The DNA replication apparatus in *Plasmodium falciparum* gametocytes

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I hereby declare that I alone have composed this thesis, and that, except where stated, the work is my own.

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ABSTRACT

DNA synthesis in gametocytes takes place at two points; at the onset of gametocytogenesis where the nucleic acid content increases from 1c to 1.8c prior to the formation of stage I gametocytes, and again 10 to 12 days later at the onset of gametogenesis when the mature male gametocyte (stage V) replicates its genome three times leading to the formation of eight haploid male gametes in less than 10 minutes.

The aim of this study was to evaluate the status of the *P. falciparum* DNA replication apparatus during gametocytogenesis, most of which no significant DNA synthesis takes place, and gametogenesis in which a sudden burst of DNA replication takes place. The proteins studied in this project were DNA topoisomerase I and II (TopoI and II), Replication factor C (Rfc) and Proliferating cell nuclear antigen (Pcna) of which *P. falciparum* homologous have been previously identified, isolated and characterised.

Standard indirect immunofluorescence assays (IFA) carried out on unsynchronised *in vitro* cultivated *P. falciparum* (3D7A) using rabbit polyclonal antiserum raised against recombinant PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopoII showed that all five proteins are present throughout gametocytogenesis. All five proteins appear to be predominantly located within the nuclear region and at significantly higher levels in stage I and V gametocytes. However PfRfc2 levels appeared to be significantly higher only in stage I gametocytes and was distinctly absent from the nucleus of stage V gametocytes. Western blot analysis showed no significant changes in the levels of these proteins occurred during gametogenesis, with the exception of PfRfc2, which appear to increase immediately after activation and then gradually decrease as gametogenesis progressed.

RT-PCR detected the presence of PfRFC2, PfRFC3, PfPCNA, PfDNA POLδ and PfTOPO I transcripts in mature gametocytes before and after activation. However, PfRFC1 and PfTOPO II transcripts were not detected in mature gametocytes either before or after activation. Similar results in protein and RNA analysis were obtained whether gametocytes were grown in AlbuMax or serum supplemented medium. *In situ* hybridisation using fluorescein-labelled PfRFC1, PfRFC2, PfDNA POLδ, PfTOPO I and PfTOPO II gene fragments showed pockets of fluorescence on the peripheral regions of schizonts away from the nuclear region stained by DAPI. PfRFC3 and PfPCNA probes appeared to show fluorescence emanating from the nuclear region of schizonts.

In conclusion, the localisation and unique expression pattern of PfRfc2 observed before and during gametogenesis, from that of the other DNA replication proteins and in particular, PfRfc1 and PfRfc3, appears to imply a significant role for PfRfc2. Further studies need to be carried out in order to get to a better understanding of the role of PfRfc2 during gametogenesis.

CONTENTS

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Dedication

I dedicate this thesis to my loving wife Cirû

and our two little jewels,

Kûi and Mathu.

God Bless.

Abbreviations

µg	Microgram
µl	Microlitre
µM	Micromolar
A	Adenosine
APB	Alkaline phosphatase buffer
APES	3-Amino-propyl-triethoxysilane
ATG	Translation start codon
ATP	Adenosine triphosphate
BB	Blocking Buffer
BCIP	Bromochloroindolyl phosphate
BSA	Bovine serum albumin
C	Cytosine
CCC	Closed covalent circular
CCMA	Complete culture medium with AlbuMaxII
CCMS	Complete culture medium with serum
CDKs	Cyclin-dependent kinases
cDNA	complementary DNA
cm^2	square centimetre
COM	Centres of movement
CTP	Cytidine triphosphate
DDT	Dichlorodiphenyltrichloroethane
DAPI	4', 6-Diamino-2-phenylindoledihydrochloride
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DEPC	Diethylpyrocarbonate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenediamine tetraacetic acid
F1	Percoll Fraction 1
F2	Percoll Fraction 2
F3	Percoll Fraction 3
F4	Percoll Fraction 4
FITC	Fluorescein isothiocynate
G	Guanosine
GLS	Gametocyte lysis solution
GSM	Gametogenesis stimulating medium
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid

ICM	Incomplete culture medium
ICMB	Institute of Cell and Molecular Biology
iDNA	Initiator DNA
IFA	Indirect immunofluorescence assay
kb	Kilobase
kDa	Kilo Daltons
kDNA	Kinetoplast DNA
LB	Lauria Bertani medium
M	Molar
MAG	Mature activated gametocytes
MCS	Multiple cloning site
MIC	Microns
MIG	Mature inactivated gametocytes
ml	Millilitres
mM	Millimolar
MOPS	Morpholinepropanesulphonic acid
mRNA	messenger ribonucleic acid
NBT	Nitro blue tetrazolium
°C	Degrees centigrade
OC	Open circular
ORF	Open reading frame
p	Prefix for plasmid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen gene
Pcna	Proliferating cell nuclear antigen protein
PCR	Polymerase chain reaction
Pf	*Plasmodium falciparum*
PMSF	Phenylmethylsulphonyl fluoride
Polα	Polymerase alpha protein
POLα	Polymerase alpha gene
POLδ	Polymerase delta gene
Polδ	Polymerase delta protein
RBC	Red blood cell
RFC	Replication factor C gene
Rfc	Replication factor C protein
RNA	Ribonucleic acid
RNase	Ribonuclease
RPMI	Rosewell Memorial Park Institute
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SB	Sample buffer
sdH_2O	sterile distilled water
SDS	Sodium dodecyl sulphate

SP	Sample protein
SSC	Standard saline citrate
T	Thymine
TAA	Translation stop codon
TB	Transfer buffer
TBE	Tris borate EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween 20
TEMED	N,N,N',N'-Tetramethylethylenediamine
TOPO I	Topoisomerase I gene
TOPO II	Topoisomerase II gene
TopoI	Topoisomerase I protein
TopoII	Topoisomerase II protein
TPCK	Tosyl-L-phenylalanine chloromethyl ketone
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCL	Tris hydrochloric acid
TRITC	Tetramethylrhodamine isothiocynate
UTP	Uridine triphosphate
UV	Ultraviolet (light)
v/v	volume per volume
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Malaria

1.1.1 Malaria the disease

Malaria is an infectious disease caused by parasitic protozoa of the genus *Plasmodium* and is transmitted to the human host through the bite of an infected female *Anopheles* mosquito. There are many species of *Plasmodium*, some of which infect reptiles, birds, rodents, and primates. There are four species of *Plasmodium* that infect man; *P. falciparum, P. vivax, P. malariae*, and *P. ovale. P. falciparum* is the most widely distributed in the world and also the most virulent species of *Plasmodium*. It is potentially lethal to humans because it causes the most dangerous complications, such as encephalopathy, renal failure and severe haemolytic anaemia leading to coma and death within a few days.

The vast majority of *P. falciparum* malaria occurs in sub-Saharan Africa. 300 to 500 million new infections and 2 to 4 million deaths annually attributed to malaria occur in residents of malaria endemic countries, the majority in sub-Saharan Africa alone where it is estimated that 90% of the infected people live (WHO, 1994; Anon, 1996). The primary population at risk is young children in sub-Saharan Africa. In western Kenya, for example, greater than 95% of children under the age of 5 years have parasites in their bloodstream during much of the year and an estimated 26,000 children die of malaria in Kenya every year which translates to 72 deaths a day (Snow *et al.*, 1999). Across the whole of Africa alone, refined figures suggest that a fraction under a million people died of malaria in 1995, of which 750,000 were children (Bloland *et al.,* 1996). Pregnant women are also at risk since the disease is responsible for a substantial number of miscarriages and low birth weight babies. *P. vivax* is also a common cause of human malaria, especially in South East Asia and in Central and South America. Though *P. vivax* rarely causes death, some patients with this form of malaria may develop severe clinical symptoms like anaemia (Tan-ariya *et al.,* 1996).

1.1.2 Malaria in history

Malaria is probably older than man is, so human *Plasmodium* species must have evolved with man in Africa and spread with human migration into Europe, the Middle East, Asia, India and China. The wide distribution of malaria is attributed to the adaptability and breeding of the *Anopheles* mosquito. Malaria control was practised long before the parasite was discovered. The Greeks and the Romans in classical times recommended that houses should be built in dry and elevated positions in order to avoid fever, and also the health benefits of draining marshes were known. Rome itself is a city on hills surrounded by the former marshes of the Campagna which were infested by mosquitoes that spread malaria to unsuspecting armies camped outside Rome and waiting to attack. Indeed, 'Mal'aria' means 'bad air' in Italian. It arose from the belief that malaria came from putrid air above stagnant water. It was not until 1880, that Alphonse Laveran, a young doctor in the French Army in Constantine, Algeria, discovered tiny microscopic parasites in the red blood cells of malaria patients and directly attributed them to being the cause of malaria. In 1897, Ronald Ross a Surgeon Major in the Indian Army in Secunderabad, India, found malaria parasites in mosquitoes. Ross declared that mosquitoes transmit malaria and received the Nobel Prize in 1902. It was after Ross's discovery of role mosquitoes in malaria transmission that malaria epidemiology became a science. Ross went on to make practical studies of malaria control and began the mathematical analysis of malaria transmission (Bruce-Chwatt, 1985).

1.1.3 Malaria eradication and control

In 1955, the Eighth World Health Assembly announced a programme for global malaria eradication. The eradication programmes were initiated in all malarious countries in the Americas and Europe, and in a majority of countries in Asia and Oceania. Only pilot projects were attempted in Africa. By 1970, malaria had been eliminated from the whole of North America, Europe, parts of Asia and South America, and several countries in the Middle East; this regions had 727 million

people now freed from malaria, and represented 53% of the originally malarious areas (Gilles and Warrell, 1995). However, seasonal and endemic malaria remained unchanged in tropical Africa where there was hardly any significant implementation of the malaria eradication programme. In 1969, the Malaria Eradication Programme was converted into a Malaria Control Program and consequently, the malaria situation greatly deteriorated. The resurgence of the disease affected several countries in southern Asia and Latin America and the number of reported cases of malaria (excluding Africa) had more than doubled by 1977. This was attributed to the reduction of malaria control activities in many countries as a result of the sudden withdrawal of external resources, which was then aggravated by the energy crisis during the 1970s and the subsequent economic strain felt by several countries. Resurgence of malaria was also due to an increase in parasite drug resistance, the rising costs of insecticides and the rapid increase in mosquito resistance to insecticides such as DDT and other organophosphates (Gilles and Warrell, 1995).

1.1.4 Malaria vaccines

The development of vaccines against malaria presents formidable obstacles in terms of parasite biology, mainly the immunogenicity and antigenic variability of some malarial antigens, combined with the fact that some host immune response are genetically restricted to certain parasite epitopes (Good *et al.*, 1998; Good and Doolan, 1999). The introduction of DNA vaccine technology has facilitated an unprecedented multi-antigen approach to developing an effective vaccine against *P. falciparum* (Watts and Kennedy, 1999). Though clinical trials of DNA vaccines in healthy human volunteers have shown them to be safe and well tolerated at all doses, the frequency and magnitude of the immune response they elicit was found to be suboptimal (Le *et al.*, 2000). Considerable research efforts are now geared towards immune enhancement strategies to improve DNA vaccines; such as the prime/boost immunisation method where the immune system is primed with a DNA vaccine and boosted with poxvirus or recombinant protein in the adjuvant. The prime/boost

strategy administered in the right order, leads to very high levels of antigen-specific cytotoxic T cells. In parallel, malaria vaccine research aims to capitalise on the data obtained from the malaria genome project (Gardener *et al.*, 1998) coupled with the recent advances in gene expression, proteomics and molecular immunology technologies (Hoffman *et al.*, 2000; Hoffmann, 2000; Carucci, 2001) to come up with a multi-valent, multi-stage and multi-immune response vaccine. Such a vaccine will, hopefully, provide high-level, long-term, sustainable protection against malaria parasites (Doolan and Hoffman, 2001).

1.1.5 Malarial chemotherapy

The spread of drug-resistant strains of *P. falciparum* since the 1960s is a major public health challenge and the principal reason for the erosion of efficacious treatments. The efficacy of chloroquine, which for several decades was a cheap, highly effective, convenient, and relatively safe drug for the chemoprophylaxis and treatment of malaria, has reduced dramatically (Gosh *et al*, 1992). The widespread use of antimalarial drugs for prophylaxis with the inevitably poor compliance and resulting underdosing has provided the selection pressure for the emergence of drug resistance parasites. Cost and the limited number of antimalarial drugs in current use impose considerable constraints on malaria control, especially in sub-Saharan Africa. In addition, of the new antimalarial drugs available, none match the safety, breadth of activity and speed of action exhibited by chloroquine when it was first introduced in the 1940s. It is against this background that the need to identify new chemotherapeutic targets in order to stop the proliferation and spread of malaria has gathered momentum over the past 20 years. Hope now lies on the multidisciplinary research on artemisinin-based combination therapy, which offers a new and potentially highly effective way to prevent or retard the development of drug resistance (Price *et al.*, 1996).

1.2 *Plasmodium falciparum*

1.2.1 Life cycle of *P. falciparum*

Plasmodium has a split life cycle, one in the human host and another in the female *Anopheles* mosquito. The entire life cycle can be better understood as a sequence of four phases: three asexual phases with multiplication and one sexual phase without multiplication (Figure 1.1).

1.2.1.1 Hepatic schizogony

Infection begins when haploid sporozoites in the salivary glands of an infected female *Anopheles* mosquito are inoculated into the bloodstream of the mammalian host during the feeding of the mosquito. The sporozoites remain in the blood for about 30 minutes during which time many are destroyed by phagocytes. However, some sporozoites invade the liver parenchymal cells and each transform into a round hepatic trophozoite. This is the second growth form of the parasite. Within the warm and nutrient-rich hepatocyte, the trophozoite grows rapidly distending and destroying the host cell as it enlarges. The hepatic trophozoite undergoes multiple cell divisions in a process known as pre-erythrocytic or hepatic schizogony and becomes a multinucleated hepatic schizont. After 6 to 16 days from the time of infection, the hepatic schizont bursts, releasing 10-30,000 invasive merozoites embedded in fragments of degenerate cytoplasm into the surrounding tissue and thence into the blood circulation. The masses are too large to be engulfed by Kupffer cells and presumably disintegrate after leaving the liver, freeing the merozoites. Until this time, the infected person has no symptoms and is unaware that infection is progressing. This is called the incubation period of infection and includes the whole of hepatic schizogony and the first few cycles of erythrocytic schizogony.

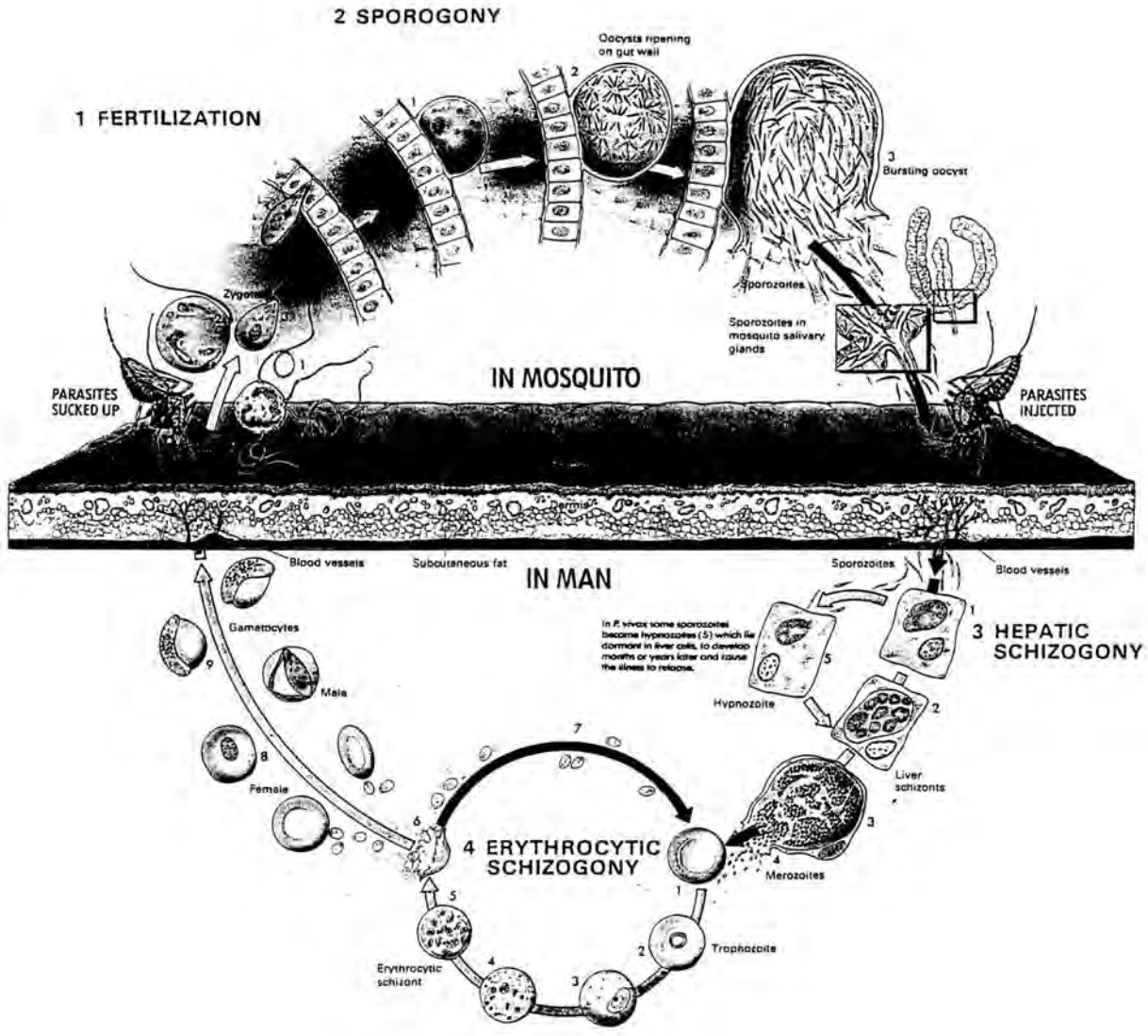
Some sporozoites of *Plasmodium vivax* upon invading liver hepatocytes do not develop immediately but instead become tiny dormant parasites called hypnozoites. These delay development for months or a few years and when they resume development cause the delayed relapses typical of *vivax* malaria. *P. malariae* may

also cause relapses sometimes twenty years after the first infection, but the resting form of this species remains obscure.

1.2.1.2 Erythrocytic schizogony

The merozoites are the smallest and shortest-lived form in the life cycle. Upon release, they immediately invade circulating red blood cells (erythrocytes) and develop into small circular forms known as rings. Antibodies present in the blood of partially immune individuals may, at this point, prevent invasion of the erythrocytes by the merozoites by causing mature merozoites to cluster. Each ring-form of the parasite grows in size as it ingests erythrocyte haemoglobin and cytoplasm, becoming irregular in shape when it is referred to as an erythrocytic trophozoite, the third growth form of the parasite. As haemoglobin is digested, an iron-haem pigment called haemozoin (often referred to as 'malaria pigment') accumulates in food vacuoles in the form of crystals. After 30-40 hours of growth, the trophozoite undergoes multiple cell divisions resulting in the formation of an intraerythrocytic schizont comprising between 8-32 merozoites in a process referred to as intraerythrocytic schizogony. The schizont bursts releasing the merozoites into the blood stream which then invade new erythrocytes and begin another cycle. This erythrocytic cycle of schizogony is repeated several times in the course of an infection, leading to progressive increase of parasitemia, hence illness, until the process is slowed down by the immune response of the host. It is these repeated asexual cycles in the blood that cause the relapsing fevers unique to malaria. The fever are induced by pyrogens released during the rupture of schizonts and are thought to cause transient suppression of the host immunity at the time of merozoite release, the period at which the parasite is most vulnerable to the host defences (Gilles and Warrell, 1995). After several asexual cycles, some merozoites invade erythrocytes and develop into either male or female sexual forms (gametocytes).

Figure 1.1 The life cycle of *Plasmodium falciparum* (Trustees of the Wellcome Trust)



1.2.1.3 The sexual phase

The gametocytes, in the human host, take between 8-10 days to mature, a process known as gametocytogenesis. The mature gametocytes taken up by a feeding anopheline mosquito, are the only parasite forms able to survive in the mosquito midgut. Within the mosquito midgut, both male (micro-) and female (macro-) gametocytes transform into gametes and emerge from the erythrocytes. The male parasites undergo rapid and repeated DNA replication to form eight haploid microgametes and show violent internal activity, as up to 8 'flagella' form within each cell and start to beat. As the formation of the flagella is completed their motion tears the cell apart, releasing them into the plasma of the blood meal. Each flagellum has a nucleus attached and swims in search of female gametes, which upon fertilisation transform into zygotes. The newly formed diploid zygote undergoes immediate meiotic division to re-establish the haploid genome (Sinden and Hartley, 1985; Sinden *et al.*, 1985; Ranford-Cartwright *et al.*, 1993). The zygote matures in the next few hours into a motile ookinete. The ookinete is a motile form, which glides through the blood meal until it reaches the stomach wall, where it passes between or through the epithelial cells to reach the basement membrane. Here it rounds up, loses the features of an invasive form, the apical complex, and transforms into an oocyst and the next phase of sporogony begins. Sexual development within the mosquito is completed quickly, in about 30 minutes. This presumably protects the parasite from the lethal mosquito proteases that are secreted into the blood as early as 5 hours after feeding (Briegel and Lea, 1975).

1.2.1.4 Sporogony

The oocyst, enclosed in a cyst wall formed of proteins secreted by the parasite and the basement membrane of the epithelial cells, grows rapidly and projects into the body cavity (haemocoel) of the mosquito. When mature, the oocyst is spherical, has a diameter of 80 microns and can be seen with a dissecting microscope. The developing oocyst feeds on haemoglobin in the blood meal and accumulates the black

malaria pigment, also seen in erythrocytic trophozoites. After about seven days or more (depending on the ambient temperature), the oocyst begins a process of internal division, with the formation of thousands of worm-shaped sporozoites, the next invasive form in the life cycle. The oocyst bursts fourteen or more days after infection of the mosquito and the sporozoites migrate through the haemocoel and invade the salivary glands. When the mosquito feeds again on a human host, she injects saliva, containing sporozoites, into the blood. Within 30 minutes, the sporozoites invade the liver cells of the human host and start a new life cycle (Gilles and Warrell, 1995).

1.3 Gametocytogenesis

1.3.1 Gametocyte development

The production of morphologically and physiologically mature *P. falciparum* gametocytes takes 8-10 days *in vivo* (Smalley, 1977) and 14-23 days *in vitro* (Carter and Beach, 1978) with identical morphological sequences during gametocytogenesis (Hawking *et al.,* 1971; Smalley, 1977; Jensen, 1979). The first definitive sign of sexual differentiation of *P. falciparum* is the appearance, *de novo*, of sub-pellicular cytoskeleton, that is, the inner pellicular membrane vesicle and microtubules (Sinden and Smalley, 1979). The assembly and loss of these components of the cytoskeleton cause gametocytes to acquire numerous characteristic changes in shape during gametocytogenesis. Developing gametocyte-infected erythrocytes sequester from the peripheral circulation to the blood spaces of the bone marrow and the spleen (Garnham, 1966; Smalley *et al.,* 1981). It is in these organs that gametocytes pass through a series of fairly distinct morphological stages, categorised I-IV. This takes 8-10 days, after which 'mature' gametocytes are released into the circulation and, after a further 2-3 days, become infectious to the mosquito (stage V).

Stage I gametocytes are round and difficult to distinguish from young trophozoites. Stage II gametocytes, produced after two days of growth, are oat grain-shaped. A new subpellicular cytoskeleton is laid down and subtended by a few microtubules

and results in the formation of the stage III gametocytes that have a characteristic elongated shape. It is at this point during gametocytogenesis (Stage III) that male and female gametocytes can be distinguished from each other. The subpellicular cytoskeleton completely encircles the gametocytes leading to the formation of symmetrical stage IV gametocytes. Sexual dimorphism is further accentuated in stage IV gametocytes, with a marked increase in the density of the ribosomes, endoplasmic reticulum, Golgi vessels and mitochondria in the macrogametocyte relative to the microgametocyte. This clearly reflects the subsequent development of the macrogametocyte as an unfertilised egg. The collapse of the pointed spindle shape of stage IV gametocytes leads to the formation of the crescent-shaped and morphologically mature stage V gametocytes (Sinden, 1982).

Gametocytes are relatively metabolically inactive, a fact that renders many anti-malarial drugs ineffective. Although gametocytes do not directly cause any clinical symptoms in humans, they are the clinical stages responsible for the continued transmission of parasites from one vertebrate host to another via a complex sexual life cycle in the mosquito vector. The process of sexual differentiation and development therefore plays a central role in the life cycle of the human malaria parasite.

1.3.2 Commitment to sexual differentiation

Factors that determine whether a merozoite differentiates into a sexual or asexual parasite upon infecting an erythrocyte remain unclear. However, the progeny of an individual schizont can either commit to asexual or sexual pathways upon re-invasion. In order to get a better understanding of parasite differentiation, *P. falciparum* schizonts, suitably diluted, were allowed to burst on monolayers of immobilised uninfected erythrocytes in order to produce separated clusters of synchronously growing sibling parasites and their development followed (Inselburg, 1983). This pioneering work by Inselburg (1983) observed, by morphological examination, that cohorts of siblings from an individual schizont had a marked

tendency to produce merozoites committed to the same developmental pathway, either asexual or sexual. Later studies, using monoclonal antibodies to distinguish between the developing sexual and asexual parasites, indicated that the progeny of individual schizonts were labelled by only one or the other monoclonal antibody (Bruce *et al.*, 1990). The marked tendency observed by Inselburg was therefore an absolute commitment of each schizont to produce a progeny of parasites with the same developmental fate, developmental choice made in the parent schizont (Bruce *et al.*, 1990).

Gametocytes exist as male (microgametocytes) or female (macrogametocytes), the precursors of male and female gametes, respectively. Until recently, gametocyte sexes were only distinguishable by morphological examination of stage IV gametocytes (Jensen, 1979). However, studies on gametocyte sex-specific gene expression have shown that micro- and macrogametocytes of *P. falciparum*, each express some proteins in a sex-specific manner. Microgametocytes express alpha-tubulin II (Rawlings *et al.*, 1992; Guinet *et al.*, 1996), where it is proposed to have a specific role in exflagellation and motility. Macrogametocytes express *Pf77* transcripts (Baker *et al.*, 1995) and, recently, the osmiophilic body protein, Pfg 377 (Severini *et al.*, 1999). Antibodies against these sex-specific proteins are valuable tools in the study of sexual dimorphism in *P. falciparum*.

Conditions for parasite growth on immobilised erythrocyte monolayers have been improved to allow for the maturation of gametocytes to stage IV (Williams, 1999) and recently, this method was used to address the question of sexual dimorphism; analysing the sex of gametocytes arising from sexually committed clusters. The use of gametocyte sex-specific markers, anti-Pfg377 and anti-alpha-tubulin II antisera, to label clusters of stage IV gametocytes arising from individual schizonts of *P. falciparum*, showed that individual schizonts commit to yield gametocytes of the same sex (Silvestrini *et al.*,2000). These observations led Silvestrini *et al.*, (2000) to suggest that commitment to sexual differentiation and the sex of the resulting gametocytes is determined prior to schizont maturation.

1.3.3 Factors that influence commitment to sexual differentiation

It has been generally believed that stress plays a great part in the switch from asexual replication to sexual differentiation of the parasite within the human host (Carter and Miller, 1979). Gametocyte production can be seen as a survival mechanism, adopted by the parasite, in response to retarded asexual growth in unfavourable conditions. The proportion of parasites that develop into gametocytes varies greatly during the course of natural infections in humans (Smalley *et al.*, 1981; Magesa *et al.*, 2000). *In vitro*, high *P. falciparum* parasite densities have been a shown to lead to a high proportion of merozoites transforming into gametocytes possibly due to the decreasing availability of nutrients or the progressive fall in pH of the medium. The addition or dilution of *in vitro* cultures of *P. falciparum* with fresh erythrocytes to dramatically decreased the proportion of parasites that transform into gametocytes (Carter and Graves, 1988). It has also been observed that in blood cultures of *P. falciparum*, the conversion to gametocytes was stimulated by the presence of lymphocytes and serum from individuals with acquired immunity to *P. falciparum*. This observation suggests that the rate of conversion of asexual parasites to gametocytes may be increased in immune individuals (Smalley and Brown, 1981). Similar results were obtained with *P. falciparum* grown in culture medium to which sera containing anti-malarial antibodies was added (Brockelman, 1979) or in a culture medium supplemented with supernatants from hybridoma cell cultures containing anti-malarial antibodies (Ono, *et al.*, 1986). Anti-malarial drugs have also been shown to increase gametocyte production; chloroquine-induced enhancement of gametocytes production has been reported *in vivo* in *P. chabaudi* (Buckling *et al.*, 1997) and *in vitro* in *P. falciparum* (Buckling *et al.*, 1999). This may help to explain the spread of drug-resistant genotypes of *P. falciparum*.

1.3.4 Genetic determinants of sexual differentiation

Experimental studies on the genetic determinants underlying gametocytogenesis have taken account of two well-established features of this phenomenon. First, different lines of parasites within a *Plasmodium* species differ markedly in their intrinsic capacity to produce gametocytes. Secondly, parasite lines propagated exclusively by asexual growth either *in vitro* or *in vivo* tend to progressively lose the capacity to produce gametocytes (Walliker, 1976; Teklehaimanot, *et al.,* 1987). Studies on mutants defective in gametocyte production have shed some light on the control of sexual differentiation in malaria parasites. Two *Plasmodium* chromosomes, 9 (Day *et al.*, 1993; Alano *et al.*, 1995) and 12 (Viadya *et al.*, 1995; Guinet *et al.*, 1996; Guinet and Wellems, 1997), have been identified as having putative genetic determinants governing sexual development.

1.3.5 Sexual-stage-specific gene expression

A variety of proteins specific for sexual-stage parasites have been identified in avian, rodent, and human malarias (Kaslow, 1990). Most of the research on the identification of sexual-stage-specific proteins has been driven by the need to identify novel targets for the induction of antibody-mediated transmission-blocking immunity. Most of these proteins are differentially expressed in various sub-stages of gametocyte development. In *P. falciparum*, Pfg27 is synthesised very early during gametocytogenesis and peaks in stage II gametocytes (Carter *et al.*, 1989; Lobo *et al.*, 1994). The transcription of the pfg27 gene is down regulated in the more mature gametocytes (stages III-V) (Alano *et al.*, 1991; 1996). Another protein known as Pfs16, an integral protein of the parasitophorous vacuole membrane, is produced in sexually committed ring-stage parasites immediately following the invasion of a red cell by a merozoite. It continues to be produced throughout gametocytogenesis and in macrogametes (Moelans *et al.*, 1991a;b; Dechering *et al.*, 1997). Pfs16 is therefore an excellent marker for sexual differentiation and development in *P. falciparum* especially since stage I gametocytes and trophozoites are morphologically

indistinguishable. In mature stage V gametocytes, Pfs230 and Pfs48/45 are synthesised and expressed on the surface of mature gametocytes (stage III-V) and gametes (Williamson *et al.*, 1996; Wizel and Kumar, 1991). Pfs25 is produced only after induction of gametogenesis within the mosquito midgut and is expressed on the surface of newly fertilised zygotes and developing ookinetes (Fries *et al.*, 1990).

1.4 Gametogenesis

Gametogenesis is a period of rapid cell division of the mature intraerythrocytic microgametocyte (male gametocyte) which occurs when it goes from the mammalian host to the mosquito gut and leads to the formation of several microgametes (male gametes) (Sinden *et al.*, 1978). Gametogenesis involves the simultaneous activation of several, apparently independent, cellular events, namely the disruption of the host cell membrane, release of the parasite, the reorganisation of the cytoplasmic structures involved in the formation of the male gametes, and lastly the replication of the genome of the male gametocyte to provide eight gametes. The last two events comprise the dynamic event classically referred to as exflagellation (Alano and Carter, 1990).

The events of gametogenesis are tightly regulated by environmental changes such as temperature and pH which are associated with the parasite's transfer to ambient conditions (Ogwang' *et al.*, 1993; Billker *et al.*, 1997). Factors in the mosquito are probably essential for gametogenesis during the natural transmission of the parasites. Induction of gametogenesis, *in vitro*, requires the simultaneous exposure to at least two stimuli; a drop in bloodmeal temperature to 5 degrees C below that of the vertebrate host, and a rise in pH from 7.4 to 8.0-8.2. However, *in vivo*, the mosquito bloodmeal has a pH of between 7.5 and 7.6. It is thought that, *in vivo*, the second inducer is present within the mosquito midgut that is able to overide the otherwise suboptimal pH within the mosquito midgut. Recently, xanthurenic acid, a side product of tryptophan metabolism, has been extracted from the mosquito midgut and

implicated as the signal that induces gametogenesis within the mosquito midgut (Garcia *et al.*, 1998; Billker *et al.*, 1998).

Both temperature and pH are critical in controlling the process of gametogenesis, though at otherwise permissive conditions of pH (pH 8.0 to 8.2), or in the presence of pharmacological activators of gametogenesis, gametocytes maintained at normal host body temperature do not undergo any of the processes of gametogenesis (Sinden, 1983; Ogwang' *et al.*, 1993). However, at a few degrees below body temperature, all three processes may be triggered. Exflagellation, and probably DNA synthesis, proceed at maximum rate, taking about ten minutes, at the highest permissive temperature; about 34°C for *P. falciparum* and about 38°C for *P. gallinaceum* (Carter and Graves, 1988).

1.5 Developmentally transcribed ribosomes

One of the remarkable features of *Plasmodium* parasites is the developmentally regulated ribosome characterised by transcription in a stage-specific manner of discrete repertoires of unlinked nuclear rRNA genes, now termed A-type (from Asexual) and S-type (from Sporozoite) (McCutchan *et al.*, 1995; Waters *et al.*, 1989; Waters, 1994; Li *et al.*, 1997). A third set of rRNA genes, the O-type, has been described in *P. vivax*, where expression is limited solely to developmental stages in the mosquito (Li *et al.*, 1997). In the human parasite, *P. falciparum*, the switch from A- to S-type transcription occurs during gametocytogenesis and zygote formation and involves accumulation of S-type rRNA precursor forms (Waters *et al.*, 1989).

To date, there has been no mRNA whose translation has been shown to be controlled by developmentally regulated ribosomes. However, the expression of the *P. berghei* 21-kDa ookinete surface protein (Pbs21) is regulated translationally in such a way that it might represent an example of control by developmentally regulated ribosomes. The mRNA for Pbs21 accumulates in the cytoplasm of female gametocytes, zygotes, and ookinetes, but the protein is translated only in the ookinetes (Paton *et al.*, 1993; Vervenne *et al.*, 1994). It is not known why the

translation of Pbs21 mRNA is repressed prior to the ookinete stage of parasite development, but it is possible that translation of Pbs21 mRNA is linked to the synthesis of the S rRNAs which begins in the ookinete (Waters *et al.*, 1997). It has not been reported yet whether the *P. falciparum* homologue of Pbs21 (Pfs28) also undergoes translational regulation.

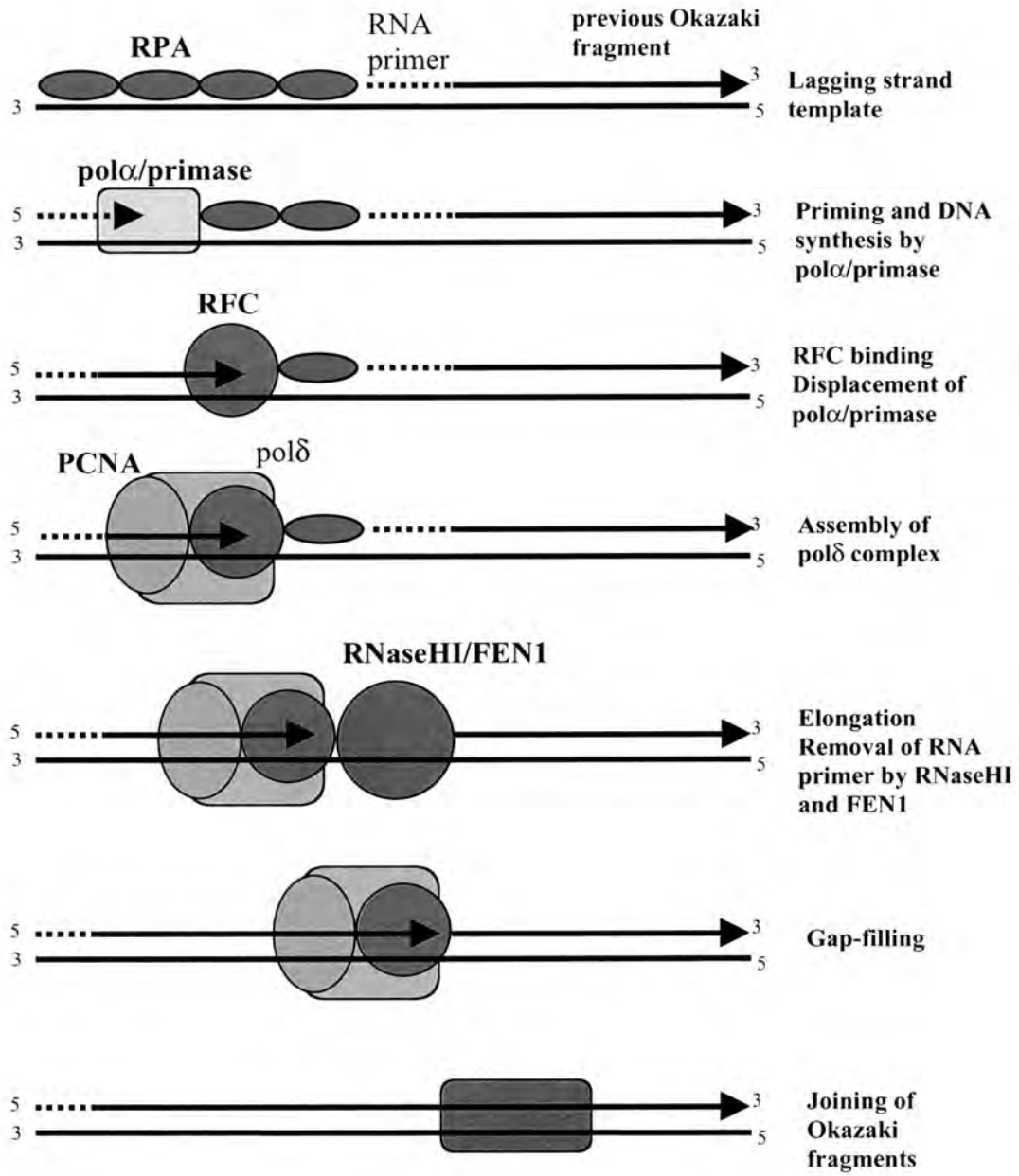
1.6 DNA replication in eukaryotes

To maintain euploid gene balance, DNA sequences must be replicated in every cell cycle but not more than once. Recent evidence indicates that cell cycle control of DNA replication is effected by a two-step mechanism (Diffley 1996). Origins first become competent to replicate by assembling proteins comprising pre-replication complexes onto chromatin in G1, and then later, during S, those origins initiate replication (Diffley et al. 1994; Bambara *et al.*, 1997). Replication from or through an origin dissociates functional pre-replication complexes. Once destroyed, these complexes cannot reassemble until the subsequent G1, thereby precluding re-firing of an origin in a single cycle (Wang, 1996; Waga and Stillman, 1998).

Knowledge of DNA replication in eukaryotes comes from extensive studies performed using cell extracts from mammalian cells that support the complete replication of plasmid DNA containing the Simian Virus 40 DNA replication origin (SV40 *ori*) (Li and Kelly, 1984;85; Stillman and Gluzman, 1985; Wobbe *et al.*, 1985). Replication of the SV40 DNA, a circular dsDNA virus with a single origin of replication, requires a single virus-encoded protein, the SV40 large tumour antigen (T antigen), which functions both as an initiator protein by binding to the SV40 *ori* and as a DNA helicase at the replication fork (Figure 1.2). Unwinding of the origin by large T antigen is stimulated by the ssDNA-binding protein known as replication protein A (RPA) that binds to single stranded DNA and promotes extensive unwinding of duplex DNA (Hubscher *et al.*, 1996; Wold, 1997). After unwinding, DNA polymerase alpha/primase complex (DNAPol alpha/primase) then loads onto the single stranded DNA and synthesises an RNA primer of approximately 10

nucleotides (nt) long then extends the primer by polymerisation to produce a short DNA extension of approximately 30 nt (Bullock *et al.*, 1994). The loading of DNAPol alpha /primase onto the DNA is called primosome assembly. Once the RNA-DNA primer is synthesised, a protein known as replication factor C (RFC), a complex of five subunits, binds to the 3'-end of the initiator DNA (iDNA) displacing DNAPol alpha/primase in an ATP-dependent process known as polymerase switching (Hubscher *et al.*, 1996). The binding of RFC to the iDNA triggers the assembly of the primer recognition complex, which is accomplished through the loading of another protein known as proliferating cell nuclear antigen (PCNA). PCNA forms a homotrimeric complex that is topologically linked to DNA by the RFC thereby allowing PCNA to track along the DNA (Tinker *et al.*, 1994). The current model suggests that RFC transiently opens the toroid structure of PCNA and then allows PCNA to re-close, encircling the double helix adjacent to the primer terminus (Tsurimoto and Stillman, 1990; Burgers and Yoder, 1993). PCNA subsequently associates with DNA polymerase delta (DNAPol delta) and acts as a sliding clamp holding the polymerase on the primer terminus. The clamped DNAPol delta is highly processive, adding thousands of nucleotides without dissociating (Waga *et al.*, 1994). For the initiation of leading-strand DNA replication, the DNA synthesis by the DNAPol delta/PCNA complex is processive and continuous, at least for 5-10 kb of DNA. For the lagging-strand, DNA synthesis continues until the polymerase encounters the previously synthesised Okazaki fragment. A complex processing reaction then removes the RNA primer from the preceding Okazaki fragment and the remaining nick is sealed by DNA ligase I (Murante *et al.*, 1998; Turchi *et al.*, 1994).

Figure 1.2 **Polymerase switching and maturation of Okazaki fragments on a lagging-strand DNA template (Waga and Stillman, 1998).**



1.7 DNA synthesis in *Plasmodium*

1.7.1 DNA synthesis in asexual parasites

The most extensive studies of DNA synthesis in malaria parasites have been carried out in rodent malaria parasites, especially *P. berghei.* These studies have included analyses of structure (Sinden *et al.*, 1985) and cytology (Canning and Sinden, 1975; Cornelissen *et al.*, 1984a;b; Janse *et al.*, 1986a;c) and the effects of DNA synthesis inhibitors (Janse *et al.*, 1986b;c; Toye *et al.*, 1977). During the life cycle of *Plasmodium*, DNA replication takes place at five points. The first takes place during exo-erythrocytic schizogony inside the liver hepatocytes of the vertebrate soon after sporozoite invasion. The second takes during intra-erythrocytic schizogony in the vertebrate blood. The third replication of DNA takes place during gametogenesis within the mosquito midgut and is followed by cytokinesis, fertilisation and formation of a diploid zygote. The fourth DNA replication takes place in the diploid zygote prior meiosis and leads to the formation of a haploid ookinete. Finally, the fifth DNA replication takes place in the oocysts during the formation of sporozoites (for review, see White and Kilbey, 1996).

1.7.2 DNA synthesis in sexual parasite

1.7.2.1 DNA synthesis during gametocytogenesis

DNA synthesis during sexual differentiation has been studied by the use of direct microfluorometry of Feulgen-pararosaniline stained parasites. In *P. berghei,* mature macrogametocytes and microgametocytes have been shown to have 1.4C and 1.8C respectively, of the haploid DNA (1C) content (Janse *et al.*, 1986a; Janse *et al.*, 1986b). In *P. falciparum*, the DNA content of Stage I gametocytes is about twice (1.8C) as high as that of haploid (1C) ring-forms implying DNA synthesis during transformation of the ring-forms into Stage I gametocytes (Janse *et al.*, 1988). In both cases, the increase in the DNA content at the onset of gametocytogenesis is probably as a consequence of partial replication or selectively amplification of some parts of their genome. Synthesis of long-lived mRNAs during gametocytogenesis and

their translation only during gametogenesis has been strongly suggested following studies using metabolic inhibitors of nucleic acid synthesis (Toye *et al.*, 1977). It is therefore possible that these long-lived mRNAs may account, in part, for the observed increase in the nucleic acid content in stage I and later in stage V gametocytes. Transcription in *P. falciparum* occurs in three compartments, the nucleus, the mitochondrion, and the plastid (Feagin and Drew, 1995; Lanzer *et al.*, 1992). Actinomycin D, a general inhibitor of RNA synthesis and presumably acting in all three compartments, effectively inhibits the growth of asexual erythrocytic stages and early gametocyte stages of *P. falciparum*, suggesting that RNA synthesis is essential in these parasite stages (Geary *et al.*, 1989).

1.7.2.2 DNA synthesis during gametogenesis

The synthesis of DNA, RNA and protein is essential for microgametogenesis as shown by the use of metabolic inhibitors (Toye *et al.*, 1977; Janse *et al.*, 1986b). Inhibitors of protein synthesis, specifically those affecting peptide elongation, like emetine, were found to effectively inhibit exflagellation. However, the partial inhibitory effects of actinomycin D, an inhibitor of DNA-directed RNA synthesis in both prokaryotes and eukaryotes, brought to light the possibility that transcription does occur to some extent during microgametogenesis and requires *de novo* synthesis of mRNA. Due to this partial inhibitory effect of actinomycin D on exflagellation, the existence of a long-lived mRNA, presynthesised in the maturing microgametocyte and translated only at the onset of exflagellation cannot be excluded. Colchicine, an antimitotic agent that acts by preventing microtubule assembly, was shown to effectively inhibit exflagellation. This suggests that a protein critical to the assembly of the mitotic spindle is synthesized or activated at the initiation of gametogenesis.

Studies on DNA synthesis in *Plasmodium* gametocytes showed that significant DNA synthesis occurs during gametogenesis (Janse *et al.*, 1986a;b). Aphidicolin, a proven specific inhibitor of eukaryotic DNA polymerases, was shown to inhibit gametogenesis in contrast to mitomycin C (Janse *et al.*, 1986b). These results

indicated that rapid and repeated aphidicolin-sensitive DNA synthesis does occur during microgametogenesis. Further studies on the cytofluorometric analysis of the DNA content of *P. falciparum* gametocytes during gametocytogenesis and gametogenesis established that micro- and macrogametocytes contain less than twice the amount of DNA of haploid sporozoites and rings-stages (Janse *et al.*, 1988). This suggests that DNA synthesis does occur to some extent during the transformation of ring-forms into stage I gametocytes.

Within 8-10 minutes of induction of microgametogenesis, three endomitotic nuclear divisions take place. These division are preceded by a very rapid and repeated replication of the entire parasite genome to 8C (Janse *et al.*, 1986a). It has been suggested that in order to achieve this rapid and repeated DNA replication, approximately 1300 replication forks are activated simultaneously in the mature gametocytes at the onset of microgametogenesis (Janse and Mons, 1987). The induction of so many independent replication forks at the onset of microgametogenesis itself not only suggests that mature microgametocytes are heavily precommitted to the rapid production of male gametes in the mosquito midgut but also that the induction mechanism is highly amplified within the parasite nucleus.

1.8 Genes involved in *P. falciparum* DNA replication

Several genes that code for proteins involved in DNA replication machinery in *P. falciparum* have been cloned and characterised. Most of these genes were identified by screening genomic and cDNA libraries with either oligonucleotides designed to conserved regions or heterologous screening with *Saccharomyces cerevisiae* gene fragments.

1.8.1 Replication factor C (RFC)

RFC is a complex of five-subunits of high sequence similarity that bind to the primer-template junction and load PCNA onto the DNA in the presence of ATP

(Cullman *et al.*, 1995). All five subunits have been identified in both humans and yeast. In yeast, the five subunits are referred to as RFC1, RFC2, RFC3, RFC4 and RFC5 whereas in humans, these subunits are referred to as p140, p37, p36, p40 and p38. In both the yeast and humans, the proposed function of individual subunits within the RFC complex is similar but not identical (Table 1.1).

The genes encoding PfRFC1, PfRFC2 and PfRFC3 have been isolated and characterised (Jill Douglas, Ph.D thesis, 1999). PfRFC1 is a single copy gene on chromosome 2, has open reading frame is 2712bp, which predicts a protein of 904 amino acids with a molecular weight of 104kDa. There are no introns present in this gene. Northern analysis detected a transcript of 4kb in size, present predominantly in trophozoites. PfRFC2 is also a single copy gene on chromosome 2. It is transcribed in the opposite direction to and approximately 40kb apart from PfRFC1. PfRFC2 has an open reading frame of 990bp, which predicts a protein of 330 amino acid residues with a molecular weight of approximately 38kDa. There are no introns present in this gene. Northern analysis detected a transcript of 1.6kb in size, present in trophozoites and two larger transcripts of 2.5 and 4kb in schizonts. The third subunit, PfRFC3, is a single copy gene on chromosome 14. PfRFC3 has an open reading frame of 1032bp, which predicts a protein of 344 amino acid residues with a molecular weight of approximately 39kDa. There is one intron of 250bp present at the 5' end of the gene. PfRFC3 has two transcripts of 1.4 and 1.8kb present in trophozoites and schizonts. All the three proteins, PfRfc1, PfRfc2 and PfRfc3 accumulate in the trophzoite stage of the asexual parasites. Immunofluorescence assays showed that all three PfRfc proteins localised to nucleus of both trophozoites and schizonts.

The genes encoding PfRFC4 and PfRFC5 have been identified and their characterisation is in progress (Spielmann and Beck, 2000). *P. falciparum* RPA (PfRPA) has been identified and is at present being characterised (Kilbey B, personal communication).

1.8.2 Proliferating cell nuclear antigen (PCNA)

PCNA was identified as a protein that accumulated during the S-phase of the cell cycle (Bravo and Cellis, 1980; Bravo, 1986) and was therefore thought to be a cyclin. PCNA was later shown to associate with DNAPol delta and acts as a sliding clamp holding the polymerase on the primer terminus. PCNA is a protein with an apparent molecular weight of 36kDa. PCNA forms a homotrimeric complex that is topologically linked to DNA by the RFC thereby allowing PCNA to track along the DNA (Tinker *et al.*, 1994).

PfPCNA has been cloned (Kilbey *et al.*, 1993) and was found to be located on chromosome 13 as a single copy with no introns. The gene is 825bp long and encodes a polypeptide of 275 amino acid residues with a predicted molecular weight of approximately 30.5kDa. Two transcripts were detected; a major one of 1.6kb and a minor one of 2.2kb. PfPCNA protein and transcript accumulate in trophozoites and persist through to schizonts. However, nuclear run-on analysis showed that PfPCNA promoter activity was present throughout the intraerythrocytic asexual cycle. This led Horrocks *et al.*, (1996) to suggest a post-transcriptional regulation of PfPCNA.

1.8.3 DNA polymerases

1.8.3.1 DNA polymerase alpha (Pol alpha)

DNA polymerase alpha is responsible for initiating DNA replication on both the leading and the lagging strands. The enzyme consists of a subunit containing polymerase activity (205kDa), two subunits with primase activity (55-60kDA and 48-50kDa) and a 70kDa subunit with unknown function (Wang, 1991).

PfDNA POL α has been isolated and characterised (White *et al.*, 1993) and encodes for a protein of a predicted molecular weight of 205kDa. The gene is present as a single copy on chromosome 4 and has a length of 5.7kb with a single intron of 204bp. A transcript of 7kb has been detected with an open reading frame of 1855 amino acid residues. Examination of the polypeptide sequence revealed the presence of seven

sequence motifs that characterise eukaryotic DNA polymerases. Of the five motifs specific for DNA polymerase α, four have been identified in PfDNA Polα. One of the two primase subunits with primase activity has also been isolated from *P. falciparum* (Prasartkaew *et al.*, 1996). The gene has an open reading frame of 1356bp encoding a protein of 452 amino acids with a molecular weight of 53kDa. The gene was found to contain 15 introns and a single transcript of 2.1kbp. The coding sequence was expressed in baculovirus and the purified recombinant primase protein was able to initiate *de novo* primer formation.

1.8.3.2 DNA polymerase delta

DNA polymerase delta is required for the elongation of both the leading and lagging strands of DNA (Tsurimoto and Stillman, 1990). The enzyme consists of two subunits; a catalytic subunit of 125kDa with both polymerase and 3'-5' exonuclease activity and a second subunit of 50kDa with an unknown function (Syuaoja *et al.*, 1990).

PfDNA POLδ has been cloned (Fox and Bzik, 1991; Ridely *et al.*, 1991). The gene exists as a single copy on chromosome 10 and contains no introns. A transcript of 5.2kb encodes a polypeptide of 1094 amino acid residues with a predicted molecular weight of 120kDa. The amino acid sequence of PfDNA Polδ contains all seven motifs used to identify DNA polymerases. PfDNA Polδ protein and transcript accumulate in trophozoites and persist through to schizonts. However, nuclear run-on analyses showed that PfDNA Polδ promoter activity was absent in rings but present in trophozoites. This was thought to suggest pre-transcriptional regulation of PfDNA Polδ (Horrocks *et al.*, 1996).

1.8.4 Topoisomerases

DNA topoisomerases alter the topological state of DNA by catalysing the breaking and rejoining of DNA strands. DNA topoisomerases are therefore essential for DNA

replication, transcription, recombination and chromosome structure. They are classified as type-I enzymes if they cleave a single strand of the DNA duplex and as type-II if they cleave both strands (Wang, J. C, 1996).

1.8.4.1 Topoisomerase I (TopoI)

Prokaryotic and eukaryotic topoisomerase type-I enzymes are active as monomers and their biological function of topoisomerase I has been studied extensively (Gupta *et al.,* 1995). Studies in cell-free SV40 DNA replication system have shown that free topoisomerase I binds to a specific site on double strand DNA, causes a transient single-strand DNA break and becomes covalently linked to the 3'-phosphoryl end of the nicked DNA. The intact strand passes through the nick; DNA relaxation results from swivelling at this nick. Topoisomerase I then reseals the cleaved strand and dissociates from the DNA (Dean and Cozzarelli, 1985). Topoisomerase I probably functions as a swivel to release the local supercoils generated during transcription elongation. Increase in levels of topoisomerase I in some tumours especially those of epithelial origin, as compared with normal tissues, suggest that this enzyme can be a target for anti-cancer agents (Liu, 1989) .

PfTOPO I has been isolated (Tosh and Kilbey 1995) and was found to be located on chromosome 5 as a single copy with no introns. The gene has an open reading frame of 2520bp that encodes a polypeptide of 839 amino acid residues with a predicted molecular weight of 116kDa. A single transcript of 3.8kb was identified by northern analysis. PfTOPO I transcript levels are highest in trophoziotes and decrease in schizonts. Nuclear-run-on assays indicated that the promoter is only active in trophoziotes and schizonts and not in early ring-stages. PfTopoI protein was shown to present at low levels in rings, but accumulating to equal levels in trophozoites and schizonts. TopoI activity assays showed schizonts had the highest level of PfTopoI activity (Tosh *et al.,* 1999).

1.8.4.2 Topoisomerase II (TopoII)

The eukaryotic type-II topoisomerase is active as two identical subunits forming a homodimer, each subunit acting on complementary DNA strands. Type-II topoisomerases catalyse the formation of transient double-stranded breaks in a DNA duplex and each duplex becomes covalently linked to each subunit of the enzyme via the 5'-phosphoryl end. The second intact DNA duplex is then passed through the double-stranded DNA gate, type-II topoisomerase reseals the gap and dissociates from the duplex DNA. Type-II DNA topoisomerases, unlike type-I, appear not play a direct role during early DNA replication (transcription) but are needed for the resolution of late replication intermediates (Ishimi *et al.*, 1992; Clarke *et al.*, 1993).

PfTOPOII has been isolated and characterised (Cheesman *et al.,* 1994). It has an open reading frame of 4194bp and is located on chromosome 14 as a single copy and contains no introns. Three transcripts of approximately 6, 7 and 8kb are seen in trophozoites but only the major 6kb transcript is seen in the ring-forms. The major 6kb transcript encodes a polypeptide of 1398 amino acid residues with an approximate molecular weight of 160-175kDa. PfTOPOII shares a 65% sequence similarity with the human TOPOII alpha isoform (Cheesman *et al.*, 1994).

PfTopoII is present in both trophozoites and schizonts as a triplet of proteins and at similar levels and localises to the nucleus. The PfTopoII transcripts accumulate principally in the trophozoites but are not detectable in the schizonts. However, nuclear-run-on analysis showed that the PfTOPOII promoter remained active throughout the asexual cycle. Topoisomerase II activity was found to be higher in schizonts than in trophozoites with no activity present in the ring-forms (Cheesman *et al.*, 1998).

1.9 Rationale and scope of thesis

DNA synthesis in gametocytes takes place at two points; at the onset of gametocytogenesis where the nucleic acid content increases from 1c to 1.8c prior to the formation of stage I gametocytes, and again 10 to 12 days later at the onset of gametogenesis when the mature male gametocyte (stage V) replicates its genome three times leading to the formation of eight haploid male gametes in less than 10 minutes. Naturally, questions then do arise as to the status of the parasite's DNA replication apparatus during most of gametocytogenesis; from stage I through to stage V gametocytes. What happens to the components of the parasite's DNA replication apparatus after the initial DNA synthesis at the onset of gametocytogenesis; are they broken down or do they still remain present and active throughout gametocytogenesis? What happens upon activation of mature gametocytes? Are new components of the DNA replication apparatus synthesised or are pre-existing proteins activated?

The primary objective of this study was to answer some of these questions. The proteins investigated in this study were, replication factor C, proliferating cell nuclear antigen and topoisomerase II. The *P. falciparum* homologues of these proteins, PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopoII, had already been identified in Prof. Kilbey's lab, expressed as recombinant proteins and rabbit antisera against all of them was available. The detection of these proteins *in situ* during gametocytogenesis by the use of indirect immunofluorescence assays and Western blot analysis during gametogenesis will be described. Gametocyte RNA will also be analysed by RT-PCR for the presence of transcripts of these genes before and after activation. An attempt to carry out RNA *in situ* hybridisation on asexual parasites using probes specific for genes involved in *P. falciparum* DNA replication will also be described.

At present, three components of the DNA replication apparatus, Primase, PfTopoI and PfTopoII, can be reliably assayed for activity. The activity of PfTopoII in mature gametocytes before and after activation will also be described.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Unless specified otherwise, Sigma Chemical Co. Ltd, UK, supplied all chemicals used. Cell culture reagents were supplied by Gibco Life Sciences, UK. FSA Laboratory Supplies, UK, supplied solvents.

2.1.2 Equipment

Bench top centrifugation was carried out using a Heraeus Biofuge 13 (Eppendorf, Germany) or a Jouan CR322 (Saint-Herbian, France).

PCR was carried out using a Techne PCR machine from Techne Instruments, UK. Beckton Dickinson Labware, UK, supplied the plasticware.

RNA hybridisations were carried out in Techne Hybridiser HB-1 oven from Techne Instruments, UK.

In situ hybridisations were carried out on a Biometra UNO-Thermoblock™ from Biometra, UK.

Fluorescence was observed using a Leitz fluorescence microscope and images captured using a Hamamatsu digital camera C4742-95.

2.1.3 Bacterial strains

Bacterial strains used were TOP10 (Invitrogen)and XL1-Blue (S) (New England Biolabs). Genotypes were:

TOP10;

F$^-$*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Δ80*lacZ* ΔM15 Δ*lac*X74 *rec*A1 deoR *ara*D 139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G.

XL1-Blue (S);

F'::Tn*10 proA*$^+$*B*$^+$ *lacI*^{q} *Δ(lacZ)M15/recA1 endA1 gyrA96*(Nal^r) *thi hsdR17* ($r_K^-m^+$) glnV44 relA1 lac.

2.1.4 Plasmids

Invitrogen, UK, supplied the pCR®2.1 (3.9kb) plasmid used in cloning of PCR products. Roche Diagnostics and Biochemicals, Germany supplied pST18 plasmid used in the *in vitro* transcription (DIG RNA labelling) experiments.

2.1.5 Parasites

In this study, *Plasmodium falciparum* clone 3D7A strain was used exclusively to grow gametocytes *in vitro*. *P. falciparum* 3D7A is a clone of isolate NF54. NF54 was isolated from a naive patient living near Schipol Airport, Amsterdam, and was propagated *in vitro* and distributed to laboratories all over the world. It is mainly used by researchers interested in the study of *P. falciparum* gametocytes as it easily forms gametocytes in larger quantities than other isolates (Teklehaimanot, *et al.*, 1987).

2.1.6 Human red blood cells and plasma/serum

Fresh whole human blood of blood group O Rhesus + was obtained from the Edinburgh and Southeast Scotland Blood Transfusion Services.

2.1.7 DNA size markers

New England Biolabs supplied 100 base pair (bp) and 1 kilo base pair (kbp) DNA markers that were used. The 100bp markers produced DNA fragments of 100, 200, 300, 400, 500/517, 600, 700, 800, 900, 1000, 1,250, 1,500bp. The 1kbp markers produced DNA fragments of 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0 and 10.0kbp. 0.5µg of DNA molecular weight marker in 1X DNA loading buffer was run per lane.

2.1.8 Imaging

Following electrophoresis, nucleic acids on ethedium bromide gel visualised by placing gel on an UV-transillunminator and a photographed taken using a Mitsubishi Video processor to heat sensitive film.

2.2 Buffers and media

Luria-Bertani medium (LB)

1% bacto-tryptone; 0.5% Bacto-yeast extract; 1% sodium chloride adjusted to pH 7.2, using 1M sodium hydroxide. LB was supplied by ICMB media services.

LB agar

LB supplemented with 1.5% agar. LB agar was supplied by ICMB media services.

MOPS SDS Running Buffer (20X)

1M MOPS; 1M Tris Base; 69.3mM SDS; 20.5mM EDTA.

NuPage Transfer Buffer (20X)

500mM Bicine; 500mM Bis-Tris; 20.5mM EDTA; 1mM Chlorobutanol.

NuPage Sample Buffer (4X)

4.00g Glycerol; 0.682g Tris Base; 0.666g Tris-HCl; 0.006g EDTA; 0.75ml of a 1% (w/v) Serva Blue G250 and 0.25ml of a 1% (w/v) Phenol red solution. Total volume of 10ml. Novex Electrophoresis GmbH, Germany supplied constituted buffer.

RNA Sample buffer

250µl Formamide (deionised), 83µl of 37% (v/v) Formaldehyde, and 50µl of 10X MOPS buffer.

RNA Loading buffer (5X)

50% (v/v) Glycerol, 1mM Na_2EDTA, and 0.4% Bromophenol blue.

RNA pre-hybrisation solution

50% (v/v) formamide, 5X SSC, 1% (w/v) blocking reagent, 0.02% (v/v) SDS and 0.1% (w/v) lauryolsarcosine.

RNA Blocking reagent (10% (w/v))

10% (w/v) blocking reagent dissolved in Maleic acid buffer pH 7.5, autoclaved

Maleic acid buffer pH 7.5

0.1mM Maleic acid, 0.15M NaCl; pH 7.5 (+20°C); adjusted with solid or concentrated NaOH and autocalved.

RNA Washing buffer

100mM Maleic acid, 150mM NaCl; pH 7.5; 3% (v/v) Tween® 20

RNA-DIG Detection buffer

0.1M Tris-HCl, pH 9.5; 0.1M NaCl

Nitro blue tetrazolium (NBT) formulation (Promega, UK)

50mg NBT in 70% formamide

5-bromo-4-chloro-3-indolyl-phosphate (BCIP) formulation (Promega, UK)

50mg/ml in 100% dimethylformamide

Tris-Buffered Saline with Tween® 20 (TBST)

50mM Tris-HCl (pH 7.5), 150mM NaCl and 0.1% (v/v) Tween 20

Alkaline Phosphatase Buffer (APB)

100mM Tris-HCl, pH 9.5; 100mM NaCl, and 5mM $MgCl_2$.

DNA loading buffer (5X)

25% (v/v) Glycerol; 5% Sarkosyl; 0.0025% Bromophenol blue.

Parasite lysis solution

50mM Tris-HCl; 150 mM NaCl; 5 mM EDTA; 0.5% Triton X-100; 0.01% sodium azide in distilled water. The following protease inhibitors were added: 5mM iodoacetamide, 10µg/ml Soybean trypsin inhibitor; 1 µg/ml aprotonin; 10 µg/ml Tosyl-L-phenylalanine chloromethyl ketone (TPCK), 75 µg/ml Phenylmethylsulphonyl fluoride (PMSF) and the pH adjusted to 7.4.

Parsite culture medium

Incomplete culture medium (ICM)

25mM HEPES; 50µg/ml of hypoxanthine; 50µg/ml gentamycin; 25 mM $NaHCO_3$ in RPMI 1640. The medium was filtered through a 0.22µm Nalgene filter and stored at 4°C for up to 4 weeks.

Complete culture medium (CCMA)

25mM HEPES; 50µg/ml of hypoxanthine; 50µg/ml gentamycin; 25mM $NaHCO_3$; 10g/L AlbuMaxII in RPMI 1640. The medium was filtered through a 0.22µm Nalgene filter and stored at 4°C for up to 4 weeks.

Complete culture medium (CCMS)

To a suitable amount of ICM, heat-inactivated human serum was added to make 10% (v/v) heat-inactivated human serum in ICM. It was filtered through a 0.22 µm Nalgene filter and stored at 4°C for up to 4 weeks.

Gametogenesis stimulating medium

0.5µM xanthurenic acid in RPMI medium, set to pH 7.4 using NaOH.

2.3 Parasite culture

2.3.1 Preparation of red blood cell

The blood was centrifuged at 1,500 g for 10 minutes at 4°C. The plasma was carefully aspirated and kept at -20°C while the "buffy coat" of white blood cells at the top of the cell pellet was aspirated and discarded. The red cell pellet was washed two times with 2 volumes of ICM and the resulting suspension centrifuged at 1,500g for 5 minutes at 4°C. After the last wash, the red blood cell pellet was re-suspended in one volume of ICM to make a 50% red blood cell suspension (50% haematocrit) which was kept at 4°C and used within two weeks.

2.3.2 Preparation of human plasma

Plasma isolated from whole human blood collected each week from the Edinburgh and Southeast Scotland Blood Transfusion Services was pooled and carefully dispensed into sterile 50ml centrifuge tubes, and heat-inactivated in a water bath at 56°C for 40 minutes. The heat-inactivated plasma was then centrifuged at 1,500 g (3,000 rpm) for 10 minutes at 4°C. Plasma was removed and stored at -20°C.

2.3.3 Establishment and maintenance of asexual blood cultures

Plasmodium falciparum strain 3D7A was cultured *in vitro* using the method of Trager and Jensen (1976) as modified by Ifediba and Vandeberg (1981). The culture procedures and related activities were carried out under aseptic conditions. A vial of *P. falciparum* strain 3D7A was removed from liquid nitrogen storage and allowed to thaw quickly at 37°C. The content was transferred to a 15ml centrifuge tube and centrifuged at 1,500g for 10 minutes at room temperature. The supernatant was discarded and 0.3ml of 3.5% (w/v) NaCl added and the suspension centrifuged at 1,500g for 10 minutes at room temperature. The supernatant was discarded again and 1ml of complete culture medium (CCMA) (section 2.2) added and the suspension re-centrifuged at 1,500g for 10 minutes at room temperature. The

supernatant was discarded and the cell pellet size was visually estimated. The appropriate amount of 50% washed human erythrocytes were added to make a packed cell volume of 0.3ml to which 4.5ml of CCM were added to make a 5ml culture at 6% haematocrit. This suspension was transferred into a $25cm^2$-base canted-neck culture flask, aerated with culture gas mixture (92% N_2, 5% O_2, and 3% CO_2) and maintained in an incubator at 37°C. Spent medium of the culture was removed every 24 hours by tilting the flask towards the front and aspirating the medium and replacing with 4.5ml of fresh pre-warmed complete medium. The parasitemia of the culture was monitored daily by observing Giemsa-stained thin films (section 2.3.9 and 2.3.10) of the culture on an Olympus BH-2 Microscope at X1,000 magnification (oil immersion). When the parasitemia reached 5-6%, the culture was diluted by adding fresh RBCs to reduce the parasitemia to around 1%.

2.3.4 Growth of blood cultures for gametocyte production

The method used was as described by Carter *et al.,* (1993). The parasitized RBCs from an asexual culture at a parasitemia above 4% were diluted with fresh RBCs to give a starting parasitemia of 0.5%. The diluted culture was dispensed in 5ml volumes at 6% haematocrit in $25cm^2$-base, canted-neck, cell culture flasks and the flasks aerated with culture gas mixture and maintained in an incubator at 37°C. Medium was replaced in each culture flask every 24 hours with 4.5ml of pre-warmed complete medium. About 2-3 days after the dilution of the cultures, thin films of all the parasites cultures were made and stained with Giemsa (section). Parasites were examined microscopically for morphological changes associated with stress, such as slightly triangular ring-forms. When these signs appeared, the haematocrit was reduced to 3.6% by increasing the volume of complete medium from 5ml to 8.5ml per flask. At the first sign of the presence of stage I gametocytes (usually 72 hours after lowering the culture haematocrit), the gametocyte cultures were maintained in CCMA containing 50mM N-Acetyl glucosamine (GluNAc) for 72 hours with medium changes every 12 hours (Ponnudurai *et al.,*1986). Thereafter, the gametocyte

cultures were maintained with 24 hourly medium changes in standard CCMA until mature stage V gametocytes (Hawking *et al.*, 1971) were present, typically 14 to 16 days after the last dilution of the cultures.

Mature gametocytes undergo gametogenesis at temperatures below 30°C. They can also be triggered to exflagellate by suspension in a bicarbonate ion-containing buffer at pH 8.0 (Ogwang' *et al*, 1993). In order to minimize the risk of activating the gametocytes, all efforts were made to keep the gametocytes at 37°C prior to the induction of suspended animation in bicarbonate-free medium (ICM).

2.3.5 Testing for activation/exflagellation

Fifty microlitres of a 16 day-old gametocyte culture were placed in a sterile microfuge tube and micro-centrifuged for 4 seconds at room temperature. The supernatant was discarded and cell pellet was re-suspended in 50µl of Gametogenesis stimulating medium (GSM). 20µl of this suspension was placed on a haemocytometer and examined under a phase contrast microscope (x400 magnification). The gametocyte cultures were taken to reached full morphological and physiological maturity when at least 20 percent of the gametocytes exflagellated, that is, at least two centres of movement (COM) per ten gametocytes. A COM represented an exflagellating body with several microgametes.

2.3.6 Preparation of purified mature gametocytes of P. falciparum using Percoll

This was done according to the method initially described by Knight and Sinden (1982) and modified by Kariuki *et al* (1998). Percoll (1.130 g/ml) was made isotonic by adding 1 volume of 10X ICM to 9 volumes of stock Percoll to make a 90% Percoll isotonic solution. This solution was then diluted to 80%, 65%, 50%, and 35% with ICM and allowed to equilibrate to room temperature prior to use. A discontinuous gradient was prepared carefully by successively layering 1.5ml of each

Percoll dilution in a tilted 15ml centrifuge tube, starting with the highest Percoll concentration.

Spent medium was removed from a mature gametocyte culture (16 day-old) and pre-warmed (37°C) ICM added immediately. The gametocyte culture suspension was transferred into a 15ml centrifuge tube and centrifuged at 1,500g for 5 minutes at 37°C. The supernatant was discarded and the infected erythrocyte pellets washed twice, by centrifugation (1,500g for 5 minutes at 37°C), with warm ICM and re-suspended again in ICM to make a 20-30% haematocrit in a maximum volume of 1.5ml. The infected erythrocyte suspension was carefully layered over the prepared discontinuous Percoll gradient and centrifuged at 1,750g for 10 minutes at room temperature with the brakes off. The resultant band of parasites at the 35/50% Percoll interphase (Percoll Fraction 2) was carefully removed using a Pasteur pipette and washed twice in 10 volumes ICM to remove any traces of Percoll. The parasite pellet, now comprising mainly of gametocytes, was used immediately for gametogenesis (section 2.3.7) or stored as dry pellet at –70°C until required.

2.3.7 Timing of Gametogenesis

This experiment was carried out using the purified gametocytes isolated from the 35/50% Percoll interphase (Percoll Fraction 2 (F2)). A purified F2 gametocyte pellet was re-suspended in 400µl of ICM. 100µl were removed and transferred into a sterile microfuge tube and micro-centrifuged for 4 seconds at 4°C. The supernatant was carefully removed and discarded. The dry pellet was labeled as Mature Inactivated Gametocytes (MIG) and immediately stored at -70°C. The remaining 300µl parasite suspension was micro-centrifuged for 4 seconds at 4°C and the supernatant carefully removed and discarded. The pellet was then re-suspended in 300µl of Gametogenesis stimulating medium (GSM). 100µl aliquots of this suspension were withdrawn at five minutes intervals. On each occasion, the aliquot was transferred into sterile 1.5ml microfuge tube and then rapidly micro-centrifuged

for 4 seconds at 4°C. The supernatant carefully removed and discarded. The cell pellet was washed once with chilled ICM, labeled appropriately, and the dry pellet immediately stored at -70°C. These pellets were labeled as Mature Activated Gametocytes at 5, 10 and 15 minutes after activation (MAG 5, MAG 10, and MAG 15). These parasite pellet-sets were used to prepare lysates for the Western blot analysis, Topoisomerase II activity assays and northern analysis.

2.3.8 Saponin lysis of infected erythrocytes

Infected erythrocytes from *in vitro* cultivated *P. falciparum* 3D7A cultures were collected by centrifugation at 2000g for 10 minutes at 4°C and the supernatant discarded. The infected erythrocyte cell pellet was re-suspended in 5 volumes of 0.1% (w/v) saponin and incubated for 5 minutes at room temperature. The released parasites were collected by centrifugation at 2000g at 4°C for 10 minutes. The supernatant was discarded and the parasite pellet washed twice in 5 volumes of ice-cold ICM. The dry parasite pellet was stored at –70°C until required.

2.3.9 Preparation of parasite films on microscope glass slides

Parasite films were prepared as described by Dacie and Lewis (1991) and Weindy (1991). A small drop of parasitized erythrocytes from a culture flask or those obtained after Percoll step gradient centrifugation was placed in the centre line of a clean microscope glass slide about 1 or 2cm from one end. Then, without delay, a second glass slide (spreader) with a smooth edge was placed width-wise at an angle of 45° to the slide and moved back to make contact with the drop of parasitized erythrocytes. After the drop spread out along the line of contact of the spreader and the glass slide, making a rapid, smooth and forward movement of the spreader made a thin film. The thin film was air dried for 10 minutes at room temperature.

To prepare a thick film, a small drop of parasitised erythrocytes was placed at the centre of a glass slide and spread out with the corner of another slide using circular

motions to cover an area about four times its original area. The thick film was dried as described above.

2.3.10 Giemsa staining of parasite films

Parasite films were stained according to the method described by Dacie and Lewis (1991) and Wendy (1991). Once dry, the thin film was fixed by placing the slide in 100% methanol for 10 seconds at room temperature. The slide was removed and allowed to drain off and dry at room temperature after which it was placed for 10 min at room temperature in a trough containing Giemsa's stain (BDH) freshly diluted to 10% (v/v) with phosphate buffered water (pH 6.8). The excess stain was washed off with tap water and the films allowed dry in an upright position at room temperature.

The same staining procedure described above was used for thick parasite films, however, thick films were not fixed but were stained immediately after they had dried.

2.3.11 Sorbitol treatment of parasite cultures

Mature asexual parasites, trophozoite and schizonts, were eliminated by the sorbitol treament of culture according to the method described by Lambros and Vandenberg (1979). The culture was centrifuged (2000xg, 10minutes, 4°C) and the infected erythrocyte pellet resuspended in 5 volumes of sterile 5% sorbitol. The culture was incubated at room temperature for 5 minutes and the culture collected by centrifugation (2000xg, 10 minutes, 4°C). The infected erythrocyte pellet was washed in 5 volumes of in ICM (2.2), collected by centrifugation (2000xg, 10 minutes, 4°C) and resuspended in CCMA at 6% haematocrit. The culture was gassed then incubated at 37°C. The culture was examined after 48 hours, and if necessary, the protocol was repeated to ensure tight synchrony.

2.4 Protein methods

2.4.1 Isolation of total parasite protein

Parasites released from infected erythrocytes by saponin lysis (section 2.3.8) were re-suspended in 5 volumes of parasite lysis solution (see section 2.2). The lysate was passed repeatedly through a G25 hypodermic needle to aid complete lysis and reduce viscosity of the sample. Lysate was cleared of debris by micro-centrifuging at 13,500rpm for 10 minutes at 4°C. Cleared lysate was transferred to a sterile microfuge tube and stored at –70°C until required.

2.4.2 SDS-Polyacrylamide gel electrophoresis (PAGE)

2.4.2.1 Preparation of sample solution for electrophoresis

Typically, a total of 10µl of sample solution was made up. To 5µl of total parasite lysate (containing approximately 1µg of total protein) was added 2.5µl of 4X SDS Sample Buffer, 1.5µl of sterile distilled water and 10µl of reducing agent (0.5M DTT). The sample solution was mixed gently heated at 70°C for 10 minutes, vortexed to mix and used for electrophoresis. 10µl of pre-stained broad range protein molecular weight markers (New England Biolabs) were also heated to 70°C for 10 minutes, vortexed to mix and used for electrophoresis.

2.4.2.2 NuPAGE gel for electrophoresis

The NOVEX™NuPAGE Bis-Tris Electrophoresis System was used. NuPAGE Bis-Tris System is a revolutionary neutral pH, pre-cast polyacrylamide mini-gel system. 10% acrylamide pre-cast gels were used. Combs were gently removed from the pre-cast gel and the wells rinsed with sterile distilled water. The gels were assembled in the Mini-Cell following the manufacturer's instructions. 1000ml of a 1X NuPAGE MOPS running buffer was prepared and 200ml set aside. 500µl of NuPAGE Running Buffer Antioxidant was added 200ml of running buffer, mixed by inversion and poured into the upper buffer chamber of the Mini-Cell. Samples were loaded

onto the gel and the lower (outer) chamber filled with about 600ml of the remaining 800ml of 1X running buffer. The gels were electrophoresed at a constant voltage of 200V for 55 minutes at room temperature.

Coomassie-staining of gels in order to visualise protein profiles was carried out by incubating the gel in 5 volumes of 0.05% Coomassie brilliant blue R-250 in water for 10 minutes. The gel was then rinsed in distilled water and visualised over a light box.

2.4.2.3 Western blot transfer

Nitrocellulose paper was cut to the exact size of the gel and immersed for several minutes in 1X NuPAGE transfer buffer comprising of 10% methanol and 0.1% NuPAGE Sample Antioxidant. Gel was released from the plastic cassettes and placed on a pre-wet nitrocellulose paper (Hybond C, Amersham) and sandwiched between 2 sheets of blotting paper and blotting pads. The gel membrane sandwich was assembled in the Blot Module, with the gel closest to the cathode, and covered with 1X transfer buffer. The outer chamber was filled with sterile distilled water to about 2cm from the top. The gel was transferred at 30 Volts for 1 hour at room temperature.

2.4.2.4 Probing of Western blots

After transfer, the protein molecular weight markers were cut out and kept while the rest of the blot was blocked with blocking buffer (TBST containing 5% (w/v) non-fat milk powder) for 1 hour, at room temperature, with gentle agitation. The appropriate dilution of rabbit antiserum in blocking buffer was added to the blot which was then incubated for a further 2 hours at room temperature with gentle agitation. The blot was washed twice, 10 minutes for each wash, in TBST followed by incubation in a 1:7500 dilution of goat anti-rabbit IgG-AP in blocking buffer for 1 hour at room temperature. The blot was washed twice, 10 minutes for each wash, in TBST at room temperature.

2.4.2.5 Detection of Western blots

Following the washes in TBST, the membrane was incubated in APB for 5 minutes at room temperature. The blot was then incubated in 10ml of APB containing 66µl of NBT (50mg/ml) and 33µl of BCIP (50mg/ml) until a coloured precipitate formed. The blot was washed in distilled water to prevent over-development, air dried and stored in the dark.

2.4.3 Immunofluorescence microscopy

Standard double IFA was performed using rabbit pre-immune and immune (antiserum) serum produced against recombinant proteins of whole or part of PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopoII. A mixture of mouse anti-Pfs230 (12F10) and anti-Pfg (1H12) 27/25 monoclonal antibodies which specifically recognise gametocytes were included in the assays in order to help distinguish between asexual and sexual parasites. These monoclonal antibodies were kindly donated by Prof. Richard Carter, Institute of Cell Animal and Population Biology (ICAPB), University of Edinburgh.

A thin blood film of infected erythrocytes from unsynchronised cultures containing both asexual and sexual stages of *P. falciparum* at 5% parasitemia were prepared on glass microscope slides. The films were air-dried and fixed in acetone for 5 minutes at room temperature. A grid was drawn on the slide with nail varnish. Onto each section of the grid, 40µl of the primary antibody solution was added and the slide incubated in a moist chamber for 1 hour at room temperature. The primary antibody solution comprised of a 1:80 dilution of appropriate rabbit immune antiserum or pre-immune serum, 1:1,000 dilution of mouse anti-Pfg 27/25 monoclonal antibody (1H12) and a 1:2,500 dilution of mouse anti-Pfs 230 monoclonal antibody (12F10) in blocking buffer (PBS containing 1% (w/v) BSA). The slide was washed twice, 15 minutes each, in PBS at room temperature and incubated in a moist chamber for 1 hour at room temperature in secondary antibody solution. The secondary antibody solution comprised of a 1:80 dilution of Goat anti-rabbit TRITC (Sigma) conjugated

antibody and a 1:50 dilution of Goat anti-mouse FITC in blocking buffer. The slide was washed twice, 15 minutes each, in PBS at room temperature and then incubated in PBS containing 0.5μg/ml DAPI for 5 minutes at room temperature and followed with a quick rinse in sterile distilled water. The slide was mounted in Mowiol mounting medium and examined by fluorescence microscopy and images captured on a digital camera.

Trophozoites in the mixed cultures were used as internal positive controls. Mouse anti-Pf 27/25 and anti-Pfs 230 monoclonal antibodies, are gametocyte-specific antibodies, and were included in the primary antibody solution in order to help distinguish between asexual and sexual stages of the parasite.

2.4.4 Affinity purification of rabbit polyclonal antiserum

Western blot analysis, using unsynchronised parasite extract and a rabbit antiserum derived against *P. falciparum* DNA replication proteins, was carried out essentially as described in (2.4.2) but with a few alterations. Parasite extract was loaded in a central trough and pre-stained protein molecular weight markers loaded on both the peripheral wells and the protocol followed as described. Following the washing steps after the incubation with rabbit antiserum, only thin strips cut off from the peripheral ends of the blot were probed with AP-conjugated antibody and bound conjugate detected as described in section 2.4.2.4. Meanwhile, the remaining central part of the blot was left in washing buffer. Following detection, the peripheral strips were aligned with the central blot and a strip of the central blot, at the position corresponding to the prominent bands seen on the peripheral strips, was cut out and incubated in a microfuge containing 100mM glycine (pH 2.5) for 10 minutes at room temperature. Following a brief spin, the supernatent was transferred to a fresh microfuge tube and the pH neutralised by the addition of a tenth volume 1M Tris-HCl (pH 8.0). The affinity purified polyclonal antibody solution was stored at –20°C until required.

2.4.5 Topoisomerase II decatenation assays

TopoGEN, USA supplied the Topoisomerase II assay kit. Percoll purified gametocytes (F2) pellets (2.3.6), containing at least 1 X 10^6 gametocytes, were lysed in 40µl of Topoisomerase II assay buffer (). The lysates were cleared of cell debri by a brief microcentrifugation at 4°C. The lysates were split in two, one half was stored frozen at –70°C and later analysed for the presence of *P. falciparum* Topoisomerase II by Western blotting as described in section (2.4.2). To the remaining 20µl lysate, 200ng on Kinetoplast DNA (KDNA) was added and the reaction incubated at 37°C for 30 minutes. The decatenation reaction was stopped by the addition of 0.2 volumes of gel loading dye (25% (v/v) glycerol, 5% (w/v) sarkosyl and 0.0025% bromophenol blue). The whole reaction contents were loaded on a 1% agarose gel containing 0.5µg/ml of ethedium bromide and electrophoresed between 50-100V until the dye front had travelled about 5cm. Linearized, decatenated and non-decatenated KDNA were also run on the gel and acted as experimental controls. The gel was visualised by UV-irradiation.

One unit of Topoisomerase II enzyme completely decatenates 200ng of KDNA in 15 minutes at 37°C.

2.4.6 Preparation of Mowiol Mounting Medium

2.4g of MOWIOL®4-88 were added to 6.0g glycerol and solution stirred for 1hour. 6.0ml of sterile distilled water were added and the solution stirred for 2 hours. 12ml of 0.2M Tris-HCL (pH 8.5) were added and the solution further incubated in a 50°C water bath with occasional stirring. The solution was clarified by centrifugation at 2000g for 15 minutes at room temperature, then aliquoted and stored at –20°C until required.

2.5 DNA methods

2.5.1 Isolation of DNA

QIAGEN Plasmid DNA Mini and Midi Kits were used to prepare plasmid DNA from *Escherichia coli*.

2.5.1.2 Isolation of Plasmodium falciparum genomic DNA

P. falciparum 3D7A was grown *in vitro*, as described in section 2.3.3, to a culture parasitemia of at least 5% and parasites were isolated as described in section 2.3.8. The parasite pellet was resuspended in PBS containing 100µg/ml of Proteinase K and the resulting suspension incubated at 50°C for 3 hours. The solution was allowed to cool to room temperature before phenol/chloroform extraction of genomic DNA as described in section 2.5.1.3.

2.5.1.3 Phenol/chloroform extraction of DNA

DNA was extracted by adding an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) pH 8.0, and the resulting suspension mixed vigorously by hand, then microcentrifuged at 13,500rpm for 1 minute at room temperature. The upper aqueous phase was transferred to a clean microfuge tube and the sample re-extracted until the interphase was clear (up to five extractions). The aqueous phase was then re-extracted against chloroform in order to remove any traces of phenol prior to ethanol precipitation as described in section 2.5.1.4.

2.5.1.4 Ethanol precipitation

To a DNA solution, a 0.1 volume of 3M sodium acetate (pH 5.2) was added followed by 2 volumes of ice cold absolute ethanol. The sample was incubated at –70°C for at least 15 minutes and then micro-centrifuged (13,500rpm, 4°C, 30 minutes) and the pellet washed twice with 70% (v/v) ethanol. The pellet was air-dried and resuspended in TE (pH 8.0). The DNA was analysed by agarose gel electrophoresis (section 2.5.9) and UV spectrophotometry.

2.5.1.5 Minipreparation of plasmid DNA

A single bacterial colony was used to inoculate 5ml of LB medium supplemented with 50μg/ml ampicillin and grown at 37°C overnight with shaking. 1.5ml of the culture was microcentrifuged (13,500rpm, 5 minutes) at room temperature. The supernatant was discarded and the bacteria pellet resuspended in 250μl of buffer P1 (50mM Tris-HCl, pH 8; 10mM EDTA; 100μg/ml RNase A). 250μl of cell lysis buffer P2 (200mM NaOH; 1% SDS) was added and the suspension mixed by gently inverting 4-6 times. 350μl of buffer N3 (1.32M potassium acetate pH 4.8) was added and the solution mixed gently by inverting 4-6 times. The resulting cloudy solution was microcentrifuged (13,500rpm, 10 minutes) at room temperature. The supernatant was applied to a QIAprep column and column microcentrifuged for 1 minute and the flow-through discarded. 500μl of buffer PB was added to the QIAprep column and the column microcentrifuged for 1 minute. The flow-through was discarded. 750μl of buffer PE were added to the QIAprep column and the column microcentrifuged for 1 minute. The flow-through was discarded and the column microcentrifuged for an additional 2 minutes to in order to remove residual wash buffer PE. The QIAprep column was placed in a clean microfuge tube and the DNA eluted by adding 50μl of buffer EB (10mM Tris-HCl pH 8.5) to the centre of the QIAprep column, incubating for 1 minute and microcentrifuging for 1 minute. 5μl of the plasmid preparation was analysed by agarose gel electrophoresis (section 2.5.9) and another 5μl quantified by UV spectrophotometry (section 2.5.8). The remaining plasmid preparation was stored at –20°C until required.

2.5.1.6 Midipreparation of plasmid DNA

A single bacterial colony was used to inoculate 100ml of LB medium supplemented with 100μg/ml ampicillin and grown at 37°C overnight with shaking. 25ml of the culture was centrifuged (2500g, 4°C, 10 minutes). The supernatant was discarded

and the bacterial pellet resuspended in 4ml of buffer P1 (50mM Tris-HCl, pH 8; 10mM EDTA; 100µg/ml RNase A) by vortexing until no cell clumps remained. 4ml of cell lysis buffer P2 (200mM NaOH; 1% SDS) was added and the suspension mixed thoroughly but gently by inverting 4-6 times and the suspension incubated for 5 minutes at room temperature. The solution was neutralised by adding 4ml of pre-chilled buffer P3 (potassium acetate, pH 5.5). The lysate was then poured into the barrel of a QIAfilter cartridge, with a cap screwed onto the outlet nozzle, and incubated for 10 minutes at room temperature. Meanwhile, a QIAGEN tip 100 was equilibrated by applying 4ml of buffer QBT (750mM NaCl; 50mM MOPS, pH 7.0; 15% ethanol; 0.15% Triton X-100) and allowing the column to empty by gravity flow. The cap was removed from the bottom of the QIAfilter cartridge, a plunger gently inserted and the cell lysate filtered into the previously equilibrated QIAGEN-tip 100. The cleared lysate was allowed to enter the resin by gravity flow and the resin washed twice with 10ml of buffer QC (1M NaCl; 50mM MOPS, pH 7.0; 15% ethanol). DNA was eluted with 5ml of buffer QF (1.25M NaCl; 50mM Tris-HCl, pH 8.5; 15% ethanol), precipitated with 0.7 volumes of room temperature isopropanol, then centrifuged (15,000g, 4°C, 30 minutes). The pellet was washed with 2ml of 70% ethanol, air dried briefly and dissolved in 200µl EB (10mM Tris-HCl pH 8.5). 5µl of the plasmid preparation was analysed by agarose gel electrophoresis (section 2.5.9) and another 5µl quantified by UV spectrophotometry (section 2.5.8). The remaining plasmid preparation was stored at –20°C until required.

2.5.1.7 Cloning of PCR fragments

The TOPO TA Cloning® kit (Invitrogen) was used. 1µl of pCR®2.1-TOPO® vector was added to 4µl of PCR product, mixed gently and incubated for 5 minutes at room temperature. 2µl of this solution was added to a vial of TOP10 One Shot® cells (provided with the kit) and the resulting suspension mixed gently and incubated

on ice for 30 minutes. The suspension was heat-shocked for 30 seconds at 42°C without shaking and immediately placed on ice. 250µl of room temperature LB medium was added to the suspension, which was then incubated horizontally at 37°C for 30 minutes. 20µl of the transformation reaction was spread onto LB plates supplemented with 50µg/ml of ampicillin and 0.004% X-Gal. The plates were allowed to dry, inverted and incubated for 18 hours.

2.5.2 Colony screening

2.5.2.1 Blue/white colony selection

To promote full colour development, the plates were transferred to 4°C for 2 hours. The multiple cloning site of pCR®-TOPO® vector contains the *Lac*Z gene. Insertion of a DNA fragment into the multiple cloning site facilitates the blue/white colony colour selection, where white coloured colonies harbour the vector with insert present. White colonies were randomly picked for further analysis.

2.5.2.2 Double restriction analysis of white colonies

10 randomly selected white colonies were picked and individually resuspended in 5ml of LB medium containing 50µg/ml of ampicillin and incubated overnight at 37°C with agitation. The following day, bacteria were collected, by micro-centrifugation, and minipreparations of the plasmids prepared as described in section 2.5.1.5. Double endonuclease restrictions using XbaI and BamHI restriction endonucleases determined the presence of inserts in the plasmid preparations and were carried out as described in section 2.5.3. The reaction products were analysed by agarose gel electrophoresis.

2.5.2.3 PCR analysis of white colonies

The multiple cloning site on pCR®2.1-TOPO® vector is bordered by M13 Reverse primers on one end and M13 Forward primers on the other. PCR analysis using

either M13 Forward or the M13 Reverse primers and a primer that hydridizes to the PCR insert determined the orientation of the PCR inserts within the vector. A standard PCR cocktail containing PCR buffer, dNTPs, *Taq* polymerase, and either the M13 Forward or M13 Reverse primers and a primer that hydridizes to the PCR insert was made up. 10 white colonies were picked and resuspended individually in 20µl of PCR cocktail. The reaction was incubated for 10 minutes at 94°C in order to lyse the cells and inactivate nucleases followed by 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. A final extension of 72°C for 10 minutes was followed by incubation at 4°C. The reaction products were analysed by agarose gel electrophoresis.

2.5.3 Endonuclease restriction

The multiple cloning site (MCS) on pCR®2.1-TOPO® has restriction sites for *Xba*I and *Bam*HI endonucleases were located on either sides of the PCR product cloning site (Figure 1). Therefore, double enzyme endonuclease restrictions, using *Xba*I and *Bam*HI endonucleases, were carried out on pCR®2.1-TOPO® isolated from overnight cultures in order to determine the presence or absence of insert DNA fragment.

5 units each of *Xba*I and *Bam*HI endonucleases were added to 5µl of plasmid DNA and 2µl of 10X Buffer 2 (New England Biolabs) and the volume made up to 20µl with sterile distilled water. The reaction was incubated at 37°C for 2 hours. The restriction reaction (20µl) was analysed by agarose gel electrophoresis and DNA fragments, showing the expected molecular weight, purified from the gel (section 2.5.4).

2.5.4 Gel extraction of DNA fragments

QIAquick gel extraction kit (QIAGEN) was used. Gel containing DNA fragments of interest was placed on a short-wave UV transilluminator and the DNA fragment of

interest quickly excised from the agarose gel by punching the open end of a sterile 1ml blue pipette tip and the gel slice transferred to a microfuge tube and weighed. 3 gel volumes of buffer QG were added and gel dissolved by incubating at 50°C for 10 minutes. 1 gel volume of isopropanol was added, mixed and solution applied to a QIAquick spin column attached to a 2-ml collection tube. The column was centrifuged for 1 minute and flow-through discarded. 500µl of buffer QG was added to the QIAquick column and centrifuged for 1 minute and flow-through discarded. 750µl of buffer PE was added to the QIAquick column and centrifuged for 1 minute and flow-through discarded. The QIAquick spin column was centrifuged for a further 1 minute in order to remove residual buffer PE. QIAquick spin column was transferred to a sterile 1.5ml centrifuge tube and the DNA eluted by adding 30µl of EB (10mM Tris-HCl, pH 8.5) to the centre of the QIAquick membrane and the column centrifuged for 1 minute at maximum speed. The gel purified DNA fragments were stored at –20°C until required.

2.5.5 Preparation of competent Escherichia coli

A single bacterial colony of XL1 was used to inoculate 5ml of LB medium and incubated, with agitation, overnight at 37°C. The overnight culture was diluted 1:200 in LB and grown, with aeration, for 2 hours or until growth was just visible. The bacteria from the overnight cultures were collected by centrifugation (1,500g, 5 minutes, 4°C) and supernatant discarded. The bacterial cell pellet was resuspended in ice-cold 0.1M $CaCl_2$ and incubated on ice for 20 minutes. The bacteria were collected by centrifugation and pellet resuspended in 100µl ice-cold 0.1M $CaCl_2$ and used immediately for transformation reactions described in section 2.5.7.

2.5.6 Ligations

The pST18 vector, supplied by Roche, has both T7 and SP6 RNA polymerase transcription start sites. DNA fragments cloned in pST18 can, therefore be

transcribed and transcripts complementary to both strands of the inserted DNA can be obtained from the same construct.

The MCS of pST18 has restriction sites for both *Xba*I and *Bam*HI endonuclueases (Figure 5.2). pST18 was therefore double restricted using *Xba*I and *Bam*HI endonucleases, as described in section 2.5.3, and gel-purified as described in section 2.5.4.

Gel-purified *Xba*I-*Bam*HI DNA fragments, isolated from pCR®2.1-TOPO®, were cloned into a *Xba*I and *Bam*HI restricted and gel purified pST18 vector. The ligation reaction comprised; 5µl of the gel-purified XbaI-BamHI DNA fragment, 1µl of the double restricted and gel-purified vector pST18, 1 unit of T4 DNA ligase (Roche), 1µl of 10X T4 DNA ligase buffer (660mM Tris-HCl, pH 7.6; 66mM $MgCl_2$; 100mM DTT; 660mM ATP) at 16°C overnight. A control including all components except the DNA fragment was also included and treated similarly. The ligation reaction was either stored at –20°C until required.

2.5.7 Transformations

10µl of the ligation reaction (section 2.5.6) was added to 100µl of competent XL1 cell suspension and the resulting solution incubated on ice for 1 hour. The cells were heat-shocked for 90 seconds at 42°C and immediately placed on ice for 2 minutes. 1ml of LB medium (without ampicillin) was added the transformed cells which were then incubated for 1 hour at 37°C with agitation. The transformed cells were collected by microcentrifugation (13,000rpm, 1 minute, room temperature) and resuspended in 100µl of LB medium supplemented with 50µg/ml of ampicillin. The transformed cells were spread on selective medium plates containing 50µg/ml of ampicillin, the spread plates allowed to dry, inverted and incubated overnight at 37°C. The following day, 10 colonies were randomly picked and individually resuspended in 5ml of LB (with ampicillin) and incubated overnight at 37°C with agitation. Minipreparations of plasmid DNA were carried out (section 2.5.1.5)

followed by double endonuclease restriction analysis (section 2.5.3). Clones shown to posses plasmids with inserts having the expected molecular weight were grown up for midipreparation of plasmid DNA (section 2.5.1.6).

2.5.8 Polymerase Chain Reaction (PCR)

2.5.8.1 Standard PCR

Method used was as described by Saiki *et al.*, 1988 and Innis *et al.,* 1990. Roche Biochemicals, UK, supplied *Taq* DNA polymerase, PCR buffer (with Mg^{2+}) and dNTPs. The PCR was assembled on ice. A typical PCR contained; 100ng template DNA, 1 unit of *Taq* DNA polymerase, 200μM dNTPs, 1μM gene specific oligonucleotides primers and 1X PCR buffer (1.5mM $MgCl_2$, 500mM KCl; 100mM Tris-HCl, pH 9.0; 1% Triton X-100), in a total volume of 50μl. The PCR mixture was placed in a thermal cycler programmed for an initial denaturation step at 94°C for 3 minutes followed by 30 cycles of 94°C for 1 minute, 42°C for 1 minute, and 72°C for 1 minute for the actual amplification. A final extension of 72°C for 10 minutes was followed by incubation at 4°C. A tenth of the reaction product was analysed by agarose gel electrophoresis.

2.5.8.2 PCR Fluorescein labelling

Roche Diagnostics GmbH, Germany supplied PCR Fluorescein labelling Mix. The PCR Fluorescein labelling Mix is a mixture of the lithium salts of dATP, dCTP, dTTP, dGTP and fluorescein-12-UTP. This nucleotide mix is used in PCRs and leads to the direct incorporation of fluorescein-12-UTP into the PCR product. The PCR for fluorescein labelling was carried out essentially as described for standard PCR but with two alterations; the fluorescein labelled dNTP mix was used in place of the unlabelled dNTP mix and the Mg^{2+} concentration was increased to 4.0mM from 1.5mM used in standard PCR. The PCR programme for amplification was the same as that used for standard PCR (section 2.5.8.1). A tenth of the fluorescein-labelled PCR product was analysed by gel electrophoresis without the addition of ethedium

bromide in order to confirm fluorescein incorporation into the PCR product. The un-incorporated fluorescein-labelled nucleotides were removed by ethanol precipitation of the fluorescein-labelled PCR product and the resulting precipitate resuspended in 50µl of sterile distilled water. The presence of remaining un-incorporated fluorescein-labelled nucleotides within the precipitated fluorescein-labelled PCR product was monitored by agarose gel electrophoresis. The fluorescein-labelled PCR product was used as a probe for the *in situ* hybrisation described in section 2.7.4.

2.5.9 UV spectrometry

DNA or RNA samples were diluted 1 in 200 with sterile water and analysed on a Perkin Elmer 115 spectrophotometer set up to scan 200-300nm range. The concentration of nucleic acid in the samples was deduced from the formula below:

{DNA] µg/ml = absorption at 260nm (A_{260}) X 50 X dilution factor

{RNA] µg/ml = absorption at 260nm (A_{260}) X 40 X dilution factor

The relative purity of the sample was determined by calculating the A_{260}/A_{280} ratio, where a value of 1.8 or greater indicates that the sample is free of protein contamination.

2.5.10 Agarose gel electrophoresis

2.5.10.1 Casting agarose gels

Roche Diagnostics and Biochemicals, Germany supplied multi-purpose agarose.

The appropriate amount of agarose (Table 1) was dissolved in 1X TBE by heating the mixture in a microwave oven. The solution was cooled to approximately 60°C and ethedium bromide added to make a final concentration of 5µg/ml. The molten gel was poured into the appropriate size casting tray and allowed to solidify at room temperature.

Table 1. The relationship between the concentration of agarose and the separation of linear DNA molecules (Sambrook *et al.*, 1989)

Percentage agarose	Separation of linear DNA molecules
0.7	0.8 – 10kb
0.9	0.5 – 7kb
1.2	0.4 – 6kb
1.5	0.2 – 3kb

2.5.10.2 Running of agarose gels

The solidified gel was submerged in 1 X TBE buffer. Samples and DNA size markers were mixed with a 1 volume of 10 loading buffer. Samples were subjected to electrophoresis at 5V/cm until sufficient separation of the DNA fragments was achieved as judged from UV transillumination of the gel.

2.5.10.3 Imaging

Following electrophoresis, nucleic acids on ethedium bromide gel visualised by placing gel on an UV-transillunminator and a photographed taken using a Mitsubishi Video processor to heat sensitive film.

2.6 RNA Methods

2.6.1 Isolation of total RNA from *P. falciparum*

Total RNA was isolated using Total RNA Isolation Reagent (TRIR) commercially available from Advanced Biotechnologies Ltd, UK. A parasite pellet prepared as described in section 2.3.8 was re-suspended in 1ml of TRIR and lysed by passage through a G25 hypodermic needle several times. Homogenate was incubated on ice for 5 minutes to allow for complete dissociation of nucleoprotein complexes. 200µl of chloroform was added, sample shaken vigorously for 15 seconds and then incubated on ice for 5 minutes. The homogenate was microcentrifuged (13,500rpm,

15 minutes, 4°C) and 4/5th of the upper layer transferred to a fresh microfuge tube. An equal volume of isopropanol was added, mixed gently and the sample placed on ice for 10 minutes. The sample was microcentrifuged (13,500rpm, 10 minutes, 4°C), supernatant carefully removed and the visible RNA pellet washed twice with 1ml of 75% ethanol by microcentrifugation (13,500rpm, 5 minutes, 4°C). The RNA pellet was briefly air-dried and resuspended in sterile RNase free, DEPC treated, double distilled water. Purity and concentration of RNA was determined by UV spectrophotometry (2.5.9) and native agarose gel electrophoresis.

2.6.2 DNase treatment of total parasite RNA

2µg of total parasite RNA were mixed with 4 units of RQ1 RNase-Free DNase (Promega) in RQ1 RNase-Free DNase reaction buffer (40mM Tris-HCl, pH 8.0; 10mM $MgSO_4$; 1mM $CaCl_2$) and incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 1µl of RQ1 DNase Stop Solution (20mM EGTA) and the DNase inactivated by heating to 65°C for 10 minutes and the final volume was made up to 100µl with sterile RNase free, DEPC treated, double distilled water. RNA re-extracted by adding 200µl of phenol/chloroform/isoamyl alcohol (125:24:1, pH 4.1) and the resulting suspension mixed vigorously by hand, then microcentrifuged at 13,500rpm for 1 minute at room temperature. The upper aqueous phase was transferred to a clean microfuge tube and re-extracted against chloroform (2.5.1.3) in order to remove any traces of phenol prior to ethanol precipitation. RNA was ethanol precipitated (2.5.1.4), washed and re-suspended in 11µl of DEPC treated, double distilled water and stored at –70°C until required.

2.6.3 1st Strand synthesis reaction

Reverse-iT™ 1st strand synthesis kit from Advanced Biotechnologies Ltd, UK was used. 1µl of anchored oligo dT (0.5µg/µl) was added to 11µl (approximately 2µg) of DNase treated total parasite RNA, mixed and heated briefly at 70°C for 5 minutes, in

order to remove any secondary structures in the RNA, and then placed on ice. The following components were added; 4µl of 5X 1st strand synthesis buffer (), 2µl of dNTP mix (5mM each of dATP, dCTP, dGTP and dTTP), 1µl of M-MLV RTase (25U/µl) and 1µl of RNase inhibitor (5U/µl). A 'no-RT' control was performed essentially the same way but with the omission of M-MLV RTase. The reaction components were mixed and incubated at 42°C for 1 hour in order for the cDNA synthesis reaction to proceed. The M-MLV RTase was inactivated by a further incubation of the reaction at 75°C for 10 minutes and the reaction products stored at –20°C until required. 2µl of the 1st strand synthesis reaction was used as template for standard PCR (2.5.8.1) using gene specific oligonucleotide primers.

2.6.4 RNA denaturing gel electrophoresis

RNA was analysed by electrophoresis on 1% agarose in 1X MOPS and 6% (v/v) formaldehyde prepared as described below.

2.6.4.1 Formaldehyde gel preparation

1g of agarose was added to 10ml of 10X MOPS (section 2.2) and 75ml of sterile DEPC-treated water, heated to 100°C to dissolve the agarose and allowed to cool to 60°C. In a hood, 16.5ml of 37% (v/v) formaldehyde was added, mixed well and poured into a casting tray and left to solidify.

2.6.4.2 RNA samples preparation and electrophoresis

RNA sample to be analysed was thawed on ice and mixed with an equal volume of RNA sample buffer (section 2.2). The sample was denatured by heating to 65°C for 10 minutes and cooled immediately on ice. 0.2 volumes of RNA loading buffer (section 2.2) were added to the RNA sample before loading onto gel. RNA molecular weight markers were treated in similar manner but in addition, 1µl of a 5mg/ml ethedium bromide solution was added to the markers before loading onto the

peripheral wells on the gel. The sample was loaded on a formaldehyde gel submerged in 1X MOPS buffer (running buffer) and electrophoresed at 70V for 2-3 hours. Approximately, 2μg of total RNA was loaded per well.

2.6.5 Northern blotting

After electrophoresis, the gel was placed on a 'wick' consisting of 2 pieces of Whatman (3MM) paper cut to the exact width of the gel, but longer, so that the ends could be submerged in a reservoir containing 20X SSC. The gel was surrounded with saran wrap. A nylon membrane (Hybond N, Amersham), cut to the exact size of the gel, was placed on the gel and all air bubbles excluded. 4 pieces of Whatman (3MM) paper, cut to the exact size of the gel, were wet in 20X SSC and placed on the nylon membrane. A stack of dry paper towels approximately 10cm high, a glass plate and a 500g weight were placed on the nylon membrane, and the gel left to transfer overnight at room temperature.

2.6.6 Digoxigenin RNA labelling

Roche Biochemicals, UK, supplied all reagents used. 1μg of the DNA template (pST18) was linearized at a restriction site upstream of the cloned insert. Either *Bam*HI or *Xba*I restriction endonucleases were used. For single enzyme restrictions, buffer H (50mM Tris-HCl, 100mM NaCl, 10mM $MgCl_2$, 1mM Dithioerythrythol; pH 7.5) was used for *Xba*I endonuclease restrictions (Roche). Buffer B (10mM Tris-HCl, 10mM NaCl, 5mM $MgCl_2$, 100mM Potassium acetate, 1mM 2-Mercaptoethanol; pH 8.0) was used for *Bam*HI endonuclease restrictions (Roche).
The linearized pST18 was purified by phenol/chloroform extraction, ethanol precipitated and resuspended in 13μl of sterile RNase free, DEPC treated, double distilled water. The following reagents were added to the linearized pST18; 2μl of 10XNTP labelling mixture (10mM each of ATP, CTP, GTP, UTP, and 6.5mM of DIG-11-UTP, pH 7.5), 2μl of 10X transcription buffer (400mM Tris-HCl, pH 8.0;

60mM $MgCl_2$; 100mM dithiothreitol; 20mM spermidine), 1µl of RNase inhibitor (40U/µl), and either 2µl of SP6 (20U/µl) or T7 (20U/µl) RNA polymerase. The reaction components were mixed gently and incubated for 2 hours at 37°C. The reaction was stopped by adding 2µl of 0.2M EDTA (pH 8.0) and unincorporated DIG-UTP removed by ethanol precipitation. The RNA pellet was washed with 75% ethanol, dissolved on DEPC-treated water and stored at –70°C until required.

2.6.7 Determination of DIG-RNA labelling efficiency

1µl of DIG-labelled RNA, prepared as described above, was spotted on a nylon membrane and cross-linked to it by placing the membrane on a short-wave UV transilluminator and exposing it to UV for 45 seconds. The membrane was then treated as described in section 2.6.10.

2.6.8 Prehybridisation and hyrbridisation

Membrane was covered with saran wrap and the RNA cross-linked to the membrane by placing the membrane on a short-wave UV transilluminator and exposing it to UV for 45 seconds. Membrane was then placed in a hybridising tube, with 10ml of pre-hybridisation solution (50% (v/v) formamide; 5X SSC; 1% (w/v) blocking reagent; 0.02% (v/v) SDS and 0.1% (w/v) lauryolsarcosine), and incubated for at least 2 hours at 65°C. Hybridisation solution (typically, 5µl of DIG-labelled RNA in 5ml of pre-hybridisation solution) was denatured by heating to 68°C for 10 minutes and placing on ice. The pre-hybridisation solution was replaced with 5ml of hybridisation solution and the membrane incubated overnight at 65°C.

2.6.9 Post-hybridisation washes

Following hybridisation, the hybridisation solution was poured into a sterile tube and stored at –70°C for re-use later. The membrane was washed twice, 15 minutes per wash, in 2X washing solution (2X SSC; 0.1% (w/v) SDS) at room temperature. The

membrane was washed twice, 15 minutes per wash, in 0.5X wash solution (0.5X SSC; 1% (w/v) SDS) wash solution at 65°C.

2.6.10 Colorimetric detection with NBT and BCIP

Following post-hybridisation washes, the membrane was equilibrated with in washing buffer (100mM Maleic acid, pH 7.5; 150mM NaCl; 3% (v/v) Tween® 20) for 1 minute and then incubated in blocking buffer (100mM Maleic acid, pH 7.5; 150mM NaCl; (+20°C); 1% (w/v) blocking reagent) for 30 minutes at room temperature, with gentle agitation. Blocking buffer was then replaced with antibody solution comprised of 1:5,000 dilution of Anti-Digoxigenin-AP in blocking buffer and incubated for 30 minutes, with gentle agitation, at room temperature. The antibody solution was discarded and the membrane washed twice, 15 minutes per wash, in washing buffer. Membrane was equilibrated with detection buffer (100mM Tris-HCl, pH 9.5; 100mM NaCl) for 2 minutes. Meanwhile, colour substrate solution was prepared by mixing 66µl of NBT and 33µl of BCIP in 10ml of detection buffer. The detection buffer was poured off and the membrane incubated in the colour substrate solution, in the dark, until desired bands were visible. The membrane was washed in sterile distilled waster in order to prevent over-development.

2.7 *In situ* hydridisation

Unless otherwise stated the methods used were as described by Levy and Herrington, 1995.

2.7.1 Microscope glass slide coating

Microscope slides were coated with 3-Amino-propyl-triethoxysilane (APES) to enhance adhesion. In a fume hood, clean microscope slides were immersed in 100% acetone for 1-2 minutes. The acetone was drained off and slides immersed in 2% (v/v) APES in acetone for 5 minutes. The excess solution was drained off and the

slides washed in running water for 1-2 minutes and then dried overnight at room temperature. The slides were stored dry and dust-free at room temperature until required.

2.7.2 Fixation and permeabilization of parasites smears

4% paraformaldehyde fixative was prepared by dissolving the appropriate amount of paraformaldehyde on hot PBS. The fixative was then cooled rapidly on ice. Thin films of unsynchronised parasites were prepared on APES-coated microscope glass slides and air-dried. The slides were immersed in the chilled 4% paraformaldehyde fixative and incubated for 30 minutes at 4°C. The slides were washed by immersion in PBS for 5 minutes at 4°C. The slides were immersed in 0.1M Tris-HCl (pH 7.2) containing 0.25% (v/v) Triton X-100 and 0.25% (v/v) Nonidet P-40 twice for 5 minutes at 4°C. The slides where then rinsed in 0.1M Tris-HCl (pH 7.2) for 5 minutes at 4°C followed by an incubation in 2X SSC for 10 minutes at 4°C. The slides were air-dried, wrapped in aluminium foil and stored desiccated at –70°C until required.

2.7.3 Nuclease treatment of thin parasite films

Slides containing fixed and permeabialized parasites smears were removed from -70°C, and allowed to warm up to room temperature while still wrapped. 11.4cm^2 Gene Frame® (Advanced Biotechnologies, UK) with a chamber volume of 300µl, was attached to slides. 10 units of RQ1 RNase-free DNase (Promega) in 300µl of RQ1 RNase-free DNase reaction buffer (40mM Tris-HCl, pH 8.0; 10mM $MgSO_4$; 1mM $CaCl_2$) was applied to the chamber which was then covered with a plastic coverslip and the slide incubated at 37°C for 4 hours. A control experiment was carried out in which slides were incubated with the buffer only, no enzyme. All slides were incubated at 65°C for 10 minutes in order to inactivate the nuclease and then rinsed twice, for 5 minutes each, in 2X SSC at room temperature. The slides were either used immediately or stored desiccated at –70°C until required.

2.7.4 *In situ* hybridisation with Fluorescein-labelled probes

Slides containing fixed and permeabialized parasites smears were removed from -70°C, and allowed to warm up to room temperature while still wrapped. 1cm^2 Gene Frame® (Advanced Biotechnologies, UK) with a cavity volume of 25µl, was attached to each slide. Probe mix was prepared by mixing 2µl of Fluorescein-labelled PCR product with 23µl of hybridisation buffer (50% formamide; 2X SSC; 20% (w/v) Dextran sulphate; 0.2% (w/v) non-fat dried milk). The probe mix was heated to 65°C for 10 minutes then cooled rapidly on ice and applied to the Gene Frame® chamber on the microscope glass slide. A plastic cover-slip was carefully placed and firmly secured on the chamber. The slide was placed on a flat plate thermal cycler and heated to 65°C for 5 minutes and then incubated at 37°C overnight in the dark.

2.7.5 Detection of *in situ* signal

All the following incubations were carried out in the dark in order to preserve fluorescence. Following overnight incubation at 37°C, the chamber was carefully removed and the slide washed once in 2X SSC for 10 minutes at room temperature. The slide were washed once in 0.2X SSC for 10 minutes at 37°C and again once in 0.1X SSC for 10 minutes at 37°C. The slide was then incubated in 2X SSC for 5 minutes at room temperature. Parasite nucleus was counter-stained by incubating the slides in PBS containing 0.1µg/ml DAPI for 5 minutes at room temperature followed by a quick rinse in sterile distilled water. The slide was mounted in Mowiol mounting medium and examined by fluorescence microscopy and the images captured using a digital camera.

CHAPTER 3

GAMETOCYTOGENESIS AND THE DNA REPLICATION APPARATUS

3.1 Introduction

Studies on DNA synthesis in *P. falciparum* gametocytes have shown that a significant amount of DNA is synthesised at the onset of gametocytogenesis with the DNA content increasing to about 1.8c most likely as a result of DNA amplification rather than whole genome duplication (Janse *et al.*, 1988).

In this chapter, an attempt to study the pattern of expression of proteins involved in *P. falciparum* DNA replication during gametocytogenesis will be described. Indirect immunofluorescence assays (IFAs) with rabbit polyclonal antisera developed specifically against recombinant small fragments of PfRfc1, PfRfc2 and PfRfc3 (Douglas J, 1999), PfPcna (Horrocks *et al.*, 1996) and PfTopoII (Cheesman, *et al.*, 1998) were used. Gametocyte-specific antibodies, mouse anti-Pfs230 (12F10) and anti-Pfg 27/25 (1H12) monoclonal antibodies were included in the assays in order to help distinguish between asexual and sexual parasites. These monoclonal antibodies were kindly donated by Prof. Richard Carter, Institute of Cell Animal and Population Biology (ICAPB), University of Edinburgh

First, Western analysis on asexual parasite protein extracts using these antisera were carried out. Although these antisera gave at least one major band at the expected molecular mass in each case, as indicated by the arrows, several other faint bands were seen (**Figure 3.1**). These additional bands might represent degradation products of the main protein or be proteins that share some similarity with the protein against which the antiserum was raised. An attempt was first made to immunoaffinity purify these polyclonal antisera, verifying their purity using Western analysis on asexual parasite protein extracts before attempting IFAs with gametocytes.

3.2 Immunoaffinity purification of whole rabbit antisera

3.2.1 Western blots of asexual parasites probed with whole rabbit antiserum and affinity purified polyclonal antibodies

Total parasite protein (2.4.1) prepared from asexual blood cultures that contained predominantly trophozoites and schizonts (2.3.3), was size-fractionated on 10% polyacrylamide gels, blotted and probed with rabbit polyclonal antiserum and the corresponding affinity purified polyclonal antibodies against PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopoII. **Figure 3.2** shows the results of this experiments. It can be seen that affinity purified antisera (lane 2) recognised only one band (indicated by an arrow), that from which it was extracted. However, by comparing the band intensities before and after affinity purification, and using them as a measure of relative amount of antibody present, it appears that affinity purification leads to a drastic reduction in reacting antibodies. In order to roughly determine the levels of reacting antibodies present after affinity purification, a series of dilutions of each whole polyclonal antisera were used to probe asexual parasite protein blots.

3.2.2 Western blots of asexual parasites probed with serial dilutions of whole antiserum

Dilutions of the whole rabbit polyclonal antisera were prepared and each was used to probe asexual parasite protein extracts. The aim of this experiment was to determine what dilution of whole rabbit antisera was needed to produce a band of roughly comparable intensity with that observed with undiluted affinity purified polyclonal antibodies. **Figure 3.3** shows the results of this experiment. Each lane contained the same concentration of parasites proteins (2μg/lane) (2.4.2.6). The peripheral lanes to the extreme left of the blot show bands produced using the whole volume of solution recovered from the extraction of band specific antibodies along the whole breadth of a mini-blot (2.4.4). By comparing the band intensities, it can be seen that affinity purified polyclonal antibodies give similar band intensities as 1:12,800 for PfRfc1,

1:3,200 for PfRfc2, 1:12,800 for PfRfc3 and 1:12,800 for PfPcna dilution of the original whole rabbit polyclonal antisera.

3.3 Indirect immunofluorescence assays (IFA)

3.3.1 IFA on asexual parasites using affinity purified polyclonal antibodies and whole rabbit polyclonal antisera

Indirect double immunofluorescence assays (2.4.3) were carried out on thin films (2.3.9) of blood smears prepared from unsynchronised cultures (2.3.4) using the affinity purified polyclonal antibodies (2.4.4) and whole rabbit polyclonal antiserum against PfRfc1, PfRfc2, PfRfc3 and PfPcna.

IFAs carried out using affinity purified rabbit polyclonal antibodies resulted in very faint fluorescence (data not shown). However, whole polyclonal antiserum also gave very faint fluorescence (data not shown) when used for IFAs at the dilutions that were seen to give similar band intensities to purified polyclonal antibodies on Western blots, that is, PfRfc1 at 1:12,800 (Figure 3.3, lane 6), PfRfc2 at 1:3,200 (Figure 3.3, lane 4), PfRfc3 at 1:12,800, (Figure 3.3, lane 6) and PfPcna at 1:12,800 (Figure 3.3, lane 6) dilution. It was therefore concluded that the purified antibodies were present at too low a level to be effective. Previous studies on the stage-specific expression of PfRfc1, PfRfc2 and PfRfc3 (Douglas J, 1999), PfPcna (Horrocks *et al.*, 1996) and PfTopoII (Cheesman *et al.*, 1998) in the intraerythrocytic cycle have obtained successful results in IFAs using the same batch of whole rabbit polyclonal antiserum at 1:80 dilution. The study was therefore continued using diluted rabbit polyclonal antisera.

3.3.2 Immunofluorescence assays during gametocytogenesis

Thin films (2.3.9) prepared from unsynchronised cultures (2.3.4) containing gametocytes in various stages of development and asexual parasites were screened with whole rabbit polyclonal antiserum against PfRfc1, PfRfc2, PfRfc3 and PfPcna

at 1:80 dilution (2.4.3) and detected with TRITC-conjugated anti-rabbit antibodies. A mixture of mouse anti-Pfs230 (12F10) at 1:2,500 dilution and anti-Pfg 27/25 (1H12) (1:1,000 dilution) monoclonal antibodies which specifically recognise gametocytes were included in the assays and detected with FITC-conjugated anti-mouse antibodies. Anti-gametocyte antibodies helped distinguish between asexual and sexual parasites.

3.3.2.1 Immunofluorescence assays using anti-PfRfc1 polyclonal antiserum

Figure 3.4 shows IFAs carried out using rabbit polyclonal antisera against anti-PfRfc1. Pre-immune serum treated similarly is shown at the bottom of the figure. It can be seen that PfRfc1 is present throughout gametocytogenesis. By comparing the DAPI and TRITC signals, fluorescence appears to emanate primarily from the nuclear region of stages I, II, III and V gametocytes. Stage I and V gametocytes show the highest intensity of TRITC fluorescence. Diffused fluorescence is observed throughout the cytoplasm of stage IV gametocytes. No fluorescence was observed with pre-immune serum.

3.3.2.2 Immunofluorescence assays using anti-PfRfc2 polyclonal antiserum

Figure 3.5 shows IFAs carried out using rabbit polyclonal antisera against PfRfc2. Fluorescence can be seen in all the gametocyte developmental stages, suggesting that PfRfc2 is present throughout gametogenesis. Apart from stage IV gametocytes, fluorescence appears to primarily emanate from the nuclear region stained by DAPI. Stage I and II gametocytes appear to have the higher intensity of fluorescence than the more mature gametocytes. Stage IV gametocyte show a more diffuse fluorescence throughout their cytoplasm. Fluorescence is distinctly absent from the nucleus of stage V gametocytes and is mainly located within the cytoplasm. This is in contrast to PfRfc1 where stage V gametocytes show the bright fluorescence located within the

nucleus. Pre-immune serum was treated similarly but failed to show any fluorescence.

3.3.2.3 Immunofluorescence assays using anti-PfRfc3 polyclonal antiserum

Figure 3.6 shows IFAs carried out using rabbit polyclonal antisera against PfRfc3. It can be seen from this figure that PfRfc3 is present in all gametocyte stages. Unlike PfRfc1 and PfRfc2, fluorescence appears to emanate primarily from a spot deep within the nucleus of all the gametocyte developmental stages. Stage I gametocytes show significantly higher fluorescence intensity than the rest of the gametocyte stages. No fluorescence was observed with pre-immune serum.

3.3.2.4 Immunofluorescence assays using anti-PfPcna polyclonal antiserum

Figure 3.7 shows IFAs carried out using rabbit polyclonal antisera against PfPcna. It can be seen from this figure that all gametocyte stages possess PfPcna. Fluorescence appears to primarily emanate from the nuclear region, stained by DAPI, of all the gametocyte stages except stage II gametocytes. Stage V gametocytes have the highest intensity of fluorescence. Gametocyte stage II and III also show diffuse cytoplasmic fluorescence. No fluorescence was observed with pre-immune serum.

3.3.2.5 Immunofluorescence assays using anti-PfTopoII polyclonal antiserum

Figure 3.8 shows IFAs carried out using rabbit polyclonal antiserum against PfTopoII. The figure shows a high intensity of fluorescence, localised primarily in the nuclear region of all the gametocyte stages, with the exception of stage IV gametocytes. Stage IV gametocytes show the lowest and most diffuse fluorescence. Some stage V gametocyte appear to have two centres of fluorescence, one located within the nuclear region, stained by DAPI, at one end of the parasite and the other on the opposite end of the parasite. No fluorescence was observed with pre-immune serum.

3.4 Conclusion

In this chapter, immunoaffinity purification of rabbit polyclonal antisera against PfRfc1, PfRfc2, PfRfc3 and PfPcna was successfully carried out as shown by Western analysis before and after purification. However, the affinity purified polyclonal antibodies failed to give a significant fluorescence when used to screen thin films of *P. falciparum* asexual parasite smears possibly because the reacting antibodies were present at very low levels. It is possible that during immunoaffinity purification, not all the antibodies bound to the target protein were eluted hence leading to low antibody recovery. Success of the elution step would have been verified by incubating the eluted strip of blot with AP-conjugated anti-rabbit antibodies followed by NBT/BCIP detection. The use of other more sensitive detection methods, such as immunogold, may be required if such dilute solutions of affinity purified antibodies are to be used for the detection and localisation of target protein *in situ*.

The immunofluorescence experiments using whole rabbit antiserum raised against recombinant fragments of PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopoII proteins have shown that all five proteins are present throughout gametocytogenesis.

PfRfc1 is present throughout gametocytogenesis and is localised primarily in the nuclear region of stages I, II, III and V gametocytes with stage I and V gametocytes showing significantly higher levels. In contrast, PfRfc1 appears to be present in the cytoplasm and at lower levels in stage IV gametocytes than in the stages of gametocytes.

PfRfc2 is also present throughout gametocytogenesis and apart from stage IV gametocytes, it is primarily located in the nucleus. Stage I and II gametocytes appear to have the higher levels of PfRfc2 than the other gametocytes. Stage IV gametocytes have the lowest and most diffuse distribution of PfRfc2. Unlike PfRfc1, stage V gametocytes appear to have low levels of PfRfc2 and in addition, PfRfc2 is distinctly absent from the nucleus of stage V gametocytes.

PfRfc3 is present in all gametocyte stages. Unlike PfRfc1 and PfRfc2, PfRfc3 is distinctly located deep within the nucleus of all the gametocyte developmental stages. It is possible that PfRfc3 is actually within the nucleolus. It appears to and remains there throughout gametocytogenesis. Only stage I gametocytes appear to have higher levels of PfRfc 1 while the rest, including stage IV gametocytes, show similar levels of expression.

PfPcna is also present throughout gametocytogenesis and is also primarily located in the nucleus, except, surprisingly, in stage II and to some extent stage III gametocytes where it appears to be mainly in the cytoplasm. Stage V gametocytes appear to have significantly high levels of PfPcna.

PfTopoII is also present throughout gametocytogenesis and remains localised within the nucleus throughout this period with the exception of stage IV gametocytes. The latter appear to have the lowest levels of PfTopoII and diffuse distribution within the cytoplasm. Some stage V gametocytes appeared to have higher levels of PfTopoII than others. The smaller and sausage-shaped, usually male gametocytes, appear to have significantly higher levels of PfTopoII than the large sickle-shaped female gametocytes.

In general, all five proteins were seen to be present at higher levels within the nucleus of stage I and V gametocytes with the exception of PfRfc2 which was distinctly absent from the nuclear region of stage V gametocytes. Stage IV gametocytes appear to have the lowest and most diffuse levels of PfRfc1, PfRfc2, PfRfc3 and PfTopoII.

Figure 3.1 Western blot analysis of asexual parasite protein extracts probes with rabbit polyclonal antisera

Total parasite protein prepared from asexual blood cultures that contained predominantly trophozoites and schizonts was size-fractionated on 10% polyacrylamide gels, blotted and probed with rabbit polyclonal antiserum against PfRfc1, PfRfc2, PfRfc3 and PfPcna at 1:200 dilution.

Lane 1 Rabbit polyclonal antiserum

Lane 2 Pre-immune serum

M Position of molecular weight markers (sizes shown in kDa)

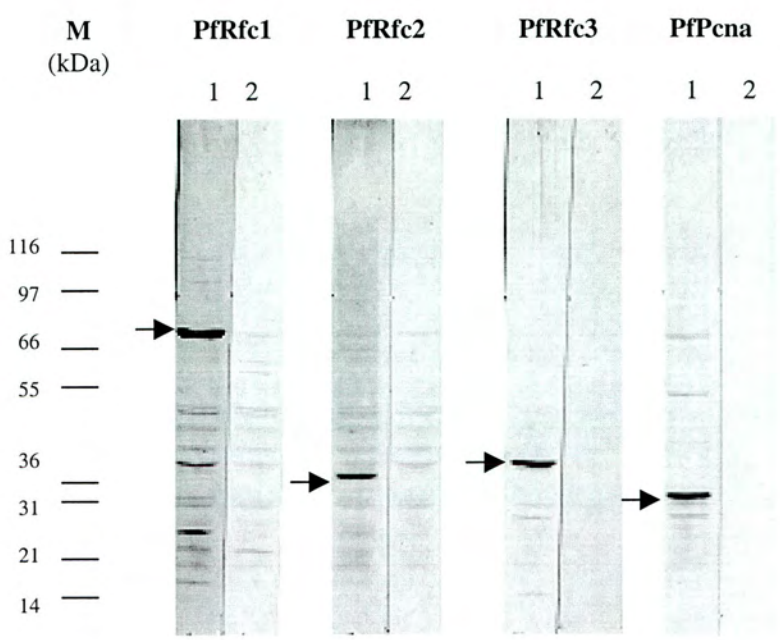

M
(kDa)
PfRfc1
PfRfc2
PfRfc3
PfPcna
1 2
1 2
1 2
1 2
116
97
66
55
36
31
21
14

Figure 3.2 Western blot analysis of asexual parasite protein extract probed with rabbit polyclonal antisera and affinity purified antibody

Total parasite protein prepared from asexual blood cultures that contained predominantly trophozoites and schizonts was size-fractionated on 10% polyacrylamide gels, blotted and probed with rabbit polyclonal antiserum and affinity purified antibody against PfRfc1, PfRfc2, PfRfc3 and PfPcna.

Lane 1 Rabbit polyclonal antiserum (1:200 Dilution)

Lane 2 Affinity purified polyclonal antibody

M Molecular weight markers (sizes shown in kDa)

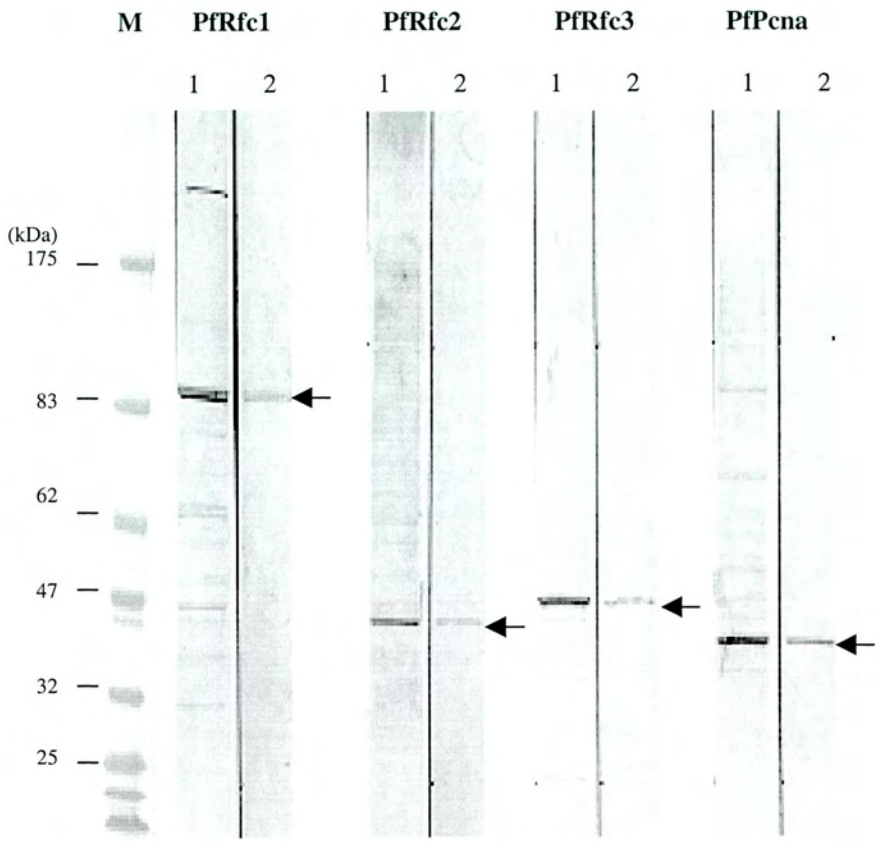

M
PfRfc1
PfRfc2
PfRfc3
PfPcna
1
2
1
2
1
2
1
2
(kDa)
175
83
62
47
32
25

Figure 3.3 Western blots of asexual parasites probed with affinity purified polyclonal antibodies and serial dilutions of whole rabbit antiserum

Dilutions of the whole rabbit polyclonal antisera were prepared and each was used to probe asexual parasite protein extracts. Each lane contained the same concentration of parasite proteins (2μg/lane). Lanes 1-6 show results obtained with serial dilutions of antiserum. Arrow indicates main band.

Lane 1 1:400 dilution

Lane 2 1:800 dilution

Lane 3 1:1,600 dilution

Lane 4 1:3,200 dilution

Lane 5 1:6,400 dilution

Lane 6 1:12,800 dilution

Lane 7 Affinity purified polyclonal antibody

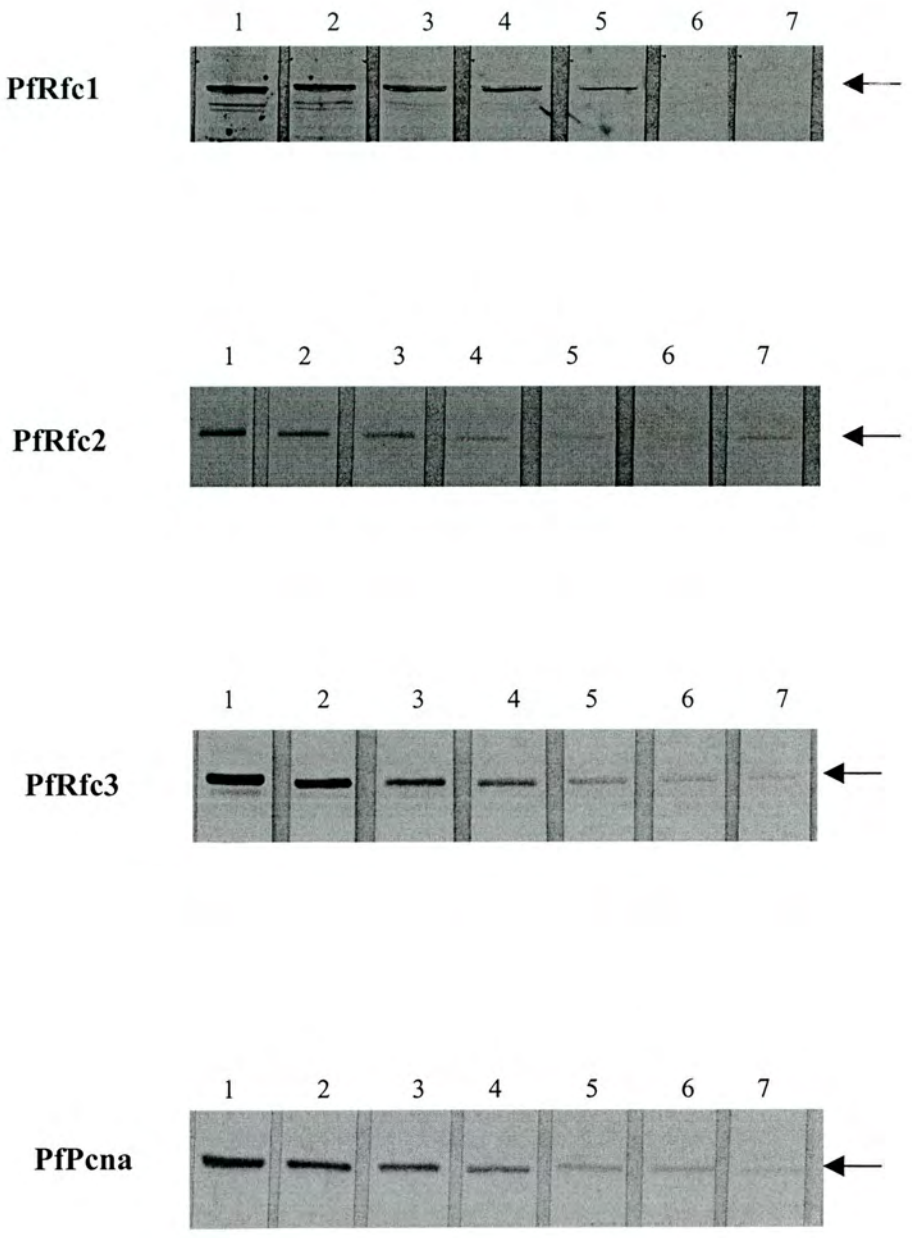

1 2 3 4 5 6 7
PfRfc1
1 2 3 4 5 6 7
PfRfc2
1 2 3 4 5 6 7
PfRfc3
1 2 3 4 5 6 7
PfPcna

Figure 3.4 Immunofluorescence assays using anti-PfRfc1 polyclonal antiserum

Thin films prepared from unsynchronised cultures containing gametocytes in various stages of development and asexual parasites were screened with whole rabbit polyclonal antiserum against PfRfc1 at 1:80 dilution. A mixture of mouse anti-Pfs230 (12F10) (1:2,500 dilution) and anti-Pfg 27/25 (1H12) (1:1,000 dilution) monoclonal antibodies, which specifically recognise gametocytes, were included in the assays in order to distinguish between asexual and sexual parasites. Pre-immune serum was treated in the same way and is shown at the bottom of the figure.

TRITC-conjugated anti-rabbit antibodies detected cross-reactive antiserum.
FITC-conjugated anti-mouse antibodies detected gametocytes-specific antibodies.
DAPI was used to counter-stain counter-stained the gametocyte nucleus.
GI, II, III, IV and V represent the gametocyte sub-stages.

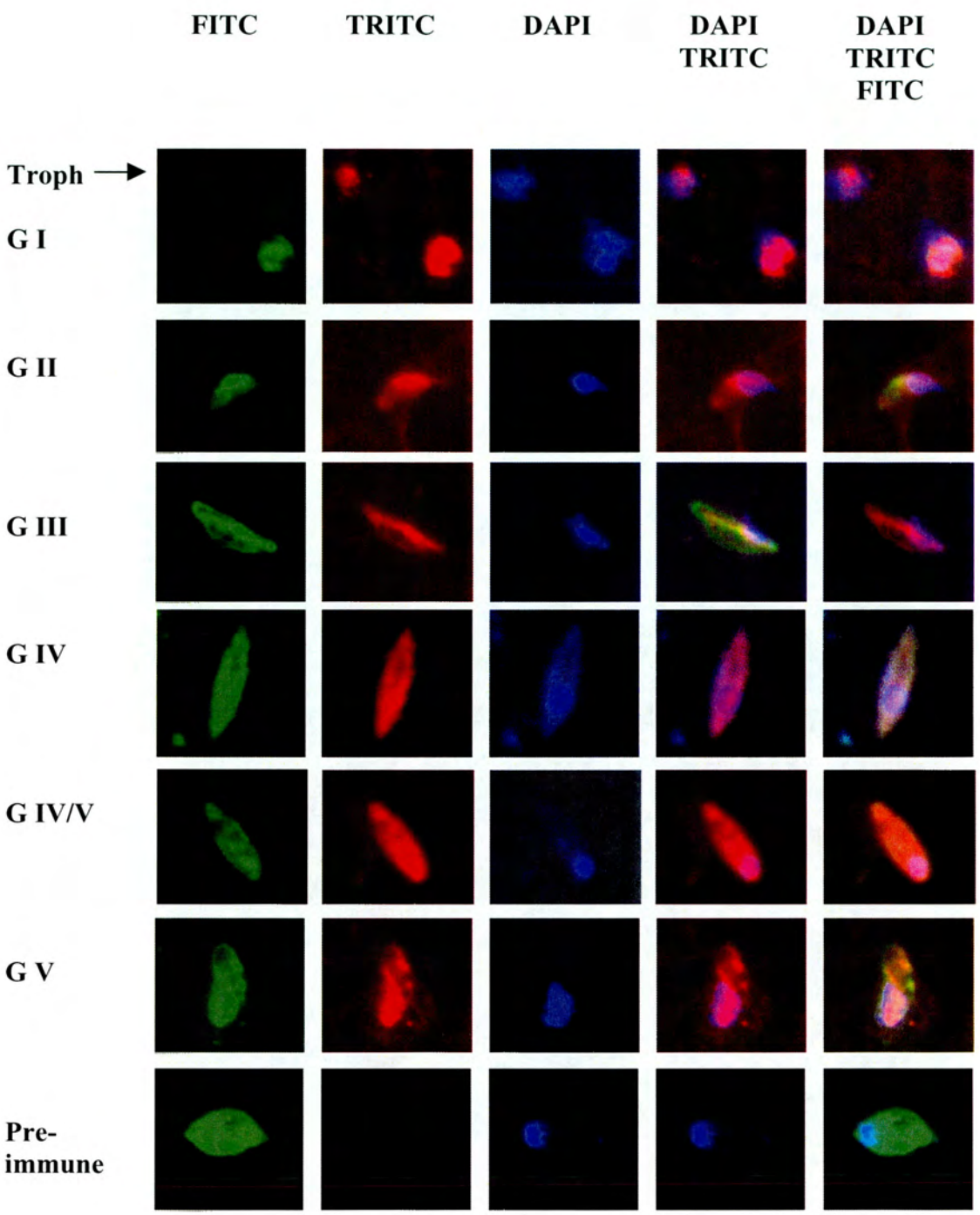

FITC
TRITC
DAPI
DAPI TRITC
DAPI TRITC FITC
Troph
G I
G II
G III
G IV
G IV/V
G V
Pre-immune

Figure 3.5 Immunofluorescence assays using anti-PfRfc2 polyclonal antiserum

Thin films prepared from unsynchronised cultures containing gametocytes in various stages of development and asexual parasites were screened with whole rabbit polyclonal antiserum against PfRfc2 at 1:80 dilution. A mixture of mouse anti-Pfs230 (12F10) (1:2,500 dilution) and anti-Pfg 27/25 (1H12) (1:1,000 dilution) monoclonal antibodies, which specifically recognise gametocytes, were included in the assays in order to distinguish between asexual and sexual parasites. Pre-immune serum was treated in the same way and is shown at the bottom of the figure.

TRITC-conjugated anti-rabbit antibodies detected cross-reactive antiserum.
FITC-conjugated anti-mouse antibodies detected gametocytes-specific antibodies.
DAPI was used to counter-stain counter-stained the gametocyte nucleus.
GI, II, III, IV and V represent the gametocyte sub-stages.

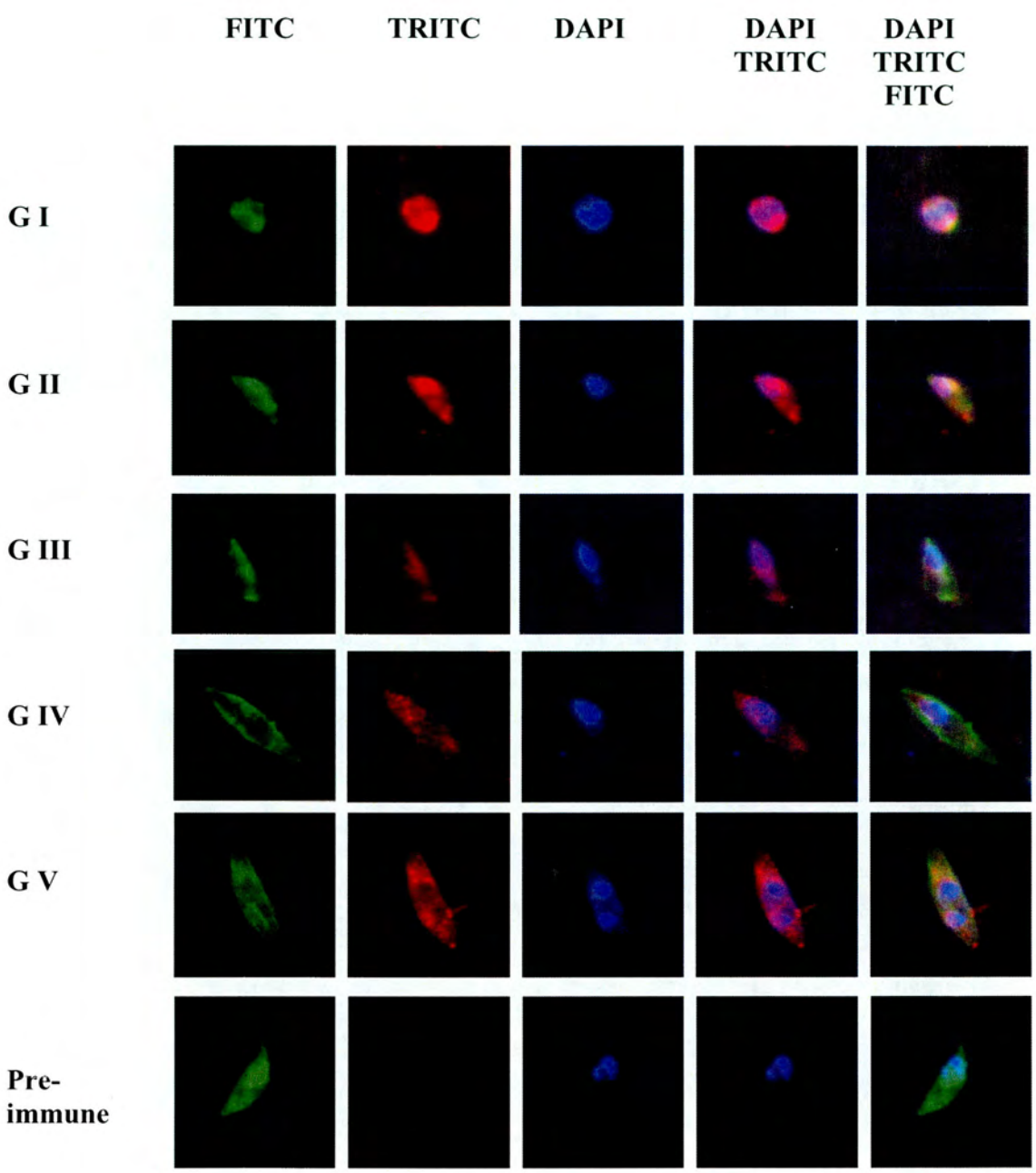

FITC
TRITC
DAPI
DAPI TRITC
DAPI TRITC FITC
G I
G II
G III
G IV
G V
Pre-immune

Figure 3.6 Immunofluorescence assays using anti-PfRfc3 polyclonal antiserum

Thin films prepared from unsynchronised cultures containing gametocytes in various stages of development and asexual parasites were screened with whole rabbit polyclonal antiserum against PfRfc3 at 1:80 dilution. A mixture of mouse anti-Pfs230 (12F10) (1:2,500 dilution) and anti-Pfg 27/25 (1H12) (1:1,000 dilution) monoclonal antibodies, which specifically recognise gametocytes, were included in the assays in order to distinguish between asexual and sexual parasites. Pre-immune serum was treated in the same way and is shown at the bottom of the figure.

TRITC-conjugated anti-rabbit antibodies detected cross-reactive antiserum.
FITC-conjugated anti-mouse antibodies detected gametocytes-specific antibodies.
DAPI was used to counter-stain counter-stained the gametocyte nucleus.
GI, II, III, IV and V represent the gametocyte sub-stages.

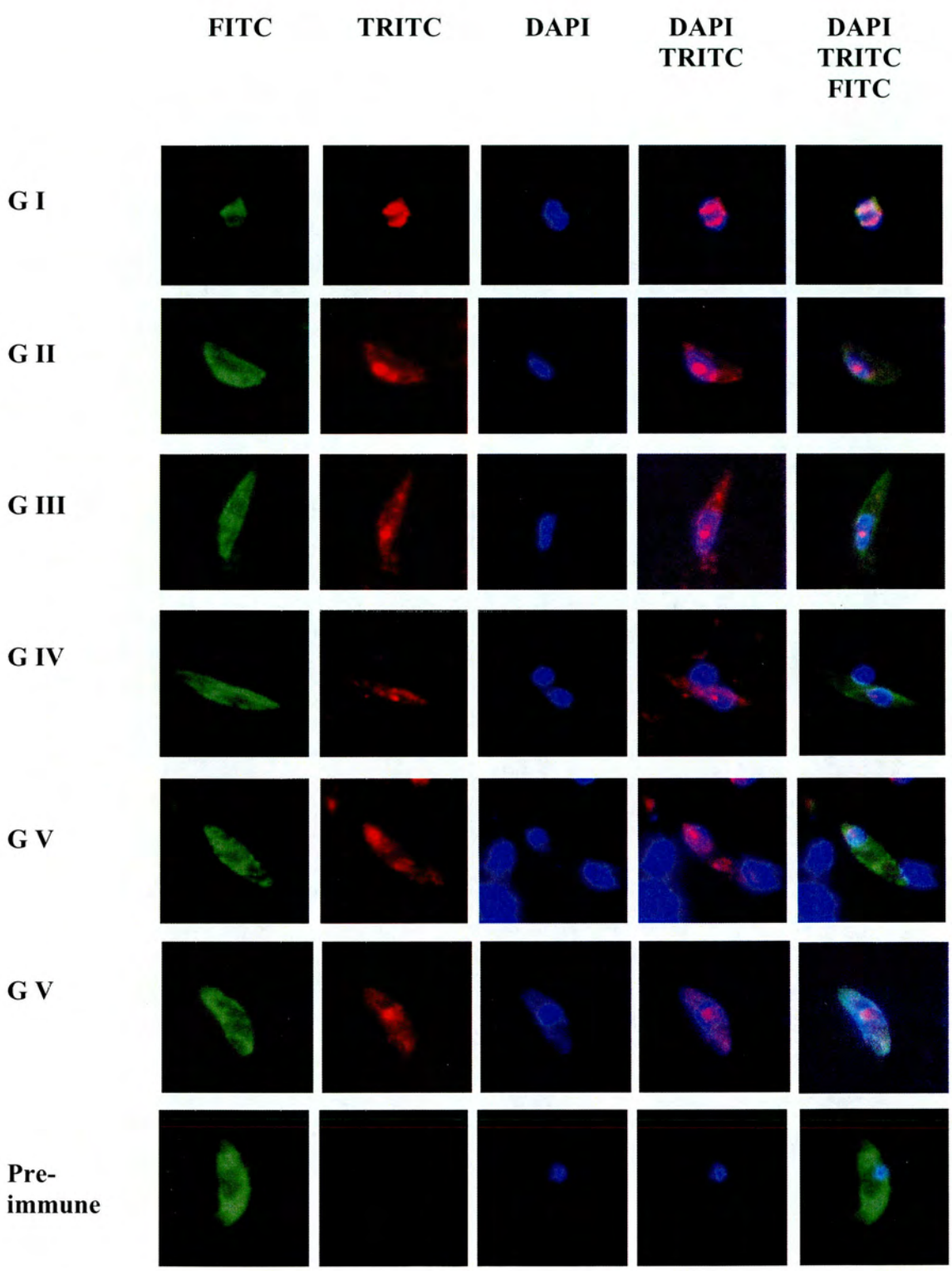

FITC
TRITC
DAPI
DAPI TRITC
DAPI TRITC FITC
G I
G II
G III
G IV
G V
G V
Pre-immune

Figure 3.7 Immunofluorescence assays using anti-PfPcna polyclonal antiserum

Thin films prepared from unsynchronised cultures containing gametocytes in various stages of development and asexual parasites were screened with whole rabbit polyclonal antiserum against PfPcna at 1:100 dilution. A mixture of mouse anti-Pfs230 (12F10) (1:2,500 dilution) and anti-Pfg 27/25 (1H12) (1:1,000 dilution) monoclonal antibodies, which specifically recognise gametocytes, were included in the assays in order to distinguish between asexual and sexual parasites. Pre-immune serum was treated in the same way and is shown at the bottom of the figure.

TRITC-conjugated anti-rabbit antibodies detected cross-reactive antiserum.
FITC-conjugated anti-mouse antibodies detected gametocytes-specific antibodies.
DAPI was used to counter-stain counter-stained the gametocyte nucleus.
GI, II, III, IV and V represent the gametocyte sub-stages.

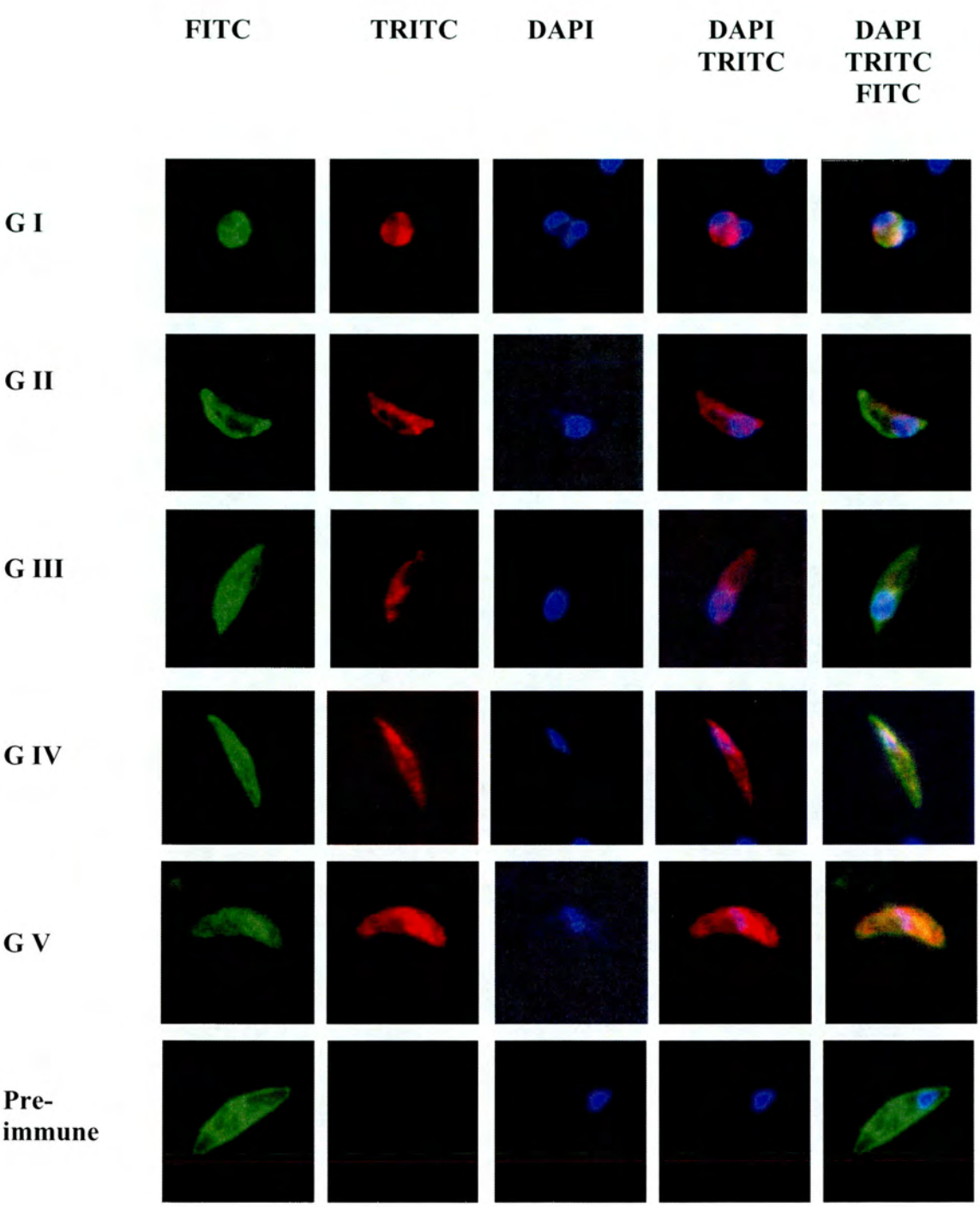
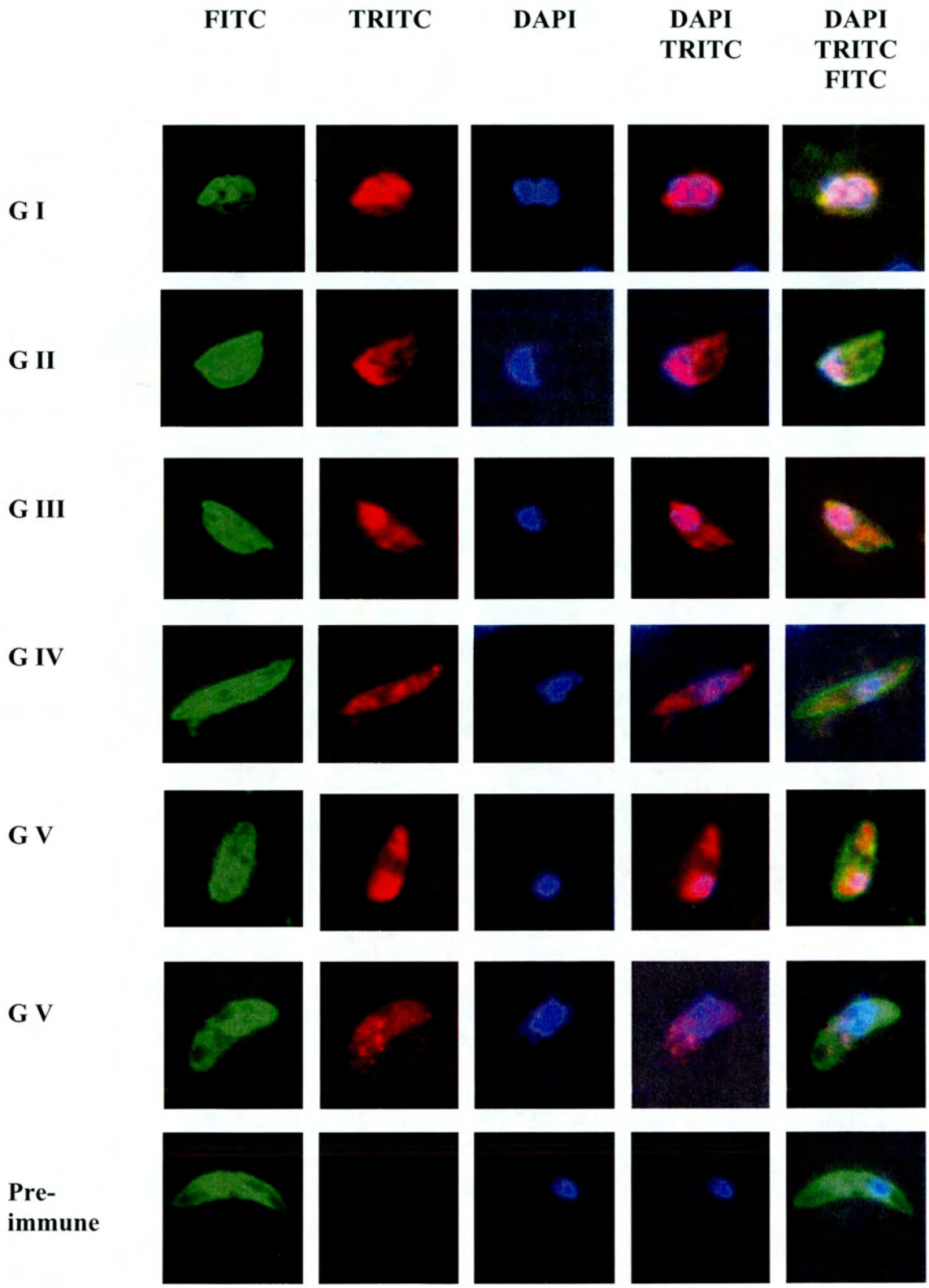

FITC
TRITC
DAPI
DAPI TRITC
DAPI TRITC FITC
G I
G II
G III
G IV
G V
Pre-immune

Figure 3.8 Immunofluorescence assays using anti-PfTopoII polyclonal antiserum

Thin films prepared from unsynchronised cultures containing gametocytes in various stages of development and asexual parasites were screened with whole rabbit polyclonal antiserum against PfTopoII at 1:80 dilution. A mixture of mouse anti-Pfs230 (12F10) (1:2,500 dilution) and anti-Pfg 27/25 (1H12) (1:1,000 dilution) monoclonal antibodies, which specifically recognise gametocytes, were included in the assays in order to distinguish between asexual and sexual parasites. Pre-immune serum was treated in the same way and is shown at the bottom of the figure.

TRITC-conjugated anti-rabbit antibodies detected cross-reactive antiserum.
FITC-conjugated anti-mouse antibodies detected gametocytes-specific antibodies.
DAPI was used to counter-stain counter-stained the gametocyte nucleus.
GI, II, III, IV and V represent the gametocyte sub-stages.


FITC
TRITC
DAPI
DAPI TRITC
DAPI TRITC FITC
G I
G II
G III
G IV
G V
G V
Pre-immune

CHAPTER 4

IN SITU HYBRIDISATION

4.1 Introduction

RNA aids the transfer of genetic information from genomic DNA to functional proteins during gene expression. This process is regulated at each step in the pathway from DNA to RNA to protein. *In situ* hybridisation (ISH) can be used to detect both DNA and RNA by the hybridisation of specifically designed nucleic acid probe sequences to intracellular complementary DNA/RNA sequences. Tissue morphology is preserved and therefore ISH has become a valuable tool for precise localisation of nucleic acids in intact cells. RNA ISH enables cell-specific mRNAs to be separated from the more abundant housekeeping proteins and allows for the investigation of gene expression and its relationship to cell differentiation. In contrast, once RNAs have been extracted from tissues or unsynchronised cell populations for northern blotting or RT-PCR, then information about the site of synthesis is lost. Therefore, knowledge of the spatial distribution and quantification of RNAs at the tissue, cellular, and subcellular levels is needed to investigate gene expression and cell differentiation successfully.

The primary objective of this chapter was to establish a working protocol for RNA ISH in order to study directly the gene expression patterns and the localisation of messages during gametocytogenesis in *P. falciparum*.

RNA ISH on thin films of asexual parasites using fluorescein-labelled gene-specific DNA probes will be described.

4.2 Fluorescent probes for used in *in situ* hybridisation experiments

Several genes involved in *P. falciparum* DNA replication have been isolated and characterised and include PfRFC1, PfRFC2 and PfRFC3 (Douglas J, 1999), PfPCNA (Kilbey *et al.*, 1993), PfTOPO I (Tosh and Kilbey, 1995) PfTOPO II (Cheesman *et al.*,1994). Northern analysis of total parasite RNA isolated from synchronised cultures showed that transcripts of these genes accumulate mainly in trophozoites and schizonts forms of the parasite. Gene-specific Fluorescein-labelled DNA probes for each gene were generated by PCR using a dNTP mix containing Fluorescein-dUTP

(2.5.8.2) and a pair of oligonucleotide primers either 500 or 1000bp apart. Two probes of different lengths were generated for each of the seven genes. It was assumed that the long probes would have more label incorporated into their sequence than the shorter probes and therefore be easier to detect *in situ*. However, this had to be balanced against the expectation that shorter probes would diffuse more readily into the parasite and therefore would be detectable *in situ* due to their high concentration. Unincorporated fluorescein-dUTP was removed by ethanol precipitation of the PCR products and subsequent washing in 70% ethanol (2.5.1.4). **Figure 4.1** shows a 1.5% agarose gel of the PCR product using oligonucleotides primers pairs either 500bp **(A)** or 1000bp apart **(B)** before (lane 1) and after (lane 2) ethanol precipitation.

4.3 Fluorescent *in situ* hybridisation (FISH)

Both the short (approx. 500bp) and the long (approx. 1000bp) fluorescein-labelled gene fragments were used in ISH on paraformaldehyde-fixed thin films of parasites prepared from an unsynchronised *P. falciparum* culture predominantly composed of trophozoites and schizonts (2.7). In all experiments where the short fluorescein-labelled gene fragments (approx. 500bp) probes were used, faint or in some cases no fluorescence was observed (data not shown). However, significantly bright fluorescence was achieved while using the long fluorescein gene fragments (approx. 1000bp). The results are shown on **Figure 4.2**.

PfRFC1 probe was 1015bp long and PfRFC2 probe was 974bp long. Both probes showed pockets of fluorescence on the peripheral regions of schizonts away from the nuclear region stained by DAPI. The PfRFC3 probe was 614bp long and, in contrast to PfRFC1 and PfRFC2, appeared to show fluorescence emanating from the nuclear region stained by DAPI. Similarly, the PfPCNA probe (804bp) showed fluorescence within the nuclear region stained by DAPI.

PfDNA POLδ, PfTOPO I and PfTOPO II probes all gave strong peripherally located fluorescence away from the DAPI stained nuclear regions in schizont forms of the parasites.

Figure 4.3 shows gametocyte at stage III of development probed with PfPCNA. Fluorescence appears to be located within the nuclear region. Diffuse fluorescence can also be seen in the cytoplasm.

4.4 FISH on DNase-treated parasite thin films

It was seen important to determine, to some extent, whether the fluorescence observed above was due to the binding of fluorescein-labelled probe to mRNA or to genomic DNA. To do this, paraformaldehyde-fixed thin films of asexual parasites were DNase-treated (2.7.3) prior to performing the ISH experiments (2.7.4). Due to time limitations, this was only attempted for PfTOPO I. The results of this experiment are shown in **Figure 4.4**. It can be seen from this figure that DAPI gave no signal implying that gDNA was almost completely eliminated following DNase-treatment. It can be seen that fluorescence was still present even after DNase-treatment. In this case, judging by the morphology, the parasite shown appears to be either a trophozoite or stage II gametocyte.

4.5 Conclusion

The result of the fluorescent *in situ* hybridisation (FISH) experiments described in this chapter using fluorescein-labelled PfRFC1 and PfRFC2 gene fragments showed pockets of fluorescence on the peripheral regions of schizonts away from the nuclear region stained by DAPI. However, in contrast to PfRFC1 and PfRFC2, PfRFC3 and PfPCNA appeared to show fluorescence emanating from the nuclear region of schizonts. PfDNA POLδ probes also showed fluorescence coming from the peripheral regions of the schizonts away from the nuclear region stained by DAPI. Probes against the topoisomerases, PfTOPO I and PfTOPO II, both gave strong peripherally located fluorescence away from the DAPI stained nuclear regions in schizont forms of the parasites. Fluorescence was still observed when the FISH experiments were repeated using fluorescein-labelled PfTOPO I gene fragments to probe DNase-treated thin films. However, the distinction between asexual and young gametocytes (stage I and II) of the parasites was difficult as no markers were used.

Although *in situ* hybridisation is theoretically particularly well adapted to the study of gene activity in specific cell types and at different stages in cell and tissue development, it has proved a difficult technique to operate in several laboratories. One obvious problem is ensuring that any signals observed are not the result of residual DNA, others have already been mentioned. These difficulties meant that there was no time to extend the analysis on each gametocyte developmental stage using fluorescein-labelled gene fragments developed for the gene of interest.

Figure 4.1 Agarose gel electrophoresis of Fluorescein PCR products

Panel **A** Short probes of approximately 1000bp in length

Panel **B** Long probes of approximately 500bp in length

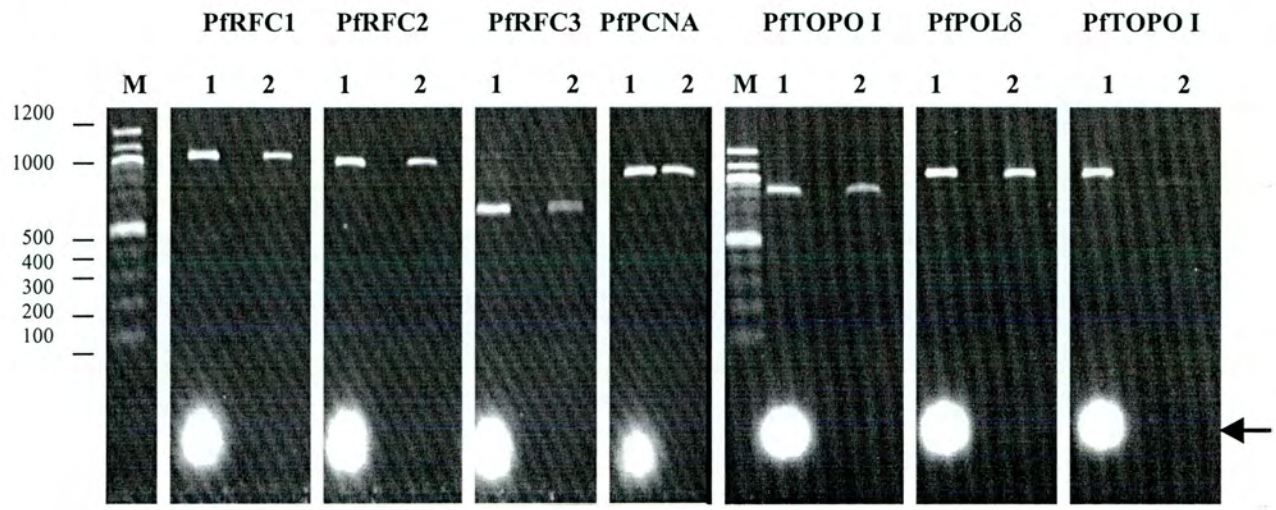
Lane1 PCR product before ethanol precipitation.

Lane 2 PCR product after ethanol precipitation and 70% ethanol wash.

M Molecular weight markers (size in kb)

Arrow shows unincorporated fluorescein-dUTP

(A)


PfRFC1
PfRFC2
PfRFC3
PfPCNA
PfTOPO I
PfPOLδ
PfTOPO I
M 1 2 1 2 1 2 1 2 M 1 2 1 2 1 2
1200
1000
500
400
300
200
100

(B)

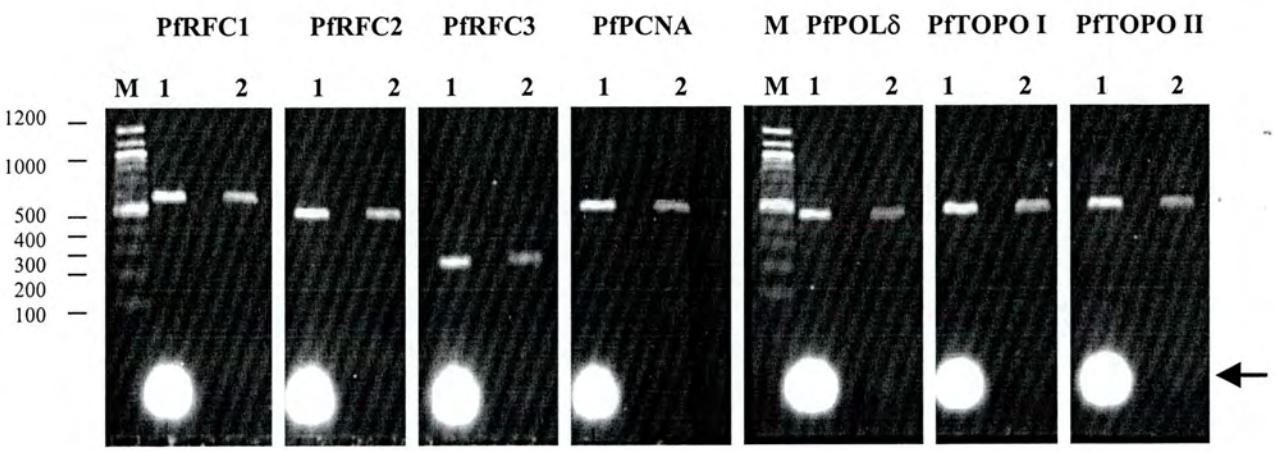

PfRFC1
PfRFC2
PfRFC3
PfPCNA
M PfPOLδ
PfTOPO I
PfTOPO II
M 1 2 1 2 1 2 1 2 M 1 2 1 2 1 2
1200
1000
500
400
300
200
100

Figure 4.2 Fluorescence *in situ* hybridisation on paraformaldehyde-fixed thin films of asexual parasites

Fluorescein-labelled gene fragments were used to screen paraformaldehyde-fixed thin films prepared from an unsynchronised *P. falciparum* culture predominantly composed of trophozoites and schizonts. Parasite nucleus was counter-stained with DAPI.

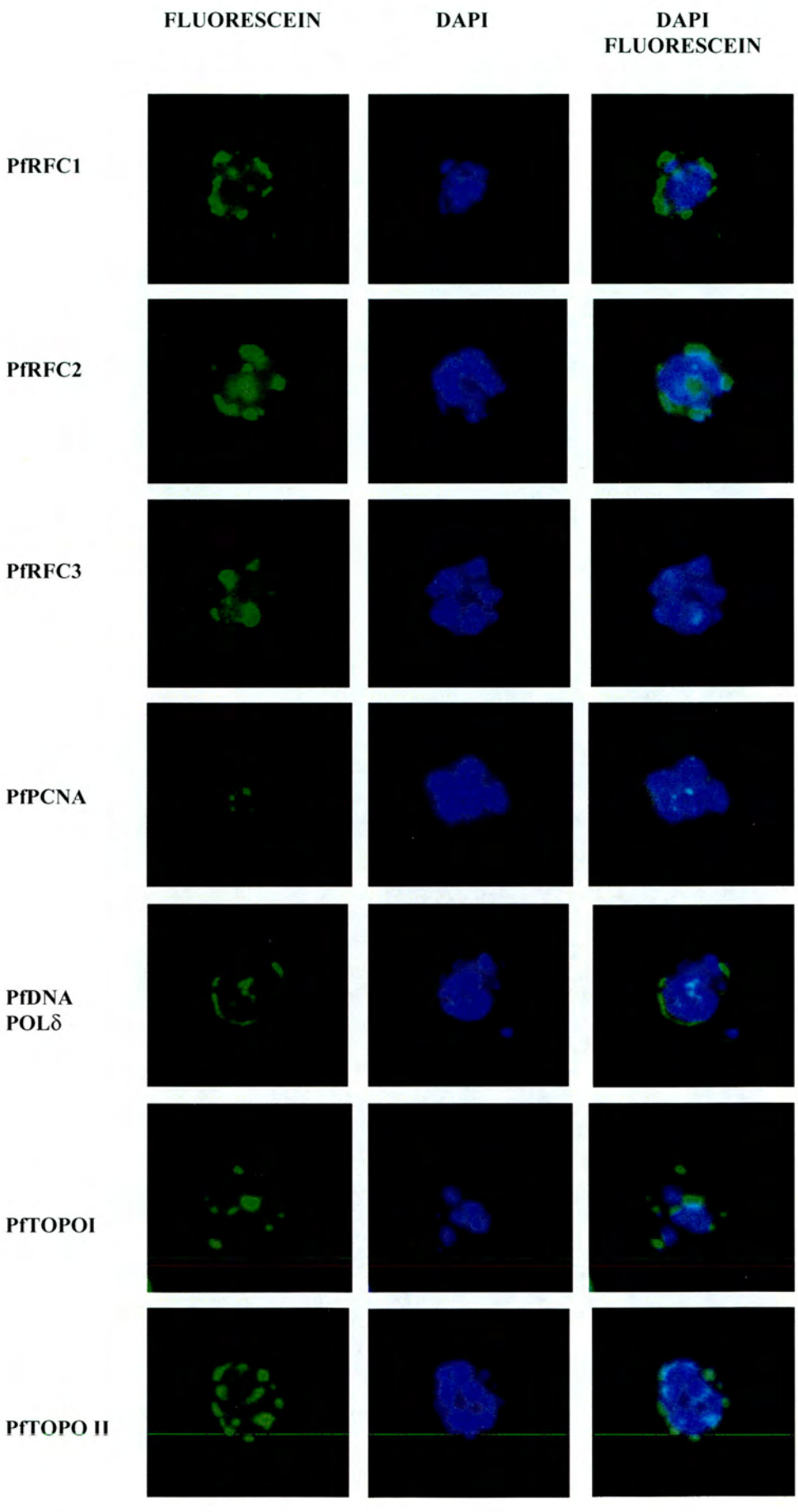
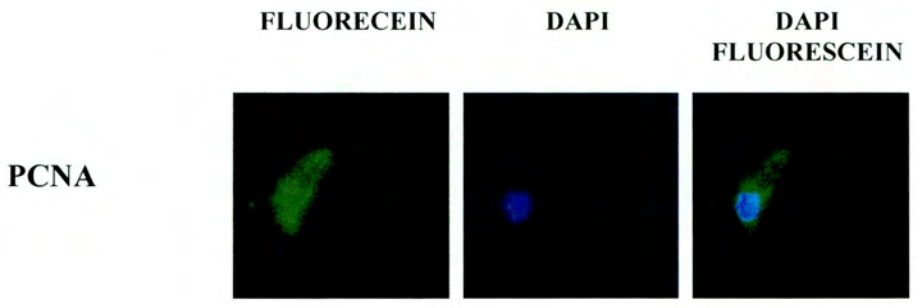

FLUORESCEIN
DAPI
DAPI
FLUORESCEIN
PfRFC1
PfRFC2
PfRFC3
PfPCNA
PfDNA
POLδ
PfTOPOI
PfTOPO II

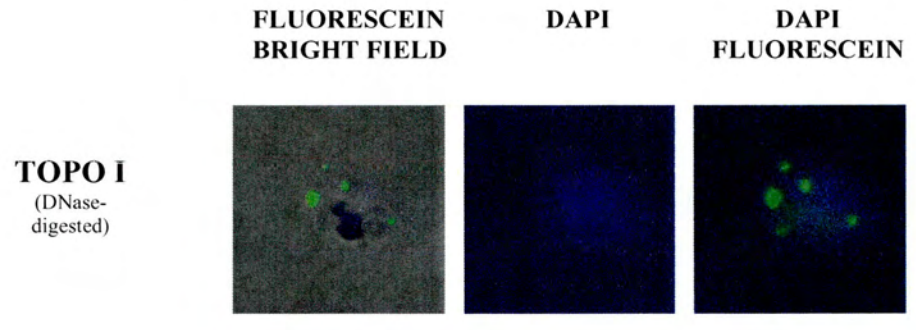
Figure 4.3 Fluorescence *in situ* hybridisation on a paraformaldehyde-fixed mixed culture using PfPCNA Fluorescein-labelled probe

A paraformaldehyde-fixed thin film prepared from a young gametocyte culture was probed with a Fluorescein labelled PfPCNA gene fragment. Parasite nucleus was counter-stained with DAPI.

Figure 4.4 Fluorescence *in situ* hybridisation on paraformaldehyde-fixed and DNase-treated thin film using PfTOPOI Fluorescein-labelled probe

A paraformaldehyde-fixed thin film was prepared from a young gametocyte culture was treated with DNase and then probed with a Fluorescein-labelled PfTOPOI gene fragment. Parasite nucleus was counter-stained with DAPI.


FLUORECEIN
DAPI
DAPI
FLUORESCEIN
PCNA


FLUORESCEIN
BRIGHT FIELD
DAPI
DAPI
FLUORESCEIN
TOPO I
(DNase-
digested)

CHAPTER 5

MATURE GAMETOCYTES AND THE DNA REPLICATION APPARATUS

5.1 Introduction

Several studies on the stage-specific expression of genes involved in DNA replication during the asexual cycle of *P. falciparum* have been carried out. Northern analysis has shown that transcripts of PfRFC1, PfRFC2 and PfRFC3 (Douglas J, 1999), PfPCNA and PfDNA POL δ (Horrocks *et al.*, 1996), PfTOPO I (Tosh *et* al., 1999) and PfTOPO II (Cheesman *et* al., 1998) accumulate primarily in the trophozoite stage of the asexual parasite cycle of *P. falciparum*. Similarly, the corresponding proteins have also been shown, by Western analysis, to accumulate in trophozoite stages. It is at the trophozoite stage of the asexual cycle that DNA synthesis takes place (Gritzmacher and Reese, 1984; Inselburg and Banyal, 1984).

In this chapter, Western analysis for the presence of PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopo II proteins in mature purified gametocytes, free of asexual parasites, will be described. RNA analysis using of northern and RT-PCR analysis of PfRFC1, PfRFC2, PfRFC3, PfPCNA, PfDNA POL δ, PfTOPO I and PfTOPO II in mature purified gametocytes, free of asexual parasites, will also be described. Gametocyte extracts, for the Western analysis, and total gametocyte RNA, for northern and RT-PCR analysis, was prepared from the same batch of gametocytes in order to permit simultaneous detection of specific proteins and their corresponding gene transcripts. Asexual parasites were used as positive controls.

5.2 Gametocyte culture and experimental conditions

Synchronised gametocyte cultures, free of asexual parasites, were cultivated to maturity (stage V) (2.3.4), purified on Percoll gradients (2.3.6), checked for absence of activation (2.3.5) and each gametocyte pellet divided into two parts. Total parasite protein was extracted from one sub-pellet (2.4.1) and total RNA was extracted from the second (2.6.1). Similarly, total protein and RNA was prepared from an asexual parasite culture. Western blot analysis was carried out on the total parasite protein (2.4.2) using rabbit polyclonal antisera against PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopo II. Northern analysis was carried out on the using

digoxigenin-labelled riboprobes specific for PfRFC1, PfRFC2, PfRFC3, PfPCNA, PfDNA POLδ, PfTOPO I and PfTOPO II (for methods see 2.6.5 to 2.6.11). Reverse transcriptase Polymerase Chain Reaction (RT-PCR) was carried out in two steps. DNase-digested total gametocyte RNA was reverse transcribed to make the 1st strand (cDNA). Small aliquots of the first strand were used as templates for standard PCR using gene-specific oligonucleotide primers approximately 500bp apart. Total RNA isolated from asexual parasites was treated in a similar manner and was to act as a positive control.

5.3 Western analysis of mature gametocytes

Protein extracts were prepared from asexual parasites and mature gametocytes and ran on 10% NuPAGE gels, transferred onto nitrocellulose and probed with rabbit polyclonal antisera raised against PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopo II (2.4.1 and 2.4.2). **Figure 5.1** shows the results of this experiment. Each lane contained equal amounts of whole parasite cell extracts (1μg/lane). It can be seen from this figure that mature gametocytes posses all five proteins, PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopo II, at relatively similar levels and bearing the same molecular weight as those detected in asexual parasites (indicated by arrows).

5.4 RNA analysis of mature gametocytes and asexual parasites

5.4.1 The preparation of digoxygenin-labelled gene-specific riboprobes

Approximately 1000bp fragment of each gene was amplified from genomic DNA (2.5.1.2) by standard PCR (2.5.8.1) using gene specific oligonucleotide primers PCR (see table 1). For ease of cloning, the PCR product was first cloned into pCR®2.1-TOPO® using a TOPO TA Cloning® kit (Invitrogen) and subsequently transformed into OneShot Cells (2.5.1.7). The PCR fragments were later re-cloned into pST18 (**Figure 5.2**) as *Bam*HI-*Xba*I fragments and grown in XL1 cells.

Figure 5.3 shows an agarose gel (1.5%) of the products following double restriction of pST18 with *Xba*I and *Bam*HI. PfRFC3 (lane 3) and PfDNA POL δ (lane 5)

fragments appear to have reduced in size from the initially cloned fragment of approximately 1000bp to about 300bp for PfRFC3 and 500bp for PfDNA Polδ. It was later discovered that both PfRFC3 and PfDNA POLδ gene fragments used contained a *Xba*I restriction site in their sequence.

The vector, pST18, contained transcription start sites for SP6 and T7 on either side of the multiple cloning site. DIG-labelled RNA fragments were synthesised using either SP6 or T7, depending on the orientation of the insert DNA fragment (2.6.7). Incorporation of DIG into the RNA probes was determined as described in section 2.6.8 and the results are shown in **Figure 5.4a**. From the figure, it can be seen that DIG had been incorporated into all the seven gene-specific RNA probes. The probes were detectable by NBT/BCIP colour substrate following cross-reaction with anti-DIG-Alkaline Phosphatase. These DIG-labelled RNA probes were used in the northern analysis described below.

However, it must be mentioned that the ability to detect these probes did not necessarily imply their sensitivity. A more appropriate test of probe sensitivity would have been carried out by spotting several dilution of the target, in this case pST18 bearing insert, on a nylon membrane, and probing these spots with a known amount of the probe.

5.4.2 Northern analysis of mature gametocytes and asexual parasite total RNA

Figure 5.4b shows the result obtained after probing northern blots, bearing equal amount of total RNA (2μg/lane) (2.2.5.9) from asexual parasites (lanes marked A) and gametocytes (lanes marked G), with the gene-specific DIG-labelled RNA probes shown in Figure 5.3a (section 2.6.5 to 2.6.11). Though faint in appearance, bands of the expected molecular weight (indicated by arrows) were detected in asexual parasites (lanes marked A) for PfRFC1 (4kb), PfRFC2 (1.6kb), PfRFC3 (1.8kb), PfPCNA (1.6kb), PfDNA POLδ (5.2kb) and PfTOPO II (7 and 8kb). No transcript was detected for PfTOPO I in asexual parasites. A blunt arrow indicates the position

at which the band for the PfTOPO I transcript was expected. In contrast, no bands were detected in the gametocytes lanes (marked G).

However, PfTOPO I transcripts have previously been detected, by northern analysis using radio-labelled probes, in total RNA prepared from asexual parasites (Tosh and Kilbey, 1995; Tosh et al., 1999). DIG-labelled RNA probes were used in this study and are thought to be as sensitive as radio-labelled DNA probes. It is possible that using the chemiluminescent substrate CSPD in place of the colour substrate NBT/BCIP used in this study (2.6.11) could have enhanced the sensitivity of the DIG-labelled RNA probes, however, CSPD was not available.

5.4.3 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

5.4.3.1 Detection of the presence of genomic DNA in the total RNA solution used for RT-PCR analysis

Unlike northern analysis, RT-PCR is very sensitive and almost always able to detect even minute quantities of genomic DNA (gDNA) within an RNA solution. It is therefore of paramount importance to reduce the amount of gDNA within a total RNA solution to undetectable levels. In this study the amount of gDNA was reduced, initially, by carrying out RNA extraction at an acidic pH of 4.1 (2.6.1) and then later on by subjecting the total RNA solution to a series of DNase-treatments, at least two treatments for total gametocyte RNA and four treatments for total asexual RNA (2.6.2).

The presence on gDNA was analysed by two methods. In the first method, two aliquots of equal amounts of total RNA were prepared; to one, reverse transcriptase (RT) was added and to the other, no RT was added. Standard PCR was then carried out using the 1st strand synthesis reaction product as template and gene-specific nucleotides as primers. The absence of a product following PCR on the minus-RT reaction implied that the RNA was not contaminated with detectable gDNA.

In the second method, a standard PCR was carried out using the 1st strand synthesis reaction product as template and oligonucleotide primers designed to anneal to the exon regions on either side of the intron.

Figure 5.5 shows the complete coding sequence of PfRFC3 gene. PfRFC3 contains an intron of 248bp located at the 5' end of the gene (Figure 4.3, intron in bold letters). The intron runs from 40bp to 298bp downstream of the proposed start **ATG**. Two oligonucleotides were designed complementary to the exons 5' and 3' to the intron. These were:

Forward oligonucleotide primer **F73488**, at position 013,

Reverse oligonucleotide primer **V7275**, at position 427.

In the **presence** of the intron, these oligonucleotides are (427-13 = 424) **424bp** apart. In the **absence** of the intron, these oligonucleotide would be (424-250 = 176) **176bp** apart. Therefore, amplification arising from genomic DNA is expected to give a PCR product of approximately 424bp whereas amplification arising from the amplification of cDNA, arising from the reverse transcription of mRNA, should give a PCR product of 176bp, a size difference of 248bp showing the absence of the intron.

5.4.3.1 RT-PCR analysis of total RNA from asexual parasites

First strand synthesis reaction (2.6.4) was carried out on 2μg each of DNase-treated total RNA (2.6.3) from asexual parasites and gametocytes; aliquoted from the same batch of total RNA used in the northern analysis described above. A control experiment without the addition of Reverse transcriptase was also carried out. The first strand synthesis reaction product was used as a template for standard PCR using gene-specific oligonucleotide primers approximately 500bp apart.

Figure 5.6 shows RT-PCR results obtained using asexual parasite RNA. All seven of the DNA replication genes, PfRFC1, PfRFC2, PfRFC3, PfPCNA, PfDNA POLδ, PfTOPO I and PfTOPO II. This findings are consistent with the result obtained from the northern analysis of the same total asexual RNA shown previously (Figure 5.3b), with the exception of PfTOPO I which was not detected on northern blots. It

is possible that failure to detect PfTOPO I transcripts in the asexual RNA by northern analysis was due to very low levels of transcripts detectable only by the more sensitive RT-PCR analysis.

5.4.3.2 RT-PCR analysis of total RNA from mature gametocyte

Figure 5.7 shows that RT-PCR results obtained using gametocyte RNA. From these results, it can be seen that gametocytes express, PfRFC2, PfRFC3, PfPCNA, PfDNA POLδ and PfTOPO I. This is in contrast to the results obtained following northern analysis of the same RNA (Figure 5.3) where only very faint bands were detected. However, no RT-PCR products were obtained when PfRFC1 and PfTOPO II oligonucleotides were used. This suggests that messages for these two genes are not present in mature gametocytes. However, while northern analysis was not able to detect transcripts of PfTOPO I in the gametocyte RNA, a product was obtained with RT-PCR. This is similar to the result found with asexual parasite RNA, and suggests that the levels of the PfTOPO I transcript in both asexual and gametocyte RNA was very low and detectable only by RT-PCR.

It is very important to stress that in both asexual and gametocyte RT-PCRs, the minus RT control experiments gave no products after PCR. In addition, there was an approximately 250bp size difference between the PCR products obtained with gDNA and RT-PCR using the same PfRFC3 oligonucleotide primer pairs F73488 and V7275. This suggests that the RNA used in the RT-PCR reaction was not contaminated with detectable gDNA and that the products observed in the 'RT-plus' lanes truly arise from the amplification of cDNA and not contaminating gDNA.

5.6 Conclusion

Western analysis has shown that PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopoII are present in mature gametocytes at similar levels and bear the same molecular mass as in asexual parasites.

Transcripts of PfRFC1, PfRFC2, PfRFC3, PfPCNA, PfDNA POLδ, PfTOPO I and PfTOPO II were difficult to detect in mature gametocyte total RNA following northern analysis. It is possible that the method used in this study was not sensitive enough to determine the presence or absence of these transcripts. It would be worthwhile to repeat the northern analysis of total gametocyte RNA but this time use radio-labelled RNA probes.

In contrast, RT-PCR carried out on the same pool of RNA used in the northern analysis, was able to detect the presence of PfRFC2, PfRFC3, PfPCNA, PfDNA POLδ and PfTOPO I transcripts in mature gametocytes. However, RT-PCR did not detect the presence of PfRFC1 and PfTOPO II transcripts. In the positive control RT-PCR experiments using asexual parasite RNA, all seven gene transcripts were detected. In this respect, the absence of PfRFC1 and PfTOPO II in the total gametocyte RNA is further evidence that it was free of contamination with asexual parasite RNA. Stringent controls using 'RT-minus' and intron-containing genes eliminated the possibility that the RT-PCR results were invalidated by contamination of the RNA with genomic DNA.

Figure 5.1 Western analysis of asexual parasites and mature gametocytes protein extracts

Protein extracts were prepared from unsynchronised asexual parasites and mature gametocytes were size separated on 10% NuPAGE gels, transferred onto nitrocellulose and probed with rabbit polyclonal antisera raised against PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopoII. Each lane contained equal amounts of whole parasite cell extracts (1μg/lane). Arrows indicate the position of protein.

Lane A Asexual parasite protein extract

Lane G Mature gametocyte protein extract

Lane M Molecular weight markers (size in kilo Daltons)

GEL Coomassie-stained gel.

IMM Immune antiserum

PRE Pre-immune serum

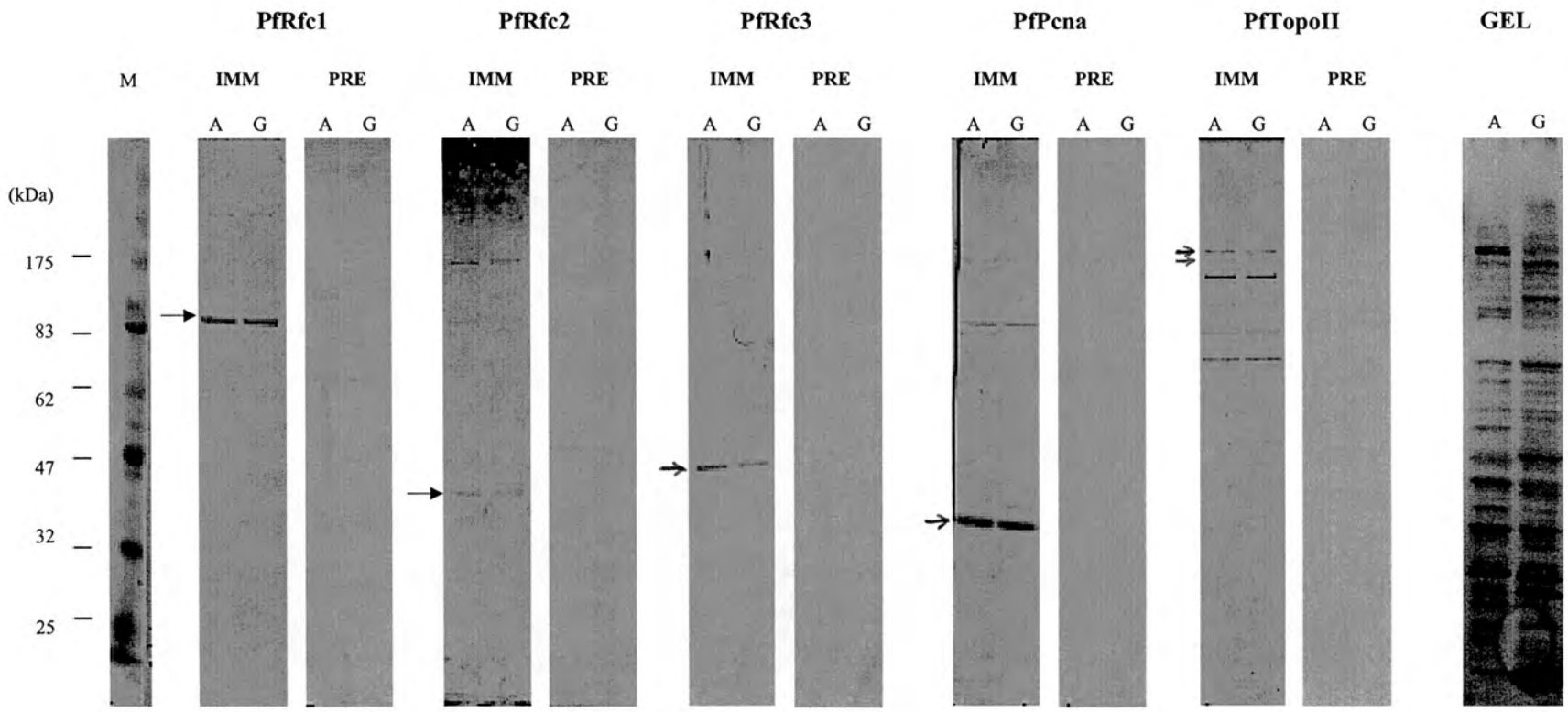

PfRfc1
PfRfc2
PfRfc3
PfPcna
PfTopoII
GEL
M
IMM
PRE
A G
(kDa)
175
83
62
47
32
25

Figure 5.2 The pST18 vector used in the synthesis of DIG-labelled riboprobes.

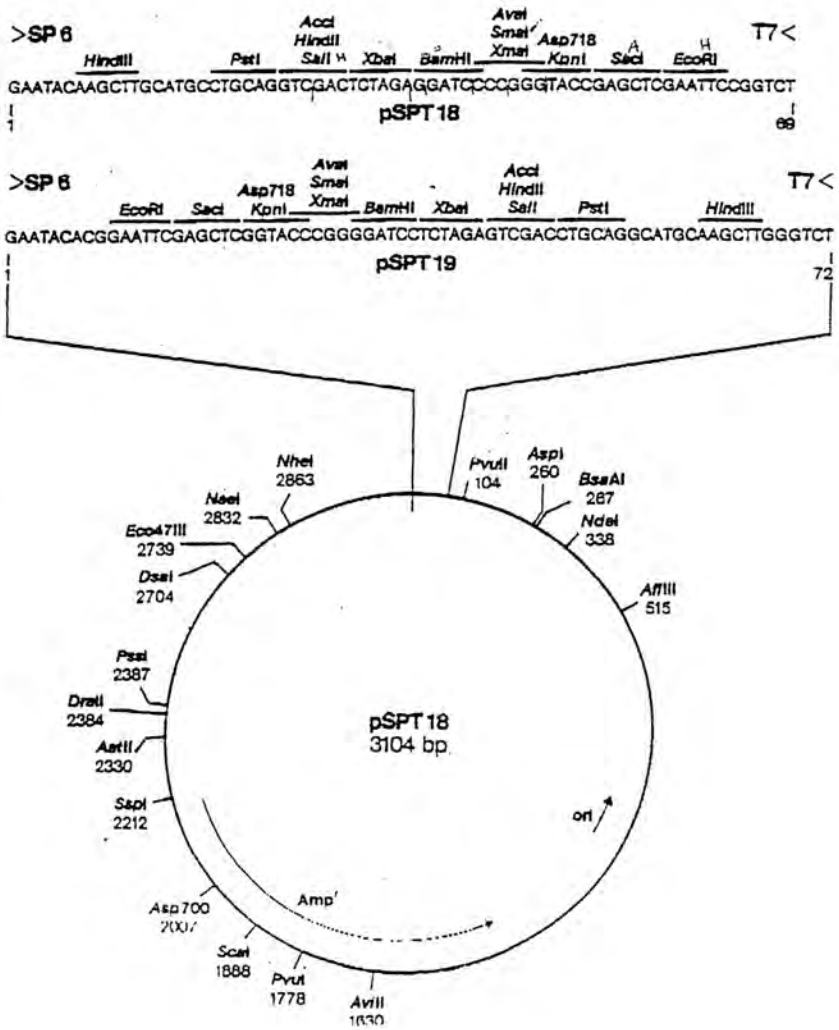


Figure 5.3 Double restriction of pST18 with *Xba*I and *Bam*HI.

Approximately 1000bp fragment of each gene was amplified from genomic DNA by standard PCR using gene specific oligonucleotide primers. PCR product was first cloned into pCR®2.1-TOPO® and later re-cloned into pST18 as *Bam*HI-*Xba*I fragments. pST18 was double restricted with *Bam*HI and *Xba*I and the reaction products analysed on a 1.5% agarose gel.

Lane1 PfRFC1

Lane2 PfRFC2

Lane3 PfRFC3

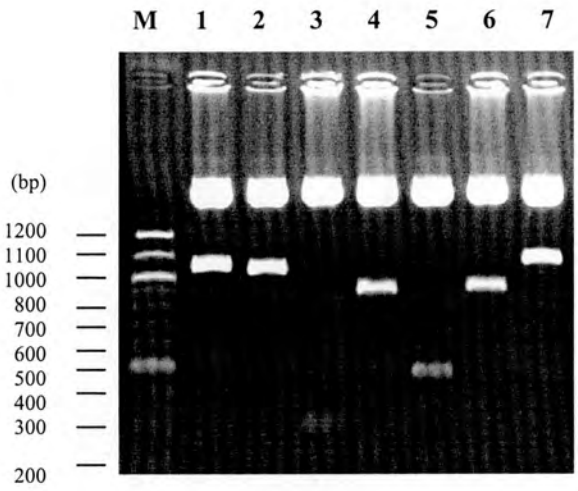
Lane4 PfPCNA

Lane5 PfDNA POLδ

Lane6 PfTOPO I

Lane7 PfTOPO II

M Molecular weight markers (sizes in base pairs)


M
1
2
3
4
5
6
7
(bp)
1200
1100
1000
800
700
600
500
400
300
200

5.4 Northern analysis of mature gametocytes and asexual parasite total RNA

(A) Confirmation of DIG incorporation into RNA probes

Incorporation of DIG into the RNA probes was confirmed by placing a small amount of RNA probe onto a nylon membrane followed by UV cross-linking. The probes were detected by NBT/BCIP colour substrate deposition following reaction with anti-DIG-Alkaline Phosphatase antibody. These DIG-labelled RNA probes were used in the northern analysis described in **B.**

(B) Northern analysis of mature gametocytes and asexual parasite total RNA using DIG-labelled gene-specific riboprobes

Northern blots, bearing equal amount of total RNA (2μg/lane) from asexual parasites and gametocytes were probed with gene-specific DIG-labelled RNA probes shown in Figure 5.4A. Hybridised probes were detected by NBT/BCIP colour substrate deposition following cross-reaction with anti-DIG-Alkaline Phosphatase.

Lane **G** Mature gametocyte total RNA

Lane **A** Asexual parasite total RNA

Sharp arrows indicate the major bands detected.

Blunt arrow indicated where a band was expected.

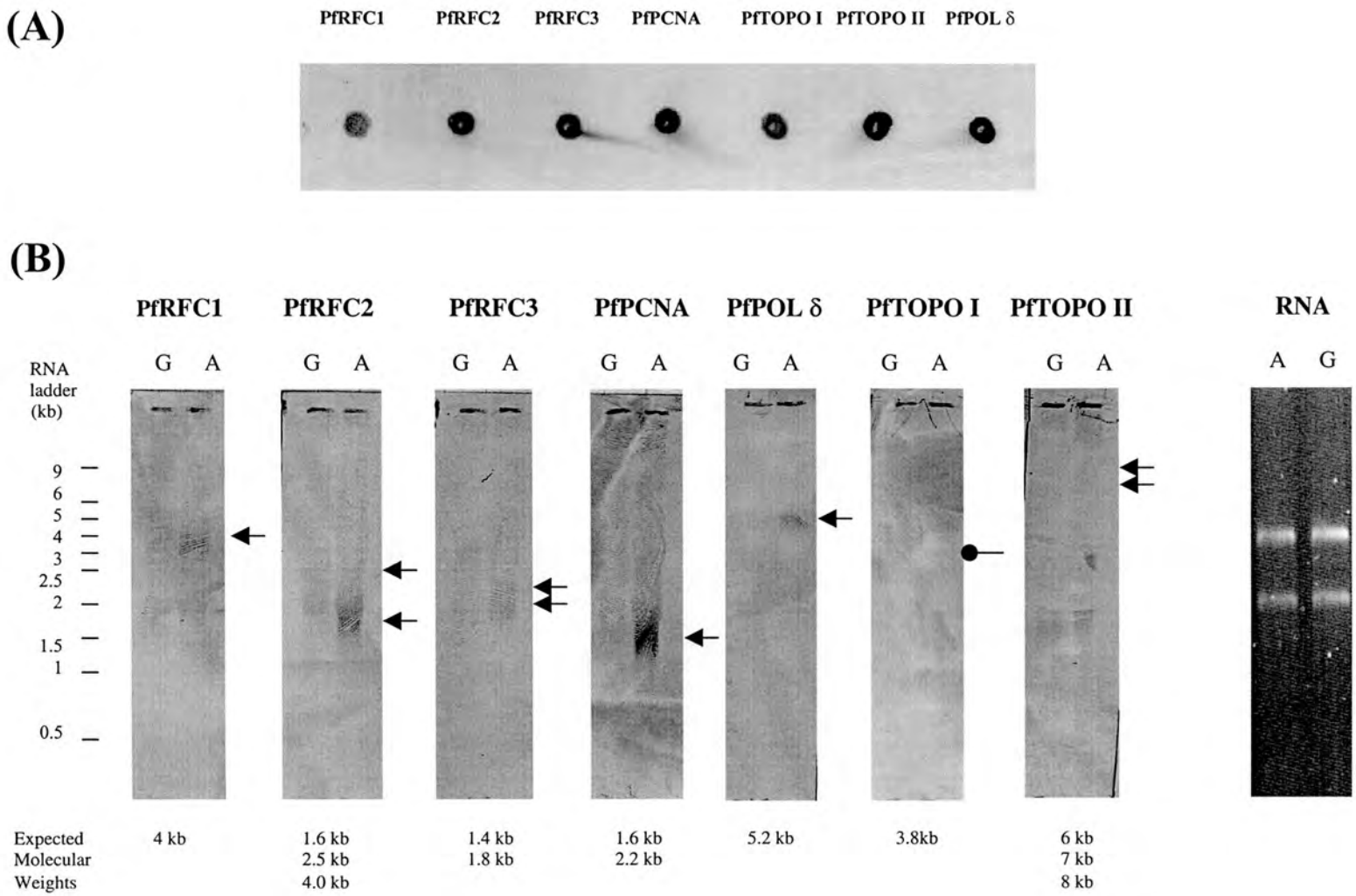

(A)
PfRFC1
PfRFC2
PfRFC3
PfPCNA
PfTOPO I
PfTOPO II
PfPOL δ
(B)
PfRFC1
PfRFC2
PfRFC3
PfPCNA
PfPOL δ
PfTOPO I
PfTOPO II
RNA
G A
A G
RNA ladder (kb)
9
6
5
4
3
2.5
2
1.5
1
0.5
Expected Molecular Weights
4 kb
1.6 kb
2.5 kb
4.0 kb
1.4 kb
1.8 kb
1.6 kb
2.2 kb
5.2 kb
3.8kb
6 kb
7 kb
8 kb

Figure 5.5 Complete nucleotide sequence of PfRFC3

The location and sequence of the synthetic oligonucleotide primers used in the RT-PCR analysis of parsasite RNA are shown.

PfRFC3 contains an intron of 248bp located at the 5' end of the gene; intron in bold letters. Two oligonucleotides were designed complementary to the exons 5' and 3' to the intron. These were:

Forward oligonucleotide primer **F73488**, at position 013,

Reverse oligonucleotide primer **V7275**, at position 427.

In the **presence** of the intron, these oligonucleotides are (427-13 = 424) **424bp** apart.

In the **absence** of the intron, these oligonucleotide would be (424-250 = 176) **176bp** apart.

The arrows point in the 5' to 3' direction of the sense and antisense strands. The sequence of the reverse primer was complementary to the sequence encased in the box.

START

```
   1 ATGACCGAAG TTGAACAACA AAGAGGAAGT GAACTAACCC CATGGGTATA TAGAAAAGAT
                 ---------->
                 F 73488

  61 TACGAATGAT TATATAAAAA GAGTTATATT TTTTATATAT ATGAAAATGT GTATTATTAT
 121 AAATTATATT TTGATTTGTT TCCATTTATA TATAATAAAA TGAGAAATGG AATTATATAT
 181 ATATATATAT ATATATATAT ATATTTATTT ATCTAATTTT TGAACACTTG TAATATTTGT
 241 TGAACAAAAA TAGTCATACA TATATTATTT TTAATTTATA TTTTTTTTTT TATTTTAGGT
 301 TGAAAAATAT AGACCAAATG TACTAAATGA TATAATATCG CATGAACAAG TAATATCAAC
 361 TATTAAAAGA TTCGTTCAGA AAGGTGAGTT ACCACATTTA CTTTTACATG GTCCCCCAGG
                                                 <.....
                                                 V7275

 421 TACAGGGAAA ACGTCTACGA TATTGGCTGT GTGTAAAGAA TTATATGGAG ATAAGAGAAG
 481 TTCATTTGTT TTAGAATTGA ATGCTTCTGA TGATAGAGGT ATAAACGTTA TTCGTGATCA
 541 AATAAAAACA TTTGCTGAAT CAAAAAATCA TTATACAACA TGTGAAAAAA CAACTTTAAA
 601 ATTAATTATT TTAGATGAAG CAGATCATAT GACATATCCT GCTCAAAACG CTATGAGAAG
 661 GATTATGGAG AACTATGCTA AAAATGTGCG TTTTTGTTTG TTATGTAATT ATGTAAATAA
 721 AATAACTCCA GCAATACAAT CTAGATGTAC GGCTTTTCGT TTTGCCCCTT TAAAAAAAGA
 781 ATATATGAAA AATAAAGCAC TAGATATAGC AAAATCTGAG AATGTAAATC TAACTGAAGG
 841 AGGAATAGAT AGTCTTATAC GTGTAGGACA TGGAGATATG AGAAGAATTT TAAATTGTTT
 901 ACAAGTAGTA TCATTAAGTC ATAAAAATCT TGTCATTGAT GAAAATGTTA TTTTATCAAC
 961 ATTAGATATC CCATTACCTA GTGAAACCAA GAAAATATTG GAATATTTTA CAAAAGGTTC
1021 TATAAAAGAA TCATACGAAT TTGTTAGTAA TTTACAATAT GATAAAGGAT ATTCAACAAA
1081 AGATATTATG ATGTGTTTAT ATGAATCAGT TTTAACATAT GATTTCCCTG ATTCGGCTTT
1141 TTGTCTTCTA CTTAAAAATT TTGGTGAAAT AGAAGAAAGA TGTTCTTCTG GAGCTAGCGA
1201 ACAAATTACT TTATCTGCTT TAATTAGTGC ATTCGTAGAA TTTCGAACAG AACTTTTCAA
1261 ATTAAAATAT GATATGAGCA ACATATAA
                              STOP
```

Figure 5.6 RT-PCR analysis of total RNA from asexual parasites

First strand synthesis reaction was carried out on 2μg each of DNase-treated total RNA from asexual parasites; aliquoted from the same batch of total RNA used in the northern analysis described above. A control experiment without the addition of Reverse transcriptase was also carried out. The first strand synthesis reaction product was used as a template for standard PCR using gene-specific oligonucleotide primers approximately 500bp apart.

\+ PCR on 1st strand synthesis reaction in which RT was added.

\- PCR on 1st strand synthesis reaction in which no RT was added

C PCR on gDNA using the same set of primers

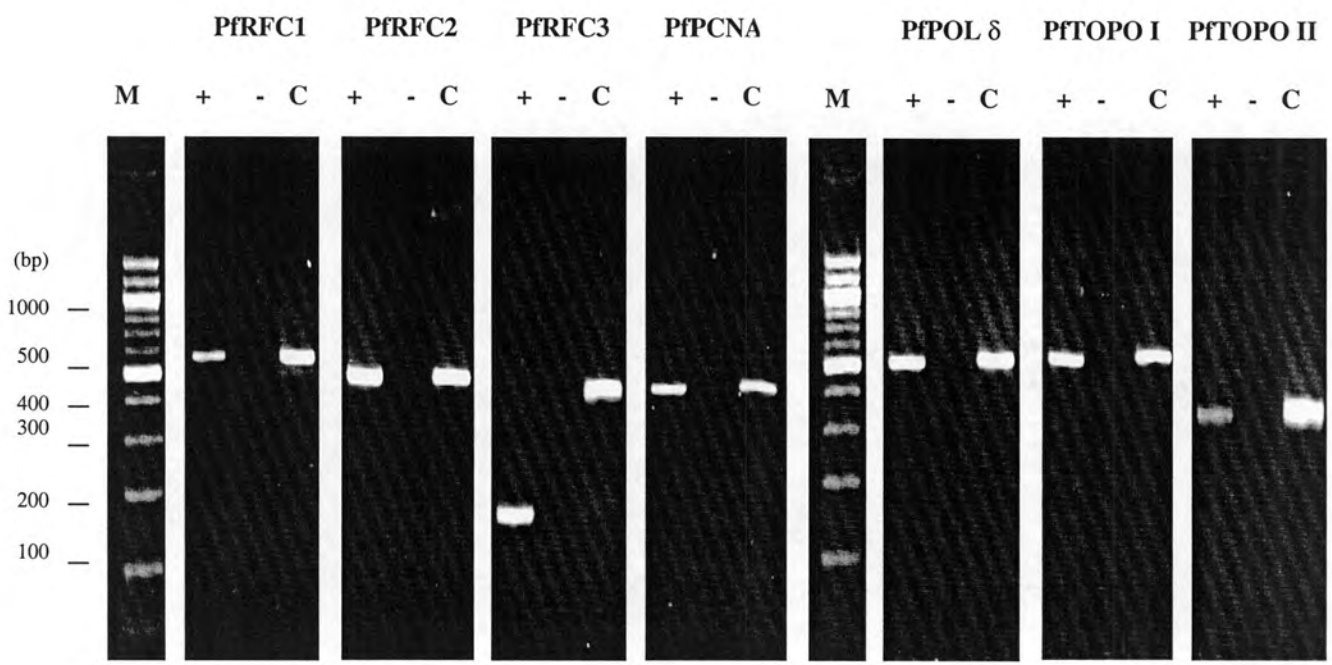

PfRFC1
PfRFC2
PfRFC3
PfPCNA
PfPOL δ
PfTOPO I
PfTOPO II
M
+ - C
(bp)
1000
500
400
300
200
100

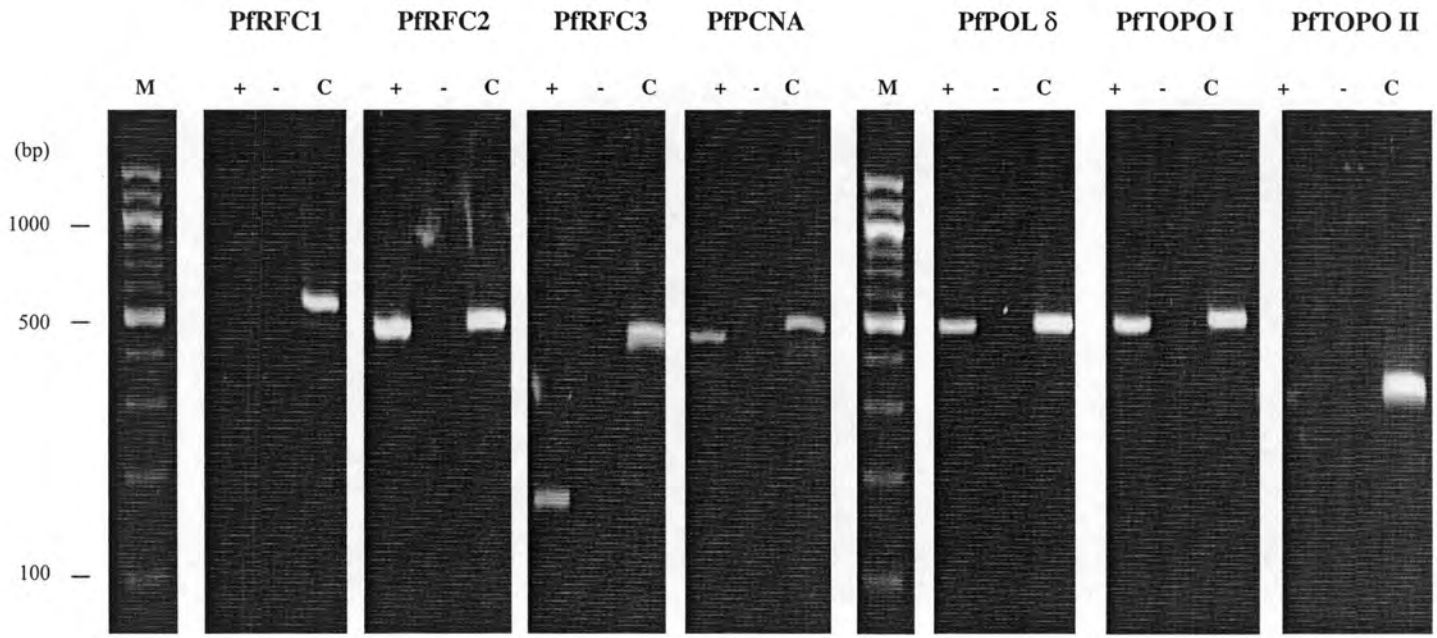
Figure 5.7 RT-PCR analysis of total RNA from mature gametocytes

First strand synthesis reaction was carried out on 2μg each of DNase-treated total RNA from mature gametocytes; aliquoted from the same batch of total RNA used in the northern analysis described above. A control experiment without the addition of Reverse transcriptase was also carried out. The first strand synthesis reaction product was used as a template for standard PCR using gene-specific oligonucleotide primers approximately 500bp apart.

\+ PCR on 1st strand synthesis reaction in which RT was added.

\- PCR on 1st strand synthesis reaction in which no RT was added

C PCR on gDNA using the same set of primers


PfRFC1
PfRFC2
PfRFC3
PfPCNA
PfPOL δ
PfTOPO I
PfTOPO II
M
+ - C
(bp)
1000
500
100

CHAPTER 6

GAMETOGENESIS AND THE DNA REPLICATION APPARATUS

6.1 Introduction

In the previous chapter, it was shown, by Western analysis, that PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopo II proteins were present in mature gametocytes before activation. It was also shown, by RT-PCR, that gene transcripts for PfRFC2, PfRFC3, PfPCNA, PfDNA POL δ and PfTOPO I were present in mature inactivated gametocytes however, PfRFC1 and PfTOPO II transcripts were not detected.

By extension of the result obtained in the previous chapter, the components of the DNA replication apparatus were analysed during the first fifteen minutes of gametogenesis by Western analysis, to follow the protein levels, and simultaneously by RT-PCR, to check for the lack of, or continued presence of gene transcripts of PfRfc1, PfRfc2, PfRfc3, PfPcna, PfDNA Polδ, PfTopoI and PfTopoII proteins.

6.2 Gametocyte culture and experimental conditions

In the experiments described in this chapter, it was critical that the gametocyte cultures used were to be as free of asexual parasite as possible. In order to achieve this, gametocyte cultures grown in CCMA (2.2) were either depleted of asexual parasites using a series of sorbitol treatments (Lambros and Vanderberg, 1979) or treated with GluNAc supplemented CCMA (Ponnudurai *et al.*, 1986). Each set of cultures was tested for its ability to activate (2.3.5) and the gametocytes concentrated and purified by Percoll gradient centrifugation (2.3.6). Gametogenesis was induced (2.3.7) and pellets from each set, were divided into two portions to enable the simultaneous assays of protein and mRNA by Western blotting and RT-PCR analysis, respectively.

Another batch of gametocytes, grown entirely in CCMS and depleted of asexual parasites by GluNAc treatment, was treated similarly. Gametocytes grown in CCMS, when resuspended in GSM, activate and progress on to exflagellate ten to 15 minutes after induction of gametogenesis. However, gametocytes grown entirely in CCMA activate but do not progress on to exflagellate. The aim of including this set of gametocytes was to determine whether the profiles of DNA replication proteins

and their transcripts during gametogenesis were different in gametocytes that retained the ability to exflagellate and those that had lost it.

6.3 Western analysis of during gametogenesis

The gametocytes used in the Western analysis experiments were grown entirely in CCMA synchronised and depleted of asexual parasites by GluNAc treatment after the onset of gametocytogenesis (2.3.4). These gametocytes, when resuspended in GSM, rounded up (activated) but did not progress on to exflagellate. **Figure 6.1** shows a Coomassie stained SDS-PAGE gel of protein extracts prepared from gametocyte pellets before and after activation, at 5 minute intervals (2.3.7). From this figure, it can be seen that each lane had similar amounts of total gametocyte extracts loaded (1µg/lane). Several sets of similar gels were prepared, blotted and probed with rabbit polyclonal antisera against PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopoII (below).

Figure 6.2a, b, c, d and **e** show Western blot analysis of gametocyte extracts before and after activation. From each of these figures, it can be seen that PfRfc1, PfRfc3, PfPcna and PfTopoII protein levels did not change markedly throughout gametogenesis, as judged from the similar band intensities (arrows). However, PfRfc2 appear to increase after 5 minutes of activation and then gradually decrease as gametogenesis progresses.

Similar results were observed when CCMA-grown and sorbitol-treated or CCMS-grown and GluNAc-treated gametocytes were analysed by Western blotting (data not shown).

6.4 RT-PCR analysis of gametogenesis

6.4.1 RT-PCR analysis of CCMA-grown and GluNAc-treated gametocytes

Figure 6.3 shows an RT-PCR analysis of total RNA prepared from the second set of twin pellets whose protein profile and Western analysis are shown on Figure 6.1

and 6.2a-e. Panel A shows RT-PCR analysis carried out using gene-specific oligonucleotide primers on 1st strand synthesised from total RNA isolated from the timed gametogenesis experiments. Transcripts for PfRFC2, PfRFC3, PfPCNA, PfDNA POLδ and PfTOPO I are detected in mature gametocytes before and after activation. Both PfRFC1 and PfTOPO II mRNA appear not to be present in mature gametocytes and are not detected even after activation. This is consistent with the findings in the previous chapter where PfRFC1 and PfTOPO II transcripts were not detected in mature inactivated gametocytes.

As mentioned earlier, PfRFC3 contains an intron of 250bp and was used to control for the absence of genomic DNA in the RNA samples. In Figure 6.3, the PfRFC3 panel shows the size difference in the PCR product obtained following the use of gDNA as template and when the 1st strand synthesis reaction product was used, is roughly 250bp; the size of the intron. This implies that the products seen on the 'RT-plus' (marked +) lanes arise from amplification of cDNA derived from mRNA. This shows that the total RNA used (**Panel B**) in each case was free of detectable gDNA and that the results can be taken to reflect the true gene expression patterns during gametogenesis. In addition, the 'minus-RT' control gave no products following PCR although the positive controls using gDNA (lanes marked C) confirmed that the oligonucleotide primers function.

6.4.2 RT-PCR analysis of CCMS-grown and GluNAc-treated gametocytes

Figure 6.4 shows the RT-PCR analysis of CCMS-grown and GluNAc-treated gametocytes. Panel A shows the RT-PCR results while Panel B shows a sample ran from the pool of total RNA used for the RT-PCR experiments. The results in this figure are similar to those seen in Figure 6.3 for gametocytes grown in CCMA and treated with GluNAc to eliminate asexual parasites.

6.4.3 RT-PCR analysis of CCMA-grown and sorbitol-treated gametocytes

Figure 6.5 shows RT-PCR analysis of total RNA prepared from CCMA-grown gametocytes and sorbitol-treated gametocytes before and during gametogenesis. **Panel A** shows the RT-PCR results while **Panel B** shows a sample ran from the pool of total RNA used for the RT-PCR experiments. From Panel A, it can be seen all seven gene transcripts, PfRFC1, PfRFC2, PfRFC3, PfPCNA, PfDNA POLδ, PfTOPO I and PfTOPO II, were detected. PfRFC1 and PfTopo II were previously not detected in CCMS-grown and GluNAc-treated gametocytes (see Figure 6.4). The size difference observed in PfRFC3 between the control, gDNA as template, and the RT-PCR product suggests that the total RNA used in each time point of gametogenesis was free of detectable gDNA. In addition, the minus RT control gave no products following PCR, further implying that signals seen in the RT-PCR arise from mRNA.

6.5 Topoisomerase II activity during gametogenesis

So far, the presence immunofluorescence assays and Western blotting analysis has confirmed the presence of PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopoII throughout gametocytogenesis and gametogenesis. However, DNA synthesis has only been confirmed to take place at the onset of gametocytogenesis and gametogenesis. This implies that the presence of components of the DNA replication apparatus does not necessarily mean that DNA synthesis is actively taking place as some components of the DNA replication apparatus may be present but not active. It is important, therefore, to carry out activity assays of some these proteins. At present, PfTopoI and PfTopoII activities can be assayed *in vitro*. In this study, the activity of PfTopoII was followed before and after activation.

6.5.1 Western analysis of gametogenesis extracts used for the PfTopoII activity assay

Gametocyte cultures, synchronised and free of asexual parasites by treatment with GluNAc, were cultivated to maturity (2.3.4), concentrated and purified on Percoll gradients (2.3.6) and pellet tested for activation (2.3.5) and divided into triplets. One pellet was stored and represented mature inactivated gametocytes. Gametogenesis was induced in the remaining two pellets one pellet collected immediately after induction and the other five minutes later (2.3.7). Protein extracts were prepared (2.4.5) from each of the pellets and triplicate sets of the ran on a 10% NuPAGE gel. Part of the gel containing one set of gametogenesis protein extracts was stained with Coomassie stain and is shown on **Figure 6.6**. From this figure, it can be seen that each lane contained similar amounts of protein extracts.

The remaining two sets were transferred onto nitrocellulose and probed with rabbit polyclonal antiserum against PfTopoII and rabbit pre-immune serum. The results are shown on **Figure 6.7**. Judging from the intensity of the triplets bands, it is apparent that the levels of PfTopoII remain unchanged as gametogenesis progressed.

6.5.2 Decatenation assay for the detection of PfTopoII activity in gametogenesis extracts

Type II topoisomerase is able to cleave both strands of a duplex DNA and hence can resolve catenated DNAs such as *Crithidia fasciculata* kinetoplast DNA (kDNA) which consists of mini and maxicircles (Marini *et al.*,1980). Decatenation of kDNA by type II topoisomerases results in the formation of nicked open circular (OC) and relaxed or covalently closed circular (CCC) decatenated kDNA. When the reaction product are run on an agarose gel, the CCC decatenated kDNA migrates fastest followed by the OC decatenated kDNA. Catenated kDNA is too dense and remains in the well. Linearized kDNA runs between CCC and OC decatenated and indicates DNase activity.

Figure 6.8 shows the decatenation activity, attributed to PfTopo II, in gametocyte protein extracts prepared before and after activation. The presence of OC and CCC decatenated kDNA implies that there is some level of decatenation activity in mature inactivated gametocytes (I) though most of the kDNA input is still retained in the well. The decatenation activity can be seen to clearly increase immediately after activation (A0) as more kDNA is released from the well. The decatenation activity is highest five minutes after activation (A5) when all the input kDNA can be seen to have been released from the well.

6.6 Conclusion

Western blot analysis of gametocyte protein extracts before and after activation showed no significant changes in the levels of PfRfc1, PfRfc3, PfPcna and PfTopoII protein throughout gametogenesis, as judged from the similar band intensities. However, PfRfc2 increased within five minutes of activation and then decreased progressively as gametogenesis progressed. In the IFA results shown earlier, PfRfc2 was seen to be distinctly absent from the nucleus of stage V gametocytes. Taken together, the IFA and Western analysis results on the expression patterns of PfRfc2 in mature gametocytes before and after activation, appear to be different from that of the other DNA replication proteins tested and in particular, that of PfRfc1 and PfRfc3. It is possible that PfRfc2 may be actively synthesised and transported to the nucleus following activation of mature gametocytes. However, further experiments, such as the *in situ* detection of PfRfc2 during gametogenesis, require to be carried out in order to get to a better understanding of the role of PfRfc2 during gametogenesis.

Analysis of total gametocyte RNA using RT-PCR detected the presence of transcripts of PfRFC2, PfRFC3, PfPCNA, PfDNA POLδ, PfTOPO I and PfTOPO II throughout gametogenesis. However, PfRFC1 and PfTOPO II transcripts were not detected. Similar Western and RT-PCR results were obtained regardless of whether the mature gametocytes were capable of exflagellating (CCMS-grown) or not (CCMA-grown). However, PfRFC1 and PfTOPO II transcripts were only detected in sorbitol treated gametocytes, possibly indicating the presence of some asexual parasites.

Topoisomerase II activity was assayed in gametocyte protein extracts prepared during the first 5 minutes of gametogenesis. Decatenation activity assays showed that topoisomerase II activity, though present in mature inactivated gametocytes, progressively increased during gametogenesis.

Figure 6.1 Coomassie stained gel of protein extracts prepared from mature gametocyte pellets before and after activation

Mature gametocytes were grown entirely in CCMA, synchronised and depleted of asexual parasites by GluNAc treatment. These gametocytes were purified and concentrated on Percoll gradients and divided into four equal portions. Three of the portions were activated by resuspension in GSM and the gametogenesis stopped at 5 minutes intervals. Each of the four portions was split into two. Total RNA was prepared from one set and total protein extract from the other set. Equal volumes of parasite extracts were size separated on 10% NuPAGE gels and stained with Coomassie Blue (2.4.2.2).

Lane I Mature gametocytes before activation

Lane A5 Mature gametocytes 5 minutes after activation

Lane A10 Mature gametocytes 10 minutes after activation

Lane A15 Mature gametocytes 15 minutes after activation

Positions of molecular weight markers are indicated (New England Biolabs)

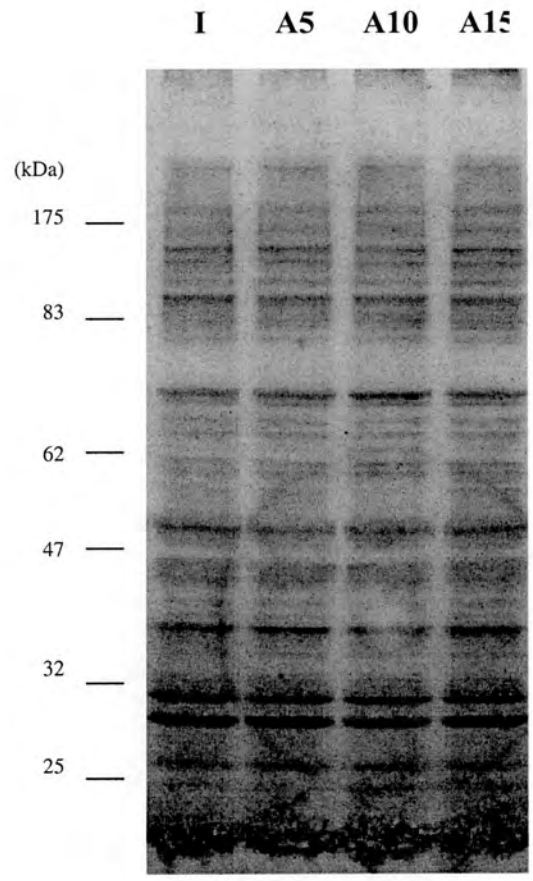

I
A5
A10
A15
(kDa)
175
83
62
47
32
25

Figure 6.2 Western blot analysis of protein extracts prepared from gametocyte pellets before and after activation

A anti PfRfc1 rabbit polyclonal antiserum at 1:200 dilution

B anti PfRfc2 rabbit polyclonal antiserum at 1:200 dilution

C anti PfRfc3 rabbit polyclonal antiserum at 1:200 dilution

D anti PfPcna rabbit polyclonal antiserum at 1:1000 dilution

E anti PfTopoII rabbit polyclonal antiserum at 1:500 dilution

Lane **I** Mature gametocytes before activation

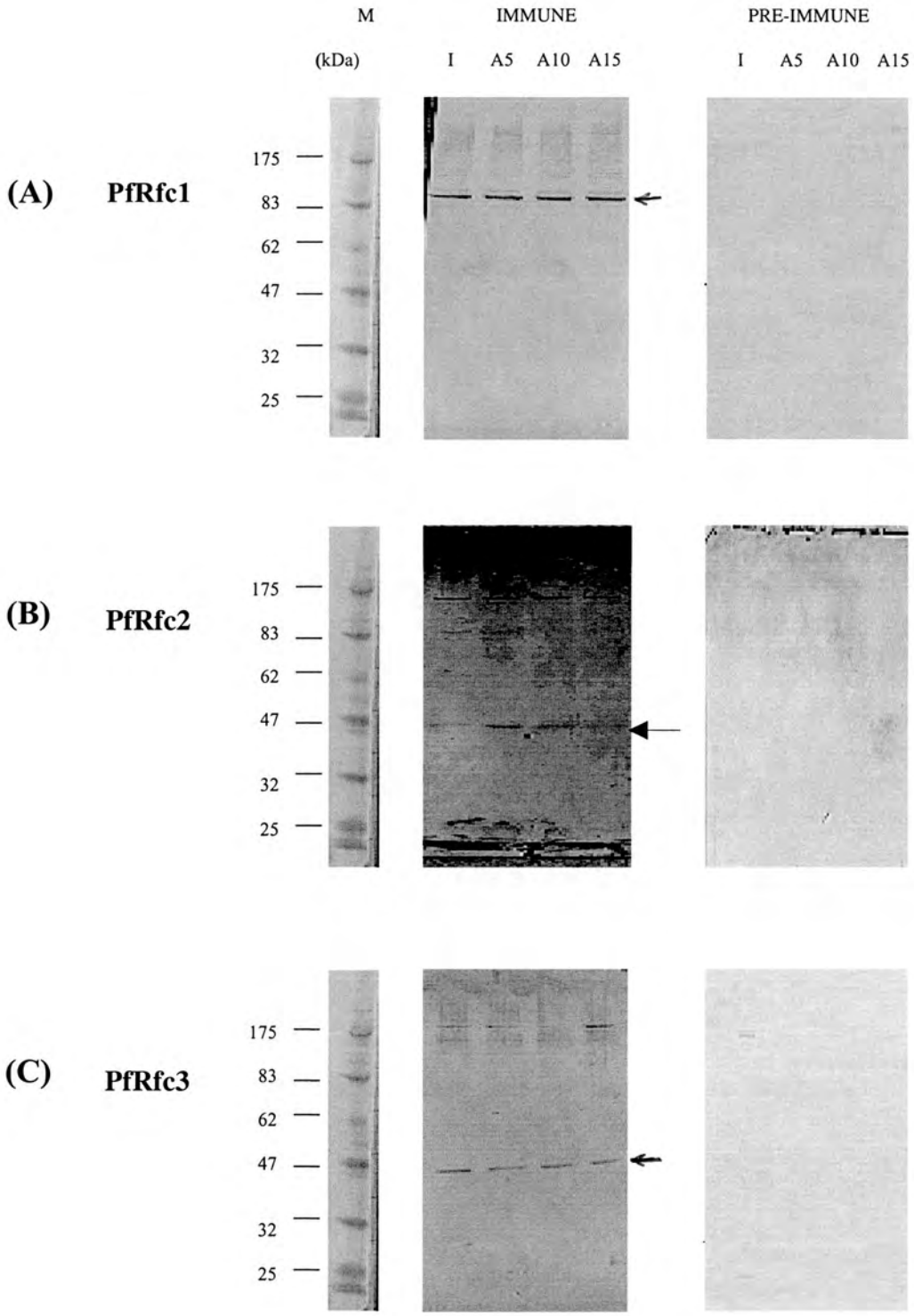
Lane **A5** Mature gametocytes 5 minutes after activation

Lane **A10** Mature gametocytes 10 minutes after activation

Lane **A15** Mature gametocytes 15 minutes after activation

Lane **M** Molecular weight markers (New England Biolabs)

Arrows indicate the positions of target proteins


M
IMMUNE
PRE-IMMUNE
(kDa)
I
A5
A10
A15
I
A5
A10
A15
(A)
PfRfc1
175
83
62
47
32
25
(B)
PfRfc2
175
83
62
47
32
25
(C)
PfRfc3
175
83
62
47
32
25

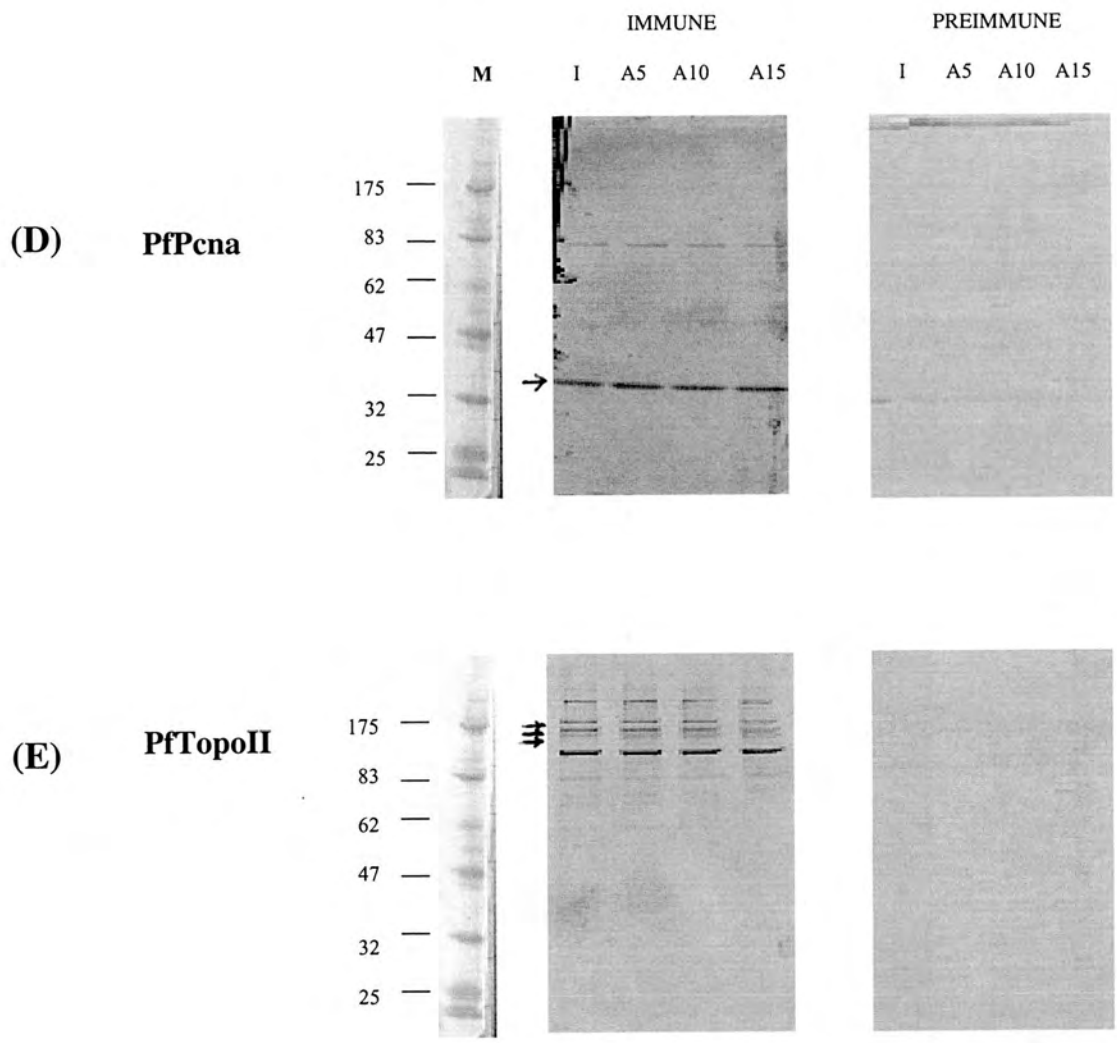

(D)
PfPcna
IMMUNE
PREIMMUNE
M
I
A5
A10
A15
I
A5
A10
A15
175
83
62
47
32
25
(E)
PfTopoII
175
83
62
47
32
25

Figure 6.3 RT-PCR analysis of CCMA-grown and GluNAc-treated gametocytes

Total RNA was prepared from the second set of twin pellets whose protein profile and Western analysis are shown in Figure 6.1 and 6.2a-e. RT-PCR analysis carried out using gene-specific oligonucleotide primers on 1st strand synthesised from total RNA isolated from the timed gametogenesis experiments. PCR products were analysed on 2% agarose gels.

A Agarose gel of the RT-PCR products

B A sample of the RNA used for the initial 1st strand synthesis reaction

Lane I	Mature gametocytes before activation
Lane A5	Mature gametocytes 5 minutes after activation
Lane A10	Mature gametocytes 10 minutes after activation
Lane A15	Mature gametocytes 15 minutes after activation
M	Molecular weight markers in kilobases (Ambion)

\+ PCR on 1st strand synthesis reaction in which RT was added.

\- PCR on 1st strand synthesis reaction in which no RT was added

C PCR on gDNA using the same set of primers

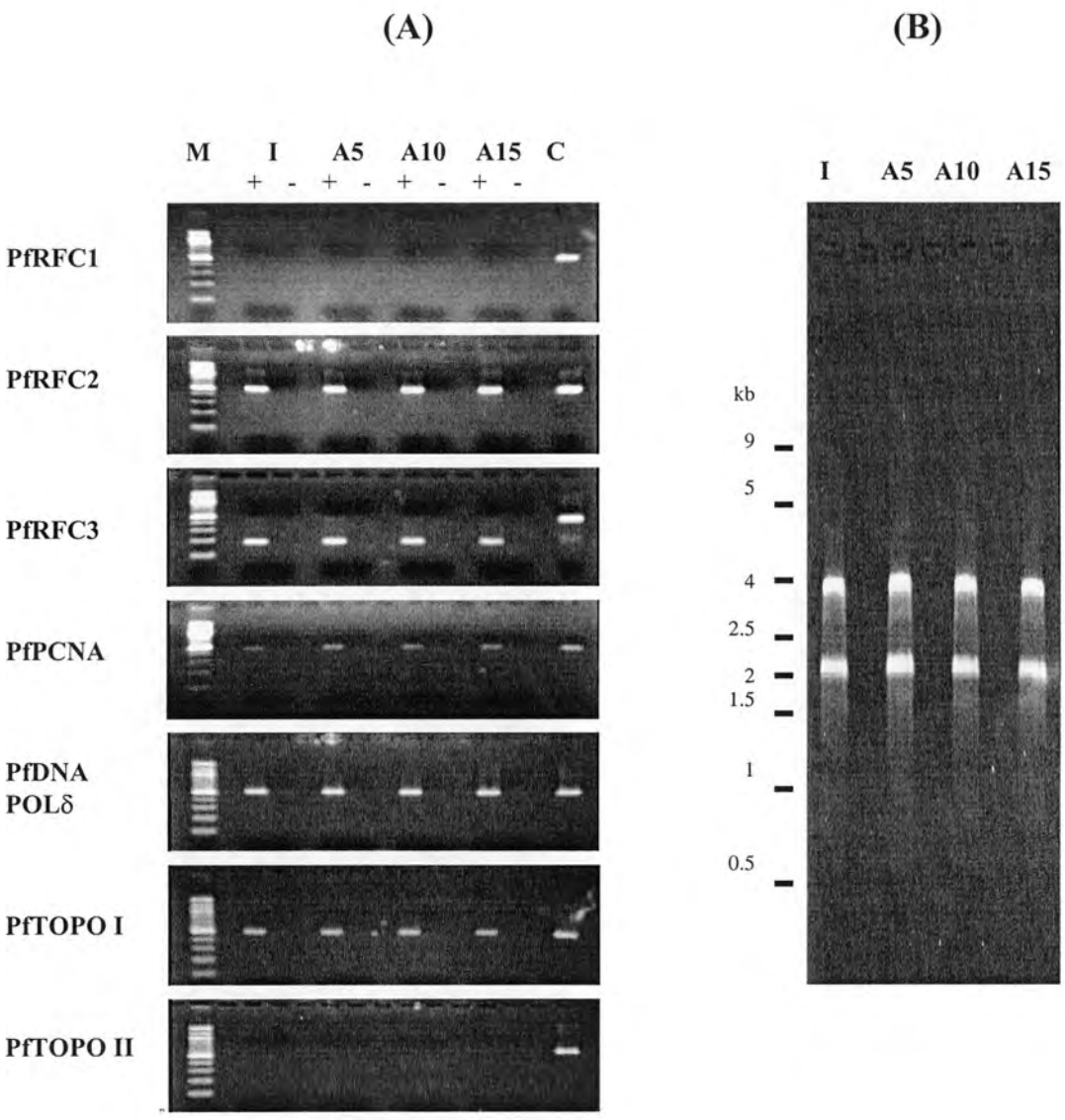

(A)
(B)
M
I
A5
A10
A15
C
+ - + - + - + -
PfRFC1
PfRFC2
PfRFC3
PfPCNA
PfDNA POLδ
PfTOPO I
PfTOPO II
I
A5
A10
A15
kb
9
5
4
2.5
2
1.5
1
0.5

Figure 6.4 RT-PCR analysis of CCMS-grown and GluNAc-treated gametocytes

Mature gametocytes were grown entirely in CCMS, synchronised and depleted of asexual parasites by GluNAc treatment. These gametocytes were purified and concentrated on Percoll gradients and divided into four equal portions. Three of the portions were activated by resuspension in GSM and the gametogenesis stopped at 5 minutes intervals. Each of the four portions was split into two. Total protein extract prepared from one set and total RNA from the other set. RT-PCR analysis carried out using gene-specific oligonucleotide primers on 1st strand synthesised from total RNA isolated from the timed gametogenesis experiments. PCR products were analysed on 2% agarose gels.

A Agarose gel of the RT-PCR products

B A sample of the RNA used for the initial 1st strand synthesis reaction

Lane I	Mature gametocytes before activation
Lane A5	Mature gametocytes 5 minutes after activation
Lane A10	Mature gametocytes 10 minutes after activation
Lane A15	Mature gametocytes 15 minutes after activation
M	Molecular weight markers in kilobases (Ambion)

\+ PCR on 1st strand synthesis reaction in which RT was added.

\- PCR on 1st strand synthesis reaction in which no RT was added

C PCR on gDNA using the same set of primers

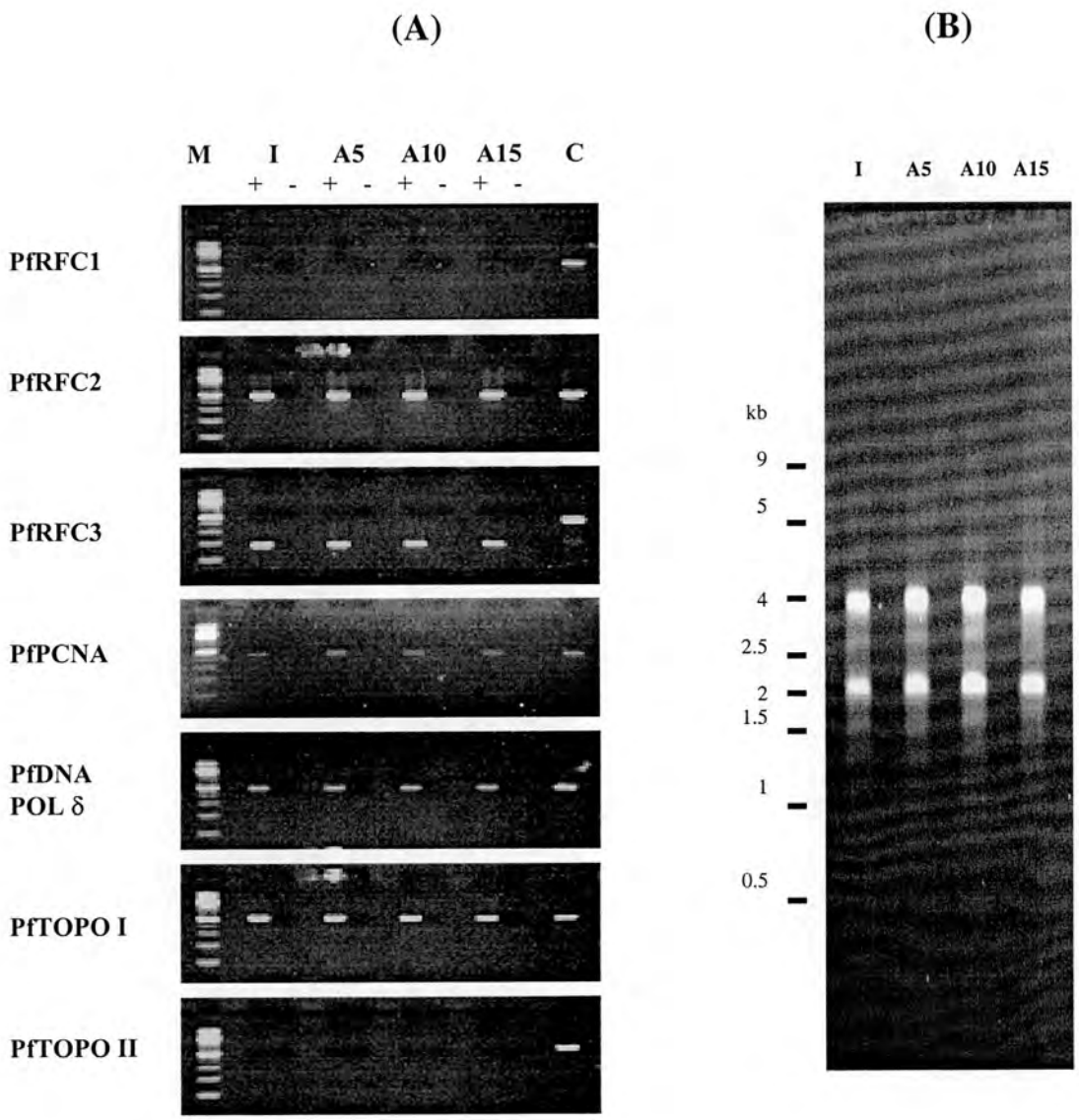

(A)
(B)
M
I
A5
A10
A15
C
+ - + - + - + -
PfRFC1
PfRFC2
PfRFC3
PfPCNA
PfDNA POL δ
PfTOPO I
PfTOPO II
I
A5
A10
A15
kb
9
5
4
2.5
2
1.5
1
0.5

Figure 6.5 RT-PCR analysis of CCMA-grown and Sorbitol-treated gametocytes

Mature gametocytes were grown entirely in CCMA, synchronised and depleted of asexual parasites by sorbitol treatment. These gametocytes were purified and concentrated on Percoll gradients and divided into four equal portions. Three of the portions were activated by resuspension in GSM and the gametogenesis stopped at 5 minutes intervals. Each of the four portions was split into two. Total protein extract prepared from one set and total RNA from the other set. RT-PCR analysis carried out using gene-specific oligonucleotide primers on 1st strand synthesised from total RNA isolated from the timed gametogenesis experiments. PCR products were analysed on 2% agarose gels.

A Agarose gel of the RT-PCR products

B A sample of the RNA used for the initial 1st strand synthesis reaction

Lane I	Mature gametocytes before activation
Lane A5	Mature gametocytes 5 minutes after activation
Lane A10	Mature gametocytes 10 minutes after activation
Lane A15	Mature gametocytes 15 minutes after activation
M	Molecular weight markers in kilobases (Ambion)

\+ PCR on 1st strand synthesis reaction in which RT was added.

\- PCR on 1st strand synthesis reaction in which no RT was added

C PCR on gDNA using the same set of primers

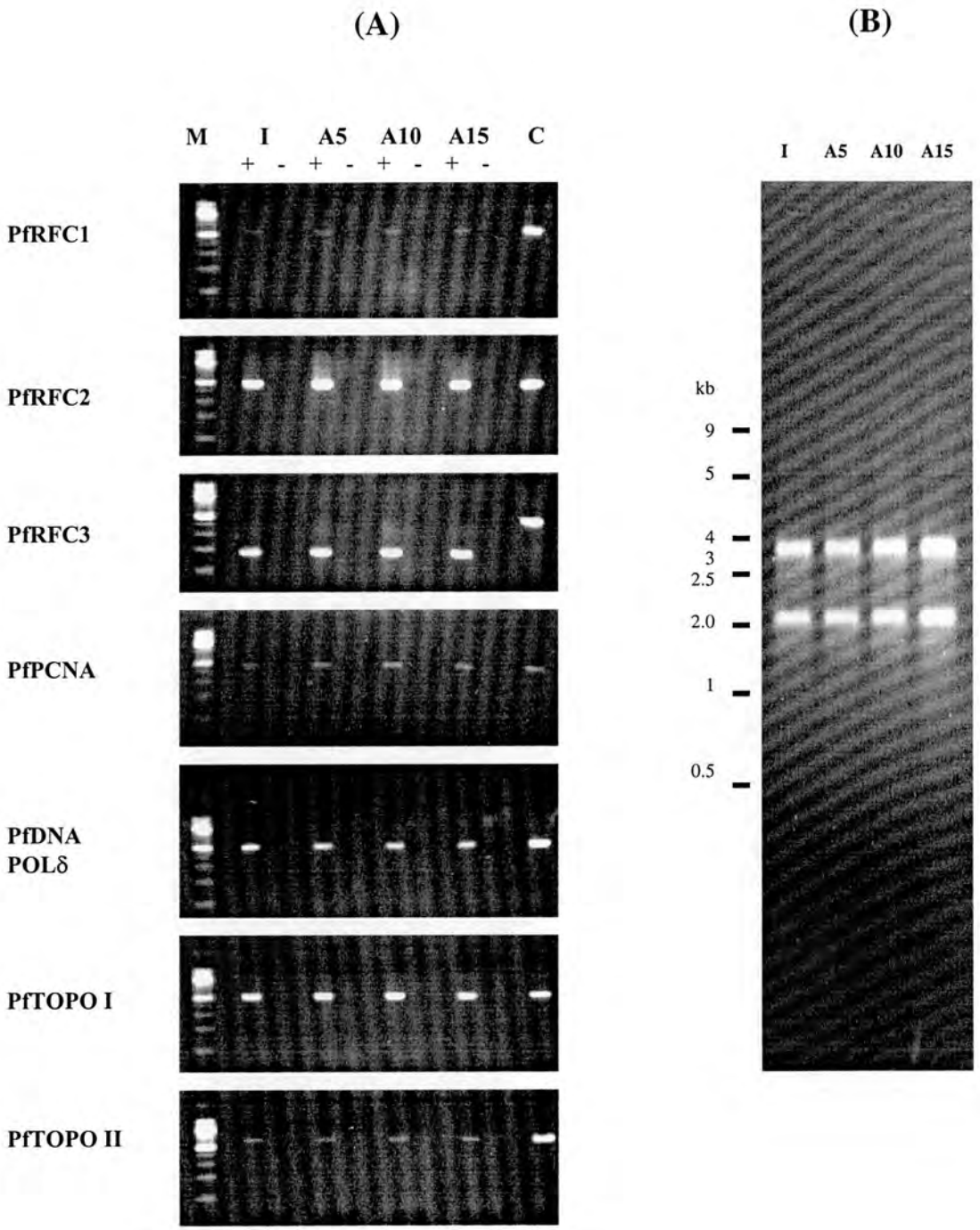

(A)
(B)
M
I
A5
A10
A15
C
+ - + - + - + -
PfRFC1
PfRFC2
PfRFC3
PfPCNA
PfDNA POLδ
PfTOPO I
PfTOPO II
I A5 A10 A15
kb
9
5
4
3
2.5
2.0
1
0.5

Figure 6.6 Coomassie stained gel of protein extracts prepared from mature gametocyte pellets before and after activation

Mature gametocytes were grown entirely in CCMA, synchronised and depleted of asexual parasites by GluNAc treatment. These gametocytes were purified and concentrated on Percoll gradients and divided into three equal portions. Two of the portions were activated by resuspension in GSM and the gametogenesis stopped at 5 minutes intervals. Total protein extract from each pellet and equal volumes of extracts were size separated on 10% NuPAGE gels and stained with Coomassie Blue.

Lane I Mature gametocytes before activation

Lane A0 Mature gametocytes immediately after activation

Lane A5 Mature gametocytes 5 minutes after activation

Positions of molecular weight markers are indicated (New England Biolabs)

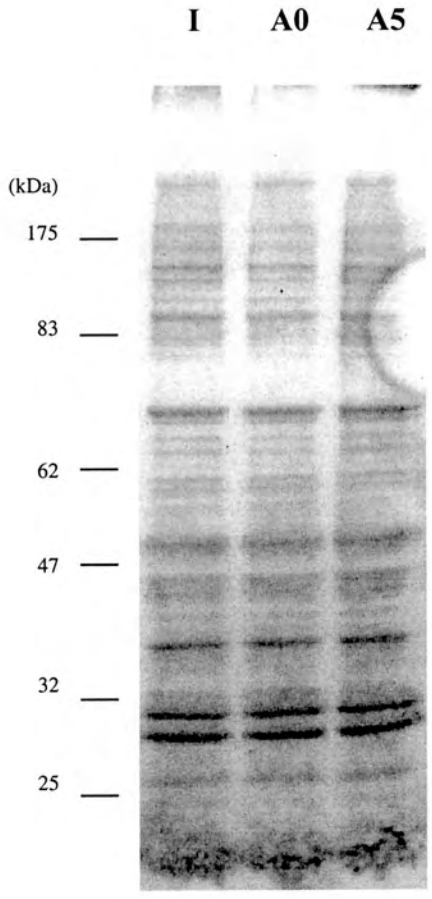

I
A0
A5
(kDa)
175
83
62
47
32
25

Figure 6.7 Western blot analysis of protein extracts prepared from gametocyte pellets before and after activation

Western blots containing equal amounts of extracts prepared from gametocyte pellets before and after activation were probed with anti-PfTopoII rabbit polyclonal antiserum at 1:500 dilution.

Lane I Mature gametocytes before activation.

Lane A0 Mature gametocytes immediately after activation.

Lane A5 Mature gametocytes 5 minutes after activation.

The extracts probed here were aliquoted from the same set used in Figure 6.6.

Figure 6.8 Decatenation activity assays in gametocyte protein extracts prepared before and after activation.

Decatenation activity assays using gametocyte extracts prepared from gametocytes before and after activation. The same set of extracts shown in Figure 6.6 and 6.7 were used.

Lanes as above. I', A0' and A5' are negative controls with extract but without kDNA.

Controls

K Catenated Kinetoplast DNA (kDNA)

D Decatenated kinetoplast DNA

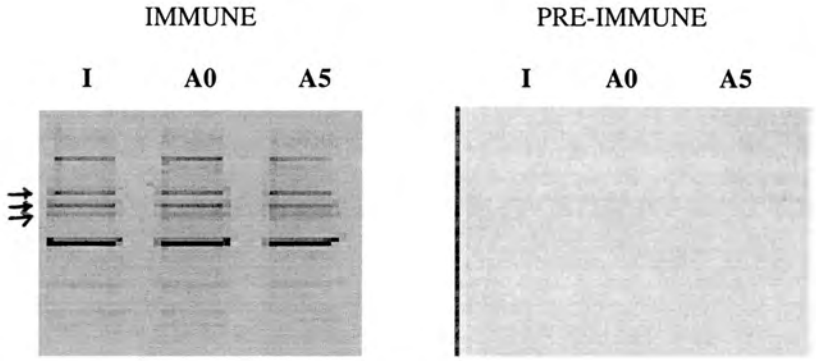
L Linear kinetoplast DNA

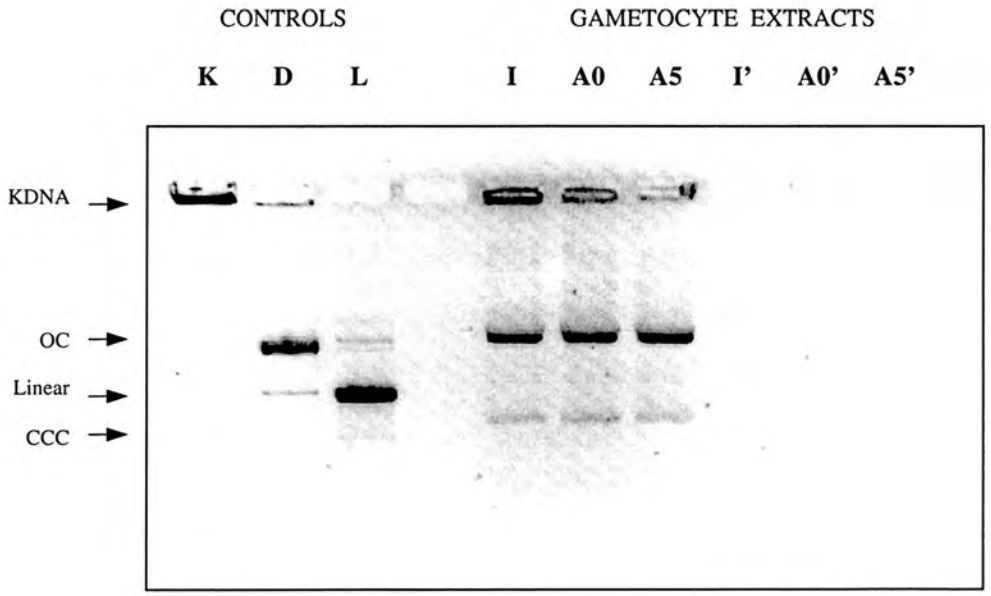
Arrows show the position of;

OC Open circular decatenated kDNA

Linear Linearized kDNA

CCC Closed covalent circular decatenated kDNA


IMMUNE
PRE-IMMUNE
I
A0
A5
I
A0
A5


CONTROLS
GAMETOCYTE EXTRACTS
K
D
L
I
A0
A5
I'
A0'
A5'
KDNA
OC
Linear
CCC

CHAPTER 7

DISCUSSION

7.1 The DNA replication apparatus during gametocytogenesis

Gametocyte maturation in *P. falciparum* takes a relatively prolonged period of 8 to 10 days *in vivo* and 14 to 17 days *in vitro* (Smalley, 1977; Carter and Beach, 1978). During this time, gametocytes appear to be metabolically inactive, relative to asexual parasites, with the only outward sign of activity being the change in their shape caused by the assembly and loss of components of their cytoskeleton (Sinden and Smalley, 1979). Studies on DNA synthesis in *P. falciparum* gametocytes have shown that a significant amount of DNA is synthesised at the onset of gametocytogenesis with the DNA content increasing to about 1.8c most likely as a result of amplification of specific genes rather than duplication of the whole genome (Janse *et al.*, 1986a).

Several genes involved in *P. falciparum* DNA replication have been isolated and their stage-specific expression during the intraerythrocytic asexual cycle analysed. PfRFC1, PfRFC2, PfRFC3 (Douglas, J, 1999), PfPCNA (Kilbey *et al.*, 1993), PfDNA POLδ (Ridley *et al.*, 1991; Horrocks *et al*, 1996), PfTOPO I (Tosh and Kilbey, 1995; Tosh *et al.*, 1999) and PfTOPO II (Cheesman *et al.*, 1994; Cheesman *et al.*, 1998) are all expressed in the late trophozoite stages of *P. falciparum*. The same studies have also shown that the corresponding proteins encoded by these genes are present in trophozoites and schizonts of *P. falciparum*. It is in trophozoites that DNA replication starts (Gritzmacher and Reese, 1984, Inselburg and Banyal, 1984) and it is to be expected that DNA replication proteins and their gene transcripts are present at this stage in the asexual parasite cycle.

As noted earlier, for much of their development, gametocytes show little evidence of DNA synthesis once an initial incomplete duplication has taken place during the formation of stage I gametocytes. It is only after activation of the mature stage V gametocytes that DNA replication resumes at a rapid pace with three rounds of replication completed in 10 minutes. There are two points to make at this stage.

First, the rapid synthesis of DNA only takes place in the male gametocytes (microgametocytes) so as to provide for eight haploid microgametes. In addition,

microgametocytes account for only 20% of the total gametocyte population, the rest being female gametocytes (macrogametocytes). Because it is not possible, at present, to apply methods such as Western blotting or northern analysis selectively to male gametocytes, many changes in the levels of replication proteins are bound to be masked by the presence of large numbers of female gametocytes.

Secondly, the changes in protein levels and/or activity are not by themselves likely be the determining factors in initiating DNA replication. Other intracellular signalling pathways, such as protein kinase cascades, that have been shown in other eukaryotes to be responsible for the transduction of extracellular stimuli to the nucleus and regulate cell proliferation and/or differentiation may play an important role *P. falciparum* DNA replication. Indeed, Pfmap-2, a homologue of the mitogen-activated protein kinase (MAPK), has been identified in *P. falciparum* gametocytes and is thought to be responsible for maintaining the gametocyte in a non-replicative state while it matures in the vertebrate host (Dorin *et al.*, 1999). It is certain, however, that DNA synthesis requires the active participation of the replication proteins studied here and it has been the object of this work to assess whether they and/or their cognate transcripts are present at all times, or only when DNA is being synthesised. Important also is the need to establish whether the proteins, when present, are active. It is, unfortunately, impossible at present to assay for the activity of most of the DNA replication proteins since they function as a multicomponent system and not many of the components have been isolated or expressed in a functional form. It has however been possible to assay for the PfTopoI (Tosh *et al.*, 1999), PfTopoII (Cheesman *et al.*,1998) and primase activity (Prasartkaew *et al.*, 1996).

The immunofluorescence results presented in this study showed that PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopo II are present throughout gametocytogenesis. All five proteins appear to be expressed at high level and are also localised to the nucleus during early gametocytogenesis, that is, in stage I and II gametocytes. However,

progression through stage III, IV and V lead to differing expression levels and changes to the localisation of these DNA replication proteins.

During eukaryotic DNA replication, RFC functions as a complex of five proteins (Hubscher *et al.,* 1996), meaning all the five proteins have to be present during the active synthesis of DNA. In this study, it was observed that PfRfc1 levels appears to be significantly higher in stage I and stage V gametocytes while PfRfc2 and PfRfc3 levels are significant only in stage I gametocytes compared to the other gametocyte developmental stages. The presence of all three PfRfc proteins in the nuclear region and at significantly higher levels in stage I gametocytes than in the rest of the gametocyte stages suggests that DNA synthesis is likely to or has just taken place. Indeed, this is consistent with the findings in earlier studies that showed that a significant amount of DNA is synthesised at the onset of gametocytogenesis, prior to the formation of stage I gametocytes (Janse *et al.,* 1986a:88). In addition, PfPcna and PfTopoII levels also appeared to be significantly higher in stage I and V gametocytes. Therefore these proteins are most likely actively aiding in the synthesis of DNA in young stage I gametocytes.

In the more mature stages III and IV gametocytes, PfRfc1, PfRfc2, PfRfc3, PfPcna, and PfTopo II proteins, though mainly present within the nuclear region, appear to be at lower levels and also present within the cytoplasm. One way of reducing the rate of DNA replication may involve inactivating one or several of its components or by disassembling its components and translocating them from the nucleus, where DNA replication takes place, to the cytoplasm. This may, in part, be why PfRfc1, PfRfc2, PfRfc3, PfPcna, and PfTopo II proteins appear to be evenly distributed throughout the cytoplasm of stage IV gametocytes in contrast to their predominant nuclear localisation observed in stage I and to some extent in stage II gametocytes and then later on in stage V gametocytes. Compared to stages III and IV, gametocytes at stage V of development, appear to have higher levels, of PfRfc1, PfRfc3, PfPcna, and PfTopo II which are primarily located within the nuclear region, implying that stage V gametocytes are putting in place the DNA replication apparatus in readiness for

the rapid and repeated DNA replication that is set to occur during gametogenesis following activation. However, the low levels of PfRfc2 and its distinct absence from the nuclear region of stage V gametocytes suggests that DNA synthesis is unlikely to be taking place. The sub-cellular localisation of components of the DNA replication apparatus of may therefore be one of the factors influencing DNA replication during gametocytogenesis and by extension during gametogenesis in *P. falciparum*.

7.2 The DNA replication apparatus during gametogenesis

Gametogenesis is a period of rapid cell division of the mature intraerythrocytic microgametocyte (male gametocyte) which occurs when it passes from the mammalian host to the mosquito gut. It leads to the formation of several microgametes (male gametes) (Sinden *et al.,* 1978). Gametogenesis involves the simultaneous activation of several, apparently independent, cellular events, namely the disruption of the host cell membrane, release of the parasite, the reorganization of the cytoplasmic structures involved in the formation of the male gametes, and lastly the rapid and repeated replication of the genome of the male gametocyte to provide eight gametes (Alano and Carter, 1990). The latter requires the replication of the entire parasite genome three times within ten to fifteen minutes at most (Janse *et al.*,1986b). The speed of replication in gametocytes contrasts greatly with that in asexual parasites, and it has been suggested that in order to achieve this rapid and repeated DNA replication during gametogenesis, approximately 1300 replication forks are activated simultaneously in the mature gametocytes at the onset of gametogenesis (Janse and Mons, 1987). The induction of so many independent replication forks at the onset of microgametogenesis itself not only suggests that mature microgametocytes are heavily precommitted to the rapid production of male gametes in the mosquito midgut but also that the induction mechanism is highly amplified within the parasite nucleus. This makes the mature gametocyte an ideal cell in which to study the mechanism of DNA replication.

In this study, western analysis, carried out during gametogenesis, showed that some components of the DNA replication apparatus of *P. falciparum* namely, PfRfc1, PfRfc3, PfPcna and PfTopoII remained at constant levels following activation. This may suggest that the rapid and repeated DNA synthesis that takes place during the first ten minutes of gametogenesis, does so without the requirement of significant additional synthesis of PfRfc1, PfRfc3, PfPcna, and PfTopo II proteins. However, Western analysis showed that the level of PfRfc2 increased immediately after activation and then gradually decreased as gametogenesis progressed. In the IFA results shown earlier, PfRfc2 was seen to be distinctly absent from the nucleus of stage V gametocytes. Taken together, the IFA and Western analysis results on the expression patterns of PfRfc2 in mature gametocytes before and after activation, appear to be different from that of the other DNA replication proteins tested and in particular, that of PfRfc1 and PfRfc3. It is therefore possible that PfRfc2 may be one of the components of the DNA replication apparatus that is actively synthesised and transported to the nucleus following activation of mature gametocytes. This may suggest that some level of protein synthesis does take place during gametogenesis. Indeed, the use of metabolic inhibitors of protein synthesis, specifically those affecting peptide elongation, like emetine, were found to effectively inhibit exflagellation in *P. yoelii nigeriensis* (Toye *et al.*, 1977; Janse *et al.*, 1986b). However, further experiments, such as the *in situ* detection of PfRfc2 during gametogenesis, require to be carried out in order to get to a better understanding of the role of PfRfc2 during gametogenesis.

Transcription of these DNA replication genes during gametogenesis appears to be very low and hardly detectable by northern analysis. However, the more sensitive RT-PCR analysis was able to detect transcripts of PfRFC2, PfRFC3, PfPCNA, PfDNA POLδ and PfTOPO I in mature inactivated gametocytes. These transcripts remained detectable even after activation. However, messages for PfRFC1 and PfTOPO II were not detected, by RT-PCR, in either mature inactivated gametocytes or after activation. At this point, it is not possible to determine whether transient

transcription and translation of PfRFC2, PfRFC3, PfPCNA, PfDNA POLδ and PfTOPO I does take place during gametogenesis especially due to the very low levels of transcripts detected by northern analysis. However, earlier studies have shown that actinomycin D, an inhibitor of DNA-directed RNA synthesis in both prokaryotes and eukaryotes, partially inhibited exflagellation in *P. yoelii nigeriensis*. This was seen to suggest that the possibility that transcription does occur to some extent during microgametogenesis and requires *de novo* synthesis of mRNA. Due to this partial inhibitory effect of actinomycin D on exflagellation, the existence of a long-lived mRNA, presynthesised in the maturing gametocyte and translated only at the onset of exflagellation was not excluded (Toye *et al.*, 1977).

The inability to separate male from female gametocytes in order to carry out protein and RNA analysis makes it difficult to track down any changes that might occur in the replication proteins and their cognate transcripts during gametogenesis. This is further complicated by the fact that only the microgametocytes undergo DNA replication and account for only 20% of the gametocyte population. In addition, at any one time not all the microgametocytes will activate upon induction of gametogenesis due to several reasons among them immaturity.

7.3 Gametogenesis and Topoisomerase II activity

The presence of the components of the DNA replication machinery at stage of the parasite development does not directly imply activity. One or more of the components of the DNA replication apparatus may require to be activated, say by postranslational modification or by phosphorylation. Indeed, the extent of phosphorylation of the mammalian TopoI has been shown to vary throughout the cell cycle (Pommier *et al.*, 1990) and it has been suggested that phosphorylation of TopoII influences activity (Ackerman *et al.*, 1985). Of the components of the DNA replication apparatus, PfTopoI and PfTopoII activity can be assayed *in vitro*. Recent studies have shown that PfTopoII activity was highest in schizont protein extracts (Cheesman *et al.*, 1998). Topoisomerase II activity was assayed in

gametocyte protein extracts prepared before activation and during the first 5 minutes of gametogenesis. Decatenation activity assays showed that topoisomerase II activity, though present in mature inactivated gametocytes, progressively increased during gametogenesis. The residual decatenation activity observed in inactivated gametocytes may have been due to some gametocytes having activated during the purification procedure. The possibility that asexual parasites contributed to this residual decatenation activity was ruled out as a combination of GluNAc-treatment and Percoll gradient centrifugation was found to completely eliminate viable asexual parasites from gametocyte preparations. Ideally, this experiment should have been carried out using serial dilution of gametocyte extracts and stopped at several shorter time points. In that way, the levels of TopoII activity within each extract would have been more clearly defined. However, larger amounts of gametocyte extracts would have been required. Western analysis of the same gametocyte protein extract showed no significant increase in the level of PfTopoII during this period. The discovery of PfTopoII activity during gametogenesis is consistent with the fact that rapid DNA replication that takes place during gametogenesis would require the functions of TopoII in separating the DNA strands.

7.4 Exflagellation and DNA replication

Exflagellation, the emergence of male microgametes, marks the end of gametogenesis (Sinden *et al.*, 1978). Gametocytes grown entirely in CCMS upon reaching maturity, activate, that is lose their sickle-shape and 'round-up', and then progress on to exflagellate when resuspended in gametogenesis stimulating medium (GSM). However, in this study it was observed that gametocytes grown entirely in CCMA, activated but did not progress on to exflagellate when resuspended in GSM. Both activated gametocytes from CCMA- and CCMS-maintained cultures were indistinguishable, when viewed through a microscope, and exhibited the characteristic internal violent activity. It was observed, in the course of this study, that the capacity of CCMA-grown mature gametocytes to exflagellate could be regained by an

overnight incubation in CCMS. This implies that there are factors in serum that are critical for exflagellation, and that these factors may not be present in AlbuMaxII. With regard to DNA synthesis and the capability to exflagellate or not, the status of the DNA replication apparatus was analysed in the period prior to the onset of exflagellation; exflagellation leads to the disruption of the male 'gametocyte', hence the loss of most of its cellular contents (Sinden *et al.*, 1978). The results obtained in this study appear to show that no significant difference, with regard to the presence of DNA replication proteins or their gene transcripts, between gametocytes that do activate only or those that activate and also have the capacity to exflagellate. This suggests that DNA replication and exflagellation may be, to some extent, totally independent events.

7.5 *In situ* hybridisation

In situ hybridisation (ISH) is a powerful technique, which enables the study of gene expression at the single-cell-level. ISH makes it possible to study gene expression in unsynchronised cell populations. *P. falciparum* is notoriously difficult to maintain in synchronised growth for more than 48 hours and *in vitro* cultures become progressively unsynchronised with time. Synchronised cultures of *P. falciparum* are routinely achieved by eliminating the mature asexual forms, trophoziotes and schizonts, by sorbitol treatment (Lambros and Vandenberg, 1979) or by the separation of the mature forms from the ring-forms by gelatin flotation (Ponnudurai *et al.,* 1986). The use of northern analysis to determine stage-specific differential gene expression during asexual or sexual differentiation in *P. falciparum* relies heavily on the need to have tightly synchronised parasite cultures at high parasitemia. This is laborious and expensive to achieve but, however, possible when dealing with asexual parasites. Gametocytes, however, present a more difficult situation. Conversion to gametocytes only starts when healthy asexual parasites cultures undergo some form of stress brought about by several factors, among them, high parasite densities (Carter and Miller, 1979). Even then, only a maximum of 20-30%

of the parasites start to differentiate into gametocytes. Unlike asexual parasites, gametocytes do not replicate and therefore the number of gametocytes only increase, in culture, as a result of further conversion of some asexual parasites. The periodic conversion of asexual parasites into gametocyte further creates a culture composed of gametocytes at different stages of development. The low gametocyte yields due to poor and unpredictable conversion rates, combined with the continuous presence of asexual parasites within gametocyte cultures makes the study of gene expression in gametocytes *per se,* rather difficult especially during gametocyte development.. Therefore, the study of transient gene expression during gametocytogenesis by the use of northern analysis would be at best, difficult, especially if the simultaneous expression of several genes is to be sought during gametocyte development.

ISH is difficult to set-up but once the right conditions are established, the simultaneous expression of several genes in a population of mixed cells should be readily determined. It is in light of this that an attempt was made to establish a working protocol to use in the study of gene expression of in *P. falciparum* by the use of RNA *in situ* hybridisation. As a start, fluorescein-labelled gene fragments of PfDNA replication genes were used to probe paraformaldehyde-fixed thin films of asexual parasites. Probes against PfRFC1, PfRFC2, PfPCNA, PfDNA POLδ, PfTOPO I and PfTOPO II all showed pockets of distinct fluorescence mainly on the peripherals of young and mature schizonts away from the nuclear region stained by DAPI. However, PfRFC3 showed fluorescence located within the DAPI-stained nuclear region. During gene expression, DNA is transcribed into heterogeneous nuclear RNA (hnRNA), hnRNA is processed to produce mature mRNA via the spliceosomes, mRNA is transported into the cytoplasm where it is translated into protein. It is therefore possible to detect fluorescence within the nuclear region as a result of hybridisation of the probe to hnRNA. The detection of hnRNA confirms that gene expression is switched on or up regulated. In this study, the fluorescence primarily appeared to emanate from the region outside the nucleus

Shorter probes gave very low fluorescence and this was attributed to the presence of fewer detectable reporter molecules, in this case fluorescein. Short probes are more prone to loss during the washing steps and this would also contribute to the low fluorescence observed. Though longer probes are easier to detect, especially by the direct method, they suffer from the risk of non-specific hybridisation and more stringent washes are required. In this study, the direct *in situ* detection method used had it advantages and disadvantages. Although RNA is inherently more prone to degradation that DNA, it is usually more abundant than its homologous DNA sequence and is, therefore, easier to detect. As this was the first time this was being attempted, the need for a quick detection method was preferred. The ease of probe preparation using fluorescein-PCR labelling meant that probes of different lengths could be generated tested. However, most of the steps from the application of the probe onwards had to be carried out in the dark so as to preserve the fluorescence of the fluorescein-labelled probes. Fluorescence quickly faded away during examination on a fluorescent microscope even in the presence of antifade reagents such as DABCO.

FISH experiments were also carried out on nuclease-treated thin parasite films using PfTOPO I probes. Fluorescence was observed in DNase-treated thin smears even when DAPI staining confirmed the complete degradation of gDNA. This would imply that the fluorescence was possibly the result of probe hybridization to mRNA. However, additional controls are required, such as the RNase-treatment of thin films prior to FISH experiments. The absence of a nuclear staining by DAPI following DNase-treatment made it difficult to distinguish the different stages of the parasites.

There are very few studies reported that have used ISH to study gene expression in *Plasmodium*. In *P. berghei*, the expression of Pbs21 mRNA, a gene encoding a parasite surface antigen, was shown by ISH, to be expressed in a sex-specific manner exclusively in the female gametocyte (Thompson and Sinden, 1994). ISH has also been used to show the sexual-stage-specific mRNA expression of *Pf*77 *P. falciparum*

(Baker *et al.*, 1995). In both these studies, riboprobes were used for ISH and the signal from gDNA was distinguished from that of from the mRNA by the use of sense and antisense probes.

7.6 Conclusion

1. *Plasmodium falciparum* DNA replication proteins, PfRfc1, PfRfc2, PfRfc3, PfPcna and PfTopoII, are present in gametocytes throughout gametocytogenesis despite the fact that DNA synthesis only takes place at the onset of gametocytogenesis.
2. During gametocytogenesis, PfRfc1, PfRfc3, PfPcna and PfTopoII are predominantly located in the nuclear region and at higher levels in stage I and V gametocytes.
3. PfRfc2 is distinctly absent from the nucleus of stage V gametocytes and is instead predominantly located in the cytoplasm. PfRfc2 is present and at higher levels within the nuclear region of stage I gametocytes.
4. During gametogenesis, the levels of PfRfc1, PfRfc3, PfPcna and PfTopoII remain markedly unchanged whereas PfRfc2 levels increase markedly at the onset of gametogenesis and then gradually decrease as gametogenesis progresses.
5. Topoisomerase II activity is present in mature gametocytes but increases markedly during gametogenesis however, the amount of PfTopoII remains relatively unchanged.

7.7 Future work

1. In general, only one in eight gametocytes is male and in addition, only the male gametocyte undergoes DNA replication during gametogenesis. It would therefore be worthwhile to find a way in which male and female gametocytes can be separated or clearly distinguished from one another. One way would be to include gametocyte sex-specific markers during the *in situ* detection of the components of the DNA replication apparatus in malaria gametocytes.
2. PfRfc2 showed a unique expression pattern from that of the other DNA replication proteins and in particular PfRfc1 and PfRfc3, before and during gametogenesis. It would be interesting to carry out further studies focussing specifically on PfRfc2 in order to get to a better understanding of its role during gametogenesis.
3. The results of this study also showed that Topoisomerase II activity increases during gametogenesis while the PfTopo II protein levels remain unchanged. Other studies have shown that topoisomerase II activity is influenced by phosphorylation. It would be interesting to check whether the phosphorylation state of PfTopoII changes upon activation of mature gametocytes. Identification of the protein kinase(s) responsible would also be worth looking into.
4. It would also be interesting to link DNA replication in mature gametocytes to the known eukaryotic cell cycle by looking into the presence and activity level of cyclins and cyclin-dependent kinases (CDK) respectively, before and after gametogenesis. Establishing the status of the origin of replication complex would also shed more light on the how the mature gametocyte is able to set off into a rapid and repeated replication of its genome thrice in less than ten minutes.
5. The promoter activities of most of the genes involved *P. falciparum* have been determined during the asexual cycle. It would also be interesting to determine whether the promoters of all the seven genes considered in this study are active during gametocytogenesis by carrying out nuclear-run-on analyses.

CHAPTER 8

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