection in yeast. Protein expression in yeast transfected with the YEp vector is regulated by the alcohol dehydrogenase promoter (ADH2). ADH2 is a strong yeast promoter capable of directing extremely high intracellular levels of (foreign) protein expression. This could be a problem if the recombinant, foreign protein is toxic to the yeast (Tuite and Oliver, 1991). To alleviate this, the ADH2 promoter is repressed in the presence of glucose while initial yeast cell growth occurs. Once the yeast have metabolized the glucose the ADH2 promoter is derepressed to a high level and protein expression begins. YEp vector also encodes an α leader sequence which targets the ribosome to the rough endoplasmic reticulum membrane. After translocation across the rough endoplasmic reticulum, the signal sequence is cleaved by a membrane bound protease. This cleavage leaves the immunogenic FLAG peptide on the exposed amino terminus of the recombinant protein.

YEp was modified to mYEp by adding a Nhe I restriction site and six histidines at the 3' end of the MCS using a synthetic oligonucleotide. The addition of the six histidines to the carboxy terminus of recombinant yeast-expressed C-Nterm protein provided the option of purifying the recombinant protein with a Ni^{2+}-NTA column. Proteins with six histidines at either the amino or carboxy terminus bind to a Ni^{2+}-NTA affinity column with a greater affinity than most antibodies and antigens (Qiagen Inc., 1992). The FLAG peptide at the amino terminus of C-Nterm could also be used to purify the recombinant using a Anti-FLAG M1 affinity column. Together the FLAG and six histidines could be used as indicators to determine if full length protein was present. The modified vector

(mYEp) is 7.2 kB and was distinguished on an agarose/TAE gel from unmodified YEp by a restriction endonuclease digestion with Nhe I, a site unique to mYEp.

Using the modified vector, the C-Nterm/mYEp construct was developed. C-Nterm was PCR amplified using oligonucleotide primers corresponding to base pair #1339 to base pair #1752 (encoding amino acid 447 to amino acid 584) in Pfs230. C-Nterm was then digested with Xho I and Apa I restriction enzymes and ligated into the corresponding sites in mYEp. Transformation efficiency was calculated as a measure of quality control for future ligations. The 1.5 x 10^9 CFU transformation efficiency of pUC19 indicated that the technique was performed correctly. The efficiency for the C-Nterm/mYEp ligation mix (2.56 x 10^4 CFU) was low compared to the controls but this was expected since the control plasmid was supercoiled. The presence of the C-Nterm insert in mYEp was confirmed by restriction analysis using Kpn I and Spe I. Results showed two bands on an agarose/TAE gel which were by molecular size standards using linear regression and found to be 7.95 kB and 931 base pairs. These are the approximate sizes of mYEp and C-Nterm plus vector sequence, respectively, and thus the presence of these bands indicated that the construct had been produced.

Manual sequencing confirmed that C-Nterm/mYEp was produced and more importantly that C-Nterm/mYEp was in the proper reading frame. Sequence was obtained starting with 15 base pairs of the vector (corresponding to vector base pair #1493 to base pair #1507). These base pairs were read and found to correlate with the actual sequence of mYEp (Figure 7). The vector sequence ended at the Xho I restriction site (base pair #1502 to base pair #1507) and the sequence corresponding to Pfs230 started. This indicated that C-Nterm was ligated into mYEp at the intended site and that the reading frame was maintained. For Pfs230.C-Nterm, base pair #1 to base pair #263 of the 404 base pair C-Nterm were readable on the autoradiograph. Within that sequence only one mismatch with the predicted sequence of Pfs230 was found. Base pair #149, which should have been nucleotide **A**, was not seen on the autoradiograph. All other nucleotides read from

the autoradiograph conformed to the reported sequence of Pfs230.C-Nterm. Base pairs #148 and #150 (like base pair #149) are also **A**'s and there is a visible gap between them on the autoradiograph indicating that a base was probably present but no ddATP was incorporated. If, however, this base pair was missing, the reading frame of C-Nterm/mYEp downstream to base pair #149 would have been altered. This seems unlikely because base pair #149 was identified later in automated sequencing of yC-Nterm/mYEp, to which C-Nterm/mYEp was a predecessor. Additionally, amino acid 50 which is encoded by base pairs #148-#150 was a lysine on the peptide sequencing data. This conforms with the reported sequence of Pfs230. Furthermore, a pattern on the autoradiograph near the 3' end of the template appeared to be several consecutive **GTC** codons. The **GTC** codon encodes the amino acid histidine. This region probably corresponds to the six histidine tag encoded at the 3' end of the multiple cloning site in mYEp and suggested that the full length C-Nterm/mYEp DNA was present.

Since the restriction digest and sequencing results confirmed that the construct had been produced, the next stage of the project involved transfecting yeast with C-Nterm/mYEp. Yeast was transfected with C-Nterm/mYEp, grown on SCM minus tryptophan/agar plates, resultant colonies were selected and then amplified further in SCM minus tryptophan liquid culture. Protein expression was induced in YPHSM expression media which contained a fixed amount of glucose, the preferential energy source of yeast. Once glucose is depleted by yeast metabolism (~24 hours) the yeast alcohol dehydrogenase promoter (ADH2) is derepressed and protein expression begins. TCA-precipitated aliquots of C-Nterm/mYEp transfected yeast supernatant were loaded on a SDS-polyacrylamide gel to evaluate protein expression. As reported in the results and as seen in Figure 9, there were several bands between 30 kDa-16 kDa (with a major band at 28 kDa +/- 0.5 kDa) when interpolated by Amersham standards. These bands were present in the lanes (2-4) which contained C-Nterm/mYEp transfected yeast supernatant and were absent in the yeast transfected with YEp vector alone (lane 1). This suggests that

these unique bands correspond to C-Nterm. Many higher molecular weight proteins were present in all four lanes (including lane 1), but these were not as visible as the lower molecular weight bands and did not reproduce well in the photograph. The theoretical size of C-Nterm is 16,270 Da.

Samples of yeast supernatant transfected with YEp alone and yeast transfected with C-Nterm/mYEp were electrophoretically blotted onto nitrocellulose and probed with Anti-r230.MBP.C antibodies and Anti-MBP antibodies. In the Anti-r230.MBP.C immunoblot, no reactivity was seen in the lane which contained YEp transfected yeast supernatant. Several bands were present, however, in the lane which contained C-Nterm/mYEp transfected yeast supernatant and were interpolated to migrate at 26.39 kDa, 23.31 kDa and 19.35 kDa. These values are bracketed by the same molecular weight range seen in the original Coomassie gel on which C-Nterm/mYEp transfected supernatant was electrophoresed (Figure 9). The reactivity of bands unique to C-Nterm/mYEp transfected yeast supernatant with Anti-r230.MBP.C antibodies strengthened the suggestion that C-Nterm was being expressed.

As a control, YEp transfected yeast supernatant and C-Nterm/mYEp transfected yeast supernatant were probed with Anti-MBP antibodies and no reactivity was seen. Taken together, the Anti-r230.MBP.C and Anti-MBP immunoblots indicated that the reactivity of Anti-r230.MBP.C with C-Nterm/mYEp transfected yeast proteins was not due to immuno-crossreactivity of the proteins with antibodies to MBP. This strongly suggests that the protein recognized by Anti-r230.MBP.C has amino acid homology to Pfs230.C, as would C-Nterm.

Ni^{2+}-NTA purification was used to isolate full length C-Nterm from the transfected yeast supernatant. As mentioned previously, the Ni^{2+}-NTA column binds proteins with six consecutive histidines. While this sequence rarely occurs in nature, it is present at the carboxy terminus of full length C-Nterm. After washing the column to remove impurities, 0.5 M imidazole was applied to elute the protein bound to the column (Figure 10). The

sizes of the eluted protein bands were interpolated against Sigma molecular weight standards by linear regression. Protein bands present in samples of C-Nterm/mYEp transfected yeast dialysate and in material that did not bind to the affinity column were compared with protein bands in the eluant. In the eluted fraction, there were distinct protein bands between 25 kDa and 18 kDa, with the bands at 25.61 kDa and 18.78 kDa being the most intensely stained. Similar bands (25 kDa-18 kDa) were seen in the C-Nterm/mYEp transfected yeast supernatant dialysate and were also seen, though noticeably less intense, in the C-Nterm/mYEp transfected yeast supernatant which flowed off the column. Higher molecular weight proteins (75 kDa to about 42 kDa) present in C-Nterm/mYEp transfected yeast dialysate and the material which flowed through the column were not present in the eluted fractions. This data further suggests that the high molecular weight bands which did not bind to the Ni^{2+} affinity column represent yeast proteins. These data indicated that the same size proteins eluted from the column (25 kDa-18 kDa) were present in the initial C-Nterm/mYEp transfected yeast supernatant and that some of the C-Nterm protein in the C-Nterm/mYEp transfected yeast supernatant did not bind to the affinity column.

Protein eluted from the Ni^{2+}-NTA column was electrophoresed on a 15% gel and stained with Coomassie. This experiment was done to better evaluate the size of the lower molecular weight bands associated with C-Nterm. The bands were interpolated against Amersham and Sigma molecular weight standards to determine if there was a difference in size when using different standards. As mentioned in the results, the Sigma standards are not pre-stained and therefore provide a more accurate weight of C-Nterm. The size of C-Nterm using Sigma standards ranged between 26 kDa-16 kDa whereas Amersham standards reported a range of 28 kDa-15 kDa. One interesting observation noted on other gels and confirmed on this higher percentage gel was that C-Nterm does not run below 15 kDa-16 kDa (close to its reported size of 16,270 Da). This may suggest that the multiple

bands associated with C-Nterm represent an unmodified form (15 kDa-16 kDa) as well as posttranslationally modified forms (>16, 270 kDa).

To characterize the identity of the eluted protein, immunoblots with Anti-r230.MBP.C, Anti-FLAG M1, and Anti-MBP were performed (Figures 13 and 14 and data not shown, respectively). The Ni^{2+} purified protein probed with Anti-MBP showed no reactivity. Ni^{2+} purified protein was recognized, however, by both antibodies generated against Pfs230 region C and the antibodies to the amino terminal FLAG peptide encoded by mYEp. These results suggested that all the bands present in the C-Nterm/mYEp transfected yeast supernatant contained amino acids corresponding to Pfs230 region C and that the FLAG peptide was present at the amino terminus of each. The Anti-MBP blot suggested that the reactivity between C-Nterm and Anti-r230.MBP.C is not due to immuno-crossreactivity of the recombinant with Anti-MBP antibodies.

Additionally, YEp transfected yeast supernatant was blotted then probed with Anti-r230.MBP.C, Anti-FLAG M1 and Anti-MBP antibodies as a negative control. The high molecular weight bands associated with YEp transfected yeast in other gels (Figure 10), which probably represent endogenous yeast secreted proteins, showed no reactivity with these antibodies. This indicated that YEp transfected yeast proteins showed no immuno-crossreactivity with Anti-r230.MBP.C, Anti-FLAG M1 and Anti-MBP.

In summary, the purification of C-Nterm protein by way of Ni^{2+}-NTA indicated that the carboxy terminus was present because purification required the presence of the six consecutive histidines on the carboxy terminus of C-Nterm. The reactivity of purified protein with Anti-r230.MBP.C and Anti-FLAG M1 indicated that the eluted protein contained amino acids corresponding to Pfs230 and that the amino terminal FLAG of C-Nterm was present. Together, these data indicated that full length C-Nterm protein was expressed.

This raises the question of why there are so many bands observed by SDS-PAGE. Several possible reasons, such as improper folding, mutations in the coding sequence, and

posttranslational modification, were considered. The possibility of disulfide bonds between cysteines causing non-linear migration on the SDS-PAGE gel could be ruled out immediately because there are no cysteines in Pfs230.C-Nterm. Heterogeneous folding of C-Nterm was also unlikely on the basis that the protein was evaluated on a SDS denaturing gel and distinct bands (in opposed to aggregate protein smears) were observed.

Other possibilities considered for the large size of C-Nterm were base pair substitutions, deletions, or mutations. The C-Nterm/mYEp construct transfected into *E. coli* was manually sequenced. The first 263 base pairs of C-Nterm were read and found to match the reported sequence of Pfs230 with the exception of base pair #149. While this base pair was not seen on the autoradiograph it was confirmed to have been present by subsequent peptide and automated DNA sequencing. The possibility that the yeast had caused mutations in the protein sequence that would result in a dramatic size shift of C-Nterm was next considered. Two peptides obtained from a *Staphylococcus aureus* digestion of the highest molecular weight C-Nterm band showed perfect sequence homology with amino acid 8 to amino acid 17 and amino acid 42 to amino acid 58 of the reported sequence of Pfs230.C-Nterm. This suggests that no mutations that could be responsible for a dramatic size shift in C-Nterm had occured in the first 58 amino acids of the protein. However, this did not rule out the possibility of mutations downstream of amino acid 58. Immunoblot analyses of protein isolated from C-Nterm/mYEp transfected yeast showed positive reaction when probed with antibodies which recognize Pfs230 region C. Thus, the possibility of mutations present in the first 58 amino acids of the construct was probably not the explanation for the various sizes associated with C-Nterm.

To determine if the yeast had caused DNA base pair substitutions, deletions, or mutations which resulted in a size shift of the protein, the C-Nterm/mYEp DNA transfected into yeast was reharvested and the resultant yC-Nterm/mYEp construct was digested with restriction enzymes, Kpn I and Spe I. The size of the insert recovered from yC-Nterm/mYEp after the digest was compared with the size of the insert present in the

original C-Nterm/mYEp and the bands (as interpolated against DNA size standards) were confirmed to be the same size. This suggests that the yeast did not drastically rearrange C-Nterm to cause a size shift. To be absolutely certain, however, that no major rearrangement had taken place in the yeast, the yC-Nterm/mYEp construct was sent for automated sequencing. As indicated by accurate peptide sequence and purification by a Ni^{2+} affinity column, mutations, had they occured, would have had to occur in the center of C-Nterm, thus restoring the reading frame for the stop codons and the six histidine tag. Plasmid DNA was isolated from C-Nterm/mYEp transfected yeast and was used to transfect *E. coli.* Ampicillin resistant *E. coli* was selected and purified plasmid was then sent to the University of Chicago Sequencing Facility for automated sequencing. The entire Pfs230 insert and the adjacent vector regions were sequenced from both directions. This included the region of the mYEp vector in the 3' end of the insert that encoded two stop codons as well as the base pairs which encoded the Nhe I restriction site and the six histidine tag. All but three of the nucleotides in the yC-Nterm/mYEp construct conformed to the reported sequence of Pfs230.C-Nterm. However, because the two stop codons present at the 3' end of C-Nterm remained intact it is unlikely that vector sequence was translated into protein, thus causing a larger protein. This hypothesis is also unlikely on the basis that the C-Nterm protein would not have been purified if any sequence existed downstream of the six histidine tag. The six histidine tag must be on the exposed amino or carboxy terminus to bind to the Ni^{2+}-NTA affinity column (Qiagen Inc., 1992). Clearly the DNA sequence showed that no major rearrangements occured that dramatically altered the size of C-Nterm.

The three substitutions that occured in automated sequencing of Pfs230.C-Nterm were present in the sequencing data from both the 5' and 3' direction. While these substitutions were probably not responsible for a 10 kDa size shift of C-Nterm, they may present problems for future work with C-Nterm. The data indicated that base pair #170 in C-Nterm was a cytosine while the base reported in Pfs230 was an adenine. Base pairs

#329 and #392 were guanines on the chromatogram while the bases were reported as adenines in Pfs230. These base pair substitutions, if they were accurate, would have changed the primary amino acid structure of Pfs230.C-Nterm. First, amino acid 57 would be a serine instead of the reported leucine. Peptide sequence indicated that this amino acid was in fact a leucine. This raises the possibility about the validity of the base pair substitution reported in the automated sequencing. The two remaining base pair substitutions, assuming they were correct, would have caused amino acid 110 to be a glutamine instead of the reported glycine and amino acid 131 would be glycine and not the reported aspartate. Because the difference of molecular weight between these amino acids is negligible, it is doubtful that they are responsible for the shift in molecular weight observed with C-Nterm. The isoelectric charges on the amino acids that may have been substituted are consistent in glutamine and glycine, but glycine (pI 5.97) and aspartate (pI 2.77) differ substantially. The change in pI of one amino acid would probably not alter the entire pI of a protein and could probably not cause a shift of up to 10 kDa of the C-Nterm protein on a denaturing polyacrylamide gel. Also, the running buffer used for the gel was pH 6.8. This is a higher pH than the pI value of both glycine and aspartate. When pH is higher than pI these residues are negatively charged and migrate towards the positive electrode. While aspartate is slightly more negative than glycine this difference would be negligible with respect to migration pattern assuming the SDS had adequately bound to C-Nterm.

Since C-Nterm multimers were unlikely because it does not contain cysteines and because C-Nterm was evaluated on a SDS denaturing gel, and no major sequence rearrangements existed that could explain the large size of C-Nterm, posttranslational modification of the protein was considered. The most common modification of secreted yeast proteins is glycosylation, specifically N-linked to asparagines. C-Nterm lacks the requisite asn-x-ser sequence so it is unlikely that it was N-link glycosylated but O-link glycosylation was still a possibility. A glycoprotein detection kit was used to determine if

carbohydrate moieties were present on C-Nterm. No staining was seen in the purified fractions of yeast transfected with C-Nterm/mYEp. Only high molecular weight proteins (specifically proteins from 98 kDa to 41 kDa) present in crude C-Nterm/mYEp transfected yeast supernatant and material which flowed through the affinity column were stained (Figure 16). C-Nterm migrates from roughly 30 kDa to 16 kDa and no bands below 41 kDa were stained. It was certain that the transfer of C-Nterm protein was successful because staining of the nitrocellulose with ponceau S revealed bands in the 30 kDa to 16 kDa range (data not shown). Taken together, these facts suggested that the detection kit used was able to identify glycoproteins produced by yeast and that C-Nterm was not glycosylated.

Other possible posttranslational modifications, notably fatty acylation (i.e. isoprenylation, palmitoylation, and myristoylation (cotranslational)), sulfation, and phosphorylation could also lead to a larger protein. Isoprenylation is a type of fatty acid addition which takes on two forms: a 15 carbon isoprenyl lipid, farnesyl; a 20 carbon lipid, geranylgeranyl (Schafer and Rine, 1992). Both isoprenoids add in thioester linkage to cysteines at the carboxy terminus of a protein. Consensus sequences for isoprenyl addition are: CAAX (where X determines which type of prenyl addition occurs); CXC and CL in which one or both cysteines are geranylgeranylated (Caldwell *et al.*, 1995). Palmitoylation is another type of fatty acylation which involves the posttranslational attachment of a 16 carbon chain to the thiol group of any cysteine throughout the body of a polypeptide (Casey, 1995). Because C-Nterm contains no cysteines isoprenylation and palmitoylation could not have occured. Myristoylation, which actually occurs cotranslationally, involves the addition of a 14 carbon chain to the amino terminal glycine residue in the consensus sequence Met-Gly-X-X-X-Ser/Thr, where the Met is cleaved (Resh, 1994). C-Nterm, with the FLAG peptide as its first 15 amino acids, has an amino terminal aspartate not glycine and therefore myristoylation could not have occured Fatty acylation is also an unlikely explanation because it is commonly a modification of cytosolic

proteins which appears to mediate interactions between proteins and cellular membranes exposed to the cytosol (Alberts, *et al.*, 1994). C-Nterm, of course, is a secreted protein isolated from the yeast media with no transmembrane domain. Once a protein is in the secretory pathway it remains inside the endomembranous system and does not appear in the cytosol (Stryer *et al.*, 1995).

Sulfation and phosphorylation may be possible contributing factors for the increased size of C-Nterm. Sulfate groups are covalently added to tyrosines and/or carbohydrate residues of secretory proteins in the Golgi complex (Rosa *et al.*, 1985). Phosphate groups are added to the hydroxyl group of serine, threonine or tyrosine residues. Gel shift assays have shown three phosphoryl additions can correlate with as much as a 20 kDa increase in size (Izumi and Maller, 1993). C-Nterm has 13 serines, seven threonines, and eight tyrosines. It is possible that sulfation and phosphorylation are modifications that take place in parasite-produced Pfs230. In the 360 kDa form of the protein and the processed 310 kDa form of the protein it is difficult to detect a 10 kDa size shift. Both of these forms of the protein migrate as a doublet which could be due to sequential proteolysis. However, these doublets may be due to posttranslational modification by the parasite in the form of O-linked glycosylation, sulfation, or phosphorylation.

Due to the uncertainty associated with the size of C-Nterm, the question remains is this project worth exploring further and how can the results of this project be of future use? It is clear that the mYEp vector constructed in this project will be useful in future studies. The vector has already been sent to two other investigators to be used in their experimental work. The six histidine tag and the Nhe I restriction site as well as the additional stop codon added provide this vector with unique advantages absent in its unmodified counterpart. Proteins expressed with this vector have the ability of being purified through the amino terminus (FLAG) and the carboxy terminus (6 his). Alternatively, a construct could be created using the Kpn I restriction site (outside of the

MCS) to generate a protein with only a six histidine tag. In addition, the Nhe I site can serve the dual purpose of distinguishing mYEp from unmodified YEp and can be used for compatible end ligations to generate future constructs. The additional stop codon serves as a precaution and is also an asset to mYEp.

With respect to C-Nterm, this project has demonstrated that under the reaction conditions used, proteins of this size can be efficiently expressed. This is evident by the amount of protein eluted from the affinity column. Like mYEp, C-Nterm may also play a role in future studies. C-Nterm has the FLAG peptide at the amino terminus which may prove useful for amino terminal purification and immunoblot analyses. Furthermore, the FLAG/C-Nterm fusion protein may also be used to determine if C-Nterm contains the site where the 360 kDa form of Pfs230 is processed to the 310 kDa form. Conversely, C-Nterm may be more beneficial without the FLAG because the FLAG peptide is highly immunogenic and may overpower any immune response generated to recombinant C-Nterm.

The question as to whether C-Nterm will be useful as an immunogen at all remains unclear. It is possible that the multiple band pattern associated with C-Nterm represents posttranslational modifications added by the yeast. There is a chance that these modifications might affect conformationally dependent epitopes in the protein. However, if the multiple band pattern represents posttranslational modifications and these modifications mimic modifications that actually occur in the parasite, this could be advantageous. The nucleotide substitutions which occured in C-Nterm according to automated sequencing, however, need to be seriously considered. While there was no indication that these substitutions caused a shift in the reading frame, it is possible that they may interfere with antibody recognition if the substitutions occured at crucial epitopes.

Because C-Nterm was designed to be an immunogen, future projects in the lab with the protein would probably first involve mice immunizations. If it is determined that

C-Nterm solicits an immune response with a good IgG titer, studies to resolve the size issue will then be conducted. These experiments might include: 1) Detecting sulfation or phosphorylation in C-Nterm by labeling the recombinant with ^{35}S sulfate or ^{32}P orthophosphoric acid, respectively ; 2) Running C-Nterm on a tricine gel to determine if SDS-PAGE anomalies are contributing to the various sizes associated with C-Nterm; 3) Isoelectric focusing - if different pI's are associated with different bands this may make more of a case for phosphorylation or sulfation because these modifications would alter the pI; 4) Creating a C-Nterm/mYEp construct without the FLAG peptide which may be a better candidate for immunizing.

CHAPTER V

CONCLUSION

In conclusion, Western blot analysis and sequencing results indicated that Pfs230 amino acid 447 to amino acid 584 (C-Nterm) was expressed as a secreted protein in *S. cerevisiae*. The protein was targeted to the yeast secretory pathway via the α signal sequence encoded by the mYEp vector. The leader sequence was properly removed in the yeast leaving the FLAG peptide at the amino terminus of C-Nterm. This was determined because Anti-FLAG M1, which is specific for FLAG on the exposed amino terminus of a protein, recognized recombinant C-Nterm. The yeast secreted protein was further shown to contain amino acids from region C of Pfs230 by immunoblot analysis with Anti-r230.MBP.C and amino acid sequencing. The recombinant protein was also determined to have the six histidine sequence at its carboxy terminus due to the fact that it was purified using a Ni^{2+}-NTA affinity column. This, coupled with the Anti-r230.MBP.C and Anti-FLAG M1 reactivity, suggested that full length yeast produced Pfs230.C-Nterm was in hand.

The primary DNA sequence of C-Nterm/mYEp was shown to be full length and in the proper reading frame by DNA sequencing. Protein sequencing of recombinant C-Nterm generated two peptides with perfect sequence homology to amino acid 8 to amino acid 17 and amino acid 42 to amino acid 58 of the reported sequence of Pfs230.C-Nterm. Automated sequencing of yC-Nterm/mYEp showed three base pair substitutions when sequenced from both the 5' and the 3' direction, one of which was corrected in protein sequencing.

Secreted C-Nterm migrates on SDS-PAGE as a number of distinct bands. The precise reason for this remains unclear. All of the bands appear to be full length, containing both the amino terminal FLAG sequence and the six histidine tag. There is no evidence for a rearranged internal DNA sequence. While the protein does not appear to be glycosylated, phosphorylation may be a possible explanation as to why C-Nterm migrates as so many bands. It is difficult to find a pattern among the bands on every gel. However, many gels seem to show a one to three kDa increase in size between the bands. It is possible that each of these bands represents a different degree of modification and that the lowest molecular weight band (which seems to migrate between 15 kDa-16 kDa depending on the gel) represents the unmodified C-Nterm protein. Currently, little is known about specific modifications of parasite-produced Pfs230, but it is possible that Pfs230 is posttranslationally modified by the parasite.

Possible immediate uses of C-Nterm include testing its immunogenicity in mice and determining whether the antibodies generated will recognize parasite-produced Pfs230. If antisera generated to C-Nterm recognizes parasites by immunofluoresence assay, it would then be tested for its ability to block parasite infectivity in the mosquito. Because C-Nterm is designed to be used as an immunological reagent and not a chemical reagent it will first be necessary to determine if decent antibody titer can be generated in mice. If C-Nterm proves to be immunogenic, the issue of posttranslational modification as a possible reason for the various sizes will be dealt with further.

Another possibility for C-Nterm is to test this region for its role in stage-specific processing of Pfs230 from the 360 kDa form to the 310 kDa form. It is predicted that the clip site for Pfs230 exists within C-Nterm. To test this, recombinant protein could be incubated in parasite extract then size fractionated to determine if the parasites have protealyzed the protein to its smaller form. If the size of the protein recognized by Anti-FLAG M1 decreases after incubation with the parasite extract this would indicate that processing of the molecule had occured.

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