FUNCTIONAL CHARACTERIZATION OF A PUF FAMILY RNA-BINDING PROTEIN DURING SEXUAL DEVELOPMENT OF THE MALARIA PARASITE *Plasmodium falciparum*

A Thesis in

Entomology

by

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Despite tremendous worldwide efforts to control malaria, it is still a major public health problem and a heavy social and economic burden in over 90 countries. Among the four protozoan species that cause malaria in humans, Plasmodium falciparum is the most fatal, accounting for 1.5-2.7 million deaths and 300-500 million clinical cases each year. Drug resistance in Plasmodium and insecticide resistance in vector mosquitoes are probably responsible for the resurgence of the disease in the world. Novel drugs and control measures are desperately needed to bring this disease under control. To this aim, we need to better understand the fundamental biology and the molecular mechanisms governing the complicated developmental cycle of the parasite. The recently completed genome sequence of the P. falciparum is a milestone in malaria research, which has contributed greatly to our understanding of the molecular biology of the parasite, and led to the discovery of parasite-specific metabolic pathways and identification of potential targets for the development of new drugs and vaccines. Meanwhile, deciphering the functions of individual genes remains a formidable task of the post-genomic era.

Malaria is caused by the bite of an infected mosquito, which injects Plasmodium sporozoites into the human circulatory system. These sporozoites invade liver cells and undergo multiple rounds of schizogony to produce thousands of merozoites, which are released into the blood to invade erythrocytes. While the majority of the parasites undergo cycles of asexual multiplication in the erythrocytes, which is responsible for the clinical symptoms of the disease, some merozoites initiate sexual development to produce gametocytes. Gametocytes are the only form that initiates further sporogonic development in
the mosquitoes, an obligatory process for the transmission of the parasite. The complex life cycle of the malaria parasites is a challenge for vaccine designs, which preferentially target multiple developmental stages. Among them, transmission-blocking vaccine that targets parasite antigens of sexual stages aims at interrupting the transmission cycle of malaria. Although morphological changes during sexual development of the malaria parasite were known almost a century ago and a number of sexual stage-specific genes have been identified and functionally characterized, a comprehensive understanding of the molecular and cellular biology of malaria sexual development is still lacking.

During studies examining the sexual development of the malaria parasite, members of a family of RNA-binding proteins were identified in *P. falciparum* gametocytes by using RNA differential display. This family was named Puf after the two first-characterized proteins *Pumilio* in *Drosophila melanogaster* and *fem*-3 binding factor (FBF) in *Caenorhabditis elegans*. Puf is an evolutionarily conserved RNA binding protein family in eukaryotes. The eight imperfect tandem repeats in Puf proteins constitute the essential RNA binding domain (RBD), which forms a rainbow structure with the concave surface interacting with the target mRNA. The target mRNAs characterized to date contain conserved elements in their 3’ untranslated regions (UTRs), and binding of Puf to these elements imposes translational repression on the target mRNA. The biological functions of Puf family proteins have been characterized in a few biological systems. Although varying from system to system, it has been postulated that the ancestral function of Puf is to promote proliferation and suppress differentiation.

The two Puf members in *P. falciparum*, *PfPuf1* and *PfPuf2*, are preferentially expressed in gametocytes. The finding that Puf proteins in *D. melanogaster* and *C. elegans* have a
conserved function in germline stem cell maintenance has prompted us to investigate the functions of PfPuf1 and PfPuf2 during *P. falciparum* gametocytogenesis. PfPuf1 and PfPuf2 mRNAs are spliced and located on chromosome 5 and 4, respectively. PfPuf1 encodes a much larger protein (1894 amino acids) than PfPuf2 (514 amino acids). Phylogenetic analysis of Pufs showed that the RBDs of PfPuf1 and PfPuf2 only share 27% amino acid identity, with PfPuf1 being more related to Pufs in plants and fungi and PfPuf2 more related to FBF in *C. elegans*. Reverse transcriptase polymerase chain reaction (RT-PCR), northern blots and western blots confirmed the preferential expression of both Puf genes in gametocytes. Using RNA ligase-mediated rapid amplification of cDNA end and primer extension, the transcription initiation site of PfPuf2 was mapped to 297 bp upstream of the translation start codon. Since the target genes for PfPuf proteins remain to be identified, the *Drosophila* nanos-responsive element (NRE) sequence in the *hunchback* 3’ UTR was used as a conserved artificial target sequence to evaluate the RNA-binding activity of PfPufs by using gel mobility shift assays and the yeast three-hybrid system. The results showed that both PfPuf RBDs displayed specific RNA binding activity to the conserved NRE sequence. This result suggests that both Puf members in *P. falciparum* utilize a similar mechanism of translation control of their target genes.

To further explore the functions of PfPuf genes, the newly developed parasite transfection technology was applied to disrupt PfPuf2. A plasmid construct was designed to truncate PfPuf2 at the 8th Puf repeat. After electroporation of the ring-stage parasites, pyrimethamine was used to select resistant parasites. Parasites with the construct stably integrated into the PfPuf2 locus were obtained and cloned by limiting dilution. Disruption of PfPuf2 in these single parasite clones was confirmed by integration-specific PCR, genomic
Southern blot, and RT-PCR. Phenotypic analysis showed that \( P/Puf2 \) disruptions did not affect asexual growth of the parasite, but gametocytes in the disruptants were formed at a much accelerated rate. The disruption of Puf genes provides us an opportunity to identify the Puf target genes through genome-wide microarray analysis.
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CHAPTER ONE: INTRODUCTION

MALARIA BIOLOGY AND TRANSLATIONAL REGULATION

IN *PLASMODIUM FALCIPARUM*
MALARIA

Current status of human malaria

Human malaria is a disease caused by protozoan parasites and transmitted by Anopheles mosquitoes. The malaria global eradication campaign in the mid-1950s was most successful in temperate endemic countries, which temporarily curtailed the distribution of malaria. However, the incidence of malaria is on the rise, largely because of drug resistance in the parasites and insecticide resistance in vector mosquitoes (Greenwood and Mutabingwa, 2002; Mendis et al., 2001). Currently, it is estimated that malaria causes 300-500 million cases and 1.5-2.7 million deaths each year (Global Health Council, 2003). While malaria is an important health problem in parts of Asia and South America, it exerts the greatest impacts in sub-Saharan Africa, where at least 90% of malaria deaths occur. Four species in the genus Plasmodium, P. falciparum, P. vivax, P. ovale, and P. malariae, are the etiological agents of human malaria. The benign tertian malaria caused by P. vivax is perhaps the most widely distributed, whereas the malignant tertian caused by P. falciparum is the most deadly, responsible for almost all malaria-associated deaths.

The social and economic burden of malaria in developing countries has resulted in the renewed interest in malaria in the developed world in recent years. Among the many new initiatives for malaria control is the Roll Back Malaria program by the World Health Organization, which aims at halving the death tolls from malaria by 2010 (WHO: Roll Back Malaria Program, 1998; http://www.rollbackmalaria.org/). Towards this goal, international research communities are undertaking tremendous efforts to develop malaria vaccines, new drugs, and insecticides. The recent complete sequencing of the genomes of P. falciparum
(Gardner et al., 2002), its vector *Anopheles gambiae* (Holt et al., 2002), and the human host
(Lander et al., 2001; Venter et al., 2001) provides new insights into the biology of these organisms and immune and evolutionary relationships among them. This comprehensive knowledge will allow an integrated analysis for a better understanding of the molecular basis of the tripartite interactions. The available global analytic tools such as DNA microarrays and proteomics have offered an unprecedented opportunity for studying these interactions at the genome-wide level.

The complexity of the malaria life cycle involving a vertebrate and an invertebrate host and numerous developmental stages (Fig. 1-1) presents great difficulties in the development of control measures. For more effective vaccine designs, it is ideal to simultaneously target multiple antigens on different developmental stages (Richie and Saul, 2002). While anti-infection vaccines targeting the sporozoites and/or liver stages aim to prevent blood cell infection, anti-morbidity and mortality vaccines are designed to reduce clinical severity of the disease. Transmission block vaccines (TBVs) are directed against mosquito stages to reduce re-infection of the human population (Carter, 2001). In the past, most research has centered on the asexual blood stages of *P. falciparum* because asexual multiplication of the parasite is responsible for the clinical symptoms and the morbidity and mortality of the disease. Research on the sexual development process of the parasite in the human host, the production of gametocytes, has been less well studied (reviewed by Alano, 1991; Lobo and Kumar, 1998).
Fig. 1-1. The life cycle of *P. falciparum*. (http://www.dpd.cdc.gov/). There are three developmental cycles for malaria parasites: exo-erythrocytic cycle in the human liver (A), erythrocytic cycle in human blood (B), and sporogonic cycle in the mosquito. The infection (i) of malaria begins with a bite of an infected anopheline female mosquito (1), which injects sporozoites into human bloodstream. The sporozoites migrate into human liver (2) within minutes and undergo asexual multiplication to schizonts (3). Upon rupture from liver cells, schizonts release merozoites to invade red blood cells (5) and erythrocytic cycle starts. This stage is responsible for clinical symptoms and used for diagnosis using thin or thick blood smears (d). Some merozoites will continue asexual multiplication through rings, trophozoites until mature schizonts (6), while others stop dividing and differentiated into sexual forms (7) of female (macrogametocytes) and male (microgametocytes). This process is known as gametocytogenesis. These sexual forms are solely responsible for malaria transmission. When another compatible *Anopheles* female mosquito bites the person (8), these gametocytes first develop into gametes by a process of gametogenesis, during which male gametocytes exflagellate, while female rarely change morphologically. Mature male gametes fertilize females in mosquito midgut to form motionless zygotes (9), then transform to mobile ookinetes (10). These ookinetes penetrate midgut and develop into oocytes (11), who release thousands of sporozoites (12). Later sporozoites migrate into salivary glands (1) and are the infectious form in mosquitoes.
Malaria parasite life cycle and developmental biology

The malaria parasite life cycle encompasses three major stages as it alternates between a human host and an anopheline mosquito vector: sporogonic development in mosquito, pre-erythrocytic or exo-erythrocytic liver stages and erythrocytic stages in human (Fig. 1-1). During blood feeding by a sporozoite-infected female mosquito, sporozoites residing in the mosquito salivary glands are injected into the human blood stream. Shortly after the inoculation, sporozoites enter the liver and infect hepatocytes, where they multiply by schizogony to produce thousands of merozoites. Upon release from the liver cells, the merozoites infect red blood cells to initiate the erythrocytic cycle. In an erythrocyte, the parasite grows through ring, trophozoite and schizont stages. When the schizont-containing erythrocyte ruptures, 16-32 merozoites are released to invade new erythrocytes. In response to unknown cues, a small proportion of the parasites differentiate into male and female gametocytes, a process known as gametocytogenesis. Within a few minutes after being taken up by a compatible anopheline mosquito, the gametocytes emerge from the erythrocytes to form male and female gametes (micro- and macrogametes), a process termed gametogenesis. The formation of microgametes, referred to as exflagellation, is a dramatic event with eight haploid motile microgametes being produced from each male gametocyte. The fertilization of a macrogamete by a microgamete leads to the formation of a diploid zygote. Within 24 hours, the zygote transforms into a mobile ookinete, which traverses the mosquito midgut wall. Once the ookinete reaches the space between midgut epithelium and the basal lamina, it transforms into an oocyst. Normally within two weeks, thousands of haploid sporozoites develop in the oocyst. After being released into the mosquito hemocoel, the sporozoites invade salivary glands, where they await to be injected into another person.
**Biology of *P. falciparum* sexual stages**

Sexual development occupies a central place in the life cycle of the malaria parasite and is responsible for transmission of malaria in human hosts through mosquito vectors (Sinden, 1983; Carter et al, 1988; Alano and Carter, 1990; Lobo and Kumar, 1998). However, the molecular mechanisms governing the processes of sexual development are poorly understood. Upon invasion of red blood cells, certain merozoites stop dividing and differentiate into micro (male) or macro (female) gametocytes. It is commonly believed that gametocytogenesis is a genetically inherited trait of the parasite (Lobo and Kumar, 1998), which is triggered by environmental cues or ‘stress’ (Dyer and Day, 2000; Paul et al., 2002). Gametocytogenesis is a lengthy process, which takes about 9-12 days for *P. falciparum* gametocytes to become mature. Mature gametocytes can survive as long as 20 days in human blood. Once gametocytogenesis begins, cellular organization of the parasite changes by reshuffling or assembly of actin filaments and microtubules, resulting in five morphologically distinct stages (stage I through V). Accompanying these morphological changes, asexual developmental activities are turned off and sexual stage-specific genes are activated (Kongkasuriyachai and Kumar, 2002).

With recognition of the significance of sexual stage antigens for transmission-blocking vaccine development (Kaslow, 1990; Targett, 1990), various sexual stage-specific genes have been characterized (Table 1-1) (Kumar and Carter, 1985; Vermeulen, et al., 1985; Alano et al.; 1991; Rawling et al., 1992; Paton et al., 1993; Bruce et al., 1994; Alano, et al., 1995; Baker et al., 1995; Williamson et al., 1995; Li, 1996, 1997, 2000, 2001a, 2001b, 2003; Muhia et al., 2003). Yet, only a few of them have been functionally analyzed through targeted gene disruption.
Table 1-1. Sexual stage-specific genes in *P. falciparum*  

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Expression Profile</th>
<th>Comments</th>
<th>KO</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf11-1</td>
<td>Parasitophorous vacuole of gametocytes</td>
<td>Associated with erythrocytic rupturing during gametogenesis</td>
<td>No</td>
<td>Scherf et al., 1993</td>
</tr>
<tr>
<td>Pfs16</td>
<td>Stage I onward, integral membrane protein</td>
<td>Potential TBV candidate</td>
<td>Yes</td>
<td>Kongkasuriyachai et al., 2004</td>
</tr>
<tr>
<td>Pfg27</td>
<td>Stage I, II onward</td>
<td>Potential TBV candidate</td>
<td>Yes</td>
<td>Lobo et al., 1999</td>
</tr>
<tr>
<td>Pfs48/45</td>
<td>Stage III onward cytoplasm, gamete surface, zygotes</td>
<td>Potential TBV candidate</td>
<td>Yes</td>
<td>van Dijk et al., 2001</td>
</tr>
<tr>
<td>Pfs230</td>
<td>Stage III onward and gamete surface, zygotes</td>
<td>Potential TBV candidate</td>
<td>Yes</td>
<td>Eksi et al., 2002</td>
</tr>
<tr>
<td>Pfs25/28</td>
<td>Surface of gametes, zygotes, ookinetes, young oocysts</td>
<td>Potential TBV candidate</td>
<td>Yes</td>
<td>Tomas et al., 2001</td>
</tr>
<tr>
<td>Pf77</td>
<td>Macrogametocytes</td>
<td>Function unknown</td>
<td>No</td>
<td>Baker et al., 1995</td>
</tr>
<tr>
<td>Pfg377</td>
<td>Mature macrogametocytes</td>
<td>Function unknown</td>
<td>No</td>
<td>Alano et al., 1995</td>
</tr>
<tr>
<td>α-tubulin II</td>
<td>Microgametocytes</td>
<td>Associated with morphologic changes during exflagellation, parasite motility</td>
<td>No</td>
<td>Rawlings et al., 1992</td>
</tr>
<tr>
<td>Pfmrk</td>
<td>Gametocytes</td>
<td>Homolog of Cyclin-dependent kinase</td>
<td>No</td>
<td>Li et al., 1996</td>
</tr>
<tr>
<td>PP-2A</td>
<td>Gametocytes</td>
<td>Protein phosphatase-2A</td>
<td>No</td>
<td>Li and Baker, 1997</td>
</tr>
<tr>
<td>PfCDPK</td>
<td>Gametocytes</td>
<td>Calcium-dependent protein kinase</td>
<td>No</td>
<td>Li et al., 2000</td>
</tr>
<tr>
<td>PfLAMMER</td>
<td>Gametocytes</td>
<td>PfLAMMER protein kinase</td>
<td>No</td>
<td>Li et al., 2001a</td>
</tr>
<tr>
<td>PfMCM4</td>
<td>Gametocytes</td>
<td>Mini-chromosome maintenance protein</td>
<td>No</td>
<td>Li and Cox, 2001b</td>
</tr>
<tr>
<td>PfPCNA2</td>
<td>Gametocytes (1 transcript)</td>
<td>Proliferating cell nuclear antigen</td>
<td>No</td>
<td>Li et al., 2002</td>
</tr>
<tr>
<td>PfORC1</td>
<td>Gametocytes</td>
<td>Origin recognition complex</td>
<td>No</td>
<td>Li and Cox, 2003</td>
</tr>
<tr>
<td>CTRP</td>
<td>Ookinetes, oocysts</td>
<td>Mosquito midgut invasion</td>
<td>Yes</td>
<td>Templeton et al., 2000</td>
</tr>
<tr>
<td>Pfgig</td>
<td>Asexual stages, Not in gametocytes</td>
<td>Promotes gametocytogenesis</td>
<td>Yes</td>
<td>Gardiner et al., 2005</td>
</tr>
<tr>
<td>PSLAP</td>
<td>Mature gametocytes</td>
<td>Modulates and protects from mosquito immunity</td>
<td>No</td>
<td>Delrieu et al., 2002</td>
</tr>
<tr>
<td>Chitinase</td>
<td>Ookinetes</td>
<td>Mosquito midgut invasion, potential TBV candidate</td>
<td>Yes</td>
<td>Tsai et al., 2001</td>
</tr>
</tbody>
</table>

*KO, knockout; TBV, transmission-blocking vaccine. Citations are for those who first identified the genes or functionally analyzed the genes when applicable. Some sexual stage specific genes in mosquitoes are also shown.*
The commitment to sexual development appears to occur in the schizont stage with the parasite developing into either male or female gametocytes (Williams, 1999, Silvestrini et al., 2000). Spontaneous loss of the ability to produce gametocytes during continuous parasite culture is often associated with chromosomal deletions. For example, mutations in chromosome 9 are linked to defects in sexual development (Day et al., 1993; Barnes et al., 1994; Bourke et al., 1996). Based on the analysis of chromosome 9 deletion of one such parasite clone, Gardiner et al. (2005) identified a gene, \textit{Pfgig} (\textit{P. falciparum} gene implicated in gametocytogenesis), which seems to play a role in the switch from asexual to sexual development. \textit{Pfgig} is expressed in asexual stages and its disruption resulted in decreasing numbers of gametocytes (Gardiner et al., 2005). Although complementation of the parasite line with a chromosome 9 deletion by expressing a \textit{Pfgig} transgene did not restore gametocytogenesis, the expression of \textit{Pfs16}, an early marker of sexual development, was detected (Gardiner et al., 2005).

Expression of sexual stage specific genes in \textit{P. falciparum} seems to be regulated in an ordered fashion with certain genes turned on as soon as gametocytogenesis begins. The expression of \textit{Pfs16} is regarded as the first sign of sexual differentiation, which has been detected in stage I gametocytes. \textit{Pfs16} encodes a 16 kDa protein located at the parasitophorous vacuole membrane (Dechering et al., 1997). Disruption of \textit{Pfs16}, which results in the expression of a truncated version of the gene (14 kDa), led to significant reduction in gametocyte formation (Kongkasuriyachai et al., 2004). In addition, male gametocytes were disabled in exflagellation \textit{in vitro} though they appeared morphologically normal. \textit{Pfg27} is an abundantly expressed gene in gametocytes with peak expression in stage I and II gametocytes (Lobo et al., 1994). \textit{Pfg27} is also an essential gene for
gametocytogenesis and its disruption led to a dramatic decrease or elimination of both male and female gametocyte populations (Lobo et al., 1999). Structural analysis indicates that Pfg27 is a non-specific RNA binding protein and interacts with Src homology 3 (SH3) modules, although the RNA targets and protein partners of this protein has not been determined (Sharma et al., 2003).

Many sexual stage-specific genes have been identified due to their potentials as transmission-blocking vaccine candidates (Sherman, 1998; Kongkasuriyachai and Kumar 2002) (Table 1-1). *Pfs48/45* encodes a protein that is localized on the surface of gametes and zygotes (Lobo and Kumar, 1998). Disruption of *Pfs48/45* resulted in very few zygotes/oocysts, although there was no quantitative change of male and female gametocytes (van Dijk et al., 2001). Further investigation showed that the reduction of zygotes was due to impaired male gametocyte fertility, which implicated *Pfs48/45* as important in maintaining the ability of male gametes to attach to, and penetrate, female gametes. Another surface protein, *Pfs230*, has a similar expression profile to *Pfs48/45* (Williamson et al., 1995). Disruption of *Pfs230* did not affect gamete morphologically, although truncated *Pfs230* proteins with no C-termini were unable to attach to the surface of gametocyte or gametes, which indicated that neither C-terminus of *Pfs230*, nor anchoring of *Pfs230* to the surface of gametocytes, was obligatory for gametogenesis (Eksi et al., 2002). Further study is needed to elucidate the functions of *Pfs230*.

Two functionally redundant ookinete surface proteins, *Pfs28/25*, are encoded by two adjacent genes with a head-to-tail organization. The transcripts of these two genes already exist in gametocytes, whereas the proteins are only found in later stages starting from gametes. Knockout mutants of the orthologues of these two genes, *Pb28/25*, in the rodent
malaria, *P. berghei*, showed that these two proteins might play roles in ookinete development (Tomas et al., 2001).

Despite all of these studies, the process of gametocytogenesis remains poorly understood. Therefore, sexual development in *Plasmodium* is an area in need of research, not only to understand the fundamental biological processes of protozoan parasites, but also to develop novel malaria control strategies.

**Functional genomics in the *P. falciparum* post-genomic era**

Despite the difficulties involved in sequencing the highly AT-rich parasite genome, the final version of the 22.8-megabase (Mb) *P. falciparum* genome was resolved in 2002 through international collaborative efforts (Gardner et al., 2002). There are 14 linear chromosomes in *P. falciparum* along with two extrachromosomal DNAs: a 6-kb mitochondrial DNA and a 35-kb circular plastid DNA in the organelle apicoplast (Wilson and Williamson, 1997). The sizes of chromosomes vary from 0.643 to 3.29 Mb with the smallest chromosome 1 and the largest chromosome 14. The AT content is about 80.6% in general, and greater than 90% for introns and intergenic regions. For some regions composed of repetitive DNA, AT contents can reach over 97%. A total of 5268 genes were predicted to encode proteins, 54% of which contain introns. The annotated proteins are mainly involved in parasite metabolism, life stages and immune evasion. Both mitochondrial DNA and plastid DNA encode a few proteins, while most proteins necessary for the functions of these organelles are encoded by the nuclear genome and transported to these organelles.

The completion of genome sequencing is truly a milestone in malaria research, which not only contributed greatly to our understanding of the fundamental biology of the parasite,
but also accelerated drug and vaccine development (Hoffman et al., 2002; Carucci, 2004).
Yet, more challenges lie ahead, as we apply these data to research and field practice (Cooke and Coppel, 2004). Since more than 60% of the genes have no known function and were annotated as hypothetical genes, functional genomics, the determination of the function of individual genes, constitutes an enormous task for the post-genomic era (Carucci, 2004; Sinden, 2004; Christophides, et al., 2004). Nevertheless, the genome sequence has allowed the application of global analytic tools such as DNA microarrays and proteomics to the study of malaria parasites (Le Roch et al., 2003; Hall et al., 2005). These studies provided us the opportunity to comprehensively view the complete transcriptome and proteome of the gametocyte stages.

**TRANSFECTION OF MALARIA PARASITES**

Besides the completion of genome sequencing, another major breakthrough in malaria research is the adaptation of transfection technology to malaria parasites (Goonewardene et al., 1993; van Dijk et al., 1995; Wu et al, 1995, 1996; Crabb and Cowman, 1996). Transfection technology has created a means to determine the functions of parasite genes by targeted gene disruption and knockout. In addition, it also allows transgene expression and the study of the activities of malaria promoters (Crabb and Cowman, 1996; Wu et al., 1996). Although the haploid parasite erythrocyte stage makes the knockout of essential genes almost impossible, gene disruption has been achieved for many *P. falciparum* genes since the first success in transient (Wu et al, 1995) and stable transfection (Wu et al., 1996). In particular, this technology is ideal for studying the functions of sexual stage-specific genes because they are not required for the asexual erythrocytic cycle.
Transfection in *P. falciparum* requires circular constructs rather than linearized plasmids, which makes the process much longer (more than 3 months) than transfection in other malaria parasites (3-4 weeks). In general, a vector for transfection in *P. falciparum* contains at least two selectable markers, one for amplification of the plasmids in bacteria and the other for selection in parasites (Fig. 1-2). A drug resistance cassette contains a promoter and a terminator from malaria parasites to ensure the expression of the selectable marker. Preferably, the gene of interest is cloned in a head-to-head orientation with the drug resistant cassette (Crabb et al., 1997).

To date, there are two ways to deliver DNA into the parasites. The most commonly used method is electroporation of DNA into the ring stage parasites (Wu et al., 1995; Crabb and Cowman, 1996), while another way is to electroporate DNA into fresh erythrocytes and let parasites take up DNA spontaneously (Deitsch et al., 2001). The most efficient setting for electroporation is 0.31 kV and 960 µF (Fidock and Wellems, 1997). After electroporation, the drug for a selective marker is added after one asexual erythrocytic cycle of the parasites (48 hours). Under selective conditions, most parasites die because the transfection efficiency is generally low, ~ 1:100 for transient transfection (Vanwye and Haldar, 1997) and 1:10000 for stable transfection (Crabbs et al., 1997). It usually takes about two weeks for resistant parasites to appear. Drug selection is maintained until the parasites grow very well for another week or two. At this stage, most of the resistant parasites contain episomes of the plasmid, which replicates in the parasites for generations. To enrich parasite population with stable integration, the drug is removed for 3 to 4 weeks. During this period, some episomes are lost due to incomplete segregation in the daughter cells (Waterkeyn et al., 1999). Reapplication of the drug will favorably enrich the parasites with stable integration of the
Figure 1-2. The first successful transfection vector in *P. falciparum* (Wu et al., 1996).

*T.g.dhfr-ts* is a mutant dihydrofolate reductase-thymidylate synthase gene from *Toxoplasma gondii*, which confers resistance to the drug pyrimethamine as a selective marker in malaria parasites. The promoter and the terminator are from 5’ sequence of the histidine-rich protein-3 (hrp3) and 3’ hrp2 of *P. falciparum*.
selectable marker in the chromosome. Cycling of the drug on-off scheme 2 to 3 times eventually leads to the predominance of parasites with targeted integration of the transgene. Single clones of resistant parasites are screened by limiting dilution (Trager and Jensen, 1976).

Despite the achievements in malaria transfection, there is much to improve. Among the multiple selectable markers tested for transfection of *P. falciparum*, including *Toxoplasma gondii* DHFR (Tg-DHFR-TS) (Wu et al., 1996), human DHFR (Fidock and Wellems, 1997), Blasticidin S deaminase of *Aspegillus terreus* (BSD), neomycin phosphotransferase II from transposon Tn 5 (NEO) (Mamoun et al., 1999), puromycin-N-acetyltransferase from *Strptomyces alboninger* (PAC) (de Koning-Ward et al., 2000), and a negative selectable marker thymidine kinase from *Herpes simplex* virus (Duraisingh et al., 2002), only a few have been thoroughly tested and adopted by malaria laboratories. Besides, the transfection procedure in *P. falciparum* is very lengthy and the whole drug selection cycles take almost 6 months to complete.

**REGULATION OF TRANSLATION**

**Regulation of translation in malaria parasites**

In all eukaryotic cells, the regulation of gene expression is accomplished at both the transcriptional and translational levels. For a process as complex as translation, regulation is exerted at various levels and through multiple mechanisms (Sonenberg et al., 2000). In *Plasmodium*, the process of translation and its regulation are poorly understood. Sequencing of the *P. falciparum* genome has revealed the presence of conserved translational factors
(Gardner et al., 2002). Five have been partially characterized in *P. falciparum*. The first reported translation factor was two identical polypeptides homologous to eukaryotic elongation factor (EF-1α) encoded by two identical genes in a head-to-head orientation (Vinkenoog et al., 1998). Another elongation factor, PfEF-1β, is encoded by a single copy gene, which has an expression pattern similar to that of the EF-1α homologues (Mamoun and Goldberg, 2001). A homologue of eukaryotic translation initiation factor 5A (eIF-5A) has been identified recently (Molitor et al., 2004). Malaria parasites and perhaps all other apicomplexan species all possess an organelle called an apicoplast, apparently the result of secondary endosymbiosis. In addition, the mitochondrion genome is only ~ 6 kb in malaria parasites. Most of the genes required for organelle function have been transferred to the nuclear genome of the parasite. As a result, the parasite nuclear genome contains genes more homologous to prokaryotic than eukaryotic organisms. For example, a gene with a C-terminal domain resembling the GTP-binding domain of the prokaryotic translation initiation factor 2 is expressed in all stages of the parasite life cycle. Interestingly, this protein is distributed as a 120 kDa fragment on the surface of sporozoites and imported to the nucleus in blood stage parasite as a 66 kDa fragment (Nguyen et al., 2001). The study of the translational machinery and its regulation in malaria parasites may lead to the identification of new targets for malaria chemotherapy.

Post-transcriptional control has been described in malaria parasites for over a decade (Kaslow et al, 1988; Paton et al., 1993); however, not until the recent microarray and proteomic studies did people realize that many genes in malaria parasites are probably regulated post-transcriptionally (Hall et al., 2005). The best example of translation repression is the sexual stage-specific gene, Pfs25, and its *P. berghei* orthologue Pbs21 (Kaslow et al,
The Pfs25 mRNA is already present in gametocytes but the proteins are detectable only after fertilization, indicating that the mRNAs are not translated immediately after synthesis. Recent studies suggest that myosin genes in P. falciparum expressed in erythrocytic asexual stages are regulated at both transcriptional and translational levels (Chaparro-Olaya et al., 2005). Recently, a comprehensive analysis of transcriptomic and proteomic data led to the identification of at least 16 sexual stage-specific genes that are transcribed in gametocytes but translated in later stages (Florens et al., 2002; Le Roch et al., 2003; Hall et al., 2005) (Table 1-2). These genes are orthologues of those in P. berghei, which contain a 47-base motif in their 3’ UTRs of mRNAs. A submotif within this 47-base motif was found in Pbs28 and it is predicted to play a role in translational repression of Pbs28. However, the motif was not found in Pfs28 and Pfs25 in P. falciparum (Hall et al., 2005). In P. gallinaceum, a similar U-rich element was found within the 3’ UTR of Pgs28 (Cann et al., 2004), a homologue of Pbs28. Various mutations of this element did not abolish the expression of Pgs28, but negatively and distinctly affected the expression levels of Pgs28, suggesting that this element in Pgs28 mRNA might play roles in optimizing the expression efficiency if not repressing its translation (Cann et al., 2004). A smaller region within the U-rich element in Pgs28 has also been found within the 3’ UTRs of Pfs25 and Pfs28 (Cann et al., unpublished), which might be possible target for translational regulation in these two P. falciparum genes. Collectively, differential expression of these genes suggests that post-transcriptional repression might be an important mechanism in gene expression in Plasmodium (Coulson et al., 2004). However, the mechanisms of translational control in Plasmodium remain to be investigated.
Table 1-2. Translationally-repressed genes in gametocytes in *P. falciparum*.

<table>
<thead>
<tr>
<th>Genes in PlasmoDB</th>
<th>Stages of transcripts</th>
<th>Stages of proteins</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF10_0302</td>
<td>Gametocytes</td>
<td>Gametes, zygotes, ookinetes</td>
<td>Pfs25</td>
</tr>
<tr>
<td>PF10_0303</td>
<td>Gametocytes</td>
<td>zygotes, ookinetes</td>
<td>Pfs28</td>
</tr>
<tr>
<td>PFL0960w</td>
<td>Gametocytes</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>PFL2320w</td>
<td>Gametocytes</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>PF13_0266</td>
<td>Gametocytes</td>
<td>Sporozoites</td>
<td></td>
</tr>
<tr>
<td>PF14_0522</td>
<td>Gametocytes</td>
<td>Sporozoites</td>
<td></td>
</tr>
<tr>
<td>PFC0495w</td>
<td>Gametocytes</td>
<td>Sporozoites</td>
<td></td>
</tr>
<tr>
<td>PF11_0087</td>
<td>Gametocytes</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>PF14_0508</td>
<td>Gametocytes</td>
<td>Sporozoites</td>
<td></td>
</tr>
<tr>
<td>MAL13P1.195</td>
<td>Gametocytes</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>PFE0715w</td>
<td>Trophozoites, gametocytes</td>
<td>Sporozoites</td>
<td></td>
</tr>
<tr>
<td>PF11_0148</td>
<td>Gametocytes, sporozoites</td>
<td>N/A</td>
<td></td>
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<td>PFI0440w</td>
<td>Gametocytes</td>
<td>N/A</td>
<td></td>
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<tr>
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<td></td>
</tr>
<tr>
<td>PF14_0507</td>
<td>Trophozoites, gametocytes</td>
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<td></td>
</tr>
<tr>
<td>PFI1390w</td>
<td>Gametocytes</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

* Based on Hall et al., 2005. N/A: not available
The Puf RNA-binding protein family in eukaryotic organisms

Translational control in eukaryotes can take place in a wide range of mRNAs with assistance from translational regulators. At the translation initiation step, both 5’ UTRs and 3’ UTRs contribute to initiation control by interacting with sequence-specific mRNA binding proteins and/or recruiting associated proteins (Gray and Wickens, 1998). One of the well-studied translational control mechanisms in eukaryotes is translation regulation through the binding of trans-acting factors to the 3’ UTR of mRNAs (de Moor et al., 2005). Of particular interest is the Puf family of RNA binding proteins (RBPs) named after Pumilio protein in Drosophila melanogaster and fem-3 binding factor in Caenorhabditis elegans (Wickens et al., 2002; Spassov and Jurecic, 2003).

Puf is a protein family of evolutionarily conserved RBPs that are widely distributed among animals, plants, fungi, and protists. So far, there are ~100 members deposited in the GenBank. Most organisms have multiple Puf genes, whereas the first identified Pum in Drosophila is encoded by a single gene. Sequence analysis and genetic studies have shown that the Puf RNA-binding domains (RBDs) are comprised of eight conserved tandem imperfect repeats of about 36 amino acids plus short flanking sequences (Zhang et al., 1997; Zamore et al., 1997; Barker et al., 1992). The entire RBD is required for its activity to bind to the 3’ UTR of target RNAs and to recruit associated proteins (Zamore et al., 1997). The crystal structures of Drosophila Pumilio (Edwards et al., 2000, 2001) and its homologue in human (Wang et al., 2001) have revealed that each repeat contains three helices and links to each other by head-to-tail to form a rainbow-like frame. Mutational analysis suggests that the convex surface is responsible for RNA binding (Edwards et al, 2001; Wang et al., 2001), while interactions with the specific sequence in the 3’ UTR of the mRNA are fulfilled by the
aromatic and basic amino acids highly conserved among repeats and across species (Wharton and Struhl, 1991; Murata and Wharton, 1995; Sonoda and Wharton, 1999). The convex surface, especially a loop lying between repeat 7 and 8, has been implicated to mediate the interactions between Pufs and associated proteins (Sonoda et al., 1999, 2001; Edwards et al., 2001; Wang et al., 2001).

Despite the conserved RBDs, Puf members appear to have very diverse functions within or among eukaryotic organisms during development. For the first studied function, Pum in Drosophila embryos determines the formation of posterior body pattern by repressing the translation of hunchback (hb) mRNA. This function is accomplished by the binding of Pum to the Nanos response elements (NREs) in the 3’ UTR of hb mRNA within the egg, along with another protein Nos (Murata and Wharton, 1995; Wharton et al., 1998; Sonoda and Wharton, 2001). As a translation repressor, Pum also plays roles in the proper asymmetric division and maintenance of germline cells during development by repressing mitotic proliferation of the pole cells (Lin and Spradling, 1997; Forbes and Lehmann, 1998; Asaka-taguchi et al., 1999; Parisi and Lin, 1999; Gilboa and Lehmann, 2004). In addition, Pum is pluripotent and also involved in the nerve tissue for long-term memory (Dubnau et al., 2003) and synaptic growth (Menon et al., 2004; Ye et al., 2004). In C. elegans hermaphrodites, one of the sex-determination genes, fem-3, is critical in the sperm-egg switch. Oogenesis is turned on through two functionally redundant Puf proteins, FBF1 and FBF2, to repress fem-3 mRNA translation by binding to the 3’ UTR (Zhang et al., 1997). Beside these two Puf homologues in C. elegans, other Puf members play essential roles in maintaining the germline stem cells (Subramaniam and Seydoux 1999, 2003; Crittenden et al., 2002, 2003). In the slime mold, Dictyostelium discoideum, PufA represses the translation
of a protein kinase A mRNA by binding to its 3’ UTR, keeping cells in the state of vegetative division (Souza et al., 1999). In the yeast *Saccharomyces cerevisiae*, the Puf protein Mpt5 controls mating-typing switch by binding to *HO* mRNA 3’ UTR to represses its expression and stimulates mating-typing switch (Tadauchi et al., 2001). In the amphibian *Xenopus laevis*, the Puf member X-Puf1 represses translation of *cyclin B1* mRNA. Inhibition of X-Puf1 leads to translation activation of *cyclin B1* and accelerates oocyte maturation (Nakahata et al., 2003). In the planarian *Dugesia japonica*, DjPum is essential for the maintenance of stem cells (Salvetti et al., 2005) through a similar mechanism of translational repression. From these examples of Puf function, it appears that Puf proteins share a common role in promoting cell proliferation and repressing differentiation (Wickens et al., 2002).

Translational repression of target mRNAs requires the binding of Puf members to their 3’ UTRs. In *Drosophila*, the specific sequence has been characterized, which is a pair of 32 U-rich sequences in the 3’ UTR of *hb* mRNA, named Nanos responsive elements (NREs) (Wharton and Struhl, 1991; Murata and Wharton, 1995). In particular, both UGU sequences in each NRE are critical for monomer Pum domain binding specificity and Pum function (Zamore et al., 1997, 1999). Similar sequences with the UUGU motif have been identified in *C. elegans*. A similar element, point mutation element (PME), residing in the 3’ UTR of *fem-3* mRNA is the target sequence for FBFs (Gallegos et al., 1998). In *P. berghei*, a 47-mer motif in the 3’ UTRs of a number of mRNAs might function as target sequences in translational repression of these genes (Hall et al., 2005). Since only a few Puf targets mRNA have been reported, studies of binding specificity of recombinant Puf have been performed using NRE sequences as an artificial target element (Zamore et al., 1997; White et al., 2001; Cui et al., 2002).
The binding of Puf memebers to 3’ UTRs of their target mRNAs is not sufficient for translational repression. It requires the formation of protein complexes through the interaction between Puf and other ancillary proteins. The first known ancillary protein in the Puf machinery is Nanos (Nos) protein in Drosophila (Sonoda et al, 1999), which also binds to RNA nonspecifically (Curtis et al., 1995, 1997). Nos appears to be conserved in metazoans and homologues have been found in C. elegans (Kraemer et al., 1999) and Xenopus (Nakahata et al., 2001). In C. elegans, there are at least three Nos members, (nos-1, 2 and 3) that interact with different FBFs. Xcat-2, a X. laevis Nos homologue, is recruited by X-Puf1 to repress cyclin B1 mRNA together with cytoplasmic polyadenylation element binding protein. Other than Nos, brain tumor (Brat) is another ancillary protein required for Pum to suppress translation of hb mRNA in Drosophila (Sonoda and Wharton, 2001).

Studies of the structures of Pum and Brat indicated that Brat might interact with Pum repeats 7 and 8 (Edwards et al., 2001, 2003). Taken together, a model of the protein-RNA complex was proposed to explain the interactions between Pum and 3’ UTRs of target mRNAs along with other associated proteins (Gavis, 2001). This complex might use two paths to suppress translation by controlling translation initiation and promoting deadenylation or turnover of mRNA. Pufs might recruit deadenylase or regulate the organization of messenger ribonucleoprotein (mRNP), though the exact mechanisms are still under investigation (Wickens et al., 2002; de Moor et al., 2005).

**Pufs in P. falciparum**

So far, only a few Puf members have been identified in protozoan species: TbPuf1 in Trypanosoma brucei (Hoek et al., 2002), TcPUF6 in Trypanosoma cruzi (Dallagiovanna et
al., 2005) and Pufs in *Plasmodium*. PfPuf1 in *P. falciparum* was identified in our laboratory by differential display (Cui et al., 2001), while PfPuf2 was retrieved from PlasmoDB database (www.PlasmoDB.org) by TBLASTN search (Cui et al., 2002). We also identified Puf members in other *Plasmodium* by BLASTP with PfPuf1 and PfPuf2. Apparently, there are two distinct *Plasmodium* Puf members within each species (detailed discussion in chapter 2).

In this thesis, I will first report the molecular characterization of two PfPufs in *P. falciparum* in chapter two. PfPuf1 and PfPuf2 are located in chromosome 5 and 4, respectively, encoding two distinct proteins. Both genes were preferentially expressed in sexual stages, as confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR), northern and western blots. Rapid amplification of cDNA end (RACE), RNA ligase mediated RACE (RLM-RACE) and primer extension showed that PfPuf1 transcript was ~7 kb with ~150 bp of both 5’ and 3’ UTR, while PfPuf2 mRNA was about 2.2 kb with a major transcription initiation site at 297 bp upstream of the start codon and a major polyadenylation site at 258 bp downstream of the stop codon. Phylogenetic comparison showed that PfPuf RNA-binding domains were highly homologous to those of other Puf members. Using *in vivo* yeast three-hybrid assay, we demonstrated that PfPuf2 RNA-binding domain had specific RNA-binding activity to the conserved target RNA sequence of the Puf family. This suggests that both PfPuf1 and PfPuf2 utilize a similar mechanism of translational control of its target genes.

In chapter 3 I will describe the functional analysis of PfPuf2 by genetic disruption. The *P. falciparum* clone 3D7 was transfected with a construct designed to disrupt repeat 8 of PfPuf2 RBD. Resistant parasites were selected with pyrimethamine, and clones with stable
disruption at the PfPuf2 locus were obtained by limiting dilution. Disruption of PfPuf2 in these parasite lines was confirmed by integration-specific PCR, genomic Southern blot, and RT-PCR. Two clones were used for phenotypic analysis, which showed that PfPuf2 disruption did not affect asexual growth of the parasite, but accelerated the formation of gametocytes. This is consistent with the ancestral function of this protein family in promoting proliferation and suppressing differentiation. This study will allow us to investigate the global gene expression profiles of wild type 3D7 and mutant strains, which may lead to the identification of PfPuf2 target genes.
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CHAPTER TWO: CHARACTERIZATION OF TWO MEMBERS OF PUF RNA BINDING PROTEIN FAMILY, PFPUF1 AND PFPUF2, IN THE MALARIA PARASITE, *PLASMODIUM FALCIPARUM*
During the development of eukaryotic organisms, temporal and spatial expression of decision-making proteins is essential. Expression of many of these proteins are controlled translationally rather than transcriptionally. The signals for translational control often reside within the 3’ untranslated regions (UTRs) of the mRNAs. Puf is an evolutionarily conserved RNA-binding protein (RBP) family that suppresses target mRNAs expression by binding to specific sequences at the 3’UTRs of these mRNAs during development. Members of this family contain a conserved RNA binding domain (RBD) with eight imperfect tandem repeats of ~36 amino acids (aa). Here we report molecular characterization of two Puf members, PfPuf1 and PfPuf2, in Plasmodium falciparum. PfPuf1 and PfPuf2 are spliced and located on chromosome 5 and 4, respectively. PfPuf1 encodes a protein of 1894 aa, whereas PfPuf2 encodes a protein of 514 aa. In addition to the size difference, PfPuf1 and PfPuf2 only share 27% homology in their RBDs. Reverse transcriptase polymerase chain reaction (RT-PCR), northern blot and western blot assays showed that both genes are preferentially expressed during gametocytogenesis of the parasite. Rapid amplification of cDNA end (RACE) and RNA ligase-mediated RACE (RLM-RACE) identified the 5’ and 3’ UTRs for both mRNAs. Surprisingly, PfPuf1 mRNA contained smaller UTRs of ~150 bp upstream and downstream of its open reading frame (ORF), whereas PfPuf2 mRNA had a ~300 bp 5’ UTR and a ~250 bp 3’ UTR. Using primer extension, a major transcription initial site at 297 bp upstream of the putative translational start codon was mapped for PfPuf2. Since there is no target mRNA identified for PfPuf1 and PfPuf2, the Nanos response element (NRE) from hunchback mRNA of Drosophila melanogaster was used as an artificial target sequence to test the
binding ability of both PfPuf RBDs using *in vitro* binding assays and yeast three-hybrid analysis. The results showed that both RBDs specifically bound to this artificial RNA target sequence, which suggests that PfPufs in *P. falciparum* might be involved in sexual development through a conserved mechanism of translational regulation of their target mRNAs.
INTRODUCTION

In spite of enormous global programs to eliminate malaria, it remains a public health problem in over 90 countries. Malaria severely hinders economic and social development in tropical areas, especially in Africa. The resurgence of malaria in the world partially resulted from drug resistance in malaria parasites and insecticide resistance in mosquito vectors. New control measures are desperately needed. One of the strategies is to develop transmission-blocking vaccines (Carter, 2001) that disrupt transmission of the parasite from human hosts to vector mosquitoes. For the obligatory transmission in mosquitoes, malaria parasites have to undergo sexual development and differentiation to form male and female gametocytes, a process known as gametocytogenesis (Garnham, 1988; Alano and Carter, 1990; Sinden et al., 1996). Because the molecular mechanisms that underlie this biological process remain elusive (Williams, 1999), a better understanding of the molecular biology of Plasmodium sexual development may help in designing new measures to disrupt the parasite transmission cycle.

To understand the mechanism of sexual development in P. falciparum, several gametocyte stage-specific genes have been identified using mRNA differential display (Cui et al., 2001). Among them, PfPuf1 is highly homologous to the Puf RNA binding protein (RBP) family. By searching the Plasmodium genome database (www.PlasmoDB.org), we identified another Puf homologue, PfPuf2. This RBP family was named Puf after the two best-characterized proteins Pumilio in Drosophila melanogaster and fem-3 binding factor (FBF) in Caenorhabditis elegans (Zamore et al., 1997; Zhang et al., 1997). Puf is evolutionarily conserved and widely distributed among eukaryotes. To date, the functions of Puf members have been elucidated among several genetically amenable organisms including
D. melanogaster, C. elegans, Dictyostelium discoideum, Saccharomyces cerevisiae, Xenopus laevis and Dugesia japonica. During the embryotic development of Drosophila, Pumilio (Pum) binds to the Nanos response elements (NREs) at the 3’ UTR of the evenly distributed hunchback (hb) mRNA in the egg. Once bound, Pum recruits posterior morphogen Nos and uniformly distributed Brain tumor (Brat) protein, leading to translational repression of hb mRNA at the posterior of Drosophila embryo, a prerequisite for the correct body patterning (Wharton et al., 1998; Sonoda and Wharton, 2001). In Drosophila, Pum is pluripotent and is also involved in long-term memory of nerve tissue (Dubnau et al., 2003) and synaptic growth (Menon et al., 2004; Ye et al., 2004). More importantly, Pum maintains germline stem cells by interacting with Nos and cyclin B1 mRNA to arrest mitotic proliferation of the pole cells (Lin and Spradling, 1997; Forbes and Lehmann, 1998; Asaka-taguchi et al., 1999; Parisi and Lin, 1999; Gilboa and Lehmann, 2004). In C. elegans, two Puf proteins, FBF1 and FBF2, which show 93% identity to each other, control the sperm-oocyte switch in the reproductive tract of hermaphrodites by binding to the 3’ UTR of fem-3 mRNA and repressing its translation (Zhang et al., 1997). Analogous to Pum function in the germline, other Puf members in C. elegans play essential roles in maintaining the germline stem cells (Subramaniam and Seydoux 1999, 2003; Crittenden et al., 2002, 2003). In the slime mold, D. discoideum, PufA represses translation of a protein kinase (PKA) mRNA, which keeps cells in the vegetative state of division (Souza et al., 1999). Starvation relieves repression of PKA, promoting development and fruiting body formation. In the yeast S. cerevisiae, the Puf protein Mpt5 represses expression of the HO gene, which encodes an endonuclease that stimulates mating-typing switch (Tadauchi et al., 2001). In the amphibian X. laevis, X-Puf1 binds to the 3’ UTR of cyclin B1 mRNA and interacts with the Nos homologue Xcat-2 and
cytoplasmic polyadenylation element binding protein (CPEB) to retard oocyte maturation by repressing translation of cyclin B1 mRNA (Nakahata et al., 2003). In the planarian *D. japonica*, DjPum is crucial for the maintenance of stem cells (Salvetti et al., 2005). Despite all of these diverse functions, Puf proteins seem to share a common role in promoting cell proliferation and repressing differentiation (Wickens et al., 2002).

In protozoans, two Puf members have been reported in *Trypanosoma*, TbPuf1 in *T. brucei* (Hoek et al., 2002) and TcPUF6 in *T. cruzi* (Dallagiovanna et al., 2005). From the *Plasmodium* genome sequencing projects, all malaria parasite species appear to have two conserved Puf proteins that differ dramatically in size and structure (Cui et al., 2002). In this study, we identified two Puf members, PfPuf1 and PfPuf2 in *P. falciparum*, which share 27% identity in their Puf domains. These two genes are preferentially expressed in gametocytes during erythrocytic development as confirmed by RT-PCR, northern blot and western blot assays. We further mapped the transcription initial sites of *PfPufs* by RLM-RACE and primer extension. Using the *Drosophila* NRE as an artificial binding sequence, we demonstrated the RNA binding activity of PfPuf1 and PfPuf2 in the yeast three-hybrid system. Taken together, these results confirmed the specific RNA binding activities for both PfPufs, which suggest that PfPuf1 and PfPuf2 are members of the Puf RBP family and might play a role in *P. falciparum* gametocytogenesis.
MATERIAL AND METHODS

Parasites culture and nucleic acid extraction

*Plasmodium falciparum* clone 3D7 was maintained in human O⁺ blood and AB serum as previously described (Trager and Jensen, 1976). Mixed asexual stages were obtained at 2 days after subculturing. Gametocytes were harvested and purified at 14–16 days by Percoll gradient centrifugation (Kariuki et al., 1998). Synchronization of parasite culture and total RNA isolation were done as described previously (Cui et al., 2001). Parasite DNA was isolated from the parasite pellet by proteinase K digestion and phenol/chloroform extractions.

Phylogenetic comparisons

A total of 75 GenBank entries with complete Puf domains were retrieved for phylogenetic analysis. The sequences of the Puf RNA binding domains of PfPufs were trimmed and used to generate the data matrix to infer the phylogenetic relationships among Puf family members. Putative Puf members from other *Plasmodium* species were obtained from the *Plasmodium* database (www.PlasmoDB.org). Their protein sequences were deduced based on the splicing patterns observed in the two PfPufs. Multiple alignment was performed using the CLUSTALW program (version 1.82) (http://www.ebi.ac.uk/clustalw) and the phylogenetic tree was drawn from the alignment using MEGA 3 (http://www.megasoftware.net/). Another three phylogenetic trees of full length, RNA binding domains and non-domain regions of *Plasmodium* Pufs were also constructed using MEGA 3.
**Nucleic acid hybridization**

For northern analysis, 10 µg of total RNA from mixed asexual stages and the Percoll gradient-purified gametocytes, respectively, were electrophoresed in 1% agarose/formaldehyde gels. RNA was then transferred to a nylon membrane. For PfPuf1, the membrane was hybridized under high stringency conditions to $^{32}$P-labeled 244 bp PfPuf1 cDNA fragment identified from the initial differential display work (Cui et al., 2001). Probe labeling was done with [$\alpha$-$^{32}$P] dATP using a random priming kit (Promega, WI). Northern analysis for PfPuf2 was performed using the DIG labeling and detection kit (Roche, IN) following the manufacturer’s instructions. The cDNA probe corresponded to the eight repeats of PfPuf2 RBD.

**RT-PCR**

To study PfPuf expression in synchronized blood stages, RT–PCR was performed on cDNAs from individual asexual stages (ring, trophozoite and schizont) and gametocytes (stages I, II and IV–V) (Cui et al., 2001). PCR conditions (30 cycles) were as described previously and the actin I gene was included as a control. PfPuf1-specific primers (GTTCAGAAATGTITATTACC and GACATTCTCTACAACATTAG) amplified a 337 bp fragment from cDNA and a 544 bp fragment from genomic DNA due to the presence of an intron. PfPuf2-specific primers (ACTGATGAAATTGTAAATG and GTTGGAATAATTCTGCATC) amplified a 209 bp fragment from cDNA and a 567 bp fragment from genomic DNA with two introns. PCR products were separated on a 1.5% agarose gel and documented using a Kodak digital camera.
RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE)

To identify 5’ and 3’ UTRs for *PfPuf1* and *PfPuf2* mRNA, RLM-RACE (Ambion, TX) was used following the protocol provided by the manufacturer. In brief, 10 µg of total RNA from *P. falciparum* late gametocyte stages (stage III-V) was dephosphorylated by calf intestinal phosphatase (CIP) to remove 5’ phosphate group from degraded mRNA, rRNA, tRNA and DNA. CIP was subsequently removed from the treated total RNA using phenol:chloroform extraction. The intact RNA was decapped using tobacco acid pyrophosphatase. An RNA adapter was then ligated to the 5’-end of the mRNA with the monophosphate using T4 RNA ligase. Afterwards, the RNA was reverse transcribed to cDNA. For the 5’ UTR of *PfPuf1*, nested PCR was performed using gene-specific primers (puf 56: 5’GTTCTCATCATCATTCCA-CATATTTG3’ and puf 52: 5’CATTTGTGTATTCCCCTTCATC3’) and the RACE adapter primers (Ambion, TX). For the 5’ UTR of *PfPuf2*, gene specific primers for nested PCR were Pub2 (5’CGTGCTCAATTGCATGTAG3’) and Pub3 (5’AAGCTTAAGGGATATTAAC-G3’). For the 3’ UTRs, Oligo-dT was used for cDNA synthesis, nested PCR was performed with Oligo-dT and two primers in the ORFs from *PfPuf1* (primers puf22: 5’ATAAGTTTTTATCCCATGAG and puf39: 5’CCCCAAAAAAAATTACGAAG3’) and *PfPuf2* (pb33: 5’CTT-AGACAAGTTAAAAGACG3’ and pb34: 5’GAGAGCTATTAACACTTGG3’), respectively.

**Primer extension**

To determine the transcription initiation site, primer extension was conducted according to the standard protocol (Sambrook et al., 1989). Briefly, 20 µg of the total RNA was extracted from mixed asexual stages and gametocytes of *P. falciparum*. The primer (5’GTATAT-
TATGAAAAAGATCATAACCCAT3’) was end-labeled by T4 polynucleotide kinase and $[^\gamma-^{32}P] \text{ATP}$ (Amersham-Pharmacia, IL), purified, and annealed to total parasite RNA. The primer was subsequently extended with Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and the RNA was digested with RNase A and T1 cocktail. The same primer was used for dideoxy termination sequencing with the cloned PfPuf2 promoter in Topo-TA vector (Invitrogen, CA) as the template with T7 DNA polymerase and $^{35}$S dATP. Both primer extension product and the sequencing reaction were electrophoresed in parallel on a 6% denaturing gel. The gel was then dried and exposed to X-ray film.

**Expression of recombinant PfPuf proteins in *Escherichia coli***

Expression of PfPuf1 RBD in *E. coli* as was described previously (Cui et al., 2002). Various truncated PfPuf1 domains were also expressed using the same strategy that corresponding cDNA sequences of these polypeptides were inserted at Bam HI and Xho I restriction sites of vector pET28 (Novagen, WI). To expression full length (aa 161-512) and partial (aa 362-512) PfPuf2 RBD, PCR was performed with gametocyte cDNA using two pairs of primers (5’CGGGGATCCGAGAATTACCTGAACAAAG3’ and 5’CGAGCTCGAGCCAATTGTT-AATAGCTCTC-3’; 5’CGGGGATCCCATAGGAAACGAGCAACAAA3’ and 5’CGAGCTCG-AGCAAAATTGTTAATAGCTCTC3’) and the PCR products were cloned at the Bam HI and Xho I sites. Recombinant proteins were expressed in *E. coli* strain BL21 after isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) induction and purified with Ni-NTA resin (Qiagen, CA). The recombinant protein was further purified from a 10% preparative SDS-polyacrylamide gel. The protein bands were excised from the gel, electroeluted, and used to raise polyclonal
antisera in rabbits (Pocono Rabbit Farm and Laboratory Inc. PA) and mice (Hybridome lab, the Pennsylvania State University, PA).

**Western blot**

Parasite pellets were resuspended in 1 X proteinase inhibitor mixture (Sigma, MO) and lysed by passing through a 1 cc syringe for 3 to 4 times. SDS loading buffer was added to the lysate at the final concentration of 50 mM Tris-HCl (pH6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol. The lysate was heated at 95°C for 5 min and ~50 µg of proteins was loaded on a 4-20% gradient polyacrylamide gel per lane (Invitrogen, CA). After electrophoresis, proteins were transferred to nitrocellulose membranes. For immunoblots, the primary anti-PfPuf2 rabbit serum was used at 1:500 and HRP-conjugated secondary antibodies were added at 1:5000. The blots were visualized with DAB substrate (Roche, IN) at room temperature.

**In vivo interaction of PfPuf RBDs with the NRE**

To confirm the results of *in vitro* binding assays of PfPuf1 and extend such observation to PfPuf2, yeast three-hybrid assays (SenGupta et al., 1996) were performed. The RBDs of PfPuf1 and PfPuf2 were cloned into the protein hybrid vector pYESTrp2, at the *Bam* HI and *Xho* I sites (Invitrogen, CA) to generate plasmid pYESTrp2-PfPuf1 or pYESTrp2-PfPuf2. In these constructs, PfPuf1 or PfPuf2 was expressed as a fusion protein with the B42 transactivation domain. For the bait RNA, both orientations of *Drosophila* NRE sequence was inserted at the *Sma* I and *Bam* HI sites of the hybrid RNA vector pRH5′ in order to fuse both sense and antisense NRE to the MS2 RNA sequence. The yeast host strain L40*ura*MS2, where expression of Lex–MS2 fusion protein was stably integrated, was co-transformed with
both pYESTrp2-PfPuf1 (or pYESTrp2-PfPuf2) and one of the bait plasmids (pRH5'-antisenseNRE or pRH5'-NRE). Four negative controls were used for PfPuf1 to exclude the non-specific binding between NRE to other RBP. For two of them, the baits were empty plasmid pRH5' or pRH5'-IRE to exclude the binding between Puf and non-specific 3’ UTR of IRE (iron responsive element). For the other two negative controls, the bait was either sense or antisense NRE, while the prey plasmid pYESTrp2-IRP contained a RBP, iron responsive protein (IRP). For PfPuf2, empty bait plasmid pRH5' or pRH5'-IRE was co-transformed with the plasmid pYESTrp2-PfPuf2 and served as two negative controls. As a positive control, pYESTrp2-IRP and pRH5'-IRE were co-transformed into the yeast. Transformed yeast was plated onto a synthetic medium lacking Ura, Trp and His. Positive transformants were selected for β-galactosidase activity using both filter and liquid assays (Te Heesen and Stagljar, 2000). For each assay, at least three yeast colonies were used.
RESULTS

Genomic organization of \textit{PfPuf1} and \textit{PfPuf2}

A single copy of \textit{PfPuf1} was confirmed by Southern blot analysis. TBLASTN search in the PlasmoDB database identified another Puf homologue in \textit{P. falciparum}, which was referred to as PfPuf2 (Cui et al., 2002). Comparison of the cDNA and genomic DNA sequences led to the identification of four (129, 147, 196 and 207 bp) and three introns (233, 125 and 124 bp) within PfPuf1 and PfPuf2, respectively (Fig. 2-1A). On chromosome 5 of the \textit{P. falciparum} genome, \textit{PfPuf1} is flanked by an upstream gene encoding a hypothetical protein and a downstream gene with homology to the yeast WD repeat gene. \textit{PfPuf2} is located on chromosome 4, flanked by dihydrofolate reductase-thymidylate synthase (\textit{DHFR-TS}) and an unknown ORF. All of the introns and putative intergenic regions (3 kb 5’ and 1 kb 3’ of the PfPuf1 ORF; 2 kb 5’ and 3.5 kb 3’ of the PfPuf2 ORF) are highly AT-rich (86-88%), containing homopolymer stretches of A or T.

Predicted PfPuf proteins and their RBDs

Regardless of the homology of the conserved Puf domains, there are considerable differences between these two PfPufs (Table 2-1). These two proteins differ in size, composition of amino acids and the locations of their respective RBDs. Besides the Puf repeats, PfPuf1 has additional short repeats of 7 or 8 aa ranging from 56 to 133 aa in length located both upstream and downstream of the RBD. Furthermore, PfPuf1 contains 27% of Asn overall and a sequence of 33 asparagines upstream the RBD from aa 613–645. In contrast, the deduced
PfPuf2 lacks these features. Both PfPuf proteins lack the Glu/Ala-rich and Ser-rich regions that were newly identified upstream of the Puf RBDs in other organisms (White et al., 2001).

A salient characteristic of the Puf RBP family is the RBD composed of eight copies of an imperfect repeat of ~36 amino acids (Fig. 2-1B) (Zamore et al., 1997; Zhang et al., 1997). BLAST search of the GenBank identified significant homology between PfPufs and other Puf family members (Fig. 2-2). As shown in the alignments, the most conserved regions correspond to the core consensus sequences of the Puf RBD (Barker et al., 1992; MacDonald, 1992) (Fig. 2-2 A and B). Recently, elucidation of the crystal structure of Pum and human Pumilio1 RBDs revealed that these consensus sequences form a basic crescent concave surface, which presumably binds to target mRNA (Edwards et al., 2001, Wang et al., 2001). The RBD sequences of PfPuf1 and PfPuf2 share 41% and 52% identity to that of Pum in D. melanogaster, respectively. The conservation of the repeats of the PfPuf RBDs suggests that PfPuf s may form a similar 3-dimensional structure and possess similar RNA binding activity, albeit the divergence between the Puf domains of PfPuf1 and PfPuf2 (27% identity) may imply distinct mRNA targets and different roles during parasite development.

The Puf RBP family

Puf RBPs are widely distributed in various eukaryotic organisms such as animals, plants, fungi and protists. At least 100 sequences have been retrieved from GenBank. Most organisms contain more than one Puf homologue. There are six in the baking yeast, at least 10 in C. elegans and more than 9 in Arabidopsis. In Plasmodium, Puf homologues have been identified in six species: two Puf homologues from the each species of rodent malaria
Figure 2-1. Genomic organization of PfPuf1 and PfPuf2 and structures of PfPuf1 and PfPuf2 (not to scale). (A) A schematic representation of the genomic organization of PfPuf1 and PfPuf2. Exons are indicated as boxes and introns and intergenic regions as solid lines. The conserved RBDs are shown as checkered boxes. The thick black arrows show the flanking genes and their orientations. (B) Comparison of the structures of PfPuf1 and PfPuf2 proteins to that of Pumilio in Drosophila. RBDs for each protein are shown with 8 repeats (checkered boxes) and two short flanking sequences (hatched boxes) on both termini. Pumilio contains 1593 aa with the RBD at C-terminus. PfPuf1 is larger than Pumilio, containing 1894 aa, and the RBD is in the central region, whereas PfPuf2 is much smaller with only 514 aa and the RBD is at the C-terminus.
Table 2-1. Comparison of PfPuf1 and PfPuf2

<table>
<thead>
<tr>
<th></th>
<th>Length (aa)</th>
<th>pI</th>
<th>M.W. (kDa)</th>
<th>% identity to Pum</th>
<th>RBD (aa)</th>
<th>aa composition</th>
<th>Other repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfPuf1</td>
<td>1894</td>
<td>8.2</td>
<td>224.1</td>
<td>41</td>
<td>823-1109</td>
<td>27% N₄, 33 N</td>
<td>Repeats of 7-8 aa</td>
</tr>
<tr>
<td>PfPuf2</td>
<td>514</td>
<td>6.2</td>
<td>61.4</td>
<td>52</td>
<td>184-476</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Notes: 1. aa, amino acids.
2. pI: isoelectric point.
5. 33 N: a sequence of 33 asparagines.
6. N/A, not applicable.
Fig. 2-2. Alignment of the deduced RNA-binding domains of Puf family. For all the alignments, “*” indicates identical amino acids, while “.” and “:” show semi-conserved or conserved amino acids, respectively. Gaps (-) were introduced to optimize the alignments. (A) Alignment of predicted PfPufs and other known Puf members from representative organisms: *Drosophila* pumilio protein (Pum), *C. elegans* FBF1 and FBF2, murine Puf1 and human Puf1. Only the conserved RNA-binding domains are shown. Human1, BAA07895; *Xenopus*, AAL14121.1; Mouse1, NP109648; AtPuf1, *Arabidopsis*, BAB01884; FBF1, AAF39879; FBF2, Q09312. (B) Alignment of RBDs of Pufs from different *Plasmodium* species. Three Puf1s and seven Puf2s were aligned. These are from the malaria parasites that infect humans (*Pf, P. falciparum*; *Pv, P. vivax*), rodents (*Py, P. yoelii*; *Pb, P. berghei*; *Pc, P. chabaudi*), chickens (*Pg, P. gallinaceum*), and primates (*Pk, P. knowlesi*). The underlined columns and letters in bold indicate amino acids that likely confer RNA binding specificity (Cui et al., 2002).
A. Alignment of Puf RBDs from representative organisms

Human1  FRNNRYPNLQLREIAGHIMEFSQ-DQHGRFQILKERATP-AERQLVFNEIQ---AAYQLMVDVFNY
Xenopus  -RRNRPNQLRLREIAGHIMEFSQ-DQHGRFQILKERATP-AERQLVFNEIQ---AAYQLMVDVFNY
Mouse  -RRNRPNQLRLDLHIFVSEFSQ-DQHGRFQILKERATP-AERQLVFNEIQ---AAYQLMVDVFNY
Pum  -RQNPYPNQLRLHIDVSEFSQ-DQHGRFQILKERATP-AEKMVFSEIIA---AAYSMTDVFDNG
AtPuf1  LKSNPARKLLESDIAGRVFEHVDHGSRFIQKLERSCD-EQKASVSEVL---QASKLMTDVFNY
PfPuf1  -YNKENPQTVGTCLLRPQK-LEKNNP-KHIEEYNAELD-HIELMDVFNY
FBF1  -EMKRSRLSEVQLSDMFLKAV-DKFGQLEVAKSLSLTYQFQFQVIGRDKDLKLSTNIFNG
FBF2  -EMKRSRLSEVQLSDMFLKAV-DKFGQLEVKSLKSTYQFQFQVIGRDKDLKLSTNIFNG
PfPuf2  --EQQVIVIDSKVMYDIVILCF-HKNGCEYI1KLENDK-EKEEIIINLILE-DTMSLCPDIYSGY

Human1  VIQKFFEFGSLE------QKLALAEIRGHRVTLSLQLQMYGCRVIQKEFIPSDQQ--NEMVRELDGNV
Xenopus  VIQKFFEFGSLE------QKLALAEIRGHRVTLSLQLQMYGCRVIQKEFIPSDQQVIQMENMRELDGNV
Mouse  VIQKFFEFGSLE------QKLALAEIRGHRVTLSLQLQMYGCRVIQKEFIPSDQQVINMRELDGNV
Pum  VIQKFFEFGSLE------QKLALAEIRGHRVTLSLQLQMYGCRVIQKEFIPSDQQVINMRELDGNV
AtPuf1  VIQKFFEFGSLE------QKLALAEIRGHRVTLSLQLQMYGCRVIQKEFIPSDQQVINMRELDGNV
PfPuf1  LQKIKMEVCTSE------QIEKIDKSSQNISVHGSRTVQKLIQMEKTQPSO--IIKATKLKNS

Human1  LKCVKDQNGNHVVQSCKIECQPPSLQFIDAFKQ---QVFVLSPIYCRCRVQIREHCPLS---
Xenopus  LKCVKDQNGNHVVQSCKIECQPPSLQFIDAFKQ---QVFVLSPIYCRCRVQIREHCPLS---
Mouse  LKCVKDQNGNHVVQSCKIECQPPSLQFIDAFKQ---QVFVLSPIYCRCRVQIREHCPLS---
Pum  LKCVKDQNGNHVVQSCKIECQPPSLQFIDAFKQ---QVFVLSPIYCRCRVQIREHCPLS---
AtPuf1  LKCVKDQNGNHVVQSCKIECQPPSLQFIDAFKQ---QVFVLSPIYCRCRVQIREHCPLS---
PfPuf1  ITILKDINGNHVQKCLENLQIDQFVDDMRLMSLQYFGHCWTVQDMSTSA---

Human1  ------DQTLPILEELHQHTEQLVQYYQNYIQHVHLHGRPED--SKIVAEGRIWVLVSQKHFA5NVV
Xenopus  ------EQTPLILEELHQHTEQLVQYYQNYIQHVHLHGRPED--SKIVAEGRIWVLVSQKHFA5NVV
Mouse  ------EQTPLILEELHQHTEQLVQYYQNYIQHVHLHGRPED--SKIVAEGRIWVLVSQKHFA5NVV
Pum  ------EQTPLILEELHQHTEQLVQYYQNYIQHVHLHGRPED--SKIVAEGRIWVLVSQKHFA5NVV
AtPuf1  ------EQTPLILEELHQHTEQLVQYYQNYIQHVHLHGRPED--SKIVAEGRIWVLVSQKHFA5NVV
PfPuf1  ------AQKELIFRINSNALLNLQVDAGNYVYQVILNLNLVQKMN--LEIANKLPNEELAYQKRF5NVV

Human1  ------EQTIPLEELIQHTEQLVQYYQNYIQHVHLHGRPED--SKIVAEGRIWVLVSQKHFA5NVV
Xenopus  ------EQTIPLEELIQHTEQLVQYYQNYIQHVHLHGRPED--SKIVAEGRIWVLVSQKHFA5NVV
Mouse  ------EQTIPLEELIQHTEQLVQYYQNYIQHVHLHGRPED--SKIVAEGRIWVLVSQKHFA5NVV
Pum  ------EQTIPLEELIQHTEQLVQYYQNYIQHVHLHGRPED--SKIVAEGRIWVLVSQKHFA5NVV
AtPuf1  ------EQTIPLEELIQHTEQLVQYYQNYIQHVHLHGRPED--SKIVAEGRIWVLVSQKHFA5NVV
PfPuf1  ------AQKELIFRINSNALLNLQVDAGNYVYQVILNLNLVQKMN--LEIANKLPNEELAYQKRF5NVV

Human1  EKCVTHASRTEAVLIDEVCT---MDNPGPHSALYTMKMDQYANVV
Xenopus  EKCVTHASRTEAVLIDEVCT---MDNPGPHSALYTMKMDQYANVV
Mouse  EKCVTHASRTEAVLIDEVCT---MDNPGPHSALYTMKMDQYANVV
Pum  EKCVTHATGERTGLIDEVCT---MDNPGPHSALYTMKMDQYANVV
AtPuf1  EKCKLEHADSTERFLIEEIMG---KSEE--DNHLLAMMKDQFANVVQYKVLLEISKDQ
B. Alignment of RBDs of Pufs in *Plasmodium* species

<table>
<thead>
<tr>
<th>Puf</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PbPuf2</td>
<td>TKEIKTKVDELDNKMIDIFLFCFHKNGCEYIKKKLEND---TKEKIQILNSLLIDASL 59</td>
</tr>
<tr>
<td>PcPuf2</td>
<td>TKEIKTKVDELDNKMIDIFLFCFHKNGCEYIKKKLEND---TKEKIQILNSLLIDASL 59</td>
</tr>
<tr>
<td>PyPuf2</td>
<td>------QTKINDLNDNTFLCDEYIKKKLEND---TAEKIQILNSLLIDPSKL 55</td>
</tr>
<tr>
<td>PgPuf2</td>
<td>------MDDEKKVVELDNKMIDIFLFCFHKNGCEYIKKKLEND---NEQRIQILNSLLIDASL 57</td>
</tr>
<tr>
<td>PfPuf2</td>
<td>------EEQVHDMSKVDLCLCFHKNGCEYIKKKLEND---KEEIKIQILNSLLIDMTSL 55</td>
</tr>
<tr>
<td>PvPuf2</td>
<td>DAAPPPAGNAGRMRLDTFLCDEYIKKKMKESENEETKRIIQKSSLLDAASL 60</td>
</tr>
<tr>
<td>PkPuf2</td>
<td>------PANEKEKLDDIFLFCFHKNGCEYIKKKLEND---TEEKEKIQILNSLLIDAVSL 55</td>
</tr>
<tr>
<td>PbPuf1</td>
<td>------YFISIHLTIPILHFTLFNLDKQSCRLQKIIIQN---QSCIDEYNEALEHIIE 58</td>
</tr>
<tr>
<td>PcPuf1</td>
<td>------YFISIHLTIPILHFTLFNLDKQSCRLQKIIIQN---QSCIDEYNEALEHIIE 58</td>
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<td>PyPuf1</td>
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</tr>
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<td>PfPuf1</td>
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<tr>
<td>PbPuf2</td>
<td>CPDMYGSYVAQSIFDLKDEKYKERFTDEFLKHTSFLTLHGYCLIQKSLESLSN---EYKC 118</td>
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<tr>
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<td>CPDMYGSYVAQSIFDLKDEKYKERFTDEFLKHTSFLTLHGYCLIQKSLESLSN---EYKC 118</td>
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</tr>
<tr>
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53
parasites *P. yoelii* (*PyPuf1* and *PyPuf2*), *P. chabaudi* (*PcPuf1* and *PcPuf2*) and *P. berghei* (*PbPuf1* and *PbPuf2*), the avian malaria parasite *P. gallinaceum* (*PgPuf1* and *PgPuf2*), the human malaria parasite *P. vivax* (*PvPuf1* and *PvPuf2*), and the primate malaria parasite *P. knowlesi* (*PkPuf2*) (Fig. 2-2). To study the evolutionary relationship of Pufs, a phylogenetic tree was constructed based on CLUSTALW alignment of 75 GenBank sequences with complete Puf domains and 11 *Plasmodium* Puf sequences (Fig. 2-3). In general, three clusters formed with first one dominated by *Puf* genes from the plants (*Arabidopsis*, rice and *Populus*), second one by those mostly from metazoan species (human, mouse, *Xenopus* and insects), and third one by nematodes and protozoans (*Trypanosome* and *Plasmodium*). Surprisingly, *Plasmodium* Pufs fell into two groups. The four *Plasmodium* *Puf1* genes are more related to *Puf* genes from *Arabidopsis* and fungi, whereas the seven *Plasmodium* *Puf2* genes are more closely related to the *FBF* genes from *C. elegans* (Fig. 2-3). Moreover, the homology was higher within *Plasmodium* groups but lower between the two groups. In addition, *Plasmodium* *Puf* genes within the same group are not only conserved in their Puf domains, but also in their genomic organization (in both flanking sequences and introns), conforming to the synteny among *Plasmodium* species (Carlton et al., 1998). The synteny of genomic organization is true for at least five *Plasmodium* *Puf2* sequences since they are all flanked by an upstream *DHFR-TS* gene and a downstream unknown ORF. Even these flanking sequences are highly homologous. We speculate that all of the malaria parasite species are likely to have at least the homologues corresponding to *PfPuf1* and *PfPuf2*.

Three additional phylogenetic trees were constructed for *Plasmodium* Puf1s and Puf2s: full length Pufs, the RNA binding domains and the other was the elsewhere regions of the proteins (Fig. 2-4). Pumilio in *Drosophila* was used as a standard Puf member in these
Figure 2-3. A phylogenetic tree showing the relationship between the Puf members.

GenBank entries with significant homology to the Puf RBDs of PfPuf1 were retrieved for detailed analysis. Among these, 75 members with complete Puf domains and 11 *Plasmodium* Pufs were used for phylogenetic analysis. Only the Puf domains were used for alignment. Each entry is identified by its GenBank accession no. *Plasmodium* Puf1 and Puf2 are highlighted along with those evolutionarily close related Pufs from plant, fungi and *C. elegans.*
Figure 2-4. Phylogenetic trees showing the relationship of *Plasmodium* Pufs. *Plasmodium* Pufs were retrieved from PlasmoDB database. For each phylogenetic tree, pumilio (Pum) in *Drosophila* is included as a standard Puf. (A) Full length Pufs, (B) The RNA binding domains and (C) The polypeptides of Pufs without the RNA binding domains.
trees. Since only a few *Plasmodium* species have been sequenced and Puf1s are much larger around 2000 aa, fewer full length Puf1s were retrieved to draw the phylogenetic trees than Puf2s. In general, *Plasmodium* Puf1s formed a cluster and Puf2 formed another one. Puf1s were more closed to Pumilio in *Drosophila*, the founding member of Pufs (Fig. 2-4A and 2-4B). The non-RNA binding regions of *Plasmodium* Pufs formed slightly different clusters, in which Puf1s and Puf2s were not grouped in two obvious clusters (Fig. 2-4C.), which probably due to the incomplete of genomic sequences for some of the *Plasmodium* species since the outgroup control, Pum, was included in *Plasmodium* Puf1 group.

**PfPuf1 and PfPuf2 expression during erythrocytic developmental stages**

Expression of the Puf family members, as shown for Pum and FBF, is developmentally regulated. To confirm the results of differential display, northern analysis was performed using RNA from asexual and gametocyte stages. The labeled *PfPuf1* cDNA fragment hybridized to an 8 kb mRNA from gametocytes but not to RNA from asexual stages (Fig. 2-5A). The discrepancy between the size of the predicted *PfPuf1* cDNA (7 kb) and that observed in the northern analysis may be due to the presence of a longer 5' UTR sequence and a long poly (A) tail. For *PfPuf2* expression, northern analysis showed its transcript was about 2.1kb (Fig. 2-5B).

For RT–PCR analysis of *PfPuf* expression in blood stage parasites, cDNAs were synthesized using total RNA isolated from synchronized parasite stages (Fig. 2-5C). Comparing to the actin I gene, which is constitutively expressed throughout parasite erythrocytic development stages, both *PfPuf1* and *PfPuf2* showed preferential expression in gametocyte stages, consistent with their expression profiles determined by microarray
analysis (Le Roch et al., 2003). Interestingly, RNA expression patterns of the two \textit{PfPuf} genes resembled that of the sexual stage-specific gene \textit{pfs16} (Dechering et al., 1999), which is immediately expressed from stage I gametocytes.

The transcription of \textit{PfPuf1} and \textit{PfPuf2} were further studied using RLM-RACE (Fig. 2-6) and RACE. The results showed that \textit{PfPuf1} had within 100 bp UTRs flanking both ends of the ORF (Fig. 2-7). For \textit{PfPuf2}, analysis of seven sequences from RLM-RACE showed that the 5’ UTR was distributed from 297 to 427 bp upstream of the putative start codon with the major one (5/7) at 297 bp (Fig. 2-8A). To verify this major transcription initiation site (TIS), we performed primer extension for \textit{PfPuf2} using RNA extracted from gametocytes (Fig. 2-9). A predominant cDNA product showed that the major TIS was consistent with the result from RLM-RACE. For 3’ UTR of \textit{PfPuf2}, we examined the polyadenylation site by RACE, which showed that the sites resided between 246 and 258 bp downstream of the stop codon. These analyses predicted the size of \textit{PfPuf2} mRNA as ~2.1 kb, close to the northern blot result (~2.3 kb).

**Expression of PfPuf RBD recombinant proteins in \textit{E. coli}**

For Pum protein in Drosophila, the eight 36 amino acid repeats plus short sequences on each side (334 amino acids) are essential for RNA binding activity (Zamore et al., 1997). To study the RNA binding activity of PfPuf1 and PfPuf2, the putative RBDs of PfPuf1 (344 amino acids) and PfPuf2 (351 aa) were expressed in a bacterial expression system as shown (Fig. 2-10). Partial His-tagged RBDs from both PfPufs were also expressed in \textit{E. coli} and purified (Fig. 2-11A and B) with Ni-NTA resin (Qiagen, CA). Recombinant protein expression was
Figure 2-5. Expression of *PfPuf* genes in blood stage parasites. (A) Northern analysis of *PfPuf1* expression. Ten micrograms of total RNA from *P. falciparum* asexual stages (A) or purified stage IV–V gametocytes (G) were electrophoresed through 1% agarose/formaldehyde gels and transferred to nylon membranes for hybridization to a $^{32}$P-labeled 244 bp *PfPuf1* cDNA fragment. The upper panel shows the autoradiograph of the northern blot and the lower panel shows the rRNAs in the ethidium bromide stained gel as loading controls. RNA sizes (in kb) are indicated. (B) Northern analysis of *PfPuf2* expression. Total RNA from mixed asexual stages and purified gametocytes was hybridized with a probe corresponding to aa 162-512. Molecular standards are indicated in Kb. The lower panel shows the rRNAs in the ethidium bromide stained gel as loading controls. (C) RT–PCR analysis of *PfPuf* expression during erythrocytic development of the parasite. RT–PCR was performed on total RNA isolated from synchronized asexual parasites as rings (R), trophozoites (T), schizonts (S) and gametocytes at stage I (I), stage II (II) and mixed stages IV and V (V). G indicates PCR amplification from *P. falciparum* genomic DNA. The actin I gene was used as an internal control, which showed constitutive expression in erythrocytic stages. The arrows in (A) and (B) indicate the specific band for *PfPuf1* and *PfPuf2* RNAs.
Fig. 2-5.

A.

B.

C.
Figure 2-6. The procedure of RLM-RACE. About 10 µg of total RNA was first treated with calf intestinal phosphatase (CIP) to dephosphate the phosphate groups in fragmental RNA. The CIP was removed by phonel/chloroform extraction. Afterward, the CIP-treated RNA mixture was treated with TAP to decapp full-length or 5’ intact RNA. A 5’ RACE adapter was then ligated to the purified decapped mRNA using RNA ligase, which resulted in 5’ adaptered-mRNA. First stranded cDNA was synthesized by reverse transcriptase using gene specific primers such as Pub2 for PfPuf2. Nested PCR was further performed. A specific PCR product was obtained and then cloned and sequenced to determine the location of TIS.
Figure 2-7. Mapping of 5’ and 3’ UTRs of PfPuf1 transcript. (A) PfPuf1 5’ end. Puf52 and Puf57 (underlined) were used for 5’ RLM-RACE. The TIS from RLM-RACE are in bold with arrows on top. Three clones (3) were sequenced and the same TIS was at –88 bp of ATG codon. (B) PfPuf1 3’ end. Puf22 and Puf39 upstream (underlined) of stop codon (in bold) were used for 3’ RACE, and three clones were sequenced with all clones at 53 bp downstream of the stop codon TGA.
Figure 2-8. Mapping of 5’ and 3’ UTRs of *PfPuf2* transcript. (A) *PfPuf2* 5’ end. Pub2 and Pub3 (underlined) were used for 5’RLM-RACE, Pub6 (underlined) was used for primer extension. The TIS from RLM-RACE are in bold with arrows on top. The (5) in the braces indicates the number of clones sequenced. (B) *PfPuf2* 3’ end. Pb33 and Pb34 upstream (underlined) of stop codon (in bold) were used for 3’ RACE, and four clones were sequenced with three clones at 246 bp.
Fig 2-8

**A. PfPuf2 5’ end**

\[ \text{TATGTAATATCTTGTATATATAATTGTTTTTCATTTTAATGTCTATTACTTCTACATATTTTATAAA} - 838 \]
\[ \text{AATTCATGTAAATATGTGAAAAGGGAAAGAAAAAAAAAAAAAAAAAAGAAACAAATTGCATGT} - 768 \]
\[ \text{CATTTTGAGAGAAAGTTAATTTTTTATTTTTTTAACTTCAAGGAAATACAAAGAAAGAAAGAA} - 698 \]
\[ \text{ACATATGTGTTGTCAGCTATATGTAGCTATAATATGTGAAATTTATATGTTATATGTTATAT} - 628 \]
\[ \text{AATTTTAAATAAAGTGCTCAATAAATAAATAAATAATGATATATATATGCTCTCAATTATA} - 558 \]
\[ \text{AAGTTCTACTAATTTTTTTTTTTTTTTTTTATAGTAATACTTATATGGTTATATTTATATT} - 488 \]
\[ \text{GTTATTTAAAACACACCTTTTATAATATATATATATATATATATATATATATATATATATATATATAT} - 427 \]
\[ \text{GTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTATCCCATG} - 348 \]
\[ \text{GAACATATATATATATATATGTACTTGTTAATTTGTGGATTTATAT} - 278 \]
\[ \text{TTATATGTGCTGGGTAAGGTTATGATCTTTTTTCATAATATAC} - 208 \]
\[ \text{GTTCAATGTTTCGTACATTTATTATTACATATTTATGGTTTGATCTAATTTTATTTTTTTATCCCATG} - 138 \]
\[ \text{AGAAATTTTATTATTAAAACTATTATAGAAAACTTAGACAAGTTAAAAGACGAAACATATGGTAATATAA} - 264 \]
\[ \text{TTATATGTGCTGGGTAAGGTTATGATCTTTTTTCATAATATAC} - 208 \]
\[ \text{GTTCAATGTTTCGTACATTTATTATTACATATTTATGGTTTGATCTAATTTTATTTTTTTATCCCATG} - 138 \]
\[ \text{GACTCCATTTTTTTAGAGAGATGATGCAATTGGACACGT} - 194 \]

**B. PfPuf2 3’ end**

\[ \text{AGAAATTTTATTATTAAAACTATTATAGAAAACATATGGTAATATAA} - 264 \]
\[ \text{TTATATGTGCTGGGTAAGGTTATGATCTTTTTTCATAATATAC} - 208 \]
\[ \text{GTTCAATGTTTCGTACATTTATTATTACATATTTATGGTTTGATCTAATTTTATTTTTTTATCCCATG} - 138 \]
\[ \text{GACTCCATTTTTTTAGAGAGATGATGCAATTGGACACGT} - 194 \]
confirmed by immunoblotting using a monoclonal anti-His tag antibody. The result of the blot assay was consistent with the predicted molecular size for each recombinant protein. The expressed proteins were further purified from preparative SDS-PAGE gels for raising antisera (Fig. 2-11). For some eluted proteins, two more bands with molecular sizes two and three times that of the expressed monomeric protein were also observed. These proteins were likely dimers and trimers of the recombinant protein because they also reacted with the monoclonal anti-His tag antibody (data not shown). The yield of the recombinant protein was 0.5–0.8 mg/l of bacterial culture for PfPuf1, but lower for PfPuf2 with less than 0.2 mg/l of bacterial culture.

To investigate protein synthesis of PfPuf1 and PfPuf2 in parasites, western blots were performed. Since PfPuf1 and PfPuf2 share 27% homology between their RBDs, the specificity of the antisera was first determined using recombinant PfPuf1 and PfPuf2 proteins. Full length his-tagged PfPuf1 RBD and GST-fused PfPuf2 RBD were fractionated in parallel in a SDS-PAGE gel and the immunoblots showed that there were no cross reactivities between these two antisera (Fig. 2-12A). Western blots using parasite lysates confirmed that PfPuf1 and PfPuf2 were preferentially expressed in gametocytes. For PfPuf1, a specific band of ~210 kDa corresponding to the predicted molecular weight of 224 kDa was identified in lysates from gametocytes, but not from asexual stages (Fig. 2-12B). However, for PfPuf2, the specific band of 73 kDa is larger than predicted molecular weight of 61.4 kD (Fig. 2-12C). It is not clear whether there are post-translational modifications, which might have slowed down the protein migration in SDS-PAGE gel. Abnormal migrations also have been found for other proteins in *P. falciparum* (Anders et al., 1988; Topolska et al., 2004).
Figure 2-9. The TIS of *PfPuf2* determined by primer extension. Primer extension was performed using 20 µg of total RNA from mixed erythrocytic stages including asexuals and gametocytes. Both the product and the sequencing reaction were run in parallel. The sequence reading was listed on the left. The major initiation site (T) is in bold and its position indicated by an arrow.
Figure 2-10. The procedures of expression and purification of recombinant proteins in bacterial system. To express PfPuf proteins in *E. coli*, their cDNA was first cloned into expression vectors with 6 X His or Glutathione S-transferase (GST) and then transformed into BL21 *E. coli*. After induction with isopropyl β-D-1-thiogalactopyranoside (IPTG), bacterial pellets were harvested and fractionated on a SDS-PAGE gel. The recombinant PfPuf proteins were identified by antibodies specifically reactive to tags, either 6 X His or GST. To scale up, *E. coli* pellets were prepared in the same way with larger volumes of bacteria. Lysed pellets were then bound to tag specific agarose (for 6 X His tag) or sepharose (for GST tag) and loaded in affinity columns. Nonspecific proteins were washed off and PfPuf protein was eluted from the column by way of changing pH of buffer or adding competitive solute such as imidazole. The purified proteins were confirmed on a SDS-PAGE gel and western blots.
Figure 2-11. Expression of the PfPuf1 and PfPuf2 RNA-binding domains in a bacterial expression system. (A) A Coomassie blue stained SDS-PAGE gel showing the purified recombinant protein (P) using a resin column and an electroeluted recombinant PfPuf1(E) protein for in gel purification. The arrow indicates the major elution product, while arrowheads show the dimmer or trimmer of the eluted protein. (B) A Coomassie blue stained SDS-PAGE gel showing the purification of recombinant PfPuf2 proteins. A full length of PfPuf2 RBD (lane 1) and partial RBD of repeats 5 through 8 (lane 2) were expressed as 6X His-tagged protein.
Figure 2-12. Synthesis of PfPuf proteins in gametocytes of *P. falciparum*. (A) Specificity of the antisera. To exclude the cross activity between two anti-PfPuf antisera, their specificity was confirmed by western blot. Both full-length 6X His-tagged PfPuf1 (2) and GST-tagged PfPuf2 (1) proteins were run in a SDS-PAGE in parallel. The blots were probed with anti-PfPuf1 (α-PfPuf1) and anti-PfPuf2 (α-PfPuf2). (B) PfPuf1 and (C) PfPuf2 protein synthesis in gametocyte stages in parasites. For both proteins, mixed stages of asexual parasites (A) or gametocytes (G) was purified, and then prepared in protein inhibitor buffer by several passages through a 1 cc syringe needle. ~50 µg protein per lane was fractioned through a 10% SDS-PAGE gel, then transferred to a nitrocellulose membrane and detected by preimmune sera, anti-PfPuf1 and anti-PfPuf2 antisera, respectively, for the blots. Markers (M) are shown in kDa.
Fig. 2-12

A.

![Western blot images for PfPuf1 and PfPuf2 with anti-PfPuf1 and anti-PfPuf2 antibodies.](image)

B.

![Western blot images for PfPuf1 and PfPuf2 with anti-PfPuf1 and preimmune sera.](image)

C.

![Western blot images for PfPuf2 with anti-PfPuf2 and preimmune sera.](image)
**In vivo interactions between PfPuf RBDs and the NRE sequence in yeast**

The results from *in vitro* binding assays provided us with valuable information about the binding activity of PfPuf1 RBD to the artificial target mRNA (Cui et al., 2002). Although there is only 27% identity between RBDs of PfPuf1 and PfPuf2, the residues in the eight repeats that are predicted to mediate the protein-RNA interactions are conserved (Fig. 2-2B). To further confirm the results of the *in vitro* binding assays for PfPuf1 and extend our finding to PfPuf2, we performed yeast three-hybrid analysis (SenGupta et al., 1996) to study the binding activity of the PfPuf1 or PfPuf2 RBD to the same artificial NRE from *Drosophila*, the same sequence used for the *in vitro* binding assays. For a yeast three-hybrid system, there are two protein hybrids and one RNA hybrid (Fig. 2-13). The protein hybrid one is fusion of proteins LexA and MS2, where LexA binds to the LexA operator to control expression of a reporter gene *Lac Z*. This protein hybrid is expressed in the yeast strain L40*ura*MS2. The protein hybrid two is expressed in a yeast plasmid pYESTrp2, where it integrates B42 transactivation domain and a target protein, i.e. PfPuf1 or PfPuf2. The RNA hybrid fuses a MS2 RNA, which interacts with MS2 protein, and a target RNA sequence, which is expected to interact with the target protein. A vector pRH5' is used to express the RNA hybrid. When a target protein interacts with a target RNA, B42 AD will activate the expression of *Lac Z* reporter gene.

In brief, a series of hybrid protein and RNA bait plasmids were constructed (Table 2-2). The yeast strains transformed with the plasmid, either pYESTrp2-PfPuf1 or pYESTrp2-PfPuf2, grew well on the selective medium and produced the fusion protein, B42–PfPuf1 or B42–PfPuf2. The expressed proteins were confirmed by their specific antibodies. As
Figure 2-13. Yeast three-hybrid system to detect the interaction between PfPuf2 and NRE RNA. Commonly used protein (MS2 and B42 AD) and RNA components (MS2) are depicted. LacZ is present in strain L40uraMS2, and is placed under the control of a LexA operator. Protein hybrid of MS2 and LexA is expressed in the yeast strain L40uraMS2, while protein hybrid of BS42 and PfPuf2 and RNA hybrid of MS2 and NRE are expressed in plasmids pYESTrp2-PfPuf2 and pRH5'-NRE, respectively.
expected, the yeast transformed with the positive control constructs to produce MS2-IRE and B42-IRP could grow well on the selective medium with 5 mM 3-aminotriazole. Yeast that was transformed with either pYESTrp2-PfPuf1 or pYESTrp2-PfPuf2 (producing B42-PfPuf1 or B42-PfPuf2) and the sense NRE RNA bait construct grew well and produced β-galactosidase at the same or similar levels as the positive control (Table 2-3). None of the yeast strains grew well when the PfPuf constructs transformed with the vectors pRH5', pRH5'-IRE or pRH5'-antisenseNRE, which were plasmids with an empty RNA bait, RNA bait for iron regulatory protein (IRP). To exclude non-specific protein-RNA interaction, we tested one RBP, IRP. The yeast strain transformed with pYESTrp2-IRP did not grow at all with RNA baits of either the sense NRE or the antisense NRE. These results demonstrated the binding specificity of RNA binding proteins of PfPuf1 and PfPuf2 to the sense NRE RNA.
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Table 2-3. β-galactosidase assays to determine the interactions between PfPufs and the Drosophila NRE sequence

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<td>B42-PfPuf2</td>
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<td>B42-IRP</td>
<td>0.222</td>
<td>B42-IRP</td>
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</table>

Note: Yeast strain L40uraMS2 was transformed with the plasmid pYESTrp2-PfPuf1 or pYESTrp2-PfPuf2 to express the hybrid protein of the B42 transactivation domain and the respective RBDs of PfPuf1 and PfPuf2. One of the bait plasmids expressing MS2, MS2–NRE, MS2–IRE or MS2–antisense NRE (MS2–asNRE) was co-transformed with the hybrid protein plasmid. For positive control, the bait RNA MS2–IRE was co-transformed with the plasmid pYESTrp2-IRP to express hybrid protein B42–IRP. In the assay for PfPuf1, two more negative controls were also included to exclude non-specific binding between protein-RNA. One was a plasmid with sense NRE and the other was one with antisense NRE (asNRE). Either plasmid was co-transformed with pYESTrp2-IRP. Only liquid assays for β-galactosidase are shown. For liquid assay, the number represents the mean using at least three yeast colonies. The in vivo yeast three-hybrid assays were preformed separately for PfPuf1 and PfPuf2 (Cui et al., 2002; Fan et al., 2004) and the data were not comparable between the two sets of experiments. N/D, not detectable. The units are arbitrary light units recorded by the luminometer.
DISCUSSION

Evolutionary conservation of Puf RBP family

Translational regulation is critical during development (Wickens et al., 1996). One of the well-studied translational regulatory protein groups is the Puf family (Zhang et al., 1997; Zamore et al., 1997; Barker et al., 1992). Thus far, over 100 Puf members have been identified in various eukaryotes from unicellular species to human. One of the conserved features of this RBP family is an RNA-binding domain (RBD) comprised of eight tandem imperfect repeats, which is mostly located at the C-termini of the proteins. Detailed sequence and crystal structure studies have shown that Puf RBD consists of eight repeats of ~ 36 amino acids flanked by short sequences on both termini, which is required for its RNA-binding activity (Zamore et al., 1997; Wang et al., 2001; Edwards et al., 2001). Each repeat forms three α-helices with a shorter helix (H2) in the middle forming the ridge and two longer ones (H1 and H3) covering the outer and inner surfaces of the RBD. A consensus sequence of aromatic and basic amino acids resides within each repeat. Reiteration of certain residues mainly happens in H3 where a putative RNA-binding surface is formed by stacking the eight repeats one on another. Conservation of these residues explains the binding specificity to certain sequence in 3’ UTRs of target mRNAs (Wickens et al., 2002). These eight repeats link each other tail-to-head (H3-H1) to form a three-layered rainbow-like structure (Wang et al., 2001; Edwards et al., 2001). The basic charge of inner surface is speculated to bind to target mRNA, while acidic outer surface interacts with other partner proteins. In particular, the eighth repeat is critical for interaction between Puf and associated protein regulators (Sonoda and Wharton, 1999).
Two PfPufs exist in *P. falciparum*. Phylogenetic comparison of the predicted RBDs of Puf proteins in other *Plasmodium* species showed high levels of homology to Puf members in other eukaryotes (Fig. 2-3). The most conserved regions are in the RBDs that are experimentally proved to bind RNA in several biological systems. Further scrutiny of H3 revealed the consensus residues of F/Y-G-X₄-Q-K/R-X₂-E among *Plasmodium* Puf RBDs (Fig. 2-2B). This suggests a similar binding mechanism of PfPufs to their target mRNAs. In *Drosophila*, Pum domain binds to two short consensus sequences of mRNAs’ 3’ UTRs named Nanos response elements (NREs) (Wharton and Struhl, 1991; Murata and Wharton, 1995; Sonoda and Wharton, 1999). Using the NRE of *hunchback* mRNA in *Drosophila* as an artificial target sequence, we showed that PfPuf RBDs had conserved RNA-binding activities by in vitro binding assay and yeast three-hybrid system. All these results indicated that the conserved UUGU motif in known target genes (*hb, fem-3, Cox17* and *HO*) might be a general recognition motif for Puf RNA-binding residues. However, the binding specificity of Puf to RNA may depend on the flanking sequences in the target mRNAs.

**Functional diversity among Puf members**

Except for Pumilio in *Drosophila*, which has two protein isoforms encoded by a single *Pum* gene, most organisms have more than one Puf. For instance, there are at least 10 Puf members in *C. elegans* (Zhang et al., 1997), six Puf homologues in *S. cerevisiae* (Gu et al., 2004) and nine in plant *A. thaliana*. In all malaria parasites, it is very likely that there are two distinct Pufs in each species (Cui et al., 2002). This suggests that gene duplication event that produced two PfPuf members must have predated the *Plasmodium* speciation events. The presence of multiple *Puf* genes in an individual organism suggests that Puf homologues may
have diverse biological functions. It is plausible that some are functionally redundant as for the two FBF genes in *C. elegans*, whereas others may act in a tissue- and/or time-specific manner to regulate different mRNAs and perform distinct biological functions. In *Drosophila* embryo, Pum regulates the anterior-posterior body patterning by interacting with *hb* mRNA (Barker et al., 1992), while it arrests pole-cell division by binding to *cyclin B* mRNA (Asaoki-Taguchi et al., 1999). In addition to other functions in somatic tissues, Pum is also involved in maintaining germline cells through unknown target RNAs (Lin et al., 1997; Forbes et al., 1998). In *C. elegans*, FBF1 and FBF2 seem redundant in controlling spermatogenesis (Zhang et al., 1997; Kraemer et al., 1999; Luitjens et al., 2000), whereas other Puf homologues regulate development of primordial germ cells (Subramaniam et al., 1999). In the baking yeast, two mRNAs, *HO* and *COX17* are translationally repressed by two Puf homologues, Mpt5 and Puf3p, respectively (Tadauchi et al., 2001; Olivas et al., 2000). Mpt5 also have multiple functions on lifespan, mating-type switch and pheromone response (Tadauchi et al., 2001; Kennedy et al., 1997; Chen et al., 1997). All these diverse functions seem to depend on specific target RNA and their associated protein partners. For instance, in *Drosophila*, though Nos is required in repressing translation of both *hunchback* and *cyclin B* mRNA, Brat is another protein partner needed for suppressing *hunchback* mRNA translation, but not for *cyclin B* mRNA translation (Sonoda and Wharton, 2001).

In *P. falciparum*, both *PfPufs* have a similar gametocyte stage-specific expression pattern (Fig. 2-5 and 2-11), but only share 27% homology and have dramatically different flanking sequences (Fig 2-1). Phylogenetic comparisons of all Puf RNA binding domains and within Plasmodium Pufs showed the different clusters which PfPuf1 and PfPuf2 belong to (Fig. 2-3 and 2-4). Thus, it is very likely that PfPuf1 and PfPuf2 have divergent other than
redundant biological functions during development. It is plausible that the assumed different functions might result from either their different target mRNAs or associated proteins. Several lines of evidence supported our assumption in this study. Except for the divergence of their structures, PfPuf1 and PfPuf2 have totally 14 different amino acids responsible for binding specificity (Fig. 2-2B), which might recognize separate target mRNAs. Moreover, the eighth repeats of both PfPufs differ, indicating that they may interact with different partners to execute their functions (Wang et al., 2001; Edwards et al., 2001).

**Common mechanism and ancestral role**

With regard to their conserved Puf RBDs, Puf RBP family restricts the translation of mRNAs through a universal mechanism of binding to their 3’ UTRs either to deactivate translation or promote RNA decay or both (Wickens et al., 2002). Although the exact mechanisms are still under investigation, studies of known Puf-RNA pairs or complexes in different organisms (Pum/hb, Pum/cyclin B, FBF/fem-3, PufA/PKA, Mpt5/HO, Puf3p/COX17, X-Puf1/cyclin B) support these common mechanisms (Zamore et al., 1997; Asaoki-Taguchi et al., 1999; Zhang et al., 1997; Souza et al., 1999; Tadauchi et al., 2001; Olivas et al., 2000; Nakahata et al., 2001). In addition, one of the associated protein, Nos, appears to be evolutionarily conserved in several metazoan species and characterized in *Drosophila, C. elegans* and *Xenopus* (Sonoda and Wharton, 1999; Kraemer et al., 1999; Nakahata et al., 2001). Accordingly, a proposed model is that Puf RBD binds to consensus sequences of specific mRNA 3’ UTR and recruits associated proteins to form a combinational complex. This complex either accelerates mRNA turnover through negatively functioning or alternates the mRNP organization to inhibit its positive activity during
translation (Wickens, et al., 2002). Most research on Puf to date has dealt with Puf RBP functions in maternal or germline cells during development. Its translation regulation determines correct development decisions. Collectively, from all known function of Puf RBPs, they seem to be decision-making chaperons during development by arresting differentiation and promoting mitotic proliferation.

In *P. falciparum*, the identified two Puf members, PfPuf1 and PfPuf2, are highly conserved suggesting that they might use similar mechanisms to regulate gametocytogenesis, though we do not know their exact biological functions yet. While the *in vitro* binding assays and yeast three-hybrid analysis have demonstrated that PfPufs are authentic RNA-binding proteins, it did not lead us to identify the putative ‘NRE’ due to the nucleotide bias in *P. falciparum* genome. The unsuitability of many research tools for identifying Puf targets in *P. falciparum* has prompted us to further investigate the function of PfPuf2 through targeted gene disruption (Chapter 3). With the elucidation of both biological functions and possible mechanisms of PfPuf in developmental regulation, it will provide insights into the biology of *P. falciparum* gametocytogenesis.
ACKNOWLEDGEMENTS

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CHAPTER THREE: PFPUF2, A SEXUAL STAGE-SPECIFIC PROTEIN, REGULATES SEXUAL DEVELOPMENT IN THE MALARIA PARASITE PLASMODIUM FALCIPARUM
ABSTRACT

Sexual development in malaria parasites is an obligatory process for the transmission of the parasite through mosquito vectors to humans. Knowledge of the molecular mechanism of this biological process may offer new venues for the control of the disease. Recently, we have identified members of the Puf RNA-binding protein (RBP) family in *Plasmodium falciparum*, which have conserved RNA binding activity and preferential expression in gametocyte stages. Research with genetically amenable organisms such as *Drosophila* has shown that the RNA binding domains of these proteins are conserved both in structure, with eight imperfect tandem repeats, and in function, to maintain germline stem cells through translational repression of the target RNAs. To investigate the role of Puf proteins in *P. falciparum* development, we have characterized the expression, RNA binding activity and biological function of *PfPuf2*. To functionally characterize *PfPuf2* in parasite sexual development, we performed targeted gene disruption of *PfPuf2*, which resulted in the expression of truncated protein with repeat 8 and the C-terminus of the RNA binding domain being deleted. Disruption of *PfPuf2* in these parasite lines was confirmed by integration-specific PCR, genomic Southern blot, and RT-PCR. Phenotypic analysis showed that *PfPuf2* disruption did not affect asexual growth of the parasite, but promoted the formation of gametocytes. The early formation of gametocytes resulted in early maturation, which was indicated by the observation of early exflagellation of mature microgametocytes and the subsequent increase of mature female gametocytes in *PfPuf2* mutants. This is consistent with the ancestral function of this protein family in promoting proliferation and suppressing...
differentiation. The conservation of this protein family in other apicomplexan parasites may implicate a similar function governing sexual development in *P. falciparum*. 
INTRODUCTION

The malignant malaria, caused by the protozoan parasite *Plasmodium falciparum*, continues to be a global health threat in over 90 countries, especially those in Sub-Saharan Africa. The annual 500 million clinical cases and up to 3 million deaths demand much greater efforts to reach the goal of halving the deaths caused by malaria by 2010 set by the Roll Back Malaria campaign (www.rollbackmalaria.org). Failure to control malaria is largely due to the development and spread of drug resistance in parasites and insecticide resistance in mosquito vectors. To combat the resurgence of malaria, new measures including effective vaccines and drugs are needed. To this end, comprehensive knowledge of the developmental biology of *P. falciparum* may provide insights into the intervention strategies. For this unicellular protozoan to complete its life cycle and switch hosts from human to mosquito, it has to transform from asexual to sexual stages in human blood, a process known as gametocytogenesis (Garnham, 1988; Alano and Carter, 1990; Sinden et al., 1996). The sexual stages have two morphologically distinct sexes, male (micro) and female (macro) gametocytes. In the mosquito midgut, gametocytes further differentiate into gametes, which fertilize to form diploid zygotes. Transformation of zygotes into ookinetes ensues, which transverse the midgut epithelium to initiate the sporogonic development. Therefore, gametocytes and gametes are crucial stages responsible for the natural transmission of the parasites by mosquitoes.

The critical role of gametocytes in malaria transmission makes sexual development of the parasite one of the promising control targets. However, how gametocytogenesis initiates is unknown (Williams, 1999), although it is believed that both genetic and environmental
cues are determinants of this process (Lobo and Kumar, 1998; Dyer and Day, 2000; Paul et al., 2002). The majority of asexual haploid parasites in human blood continue to divide asexually, while a very small portion differentiates into gametocytes. After commitment to sexual development, parasites not only change their morphology, but also their gene expression profiles with a number of sexual stage-specific genes being turned on (Florens et al., 2002; Hall et al., 2005).

Among the sexual stage-specific genes identified in *P. falciparum*, only a few have been functionally analyzed. Some of these genes, including pfs16, pfg27, pfs48/45, pfs230 and pfs25/28, have been chosen as vaccine candidates. *Pfs16* and *Pfg27* are expressed in stage I gametocytes (Lobo et al., 1994; Dechering et al., 1999), which have been used as genetic markers for the initiation of gametocyte development prior to distinct morphological change. Disruption of *Pfg27* abolishes the formation of both male and female parasites, indicating that pfg27 is essential for gametocyte development (Lobo et al., 1999). *Pfs16* is also critical for gametocyte development since its disruption leads to a dramatic reduction of gametocyte population and a defect in male gametocyte exflagellation *in vitro* (Kongkasuriyachai et al., 2004). *Pfs48/45* and *pfs230* are expressed starting from stage III gametocytes. *Pfs48/45* is important for fertilization since its disruption leads to defects in male gamete fertilization and results in very few zygotes/oocysts (van Dijk et al., 2001). However, the function of *pfs230* is less clear since its disruption is not critical for gametocytogenesis, although deletion of its C-terminus disabled pfs230 from attaching to gametocytes or gametes (Eksi et al., 2002). The expression of pfs25/28 is apparently under translational control with mRNAs being detected in gametocytes, while protein is only detected later in gametes, zygotes, ookinetes and young oocysts. Disruptants of its
orthologoue in the rodent malaria parasite, *P. berghei*, impaired ookinete/oocyst development (Tomas et al., 2001).

Recent studies using the genetically more amenable rodent malaria species, *P. berghei*, have gained tremendous insights into how protein kinases regulate gametocytogenesis and gametogenesis. The *P. falciparum* genome encodes six plant-like calcium dependent protein kinases (CDPKs) (Zhao et al., 1993; Farber et al., 1997; Li et al., 2000). Billker et al. (2004) have shown that deletion of CDPK4 crippled the male gametogenesis process, and male gametocytes lost the ability to exflagellate in response to external stimuli, implicating CDPK4 as a Ca$$^{++}$$ effector playing a role in controlling formation of male gametes and later zygotes. Another kinase, mitogen activated protein kinase (MAPK), also controls male gamete formation in *P. berghei* (Rangarajan et al., 2005), while NIMA-related kinase (NEK4) is female gametocyte-specific and might play a role in controlling the development of the zygote and progression of meiosis (Khan et al., 2005). All of these kinases appear to be involved in signaling pathways as their homologues do in other organisms; however, an integrative picture of the mechanisms in *Plasmodium* parasites is still far from completion.

These previous studies established that sexual stage-specific genes play critical roles in controlling sexual development of *P. falciparum*. Translational control appears to play a role in stage-specific regulation. A good example in *P. falciparum* is *pfs25*, whose protein is synthesized in gametes, ookinetes and young oocysts, whereas its RNA transcript exists in earlier stage (gametocytes) (Kaslow et al, 1988; Paton et al., 1993), although the mechanism has not been investigated yet. With the completion of *P. falciparum* genome sequencing (Gardner et al., 2002), more unknown genes identified are translationally regulated,
indicating that translational regulation of sexual-stage specific genes might be one way to
control gametocytogenesis or gametogenesis (Hall et al., 2005). In a previous study, we
identified two gametocyte stage-specific genes, \textit{PfPuf1} and \textit{PfPuf2}, which are expressed
throughout the entire gametocytogenesis process (Cui et al., 2002; Fan et al., 2004). These
two genes are members of the Puf translational repressor family, which encode RNA binding
proteins. This evolutionarily conserved protein family appears to use a similar mechanism to
fulfill their diverse functions by binding to the 3’ UTRs of their target mRNAs and
repressing their translation. Functional studies of certain Puf members suggest that they are
involved in decisional switches during development (Wickens et al., 2002). Although the
biological processes these proteins participate in are diverse, their unifying ancestral role
might be in promoting mitotic proliferation and repressing differentiation (Wickens et al.,
2002). The findings of the differential expression of \textit{PfPuf1} and \textit{PfPuf2} led us to further
explore their potential functions during gametocytogenesis in \textit{P. falciparum}.

In this study, we examined the functions of \textit{PfPuf2} through targeted gene disruption.
Disruption of \textit{PfPuf2} did not affect asexual development, but accelerated gametocyte
formation. Moreover, the male gametocyte maturation period was shortened compared to that
of wild type 3D7, which resulted in a higher proportion of mature female gametocytes in
later stages. The gametogenesis appeared normal. These results suggested that PfPuf2 might
play a role in maintaining asexual development while repress gametocytogenesis.
MATERIALS AND METHOD

Construction of plasmid pPfPuf2Δrep8

Plasmid pPfPuf2Δrep8, was made to disrupt repeat 8 of the RNA binding domain and the C-terminus of PfPuf2 through a single crossover event. Primers F2 (5’CGGGATCCGAGAATTACCTGAACAAG3’) and R2 (5’CTAGTCTAGATTAATCTTCTTCTCTGTTATAGCTTC3’) were used to amplify nt 491-1553 of PfPuf2 by PCR. The PCR product was digested Bgl II and Bam HI (italics in primers), and then cloned at the Bam HI site of pDT.Tg23 (Wu et al., 1996) downstream of the selectable dihydrofolate reductase-thymidylate synthase (DHFR) gene of Toxoplasma gondii (TgDHFR). The construct was confirmed by restriction enzyme digestion and automated sequencing.

Parasite culture and transfection

P. falciparum clone 3D7 was maintained as described previously (Trager and Jensen, 1976) and synchronized by sorbitol treatment (Lambros and Vanderberg, 1979). Two sets of plasmid construct pPfPuf2Δrep8 and one set of control plasmid pDT.Tg23 were purified using a Qiagen Maxiprep kit (Qiagen, CA) and ~100 µg of plasmid DNA were electroporated into 3-4% of synchronized ring stage cells using an established procedure (Fidock and Wellems, 1997). Two days later, for the control and one set of pPfPuf2Δrep8 parasites were selected with 100 ng/ml of pyrimethamine for 48 h, and subsequently 20 ng/ml of pyrimethamine for ~3 weeks with weekly replenishment of fresh red blood cells until resistant parasites were obtained. For the other set of pPfPuf2Δrep8 parasites, 20 ng/ml of pyrimethamine was added on day 2 post-electroporation throughout the first drug selection
cycle. Parasites containing integrated forms of the constructs were enriched using three on-off cycles of drug treatment and the subsequent use of 500 ng/ml of pyrimethamine essentially as described (Crabb and Cowman, 1996). Drug-resistant parasites were screened by PCR and Southern blots to detect plasmid integration at the \textit{PfPuf2} locus. Single clones of parasites with stable integration of the mutant \textit{pPfPuf2\Delta rep8} allele were obtained by limiting dilution. Four clones from two different transfection experiments were randomly selected for the characterization of the integration events and two were subsequently used for phenotypic analysis.

**Nucleic acids**

Genomic DNA was extracted from mixed asexual parasites using a proteinase K digestion and phenol:chloroform extraction procedure (Cui et al., 2002). Briefly, parasite pellets resulting from the lysis of the red blood cell membrane by treatment with 0.15% saponin were digested overnight in digestion buffer (100 mM NaCl, 10 mM Tris, pH 8, 25 mM EDTA pH 8; 0.5% SDS, 0.1mg/ml proteinase K). After extraction with phenol:chloroform, DNA was precipitated with 0.5 volumes of 7.5 M ammonium acetate and an equal volume of isopropanol. To isolate RNA from gametocytes, cultures were induced to undergo gametocytogenesis, and gametocytes were then cultured for approximately 16 days until the majority were mature. Gametocytes were purified by centrifugation in Percoll gradients (Kariuki et al., 1998). Total RNA was isolated from gametocytes using Trizol Reagent (Invitrogen, CA).
Confirmation of stable integration of \( PfPuf2\delta\text{rep8} \) by PCR and Southern blot

To verify the presence of the plasmid \( PfPuf2\delta\text{rep8} \) in the \( P. falciparum \) chromosome 4, genomic DNA from resistant parasites was amplified using F2 and M13R (Fig. 1).

Integration-specific PCR was performed using one primer upstream of the integration site (F2d, 5’GATCCAGAGAA-TGACATATT3’) and M13R (Fig. 1). Another set of primers F2d and Puf2R (5’GTTGTCAATAATTCTGCATC3’) were used to further confirm that the obtained parasites were from single clones without contaminating parasites with plasmid integrated elsewhere.

For genomic Southern blotting, 3 µg of genomic DNA from each clone (4 clones from each disruptant) and 6 µg from wild type 3D7 were digested with \( Xba \) I and separated on a 0.6% agarose gel. DNA was transferred to a nylon membrane (Osmonics, MA), denatured, and UV-crosslinked to the membrane using a Stratalinker 2400 (Stratagene, CA). About 25 ng of PCR product using primers F2 and R2 were labeled with \( [\alpha^{-32}\text{P}]\)-dATP using the Prime-a-gene system (Promega, WI). The membrane was prehybridized in 7% SDS, 0.5 M sodium phosphate monobasic (NaH\(_2\)PO\(_4\), pH 7.2), 2% dextran sulfate for 3 hrs at 65°C (Keys et al., 2000) and then hybridized with the denatured probe for 12 h at 65°C. Washes were done twice at 60°C for 20 min with 2X SSC/0.5% SDS and once for 50 min with 1X SSC/0.1% SDS. The membrane was exposed to a Kodak X-ray film at –80°C for autoradiography.

RT-PCR

Two micrograms of total RNA from gametocyte pellets were reverse transcribed with superscript III (Invitrogen, CA) at 50°C for 60 min (Cui et al. 2002). RT-PCR was performed
with Fd2 and DR1 (5’CGCGATCCTAATCTATAACTTGTTCAGGTGA3’) to detect the expression of PfPuf2 upstream from the integration site on Chromosome 4, and generated a 195 bp PCR product from both genomic DNA and cDNA. To verify the lack of expression of PfPuf2 downstream of the integration sites in the disrupted parasite clones, RT-PCR was performed with PB3A (5’TTTGCAAAGACTGCTATGG3’) and Puf2C (5’CCAAATTGT-TAATAGCTCTC3’), which produced a 282 bp of PCR product from genomic DNA and a 160 bp from cDNA in the control clones.

**Western blot**

Parasite pellets were resuspended in 1 X protein inhibitor mixture (Sigma, MO) and passed through a 1cc syringe 3-4 times, and clarified by centrifugation. SDS loading buffer was added to the lysate to the final concentration of 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol. About 50-100 µg of protein was boiled at 95°C for 5 min, separated on a 4-20% polyacrylamide gel (Invitrogen, CA), and transferred to a nitrocellulose membrane. The membrane was incubated with primary anti-PfPuf2 mouse serum at 1:500 and then alkaline phosphatase-conjugated secondary antibodies at 1:5000. Proteins were visualized with 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT) (Promga, WI) at room temperature.

**Parasite growth and development**

One milliliter of synchronized ring stage parasites at an initial parasitemia of 0.5% and haematocrit of 5% was placed in a 24-well plate (Corning Costar, NY) in triplicates for each
parasite clone. Medium was changed daily and thin smears were made to determine parasite growth and development for 16 days. Asexual parasitemia and gametocytemia were determined by counting at least 5,000 red blood cells from each culture. The sex ratio of microgametocytes (male) to macrogametocytes (female) was determined by counting at least 500 mature gametocytes. Exflagellation of mature microgametocytes from each clone was examined using a standard procedure (Kawamoto et al., 1990; Bhattacharyya and Kumar, 2001). In brief, the gametocytes was pelleted at 37°C at 400 x g, washed twice with warm PBS, and resuspended in warm PBS at a final haematocrit of 50%. Twenty microlitres of suspension with 5 µM of xanthurenic acid was dispersed into an erythrocytic monolayer under the cover slip of a hemacytometer at room temperature. The exflagellation centers were observed under a microscope about 5 to 10 minutes later. Ten random fields were counted at 200X magnification and the exflagellation centers were standardized after dividing by mean gametocytemia of each parasite clone.

**Statistical analysis**

The data were analyzed using Minitab 13.0 and SAS 8.0. The data were transformed to satisfy normal distribution when needed. For asexual growth, data were analyzed by a one-way ANOVA, followed by Tukey’s pairwise comparison. For gametocyte development, a two-way ANOVA was used to analyze the data, and the interaction term (day by treatment) was included. Orthogonal contrasts were used to pair-wise compare between the treatments within day 7, day 8 and day 10, respectively. For mature gametocytes ratio and male gametocyte exflagellation, a one-way ANOVA was used to analyze the data.
RESULTS

Targeted disruption of *PfPuf2*

To study the function of PfPuf2, the plasmid *PfPuf2*Δrep8 was constructed in order to disrupt *PfPuf2* after repeat 7 of the RNA binding domain. Two sets of drug selection for positive clones were performed (Fig. 3-1). In brief, the plasmid was transfected into WT3D7 parasites in two parallel experiments, and different concentrations of pyrimethamine were applied to the transfected cells at the beginning of the first selection cycle. The culture was maintained on alternating cycles of drug on-off treatment to facilitate the loss of the episomal plasmid and enrich parasites with integrated forms of the construct. Integration events were monitored for each cycle by using integration-specific PCR. With the progression of selection cycles, stable integrants were enriched. By limiting dilution (Fig. 3-2), more than 20 single clones were obtained from two independent transfection experiments. There was no difference for the quantity of positive clones for two sets of selection. To determine whether the plasmid had integrated at the *PfPuf2* locus, parasite DNA was used for PCR analysis using three sets of the primers. To prove that pyrimethamine-resistant clones did not result from complete loss of plasmid, PCR were performed using F2 and M13R. The generation of a 1370 bp DNA fragment in all selected parasite clones demonstrated the existence of the plasmid backbone (Fig. 3-3A). Integration-specific PCR using primers F2d and M13R established that the construct was stably integrated at the *PfPuf2* locus in all 10 parasite clones (Fig. 3-3B). Further PCR with F2d X Puf2R produced a band of 1515 bp in wild type 3D7 and in two F2d X M13R-negative clones (presumably integrated elsewhere) (data not shown), but not in the eight F2d X M13R-positive clones, suggesting that single clones were obtained.
Figure 3-1. The procedure of genetic disruption of *PfPuf2*. Two sets of parallel transfections were performed at the same time. Purified plasmids were electroporated into ring-stage parasites. The culture medium was changed next day. On day 2 post-electroporation, one set of transfection was treated with 20 ng/ml pyrimethamine, while the other was treated with 100 ng/ml. On day 4, 20 ng/ml of pyrimethamine was added for both transfections until resistant parasites appear at a moderate parasitemia in the culture. Then drug was off for 3-4 weeks. For later selection cycles, pyrimethamine was kept at 500 ng/ml. After 2 or more drug on-off cycles, when treated with pyrimethamine, parasites rarely died or revived very quickly indicating resistant parasites dominated the parasite population.
Figure 3-2. Cloning and screening of positive disrupted parasites. (A) Cloning parasites by limiting dilution. First, smear was made to determine parasitemia (x%, x is about 1-3) and red blood cells (RBC) was quantified with a hemacytometer. Then 10^6 RBC was diluted in 10 ml of culture medium, in which there was 10^4X parasites. One hundred microliters of the above mixture was added to another 10 ml of fresh medium with 2% fresh RBC with 10^2X parasites in the new mixture. Then 100 µl of the mixture was aliquoted into each well of a 96-well plate to keep x parasites per well. (B) Screening of positive parasites. The diluted parasites from step (A) were kept in the 96-well plate for two weeks and fed with fresh RBCs every 6-7 days before 500 ng/ml of pyrimethamine is added to the culture. 2-3 days after the addition of drug, smears were made for each well and screened under a microscope. The culture with parasites was transferred to a 24-well plate with 4% hematocrit in 1 ml of culture for each well. About 1 week later, parasites were monitored under a microscope, and then transferred to a 5 ml-culture flask when parasitemia was about 2-5%. Stocks were made for each clone at ring stages, and then the cultures were kept to obtain DNA and RNA for further confirmation by integration specific PCR, Southern blot and RT-PCR.
Figure 3-3. Scheme of homologous recombination at *PfPuf2* locus. (A) Predicted disruption of *PfPuf2* from a single crossover event. Primers are shown for their positions and orientations on chromosome 4 and the plasmid. Restriction enzyme *Xba* I (Xb) sites and the expected sizes of DNA fragments after *Xba* I digestion are shown. Top: *PfPuf2* locus in chromosome 4. Solid lines indicate introns or intergenic regions; filled boxes are exons; and checkered boxes are RNA binding domain. Primers F2 and R2 were used to construct the disruption plasmid. Middle: The disruption construct p*PfPuf2*Δrep8 is shown with the DNA fragment from *PfPuf2* genomic DNA. Restriction sites *Bam* HI (B) and *Xba* I (Xb) and M13R primer are shown. Bottom: The resultant single crossover event at the *PfPuf2* locus with integration of one copy of the plasmid construct. The primers F2d and MR13 were used for identifying integration and the primers F2d X Puf2R were used to confirm single clones. (B) Confirmation of integration at *PfPuf2* locus with PCR. Results from four clones are shown. Wild type (WT) 3D7 was used as a negative control. A 1062 bp PCR product using primers F2 X R2 and genomic DNA as template indicated the existence of episomes, while a 1370 bp PCR product from primers F2d X MR13 using genomic DNA implied the occurrence of integration. (C) Confirmation of integration with Southern blot. Six micrograms WT3D7 genomic DNA, 3 µg of genomic DNA from four clones and 100 ng of the plasmid construct (P) were digested with 0.5 µl of *Xba* I and then was electrophoresed through a 0.6% agarose gel. The blot was probed with a 32P-labeled PCR product using primers F2 X R2 (shown in Fig. 1A). DNA markers are shown in kb.
Fig. 3-3.

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C.

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F2d X M13R

F2 X M13R

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clones with PfPuf2 site integration were selected for further analysis by genomic Southern blot (Fig. 3-3C). As expected from a single crossover event, a 10.8 kb and a 4.4 kb band were observed in the PfPuf2-mutants, in contrast to the 5.7 kb band in wild-type 3D7. The occurrence of an extra 8.5 kb band of the same size as the plasmid construct in clone 1 and 2 indicated that these clones most likely resulted from integration of plasmid concatamers (O'Donnell et al., 2001).

Expression of PfPuf2 downstream is abolished in transformed clones

To further confirm that the PfPuf2 disruption was successful, transcript and protein levels were examined in mutant clones as well as WT3D7. RNA or protein was extracted from the mixed gametocytes stages withdrawn from parasite culture on day 14-16. To examine RNA levels, three sets of primers were used to perform RT-PCR in disrupted parasites, WT3D7 and a vector control (Fig. 3-4). C341 (PF11170c) is a housekeeping gene in P. falciparum, thus a 238 bp PCR product using C341 as a template was used as an internal control. For RNA expression upstream of the disruption of PfPuf2∆rep8, primers Fd2 X DR1 were used to amplify a 195 bp DNA fragment by RT-PCR; while downstream primers PB3A X Puf2C produced a 160 bp band with RT-PCR. The expression of truncated 5’ RNA in PfPuf2∆rep8 did not show a significant difference compared to WT3D7 and a transfection control, while that of 3’ RNA in PfPuf2∆rep8 was completely deleted, which suggested the disruption of repeat 8 at the PfPuf2 locus in parasites.

The synthesis of truncated proteins in parasites was tested by western blotting. Mixed stages of gametocytes from four clones were examined, and the anti-PfPuf2 antisera raised in mice were used to detect the expected band. A 73 Kd band corresponding to PfPuf2 in
Figure 3-4. Confirmation of *PfPuf2* disruption with RT-PCR. Top scheme represents *PfPuf2* genomic organization and the hatched box is the DNA fragment to construct a plasmid to disrupt repeat 8. Solid lines are intergenic regions and introns, while boxes are ORF and checkered boxes indicate PfPuf2 RNA binding domain. The primers (Fd2 and DR1 at 5’ terminus; PB3A and Puf2C at 3’ terminus) are shown for both their positions and orientations. Bottom panel shows the RT-PCR result: a 238 bp band was from a housekeeping gene C341 in parasites as an internal control; bands of 195 bp and 160 bp are the RT-PCR products from upstream and downstream of *PfPuf2*, respectively. Two representative clones (1 and 3) for disruption of repeat 8 were tested for their RNA expression. Wild type 3D7 was used as a positive control. The transfection control was from a parasite clone containing an integration event elsewhere in the genome.
WT3D7 was observed (Fan et al., 2004). Two bands were observed in samples corresponding to the mutant clones at a slightly smaller position in the blot (Fig. 3-5). Since the molecular weight for the repeat 8 and C-terminus was 9 kDa and the migration of PfPuf2 in WT3D7 was abnormal (predicated molecular weight is 64 kDa), we speculated that the bands slightly smaller than 73 kDa in the disruptants were the truncated PfPuf2.

**Gametocytogenesis is accelerated in PfPuf2 mutants**

To assess the effect of PfPuf2 mutations (d1 and d3), parasite morphology and development for both asexual and sexual stages were examined daily. The parasites appeared normal and there was no obvious morphological abnormality observed for both asexual and sexual stages in both disruptants.

Disruption of PfPuf2 did not affect asexual growth, (Fig. 3-6A) since PfPuf2 is only expressed in erythrocytic sexual stages in *P. falciparum*. The parasites at initial parasitemia of 0.5% were equally aliquoted in three wells for each clone and thin smears were made daily. The asexual growth curve for four clones (two disruption clones and two controls) showed very similar patterns. Statistical analysis confirmed that there was no significant difference in asexual growth among the four clones (P>0.05, ANOVA).

However, gametocytogenesis of the mutants were significantly different from that of transfection control and WT3D7 (Fig. 3-6B). Starting from the same initial parasitemia of 0.5%, gametocytes in mutants appeared earlier on day 2 or 3, whereas gametocytes appeared on day 4 or 5 in controls. The gametocytemia was significantly different (P<0.05, ANOVA) between the disruption groups and the two controls on day 7. At this point, about 80%
Figure 3-5. Synthesis of proteins in *PfPuf2* mutant clones and two controls by western blotting. Two nitrocellulose membranes blotted with parasite proteins were detected by either a mouse preimmune serum or an anti-*PfPuf2* antiserum as primary antibodies. Bands corresponding to *PfPuf2* were not observed in the blot with preimmune sera, whereas a 73 kDa band was identified in the blot with the anti-*PfPuf2* antiserum in controls. The protein marker is in the middle of the blots, while the molecular weights are shown in the left in kDa. The left arrow indicates proteins of *PfPuf2* in WT3D7 and transfection control at 73 kDa, while the up-arrows show the proteins of *PfPuf2* in the two mutants.
Figure 3-6. Asexual development and induction of gametocytogenesis. Four clones, WT3D7, transfection control and two disruptants (d1 and d3) were used for the assay. (A) Asexual growth. Parasites were aliquoted in triplicates for each clone at the initial parasitemia of 0.5% on day 1. Giemsa-stained thin smears were made daily and the parasitemia was the average of the parasites per 1,000 RBCs counted from triplicates. The data were analyzed separately by a one-way ANOVA. The treatment groups were compared by Tukey’s pairwise comparison. The result showed that the daily parasitemia was not significantly different between the clones (P>0.05). (B) Gametocytogenesis. Induction of gametocytogenesis was conducted without replenishment of fresh RBCs. The gametocytes were counted as the number per 1,000 red blood cells. And gametocytemia was the average from the triplicates. A two way ANOVA was used to analyze the data, and interaction term (day by treatment) was included. Orthogonal contrasts were used to pair-wise compare between the treatment within day 7, day 8 and day 10, respectively. The gametocytemia was significantly different between the disruptants and controls (p<0.001; F=34.4, df =4,40). For each group, data labeled with different letters indicates significant difference between two clones at P<0.05 (ANOVA).
Fig. 3-6

A. Asexual Growth

![Asexual Growth graph](image)

B. Gametocytemia

![Gametocytemia graph](image)
gametocytes were in stage III and IV in mutants, whereas over 75% gametocytes were in earlier stage II in controls.

The elevated gametocytemia continued for a few days from day 7 until day 10. Although gametocytemia were still different between clones and controls on day 10, there were no statistical differences. In the meanwhile, over 85% of gametocytes in controls were in stage III and IV, whereas mature gametocytes were already seen in *PfPuf2* disrupted clones. A small number of male gametocytes started exflagellation as early as on day 9, whereas exflagellation was observed until day 14 in control groups (data not shown).

Even though mutants and controls reached similar gametocytemia at last, the structure of mature gametocyte population was changed over time. It appeared that in mutants, male gametocytes dominated the population on day 12 or earlier, whereas mature females in mutants were more than males later on day 16 (Fig. 3-7). Comparing to WT3D7 and transfection control, the ratios of two mutants varied before and after the majority of gametocyte maturation. Starting from day 7, a few mature gametocytes appeared in mutants but not in the controls. When gametocytes reached mainly stage III and IV on day 12 in controls, mature female gametocytes were significantly higher in the mutants. When all the gametocytes started to become mature on day 14 or later, the ratio shifted and it became significantly lower in the mutants than controls. To unravel the mystery of mature gametocyte population shift, we further observed mature gametocytemia from the first appearance of mature gametocytes (day 8) in mutants until almost all gametocytes reached their maturation on day 16 (Fig. 3-8). The mature gametocytes in mutants increased at the beginning as those in the two controls. However, later, while mature gametocytes were on the rise in the controls, they decreased in the mutants (Fig. 3-8A). We then differentiated
female and male mature gametocytes in the population. To our surprise, female gametocytes developed in a very similar pace and pattern in the mutants to those in the controls (Fig. 3-8B). Then we found that reducing male gametocytes were due to the loss of mature ones, not the immature ones (Fig. 3-8C).

We also observed male gametocyte exflagellation ability. Starting from day 9, sporadic exflagellation occurred in mutants. With time, male gametocytes showed continuous moderate exflagellation. Even though there was a reduction in later mature male gametocytes, exflagellation did not show any significant differences between mutants and controls (P=0.125, ANOVA) (Fig. 3-9).
**Figure 3-7. The ratio of male:female gametocytes.** After induction of gametocytogenesis, gametocytes were maintained in same triplicates for maturation. Media was changed and thin smears were made daily. Mature gametocytes on day 12 and day 16 were counted to differentiate mature female and male gametocytes. The total numbers of 30 fields from triplicates were counted for both female and male gametocytes, and then the ratios were calculated with those numbers. A two-way interaction term (Day by Clone) was included in the two-way ANOVA. Two one-way ANOVAs were used to analyze the effect of clone within day 12 and day 16, respectively. When the effect of clone was significant, Tukey’s pairwise comparisons was used to compare among the clones within day 12 and day 16, respectively. The significance can be differentiated by the different letters in each group. * indicates P<0.001.
Fig. 3-8

A.

B.
C.

Figure 3-8. Time course of gametocyte maturation. Mature gametocytes starting at the first sight in the culture was quantified in the initial one hundred all-stage gametocytes from day 8 of culturing until most of gametocyte became mature on day 16. (A) mature gametocytes for both males and females (B) mature female gametocytes (C) mature male gametocytes in the initial one hundred all-stage gametocytes were quantified from triplicates for each sample. The average is shown for each sample.
Figure 3-9. Comparison of gametocyte exflagellation in vitro. Mature gametocytes on day 14-16 were spun and diluted into warm PBS at 50% haematocrit. A total of 20 µl of parasites with 2 mM of xanthurenic acid were spotted on a glass slide and then covered with a slip. Five to ten minutes later, the active exflagellation centers were counted in 10 random fields. The final counts were the mean standardized by the gametocytemia and gametocyte sex ratio. A one-way ANOVA was used to analyze the data. The result showed that there were no significant differences among these clones (p=0.125; F=2.04, df=3,36).
DISCUSSION

The completion of the *P. falciparum* genome sequence (Gardner et al., 2002) enabled the application of global analytical tools such as DNA microarrays and proteomics to study the developmental biology of the malaria parasites (Bozdech et al., 2003; Daily et al., 2004; Florens et al., 2002). These studies allowed direct comparison of genes and proteins expressed between asexual blood stages and gametocytes, and even between different sexes of gametocytes (Eksi et al., 2005; Hall et al., 2005; Silvestrini et al., 2005; Young et al., 2005). To study the initial process of gametocytogenesis, Silvestrini et al. (2005) employed genome-wide microarray analysis to compare gene expression in gametocytemic and non-gametocytemic lines. While these studies identified genes that are transcriptionally regulated during stage transition, several genes have also been identified to potentially subject to translational control, as these genes are transcribed in gametocytes without protein products (Hall et al. 2005). These results have underscored that translational regulation, once considered an uncommon phenomenon in malaria parasites, may play an essential role in parasite development. We have characterized two genes of the Puf RNA-binding protein family, *PfPuf1* and *PfPuf2*, which are preferentially expressed in gametocytes of *P. falciparum* (Cui et al., 2002; Fan et al., 2004). We previously showed that these proteins possess a conserved RNA binding activity and are likely translational repressors. Here we further analyzed the functions of *PfPuf2* by targeted gene disruption, which did not affect parasite asexual growth but appeared to promote the gametocytogenesis process.

Puf proteins are a family of evolutionarily conserved RNA binding proteins in eukaryotes, which appear to have a unifying role of suppressing differentiation and promoting proliferation despite their diverse functions in different organisms (Wickens et al.,
The first member of the family Pum of *Drosophila* embryos was shown to determine the formation of posterior body pattern by repressing the translation of *hunchback (hb)* mRNA (Murata and Wharton, 1995; Wharton et al., 1998; Sonoda and Wharton, 2001).

Another founding member FBF in *C. elegans* hermaphrodites controls sperm-oocyte switch by repressing translation of *fem-3*, one of the sex-determination genes, (Zhang et al., 1997).

In many characterized metazoan organisms such as *Drosophila, C. elegans*, and planarian *Schmidtea japonica*, Puf proteins are involved in the maintenance of germline stem cells during development by repressing mitotic proliferation (Lin and Spradling, 1997; Forbes and Lehmann, 1998; Asaoka-taguchi et al., 1999; Parisi and Lin, 1999; Gilboa and Lehmann, 2004; Subramaniam and Seydoux 1999, 2003; Crittenden et al., 2002, 2003, Salvetti et al., 2005). In the slime mold, *D. discoideum*, PufA represses translation of a protein kinase A mRNA by binding to its 3’ UTR, keeping cells in the state of vegetative division (Souza et al., 1999). In the yeast *S. cerevisiae*, the Puf protein Mpt5 controls mating-type switch by binding to *HO* mRNA 3’ UTR to represses its expression and stimulates mating-type switch (Tadauchi et al., 2001). In the amphibian *X. laevis*, the Puf member X-Puf1 represses translation of *cyclin B1* mRNA; inhibition of X-Puf1 leads to translation activation of *cyclin B1* and accelerates oocyte maturation (Nakahata et al., 2003). In addition to these diverse functions, Pum is pluripotent and also involved in long-term memory of nerve tissue (Dubnau et al., 2003) and synaptic growth (Menon et al., 2004; Ye et al., 2004). Regardless of the functions, Puf proteins use a similar mechanism by binding to the 3’ UTR of their target genes and repressing their translation. Both in vitro deletion analysis and structural resolution have shown that the intact eight repeat structure plus short N- and C-terminal extensions are necessary for its RNA binding activity and function (Sonoda and Wharton,
Furthermore, repeat 8 plays an essential role in recruiting associated proteins to facilitate translation repression of target RNA (Wang et al., 2001). Therefore, we disrupted *PfPuf1* and *PfPuf2* to eliminate repeat 8 plus the C-terminus in *PfPuf1* and *PfPuf2*.

Unfortunately, the disruption for *PfPuf1* was not successful. Resistant parasites were seen in the initial selection cycles and PCR confirmed the existence of plasmids in the parasites. However, integration specific PCR was never shown the expected band, thus a single positive clone was not obtained at all by limiting dilution. Several attempts have been tried, but all failed. We suspected that the genomic composition of *PfPuf1* was not favor in the homologous recombination, or *PfPuf1* might have either deleterious or lethal function during asexual development, which made the disruption unfeasible.

The failure of disruption of *PfPuf1* led us to switch the function analysis to *PfPuf2*. To our surprise, it was much easier to disrupt *PfPuf2*. After the confirmation of single clones (Fig. 3-3 and 3-4), we further examined the potential function of *PfPuf2*. As expected, the disruption did not have any effect on asexual growth since the development of asexual parasites in mutants had very similar pattern to those in controls (Fig. 3-6A). However, in the mutated clones, gametocytogenesis was apparently turned on earlier in development (Fig. 3-6B) and gametocytes, especially microgametocytes, developed at a more accelerated pace than the wt3D7 clones and the transfection control (Fig. 3-7). The repressive effect of PfPuf2 on gametocyte development is reminiscent of the role of X-Puf1 in *X. laevis* in repressing the maturation of the oocyte (Nakahata et al., 2003). This effect appears to conform to the unifying ancestral role of Puf family members in repressing differentiation and promoting proliferation (Wickens et al., 2002).
More rapid gametocyte development in all the PfPuf2 mutant clones was observed during routine culture. The more detailed analysis of the phenotypic changes in these clones showed that the accelerated rate of gametocytogenesis process is more prominent in the early stages, especially in the development of microgametocytes (Fig. 3-7 and 3-8). Eventually the gametocytemia leveled off and both the gametocytemias and the sex ratios were similar between the PfPuf2 mutants and the two controls on day 16. The drop of sex ratios in mutants on day 16 was disclosed with further detailed every other day observation of mature gametocyte from day 8, when mature gametocytes were seen in the culture (Fig. 3-8). The increasing female gametocytes in the mature gametocyte population was not due to the fast development of themselves, instead, male gametocytogenesis was initiated earlier than females and male gametocytes developed much faster towards maturation. While most of gametocytes reached maturation, some mature male gametocytes disappeared from the culture (Fig. 3-8C), which resulted in the shift of female and male gametocyte population on day 16. Since mature gametocytes are more sensitive to the change of environment, such as temperature and pH value, the early mature microgametocytes might have been lost during daily changes of culture medium as the result of exflagellation.

Collectively, PfPuf2 not only accelerates the initiation, but also the maturation of male gametocytes. Based on the effect of PfPuf2 on male gametocytogenesis, it would be interesting to test whether PfPuf2 has differential expression in different sexes of the gametocytes. Furthermore, it would be important to test whether PfPuf1 plays a similar role in gametocytogenesis, and whether they have redundant functions although they only share 25% identity in the RNA binding domain.
The conserved structural property of PfPufs and their authentic RNA binding activity suggest that PfPufs exert their functions using a similar mechanism of the Puf family by binding to the 3’ UTRs of their target mRNAs and blocking translation. The RNA target sequence of Puf proteins are conserved to some extent, and bioinformatic analysis has identified more than 90 genes in \textit{P. falciparum} genome with a similar motif in their 3’ end. However, the expression profiles of most of these genes either do not fit the expression profiles of the PfPuf genes, or these genes have no signs of translation regulation as revealed by proteomic analysis. One well-characterized, translationally regulated gene in malaria parasite gametocytes is \textit{pfs25}, which encodes a surface protein of the zygotes and ookinetes. However, in vitro binding assay and yeast three-hybrid analysis showed that PfPufs do not bind to the 3’ end of this gene. In addition, bioinformatic analysis did not identify conserved binding motifs in the 3’UTR of \textit{pfs25} gene, further suggesting that PfPufs do not contribute to the translation control of \textit{pfs25}. Recent analysis of the rodent malaria Pufs, combined with the data from the microarray and proteome analyses in \textit{P. falciparum}, has led to the identification of a dozen genes with potential posttranscriptional and translation regulation in gametocytes (Florens et al. 2002, Le Roch et al. 2003, Hall et al. 2005), attesting to the premise that post-transcriptional regulation might be an important mechanism of gene regulation in gametocytes. It still remains to be tested how these genes are controlled and whether they are targets of the PfPuf proteins. The success in generating PfPuf mutant lines will provide a useful tool for dissecting the Puf regulatory pathway.
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CHAPTER FOUR: CONCLUSIONS AND PERSPECTIVE
POST-GENOME ERA OF P. FALCIPARUM

Malaria has plagued human for centuries and remains a major public health problem in many regions of the world. A historic event in 2002 for malaria researchers was the completion of the genome sequences of *P. falciparum* (Gardner et al., 2002), its vector, *Anopheles gambiae* (Holt et al., 2002), and the human host (Lander et al., 2001; Venter et al., 2001). These genome sequences have provided a useful foundation for all aspects of malaria research. The 22.8 megabases of *P. falciparum* clone 3D7 nuclear genome is distributed on 14 chromosomes of different sizes. The overall AT content is over 80%, with the highest about 97% for repetitive DNAs. Fifty-four percents of the 5,268 annotated genes contain introns and 60% of these genes have no function.

Functional genomics has begun to unravel the biological processes of *P. falciparum* and their underlying mechanisms. Various techniques including bioinformatics, gene mapping, genome modifications and large-scale transcript analysis have been adapted for *P. falciparum* (Roos, 2001; Wellems et al., 1999; Cowman, 2001; Munasinghe et al., 2001; Dessens et al., 2000; Hayward et al., 2000; Ben Mamoun et al., 2001; Kappe et al., 2001). However, the high AT content in *P. falciparum* genome presents substantial hurdles. Adaptation of these techniques needs to be carefully revised for this problematic genome. Nevertheless, all these techniques have helped malaria research. DNA microarrays make it possible to study transcriptional profiling of thousands of genes at a time (Rathod et al., 2002). Comparative genomics between closely related species provides shortcuts to predict unknown genes and their potential functions (Waters, 2003). High-throughput proteomics is
useful to identify a large number of proteins in different developmental stages of \textit{P. falciparum} (Florens et al., 2002).

To further confirm the predicted functions of unknown genes experimentally, transfection technology has been widely used in malaria parasites for over a decade (Goonewardene et al., 1993; van Dijk et al., 1995; Wu et al., 1995, 1996; Crabb and Cowman, 1996). Since the inception of this technology in Plasmodium, a considerable number of genes have been disrupted or knocked out for functional characterization, with the main focus on the erythrocytic asexual stages. Many of these functional studies have been performed using the rodent malaria model, \textit{P. berghei}, which has much higher homologous recombination rate than \textit{P. falciparum}. Sexual stages of \textit{P. falciparum} as the sole form for transmission to humans have led to increase to attention towards the task of its life cycle. Large-scale gene analysis technology, in combination with the use of transgenic parasite lines that can be used to sort the male and female gametocytes by flow cytometry, have led to the identification of several genes specifically expressed in different sexes of the gametocytes (Le Roch et al., 2003, Hall et al., 2005, Young et al. 2005). Functional studies of these genes will ensue.

Another alternative for functional analysis is the use of RNA interference (RNAi), or rather, double-stranded RNA (dsRNA) treatment, which provides a simple and fast way to study genes in \textit{P. falciparum}. RNAi is a widely used technique to silence or down regulate gene expression within a very short period of time for many eukaryotic organisms. However, in spite of a few sporadic reports about the successful applications of RNAi in reducing malaria gene expression (Gardiner et al., 2000; McRobert and McConkey, 2002; Kumar et al., 2002; Malhotra et al., 2002; Gissot et al., 2005), most attempts have failed. This may be
attributable to the lack of the components of the RISC complex in the malaria genome, which is responsible for traditional RNAi (Aravind et al., 2003; Kumar et al., 2004).

The completion of the *P. falciparum* genome sequences brought excitement, as well as challenges, to malaria researchers. The vast volumes of data, and the adaptation of technologies applied to other organisms, provide many new avenues to examine the biology of malaria parasites. Importantly, these future studies may provide promising ways to optimize future malaria intervention strategies.

**POST-TRANSCRIPTIONAL REGULATION IN *P. FALCIPARUM***

While the first description of post-transcriptional control in malaria parasites occurred really in the past two decades (Kaslow et al., 1988; Paton et al., 1993), the significance of this discovery was not generally appreciated until recent microarray and proteomic studies (Hall et al., 2005) (Table 1-2, Chapter 1). The transcripts of these genes were detected in early life cycle stages, while protein synthesis were delayed until later stages. Interestingly, the majority of these 16 genes are sexual stage-specific. For example, the only genes among them of known function are *Pfs25* and *plasmepsin*, whose transcripts are found in gametocytes, whereas the proteins are synthesized in gametes and sporozoites, respectively. This finding implies that post-transcriptional control mostly occurs in erythrocytic sexual stages in *P. falciparum*. Further scrutiny showed that these genes are orthologues of those in *P. berghei*, which contain a putative 47-base motif within the 3’ UTR of their mRNAs. This motif has been predicted to play a role in translational repression. *Pbs28* is one of these genes in *P. berghei* and a similar U-rich element was also found in its homologue, *Pgs28*, in *P. gallinaceum* (Cann et al., 2004). Mutational study of this element in *Pgs28* indicated that it
could reduce expression of Pgs28. However, no such element was found in either \textit{Pfs25} or \textit{Pfs28} of \textit{P. falciparum} (Hall et al., 2005). Thus, the mechanism of translational control in \textit{P. falciparum} might use alternative pathways and they remain to be investigated.

Coincidentally, our work on sexual stage specific genes in \textit{P. falciparum} has resulted in the identification of two genes, \textit{PfPuf1} and \textit{PfPuf2}, encoding members of the Puf RNA binding protein family (Cui et al., 2002; Fan et al., 2004). This protein family contains a conserved RNA-binding domain through which these proteins repress translation of target genes by binding to the 3’ UTRs of target mRNAs (Wickens et al., 2002). Moreover, these proteins share a common ancestral role in promoting cell proliferation and repressing differentiation (Wickens et al., 2002). The mechanism of post-translational regulation through 3’ UTRs has gained more and more attention since it was first described in \textit{Drosophila} (Zhang et al., 1993).

To investigate the potential roles of PfPuf1 and PfPuf2 in \textit{P. falciparum} the two proteins, we first molecularly characterized these two genes. These two genes are preferentially expressed in gametocytes during erythrocytic development as confirmed by RT-PCR as well as northern and western blotting. Further study showed that the major transcription initiation site of \textit{PfPuf2} was 297 bp upstream of the start codon by RLM-RACE and primer extension. These two proteins share 27% identity in their Puf domains and show high homology to other Puf members. To investigate the RNA-binding activity of PfPuf1 and PfPuf2 to an artificial binding sequence, \textit{Drosophila} NRE, we conducted \textit{in vivo} yeast three-hybrid assays, which confirmed our hypothesis. Results from these analyses confirmed the RNA binding activities for both PfPufs, which suggests that PfPuf1 and PfPuf2 are members of Puf RBP family and might play a role in \textit{P. falciparum} gametocytogenesis. This
fundamental study led us to further investigate the possible roles of these genes in *P. falciparum*.

We examined the function of *PfPuf2* through targeted gene disruption at repeat 8. Two independent chromosomally-disrupted clones were used for phenotypic analysis for asexual development, gametocytogenesis, and sexual development. Collectively, our studies showed that *PfPuf2* did not affect asexual development, but accelerated gametocyte formation. Moreover, male gametocytes attained maturation earlier than wild type clone 3D7. Together, these results implicated that *PfPuf2* is a member of the Puf RNA binding protein family, and it might maintain asexual development while suppressing male gametocytogenesis.

Though we do not yet know how *PfPuf2* repress gametocyte differentiation, we speculate that it may fulfill its role through post-transcriptional regulation as other Puf RNA binding protein members are known to do. This provided us a new way to understand the transition of developmental stages in *P. falciparum*, although further study is still needed.

**IMPLICATIONS AND FUTURE OUTLOOK**

Translational control plays an essential role in the regulation of gene expression during development. The Puf family RNA binding proteins found in a variety of eukaryotic organisms regulate gene expression by translational repression of specific target genes. The conservation of the structure of Puf family proteins RNA binding domains, functioning mechanism and ancestral role provides useful information for further study of the *P. falciparum* homologues. The speedy maturation of male gametocytes in *PfPuf2* disruptants
led to the shift of population structure for mature gametocytes, and might affect mosquito infection (Burkot et al., 1984; Robert et al., 1996; Paul et al., 2002).

To further elucidate the functions of PfPuf2 and/or PfPuf1 in P. falciparum sexual development and translational regulation, several lines of evidence from the following aspects would corroborate the results stated in Chapter 2 and 3.

1. Further confirmation of the impact of PfPuf2 on asexual and erythrocytic sexual development. Over 20 positive clones for each transfection were identified by integration specific PCR. Two more independent chromosomally integrated clones will be selected from each transfection to repeat the asexual and sexual development to enforce the consistency of assays. Also, it implied that PfPuf2 only affected male gametocytogenesis. Therefore, it is interesting to test if PfPuf2 is also a sex-specific as well as stage-specific protein using IFA and male specific antibody.

2. Effect of PfPuf2 disrupted isolates on sporogonic development. This will explain if the function of PfPuf2 in P. falciparum is limited in erythrocytic sexual developmental stages or extended to sexual development in mosquitoes. To this aim, gametocytes on day 12 and day 16 will be fed to susceptible mosquitoes using artificial membrane feeding. Four samples will be used, two controls and two isolates, as mentioned in Chapter 3. On day 12, mature gametocytes are seen in isolates, but not in controls. Development of oocysts would indicate that PfPuf2 might not have effect on sporogonic development and/or the population structure of mature male:female might have changed, which would affect mosquito infectivity (Burkot et al., 1984; Robert et al., 1996; Paul et al., 2002).

3. Determination of PfPuf2 target genes. So far, we do not yet known how PfPuf2 functions as a translational repressor since we do not know which genes are under its control.
RT-PCR, quantitative RT-PCR, RNA microarray and proteomics will be performed to determine the potential target genes. Large quantity of high quality RNA from four clones used in Chapter 3 will be prepared from mature gametocytes. Several potential target genes were identified by Dr. Qi Fan (personal communication) and Dr. Neil Hall et al., (Science, 2005). Comparison of expression of these 17 genes by semi-quantitive RT-PCR will be further confirmed by quantitative RT-PCR.

To screen more potential target genes, RNA microarray will identify changes of abundance of certain RNAs, which might be potential PfPuf2 targets.

Proteomics is the best way to determine target genes. However, the low efficiency in expression of PfPuf2 protein both in vitro and in vivo increases difficulty of this method. Tandem affinity purification (TAP) allows for rapid and efficient purification of epitope-tagged protein complexes from crude extracts under native conditions. The method was established in yeast (Rigaut et al., 1999; Gavin et al., 2002) and has been successfully applied to other organisms, including mammals and trypanosomes (Schimanski et al., 2005). Modification and improvement of TAP has been adapted to diverse organisms (Drakas et al., 2005). To study the proteomics influenced by PfPuf2 in P. falciparum, a plasmid flanking with a TAP tag at 3’ UTR will be constructed. This plasmid will be transfected into clone 3D7 and homologous recombination is expected to substitute 3’ UTR of PfPuf2, where TAP tag will be added to PfPuf2 in vivo. Thus, PfPuf2 can be purified in a large quantity using TAP affinity column under native condition to perform proteomics.

4. Genetic disruption of PfPuf1. Since PfPuf1 is more related to Pumilio in Drosophila, we speculate that it might also play roles in P. falciparum development. Targeted gene disruption has been tried for quite a few times and still failed, antisense
double-stranded RNA (dsRNA) might be an applicable alternative. Sense and antisense RNA encoding PfPuf1 RNA-binding domain will be synthesized in vitro and will be annealed to dsRNA. The dsRNA will be delivered into the culture to feed ring stage parasites and the effect on *P. falciparum* will be observed as described in Chapter 3.

All above assays will by all means help us understand the functions of PfPuf2 and/or PfPuf1 in sexual stage development, and the roles of these two proteins in translational control of *P. falciparum*. These exciting findings will provide not only insights into the life cycle stage transition and sexual developmental biology of the virulent parasite, but also useful references for malaria intervention design.
REFERENCES


Appendix A: Culturing of malaria parasites

1. Culturing of asexual erythrocytic P. falciparum

**Materials:** Incomplete 1640 Medium: homemade good for up to 4 wks
- Sodium bicarbonate
- Hypoxanthine
- Gentamycin
- RBCs O+: get fresh blood and good until the expiration date; if using albumax, any blood type can be used.
- Human serum, pooled of all types, inactivated at 56ºC for 1hr. Do not sterilize.

The aseptic procedure is very important. If use bovine serum albumin, Albumax (Invitrogen, CA), the solution is need to be sterilized by filtration.
- Flasks of 25 cm³; 75 cm³ and 175 cm³
- Gas mixture: 5% O₂; 2-3% CO₂; balance N₂ (or a CO₂ incubator or candle jar)

**Complete medium:** (For 2 liters of total volume):
- 11.9 g of Hepes (Acidic)
- 4.2 g of sodium bicarbonate
- 0.1 g of hypoxanthine  (very hard to be dissolved, takes at least 3-4 hrs)
- 1 ml of  10 mg/ml gentamycin
- 2 X 10.4 g packets of RPMI 1640

Sterilization through a 0.22 µm filter.

Do not need to adjust pH value. However, it should be orange/pink, NOT pink (pH 7.2-7.4, not higher than 7.6). Use the media within 4 weeks. If it is too pink, throw it out.
Preparation of red blood cells:

We get blood from Hershey blood bank, usually the majority of buffy coat has been removed. However, there might be anticoagulant, which also needs to be removed.

1) Aliquot red blood cells from the pack to 50 ml of sterile tubes. Fill half of it (25 ml). Store the rest blood in 4°C if it is not be used immediately.
2) Prepare red blood cells right before you are using. Use fresh cool (4°C) RPMI 1640 media to fill up the 50 ml tube containing blood. Invert to mix it well.
3) Centrifuge at 3, 700 rpm (around 3000 g) for 10 min at 4°C.
4) Carefully remove the supernatant and the very thin white buffy layer between red blood cells and supernatant with a sterile Pasteur pipette.
5) Wash it again with 25 ml of cold RPMI 1640 if buffy coat is not removed completely.
6) Add equal volume of RPMI 1640 to the blood pellet and make it to 50% haematocrit.

Thawing parasite stocks from liquid nitrogen

Thawing solution I: 12% NaCl; II, 1.8% NaCl; III, 0.2% glucose (dextrose)/0.9% NaCl.
All solutions need to be sterilized by filtration. (0.45 or 0.22 µm filter)

1) Remove the ampoule from the liquid nitrogen and thaw quickly at 37°C (need about 10 min for over 0.5 ml).
2) Transfer the contents of the ampoule to a 10 ml centrifuge tube.
3) Add Solution I at 0.2 volumes of freezing mixture. We usually add 100 µl if $V \leq 0.5$ ml; 200 µl if $V > 0.5$ ml slowly with drop by drop and mix or shake gently. Stand the tube for 5 min at room temperature to draw out the freezing mixture.
4) Add Solution II at 10 volumes of freezing mixture. We usually add 1 ml if $V \leq 0.5$ ml; 2 ml if $V > 0.5$ ml slowly with drop by drop and mix or shake gently. Stand the tube for 5 min at room temperature.
5) Add Solution III at 10 volumes of freezing mixture. We usually add 1 ml if $V \leq 0.5$ ml; 2 ml if $V > 0.5$ ml slowly with drop by drop and mix or shake gently. Stand the tube
6) Centrifuge at 1500 X g (~ 2000 rpm) for 5 minute.
7) Remove supernatant, and transfer the pellet to prepared small flask with 5% final hematocrit in 5 ml.
8) Change media next day and make a thin smear. If the parasitemia is >2.5%, dilute to another flasks.
9) Maintain the culture between 2-6% parasitemia for the first week, as the growth can be erratic if you overdilute early on.

**Culturing of parasites:**

1) Before changing media, warm media to 37°C.
2) Remove spent media with sterile Pasteur pipette. Make a thin smear if needed.
3) Replace fresh warm complete media to the flask. Shake the cultures gently to resuspend. This helps to reach a high parasitemia in a short time.
4) Gas the flask about 10 seconds at 5lb/sq. inch for small flask (25 cm³) or 10lb/sq.inch for larger flask.
5) When growing well, culture will need to dilute down 2-3 times a week.
6) Do not dilute cultures below 1% as this can stop them growing well for a few days. You can let the culture get quite high (8-10%) for static culturing or ~20% for shaking culture. But do not let it crash.
7) When the parasitemia reaches 6-8%, it’s ready to dilute according to the experiment. Keep the final hematocrit at 5% and parasitemia above 1% if there is no special requirement.
8) Change media **every day**. It is a good idea to make this at the **same time** of each day, if possible. Keep the cultures **as warm as possible** at all times.
References:


2. Culturing of sexual erythrocytic *P. falciparum*

You need quite a lot of flasks for decent amounts of RNA. It needs at least 4-5 large flasks (175 cm$^3$) per Affymetrix slide.

1) For the first few days before. Make sure culture is in a happy condition and **low %** parasiteamia (2-5%). Make sure you have a culture from **fresh** tube of **3D7** growing that hasn't been continuously cultured.

2) Keep the culture very synchronized to minimize the production of any early gametocytes

3) Culture in T75 flasks with 40-50 ml medium.

4) Adjust the parasites at trophozoite stage to about 1-1.5% at about 3% hematocrit and then shake them overnight

5) Next day you should have about 5-6% parasitemia rings - adjust to 5% if necessary, but keep about 1/4 to 1/3 of the old medium in the flask

6) When the reach schizont, split them into four to five flasks with **VERY fresh** blood with 1ml-1.5ml RBC per flask. Place them on a shaker overnight.
7) Next day you should have about 8-10% rings. You can add 50mM N-acetyl glucosamine to get rid of the asexuals - takes about 5 days (see Ponnudurai's work)
8) I find the stages I-V will peak every 2 days - i.e. 2, 4, 6, 8, 10. However, everyone does have a few differences.

The protocol was according to Dr. Quinton Fivelman
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3. Harvest gametocytes by Percoll gradient

1) Make Percoll (1.130 g/ml) isotonic by adding 1 volume of 10X RPMI 1640 to 9 volumes of stock Percoll to make a 90% Percoll isotonic solution.
2) From the 90% Percoll isotonic solution, make dilutions of 80%, 65%, 50%, and 35% Percoll with 1X ICM and allow these solutions to equilibrate to room temperature prior to use.
3) Prepare a discontinuous gradient by carefully and successively layering 2.0 ml of each Percoll dilution in a tilted 15ml centrifuge tube, starting with the highest Percoll concentration.
4) Remove as much as spent medium from a mature gametocyte culture (16 day-old) as possible and transfer the infected red blood cells to 15 ml (for few flasks) or 50 ml tubes (for more than 3 flasks).
5) Rinse flasks with sterile warm PBS or RPMI and transfer the suspension to the same above tubes.
6) Centrifuge the tubes at 1,500 g for 5 min at 37°C.
7) Remove the supernatant and Rinse the pellet with pre-warmed RPMI. Repeat step 6.
8) Remove the supernatant and resuspend the pellet with warm RPMI, and make the final hematocrit <= 50% (20-30% is better).
9) Carefully lay 2 ml of gametocyte infected erythrocytes on top of the already prepared discontinuous Percoll gradient and centrifuge at 1,750 g for 10 min with the break off.
10) Carefully remove the resultant bands of parasites at each interphase (the 35/50% Percoll interphase contains mainly gametocytes; 50/65% interphase contains some gametocytes within red blood cells) using a Pasteur pipette and place in separate centrifuge tubes.
11) Add 10 volumes of RPMI to wash the gametocyte by centrifuge at 1,500 g for 5 min twice to remove any traces of Percoll.
12) Spot a little amount of each preparation (~ 1 µl) to make a smear, stain and examine under a microscope in order to ascertain the composition of each Percoll fraction.
13) Proceed to use the preparations or freeze at –70°C.
Appendix B: Transfection of *P. falciparum*

1. Transfection of *P. falciparum*

**Preparation of DNA for transfection**
1) Better use human *dihydrofolate reductase (hdhfr)* as selectable marker rather than *Tgdhfr* from *Toxoplasma gondii*. The resultant construct should be prepared by maxiprep using the Qiagen Maxi prep Kit (Qiagen, CA). Six hundred milliliters of LB broth should be enough. We usually prepare 1 liter using 1 column.
2) Recheck the insert with restriction enzyme digestion. Sometimes, the maxiprep will result in a different physical map, which may affect the efficiency of transfection.
3) After confirmation of the restriction sites and the insert, Dissolve the pellet thoroughly in 200-300 µl of TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA).
4) Measure the concentration of the DNA with SmartSpec 3000 (BioRad, CA).
5) Aliquot DNA to 1.7 ml eppendorf tubes with 60 µg each tube.
6) Add 1/10 volume of 3 M sodium chloride and 2 volume of absolute ethanol. Keep the tubes in −20ºC until needed for transfection.
7) Before transfection, remove 1 tube from the freezer and centrifuge to recover DNA.
8) Discard the supernatant.
9) Wash the pellet thoroughly with 75% ethanol.

The following steps should be preceded in the laminar hood to keep the DNA sterile.
10) Remove the spent 75% ethanol in the hood and let the pellet dry in the hood. Do not be too dry; otherwise it is very hard to be dissolved.
11) Redissolve the pellet thoroughly in 400 ul of Cytomix (120 mM KCl, 0.15 mM CaCl₂, 2 mM EGTA, 5 mM MgCl₂, 10 mM K₂HPO₄/KH₂PO₄, 25 mM Hepes pH 7.6). It usually takes several hours. Better prepare DNA a day ahead. The dissolved DNA can be stored at 4ºC for 1 week.

**Preparation of parasites for transfection**
1) Thaw a new ampoule of parasites from liquid nitrogen with very fresh human erythrocytes.
2) If the parasites grow well and get high parasitemia (~5%) and over 70% are ring stages within 1 week, then the culture can be used for transfection without synchronization.
3) If the parasites can not get a high parasitemia within a week, synchronization may be needed since ring stages exist but usually less than 50% the parasite population.
4) Synchronize *P. falciparum* parasites at approximately 1% ring stages using 5% sorbitol two days before transfection.
5) On the day of transfection, parasites should be 3-5% parasitemia with majority of ring stages. Too high, parasites will crash within 2 days; too low, the efficiency will be significantly affected.
6) Use 5 ml of culture at 5% hematocrit for each transfection.

**Electroporation and plating**
1) Centrifuge 5 ml of culture at 1,500 g for 5 min and remove supernatant. Better keep the pellet less than 200 µl.
2) Add the cytomix/plasmid mixture to the parasitised erythrocyte pellet and pipette up and down gently to mix.
3) Transfer the parasitised erythrocyte/DNA mixture to a GenePulser (BioRad, CA) cuvette (0.2 cm gap). Electroporate at 0.310 kv and 950 µF. The resulting time constant should be between 7 and 12 msec.
4) Immediately add the electroporated sample to a labeled 25 cm³ flask to final 5 ml culture at 5% hematocrit. Use fresh made complete media either with human sera or albumax. Gas parasite with 5% O₂, 2% CO₂, 93% nitrogen.
5) Change media next day.

**Drug selection**
1) Two days post-transfection ensure that parasitemia is **no higher than** 5% by diluting or discarding part of it.
2) At day 2, add WR99210 to 2.5 nM for *hdhfr* or 100 ng/ml of pyrimethamine for *Tgdhfr*.
3) Fresh media and the appropriate drug are added to culture for a further 48 hrs. For further drug selection, keep same concentration for WR99210 for *hdhfr*, while lower
pyrimethamine to 20 ng/ml for Tgdhfr.

4) After another change, the media can be changed every 3 days until resistant parasites establish.
5) Add fresh RBCs (~ 100 µl) every 5-7 days.
6) Usually parasites appear in two weeks, but it may take shorter (about 7 days) or much longer (a few months).
7) Upon parasites appear; make stocks as soon as possible. Grow the parasite under drug selection for 2 or 3 more cycles to assure the parasites grow well.
8) Remove drug for 3-4 weeks. Make stocks twice each week.
9) Reapply drug for selection. Use same concentration (2.5 nM) for WR99210, or 500 ng/ml for pyrimethamine. Resistant parasites should again appear about 2 weeks later.
10) Make stocks for reappear parasites and keep drugs for 1 or 2 more weeks according to the growth.
11) If there is no parasite after 3-4 weeks, restart the parasites from the latest stocks.
12) Continue two more drug off-on cycles or until no more death observed after addition of drug.
13) Check integration with PCR or Southern blot during cycles. When integrated parasite population is over 70%, it is ready for single parasite cloning.

2. Cloning of parasites
1) Do thin smear of culture to count parasitemia.
2) Change medium in stock dish, then resuspend culture & put in a 10ml tube.
3) Add 2 µl to 38 µl medium in an Eppi & count RBC on haemocytometer (x40 magnification). From the central 25 squares count 5 squares including RBC within the triple lines on 3 sides only. Calculate average count for 1 square, and then multiply by 25x20. This takes into account the numbers for the full 25 squares & the dilution factor of 20. Then multiply by 10000 to get the number of RBC/ml.
4) Add 10^6 cells to 10ml medium without RBC (usually this is 2 µl neat culture which is at ~5 x 10^8 cells/ml = 5 x 10^5/µl).

NB Always use round-bottomed microtitre plates for cloning.
5) Make required vol. of medium including 2% RBC (~10 ml/microtitre plate).
6) Add 300 µl (for 3 parasites/well) or 100 µl (for 1 parasite/well) to X x 10ml of 2% haematocrit medium, where X= % parasitaemia, and 10ml=Volume of 96 well tray (100 µl/well).

NB Scale down accordingly if want less than X x 10 ml of medium (usually only need 10 ml for 1 plate).

NB Use multipipette (8 or 12-channel) that can utilize normal autoclaved Media tips.

7) Fill the outside wells of each plate with 100 µl sterile PBS. This leaves only 60 wells for parasite clones.
8) Mix the diluted parasites then pour into sterile petri dish. Using multichannel pipette, add 100ul to each of the 60 wells.

9) Plates are set up without Pyrimethamine for 12 days & are fed every 6-7 days.

NB Need a plastic 500 ml or 1L beaker for removing tips used for feeding clones. For medium removal set multipipette to 90 µl & use new tips for each well. For medium addition set multipipette to 100 µl. You can feed whole plate with same tips.

10) Pyrimethamine is added at Day 12. Clones should appear ~Day 14 and Smear each well showing a color change by taking 1 µl RBC to a slide & smearing. Color change is not always reliable. Check smears under microscope to make sure the existence of live parasites.
11) After reading smears, transfer the parasitised erythrocytes from positive wells into 24-well plate of 4% haematocrit medium in total of 1 ml culture.

12) Smear after 5-7 days according to the initial parasitemia by taking 1µl RBC to slide. When parasitemia is >1%, change the media and transfer the parasite to a 25 cm³ flasks with 5% hematocrit in 5 ml of culture.
13) Make stocks if possible and extract DNA for further confirmation.
Appendix C: Phenotypic analysis

1. Asexual parasite growth curve and gametocytogenesis

1) Synchronize parasites twice in two consecutive life cycles in early ring stages.
2) Make a thin smear after second synchronization.
3) Count ring stage-parasites under a microscope to determine parasitemia.
3) Dilute it to triplicates of a 24-well plate at final 0.5% parasitemia in 1 ml of culture. Keep the original flasks. This is Day 1.
4) Next day count trophozoites again to reassure the parasitemia. Adjust accordingly by further diluting or get more from the original culture. The numbers can be used for Day 2.
5) The plate is then incubated in a candle jar.
6) Change media daily and make thin smears meanwhile.
7) For asexual growth, make smears from day 1 through 7.
8) For gametocytogenesis, the parasite culture needs to be maintained until mature gametocytes (stage V) appear (about 14-16 days).
9) The sex ratio of macrogametocytes (female) to microgametocytes (male) is determined by counting at least 500 mature gametocytes or 30 fields per sample.

Reference:

2. Exflagellation

1) Collect mature gametocytes from culture day 16 or older to a 15 ml centrifuge tube.
2) Centrifuge it at 37°C (or over 30°C) at 400 g for 5 min.
3) Remove the supernatant and wash twice with warmed PBS.
4) Resuspended the gametocytes pellet in warmed PBS at a final of 50% hematocrit.
5) Pipette 14 µl of suspension and drop it on a slide with 6 µl of 5 µM of xanthurenic acid.

6) Disperse the mixture into a contiguous erythrocytic layer under the cover slip of a hematometre at room temperature.

7) The mobile exflagellation centers were observed under a microscope about 5 to 10 minutes later with a decrease of temperature.

8) Count ten random fields and then standardize exflagellation centers by dividing it by mean gametocytemia in triplicates.

References:

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ACADEMIC PREPARATION

8/2000 to date Ph.D. Candidate, Department of Entomology, The Pennsylvania State University, University Park, PA, 16802, USA

Concentrations: Determination and characterization of a gene, PfPuf2, important for Plasmodium falciparum sexual development using molecular biological techniques and targeted gene disruption

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10/1998-4/2000 Master of Medicine, Department of Etiology, Peking Union Medical College & Chinese Academy of Medical Sciences (PUMC&CAMS), Beijing, P.R. China

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HONORS AND AWARDS

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6/2003-8/2003 Graduate Student Competitive Grant Program, College of Agriculture Sciences, PSU

4/2002 Participant of international workshop on malaria transfection in New Delhi, India

Practice on transfection lab and attendance of all scientific seminars and technique lectures related to malaria transfection

MEMBERSHIP IN SCIENTIFIC SOCIETY

6/2002 to date Membership of American Society of Tropical Medicine and Hygiene (ASTMH)

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PRESENTATIONS

9/2004 Molecular parasitology meeting, Woods hole, MA

Poster: PfPuf2, a sexual stage specific protein in the malaria parasite, Plasmodium falciparum.

11/2004 American Society of Tropical Medicine and Hygiene meeting, Miami, FL

Presentation: PfPuf2, a sexual stage specific protein in the malaria parasite, Plasmodium falciparum.

11/2002 American Society of Tropical Medicine and Hygiene meeting, Denver, CO

Poster: Characterization of the clathrin gene from the Plasmodium falciparum malaria parasite.

PUBLICATIONS AND MEETING ABSTRACTS


