MOLECULAR STUDIES ON SIGNAL TRANSDUCTION IN *Plasmodium falciparum*

Ali Awad ElKarim M. Sultan

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Institute of Cell, Animal and Population Biology
University of Edinburgh

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DECLARATION

The research reported in this thesis is original and my own, except where due acknowledgement is made, and has not submitted for any other degree

DEDICATION

This thesis is dedicated to my family, and especially to my mother. It has been their sincere and continuous love and support that have made everything possible.

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Abstract

The World Health Organisation estimates that more than 40% of the world's population is at risk of malaria infection, and that between 250-400 million people live in areas where malaria is endemic. Neither drug administration nor vector control has fulfilled early hopes that malaria eradication might be possible. Instead insecticide and drug resistance have become worse problems in malarious areas. A greater understanding of the genetics and molecular biology of the parasite is therefore needed in order to develop novel chemotherapeutic, and possibly vaccine based, control strategies. One aspect of the parasite's biology which is both poorly understood and represents a potential target for anti-parasite intervention is the regulation of the cell cycle during intracellular development.

The research outlined in this thesis was primarily designed to study certain genes and their encoded proteins which regulate the development of the malaria parasite, *Plasmodium falciparum,* through its intra-erythrocytic life cycle. The work has involved molecular cloning and characterisation of a *P. falciparum* homologue of the *ras*-related nuclear GTP-binding protein, Ran/TC4. This protein has been shown to play a key role in control of the cell cycle in mammalian and yeast systems. In this work, the homologous *P. falciparum* gene, *pfran*, has been cloned and characterised. Its predicted protein product is 214 amino acids long and contains consensus motifs found in the Ras superfamily. The expression of a 1.5 kb *pfran* transcript has been found to change during the cell cycle, reaching a peak at the trophozoite and schizont stages. Western blotting and immunoprecipitation using a polyclonal antiserum raised against the expressed protein have shown that *pfran* encodes a protein 27 kDa in size. Immunofluorescence assays have shown that *pfran* is localised in both the nucleus and cytoplasm of the parasite. Some experiments were undertaken to express *pfran* in yeast which had only limited success.

Ran/TC4 is proposed to function in conjunction with the chromatin-binding protein RCC1, (regulator of chromosome condensation), to couple completion of DNA synthesis with initiation of mitosis. In response to completion of DNA synthesis Ran-RCC1 complex activate the cyclin-p34 ^{CDC2} complex which initiate entry into mitosis. To understand better the regulation of the cell cycle in malaria parasites, and the role of Ran in this process, the *P. falciparum* homologue of the RCC1 has been cloned and partially sequenced. Expression of this gene and its possible association with *pfran* have been studied.

The implications of these findings are discussed in the thesis.

List of Abbreviations

Amp.	ampicillin
bp	base pair(s)
Bq	Becquerels
BSA	bovine serum albumin
°C	degree centigrade
cdc	cell division cycle
cdc	cell division cycle gene
cdk	cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
Ci	curie
cpm	counts per minute
DAPI	4'-6-diamino-2-phenylindole
dATP	deoxyadenosine-5'- triphosphate
dCTP	deoxycytosine-5'- triphosphate
(d)dATP	2', 3'-dideoxyadenosine-5'-triphosphate
(d)dCTP	2', 3'-dideoxycytidine-5'-triphosphate
(d)dGTP	2', 3'-dideoxyguanosine-5'-triphosphate
dGTP	deoxyguanosine-5'- triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
DTT	dithiothreitol
dTTP	deoxythymidine-5'- triphosphate
EDTA	ethylenediamine N,N,N',N', tetra-acetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetra-acetic acid
FITC	fluorescein isothiocynate
G1	gap 1, period after mitosis, before DNA synthesis
G2	gap 2, period after DNA synthesis, before mitosis
GCG	Genetic Computer Group

GDP	guanosine diphosphate
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HeLa	line of human neoplastic (cervical) epithelial cells
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphoic acid).
IFA	immunofluorescence assay
kb	kilobase
kDa	kilodalton
LB medium	Luria-Bertani medium
MPF	maturaion or mitosis promoting factor
mRNA	messenger ribonucleic acid
NP-40	NONIDET-40, nonionic detergent
O.D.	optical density
^{32}P	β emitting isotope of phosphorous
PBS	phosphate buffer saline
PCC	premature chromosome condensation
PCR	polymerase chain reaction
PFGE	pulsed-field gradient gel electrophoresis
PIPES	piperazine-N,N'-bis (2-ethane-sulphonic acid)
PMSF	phenylmethyl sulfonyl fluoride
RCC1	regulator of chromosome condensation
RITC	rhodaamine isothiocyanate
RPMI	Rosewell Memorial Park Institute
RNA	ribonucleic acid
^{35}S	β emitting isotope of sulphur
SDS	sodium dodecyl sulphate
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TEMED	N,N,N,N,-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminoethane
Tween 20	polxythlenesorbitan monolaurate, nonionic detergent
ts	temperature sensitive
UV	Ultra-violet
w/v	weight per volume

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CHAPTER 1

INTRODUCTION

Malaria is caused by a protozoan parasite of the genus *Plasmodium*. More than 100 *Plasmodium* species infecting a wide range of hosts, including reptiles, birds, rodents and primates, have been identified. Each parasite species exhibits a narrow host specificity. Four species; *P. vivax, P. malariae, P. ovale* and *P. falciparum*, cause disease in man. The disease is transmitted to the vertebrate host by the bite of the female *Anopheline* mosquito.

1.1 The current global malaria situation

Malaria is the most important tropical parasitic disease, causing great morbidity and mortality. Control is becoming increasingly difficult and the epidemiological situation is deteriorating at present. Some 300 million people are believed to be infected with malaria parasites every year, and around 90% of these people are living in tropical Africa (W.H.O. 1992). Of the four species that infect man, *P. falciparum* accounts for most of the infections in Africa and for over one third of infections in the rest of the world. More than 2 billion people, nearly 40% of the world's population, are at risk (W.H.O.1992). Although virtually no reliable statistics exist on malaria morbidity and mortality in Africa, extrapolations from epidemiological studies suggest that the disease is probably responsible for between 500,000 to 1.2 million deaths annually, mainly among African children below the age of 5 years (W.H.O.1992). World-wide, it is estimated that there are about 120 million clinical cases of malaria per year. This enormous total of lives and days of labour lost plus the costs of treatment and the negative impact of the disease on development make malaria a major social and economic burden.

The clinical symptoms of malaria are caused by the development of the parasite in red blood cells. The main symptom is fever, which shows a clear cyclic pattern in all but *P. falciparum* infections. Falciparum malaria is the most dangerous form of the disease largely because it can have life-threatening complications such as anaemia and cerebral malaria. Many infected people are partially protected from complications by immunity acquired from previous recent infections. Severe complications are found mostly in the relatively non-immunes, notably children, expatriates and migratory workers, entering highly endemic areas, and in the inhabitants of areas with unstable endemicity.

Control programmes which, by 1970, had been successful in eradicating malaria from some non-tropical malarious areas, have failed in the tropics (Bruce-Chwatt, 1985). This has been due to many causes, e.g. the development and spread of drug resistant parasite strains and vector resistance to insecticides. In endemic

areas, population movements have brought infected individuals into relatively malaria-free areas and as well as non-immune individuals into areas of higher malaria risk. Significantly, long term implementation of control programmes have also become beyond the administrative capacities of almost all poor countries. In the developed world, strategies of malaria control have been directed towards designing new anti-malarial drugs and vaccines. These 'knowledge intensive' strategies require a greater understanding of the biochemistry and molecular cell biology of the parasite. Metabolic or developmental pathways in the parasite that differ from those of the host, or enzymes that differ structurally or functionally from the mammalian enzymes, could be exploited as drug targets.

In this chapter I will attempt to review the current knowledge on the molecular biology of the parasite life cycle. Where appropriate, comparisons and contrasts will be drawn with other parasites and organisms to illustrate similarities, and to highlight any differences.

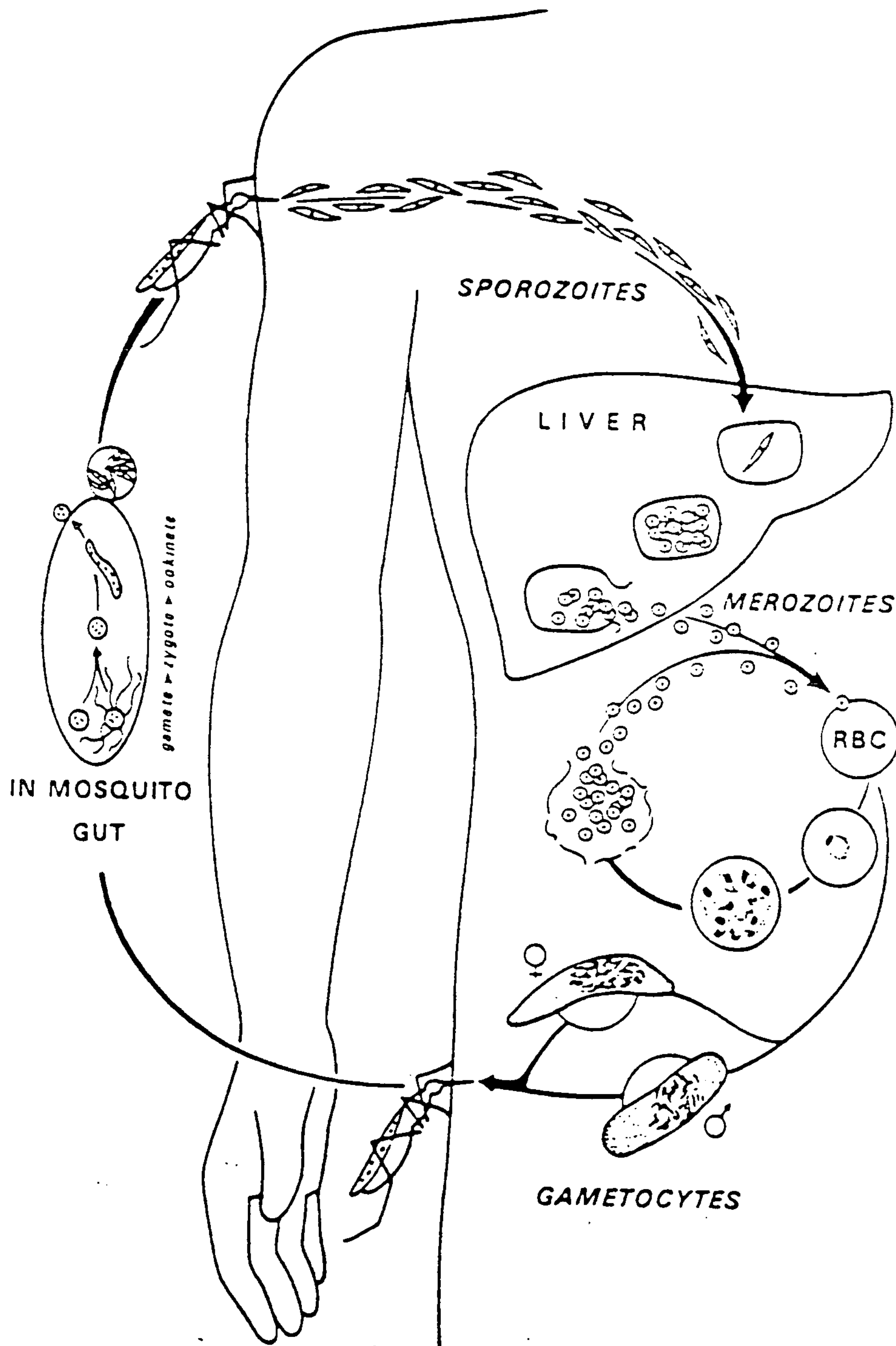
1.2 The life cycle of malaria parasites

All four human malaria species have essentially the same life-cycle (Fig.1.1), alternating between vertebrate and arthropod hosts. The part of the life-cycle which occurs in the human host is predominantly asexual, although gametocytes are formed in this host. The sexual cycle and sporogony take place in the female *Anopheles* mosquito.

Infections in man are initiated through the bite of an infected anopheline mosquito. While taking the blood meal the mosquito injects saliva containing sporozoites into the host skin capillary bed. The motile sporozoites travel to the liver, where they enter parenchymal cells (hepatocytes). Inside a hepatocyte, the parasite undergoes a series of rapid mitotic divisions to produce thousands of merozoites, a process called pre- or exo-erythrocytic schizogony. After 6-10 days, depending on species, the hepatocytes burst, releasing the merozoites into the blood stream where they rapidly enter erythrocytes and begin erythrocytic schizogony. The merozoite develops into the "ring" stage, which then grows through the trophozoite to the mature schizont, which contains 8-32 daughter merozoites. After the red cell ruptures, these merozoites initiate subsequent intra-erythrocytic cycles of development. Intra-erythrocytic development becomes synchronous, with a generation time of 48 hours for *P. falciparum*, *P. vivax* and *P. ovale*, or 72 hours for *P. malariae*. Since red blood cell rupture and the release of parasites into the blood stream causes fever, early stages of the infection are characterised by constant or fluctuating fevers, but as the

Figure 1.1 :

Life-cycle of the malaria parasite *Plasmodium falciparum*. Reproduced, with permission, from Hadley, Koltz and Miller (1986), Annual Review of Microbiology, 1986, Annu Rev. Inc.


SPOROZOITES
LIVER
MEROZOITES
RBC
gamete ▸ zygote ▸ ookinete
IN MOSQUITO
GUT
GAMETOCYTES

development of the parasite in the red blood cells becomes synchronous, typical periodic fevers occur.

After a period of asexual replication, a proportion of intra-erythrocytic merozoites differentiate into macrogametocytes (female) and microgametocytes (male), and these sexual forms, when taken up by the mosquito, continue the life cycle in this host. Only mature gametocytes survive digestion and undergo further development (gametogenesis). The microgametocyte undergoes a process of rapid DNA replication in the gut of the mosquito to produce eight flagellated cells, termed microgametes. The macrogametocyte, on shedding the red blood cell membrane, becomes the macrogamete. The microgametes exflagellate and one of them fertilises the macrogamete to form a diploid zygote. Maturation of the zygote results in the first meiotic division shortly after fertilisation, and it is assumed that a second meiotic division follows (Sinden and Hartley 1985). Over the next 12-18 hours the zygote develops into an ookinete which, on maturation, passes through the mid-gut epithelium to rest between the basement cell membrane and the basal lamina of the mid-gut wall. Here, it differentiates into an oocyst which enlarges and matures over the next 10-12 days. During sporogonic development, the nuclei undergo divisions and several thousand sporozoites are formed, each containing a single nucleus. Eventually the elongated, motile, sporozoites are released from the oocyst and move through the insect's haemocoel to penetrate the salivary glands, to await transmission to the vertebrate host.

1.3 The genome of malaria parasites

Sequences of malaria parasite genes are characteristically eukaryotic in nature with introns, capped and polyadenylated RNA and small nuclear ribonucleoproteins (Hyde *et al.*, 1984; Francoeur *et al.*, 1985). The DNA is enclosed within a nuclear envelope and bound to histones, and presumably packaged into nucleosomes (Wunderlich *et al.*, 1980; Ponzi *et al.*, 1985; Dore *et al.*, 1986), although little is known about the detailed nuclear organisation of the parasite.

1.3.1 Genome Size: The total amount of DNA of the haploid genome, expressed in picograms (pg) or in base pairs (bp), is a characteristic of each living species, and can be described as genome size, genome complexity or C-value. Table (1) shows estimates of genome sizes of different *Plasmodium* species. The estimates vary widely, even for a single species, and range from $1x10^7$ to $3.0x10^7$ bp. The genome size of 1-3 x 10^7 bp, established for *P. falciparum* has been confirmed in several laboratories by determination of the yield of isolated parasite DNA, and by the cloning of size-selected genomic DNA restriction enzyme fragments (Goman *et al.*,

1982; Pollack *et al.*, 1982; Wellems *et al.*, 1987). The best estimates result from recent genome mapping work and are in the range of 30 megabases of DNA (3×10^7 bp) (Walker-Jonah *et al.*, 1992). The malaria parasite genome is thus about 6 times the genome size of *Escherichia coli*, twice that of yeast, one third of *Caenorhabiditis elegans* and less than one hundredth of the human haploid genome.

Table 1: Estimates of the genome size of different *Plasmodium* species.

Plasmodium species	Genome size in base pairs	references
P. falciparum	1-2 $\times 10^7$ bp 2 $\times 10^7$ bp 3 $\times 10^7$ bp	Goman *et al.* (1982) Pollack *et al.*(1982) Wellems *et al.* (1987)
P. berghei	1.5 $\times 10^7$ bp 2.5 $\times 10^7$ bp	Dore *et al.*(1980) Cornelissen *et al* (1984)
P. knowlesi	1.9 $\times 10^7$ bp	Gutteridge *et al.* (1971)
P. yoelii	1.5 $\times 10^7$ bp	Dore *et al.* (1980) Birago *et al.* (1982) Casaglia *et al.* (1985a)

1.3.2 Base composition of DNA: The base composition of the nuclear DNA differs among the various mammalian malaria species. The guanine-cytosine (G+C) content of *P. falciparum* (Goman *et al.*, 1982; Pollack *et al.*, 1982; McCutchan *et al.*, 1984), *P. berghei*, *P. vinckei* and *P. chabaudi* (McCutchan *et al.*, 1984) has been reported to be around 18% which is considerably lower than the G+C content of host DNA (37%). The slightly higher values (23-24%) for *P. berghei* and *P. vinckei* found in earlier investigations (Gutteridge *et al.*, 1971; Dore *et al.*, 1980) can probably be attributed to contamination with host DNA (McCutchan *et al.*, 1984). In comparison to the these species, the primate parasites *P. knowlesi* and *P. fragile* show a much higher G+C content: 30-38% (Gutteridge *et al.*, 1971; McCutchan *et al.*, 1984) and 30% (McCutchan *et al.*, 1984), respectively, while the DNAs of *P. cynomolgi* and *P. vivax* consist of two components, one with a low (about 20%) and the other with a high (about 30%) G+C content (McCutchan *et al.*, 1984).

1.3.3 Molecular karyotype Malaria parasite chromosomes do not condense in mitotic divisions during asexual multiplication and so cannot be visualised by light microscopy (Bahr and Mikel, 1972). However, using electron microscopy, condensed chromatin has been found during the final stages of microgamete formation in the rodent malaria parasite *P. yoelii* (Sinden *et al.*, 1976).

The development of pulsed field gradient electrophoresis (PFGE) made it possible to separate *P. falciparum* chromosomes on agarose gels (Kemp *et al.*, 1985, Van der Ploeg *et al.*, 1985), showing the genome of *P. falciparum* to contain 14 distinct chromosomes (Kemp *et al.*, 1987b; Wellems *et al.*, 1987). This figure is in agreement with the number of kinetochores, the attachment sites of chromosomes to spindle microtubules, in serial sections of mitotic spindles visualised by electron microscopy (Prensier and Slomianny 1986; Sinden and Strong, 1987). All species of *Plasmodium* studied to date appear to have 14 chromosomes (Langsley *et al.*, 1987; Sharkey *et al.*, 1988; Sheppard *et al.*, 1989). Many genes have now been mapped to their chromosomes by blotting and hybridisation techniques (Kemp *et al.*, 1990; Walker-Jonah *et al.*, 1992). Currently the construction of yeast artificial chromosomes (YACs) containing large segments of *Plasmodium* chromosomes is proving to be an important technique for making a complete malaria genome map (Triglia and Kemp, 1991; Watanabe and Inselburg, 1994)

The sizes of *P. falciparum* chromosomes range from 630 kilobases (kb) for the smallest (chromosome 1) to about 3.5 megabases for the larger chromosomes (Triglia *et al.*, 1992). The sizes of homologous chromosomes vary widely among isolates; an individual chromosome may vary in size by several hundred kilobases, both in parasites recently isolated from patients, and in isolates maintained in culture over long periods of time (Corcoran *et al.*, 1986). PFGE fractionation of chromosomes from the progeny of a genetic cross carried out by Walliker and colleagues (1987), revealed considerably more chromosomal size polymorphism than in the parent clones, suggesting that size polymorphism may be generated during meiosis. This has also been found in other malaria parasites such as *P. chabaudi*, *P. vinckei* and *P. berghei* (Sharkey *et al.*, 1988; Janse *et al.*, 1986b). During asexual division which is only mitotic, DNA rearrangements can occur in the subtelomeric regions of the chromosomes (Janse *et al.*, 1992). Such size variations in the chromosomes of *P. berghei* appear to be mainly due to variation in the copy number of 2.3 kb. repeats, generated by either deletion or expansion of this repeat unit (Pace *et al.*, 1991).

The fluidity of the genome of *P. falciparum* is in accordance with observations on other protozoa. In *Trypanosoma brucei,* genes for the variant surface

glycoproteins (VSGs) of the surface coat of the parasite undergo re-arrangements that regulate their expression (Van der Ploeg *et al.*, 1984). This involves the transposition of large segments of DNA from one chromosome to another to produce marked chromosome size polymorphisms (Van der Ploeg and Cornelissen, 1984). Chromosome size polymorphisms in *P. falciparum* do not appear to involve inter-chromosomal exchange of large segments of DNA (Corcoran *et al.*, 1986), and it is possible that these polymorphisms can be attributed to changes in the abundance of repetitive DNA present in the genome in the absence of strong selective pressure. Recombination events within these blocks of repeats, particularly in the sub-telomeric repeats, have been shown to account largely for chromosome size polymorphisms (Oquendo *et al.*, 1986). Thus, in contrast to *T. brucei* , intra-chromosomal deletions and duplications rather than inter-chromosomal translocation appears to be the predominant mechanism responsible for length polymorphism. Analysis of molecular karyotypes of *Leishmania* species and strains by PFGE has also revealed chromosome size polymorphisms similar to those in *P. falciparum* (Spithill and Samaras, 1987).

1.3.4 Repetitive DNA: Repetitive DNA sequences (repDNA) have been reported for several malaria species. About 10% of the DNA of *P. falciparum* consists of repetitive sequences (Hough-Evans & Howard, 1982). In *P. berghei* and *P. yoelii* , repDNA fractions were found to be 5-11% and 9-17%, respectively (Dore *et al.*, 1980; Birago *et al.*, 1982; Casaglia *et al.*, 1985b).

Restriction enzyme analyses of total DNA of *P. falciparum* has shown significant differences in the arrangement of repetitive sequences between isolates from different geographical origins and between clones from a single isolate (Goman *et al.*, 1982; Bhasin *et al.*, 1985; Oquendo *et al.*, 1986). Even within a cloned line the arrangement of repDNA sequences changed after a six month culture period (Bhasin *et al.*, 1985), further confirming the relative plasticity of the genome. Cloned repetitive sequences from *P. falciparum* did not cross-hybridise with DNA of other malaria species (Guntaka *et al.*, 1985; Aslund *et al.*, 1985; Bhasin *et al.*, 1985; Cornelissen *et al.*, 1985; Oquendo *et al.*, 1986).

A repetitive DNA sequence, termed *rep20*, has been reported by several groups (Aslund *et al.*, 1985; Oquendo *et al.*, 1986; Rao *et al.*, 1986 and Zolg *et al.*, 1987). This 21-bp tandemly repeated sequence is very AT rich and has many of the properties of eukaryotic satellite DNA . The 21-bp repeat is not transcribed in the erythrocytic stages, is specific for *P. falciparum*, and comprises about 1% of the genome (Aslund *et al.*, 1985; Oquendo *et al.*, 1986). *Rep20* was mapped to sub-telomeric regions of the chromosomes (Oquendo *et al.*, 1986). This sequence is

present in all resolved parasite chromosomes and displays significant restriction fragment length polymorphism between different isolates of *P. falciparum* (Oquendo *et al.*, 1986).

1.3.5 Extra-nuclear DNA: There is evidence from electron microscopy that the intra-erythrocytic forms of malaria parasites have at least one mitochondrion per cell whereas the gametocyte forms appear to possess more (Sinden, 1983; Prensier and Slomianny 1986). However, biochemical studies on these organelles have been hindered by technical difficulties in isolating pure mitochondrial fractions .

Malaria parasites have two types of extrachromosomal DNA; a multi-copy 6-kb linear molecule and a lower copy number 35-kb circle (Wilson *et al.*, 1991). Subcellular fractionation suggests that the 6-kb element is mitochondrial in origin, unlike the 35-kb molecule which has yet to be assigned to a specific organelle. Crosses between two different clones of *P. falciparum* showed that inheritance of allelic forms of the mitochondrial gene cytochrome b found on the 6-kb element was uniparental (Creasey *et al.*, 1993), thus providing further support for the hypothesis that its origin is mitochondrial.

1.3.6 Chromatin structure: Little is known about the structural organisation of the DNA of malaria parasites. Electron micrographs of chromatin prepared from erythrocytes infected with schizonts of *Plasmodium knowlesi* show a beaded fibre structure suggesting the presence of nucleosome filaments similar to that of higher eukaryotes (Wunderlich *et al.*, 1980). The same nucleosomal pattern has been observed in the ring and mid-trophozoite stages of *P. knowlesi.* However, these data are not entirely conclusive, since structures that appear similar to nucleosomes in electron micrographs can be detected even in the absence of histones (Sinha *et al.*, 1982). A histone H2A gene has been identified in *P. falciparum* (Creedon *et al.*, 1992) and recently Cary and collegues, (1994) have been able to show that micrococcal nuclease digestion of chromatin in isolated *P. falciparum* nuclei yields a ladder of DNA fragments characteristic of nucleosomal organisation. In addition, they isolated a set of major proteins from *P. falciparum* nuclei that are similar in size and charge to histones of other eukaryotes.

1.4 Genetics:

The asexual forms of the malaria parasite are haploid. Proof comes from the fact that despite considerable variation in protein and DNA sequences between different strains of a species, cloned parasites always exhibit only one of these variable traits (Walliker, 1983). Furthermore, although chromosomes vary in size between different strains, it has been shown that only one form of each chromosome is found within a single clone (Walliker, 1987). In addition, crossing experiments between clones of the rodent malaria parasites *P. berghei*and *P. chabaudi* (Walliker *et al.*, 1973; Walliker *et al.*,, 1975) and the human parasite *P. falciparum* (Walliker *et al.*, 1987) have been carried out which have demonstrated that blood forms of the parasites are haploid. Recombination between alleles of different genes occurs, by re-assortment of genes on different chromosomes (Fenton and Walliker, 1990; Walker-Jonah *et al.*, 1992), and by crossing-over between linked genes (Sinnis & Wellems, 1988) during meiosis in mosquitoes. Recent work has also proved that intra-genic recombination within the *msp-1* gene has occurred in some of the progeny of the laboratory crosses referred to above (Kerr *et al.* 1994).

1.5 DNA replication and cytogenetic events during the malaria parasite life cycle

Studies on the organisation of the malarial genome, its replication and division, have been hampered by the complexity of the parasite's life cycle and the small size of the parasite and its nuclei. However, studies on DNA replication in malaria parasites have progressed rapidly in the last decade. Considerable progress has been made in understanding the patterns of DNA replication in malaria parasites and in isolating genes encoding proteins responsible for DNA synthesis.

1.5.1 DNA synthesis: Malaria parasites are incapable of *de novo* synthesis of the purines necessary for the synthesis of nucleic acids and other metabolic functions, and therefore must operate purine salvage pathways (Homewood and Neame, 1980). *De novo* synthesis of pyrimidines does occur and involves the vitamin para-amino-benzoic acid (PABA) and folate co-factors. Antimalarial drugs such as the biguanides, proguanil and pyrimethamine act on the parasite's dihydrofolate reductase with little effect on the corresponding host enzyme (Ferone *et al*,1969). Thus the main effect of these drugs on the parasite is probably to disrupt thymidine synthesis and hence DNA synthesis. Sulfonamides and sulfones compete with PABA for the active site on the enzyme dihydropteroate synthase, and thus also

starve the parasite of a vital source of DNA synthesis substrates (Ferone *et al*, 1969; Peters, 1987).

DNA synthesis in the malaria parasite takes place at five points in the life cycle: two of these are in the vertebrate host, the first one during exo-erythrocytic schizogony in the hepatocytes and the second during erythrocytic schizogony. DNA synthesis also occurs in the mosquito during microgamete formation, after fertilisation and before meiosis and finally during the oocyst stage to produce thousands of sporozoites.

DNA synthesis during asexual reproduction starts approximately mid-way through the 48 hours erythrocytic cycle (late trophozoite stage), and continues during schizogony almost to erythrocyte rupture, as demonstrated by the incorporation of radiolabelled precursors into the DNA of synchronised erythrocytic forms of the parasite (Inselburg and Banyal, 1984; Gritzmacher and Reese, 1984; and Waki *et al.*, 1985). Each round of DNA replication is accompanied immediately by mitotic division (Janse *et al.*, 1986a). DNA synthesis in this phase can be inhibited by aphidicolin (Inselburg and Banyal, 1984), an inhibitor of polymerase δ and α (Ikegami *et al.*, 1978), the enzymes primarily responsible for DNA chain elongation in eukaryotes (Scovassi *et al.*, 1980). Recently the genes encoding polymerase α and δ in *P. falciparum* have been cloned and sequenced (Ridley *et al.*, 1991; White *et al.*, 1993).

DNA synthesis during sexual reproduction has never been demonstrated directly. Early studies using mitomycin C as an inhibitor of DNA synthesis indicated that DNA replication takes place during the early stages of maturation of gametocytes (Sinden and Smalley 1979; Toye *et al.*, 1977), although later studies have suggested that the use of mitomycin C may be unreliable as a test for DNA synthesis in *Plasmodium* (Janse *et al.*, 1986a).

The DNA content of mature *P.berghei* macrogametocytes and microgametocytes has been assessed by microfluorometry. It appears that the DNA content of both macrogametocyte and microgametocyte is intermediate between haploid and diploid levels (Janse *et al.*, 1986b, 1989), and it has been suggested that this increase, mainly in microgametocytes, is due to selective gene amplification, for example of those genes encoding ribosomal RNA (Janse *et al.*, 1986b; 1988). However, no amplification of these genes was detected during gametocytogenesis (Cornelissen *et al.*, 1985), and no changes in the size of the chromosomes containing these genes were found (Van der Ploeg *et al.*, 1985). After gametocytes have been taken up by the mosquito the microgametocyte is converted into eight flagellated microgametes, over a period of only 20 minutes, during which three very rapid

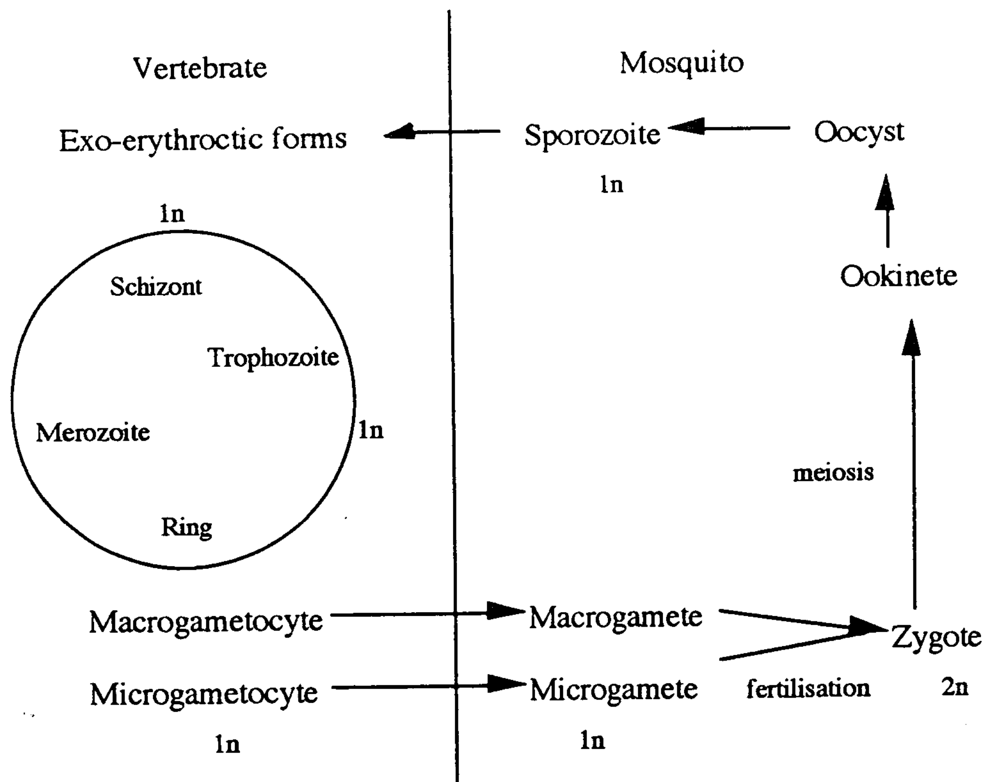
rounds of DNA replication occur. This DNA is then distributed into each microgamete, which each contains the haploid amount of DNA (Janse *et al.*, 1986c). Each macrogametocyte, in contrast, is thought to give rise to a single macrogamete which does not involve DNA synthesis. Fertilisation of haploid gametes produces a diploid zygote. A round of DNA replication follows fertilisation, that can be inhibited by the drug aphidicolin (Janse *et al.*, 1986c). The DNA content of sporozoites has also been determined by microfluorometry which indicates that sporozoites also contain the haploid content of DNA (Cornelissen *et al.*, 1984; Janse *et al.*, 1986b). Liver forms of the parasites are probably also haploid, as shown from the observation that where two clones of *P. falciparum* differed in the structure of a merozoite surface protein, the liver form in progeny derived from a cross between these clones always expressed a single allelic variant and never both (Szarfman *et al.*, 1988).

1.5.2 Mitosis: Mitosis occurs in different phases of the life-cycle of malaria parasites, in the exo-erythrocytic and erythrocytic schizonts, during microgamete formation and in the oocysts. Mitosis in malaria parasites is different from that found in higher eukaryotes: chromosomes do not condense, the nuclear membrane remains intact during mitosis and the spindle fibres are formed inside this membrane (Aikawa and Seed, 1980; Sinden, 1991). Moreover, activated microgametocytes, exo-erythrocytic schizonts and oocysts have large polyploid nuclei, containing numerous spindles. Nuclear fission in these stages appears to occur only just prior to so-called budding-off of microgametes, merozoites and sporozoites, respectively. During erythrocytic schizogony a normal mitotic sequence of genome segregation and nuclear fission is assumed to occur.

1.5.3 Meiosis: Malaria parasites are haploid during the greater part of their life cycle, and meiosis is assumed to be post-zygotic, although without direct evidence (Sinden, 1978; Walliker, 1983; Kemp *et al.*, 1985 and Casaglia 1985a). However, the exact time of the first division of meiosis has been determined in *P. berghei*, which is within 2.5 hour of fertilization (Sinden & Hartley, 1985) resulting in the synthesis of approximately 4 times the haploid DNA quantity (Janse *et al.*, 1986). These observations are consistent with duplication of the diploid chromosome set at the first stage of meiosis. Electron microscopy has shown synaptonemal complexes, structures associated with chromosome pairing during the first division of meiosis, in the early ookinete stage (Sinden & Hartley, 1985). The second meiotic division, reducing the chromosome number to 1n, has no tbeen discovered yet. Figure (1.2) is schematic illustration of the cytogenetic events during the parasite life cycle.

Figure 1.2 :

Schematic illustration of cytogenetic changes in the life-cycle of *Plasmodium*. " n" refers to the haploid chromosome complement per cell, " 2n" refers to the diploid cells.


Vertebrate
Mosquito
Exo-erythroctic forms
Sporozoite
Oocyst
1n
1n
Schizont
Ookinete
Trophozoite
Merozoite
1n
meiosis
Ring
Macrogametocyte
Macrogamete
Zygote
Microgametocyte
Microgamete
fertilisation
2n
1n
1n

1.6 The eukaryotic cell cycle and its relationship to the cell cycle of *P. falciparum*

1.6.1 The phases of the cell cycle: The 'standard' eukaryotic cell-cycle is the complete process of DNA replication, mitosis, and cytokinesis that leads to the production of two daughter cells from a single mother cell. This cycle is traditionally divided into four phases: G1(gap1), S (DNA synthesis), G2 (gap2) and M (mitosis) (Howard and Pelc, 1953) and currently two major regulatory transitions at G1/S and G2/M are recognised (Murray and Hunt, 1993). The replication of DNA and synthesis of histone proteins occurs only in the synthetic (S) phase of the interphase. In this period, each double-helical DNA molecule is replicated into two identical daughter DNA molecules, and histone and other chromosomal proteins bind quickly to newly replicated DNA. The S-phase is preceded and followed by two gap periods of interphase (G_1 and G_2 respectively) in which there is no net synthesis of DNA, although repair of damaged DNA can occur. During the mitotic (M) period, identical copies of the cellular DNA are distributed to each of the daughter cells.

All cell types undergo some version of the basic cycle described above, although details of its regulation and the size of the gap phases differ. Generally, DNA synthesis in eukaryotes is periodic and does not occupy all the cell cycle. The exception may be in the very rapidly dividing cells of early amphibian embryos (Newport and Kirschner, 1984). In most mammalian cells, the following pattern has been observed; G1 of 8-10 hours, S of 6-7 hours and G2 of 3-5 hours. The total cycle time is 18-20 hours, and mitosis takes about an hour. However this is not the universal pattern even with one cell line. The majority of S phases last for 6-8 hours, but there are cases where DNA synthesis takes longer (Murray and Hunt 1993). G2 is less variable and there are a few cases where it is less than 2 hours or greater than 6 hours. G1 is the most variable of the phases. It can be entirely absent, as in the Chinese hamster lung cells (Robbins and Scharff, 1967), or as long as 16 hours. It has been assumed that cells which grow fast generally have short G1 periods. Exactly why gap periods are required at all is not fully understood.

In mammalian tissues and cell cultures, there is a tendency for the S period to last 6-8 hours and also for the G2 period to be relatively constant. As a result, the main difference between fast and slow growing cells is in the length of G1 period, which can vary from zero to many hours. Although data on lower eukaryotes is scanty, it has been observed that cycles without G1 are quite common in for example, *Amoeba, Physarum, Schizosaccharomyces* and in the micronuclei of *Tetrahymena* and *Paramecium* (Mitchison, 1971). It is obvious that the asexual forms of *P. falciparum* have schizogony rather than a yeast or mammalian type G1/S/G2/M cycle. This might

approximate to a G1--S/M/S/M/S/M/S/M--lysis--invasion, G1 type cycle, although this is highly speculative; the truth is that little is known about the cell cycle of the malaria parasite.

1.6.2 Study of cell cycle control using yeast as a model system: Our understanding of how progression through the cell cycle is regulated has advanced enormously during the last two decades. Data from genetic, biochemical and molecular investigations of diverse organisms, especially the cloning of some key cell cycle control genes, has given new insights into the control mechanisms of the cell cycle and realisation that certain aspects are universal in all eukaryotes (Nurse, 1990; Forsburg and Nurse, 1991; Norbury and Nurse, 1992).

Because of the possibility that all eukaryotes could have similar cell cycle controls, many scientists have sensibly started to explore the cell cycle in simple experimental systems. Two of the most successfully used are *Saccharomyces cerevisiae* (budding yeast) and *Schizosaccharomyces pombe* (fission yeast). Their small nuclear genome and well characterised genetics, in addition to their suitability for gene manipulation, has made them model organisms for cell cycle studies. Since yeasts are unicellular, can be haploid and have a cell cycle that can be as short as 2 hours, they can be readily grown as large populations for both biochemical analysis of cell cycle gene products and for analysis of the effects of cell division cycle (cdc) mutants. A powerful way of interfering with the cell cycle has been the use of such cdc mutants. It has become possible to isolate conditional mutants that make altered proteins which are functional under one condition but non-functional under another. This conditional type of mutant has been widely used, with increased temperature as the restrictive condition.

1.6.3 Evolutionarily conserved elements involved in control of the cell cycle: Isolation of temperature sensitive (ts) mutants in yeasts has resulted in a collection of mutants that can be arrested at different stages of the cell cycle. Among the the key genes identified are CDC28 of *S. cerevisiae* and *cdc2* of *S. pombe* (Reed 1980; Nurse and Thuriaux, 1980). Cloning and sequencing of *cdc2* and CDC28 genes has revealed that they both encode proteins of 34kDa that both are protein kinases sharing 60% amino acid sequence homology (Lee and Nurse, 1987). In addition, it has been shown that CDC28 of the budding yeast can complement a *cdc2* mutant in *S. pombe* and vice versa (Beach *et al*, 1982; Booher and Beach 1986). The similarity between the products of *cdc2* and CDC28 genes in the distantly related yeasts *S. pombe* and *S. cerevisiae* indicated the possibility that such a conserved gene

product might also exist in other eukaryotic organisms. A human *cdc2* gene, *cdc2Hs*, was successfully cloned by complementation of a temperature sensitive mutation of *cdc2* gene in fission yeast with a human cDNA (Lee and Nurse, 1987). At the same time, Draetta and colleagues also identified a 34 kDa protein kinase in human HeLa cells using a monoclonal antibody against fission yeast $p34^{cdc2}$ protein (Draetta *et al.*, 1987;). This was confirmed later as the human homologue of the fission yeast *cdc2* gene product. To date, homologues of the *cdc2* gene or gene product have been isolated from a variety of eukaryotes including starfish (Arion *et al.*, 1988), sea urchin (Meijer *et al.*, 1989), clam (Draetta *et al.*, 1989), *Drosophila* (Lehner and O'Farrell, 1990), maize (Colasanti *et al.*, 1991), soybean (Miao *et al.*, 1993), *Leishmania* (Mottram *et al.*, 1993) and recently *P. falciparum* (Ross-McDonald *et al.*, 1994). The key cell regulating protein $p34^{cdc2}$ is present in all eukaryotes and the fundamental mechanism that controls mitotic initiation in all eukaryotes is probably universal.

At about the same time as CDC28 and *cdc2* were discovered, cytological and biochemical analysis of *Xenopus* oocytes identified an activity described as maturation-promoting factor (MPF) which was present in metaphase cytoplasm of maturing oocytes and unfertilised eggs, and which could cause immature oocytes to mature (Masui and Marker, 1971; Doree, 1990). Highly purified MPF was later found to consist of a complex of a 34 kDa serine/threonine protein kinase, identified as a *Xenopus* homologue of the *cdc2* gene product, $p34^{cdc2}$, and a 45 kDa substrate, identified as a *Xenopus* B-cyclin. When the PSTAIR antibody, antibody against the sequence EGVPSTAIREIELLKE that is highly conserved in cdc2 proteins from yeasts to human, (Lee and Nurse, 1987) was used on a Western blot of the purified MPF preparations, it revealed that the 34 kDa component of MPF co-migrated with authentic human $p34^{cdc2}$ (Gautier *et al.*, 1988). Identification of $p34^{cdc2}$ as the kinase component of MPF, which is today recognised as the regulator of G2/M transition, further confirmed the universality of these components of cell cycle control in diverse organisms.

1.7 Signal transduction in the eukaryotic cell cycle:

All biological systems have the ability to process and respond to enormous amounts of stimuli. Much of this information is provided to individual cells in the form of changes in concentration of hormones, growth factors, neuromodulators, or other molecules. These ligands interact with specific receptors, and such binding events are transduced into intra-cellular signals. Several families of cell surface receptors have been characterised that are coupled to different mechanisms of signal transduction. A family of guanine nucleotide-binding regulatory proteins (G-proteins)

is known to be involved in a variety of receptor-mediated signal transduction systems in mammalian cells (Gilman, 1984). The general significance of G-proteins in cell biology was recognised by the award of the Nobel Prize for medicine to Alfred Gilman of Texas University (USA) this year for his research in G-proteins.

1.7.1 G-proteins as signal transducers: The 'classical' guanine-nucleotide-binding proteins (G-proteins) comprise a family of homologous, membrane associated (although not transmembrane), proteins which form part of a heterotrimeric complex of molecules. They function to allow communication between cell surface receptors for hormones, growth factors and neurotransmitters and the effector systems which convert this primary message within the cell into a signal which will generate a physiological response (Gilman, 1987; Milligan, 1989). The heterotrimer consists of distinct α-(39-46 kDa), β-(35-36 kDa) and γ-(8 kDa) subunits (Valencia *et al.*, 1991). The α-subunits of the various G-proteins are assumed to define the specificity and selectivity of both receptor/G-protein and G-protein/effector interactions (Bourne, *et al.*, 1990; Simon *et al.*,1991). Very strong evidence for a positive signalling role of the β and γ subunits has been provided by the genetic analysis of G-proteins involved in the mating pheromone response in the budding yeast *Saccharomyces cerevisiae* (Marsh *et al.*, 1991). In addition, a considerable family of small (19-27 kDa) GTP-binding proteins has recently been identified, including members of the Ras, Ral, Rho, Rab, Ran, and ARF families of gene products. While there is no evidence to indicate that these polypeptides can interact with β/γ subunits of G-proteins, and may serve different functions, the potential role of at least some of these proteins as proto-oncogenes has stimulated considerable interest in the concept that they may function as transducer elements analogous to the 'classical' G-proteins (Milligan *et al.*, 1988). Members of the small monomeric GTP-binding proteins such as Rho and Ran may also be involved in signal transduction through the indirect activation of serine/threonine kinase.

1.7.2 Signal transduction and the *P. falciparum* life-cycle: Recently, it has been observed that recognition and invasion of hepatocytes by malaria sporozoites may involve multiple binding events which might be mediated by binding of the circumsporozoite protein to sulfated glyco-conjugates (Cerami *et al.*, 1992). This defined receptor/ligand interaction highlights the importance of intra-cellular and inter-celluar signalling systems and pathways in malaria parasites, although very little is yet known about them. As discussed above, key components of signalling pathways in higher eukaryotes are G-proteins and there are quite strong resemblances

between certain well understood receptor/ligand activated signal transduction pathways in yeast and some developmental processes in *P. falciparum.*

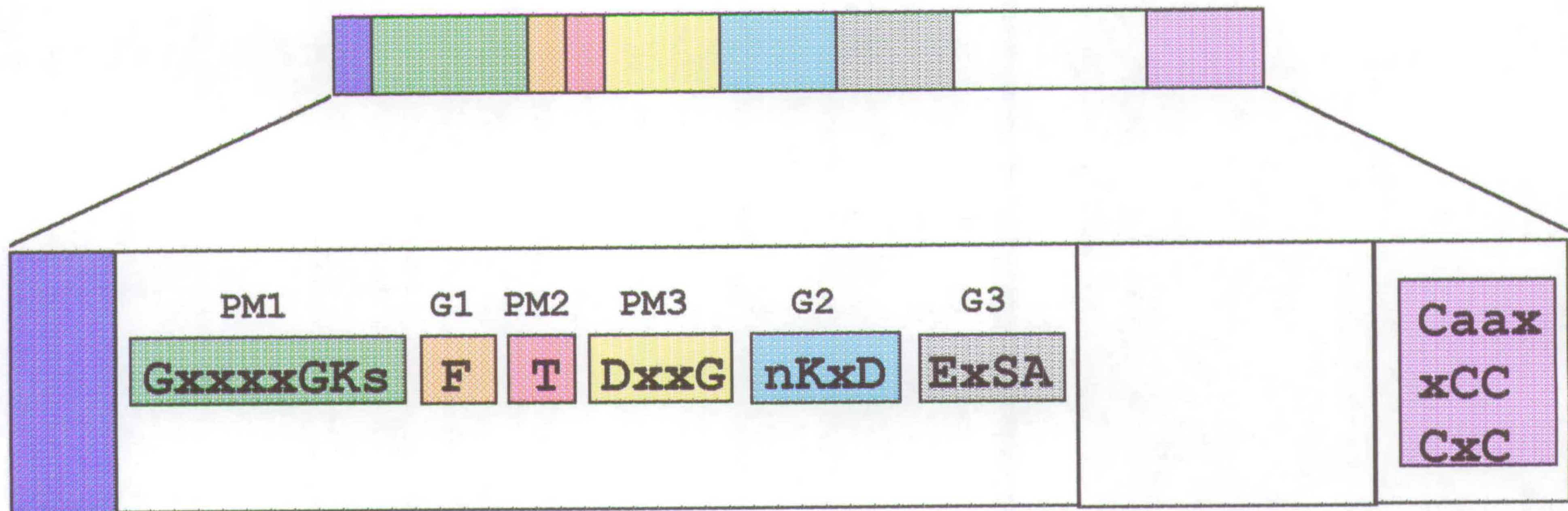
In both the budding yeast *S. cerevisiae* and *P. falciparum* single haploid cells can differentiate into gametes of both sexes. In *S. cerevisiae* cell to cell signalling leads to differentiation in preparation for mating, manifested by transcriptional induction of many genes, morphological changes and the arrest of the cell cycle at G1 phase (Marsh *et al.*, 1991). In *P. falciparum* cultures, at high parasitaemia, a high proportion of *P. falciparum* merozoites differentiate sexually into G1 arrested gametocytes, but when the culture is diluted with fresh medium and red blood cells, a marked reduction has been noticed in the proportion of parasites which develop into sexual forms (Bruce *et al.*, 1990). Malaria merozoites which invade red blood cells and become gametocytic sexual forms differentiate into elongated forms over a period of 8-10 days and some sexual stage specific proteins are known to be produced (Alano and Carter, 1990). However, neither the induction nor the signal transduction processes are understood in this parasite, at least partly because of the intractability of its genetics and life cycle and the problems associated with a difficult and fragile culture system (Cornelissen, 1988). However, it is clear that many aspects of signal transduction for sexual differentiation in yeast are evolutionarily conserved and although very little is known about the mechanisms of signal transduction in malaria parasites, it is tempting to speculate that elements of evolutionary conservation could exist between signal transduction in *Plasmodium* and yeast

1.8 The Ras superfamily of GTPases:

In 1980, two *ras* genes were discovered: the first, now known as v-H-*ras,* is the transforming gene of Harvey, BALB and Rasheed sarcoma viruses, and the second, v-K-*ras,* is the transforming gene of Kirsten sarcoma virus (Barbacid, 1987). Now more than 50 distinct Ras-like proteins controlling many different aspects of cell growth and behaviour have been identified in mammals as well as in many other eukaryotes. The primary sequence structure of Ras-related proteins may be subdivided into several main parts (Fig.1.3). The N-and C-terminal parts of the sequence form two subdomains of the structure. The N-terminal end is of variable length, ranging from 5 to more than 30 amino acids. The N-terminal subdomain contains phosphate-magnesium binding sites. The C-terminal subdomain has guanine binding sites which are highly conserved between the various Ras-related proteins.

Figure 1.3 :

Diagrammatic structure of the main sequence elements and highly conserved residues of *ras*-like proteins. GNB= guanine nucleotide binding domain, PMn= phosphate magnesium binding regions, Gn= guanine base binding regions, Caax: a=aliphatic, X= any residue. GxxxGKs: s=Serine or Threonine. Reproduced with modification from Valencia *et al.*, (1991), *Biochemistry* 30, 4637-3648.



N-terminus
4-31 aa

GNB-Domain
~160 aa

C-terminal extension
13-49 aa
~120 aa in RASY 1,2

C-terminus

The C-terminus extension is highly variable, even among closely related proteins of the Ras superfamily. The last 4-5 residues always include a cysteine-containing motif that appears to be required for *in vivo* covalent modification (Valencia *et al.*, 1991).

The Ras proteins are members of an extended family of GTPases (Bourne *et al.,* 1990, 1991) which includes proteins involved in protein synthesis (the elongation and initiation factors) and signal transduction (the α subunit of heterotrimeric G proteins). The GTPases of the Ras superfamily regulate many aspects of cell growth, differentiation and action. Their functions depend on their ability to alternate between inactive and active forms, and on their cellular localisation. Figure (1.4) shows a simplified representation of these proteins' switching cycle (Bourne *et al.*, 1990; Boguski and McCormick, 1993). The GTP-bound form is slowly converted to the GDP-bound form by the protein's intrinsic capacity to hydrolyse GTP, a process accelerated by GTPase-activating proteins (GAPs).

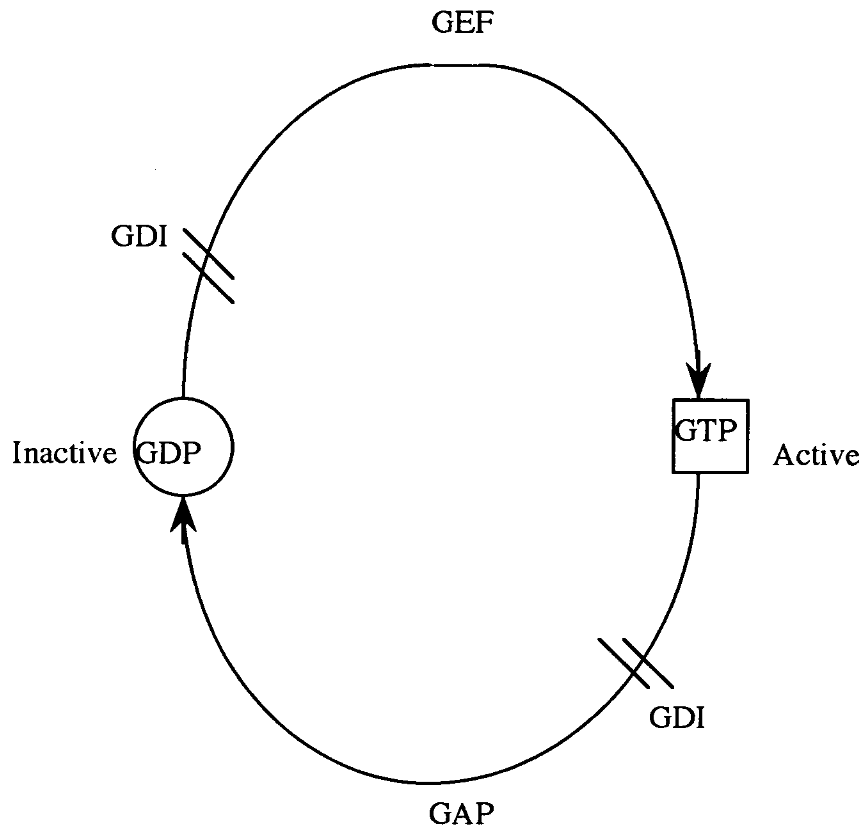
Numerous proteins affecting GTPase activity, nucleotide exchange rates and membrane localisation of Ras superfamily members have now been identified. Many of these proteins are larger and more complex than their targets, containing multiple domains capable of interacting with an intricate network of cellular enzymes and structures (Bourne *et al.*, 1990; Boguski and McCormick, 1993). Proteins that activate Ras proteins by exchanging bound GDP for free GTP, known as guanine-nucleotide exchange factors (GEFs), are being discovered at a rapid rate. In *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Drosophila melanogaster* and *Caenorhabditis elegans*, genetic loss of GEF function has similar effects to those induced by loss of the Ras-like proteins themselves, showing that GEFs are essential for Ras action (Boguski and McCormick, 1993).

1.8.1 The Ras-related proteins (the small GTPases): A large superfamily (more than 50 members) of monomeric GTP-binding proteins homologous to Ras has been described in the last few years (Bourne *et al.*, 1991). The Ras protein family consists of small proteins (20-35 KDa) with sequence similarities, especially in their guanine nucleotide-binding domains, to the products of Ras proto-oncogenes. Many of these proteins were discovered because they bind GTP or because their cDNAs hybridise with nucleotide sequences derived from *ras* genes. Not much is understood about the cellular function of individual small GTPases and therefore these proteins are classified according to similarities of primary structure. Based on sequence homology the Ras superfamily can be divided into four subfamilies : Ras and Ras-like, Rho, Rab and Ran (Downward, 1992; Hall, 1990).

Figure 1.4 :

Simplified diagram showing regulation of Ras-like GTPase by guanine-nucleotide-exchange-factors (GEF), guanine-nucleotide-dissociation-inhibitors (GDI) and GTPase-activating proteins (GAP).

GEFs catalyse exchange of the GDP for GTP; GAPs catalyse conversion of GTP-bound forms back to their inactive GDP states. GDI proteins for small GTPases, affect nucleotide dissociation and GAP activity.


GEF
GDI
Inactive
GDP
GTP
Active
GDI
GAP

The Ras-related proteins have been implicated in many cellular processes from signal transduction to vesicular transport (Bourne *et al.*, 1991), and function as oncogenes following mutational activation. These proteins transmit signals from tyrosine kinases at the plasma membrane to a cascade of serine/threonine kinases, which delivers signals to the cell nucleus (Bourne *et al.*, 1991). The Rho/Rac protein subfamily is involved in organisation of the cytoskeleton (Boguski and McCormick, 1993). Rac proteins are essential components of the NADPH oxidase system that generates superoxide in phagocytes (Segal and Abo, 1993), and may also be involved in signal transduction through the direct activation of serine/threonine kinases (Manser *et al.*, 1994). The Rab protein subgroup is involved in regulation of membrane trafficking, that is, the transport of vesicles between different intracellular compartments (Pryer *et al.*, 1992). Recently a number of Rab homologues have been identified in *Plasmodium falciparum* (G.Langsley, personal communication).

1.8.2 Ran/TC4: Ran/TC4 is the prototype of a fourth major subfamily of *ras*-related genes, first identified in human cDNA libraries (Drivas *et al.*, 1990, 1991). Homologues have since been identified in other mammals (dog, mouse), *Drosophila*, *Caenorhabditis*, *Dictyostelium*, and in yeasts (*S. cerevisiae* and *S. pombe*). The Ran subfamily is highly conserved; the predicted sequences of all known Ran-like proteins are at least 70% identical. However, the subfamily shows only limited resemblance to other Ras proteins. Outside the four stretches of sequence that specify the Ras guanine nucleotide binding site, there are no obvious homologies between Ran sequences and those of other Ras family members. One striking difference is that, while other Ras-related proteins share a carboxyterminal cysteine-rich motif that mediates isoprenylation and membrane association, Ran proteins have a stretch of acidic amino acid residues at this position (Bischoff and Ponstingl, 1991a, Ren *et al.*, 1993).

Ran-GTPases appear to be involved in cell cycle control. In the eukaryotic cell cycle, nuclear replication (S phase) and mitosis (M phase) are linked such that replication must be complete before mitosis can begin. To prevent cell division beginning prematurely, eukaryotic cells have systems for detection of unreplicated DNA which transmit signals that inhibit the activation of mitotic factors (Hartwell and Weinert,1989; Nishimoto, *et al.*, 1992). Both genetic and biochemical approaches have been taken to elucidate the pathway through which unreplicated DNA sends inhibitory signals, and a number of proteins required for signal transmission have been identified (Enoch and Nurse, 1990; Enoch *et al.*, 1992; Roberge, 1992).

1.9 The regulator of chromosome condensation (RCC1):

Ran/TC4 has been hypothesised to function mainly in the nucleus in conjuction with a guanine nucleotide exchange factor to couple completion of DNA synthesis with initiation of mitosis through the activation of a cyclin-p34^{cdc2} complex (Ren *et al.*, 1993). However, only one gene product, the regulator of chromosome condensation (RCC1), has been found to be a chromatin-associated protein (Ohtsubo, *et al.*, 1989), making it the most likely candidate for a protein which interacts with DNA directly to monitor the completion of replication.

An RCC1 mutant was first isolated in a temperature-sensitive cell line (*ts*BN2 cells) derived from BHK21/13 hamster cells (Nishimoto *et al.*, 1978). Genes homologous to RCC1 have been isolated from organisms including humans (Bischoff and Ponstingl, 1991a), *Xenopus* (Nishitani *et al.*, 1991), *S. cerevisiae* (Clark and Sprague, 1989; Aebi *et al.*, 1990), *S. pombe* (Matsumoto and Beach, 1991) and *Drosophila* (Frasch, 1991). The predicted amino acid sequences of RCC1 proteins are well conserved across vertebrate species. The RCC1 protein has a domain consisting of seven homologous and very conserved repeats of about 60 amino acids (Ohtsubo *et al.*, 1989; Ohtsubo *et al.*, 1991). RCC1 has been shown to be a nuclear-localised DNA binding protein (Ohtsubo *et al.*, 1989; Frasch, 1991). It is present at about one million copies per nucleus, equivalent to one copy per nucleosome (Seino *et al.*, 1992).

A mutation of the RCC1 gene in the *ts*BN2 cell line has been found to confer a pleiotropic phenotype, including G1 arrest and premature induction of mitosis in cells synchronised at the G1/S boundary (Ohtsubo *et al.*, 1989). Similarly, mutations of the SRM1/PRP20 gene, the *S. cerevisiae* homologue of RCC1, are pleiotropic: the *srm1* mutant shows G1 arrest and a suppression of mating defect in a mutant lacking pheromone receptors, and the *prp20* mutant shows an alteration in mRNA metabolism (Clark and Sprague 1989).

1.10 The role of Ran/TC4 and RCC1 in regulation of the cell cycle:

Genetic and biochemical evidence collectively suggest that Ran/TC4 and RCC1 are key components of a control system that monitors the progress of DNA replication and regulates mitosis.

A key component of eukaryotic cell cycle control is mitosis promoting factor (MPF). MPF is a complex of two proteins, cyclin B and a cyclin-dependent serine-threonine protein kinase denoted p34^{cdc2} (Freeman and Donoghue 1991; Norbury and Nurse 1992). Its regulation involves interactions among tyrosine and serine-threonine

protein kinases, protein phosphatases, a cyclin protease, and other activators and inhibitors. It is formed late in G1 or in S phase depending on the cell type, by the association of newly synthesised cyclin B with $p34^{cdc2}$, and is inactivated by phosphorylation of specific threonine and tyrosine residues.

Premature entry into mitosis occurs upon loss of RCC1 (see section 1.8.3) requires MPF activation and the presence of cdc25 the phosphatase that activates preMPF (Nishitani *et al.*, 1991; Seino *et al.*, 1992; Seki *et al.*, 1992). A fission yeast temperature-sensitive mutant, defective in coupling mitosis to the completion of DNA replication, contain a mutation in the RCC1-homologous gene called premature initiation of mitosis-1 (Pim1) (Matsumoto and Beach, 1991). *pim1* mutants are rescued by overexpression of a wild-type allele of suppressor of pim1 (SPI1), the fission yeast homologue of Ran/TC4 (Matsumoto and Beach, 1991, 1993). It has been shown that human RCC1 protein can be purified from HeLa cells in the form of a complex with Ran/TC4 (Bischoff and Ponstingl, 1991a). RCC1 protein specifically catalyses exchange of guanine nucleotides on the Ran/TC4 protein (Bischoff and Ponstingl, 1991b).

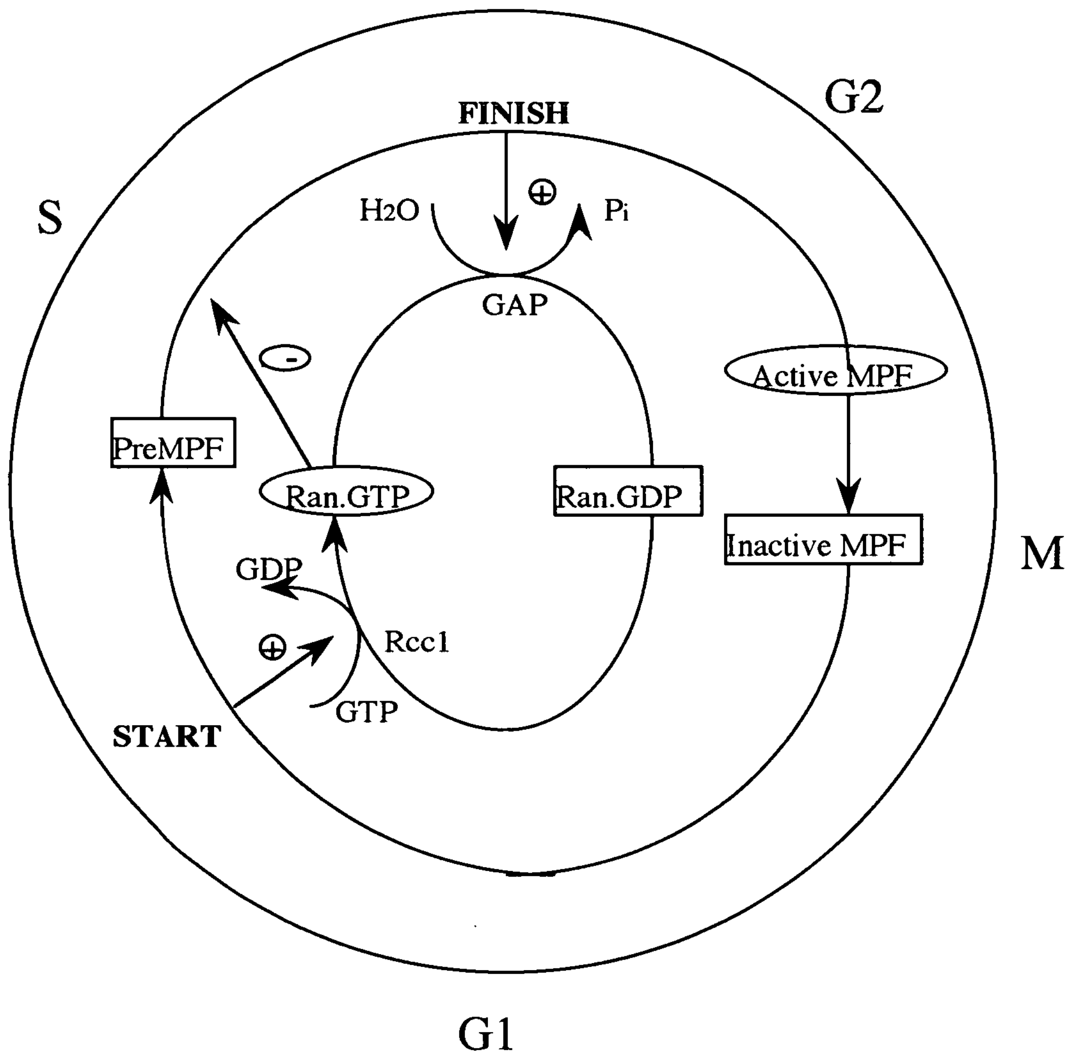
Models have been proposed, in which the cell cycle is coupled to a Ran-GTPase cycle (Bischoff and Ponstingl, 1991a, 1991b; Roberge, 1992; Ren *et al.*, 1993). Ran can switch between an active GTP-bound form and an inactive GDP-bound form, and this switch may regulate the MPF-driven cell cycle (Fig.1.5). In this figure, START, the commitment of the cell to DNA replication, stimulates the guanine nucleotide exchange function of RCC1, and this in turn converts Ran/TC4 to its active GTP-bound form. The Ran-GTP complex (active) transduces a signal that inhibits the conversion of preMPF to MPF. FINISH, the completion of DNA replication, is associated with the inactivation of RCC1 and the activation of Ran-specific GAP, restoring Ran to its inactive GDP-bound form.

The model predicts that unreplicated DNA present between START and FINISH alters the level of Ran/TC4-GTP. This may be an essential step of a pathway that leads to MPF inactivation, although there may be multiple intermediate steps. For example, Ran/TC4-GTP could stimulate, either directly or through regulatory kinases and phosphatases, activation of the kinase that phosphorylates $p34^{cdc2}$ on tyrosine to inactivate MPF (Smythe and Newport, 1992). Ran/TC4-GTP could also inhibit, directly or indirectly, the tyrosine phosphatase that dephosphorylates $p34^{cdc2}$, and that is required for MPF activation (Millar and Russell, 1992).

Figure 1.5 : A model for Ran/GTPase cycle.

A model coupling the cell cycle and a GTPase cycle via nuclear proteins Ran and RCC1. The inner circle represents the Ran-GTPase, the second circle represents the MPF (maturation-promoting factor) cycle, and the outer circle represents the cell cycle.

START is the point of commitment to DNA synthesis, as distinguished from the point at which DNA replication actually begins. FINISH is the end of DNA replication. For detailed description see section 1.10, Chapter 1. The figure is produced, with modifications, from Ren *et al.*, (1993). Journal of the Cell Biology, 313-323.



FINISH
G2
S
H2O
⊕
Pi
GAP
⊖
Active MPF
PreMPF
Ran.GTP
Ran.GDP
Inactive MPF
M
GDP
⊕
Rcc1
GTP
START
G1

1.11 The cell cycle of malaria parasites

Research on the molecular mechanisms that control the cell cycle in yeast and higher eukaryotes, has led to a detailed model of how the eukaryotic cell cycle is regulated. The control of the cell cycle is important not only for the normal growth of the cells but also in developmental processes, most notably where the cycle is interrupted in differentiated lineages.

There have been rather few studies on the biochemistry and molecular biology of the cell cycle of *P. falciparum* (Sherman 1979; Homewood and Neane, 1980; Kemp *et al.*, 1986; Janse *et al.*, 1986 a, b, c) and the genetic and molecular processes underlying and directing the extensive cyclic differentiation are poorly understood. Obviously studies of malaria cell cycle lack cell cycle mutants and it is difficult to perform experiments that seek to vary growth rates and measure cell size in the rather fragile *in vitro* culture model. However, several genes implicated in DNA replication and other cell cycle events in *P. falciparum* have recently been isolated. Genes encoding protein homologues of DNA polymerase α and δ, known to play an essential role in DNA replication in eukaryotes, have been cloned in *P. falciparum.* A group of DNA polymerases has also been identified in the rodent malaria, *P. berghei*, (Ridley *et al.*, 1991; De Vries *et al.*, 1991; White *et al.*, 1993). The gene encoding the malarial homologue of the proliferating cell nuclear antigen (PCNA), an auxiliary factor for extended DNA replication by DNA polymerase δ, has been isolated and characterised in *P. falciparum* (Kilbey *et al.*, 1993). Recently, a malarial homologue of the type II DNA topoisomerase gene homologue has been identified in this species (Cheesman *et al.*, 1994), together with several members of the kinase family, such as the *cdc-2* like protein kinase and *pfcrk*-like kinase (Ross-MacDonald *et al.*, 1994; C. Doerig, personal communication).

1.12 The aim and outline of the research presented in this thesis

The research outlined in this thesis has been primarily designed to study the signal transduction pathways involved in the regulation of the cell cycle of the malaria parasite *Plasmodium falciparum.* The specific aim of my research project, and indeed this thesis, has been to clone, sequence and characterise, from *P. falciparum*, gene members of the GTPase superfamily protein that have been implicated in regulation of cell cycle in other organisms. The work can be divided into three complementary parts, all aimed at trying to see if *P. falciparum* homologues of certain key genes involved in signal transduction for cell cycle regulation exist and, if so, whether they can be analysed in studies aimed at clarifying cell cycle events in this protozoan parasite.

The first part (chapter 3) is devoted to molecular cloning and sequencing of the *P. falciparum* homologue of the Ran/TC4 gene. Using a PCR-based approach, a fragment which displays a high degree of homology to Ran/TC4 was obtained from the genome of *P. falciparum* (Sultan *et al.*, 1994), and this fragment was used to screen genomic and cDNA libraries to obtain the full coding length of the gene. At the same time the copy number, chromosomal location and level of RNA expression of the *P. falciparum* homologue were determined (Sultan *et al.*, 1994).

The second part of the work (chapter 4) was mainly aimed at characterising the *pfran* gene product. Polyclonal antibodies against a specific segment of the predicted protein sequence were raised . The anti-pfRan serum recognises, in total protein prepared from the parasite, a band of 27 kDa which corresponds to the predicted molecular weight of the pfRan protein. In addition, the serum was used to determine the localisation of the protein in infected red blood cells prepared from parasite culture.

To gain further insight into intra-nuclear processes occurring during the cell cycle regulation in the malaria parasite, and the role of Ran in this process, the *P. falciparum* homologue of RCC1 was cloned and almost completely sequenced (chapter 5). This protein known to interact with Ran-GTP and to regulate the functional state of the GTPase-switch that monitors the progression of DNA replication as well as being involved in a variety of other intriguing events in cell development.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and equipments

Standard laboratory chemicals and solvents were obtained from British Drug House (BDH) plc., Fisons Scientific Apparatus or Sigma Chemical Co. Ltd., UK. and were of analytical grade. Radioactive nucleotides were supplied by Amersham International plc. The sources of other materials are given in the text.

Centrifugation was either carried out in a bench top centrifuge MSE,(Fisons) or IMV-13 microfuge (IBI). Higher speed centrifugations were performed in either Sorvall-RC-5B-refrigerated centrifuge or ODT-65 ultracentrifuge (DuPont Instruments).

2.1.2 Parasite source

The parasites used in this work were clones 3D7A, 106/1, 105/1, 102/1, C10, 1776, Dd2, 7G8, T9/94 and T9/96. These *P. falciparum* clones are kept in the WHO Registry of Standard Strains of Malaria Parasites in the Centre for Parasite Biology, ICAPB, University of Edinburgh.

2.1.3 Erythrocytes

Fresh whole blood, group O Rh +, used for parasite culturing was obtained from Edinburgh and South-East Scotland Blood Transfusion Service. The blood was washed and centrifuged at 1500g three times in incomplete RPMI medium to remove citrate. The 'buffy coat' of white blood cells was removed from the red cell pellet, which was resuspended in complete medium to give a haematocrit of 50%. The washed red blood cells (RBCs) were kept at 4°C for up to one week.

2.1.4 Genomic and cDNA libraries

Four *Plasmodium falciparum* cDNA and genomic libraries were screened during the course of this work. These were : asexual cDNA library made from rings and late trophozoites in plasmid pJEF14 (a gift from Dr. A. Craig, Oxford university), λgt11, gametocyte cDNA library from clone 3D7 (Alano, *et al.*, 1991), λNM 1149, *HindIII* digested genomic DNA from isolate K1 and λgt11 (Goman, *et al.*, 1982), partial *DraI* digested genomic DNA from isolate K1 (a gift from Dr. B. Kilbey).

2.1.5 Bacterial strains

The following bacterial strains were used in the course of this study : INVαF' (Hanahan, 1983b), TG-1 strain and Y1089 (Young and Davies, 1983).

2.1.6 Oligonucleotide primers used in this study:

Oligonucleotide primers used in this study were synthesised by the Oswel DNA Service, Department of Chemistry, University of Edinburgh, and HPLC purified if necessary. The sequence of the primers are given below (+: sense; - anti-sense):

M13 (-40), +	5'- GTT TTC CCA GTC ACG AC-3'
M13, -	5'- CAG GAA ACA GCT ATG AC-3'
λ gt11,+	5'-GGTGGCGACGACTCCTGGAGC CCG-3'
λ gt11, -	5'- TTGACACCAGACCAACTGGTAATG-3'
pJFE-14, +	5'- TCT AGA GAT CCC TCG ACC TC-3'
pJFE-14, -	5'- CGC GGA CGT CCA GCG CCG GC-3'

Ran Primers:

A482,+ (Ran-1)	5'- GGA(AT) GAT GG(AT) GG(AT) AC(AT) GG(AT) AA-3'
A483,+ (Ran-2)	5'- GG(AT) GAA TT(AC) GGA AA(AG) AA(AG) TA- 3'
A484,- (Ran3)	5'- GG(CT) TT(TC) TC(TG) AA(AG) TT(AG) TA(AG) TT-3'
A901,- (Ran/inv)	5'- TAT TTC TCC TTT TCA TTA CG -3'
A902,- (Ran/inv)	5'- CAA TAC GAT CTA TCT GC -3'
G2037,- (Ran5)	5'- TTT TCA ATA TCT TCT TCA TC -3'
G1701,+ (Antb/F)	5'- CCC GGA TCC GGT TAG CTA GGA GAT TG -3'
G1702,- (Antb/R)	5'- GGG GAA TTC ATT TTC AAT ATC TTC TTC-3'
G5793,+ (yes/F)	5'- CGCCATATGGATTCACAAGAATATATTCC-3'
G5794,- (yes/R)	5'- CGCGGATCCTTAATTTTCAATATCTTCTTC-3'

RCC-1/Pim-1 Primers

G5058, -	5'- GTA TC(AT) CG (AT) CC(AT) A(AG)(AT) GC(AT) CC-3'
G5146,-	5'- CG(AT)A(AG)(CT)TG(AG)(AT)(AT)(CT)TG(CT)TG(AT) CC(AG)TT(AT)CCC CA -3'
G6037,+	5'- G(AC)(AT) G(CT)(AT) GG(AT) GG(AT) ATG CA-3'
G8119,+	5'- CGG TTG ATA ATA ATG GTG-3'
G8695,+	5'- GAA TTC TTT ATG TAT GTG CAG G-3'
G8913,-	5'- CCA AAA TAA GTA TAT TCA TTT TG-3'
G8914,+	5'- ATT TCT AAA TTA CCT AAG C-3'
G8118,+	5'- GGG GCT CGA GAA ATG GAT TCA CAA GAA TAT-3'
G9075,+	5'- AAG TTA TAA TAA GCC CC-3'
G9161,- (inv)	5'- CAC CAC CTG TGC AGA TAG CC-3'
G9191,- (antb)	5'- TGC ACA TAC ATA AAG AAT TC-3'

G9192,+(antb)	5'- CCC GGA TCC TGC ACA CAT TGG CTA TCT GC-3'
H0472,+	5'- GAA GGA TGT ACA TTT TG-3'
H1041,+	5'- GTG TAT TTT TAT TAG AAA GC-3'
H1790,+	5'- GAA CCT GTT CAT GTG AAG-3'
H4252,	5'- AAT TTT GTA AAT GAC ACC-3'
H4253,	5'- GGA AGT TGT AGT GAT ATG G-3'
H4254,	5'- AAA CAT GAC TGG TTT ACT CC-3'

2.1.7 General stock solutions and media

All aqueous solutions were prepared in de-ionised glass distilled water. pH values of solutions were measured with a pH meter model PW 9410 (Philips).

TAE 50x stock solution

242g Tris base, 37.2g Na_2 EDTA, 2H2O 57.1ml glacial acetic acid, dH_2O to 1 litre. This solution was diluted x50 to give 1x working solution (40mM tris acetate, 2mM EDTA).

TBE 10x stock solution

108g Tris base, 55g boric acid, 40ml 0.5M EDTA pH 8.0, dH_2O to 1 litre This solution was diluted x10 to give 1x working solution (89mM Tris base, 89mM boric acid, 2mM EDTA).

MOPS 10x stock solution

0.2M 3-[N-Morpholino]- propane-sulphonic acid, 0.5M Na acetate pH7.0, 0.01M Na_2 EDTA, H_2O to 1 litre. 1x working solution is (0.02M morpholino-sulphonic acid, 0.05M Na acetate, 0.001M EDTA)

SSC 20x stock solution

175g NaCl, 88g Na_3 citrate.2H2O, adjusted to pH 7.0 and made up to 1 litre with dH_2O. 2x working solution is (0.3 M NaCL, 0.03M Na_3 citrate.$2H_2O$).

SDS 10% stock solution

100g was dissolved in 900ml H_2O, heated to 68°C and the pH was adjusted to 7.2 by HCl, and made up to 1 litre with dH_2O.

PBS 10x stock solution

80 g NaCl, 2g KCl, 11.5g Na_2 $HPO_4.7H2O$, 2g KH_2PO_4 , dH_2O to 1 litre. working solution, pH~7.3 was 137 mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4.7H_2O$ 1.4mM KH_2PO_4 .

TE buffer (1x)	10mM Tris-HCl pH 8.0 1mM EDTA pH 8.0
SOC medium	2% Bacto-tryptone 0.5% bacto-yeast extract 10mM NaCl, 2.5mM KCl 10mM Mg_2Cl, 10mM Mg_2SO_4 20mM glucose
SM medium (per litre)	5.8 g NaCl 2 g $MgSO_4.7H_2O$ 50ml 1M Tris.Cl (pH 7.5) 5ml of 2% gelatin
Transformation Buffer I TFB1 (amounts/litre)	30mM potassium acetate (30 ml of a 1M stock pH7.5) 100mM RbCl (12g) 50mM $MnCl_2.2H_2O$ (9.9g) 10mM $CaCl_2.2H_2O$ (1.5g) 15% (w/v) glycerol (150g) pH adjusted to 5.8 with 0.2M acetic acid, Solution sterilised by filtration.
Transformation Buffer II TBF II (amounts/litre)	10mM MOPS (20ml of a 0.5M stock pH 6.8) 10mM RbCl (1.2g) 75mM $CaCl_2.2H_2O$ (11g) 15% (w/v) glycerol (150g) pH of the solution was adjusted with KOH to 6.8 and sterilised by filtration.

Luria-Bertani medium (LB)	1% Bacto-tryptone 0.5% Bacto-yeast extract 1% NaCl adjusted to pH 7.2 with NaOH
LB agar	LB medium with 1.5% agar
LB-amp.	LB supplemented with ampicillin to 50μg/ml
EMM (per litre)	20g Glucose, 5g NH_4 Cl, 3g K phathlate 1.8g Na_2HPO_4, 1g $MgCl_2.6H_2O$ 100mg Na_2SO4, 15mg $CaCl_2.7H_2O$ 1ml Vitamins solution (Stock solution consists of 5g Inositol, 5g Nicotinic acids, 0.5g Pantothenoic acid and 5g of Biotin) 0.1ml Trace elements (stock solution is 1g boric acid, 1.04g $MnSO_4.4H_2O$, 0.8g $ZnSo_4.7H_2O$, 0.4g $FeCl_3.6H_2O$, 80mg $CuSO_4.5H_2O$, 2g Citric acid and 20mg KI dissolved in 200ml dH_2O. 20g of Bacto-agar for plates
X-Gal	Fresh dry LB-amp. plates, spread with 25μl X-Gal (40mg/ml stock solution in dimethyl-formamide)
IPTG	Stock solution (0.1M) was made up by dissolving 0.238g in 10ml dH_2O, filter sterilised, and stored in 1ml aliquots at -20°C.
Gel Loading Buffer (GLB)	50 mM Na_2 EDTA , 0.25% bromophenol blue 0.25% xylene cyanol 40% (w/v) sucrose in H_2O

	made in 20ml and stored as small aliquots at 4°C.
Protein Sample Buffer (2X concentrated)	125mM Tris-HCl, 10% SDS, 20% glycerol, 0.002% bromophenol blue, pH 6.8. (reducing) contains: 10% 2mercaptoethanol.
RPMI (incomplete) medium	10.4g of RPMI 1640,5.94 g of HEPES, dissolved in 960 ml of distilled $2H_2O$. Filtered through 0.22mM Nalgene filter and stored at 4°C for up to four weeks.
Complete RPMI medium	Was prepared by addition of 42 ml of Sodium bicarbonate, 50mg/ml gentamycin, and 40 ml heat inactivated human serum to 600ml of incomplete RPMI medium.
Protease inhibitors	Leupeptin 50mg/ml (in dH_2O) . Chymostatin 10mg/ml (in acetic acid) Pepstatin 10mg/ml in methanol. PMSF 200mM in Propanol. Idoactamide 20mg/ml in dH_2O. EDTA 500 mM, EGTA 500mM.

Percoll gradient:

Solution for gradient layers of 52.5%, 45% and 30% Percoll were made up in incomplete RPMI medium as follows: volume of 10x conc. RPMI medium was added to 9 volumes of stock Percoll (Sigma) to make an isotonic 90% Percoll solution. Then:-

a) to 2 volumes of 90% Percoll, 1 volume of 1xRPMI was added to make 60% Percoll.

b) to 1 volume of 60% Percoll, 1 volume of 1xRPMI was added to make 30% Percoll.

c) to 1 volume of 60% Percoll, 1 volume of the 30% Percoll was added to make 45% Percoll gradient.

d) to 1 volume of 60% Percoll, 1 volume of 45% Percoll was added to make 52.5% Percoll gradient.

2.2 METHODS

2.2.1 *In vitro* culture of *P. falciparum.*

2.2.1.1 Asexual parasite cultures

Freshly thawed parasites were resuspended in 5mls of complete medium containing washed RBC, to initiate a new culture at 5% haematocrit in 25ml culture flasks (Trager and Jensen, 1976; Zolg *et al.*, 1982). The flasks were gassed with a mixture of 1% O_2, 3% CO_2 and 96% N_2, and maintained at 37°C. The medium was replaced with fresh pre-warmed medium daily. Small quantities of blood were removed in a Pasteur pipette, smeared on a slide, stained with Giemsa's stain at pH 7.2 (Sorensen's buffer), and examined microscopically to measure the parasitaemia and health of the culture. The cultures were diluted every 2-3 days with fresh RBCs in order to maitain the parasites in a healthy state.

2.2.1.2 Culture synchronisation

In order to obtain synchrony of different parasite stages, cultures with low parasitaemia (about 1%), and a high proportion of ring-forms were used as starting material. Treatment with sorbitol destroys large parasites (trophozoites and schizonts) in erythrocytes (Lambros and Vandenberg, 1979), resulting in a synchronously growing population, starting from the remaining ring-forms.

Cultures were centrifuged at 1000g for 5 minutes, and the supernatant was discarded. The cells were then resuspended in 5% sorbitol solution in dH_2O (10%[v/v]), and allowed to stand at room temperature for 5 minutes. The mixture was then centrifuged as above and the cells resuspended in complete RPMI medium. The centrifugation was repeated once more, to remove any residual sorbitol, and the cells were finally resuspended in RPMI medium at 5% hematocrit (method 2.2.1.1). After 24 hours of culture, a blood smear was prepared and examined under the microscope. At this stage the culture should not contain ring-forms, but mainly trophozoites and maturing schizonts. Six hours later, another blood smear was examined and if a new generation of small rings was seen, a second sorbitol treatment was carried out as above to remove remaining trophozoites/schizonts. The culture was then monitored over 48 hours to ensure that it was synchronous. If not, more sorbitol treatments were carried out at times when the only ring-forms present were small. When cultures were fully synchronous, they were harvested for the desired experiments.

2.2.1.3 Density gradient separation of different stages of the parasite

Parasitised cells are less dense than intact red cells, mainly due to loss of

haemoglobin and lipid formed by the parasites (Heidrich, 1988). They can thus be purified on density gradients using materials such as Plasmagel, Physiogel, Ficoll, Gelatin and Percoll, (Jensen,1978; Stanley *et al.*, 1982; Dulzewski *et al.*, 1984). Such methods are also quite effective in separating red cells infected with large parasites, i.e; trophozoites and schizonts, from those with ring-forms.

Gelatin or plasmagel (Sigma) can be used to float the mature stages (trophozoites and schizonts). These stages are less dense than red blood cells or ring-forms parasites; and so remain at the top of a gelatin solution. Cultured erythrocytes (parasitaemia 10-15%) were collected in sterile screw-capped tubes. One volume of 2x gelatin solution in RPMI medium was added to the erythrocyte suspension and placed in water-bath at 37°C for 30 minutes until the suspension was separated into two portions. The upper portion (which contained mainly the mature stages) was removed carefully into a sterile tube and centrifuged at 2000rpm for 10 minutes. After washing with 1xPBS, the pellet was ready for further analysis.

2.2.1.4 Gametocyte culture

15ml cultures were set up at 6% haematocrit in 75ml flasks (Ifediba and Vanderberg, 1981). Parasitised RBCs from asexual stock cultures at parasitaemias above 4% were chosen to ensure a high rate of commitment to sexual development. These were used to give a starting parasitaemia in the cultures of 0.5 to 0.7%. Cultures were gassed as for asexual parasites (method 2.2.1.1), and maintained at 37°C. The medium was replaced daily with pre-warmed medium at 37°C. 4 to 5 days after the start of each culture, when a high parasitaemia had been reached, samples of the parasites were examined microscopically for changes in the morphology associated with gametocyte development, such as, ring forms and early trophozoites becoming somewhat triangular in shape. When such features were observed, the haematocrit was reduced to 3.6% by the addition of 25ml of complete RPMI medium instead of 15ml. The gametocyte cultures were then maintained at 25mls until the desired stage of maturity was reached according to the Hawking *et al.*, (1971) classification. Young gametocytes or mature gametocytes were usually harvested 8 to 10 days or 14 to 17 days respectively after the start of these cultures.

2.2.1.5 Purification of gametocytes

By using a high gametocyte producing line, such as 3D7A, grown manually in 75cm^2-base culture flask, a yield of 2-5x10^7 stage IV or V gametocytes can be obtained. Mature gametocytes are activated to undergo gametogenesis at temperatures below 36°C, although they also require another environmental change such as the pH

being raised above 8.0 in the presence of bicarbonate ions (Alano *et al.*, 1993). In order to minimise the risk of inducing gametogenesis, efforts were taken to keep the gametocytes at 37°C prior to resuspending in bicarbonate-free medium (incomplete RPMI medium).

Gametocyte cultures were pooled together and centrifuged at 2000g for 5 minutes in a bench centrifuge pre-warmed to 37°C. The cells were resuspended to a final haematocrit of about 20% in incomplete RPMI medium and divided into 4ml amounts in 15ml Corex tubes. Using a 10ml syringe and a 6 inch 16 gauge needle, 3ml of 30% Percoll solution, (for preparation of Percoll gradients see material) was inserted beneath the cell suspension; and the process was repeated with 3ml of 45% Percoll solution, and then with 3ml of a 52.5% Percoll solution, layering each Percoll solution underneath the previous one. The gradient was then centrifuged at 10,000rpm for 10 minutes at room temperature. After centrifugation the tubes were placed onto a vertical test tube rack and, using a syringe and 6 inch 16 gauge needle, the material at the gradient interfaces was removed in the following order: first the interface between the upper supernatant and the 30% Percoll layer was removed; this contained large amounts of acellular parasite debris, malaria pigment, and RBC ghosts; second, the interface between the 30% and 45% Percoll layers; this contained largely gametocytes whose degree of purity with respect to other cells varied from at least 90% to 60% and third, the interface between the 45% and 52.5% layers; this contained fewer gametocytes, many RBCs and some asexual parasites. The material in each layer was checked by viewing a sample under a cover-slip using phase contrast optics. The gametocyte preparation was either used immediately to prepare RNA or isolate nuclei, or it was frozen at -70°C until it was needed for use.

2.2.2. DNA isolation and purification

Unless otherwise stated in the text the methods used were based on those described in Sambrook *et al.* (1989).

2.2.2.1 Preparation of *P. falciparum* DNA *P. falciparum* parasites were cultured *in vitro* as described above. DNA was prepared from 5 ml of cultured parasites (4-10% parasitaemia) as follows. The parasitised erythrocyte pellet was collected by centrifugation, using a bench top centrifuge, at 4,000rpm for 10 minutes. After centrifugation, the pellet was resuspended in 1 ml of 0.1% saponin in phosphate-buffered saline (PBS), then incubated at room temperature for 5 minutes. The lysed erythrocyte suspension was then transferred to a microfuge tube and spun in a microfuge for 10 minutes (8,000rpm). The supernatant containing haemoglobin and red cell ghosts was removed and the parasite pellet resuspended in 1 ml of ice-cold

PBS and the centrifugation step repeated. After removing the supernatant and the residual ghosts, the parasite pellet was lysed by addition of 600μl of 10mM Tris-HCl pH 7.6, 50mM EDTA pH 8.0, 0.1% SDS, 1mg/ml proteinase K. The resulting viscous lysate was then homogenised by 30 passages through a 25 gauge needle and incubated overnight at 50^{0}C. The lysate was extracted twice with phenol/chloroform then once with chloroform. DNA was then precipitated from the supernatant by the addition of 0.1 volumes 5M sodium Acetate and 1 volume isopropanol, and left to stand at room temperature for 15 minutes. DNA was then pelleted by centrifugation, washed in 70% ethanol, dried and resuspended in 100ml of TE buffer (10mM Tris-HCl/ 1mM EDTA, pH 7.6).

2.2.2.2 Mini-prep plasmid DNA isolation: The Magic Minipreps DNA Purification System™ (Promega Corp.) was used, when necessary, for mini-prep of plasmid DNA preparation.

Single colonies were picked into 5ml of LB broth containing 50μg/ml ampicillin and grown overnight at 37^{0}C with shaking. The bacteria from 1-3ml of the overnight culture were pelleted by microcentrifugation for 2 minutes and resuspended in 200μl of cell resuspension solution (10mM EDTA, 50mM Tris-cl pH 8.0, 100μg/ml RNase A). The cells were then lysed by the addition of 200μl of cell lysis solution (0.2N NaOH, 1% SDS) and mixed by inversion of the tube several times. This was followed by addition of 200μl of neutralisation solution (2.55M potassium acetate pH 4.8), mixing and inversion. In order to pellet precipitated bacterial proteins, the solution was microcentrifuged for 5 minutes and the supernatant transferred to a fresh Eppendorf tube. Then 1ml of the Magic Minipreps DNA Purification Resin was added to the supernatant and mixed by inversion of the tube several times. The resin/DNA mix was pipetted into a syringe barrel, and the syringe plunger was applied to push the slurry into a Minicolumn™. The resin in the Minicolumn was then washed with 2ml of wash solution (200mM NaCl, 20mM Tris-HCl; pH7.5, 5mM EDTA, 50% ethanol). After that, the Minicolumn was spun for 30 seconds to dry the resin. Then 50μl of TE buffer was added to the Minicolumn and left at room temperature for 2-5 minutes, and the plasmid DNA eluted by centrifugation of the Minicolumn for 30 seconds in microcentrifuge. The plasmid DNA was collected in Eppendorf tube, and stored at 4°C or -20°C .

2.2.2.3 Maxi-prep of plasmid DNA: Large scale preparations of plasmid DNA were prepared using, QIAGEN™ commerial kit, following the manufacturer's instructions.

A single colony was picked into 250ml of LB-amp and grown overnight at 37^{0}C with vigorous shaking. The bacteria were pelleted by centrifugation at 3000rpm for 10 minutes at 4^{0}C and the pellet resuspended in 4ml of buffer P1 (100μg/ml RNase A, 50mM Tris-HCL, 10mM EDTA, pH 8.0). The cells were lysed by the addition of 4ml of buffer P2 (200mM NaOH, 1% SDS). The suspension was mixed and incubated at room temperature for 5 minutes. After the addition of 4ml of buffer P3 (2.55M potassium acetate pH 4.8) and mixing, the solution was centrifuged at 11,500rpm (SS-34 Sorvall rotor) for 30 minutes at 4^{0}C to pellet the bacterial debris. The supernatant was then centrifuged again for 10 minutes to ensure removal of all debris. Meanwhile, a QIAGEN Tip 100 column was equilibrated with 3ml of a low salt buffer QBT (750mM NaCl, 50mMMOPS, 15% ethanol pH 7.0, 0.15% Triton X-100). The supernatant was applied to the column and allowed to enter by gravity flow. The column was washed with 10ml of a medium salt buffer QC (1.0M NaCl, 50mM MOPS, 15% ethanol pH 7.0) and the DNA eluted with 5ml of a high salt buffer QF (1.25M NaCl, 50mM MOPS, 15% ethanol pH 8.2). DNA was precipitated at room temperature with 0.7 volume of isopropanol for 10 minutes and pelleted by centrifugation at 12,000rpm (SS-34 rotor, sorvall) for 30 minutes at room temperature. The DNA was washed with 1ml of 70% ethanol, air dried and dissolved in 200μl of TE buffer.

2.2.3 Purification of DNA fragments

DNA fragments obtained after PCR amplification, restriction digestion and ligation were purified by one of the following methods:

2.2.3.1 Phenol/chloroform extraction: After one of the above reactions was completed, an equal volume of phenol/chloroform was added to the reaction product in Eppendorf tube, and the mixture vortexed for a few seconds. The mixture was microcentrifuged for 5 minutes and the aqueous upper phase, containing the DNA removed. Then the residual phenol was removed by one chloroform extraction. The DNA was precipitated by addition of 0.2 volumes of 3M sodium acetate and 2 volumes of absolute ethanol, and dissolved in an appropriate volume of TE buffer.

2.2.3.2 Magic PCR Preps™ DNA purification system: DNA contained in agarose gel slices was also purified using Magic PCR Preps™ purification Kit (Promega) according to the manufacturers protocol. The gel slice was dissolved in a 1.5ml Eppendorf tube at 70^{0}C until the agarose had completely melted. 1ml of the Magic PCR Preps™ resin was added to the melted agarose and vortexed briefly. The mix was pipetted into a syringe barrel, then the syringe plunger was applied slowly into

a mini-column. The mini-column was washed with 2ml of wash solution (80% isopropanol). The syringe was removed and the mini-column centrifuged for 20 seconds at 13,000rpm in a microfuge to dry the resin. The mini-column was then transferred to a new Eppendorf tube. The DNA was eluted by application of 50μl of TE buffer to the column. After 1 minute the Eppendorf tube containing the mini-column was centrifuged for 1 minute at 13,000rpm. The mini-column was removed and discarded. Purified DNA was stored either at 4°C or -20°C until required.

2.2.4 Spectrophotometry

The concentration of DNA in solution was estimated by measuring its absorbence at 260nm in spectrophotometer. It was assumed that an OD_{260} of 1.0 is equivalent to a concentration of 50μg/ml for double stranded DNA and 35mg/ml for oligonucleotides.

2.2.5 Restriction endonuclease digestion of DNA

Restriction endonuclease enzymes were obtained from Boehringer Mannheim Chemical Company and New England Biolabs. Restriction enzyme digestions were performed in the appropriate buffer supplied by the manufacturer. The reactions were carried out at 37°C for 1-2 hours (unless otherwise indicated) and 4-12 units of restriction endonuclease were used per μg of DNA. Reactions were terminated by addition of 1/10 volume of 50mM Na_2 EDTA, 40% w/v sucrose, containing 0.25% bromophenol blue, 0.25% xylene cyanol.

2.2.6 Agarose gel electrophoresis

Agarose gels were used to check the integrity of DNA, to analyse PCR products and restriction enzyme digests. 1% - 2% agarose (IBI) was dissolved in appropriate volume of 1xTAE buffer at 90°C. After the solution had cooled to around 45°C, ethidium bromide (EtBr) was added at concentration of 0.5μg/ml, mixed and the gel was poured onto a pre-levelled electrophoresis gel plate (IBI) and left to polymerise at room temperature. The appropriate amount of DNA was mixed with 0.1 volumes of gel loading buffer (40% w/v sucrose , 50mM EDTA pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol) and loaded into the submerged gel. Electrophoresis was carried out in 1xTAE at 100 volts for the appropriate time depending on the size of the fragment being separated. Fluorescence from DNA bound-ethidium bromide was visualised by short wave-length UV light and photographed.

In order to estimate the size of DNA fragments 2μg of DNA markers were loaded adjacent to the DNA samples on agarose gels. The standards, pBR328

BglI+HinfI digest (Boehringer Mannheim) consisted of the following sized fragments 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220, and 154 base pairs(bp), and *Hind III* digest of bacteriophage λ cI_{857} (Boehringer Mannheim) consisted of the following sized fragments (bp): 23130, 9416, 6562, 4368, 2322, 2027, 564 and 125.

2.2.7 Pulsed field gradient gel electrophoresis (PFGE)

Physical mapping of *P. falciparum* has been transformed in recent years by the introduction of pulsed field gradient electrophoresis (PFGE) (Schwartz and Cantor, 1984). Southern blots of PFGE separations readily serve to assign genetic markers to chromosomes. Pulsed field gel electrophoresis of the parasite was performed essentially as described by Kemp and colleagues (1987). On a practical level, important features of PFGE methods include embedding cells in agarose blocks and removing protein and lipid so that the DNA remains intact but free within the gel matrix, and movement of the large chromosomal DNA molecules through the gel by switching direction of the applied electric field at fixed time intervals.

2.2.7.1 Preparation of parasite DNA in agarose blocks For preparation of chromosomal DNA in agarose blocks, parasites were first grown in 5ml asynchronous culture at 5% haematocrit and 10% parasitaemia (method 2.2.1.1). Parasitised red blood cells were pelleted by centrifugation at 4000rpm for 5 minutes at 4°C. Parasites were released from the red blood cell pellet by lysis with an equal volume of 0.15% saponin in 1xPBS (made up fresh). After incubation at room temperature for 10 minutes the parasites were pelleted by centrifugation as above. The supernatant was removed and the pellet was washed twice in excess of 1xPBS. The pellet was resuspended in 2 volumes of 1xPBS at room temperature, and the suspension was fixed by addition of an equal volume of 2% low-melting point agarose (IBI) made up in 1xPBS at 42°C, mixed gently and pipetted into a pre-warmed mould (Bio-Rad), quickly before the agarose had set. The mould was then placed at 4°C for 20 minutes to allow the gel to set. The agarose blocks were transferred from the mould into lysis solution (1% sarkosyl, 0.5M EDTA pH 9.0, 0.01M Tris pH 9.0, containing 0.25mg/ml proteinase K), approximately 0.5ml per block, and incubated for 48 hours at 50°C with one change of the lysis solution. The blocks were stored in the lysis solution, without proteinase K, at 4°C until they were used. Blocks can last for several years if properly made and stored.

2.2.7.2 Electrophoresis conditions: Chromosome separations were performed in 0.8% PFGE-grade agarose in 0.5xTBE buffer. Molten agarose was

cooled to about 60°C poured into the casting stand, and a comb inserted. The gel was allowed to set at room temperature for about 30 minutes. After that the comb was removed and the blocks containing parasite DNA were inserted into the wells. The blocks were sealed into the wells with low-melting agarose in 1xPBS, avoiding trapped air bubbles in the wells. The temperature of the buffer was maintained at 14°C by circulating it through a cooling manifold. Table 2.1 shows the conditions used for separating DNA of various sizes using Bio-Rad CHEF DRII system.

Table(2.1) Electrophoresis Conditions for Separation of *P. falciparum* DNA Fragments by Bio-Rad CHEF DRII PFGE systems:

Fragment size	Voltage	Switching interval	Run time
2-200 kb	120 V	2-10 sec	24 h
50-400 kb	140 V	10-20 sec	48 h
100-500 kb	180 V	20-40 sec	48 h
>500 kb	180 V	20-40 sec	72-96 h

2.2.8 Photography

After electrophoresis the agarose gels were stained with ethidium bromide and photographed under UV transilluminator (IBI) using short wave-length. A photograph of the gel was obtained by exposing it to Polaroid 667 or 665 professional film or using a gel video documentation system (Video Copy Processor, Mitsubishi ™)

2.2.9 Southern blotting

The blotting of DNA fragments from agarose gels was performed by the method of Southern (1975). The agarose gel containing fractionated DNA was soaked in 0.25M HCl for 15-30 minutes in order partially to depurinate the DNA. The gel was then rinsed in dH_2O for 5 minutes before being soaked in 0.4M NaOH twice for 15 minutes. The gel was then placed onto blotting paper pads, submerged into a reservoir containing 0.4M NaOH, overlaid with a Hybond N^+ membrane. On top of this were placed 3 pieces of 3M paper cut to the size of the gel and pre-wetted in 0.4M NaOH, then another 3 sheets of dry blotting paper, a stack of towels, a glass plate and a weight. Exposed areas of the blotting paper pads were covered with Saran wrap™ in order to minimise evaporation and to ensure that flow of 0.4M NaOH was only through the blot. After overnight transfer the membrane was removed and neutralised in 0.5M Tris-HCl for 5 minutes, air dried, and wrapped in Saran wrap, (Dow

Chemical Company), until it was required for the hybridisation reaction (method 2.2.12).

For transfer of DNA from mini-gels onto Hybond-N^+ membrane (Amersham) prior to probing the following method was used: The agarose gel was first soaked in denaturation solution (0.5M NaOH, 1.5M NaCl) for 15 minutes and then transferred into neutralisation solution (1.5M NaCl, 0.5M Tris pH 7.5) for another 15 minutes. The gel was then placed on a glass plate and overlaid with a piece of Hybond N^+ membrane and 3 sheets of blotting paper all pre-wetted in neutralisation solution. Onto this were placed 3 sheets of dry blotting paper, a stack of paper towels, a glass plate and a weight. After transfer for 4-6 hours, the membrane was removed, rinsed in 2xSSC and air-dried. The membrane was then ready for prehybridisation and probing.

2.2.10 Dot blotting

Cut plasmid DNA or *P. falciparum* genomic DNA was appropriately diluted in TE, denatured by heating to 95°C for 5 minutes and left on ice for at least 2 minutes. Then 1 volume of 20xSSC was added to the sample. A commercial dot blotting apparatus (Hybaid™) was used to spot the samples on Hybond-N^+ membrane in equal aliquots. The membrane was then placed , DNA side up, on a filter paper wad soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 5 minutes. Then the membrane was transferred to a filter wad soaked in neutralisation solution (1.5M NaCl, 0.5M Tris-HCl pH 7.2) and left for 1 minute. The DNA was then fixed by placing the membrane for 20 minutes, DNA side up, on a pad of absorbant filter paper soaked in 0.4M NaOH. After that, the membrane was rinsed in 5x SSC with gentle agitation for 1 minute. The membranes were either used immediately or wrapped in SaranWrap and stored at 4°C until needed.

2.2.11 Probe synthesis

The selection of an appropriate combination of label and labelling method for a particular experiment depends mainly on the level of sensitivity and resolution required. For most filter hybridisation applications, sensitivity is considered to be of greater importance. Accordingly, for maximum sensitivity in filter hybridisation, phosphorus-32 (^{32}P) is the most widely used radiolabel as it is available at high specific activity and can be detected with high degree of efficiency. Several methods are available for making probes. End-labelling with polynucleotide kinase is employed with short oligonucleotides, whereas longer DNA fragments are generally labelled by random priming methods.

2.2.11.1 Random priming method: In this work, random primer labelling method was used which was first described by Feinberg and Vogelstein (1983). The approach utilises Klenow enzyme, the large fragment of *E. coli* DNA polymerase I, which has 5'-3'polymerase activity. This enzyme is used because it lacks the 5'-3' exonuclease activity which would otherwise degrade the primers. The absence of the 5'-3' exonuclease also ensures that incorporated nucleotides are not subsequently removed as monophosphates. A mixture of random DNA hexanucleotides is used to prime the DNA and the complementary strand synthesised from the 3'-OH termini of the primers with the incorporation of radiolabelled dNTP. The length of the DNA fragments to be labelled influences the reaction. Linear single-stranded or denatured double-stranded DNA molecules are usually used as substrates. Covalently-closed circular DNA can also be used, but slightly lower incorporations are obtained.

In this work, the Random Primed DNA Labelling Kit (Boehringer Mannheim), was used to synthesised radioactive probes for hybridisation reactions. The DNA template (25-50ng in TE buffer) was first denatured by boiling for 5-10 minutes and chilled on ice to prevent reannealing. A mixture of 2μl hexanucleotide mixture in 10x reaction buffer [900mM HEPES, adjusted to pH 6.6 with 4N NaOH, 100mM $MgCl_2$], 1μl [0.5mM] each of dCTP, dGTP, dTTP, 5μl [50μC, 1.85MBq] of [α-^{32}P] dATP (Amersham International. Plc.), and 1μl (2 units) of Klenow enzyme were then added to the denatured DNA template and incubated at 37°C for 30-60 minutes. The reaction was stopped by addition of 0.5mM EDTA and the unincorporated nucleotides separated from the labelled DNA by chromatography column as described in the next section. This procedure can routinely be used to obtain specific activity as high as 1.8×10^9 dpm/μg.

2.2.11.2 Removal of unincorporated nucleotides and proteins: In general, the denatured labelled DNA can be used directly as a hybridisation probe without stopping the reaction or removing unincorporated label. Sometimes, if small amounts of target RNA or DNA molecules are present on the hybridisation membrane, further purification of labelled probe is necessary in order to get rid of any possible interference either from unincorporated nucleotides or from protein debris.

Removal of unincorporated nucleotides was performed by passing the labelling reaction over a Sephadex G-50 column. The column was made by packing a glasswool-plugged disposable plastic syringe with Sephadex G-50 prepared in TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). The column was washed several times with TE buffer. After the radiolabelling reaction was completed, 5μl of

gel loading buffer was added to the DNA sample. The DNA sample was applied in a volume of 100μl to the column and the column was washed with approximately 100ml of TE buffer. The column was connected to a reservoir of TE buffer so that the flow rate was about 0.5ml/min. 15-20 fractions, approximately 0.5ml, of the material were collected in Eppendorf tubes (usually the sample was collected up to the xylene cyanol band, which usually contains all incorporated material). The radioactivity in each tube was measured by a hand-held Geiger counter, and the radioactive fractions in the leading peak pooled and stored at -20°C until needed. The probe was boiled for 10 minutes before it was added to hybridisation solution.

2.2.12 Hybridisation

All hybridisations were performed in a Hybridisation oven (Hybaid™). In the course of this study all Southern blots, Northern blots and filters were probed for homologous DNA sequences and therefore hybridisation was carried out under stringent conditions throughout. In general, to maximise the rate of annealing of the probe with its target, hybridisations were usually carried out in the presence of 10% dextran sulphate in a solution of high ionic strength at a temperature that was 20-25°C below the melting temperature (T_m) (Sambrook *et al.*, 1989).

Prehybridisation was carried out in 10-20ml aqueous hybridisation buffer which was made by addition of 10g of dextran sulphate [MW~500,000], 10ml of 10% SDS and 5.8g NaCl, to 85 ml of dH_2O and heated at 65°C for 30 minutes. This buffer usually prepared in bulk and stored at -20°C. Just before prehybridization was carried out, the buffer was diluted and 100μg/ml denatured, sonicated salmon or herring sperm DNA was added. Prehybridization was performed at 65°C for 1-2 hours. The boiled, denatured probe was then added and hybridisation continued overnight at 65°C. Non-specifically bound nucleotides were removed by washing the membrane twice with excess 2xSSC at room temperature for 10 minutes. Then the membrane was washed stringently in 0.5xSSC, 0.1% SDS at 65°C (4x15 minutes). Filters were then placed between two layers of Saran Wrap and autoradiographed (method 2.2.14).

2.2.13 Stripping probes from DNA blots

Blots were stripped before were used in different hybridisation reaction. For efficient removal of probes, the membranes were not allowed to dry during the hybridisation and detection procedure. DNA filters were incubated at 45°C for 30 minutes in 0.4N NaOH and then in neutralisation solution (0.1xSSC, 0.1% SDS, 0.2M Tris-Cl pH 7.5) for 30 minutes. The filters were then exposed to X-ray films and autordiographed to check for removal of the probe.

2.2.14 Autoradiography

This was performed using X-ray film (Kodak XAR-5 or AGFA Curix) for ^{32}P probed filters, in a cassette containing intensifying screens. The cassette containing ^{32}P probed filters were stored at -70°C while those containing ^{35}S labelled gels were stored at room temperature. After the appropriate length of time the film was developed using a X-O'GRAPH X1 automatic X-ray film processor.

2.2.15 Recombinant DNA techniques

2.2.15.1 Ligation of DNA: In order to prevent the religation of the compatible ends of digested plasmid, linearised DNA was treated with calf intestine alkaline phosphatase (CIP) which removes the 5'-phosphatase residues from DNA. After digestion of the plasmid with the appropriate restriction endo-nuclease, 1 unit of CIP (New England BioLabs.) was added and the preparation incubated at 37°C for 1 hour. To inactivate the enzyme, 0.1 volume of 250mM EDTA pH 8.0 was added and the solution incubated at 75°C for 10 minutes.

Vector DNA (50ng) and insert DNA were cut with suitable restriction enzyme endonucleases (method 2.2.5), and if necessary treated with phosphatase as above and purified (method 2.2.3). Ligation of the insert to vector was performed at a ratio of approximately 3 molecules of insert to 1 molecule of vector. To the mixture of vector and insert DNA, in TE, was added 1µl of 10x ligase buffer (500mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 100mM DTT, 100mM ATP), 1 unit of T4 DNA ligase (New England BioLabs.) and the total reaction volume made up to 10µl with dH_2O. The reaction was incubated at 16°C overnight and then diluted 5-fold with dH_2O prior to *E. coli* transformation (method 2.2.15.4).

2.2.15.2 Subcloning of PCR products into pCRII plasmid vector: The TA Cloning™ System, Fig.2.1, from Invitrogen Corp. was used routinely during this study to provide a quick one-step cloning strategy for direct insertion of PCR product into a plasmid vector (Mead *et al.*, 1991). The procedure eliminates any enzymatic modifications of the PCR product such as Klenow or T4 polymerase treatment to create blunt ends, and it does not require the use of specifically designed primers containing restriction sites. The system takes advantage of the non-template dependent activity of the thermostable polymerases used in PCR that add single deoxynucleotides to the 3' end of all duplex molecules. These A-overhangs are used to insert the PCR product into specifically designed vectors providing single 3'T-overhangs ready for the insertion site.

Figure 2.1 :

Schematic diagram showing the pCRII plasmid vector map. The sequence above the diagram represents the pCRII vector sequence with a PCR product cloning site indicated.

M13 Reverse Primer — Sp6 Promoter

CAG GAA ACA GCT ATG AC C ATG ATT ACG CCA AGC T AT TTA GGT GAC ACT ATA GAA
GTC CTT TGT CGA TAC TG G TAC TAA TGC GGT TCG A TA AAT CCA CTG TGA TAT CTT

NsiI HindIII KpnI SacI BamHI SpeI

TAC TCA AGC TAT GCA TCA AGC TTG GTA CCG AGC TCG GAT CCA CTA GTA ACG GCC
ATG AGT TCG ATA CGT AGT TCG AAC CAT GGC TCG AGC CTA GGT GAT CAT TGC CGG

BstXI EcoRI EcoRI EcoRV

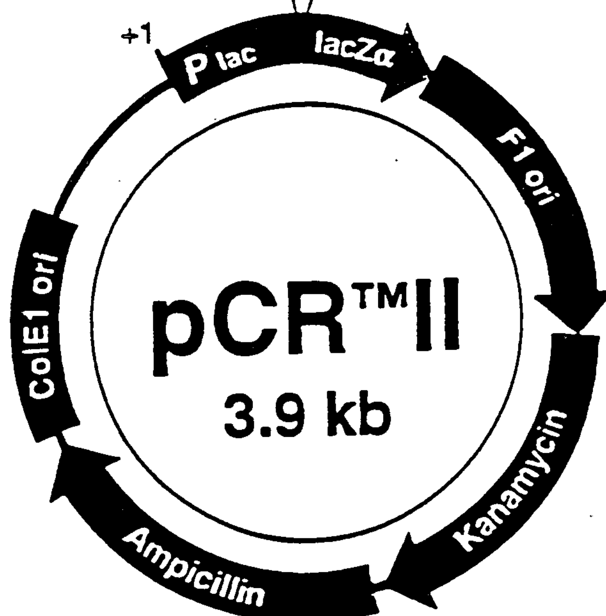
GCC AGT GTG CTG GAA TTC GGC TT **PCR Product** A A GCC GAA TTC TGC AGA TAT
CGG TCA CAC GAC CTT AAG CCG A A TT CGG CTT AAG ACG TCT ATA

AvaI
PaeR7I
BstXI NotI XhoI NsiI XbaI ApaI

CCA TCA CAC TGG CGG CCG CTC GAG CAT GCA TCT AGA GGG CCC AAT TCG CCC TAT
GGT AGT GTG ACC GCC GGC GAG CTC GTA CGT AGA TCT CCC GGG TTA AGC GGG ATA

T7 Promoter — M13 (-20) Forward Primer — M13(-40) Forward Primer

AGT GAG TCG TAT TA C AAT TCA CTG GCC GTC GTT TTA C AA CG T CGT GAC TGG GAA AAC
TCA CTC AGC ATA AT G TTA AGT GAC CGG CAG CAA AAT G TT GC A GCA CTG ACC CTT TTG



The ligation and transformation were carried out following the manufacturer's instructions. Briefly, after the final PCR amplification cycle was completed, the appropriate amount of PCR sample was removed from underneath the mineral oil overlay and transferred to a new tube. The ligations with the pCR™ vector (3.9kb) were set up as 1:1 to 1:3 molar ratio of vector: PCR insert. For each ligation reaction; 2μl (50ng) of vector, 1μl (12.5ng) of PCR product, 1μl 10x ligation buffer (0.5M Tris pH 7.4, 0.1M $MgCl_2$, 0.1M dithiothreitol, 10mM spermidine, 10mM ATP, 1mg/ml BSA), 1μl of T4 DNA ligase and 6μl of dH_2O. The ligation mix was incubated overnight at 14-16°C.

2.2.15.3 Preparation of competent Cells: The method used was modified after Hanahan (1983). Cells of the appropriate *E. coli* strain were plated on LB agar overnight at 37°C. 2-3 colonies were picked and grown in 10ml LB broth overnight at 37°C with shaking. The following morning the culture was diluted 50-fold in LB broth and incubation continued for approximately 2 hours until the cell density was 4-7 x 10^7 viable cells/ml, which corresponds to $O.D._{550}$ of 0.3-0.6. The culture was chilled on ice for 15 minutes. The bacterial pellet was then collected by centrifugation at 5,000rpm for 5 minutes at 4°C, and resuspended in ice-cold transformation buffer I (TBI) (material 2.1.7) at 1/3rd the original culture volume. The cells were then incubated on ice for 5 minutes, collected again by centrifugation as above. The pellet was then resuspended in 1/12.5 of the original culture volume ice-cold transformation buffer II (TBII) (material 2.1.7), and incubated on ice for 30 minutes. The cells could then be stored at -70°C in small aliquots (100-200μl) after the addition of glycerol to 15% v/v, or could be used the same day.

2.2.15.4 Transformation of *E. Coli* Competent cells (50-100μl) were mixed with either 25 ml of a 5-fold diluted ligation reaction or 20ng of uncut plasmid DNA. The cells were then incubated on ice for 30 minutes, heat-shocked at 42°C for 60 seconds and incubated on ice for 2 minutes. To each transformation, 400-450μl of pre-warmed LB or SOC medium was added and the mixture was incubated at 37°C, with gentle shaking, for 1 hour. 25-100μl of from each transformation were plated out on appropriate selective LB agarose plates and incubated overnight at 37°C.

2.2.15.5 Colony screening: The procedure used was that described by Hanahan and Meselson (1983) and Buluwela *et al.*, (1989) in which colony lysis, DNA denaturation and DNA fixation to the membrane is carried out in a single step.

Using sterile, blunt-ended forceps (Millipore) a sterile Hybond-N^+ filter was laid on a surface of a day-old LB agar plate containing ampicillin. When the filter was

thoroughly wet, the bacterial suspension was directly plated from the transformation mixture onto the surface of filter. The plates were left at room temperature for 10-15 minutes to allow the inoculum to evaporate. After that the plates were inverted and incubated overnight at 37°C for about 8-10 hours. The next morning, the filters were peeled off the overnight plate and laid, colony side up, on a dampened pad of sterile 3MM paper. A fresh, sterile Hybond-N^+ filter was laid on the master filter. Care were taken to prevent air bubbles from becoming trapped between the two filters. The two filters were pressed firmly together with a heavy glass plate (with a 3MM paper between the glass and the filters). The two filters were then oriented by making a series of holes in them with an 18-gauge needle and before being peeled apart. The replica filter was laid on a fresh LB-amp. agar plates and incubated at 37°C until the colonies appeared (4-8 hours). The master filter was replaced on a fresh LB-amp. agar plate and incubated for 1-2 hours, and kept at 4°C until needed.

The replica filters were removed and placed for 3 minutes, colony side up, onto filter paper pre-wetted with 10% SDS solution. This treatment, which is optional, seems to result in a sharper hybridisation signal. It probably works by limiting the diffusion of plasmid DNA during denaturation and neutralisation. The filters were then placed for 5 minutes onto a second sheet of 3MM paper that had been saturated with denaturing solution (0.5M NaOH, 1.5M NaCl). After that, filters were placed for 5 minutes onto a third sheet of 3MM paper that had been saturated with neutralising solution (1.5M NaCl, 0.5M Tris.Cl pH 8.0). Finally, the filters were rinsed briefly into 2xSSC solution and allowed to dry at room temperature for 30-60 minutes. The filters were either used immediately in hybridisation reactions or wrapped loosely in aluminium foil and stored under vacuum at room temperature. Positive signals were then identified and the appropriate colony on the master plate picked for further analysis.

2.2.16 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR), is a powerful *in vitro* method for enzymatic synthesis of specific DNA sequences (Saiki *et al.*, 1988). The double stranded DNA is first denatured, then specific primers corresponding to each end of the target sequence annealed, and the sequence extended. This cycle is repeated as much as 40 times and has the potential of amplifying the target sequence as much as 10^9 times. Due to the high temperature required for the denaturation step, a thermostable DNA polymerase called *Taq* polymerase is needed which was originally isolated from *Thermus aquaticus*.

PCR was performed in 0.5ml Eppendorf tubes. Each reaction contained 1μl of each primer at 10mM, dNTP solution (a mixture of 10mM dGTP, dATP, dTTP, dCTP) at 40mM, 10μl of 10x*Taq* polymerase buffer (500mM KCl, 100mM Tris-HCl pH 8.3, 20 mM $MgCl_2$, 0.02% gelatin), dH_2O to a total reaction volume of 99μl and 1μl (0.25 units) of *Taq* polymerase (Cetus Corp.). The reaction mixture was overlaid with 100μl of mineral oil and PCR was performed using Hybaid™ Omnigene thermal reactor. The products of the PCR reactions were analysed by agarose gel electrophoresis and ethidium bromide staining. The gels were then observed under the UV light and, if necessary, a photograph was taken.

2.2.17 Inverse Polymerase Chain Reaction (IPCR)

This method is used for rapid *in vitro* amplification of DNA sequences that flank a region of known sequence. The method uses the PCR, but it has the primers oriented in the reverse direction of the usual orientation. The template for the reverse primers is a restriction fragment that has been ligated upon itself to form a circle (Triglia *et al.*, 1988).

1μg of *P. falciparum* genomic DNA was digested in 20μl to completion with the enzyme of choice. The reaction was heat inactivated if possible, but otherwise it was phenol/chloroform extracted and alcohol precipitated. Then the DNA was resuspended in an appropriate volume of TE buffer. The product of the digestion was ligated and inverse PCR was carried out in 50μl PCR reaction using 2μl of the ligation mix as template DNA.

2.2.18 DNA sequencing

DNA sequence determination was performed by the chain termination method of Sanger *et al.*, (1977), using the Sequenase version 2.0 Kit based on T7 DNA polymerase from United States Biochemical Corporation (USB), following a modification of protocol of Winship (1989). In this protocol, the use of culture grade DMSO at 10% in the sequencing reactions is recommended to enhance the intensity of the signal and to reduce background and prevent formation of secondary structure when sequencing double-stranded DNA fragments.

For sequencing plasmid and double-stranded DNA templates the alkaline-denaturation method was used. Approximately 5μg of double stranded DNA in 20μl of TE buffer was incubated with 5μl of 0.4 NaOH, 20μl 1mM EDTA and 55μl dH_2O for 30 minutes at 37°C. After incubation, 20μl of Na acetate and 250μl of absolute ethanol were added and incubated at -70°C for 30 minutes. The DNA was collected by centrifugation at 10,000rpm for 10 minutes. The pellet was washed with 70% ethanol

and resuspended in 6μl of TE buffer. An aliquot (6μl) of denatured template was used for the subsequent sequencing reaction. It was added to 2μl of reaction buffer (200mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 250mM NaCl), mixed with 1μl of primer (1ng/μl) and 1μl of 10% DMSO. The annealing reaction was performed at 37oC for 15-30 minutes. To this was added 1μl of 0.1M DTT, 1μl of 1:5 diluted labelling mix (1.5μM each of dCTP, dGTP, dTTP), 1μl of [α-35-S]dATP,1000ci mM, $10\mu Ci^{-1}$, (Amersham), and 1μl (2 units) Sequenase (diluted in enzyme dilution buffer of 10mM Tris-Cl pH 7.5, 5mM DTT and 0.5mg/ml BSA). The solution was mixed thoroughly and incubated at room temperature for 5 minutes. Then 3.5μl of the solution was aliquoted into appropriate pre-warmed (37°C) termination mix (80μM dNTP [dGTP, dATP, dCTP, dTTP]; 8μM ddNTP [ddGTP, ddATP, ddCTP, ddTTP, respectively]; 50mM NaCl and 10% DMSO). The termination was stopped after 5 minutes incubation at 37°C by adding 4μl of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF). The samples were boiled for 3 minutes prior to loading, (3μl per well) into a 6% denaturing polyacrylamide gel.

2.2.19 Polyacrylamide gel electrophoresis

Sequence reactions were separated on a 380mm x 0.3mm, 6% polyacrylamide gel (8 M Urea, 6% acrylamide, 0.16% bis-acrylamide, 0.08% ammonium persulphate in 1xTBE pH 8.8-8.9. 30 μl of TEMED (N,N,N'N'- tetramethylethylenediamine) were added before pouring the gel between cleaned glass plates. Just before loading, samples were heated to 85oC for 3 minutes, and then loaded onto a pre-run (15-30 minutes) polyacrylamide gel. Electrophoresis was then carried at 40W constant power. Following electrophoresis, the gel was fixed in 7% acetic acid and 7% methanol for 30 minutes. Gel were dried under vacuum on a gel dryer (Model 583 Gel Dryer, BIO-RAD) for 1-2 hours at 80oC, and exposed to X-ray film (Kodak XAR-5 or AGFA CURIX) in a cassette for about 16-24 hours. Films were developed in an automatic X-ray film processor (X-ograph X1).

Sequences were analysed using the University of Edinburgh Computing facilities. Compiling of data, analysis and manipulation was performed using the University of Wisconsin Genetics Computer Group, (GCG), Sequence Analysis Software Package Version 7.1 (Devereux *et al.*, 1984).

2.2.20 RNA methods

2.2.20.1 Preparation of total RNA from parasite cultures

Total RNA was prepared from synchronous *P. falciparum* using the RNA Extraction Kit from Pharmacia (Pharmacia LKB Biotechnology). The primary problem

facing any RNA extraction procedure is to protect RNA from degradation by ribonucleases, which are widely prevalent both in tissues and in the laboratory environment. The inclusion of the chaotropic salt guanidium thiocyanate (GTC) in the extraction buffer provides a high degree of protection from RNase activity, (Chirgwin *et al.*,1979). The caesium chloride used for equilibrium centrifugation is replaced in this kit by caesium trifluoroacetate, (CsTFA), which is more effective in inhibiting RNase activity and deproteinising nucleic acids.

Cultures were centrifuged at 4,000rpm for 5 minutes to collect the parasites. Then the parasites were released from the RBCs by lysis in 0.1% saponin in 1xPBS (described in section 2.2.2.1) and centrifuged. The pellet was then homogenised in Extraction Buffer (guanidinium thiocynate, N-lauryl sarcosine, and EDTA), the cellular debris was removed by centrifugation at 7,000rpm in a Beckman JA-14 rotor for 20 minutes at 15°C, and the supernatant passed through an 18 gauge syringe needle to shear chromosomal DNA. The extract was then loaded onto a "cushion" of CsTFA solution and was ultracentrifuged for 20 hours at 33,000rpm. After centrifugation, the bulk of the liquid was aspirated off, stopping just before the DNA band (or about 1 cm from the bottom if the DNA band was not visible), and then the remaining liquid was decanted, taking care not to disturb the RNA pellet. The tube was then inverted and allowed to drain on a paper towel for 5 minutes. The RNA pellet was dissolved in 100-200μl TE buffer, heated at 65°C for 10 minutes and vortexed. Sometimes precipatation with ethanol was carried out to concentrate the RNA or to remove traces of CsTFA. In such cases 0.1 volume of 2M potassium acetate and 2.5 volumes of ethanol were added to the dissolved pellet. The mix was then chilled at -20°C for 2 hours and the RNA was collected by centrifugation at 5000g for 20 minutes. The supernatant was decanted and the RNA pellet was dissolved in 200μl of TE buffer. The total RNA was used either used immediately or stored frozen at -70°C until used.

2.2.20.2 Formaldehyde gel electrophoresis

RNA was fractionated in 1.2% w/v agarose gel prepared and run in 1xMOPS buffer (20mM MOPS, 5mM NaAc pH 7.0, 1mM EDTA, pH 7.0) containing 3% formaldehyde. The RNA in solution was mixed with an equal volume of formamide sample buffer (2xMOPS buffer, 50% formamide, 10% formaldehyde) and heated to 65°C for 10 minutes then immediately cooled on ice. After addition of 0.25 volumes of normal sample buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) the sample and an aliquot of RNA molecular weight marker (Boehringer Mannheim RNA marker II consisted of the following bands (kb) 7.4, 5.3, 1.9, 1.6, 1.0, 0.6, 0.4, and 0.3) were loaded. The gel was run at 70V for 1-2 hours. After the

electrophoresis was complete the gel was stained in EtBr. at 0.5μg/ml and photographed.

2.2.20.3 Northern blotting

The RNA gels were first rinsed in 1xSSC for 10 minutes to remove formaldehyde from gels. Large RNA molecules were slightly denatured by soaking the gels for 10 minutes in 1xSSC, 50mM NaOH solution. The gels were blotted overnight onto Hybond-N^+ membrane using 10xSSC. After overnight transfer the membrane was removed and rinsed in 2xSCC. The blot was then ready for hybridisation.

2.2.20.4 Pre-hybridisation and hybridisation

Membranes were pre-hybridised at 60°C for 16 hours in 0.25M sodium phosphate buffer pH 7.2 and 7% SDS. The buffer was changed once after 8 hours. The probe was random-primer labelled. Hybridisation was carried out at 60°C overnight using the same solution overnight. Filters were washed in 2xSSC, 0.1% SDS, then in 0.1xSSC, 0.1% SDS for 30 minutes twice before exposed to X-ray films.

2.2.20.5 Stripping of Northern blots

When necessary Northern blots were first stripped prior to probing. A solution of boiling 0.1% (w/v) SDS poured over the blot and allowed to cool to room temperature. The filter was then autoradiographed to check complete removal of the probe.

2.2.21 Nuclear run-on analysis

Nuclear run-on is an *in vitro* assay that can be used to study transcriptional activity of specific gene(s). The purpose is to prepare a cell-free extract which is active in RNA synthesis. The RNA synthesised should reflect both qualitatively and quantitatively the RNA synthesised in the intact cell. In particular, only the correct DNA strand should be transcribed, and initiation, termination and processing should occur at the same sites *in vivo* as *in vitro*. In addition, only these genes which are expressed *in vivo* should be expressed *in vitro* (Marzluff and Huang, 1989).

To obtain nuclei active in faithful RNA synthesis , it is necessary to preserve RNA polymerase activity and structure during the isolation of nuclei and to exclude degradative activities (nucleases, proteases, triphophatases) from the preparation.

All steps were carried out on ice. 10ml cultures of *P. falciparum* (clone 3D7) were set up in 25ml flasks and the cultures were allowed to reach a parasitaemia of 8

% before the contents of the flasks (10 flask per preparation) were collected by centrifugation and washed in 1xPBS. The red blood cells were lysed by addition of equal volume of 0.15% saponin, followed by three washes in E buffer (250 mM sucrose, 20mM PIPES pH 6.3, 0.5mM $Cacl_2$, 1mM PMSF, 2μg/ml apotinin, 2μg/leupeptin, 1μg/ml pepstatin A, 2μg/ml antipain, 0.1mM benzamidine, and 0.1mM metabisulfite). The pellet free of RBCs was then resuspended in 3ml of E buffer and transferred to a dounce homogeniser. 200ml of 10% NP-40 solution was added and about 10-15 strokes with a B-pestle were applied. Nuclei were collected by centrifugation at 10000rpm for 10 minutes in a pre-chilled Sorvall SM24 rotor, and washed three times in E buffer before being transcribed ($7x10^8$ nuclei) at 37°C for 10 minutes in 600μl of solution B (50mM HEPES pH 7.9, 50mM NaCl, 10mM MCl_2, 1.2mM DTT, 10mM creatine phosphate, 1mM GTP, 1mM CTP, 4mM ATP, 25% glycerol, 125U/ml rRNasin (Promega), 0.2mg/ml creatine kinase and 0.5μM [α-^{32}P] UTP 3000 Ci/mmol). Radiolabelled RNA was isolated by the Chomcyski and Sacchi, (1987) method and then purified by using chroma-100 spin column (Clontech ™).

The single stranded DNA fragments (0.2pmol) from *pfran, pfrcc1, msp1, CS, pfg27/25* and *pfs230* genes, were immobilised on Hybond-N+ membrane by the dot blotting method 2.2.10. Prehybridisation, hybridisation and washing were carried out as described in method 2.2.12. The membranes were dried and exposed at -70°C with an intensifying screen for up to a week.

2.2.22 Protein methods

2.2.22.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein extracts prepared from bacteria and parasites were fractionated by SDS-PAGE (Laemmli,1970) with a discontinuous buffer system. Both large (160mm x 180mm x 1mm) and mini-gels (80mm x70mm x 0.75mm) (Hoefer), were used in the course of this study and gel mixtures were prepared according the recipes given in Table 2.2. Large gels were prepared with a 160mm resolving gel and a 20mm stacking gel, while mini-gels were cast with a 50mm resolving gel and 10mm stacking gel.

The resolving gel was poured and overlaid with a small volume of water-saturated butanol. After gel polymerisation the overlay was removed and the gel surface rinsed with dH_2O. The stacking gel was then poured and the appropriate comb inserted. Once the gel had polymerised, the comb was removed and the wells rinsed with dH_2O. The gel was then ready for loading.

Protein samples were prepared by the addition of an equal volume of 2x sample loading buffer (material 2.1.7) and then boiled for 5 minutes prior to loading. Gels were run in an electrode buffer (25mM Tris, 190mM glycine, 1%SDS), either at

65V overnight for large gels or at 150V for an hour for mini-gels. Separated proteins were then either stained with Coomassie blue stain (method 2.2.22.3) or electrophoretically transferred onto a nitrocellulose membrane (method 2.2.22.6).

Table 2.2 SDS-PAGE separating and stacking gel recipes

Gel	Resolving (0.375M Tris)		Stacking (0.125M Tris)
Monomer Concentration	10%	12%	4%
Acrylamide/bis-acrylamide (30: 0.8)	10ml	12ml	2.6ml
dH_2O	12.5ml	10.5ml	6.1ml
1.5M Tris-HCl, pH 8.8	7.5ml	7.5ml	-
0.5M Tris-HCl, PH 6.8	-	-	5.0ml
10% SDS	600μl	600μl	200μl
10% ammonium persulphate	300μl	300μl	100μl
TEMED	50μl	50μl	20μl
Total volume	30ml	60ml	20ml

2.2.22.2 Protein molecular weight markers

Colored (rainbow) marker (Amersham) contained Myosin (200,000 blue), Phosphorylase b (92,500 brown), BSA (69,000 red), Ovalbumin (46,000 yellow), Carbonic anhydrase (30,000 orange), Trypsin inhibitor (21,000 green) and Lysozyme (14,000 magenta) was used routinely in this study. The marker was prepared in 2x loading buffer and boiled for 5 minutes before being loaded. For mini-gels 2μg of each marker protein was loaded per well, and for large gels 10μg per lane was loaded.

2.2.22.3 Coomassie blue staining of protein gels

Protein gels were stained with 0.25% Coomassie brilliant blue R-250, in order to visualise fractionated protein. The stain was prepared in water: methanol: glacial acetic acid 5:5:1 for 2 hour at room temperature with gentle agitation. Excess stain was removed by destaining the gel in water: methanol: glacial acetic acid 5:5:1 until the background was clear. Coomassie staining can detect approximately 0.5-1μg of protein per band.

2.2.22.4 Raising antisera to recombinant glutathione S-transferase (GST) fusion proteins

2.2.22.4.1 Expression of GST fusion proteins: pGEX vectors were used to express polypeptides in *E. coli* as fusions with glutathione*S*-transferase (GST). The basic protocol used for expressing, purifying and cleaving fusion proteins was that described by Smith and Corcoran (1990).

The chosen *P. falciparum* DNA fragments were subcloned into the appropriate pGEX vector (pGEX-3 in this study). TG-1 cells were used for transformation and the cells were grown on LB-amp agar plates overnight at 37°C.

Single colonies were picked and put into into LB-amp, 5ml for a small scale preparation or 100ml LB-amp. for a large scale preparation, and grown overnight at 37°C with shaking. The following day the culture was diluted 1:10 in LB-amp and grown at 37°C with shaking. After one hour, 1ml of culture was removed as a control before induction, and to the remaining culture 100mM IPTG was added to a final concentration of 0.1mM in order to induce fusion protein expression. Cells were then grown for a further 3 hours. These were determined as the optimum growth conditions to give consistently high yields of fusion protein. Cells were collected by centrifugation, at 5,500 rpm (Sorvall) for 10 minutes at 4°C. They were then resuspended in ice-cold PBS, 1:100 dilution of the original culture. In order to check expression of the fusion protein 100μl of culture was spun down, resuspended in 10ml of loading buffer and checked by SDS-PAGE and Coomassie staining.

2.2.22.4.2 Purification of GST fusion proteins: Bacterial cells, containing the expressed fusion protein, were lysed using a sonicator with a 5mm diameter probe, with lysis generally occurring after 30 seconds sonication. 10% Triton X-100 was added to the lysed cell suspension to a final concentration of 1%, in order to minimise any association of fusion proteins with bacterial proteins. An aliquot of the lysed bacterial extract was then spun and a sample of both the supernatant and pellet analysed by SDS-PAGE and Coomassie staining. If the fusion protein was soluble the protein was further purified by the following method:

If the GST fusion protein was soluble, the lysed cells were microcentrifuged for 10 minutes in 1 ml aliquots and the supernatant, containing the soluble fusion, transferred into a clean microfuge tube. To each aliquot of supernatant was added a 50% slurry in PBS of pre-swelled S-linkage glutathione-agarose beads (Pharmacia) and this mixed was gently for 1 hour at room temperature. The beads were collected by microcentrifugation for 5 seconds and pooled. The supernatant was then treated with a second aliquot of beads. Each batch of pooled beads were then washed in 50

volumes of ice-cold PBS and collected by centrifugation at 1000rpm for 1 minute at 4°C. The wash was repeated 2 times. The beads were then resuspended in loading buffer and an aliquot analysed by SDS-PAGE and Coomassie staining in order to estimate the concentration of bound protein.

For large scale purification, a colony of pGEX transformants was inoculated into 100ml LB-amp medium and grown overnight at 37°C in a shaking incubator. This culture was then diluted 1:10 into 500ml fresh LB-amp. medium and grown at 37°C. After 1 hour the culture was induced by addition of 100mM IPTG to a final concentration of 0.1mM. The incubation was then continued for an additional 3 hours. The culture was centrifuged for 10 minutes in a Beckman rotor at 5000rpm and the supernatant discarded. The pellet was resuspended in 10ml ice-cold PBS and the cells were lysed by sonication. After sonication, 10% Triton X-100 was added to give a final concentration of 1%. This was mixed and centrifuged for 5 minutes at 10000rpm at 4°C. The supernatant was collected and 1ml of glutathione agarose beads was added and mixed for 5 minutes at room temperature. The beads were washed three times with 1xPBS. The pellet was resuspended in 1ml of ice-cold PBS and then transferred to an Eppendorf tube. The fusion protein was eluted by addition of 1ml of 50mM Tris-Cl (pH 8.0)/5mM reduced glutathione. An aliquot of induced proteins were run in parallel to non-induced ones on SDS-PAGE.

2.2.22.4.3 Immunisation regime : New Zealand 5 month old male rabbits were used to raise antibodies against the GST fusion protein. Approximately 0.5mg of protein was used per immunisation emulsified in 1ml of adjuvant. A rabbit was also used to raise antibodies against the GST moiety. Prior to the first immunisation a 1ml ear bleed was taken from each rabbit as a sample of 'pre-immune' sera. Protein for the first immunisation was prepared in 1ml Freund's adjuvant and 0.25ml injected subcutansously (s.c.) into four different part of the rabbit. Three weeks later the animals were boosted with protein prepared in incomplete Freund's adjuvant. Again 0.25ml was injected s.c.into four different parts of the skin. Rabbits were boosted again 3 weeks later following an identical procedure. Two weeks after each boost a 20ml ear bleed was taken.

To separate the serum from whole blood the blood sample was left for 1 hour at 37°C, incubated at 4°C overnight and the serum decanted into a fresh tube. In order to remove any remaining traces of blood clot the serum was centrifuged for 10 minutes at 2000rpm. The clarified serum was then removed, aliquoted into Eppendorfs and stored at -20°C. The sera were tested by western blotting, IFA and immunoprecipitation.

2.2.22.5 Preparation of total proteins from *P. falciparum*

Parasites were released from infected RBCs by saponin lysis and washed in 1xPBS as described in section 2.2.2.2. The parasites were pelleted by centrifugation at 4,000 rpm for 10 minutes, washed in PBS and resuspended in SDS-PAGE loading buffer (method 2.2.4.1), 100μl per ml of original infected blood. Samples were boiled for 5 minutes prior to loading, 10-15μl per lane.

2.2.22.6 Western transfer

Proteins separated by SDS-PAGE were transferred onto nitrocellulose (Schleicher & Schuell, BA85) or Hybond-C (Amersham) by the method of Towbin *et al.*, (1979).

The following gel 'sandwich' was assembled in a blotting cassette and submerged in transfer buffer (48mM Tris-base, 39mM glycine, 0.037% SDS, 20% methanol): a sponge pad, a sheet of blotting paper cut to gel size, the gel, a nitrocellulose filter cut to size, another sheet of blotting paper and another sponge pad. The cassette containing the gel sandwich was placed, with nitrocellulose towards the anode, into the transfer chamber (Hoeffer Scientific). Transfer was conducted, in transfer buffer, at 60V for 4 hours or overnight at 30V. Filters were then ready to be probed for bound antigen.

After the Western transfer was completed the following method was used to detect antigens bound to nitrocellulose filters. The filters were first incubated in blocking solution (5% fat free dried milk in 1xPBS, 0.025% Tween 20) for 1 hour. The filters were then incubated in an antiserum in blocking solution with gentle shaking for 2 hours. The polyclonal rabbit serum was used at dilution 1/500. The filters were washed three times, 5-10 minutes per wash in 1xPBS, 0.025% Tween 20 and washed once more in TBS solution (0.12g Tris-base, 0.9g NaCl in 100ml of dH2O). The filters were then incubated for 2 hours in Horse Radish Peroxidase conjugated anti-IgG second antibody (DAKO, Denmark) diluted 1/1000 in blocking solution. The filters were washed as before and the bound second antibody detected using 4-chloro-1-naphthol (Sigma). For 50 ml of detection solution, 40ml of TBS solution, 10ml of methanol and 30μl of Hydrogen Peroxide were added to 30mg of 4-chloro-1-naphol and the solution used immediately. Colour development was continued until the desired intensity was achieved. Filters were then rinsed in dH_2O and dried. All incubations and washing was performed at room temperature with shaking.

2.2.22.7 Immunoprecipitation of radiolabelled parasite proteins

The method is based on the iodination of tyrosine groups in the parasite surface proteins (Howard *et al.*, 1982). It is therefore essential that the cells be resuspended in protein-free solution. Appropriate precautions were taken for handling ^{125}iodine, which was used in the form of sodium ^{125}iodine and therefore contains volatile ^{125}iodine. All operations involving unbound ^{125}iodine were conducted in a fume hood with the air-evacuation fan on, or in closed containers when brought into open laboratory as for centrifugation.

20ml of parasite culture was pelleted by centrifugation at 2,500rpm for 5 minutes. The pellet was resuspended in 100µl of NETT (50mM Tris-HCl, 0.15M NaCL, 5mM EDTA, 0.5% Triton X-100, 0.01% sodium azide, pH 7.4) and a cocktail of protease inhibitors (Sigma), (material 2.1.7). The solution was vortexed briefly and spun for 5 minutes at 10,000rpm. The supernatant was transferred to a microcentrifuge tube which had been treated with IODOGEN (1,3,4,6-tetrachloro-3α,6α-diphenyl glycocoluril). The tube was placed in a fume hood and 500µCi of sodium ^{125}iodide (5µl of the stock solution of sodium ^{125}iodide at 100mCi/ml in NaOH, pH 7-11), was added to the solution and agitated intermittently for 5 minutes. 50µl of the antibody was added to the tube and incubated for 1 hour at room temperature. After that, 40µl of a 25% suspension of protein A Sepharose beads in NETT were added to the tube and agitated gently on a rocker for 2 hours at room temperature. The beads were centrifuged and the supernatant was discarded. The beads were then washed once with NETT and once with NETTS (this was necessary to remove unbound antigen). 50µl of non-reducing 1x sample buffer (material 2.1.7) was added to the beads and the solution was heated at 100°C for 5 minutes. The sample was spun briefly to return condensation to the bottom of the tube and was loaded on an SDS-PAGE gel (for detailed description of the SDS-PAGE, see method 2.2.22.1).

2.2.22.8 Indirect Immunofluorescence Assay (IFA)

2.2.22.8.1 Preparation of slides : In order to prepare slides from different developmental stages of *P. falciparum*, cultures were first synchronised according to the method (2.2.1.2), and examined regularly by thin blood film, until parasitaemias of 5%-10% was obtained. Then the cultures were centrifuged at 2,000rpm for 5 minutes, and the cells were washed three times in incomplete RPMI medium. The pellet was then resuspended at 10% in complete RPMI medium. Then on a clean multispot slide (Hendly, Essex, UK), 20µl was aliquoted onto each well and allowed to dry at room temperature. Sometimes thin smears from synchronised cultures

were spread on slides. The slides were either used immediately or stored with desiccant (silica gel) at -20°C.

2.2.22.8.2 Staining of slides: Slides were fixed in acetone for 5 minutes, allowed to air dry and then grided into 12 squares using nail varnish. On each square was placed 20μl of appropriately diluted antibody, in PBS with 1% BSA and 0.05% sodium azide. Slides were incubated in a moist chamber for 30 minutes, the antibodies were then aspirated off, and washed twice in PBS and once in dH_2O, allowing 5 minutes per wash. Slides were then air dried and incubated with 20μl of the manufacturer's recommended dilution of the second conjugated antibody per square. This was either a fluorescein isothiocyanate conjugate of immunoadsorbant-purified polyvalent anti-mouse immunoglobulin (FITC-anti IgG, Sigma) or a rhodamine isothiocynate conjugate of immuoadsobant-purified anti-rabbit immunoglobulin (RITC-IgG Sigma). In addition, parasite nuclei were counter-stained with DAPI (4;6 diamidino-2-phenyl-indole, Sigma). In order to do this DAPI was included in the second antibody solution at 1:40 dilution in PBS/azide (final concentration of 5μg/ml). Slides were then incubated and washed as before, and mounted in mounting solution (50% glycerol in PBS, DABCO-25g/l) to prevent fading. The slides were then examined by fluorescence microscopy (Leitz, Germany).

2.2.23 Yeast expression methods

2.2.23.1 Expression of *pfran* into yeast vector pREP1: The *pfran* cDNA was engineered with appropriate restriction sites for cloning into the *S. pombe* expression vector pREP1 (Kindly provided by Dr. P.Fantes) by PCR.

Oligonucleotides G5793,+ 5' CGC CAT ATG GATTCA CAA GAA TAT ATT CC (29 bases, nucleotides 680-703 plus 6 additional bases at the 5' end which provided a *BamHI* recognition site) and G5794,- 5' CGC GGA TCC TTA ATT TTC AAT ATC TTC TTC (30 bases, nucleotides 1305-1325 plus additional 9 bases at the 5' end providing an *NdeI* recognition site) were used as primers. This construct contains the AUG start codon and the termination codon TAA of the *pfran* gene. These primers were used for amplification of the *pfran* construct as described in method 2.2.16. The PCR product was treated with proteinase K prior to endonuclease digestion to improve the cloning efficiency by getting rid of *Taq* polymerase which remained bound to the DNA (Crowe *et al.*, 1991). The PCR generated fragment was introduced by ligation into the restriction sites of the expression vector.

2.2.23.2 Transformation into *pim1* mutant The Pim1 mutant (Matsumoto and Beach, 1991) was obtained from Dr. D.Beach (Howard Medical Centre, Cold Spring Harbor, USA). First the mutant was grown on Edinburgh Minimal Medium (EMM) plates (material 2.1.7) at 26°C overnight. A single colony was picked and grown at 26°C into 10ml EMM broth containing leucine. 1ml from the overnight culture was subcultured into 100ml of EMM containing leucine. The growth was monitored until the cell density was $1x10^7$/ml. The cells were collected by centrifugation for 5 minutes at 2000rpm at 4°C. The cell pellet was washed three times in ice-cold 1.2M sorbitol and resuspended finally into 1ml of ice-cold 1.2M sorbitol. The construct was then transformed into the Pim1 mutant cells by electroporation.

2.2.23.3 Electroporation

100μl of the electrocompetent cells were placed in a pre-cooled 2mm cuvette on ice. The DNA was added and incubated on ice for 1 minute. Appropriate voltage (25kV/cm with time pulse of approximately 5 seconds) was applied using a Flowgen™ pulser. The cells in the cuvette were then resuspended with 1ml of 1.2M sorbitol, and an aliquot of this was plated onto selective media.

CHAPTER 3

CLONING AND CHARACTERISATION OF THE *P. falciparum* Ran/TC4 HOMOLOGUE.

3.1 Summary

Degenerate oligonucleotides based on conserved amino acid sequences in the Ran/TC4 of other organisms, were used to amplify *Plasmodium falciparum* genomic DNA, to obtain a DNA fragment encoding the *P. falciparum* Ran/TC4 homologue. The fragment was used to screen a cDNA library to obtain full-length clones. *pfran* (*P. falciparum ras*-like nuclear protein), encodes a protein of 214 amino acids with 70% amino acid sequence identity with other eukaryotic Ran/TC4 proteins. The GTP-binding sites, characteristic of the GTPase superfamily were highly conserved in the *P. falciparum* gene. Like other *P. falciparum* mRNAs, *pfran* mRNA contains an exceptionally long 5' untranslated region (679bp). Southern blotting experiments have shown that *pfran* is a single copy gene on chromosome 11. RNA hybridisation experiments have shown that *pfran* mRNA is abundant in late trophozoite and schizont stages, but present only at very low levels in gametocytes and ring stages.

3.2 Introduction

'Knowledge based' strategies for vaccine development and chemotherapy of malaria require a better understanding of the basic molecular biology of the parasite. One large gap in what we know about malaria parasites concerns the mechanisms controlling intra-erythrocytic proliferation. The control of the cell-cycle in eukaryotes employs a regulatory network of proteins (Norbury and Nurse, 1992; Draetta, 1990; Peter and Herskowitz, 1994), many of which are highly conserved across a wide range of species. Key features include protein complexes such as serine/threonine kinases, cyclins and cyclin inhibitory factors, heterotrimeric G proteins and the small GTP-binding proteins of the GTPase superfamily. This latter group of proteins has been found to play several roles in cell cycle, notably those involving signal transduction.

Ran/TC4, (Ras-related nuclear GTP-binding protein), is a component of a GTPase switch that is postulated to regulate the progression of the cell-cycle (Drivas *et al.*, 1990; Bischoff & Ponstingl, 1991b; Dasso, 1993; Ren *et al.*, 1993). In contrast to other GTPases of the Ras superfamily which play a wide variety of roles in the cytoplasm including regulation of growth and differentiation, actin polymerisation and vesicular traffic, Ran/TC4 has been considered to function mainly in the nucleus (Bischoff and Ponstingl, 1991b; Boguski and McCormick, 1993), where it interacts with the guanine nucleotide exchange factor RCC1 (regulator of chromosome condensation) that appears to be involved in chromosome condensation and the onset of mitosis (Bischoff and Ponstingl, 1991b; Ren *et al.*, 1993). The Ran/TC4-RCC1 complex may couple completion of DNA synthesis with initiation of mitosis through

the activation of a cyclin-p34^{cdc2} complex (Ren *et al.*, 1993). It is therefore possible that the Ran/TC4-RCC1 complex functions as signal transducer to regulate the cell cycle in eukaryotes.

Ran/TC4 is an abundant small GTPase which has been found to constitute about 0.36% of total HeLa cell protein (Moore and Blobel, 1993). Recently, it has been shown that Ran/TC4 is also an essential component in the transport of proteins into the nucleus (Moore and Blobel, 1993; Goldfarb, 1994; Moore and Blobel, 1994). In addition, the Ran/TC4-RCC1 complex has been implicated in the regulation of RNA processing and transport in *Saccharomyces cerevisiae* (Kadowaki *et al.*, 1993).

The gene was first identified in a human teratocarcinoma cDNA library (Drivas *et al.*, 1990), and since then, homologues have been reported from *S. pombe* (Matsumoto and Beach, 1991), *Onchocerca volvulus* and *Brugia malayi* (Dissanayake *et al.*, 1992), *S. cerevisiae* (Belhumeur *et al.*, 1993) and *Dicytostelium discodium* (Bush and Cardelli, 1993).

This Chapter describes the cloning and characterisation of the Ran/TC4 homologue of the malaria parasite *P. falciparum.* Cloning the gene encoding *P. falciparum* homologue of the Ran/TC4 is the first step towards understanding its structure and function. Apart from sequence comparison with other Ran/TC4 genes, it also allows identification of key features such as GTP-binding domains or the characteristic carboxy terminal sequences which are believed to be responsible for its nuclear localisation (Bischoff and Ponstingl, 1991a; Ren *et al.*, 1993). It will also provide the opportunity to produce specific antibodies which could be used to study its possible role in cell cycle control in *P. falciparum.*

3.3. Strategies and methods used to isolate the *P. falciparum* Ran/TC4 homologue

A PCR based approach was used to isolate the Ran/TC4 gene homologue. This type of approach has been shown to be useful in identifying the Rab and Rho genes members of the GTPase superfamily proteins from the mouse genome (Chavier *et al.*, 1992).

Three degenerate oligonucleotides were synthesised based on the conserved blocks of amino acids in previously identified Ran/TC4 sequences from human, *S. pombe* and *S. cerevisiae* (Matsumoto and Beach, 1991). Two of these oligonucleotides, A482,+ and A484,-, (Chapter 2, section 2.1.6) were based on the conserved amino acid sequences of the first and fifth domains which are known to participate in the formation of the GTP-binding site of all *ras*-related proteins (Valencia *et al.*,1991), see Fig. 1.3 (Chapter 1). The third oligonucleotide (A483,+) was

designed in order to a recognise a region outside the GTP-binding sites; this sequence is found only in the Ran/TC4 gene subfamily. To reduce the degeneracy of the oligomers, advantage was taken of the codon bias of the malaria parasites towards A+T rich codons (Weber, 1987; Saul and Battistutta 1988).

Amplification was performed with the 3 oligomers in all possible combinations. Several different PCR conditions were tried, before the following programme gave an optimal result; denaturation at 94°C for 30 seconds, annealing at 50°C for 60 seconds and extension at 72°C for 90 seconds. The template was genomic DNA isolated from asexual cultures of *P. falciparum* clone 3D7A.

The PCR amplified fragment was cloned into the pCRII vector (see Chapter 2, section 2.2.15.2). After the amplification was completed, the PCR product was ligated to the vector, and transformed into *E. coli* INVαF' cells. Positive colonies were identified by hybridisation to the PCR insert (method 2.2.12). DNA was prepared from the positive clones and sequenced, on both strands as described in method 2.2.18.

In order to identify the full coding sequence of the Ran/TC4 gene homologue of *P. falciparum*, a cDNA library was screened, as described in method 2.2.15.5, using the PCR fragment as a radioactive probe. The library (a gift from Dr. A. Craig, Oxford University) was made in the plasmid vector pJFE14 (Fig.3.1), from asexual stage mRNA (late rings/early trophozoites) of the *P. falciparum* clone ITO4.

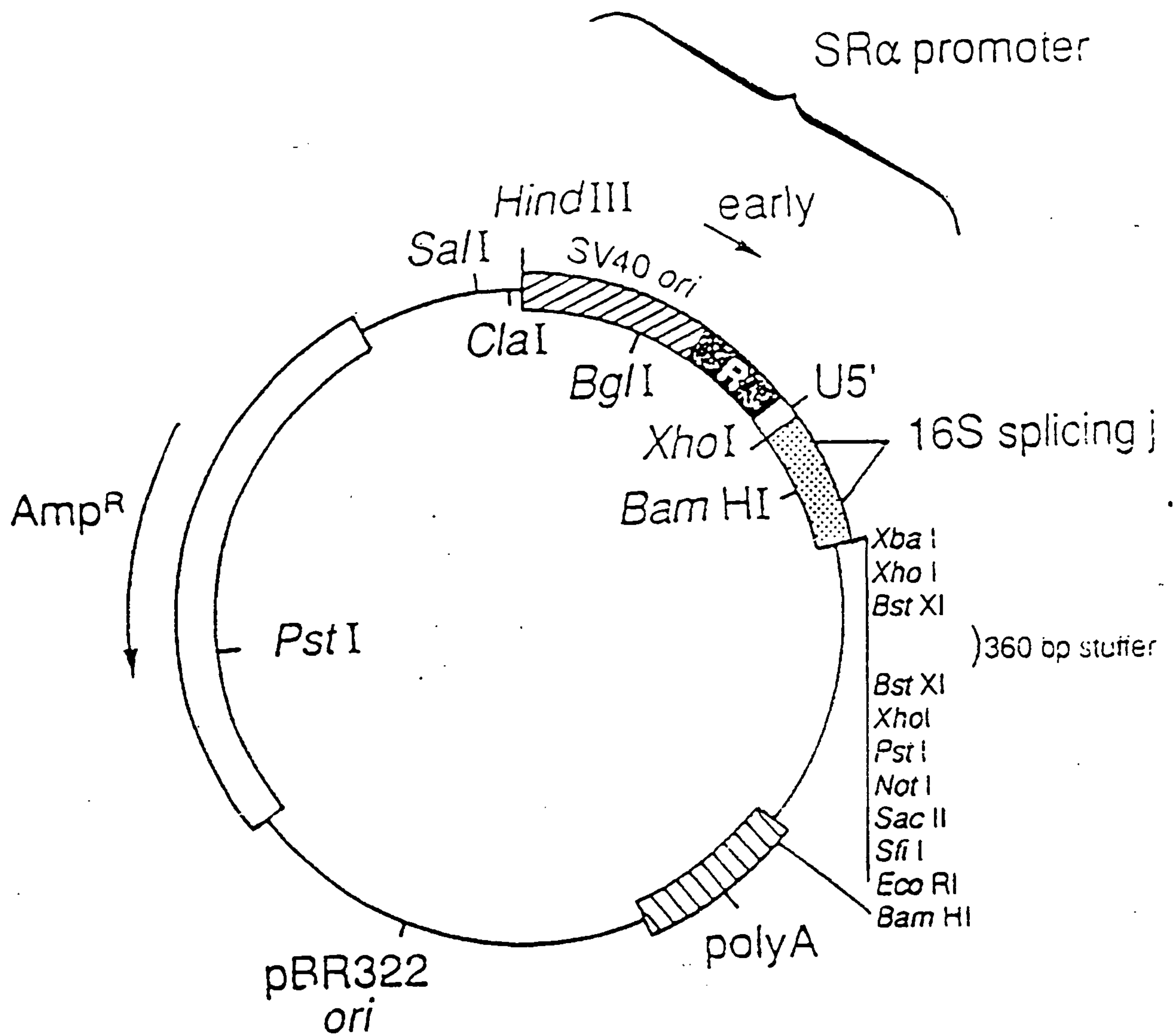
3.4 Results

3.4.1 Isolation of the *P. falciparum* Ran/TC4 homologue

A 552bp DNA fragment was obtained by amplifying *P. falciparum* genomic DNA using two degenerate primers based on conserved regions of the Ran/TC4 protein from other organisms (Fig. 3.2). Sequencing of the PCR product confirmed that the amplified fragment represented part of a gene encoding an open reading frame (ORF) with homology to published Ran/TC4 sequences. The ORF of the 552bp fragment is interrupted by an intron (165 bp long) between nucleotides 796 and 797 (Fig. 3.4B). The intron is 90% A+T which compares with 67% A+T for the coding sequence and 85% A+T for the non-coding regions. The sequences at the ends of the intron (Fig. 3.5) have the universally conserved GT and AG dinucleotides marking the intron-exon boundaries, and the 3' AG splice acceptor site is preceded by a stretch of 17 thymidine residues, an exaggeration of the pyrimidine nucleotide motif at the 3' intron boundary of all eukaryotic genes (Weber, 1987). The sequence at the 5' intron boundary (GTAAGTGAA) is also fairly well conserved among *Plasmodium* introns and quite similar to the consensus eukaryotic intron boundary sequence.

Figure 3.1:

Schematic diagram represents the map of the pJFE14 plasmid vector (Elliot *et al.*, 1990) which has been used to engineer the *P. falciparum* asexual cDNA library used during the course of this study. The asexual RNA was prepared from ring and trophozoite stages of line ITO4.



pJFE14

Sequence of the Polylinker from pJFE14 plasmid vector :

5' TCTAGAGATCCCTCGACCTCGAGATCCATTGTGCTGGC'....360bp'stuffer

.........GCCAGCACAATGGATCTCGAGGGATCTTCCATACCTACCAGTTCT

GCGCCTGCAGGTCGCGGCCGCGGGAGGCCGAATTC 3'
cgcggacgtccagcgccggc

Figure 3.2:

A.

Amplification of *P. falciparum* genomic DNA of clone 3D7 and HB3 and isolate K1, using the Ran primers. Three degenerate primers (Ran1 = primer A482+, Ran2 = primer A483+, Ran3 = primer A485-) were tried in different combinations to obtain the fragment encoding the *P. falciparum* Ran/TC4 homologues. 5µl of amplified product was run on a 1.5% IBI agarose gel in 1xTAE buffer.

Lane 1. DNA from isolate K1 with primer Ran 2+ Ran 3.

Lane 2. No DNA (control) with Ran 2+Ran 3.

Lane 3. DNA from clone HB3 with Ran 2+ Ran 3.

Lane 4. DNA from clone HB3 with Ran 1+ Ran 3.

Lane 5. DNA from clone 3D7 with Ran 2+ Ran 3.

Lane 6. DNA from clone 3D7 with Ran 1+ Ran 3.

M. DNA molecular weight marker VI (pBR 328 DNA cut with *Bgl I*+ *Hinf I*).

B.

Amplification product of genomic DNA from clone 3D7. The Ran 2 and Ran 3 primers were used on the template with different annealing temperatures to optimise the PCR conditions.

Lane 1. annealing temperature used was 37°C.

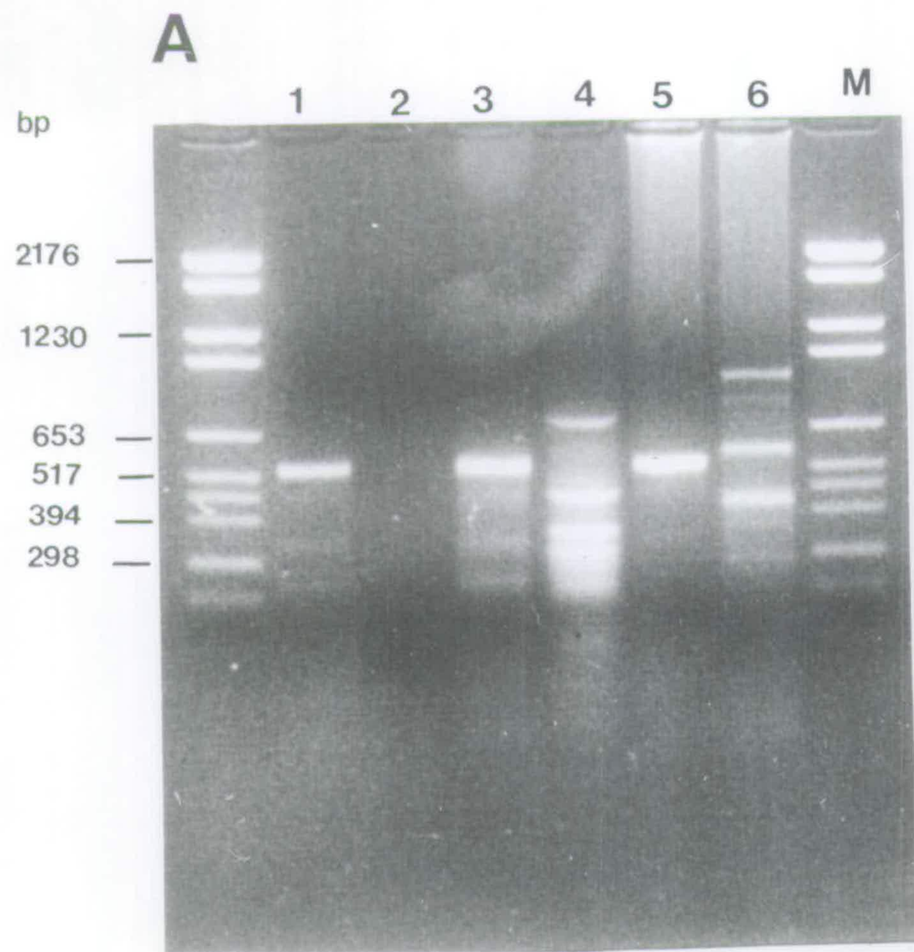
Lane 2. annealing temperature used was 40°C.

Lane 3 . annealing temperature used was 45°C.

Lane 4 . annealing temperature used was 50°C.

Lane 5 . annealing temperature used was 52°C.

M. DNA molecular weight marker (same as in figure B).


A
1
2
3
4
5
6
M
bp
2176
1230
653
517
394
298

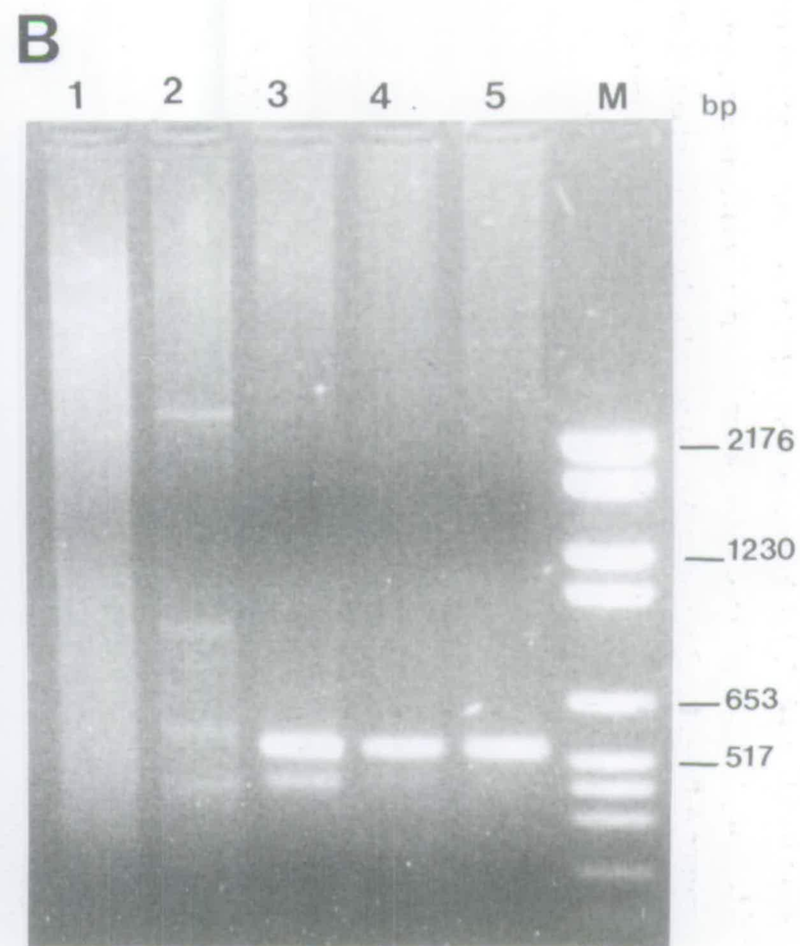
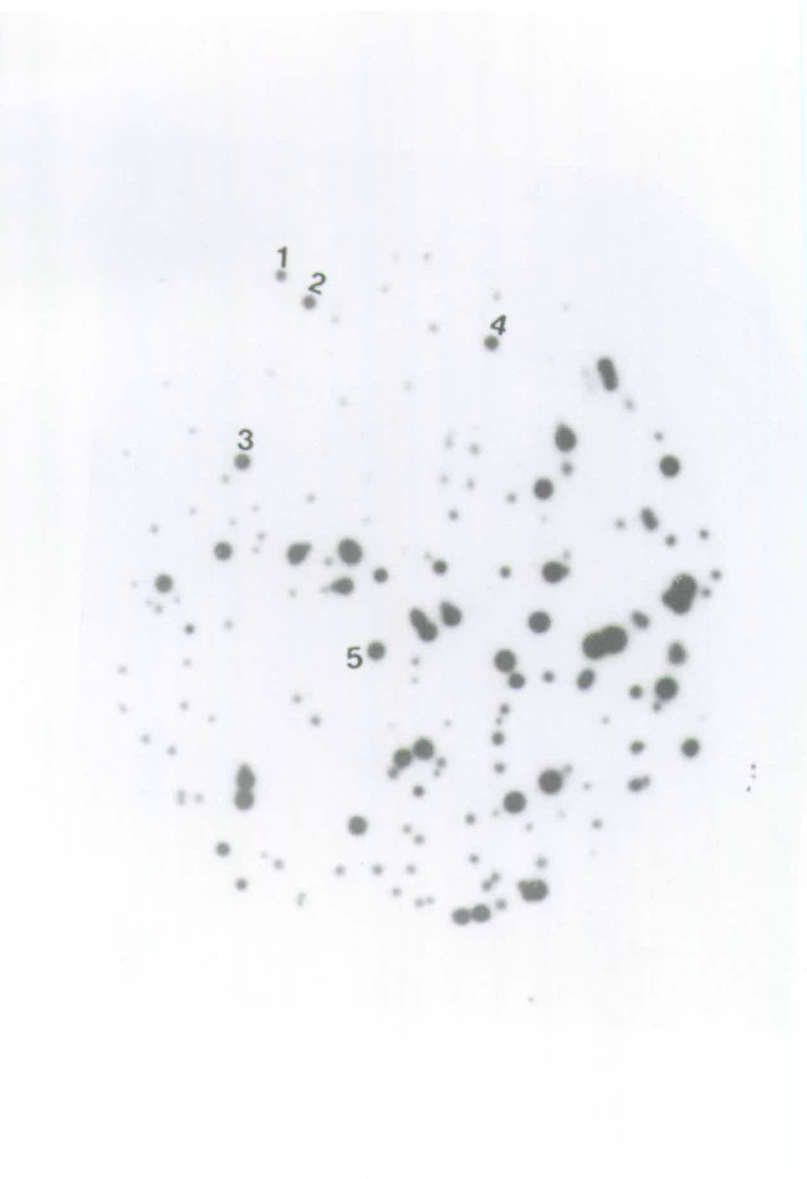

B
1
2
3
4
5
M
bp
2176
1230
653
517

Figure 3.3 :

An autoradiograph showing the result of secondary screening of the three positive clones isolated from the asexual cDNA library, probed with the PCR product (552 bp) produced by the amplification of genomic DNA from clone 3D7 with Ran2 + Ran 3 primers.

The PCR fragment was radiolabelled by random priming and used as a hybridisation probe to screen these clones. DNA from the positive clones was transferred to Hybond N+ filters, and the filters were prehybridised for 1 h at 65°C in 5xSCC/ 5xDenhardt's solution/ 0.1% SDS/ 50mM sodium phosphate buffer pH 7.0 / 100mg/ml denatured salmon sperm DNA. Hybridisation was carried out, with the radiolabelled PCR fragment, in the same buffer, with 10% dextran sulphate added, at 65°C overnight. Filters were extensively washed in 2xSCC at room temperature and then washed in 0.5x SSC/0.1 SDS at 65°C for 1h. The filters were exposed to X-ray film for 24 h before developing.
Five clones (numbered) were picked and sequenced on both strands by the dideoxy chain termination method. Clone No. 4 was found later to encode the whole *P. falciparum* Ran/TC4 homologue.



1
2
3
4
5

The PCR fragment was used as a hybridisation probe to screen the cDNA library. 50,000 colonies were screened, (method 2.2.15.5), which yielded 3 positive clones. A second round of screening was carried out on these clones, using the PCR fragment as a probe, in order to confirm the result of the primary screening and to obtain single isolated colonies (Fig. 3.3). Finally, one clone with a 1.4 kb insert, C 4.1, was selected for analysis. The clone was sequenced on both strands using the dideoxynucleotide chain termination method of Sanger *et al.*, 1977, (method 2.2.18).

The sequence of the cDNA clone C4.1 is shown in Figure (3.4A). The insert contained a single open reading frame (ORF) extending from nucleotide 677 to a stop codon 77 bases upstream from the 3' terminus. The deduced amino acid sequence had a methionine codon at nucleotide 680 which permitted a homologous alignment of the sequence with available sequences of other Ran /TC4 proteins. The C4.1 cDNA contained a long (679 bp) 5' untranslated region (UTR), and 77 bp of non-coding sequences at the 3' end. Sequences of genomic DNA adjacent to the original 552 bp PCR product were obtained by utilising the inverse PCR procedure (Triglia *et al.*, 1988) as described in method 2.2.17. The coding sequence, the intron-exon boundaries, and three hundred base pairs of 5' UTR sequence proximal to the start codon were confirmed as identical in both the genomic DNA and the cDNA clone (Fig. 3.5).

3.4.2 Sequence comparison and data-base searches

Comparison of the amino acid sequence deduced from clone C4.1 with the sequences contained in the Gene-bank data-bases was performed by a BLAST search of the Swissprot 22 data-base (Devereaux *et al.*, 1984). The search was done for predicted amino acid sequences and scored for both identity and similarity. The search showed that clone C 4.1 (hereafter known as *pfran* gene) was clearly related to proteins of the small GTPase superfamily (Fig. 3.6). The highest similarity scores were with the members of the Ran/TC4 subgroup of this family, 72% and 70% with *S. cerevisiae* Gsp1 and 2 respectively (Belhumeur *et al.*, 1993), 69% with *S. pombe* Spi1(Matsumoto and Beach, 1991), 68% with the filarial worms Ran/TC4 proteins (Dissanayake *et al.*, 1992), 67% with the human Ran/TC4 (Drivas *et al.*, 1990; Bischoff and Ponstingl, 1991a), and 66% with *Dictyostelium* Dd.TC4 (Bush and Cardelli, 1993).

Figure 3.4 : Nucleotide sequences of *pfran* gene.

A. The complete sequence of cDNA clone 4.1 containing the large 5' untranslated region and complete coding sequence of the *pfran* gene of *P. falciparum.* The predicted amino acid sequence for the coding region is shown below the nucleotide sequence, in single letter code. The site of the intron, detected in the genomic sequence, is indicated by an asterisk. The underlined sequences are the nucleotides used to designed the primers (P1, P2, and P3) used for amplification of DNA from the clone C4.1 clone 3D7 genomic DNA, to check for the presence of other introns (for the result see Fig. 3.5).

N.B. The nucleotide sequence data in this figure is deposited in the EMBL, Genbank and DDBJ data-bases under the accession number X73954.

B. Nucleotide sequence of the intron in the *P. falciparum pfran* gene obtained from genomic DNA. Intron sequence is in the lower case typescript. Numbering as in (Fig. 3.4 A).

```
                                                   1
                                                   AAGTTTTTTTTTTCATATTATTTAATAATCCTTTTTTTTCTTTCCCCCTTTTTCTGAGATTATAAATTAATATATATATAT  79

ATATATATATATAATTATTAAATTAATTAAAATTAAAATATATTTTTTTTATTTCCATTATTTTTATTTTTGCTCTAATTTCTTTCCAACTTTGCTTATTTTTCTAAAAAAAAAAATTTT  199

ATTTTTATTTGTTTAAAATATAATATAAGGAAAAAAAAAAAAAAAAAAAAAAAAGGCCTTCCTTTTTTAAACCTTGGAATTCCAATTAAAAAAAAAAAATTAAAACCATTAATTTTAGGT  319

TATTATTAATTAAAAAAAAAAAAAAAATTAAACCATTAATTTTATTGGTTGGAATTAATTTATTTTTTTTATATTAATTAACCTTAAATTATATATTATAAATTCCTTTAAAAAGAATAAC  439

CGTTTTTTACATTAAATCTAGTGCAGTTTTAAAACACACTTAAAAGAAAAATTTGCAACCTTTTAAAGCTCTGAGTACATCATTTAAGAATATAGTTTTCTATTATATATACGTATATTT  559

TTGTATATAACACATAAGCAATATGTCAGTTGATATATAAATTAAATATATATTTACAACATAGTAGTCTTATTTAATATATCATTTTTTTTTTTCTTTTTATTAGTACAATTTAAAAA  679
Primer  P1 ----->                                                                                                     *
ATGGATTCACAAGAATATATTCCACAATATAAATTAATCTTAGTCGGTGATGGTGGTGTTGGCAAAACAACCTTTGTGAAAAGACACTTGACTGGAGAATTCGAAAAAAAATATATAGCT  799
 M  D  S  Q  E  Y  I  P  Q  Y  K  L  I  L  V  G  D  G  G  V  G  K  T  T  F  V  K  R  H  L  T  G  E  F  E  K  K  Y  I  A
        Primer P3 --->
ACTCTTGGTGTGGAAGTTCACCCCTTAAAATTTCAAACAAACTTTGGAAAAACTCAATTTAACGTATGGGATACTGCAGGACAAGAAAAGTTTGGTGGTTTAAGAGATGGATATTATATA  919
 T  L  G  V  E  V  H  P  L  K  F  Q  T  N  F  G  K  T  Q  F  N  V  W  P  T  A  G  Q  E  K  F  G  G  L  R  D  G  Y  Y  I

AAAAGTGATTGTGCTATAATTATGTTTGATGTATCTTCTCGTATTACTTACAAGAACGTTCCAAATTGGTATAGAGATATTACAAGAGTGTGTGAAACAATTCCTATGGTTTTAGTTGGA  1039
 K  S  D  C  A  I  I  M  F  D  V  S  S  R  I  T  Y  K  N  V  P  N  W  Y  R  D  I  T  R  V  C  E  T  I  P  M  V  L  V  G

AACAAAGTTGATGTTAAAGACAGACAAGTAAAATCAAGGCAAATTCAATTTCACAGAAAAAGGAATTTACAATACTACGATCTATCTGCAAGATCAAACTACAATTTCGAAAAACCTTTC  1159
 N  K  V  D  V  K  D  R  Q  V  K  S  R  Q  I  Q  F  H  R  K  R  N  L  Q  Y  Y  D  L  S  A  R  S  N  Y  N  F  E  K  P  F

TTATGGTTAGCTAGGAGATTGTCCAACCAACCAAATCTTGTTTTCGTAGGAGAACATGCTAAAGCACCAGAGTTCCAAATTGATCTAAATATTGTAAGAGAAGCTGGAAAAGAATTAGAG  1279
 L  W  L  A  R  R  L  S  N  Q  P  N  L  V  F  V  G  E  H  A  K  A  P  E  F  Q  I  D  L  N  I  V  R  E  A  G  K  E  L  E
                          Primer P2 <----
CAAGCAGCAGCTGTAGCTATTGATGAAGAAGATATTGAAAATTAAATAAATAGAAAAAAAAAAAAAAAAAAAAAAAAAGGGAAACAGCCAAAATGGAAGTAAAAAAAAAAAAAAAAAAGAAA  1398
 Q  A  A  A  V  A  I  D  E  E  D  I  E  N stop
```

B.

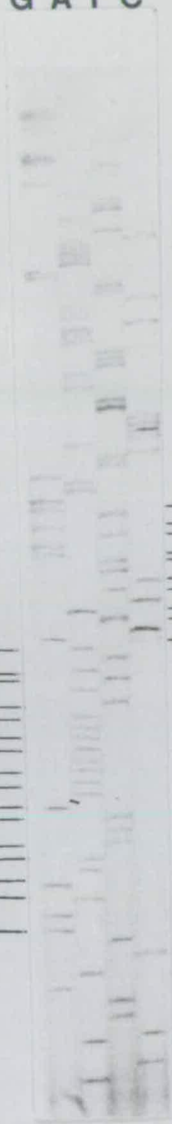
```
          796
           |
GAAAAAAAATATATA gtaagtgaaaaggagaaataataaaatgggaacattaatatatatatatatatatatatatatatatttatttatttatttatatgtttacatatatatt
 E  K  K  Y  I

                                                                     797
                                                                      |
tatatttatttttttatatttttgtatttatttattttattataattttttttttttttttttttag GCTACTCTTGGTGTG
                                                                      A  T  L  G  V
```

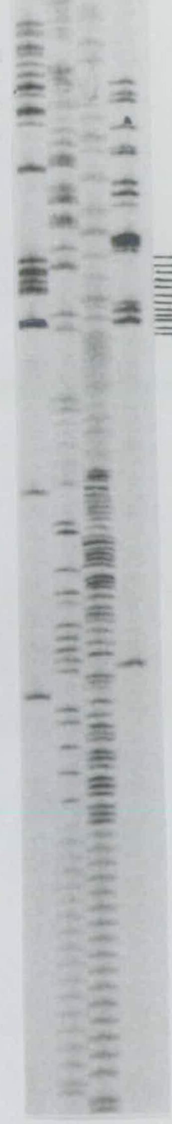
Figure 3.5 : Exon/intron boundaries of *pfran.*

Autoradiograph showing a sequence comparison of border of exon 1/intron 1 (Track A) and intron 1/exon 2 (Track C) of genomic *pfran* 3D7 DNA with the *pfran* cDNA from clone C4.1 (Track B), showing that intron 1 has been removed.

a



b



c

Figure 3.6: Amino acid sequence comparisons of pfRan.

Comparison of protein sequences from three members of Ran/TC4 subfamily with the pfRan amnio acid ssequence. Sequences are aligned relative to the human Ran/TC4 protein, the first member of the subfamily described (Drivas et al., 1990). Dd.TC4 is the Dicytostelium discoideum sequence (Bush and Cardelli, 1993) and Spi of Schizosaccharomyces pombe (Matsumoto and Beach, 1991).

Dots indicate identical residues, and dashes indicate gaps introduced to maximise similarity within the alignment. The 5 regions involved in guanine nucleotide binding, (G1 to G5), which are highly conserved between all members of the small GTPase superfamily are underlined.

```
                                    G-1                                 G-2
TC4       MAAQGEPQVQ FKLVLVGDGG TGKTTFVKRH LTGEFEKKYV ATLGVEVHPL  50
PfRan      MDSQ.YIP. Y..I...... V......... .........I ..........
Dd.TC4        MAKKE. I......... V......Q.. ......PR.I P....S....
Spi1       M..PQNVPT .......... .......... .........I ..........
                           G-3
TC4       VFHTNRGPIK FNVWDTAGQE KFGGLRDGYY IQAQCAIIMF DVTSRVTYKN 100
PfRan     K.Q..F.KTQ .......... .......... .KSD...... ..S..I....
Dd.TC4    I.Y..F.K.H .......... .......... ..GN...... .....IS...
Spi1      H....F.E.C .......... .L........ ..G..G.... .....I....
                                 G-4                            G-5
TC4       VPNWHRDLVR VCENIPIVLC GNKVDIKDSK VKAKSIVFHR KKNLQYYDIS 150
PfRan     ....Y..IT. ...T..M..V .....V..RQ ..SRQ.Q... .R......L.
Dd.TC4    .....S..T. .......... .....V..R. ..PSQ..... RY..S...V.
Spi1      ..H.W..... .......... .....V.E.. ....A.T... ..........

TC4       AKSNYNFEKP FLWLARKLIG DPNLEFVAMP ALAPPEVVMD PALAAQYEHD 200
PfRan     .R........ ......R.SN Q...V..GEH .K..-.FQI. LNIVREAGKE
Dd.TC4    .......... .V..TS..L. NKAVTL.QQ. T.KL..T.L. SN.MSL..KE
Spi1      .......... ........V. N.......S. .......QV. QQ.L...QQE

TC4       LEVAQNPALP DEDD-DL 216
PfRan     ..Q.AAV.I- ..E.IEN
Dd.TC4    VAD.AALP.. EDN.-DL
Spi1      MNE.AAMP.. ....ADL
```

3.4.3 The *pfran* gene has one intron

In order to check by PCR whether there is a second intron following the first one, three non-degenerate oligonucleotide primers were synthesised (Fig.3.4.A). Oligomer P1(20 bases, nucleotides 680-699) and P2 (19 bases, nucleotides 1304-1322) which flank the whole coding sequence of the full cDNA clone. The third oligomers, was designed immediately after the intron, P3 (17 bases, nucleotides 807-823). The primers were used in different combinations with DNA from plasmid clone C4.1 and *P. falciparum* genomic DNA from clone 3D7A as template. The PCR results are shown in figure 3.7. When primers (P1+P2) flanking the whole coding sequence of clone C4.1 were used in amplification reactions on the cDNA clone and genomic DNA size variation of about 165bp in the PCR product was found between the two templates. When primer P2 and P3 (immediately following the intron identified in the 552 bp genomic PCR product) were used on the above templates there were no size different between the PCR products from both genomic and the cDNA clone C 4.1.

The fission yeast homologue of the *ran* gene, the *spi1* (suppressor of premature initiation of mitosis) gene, contains two introns. The first intron of *S. pombe* is in the same position as for the 165bp intron that has been found in the *pfran* genomic sequence. PCR products from genomic DNA and C4.1 cDNA clone using above primers showed that no second intron is present in the genome of the parasite (Fig.3.7).

3.4.4 Copy number of *pfran* gene

Genomic *P. falciparum* DNA was digested with restriction enzymes (method 2.2.5) and blotted onto a nylon membrane. Hybridisation was carried out with the C4.1 insert as a probe. The result is shown in Fig.3.8.A.

All lanes contain DNA digested with restriction enzymes that do not cleave within the sequence recognised by the probe, (*Acc I, Bgl II, Hinc II, Hind III, Msp I. Nhe I, Xba I* or *Xho I*), show hybridisation of the probe to a single fragment. DNA digested with *Alu I, Dra I, Hinf I* and *Sau 3A*, enzymes which cleave within the probe, yielded more than one hybridising fragment, whose sizes corresponded to the sizes predicted from the sequence.

3.4.5 Chromosome mapping

To characterise further the *pfran* gene, its chromosomal location was identified. This was achieved by blotting pulsed field electrophoresis gels (PFGE) (as described in method 2.2.7) of malarial chromosomes with the coding sequence from clone C 4.1.

Figure 3.7 : *Pfran* intron study.

Amplification of *P. falciparum* genomic DNA of clone 3D7 and the *pfran* plasmid (clone C4.1) using primer P1 and P2 which flank the entire coding region of clone C 4.1 (see fig. 3.4 A) and primer P3 and P2 (primer P3 is located immediately after the 165bp intron recognised in the 552bp PCR insert). The PCR reaction was carried out using both sets of primer on the genomic and the cDNA in order to check for the presence of another intron in the genomic sequence of *P. falciparum.* 5μl of amplified product was run on a 1.5% agarose gel in 1xTAE buffer.

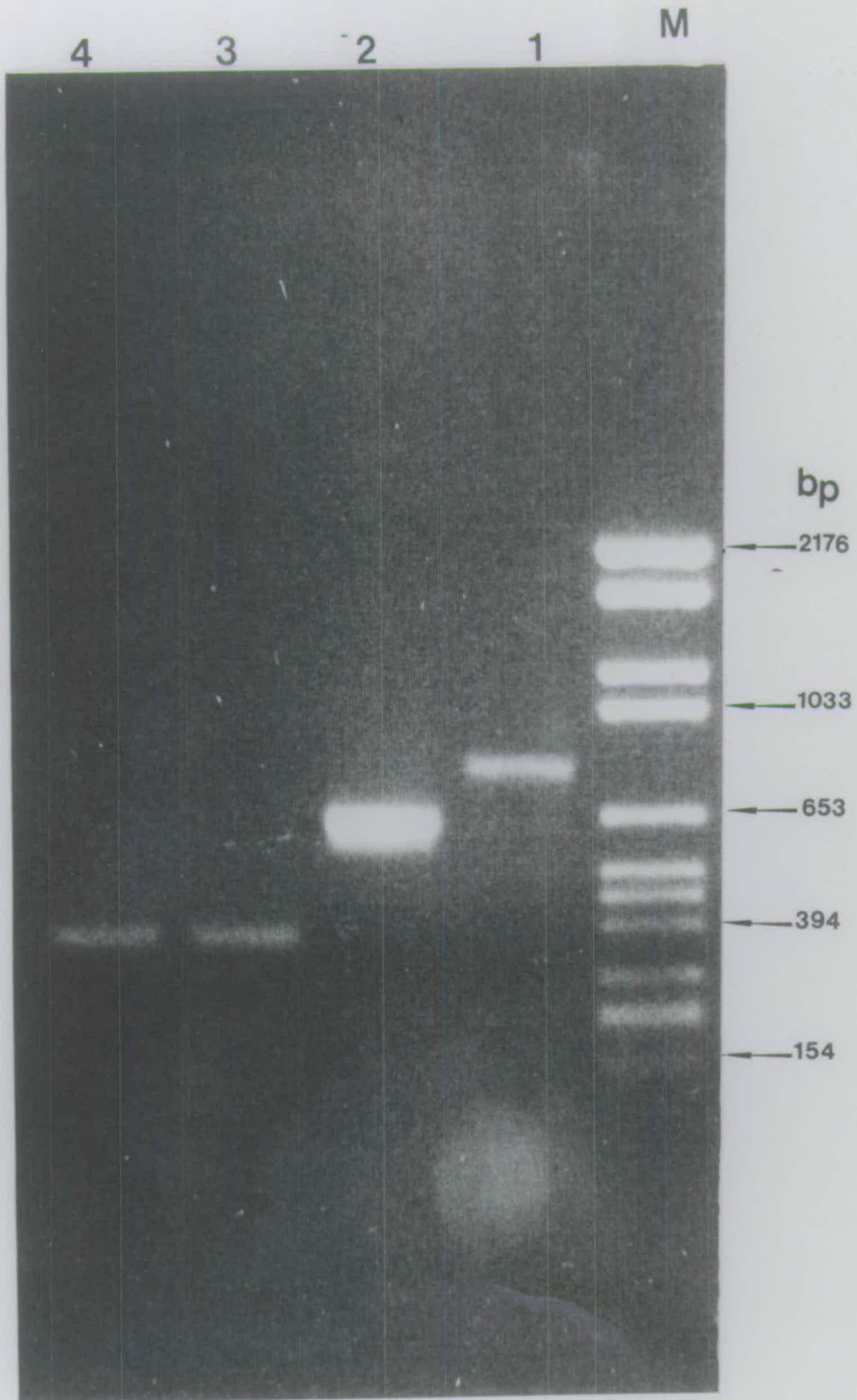
M. DNA molecular weight markers.

Lane 1. amplification product from genomic DNA from clone 3D7 using primers P1+P2.

Lane 2. amplification product from clone C4.1 using primers P1+P2.

Lane 3. amplification product from genomic DNA using primers P2+P3.

Lane 4. amplification product from clone C4.1 using primers P2+P3.

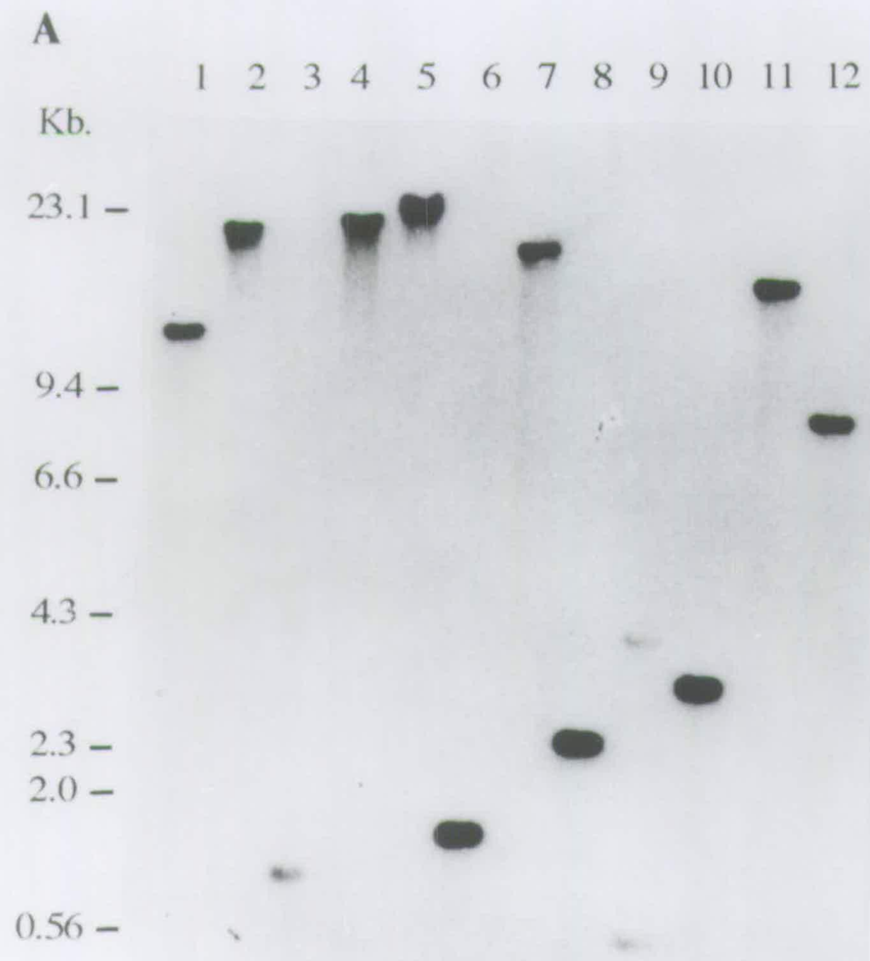


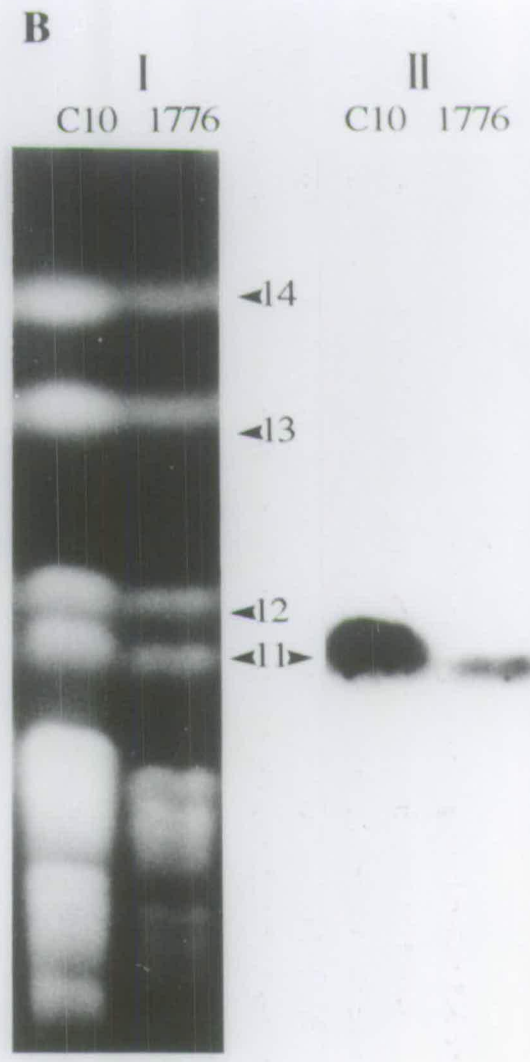
4
3
2
1
M
bp
2176
1033
653
394
154

Figure 3.8 : Southern analysis of *Pfran* gene.

A. An autoradiograph demonstrating that *pfran* is a single copy gene. Genomic DNA from *P. falciparum* was digested with various enzymes, resolved, blotted and hybridised with the*pfran* specific probe. The enzymes used were : (Lanes 1-12): *AccI, Bgl II, Sau3A, MspI, XhoI, DraI, HincII, HindIII, AluI, HinfI, XbaI* and *NheI.*

B. Chromosome assignment of the *pfran* gene. (I): Ethidium bromide-stained pulsed field gradient gel of *P. falciparum* clones C10 and 1776, with chromosomes 11, 12, 13, and 14 marked; (II): autoradiograph of the blot from the gel in panel (I) probed with the radiolabelled *pfran* cDNA fragment.


A
1 2 3 4 5 6 7 8 9 10 11 12
Kb.
23.1
9.4
6.6
4.3
2.3
2.0
0.56


B
I
C10 1776
II
C10 1776
14
13
12
11

The *pfran* gene was found to be located on chromosome 11 (Fig. 3.8.B) according to the definition of chromosome numbers proposed by Kemp *et al.*, (1987). The blots were then stripped and reprobed with marker specific to chromosome 5 and chromosome 9 ((*pfmdr-1* and *msp-1*) respectively (Foote *et al.*, 1989; Kemp *et al.*, 1987b) (data not shown) to confirm the order of the chromosomes on that particular blot.

3.4.6 Northern Blot analysis

To obtain some information on how *pfran* mRNA levels are regulated during intra-erythrocytic development, total RNAs were isolated from synchronised stage specific asexual and sexual *P. falciparum* cultures as described in method 2.2.20, and the Northern blots were probed with clone C4.1. As shown in figure (3.9) a diffuse band of approximately 1.5-1.6 kb was observed. Signals are strongest on the schizont and trophozoite lanes (Fig. 3.9B, lanes 1 and 2) and very weak signals can be seen in RNA derived from early asexual stages and from gametocytes lanes (Fig. 3.9B, lanes 3, 4 and 5). As an internal control for hybridisation to gametocyte mRNA, the same blot in figure 3.9A was stripped and hybridised to sequences from the gametocyte specific pfg27/25 gene (Alano *et al.*, 1991). As shown in figure 3.9C, intense signals were only seen in lanes containing RNA from gametocytes.

An independent Northern blotting experiment of total RNA prepared from asexual synchronous and asynchronous cultures from *P.falciparum* clone C10 and its parent line 1776 was probed with C4.1 fragment had confirmed the above result (Fig. 3.10).

3.5 Discussion

Comparison of the deduced amino acid sequence of pfRan with Ran proteins from other species has shown about 70% identity. Furthermore, the consensus motifs (G1-G5) of GTPases of the Ras superfamily (Bourne *et al.*, 1991) were identified in pfRan. Predicted molecular weight and possession of the five conserved guanine nucleotide binding regions clearly indicate that *pfran* is a member of the small GTPase superfamily. However, outside these conserved regions, there are no obvious homologies between the pfRan sequence and those of the other three main subfamilies of this group, the products of *ras* - like proto-oncogenes, the *ypt/rab* genes and the *rho* type genes (Bourne *et al.*, 1991). Unlike cytoplasmic Ras-like GTPases that are targeted to the membrane, pfRan does not have the carboxyterminal cysteine Cys-A-A-X ('A' is an aliphatic amino acid and 'X' is usually a methionine or serine residue) motif that mediate isoprenylation and membrane association in Ras family members.

Figure 3.9: Northern blot analysis of *pfran* gene.

Total *P. falciparum* RNA obtained from synchronous cultures of different developmental stages of clone 3D7 was fractionated in a 1.2% denaturing formaldehyde gel, blotted and hybridised to *pfran* gene probe and *pfg27/25* gametocyte specific gene. **A**: Ethidium bromide stained gel (5µg of total RNA loaded per lane), **B**: and **C**: autoradiographs of the gel probed with *pfran* and *pfg27/25* genes respectively.

Lane 1. schizonts.

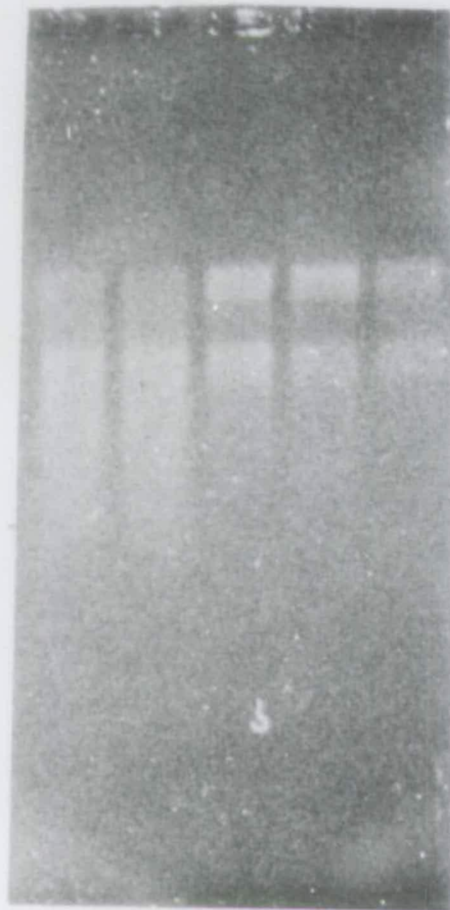
Lane 2. trophozoites.

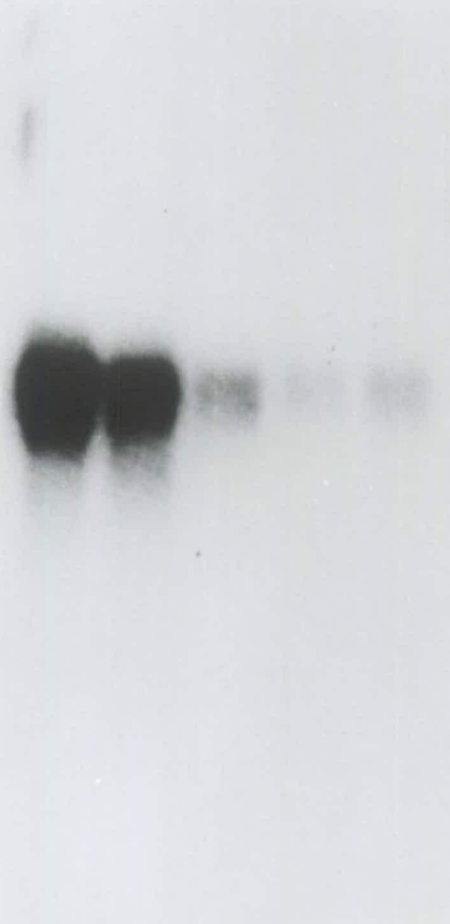
Lane 3 . rings

Lane 4. mature gametocytes (stage V).

Lane 5 . young gametocytes (stage I and II).

N. B. Gametocyte stages classification according to Hawking *et al*., 1971; Carter and Miller, 1979.


A
1 2 3 4 5
kb.
2.8
1.9
1.6


B
1 2 3 4 5

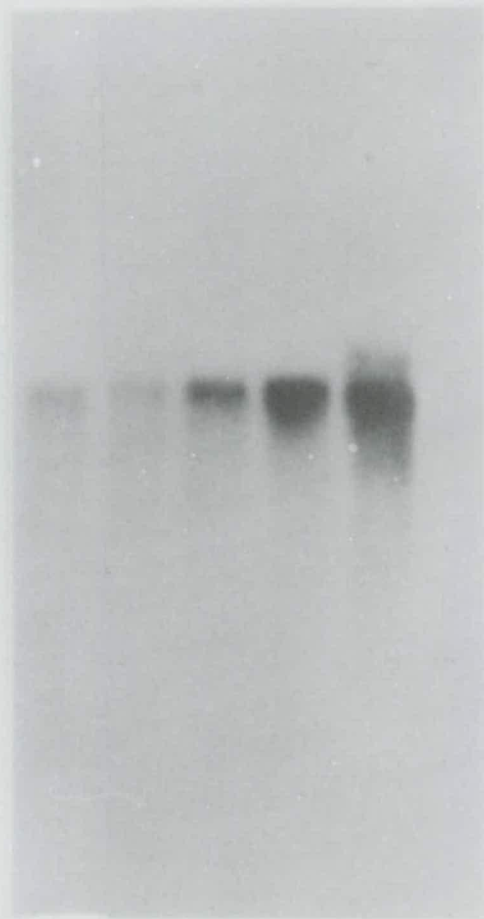

C
1 2 3 4 5

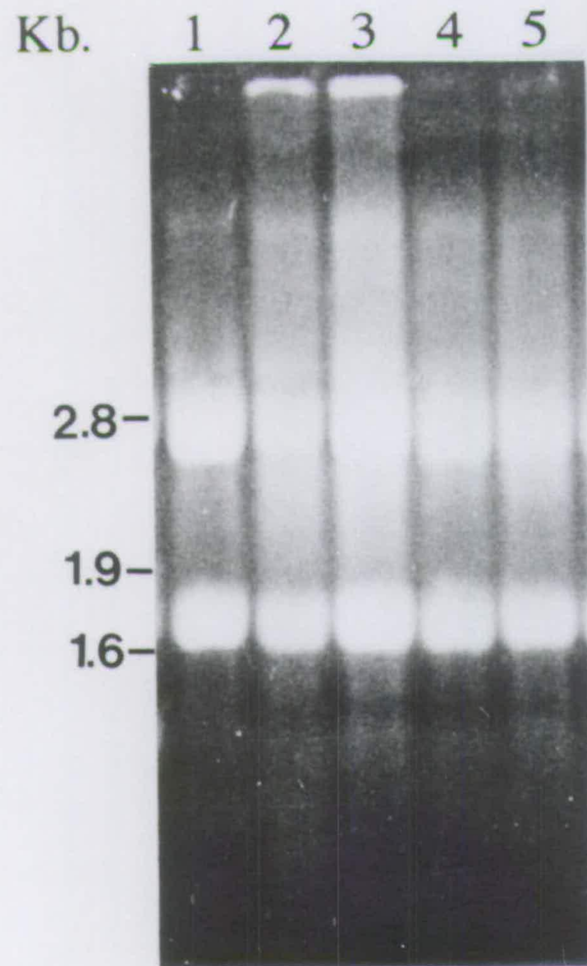
Figure 3.10: Northern blot analysis of *pfran* gene.

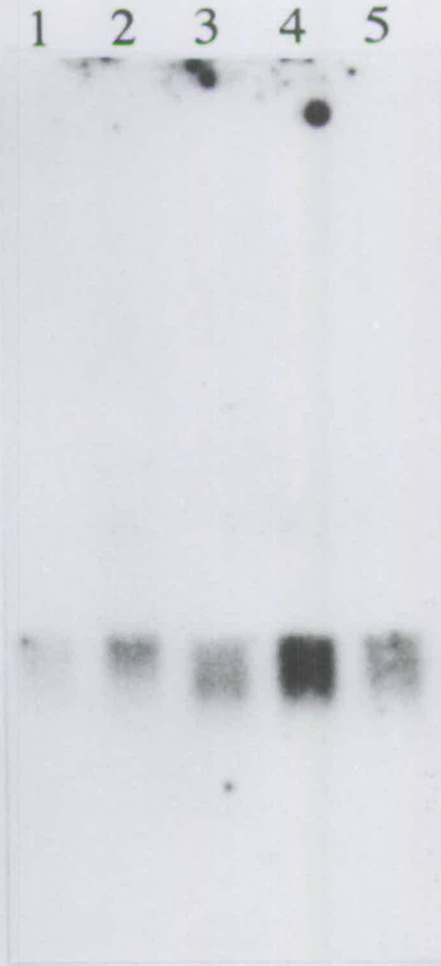
Total RNA prepared from parasite clone C10 and its parent line 1776 respectively. 10μg of RNA separated by electrophoresis in a 1.2% denaturing formaldehyde gel, blotted to nitrocellulose filter and probed with *pfran* fragment.

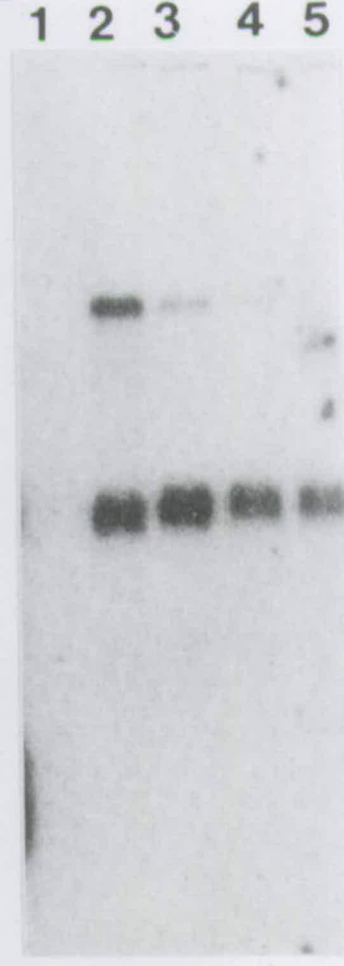
A. Ethidium bromide-stained gel :
Lanes 1 and 5 total RNA isolated from asynchronous cultures of clone C10 and line 1776. Lanes 2, 3 and 4 total RNA of rings, trophozoites and schizonts respectively obtained after culture synchronisation of clone C10. (rings; 87% pure, trophozoites; 69% pure and schizonts 95% pure).

B. Autoradiograph of the gel from panel A probe with the *pfran* specific fragment.

C. Autoradiograph of the same gel from panel A hybridised with *msp-1* specific probe in order to compare relative quantities of the mRNAs.


A
Kb.
1
2
3
4
5
2.8
1.9
1.6


B
1
2
3
4
5


C
1
2
3
4
5

However, the usual COOH-terminal Asp-X-Asp-Leu motif found in other members of Ran subfamily of Ras-like protein is absent in pfRan. pfRan shares 66-72% amino acid identity with all members of the fourth small GTPase subfamily so far characterised, the Ran/TC4 type proteins. Although the homology between mammalian representatives of this subfamily is at least 95%, this drops to around 80% amino acid identity between human and yeast homologues and 68% identity between human and *Dictyostelium* Ran/TC4 proteins. The sequence homology argument for provisionally accepting pfRan as the *P. falciparum* representative of the Ran/TC4 small GTPases is therefore strong.Therefore, the argument that the *pfran* gene is a *P. falciparum* homologue of the Ran/TC4 subfamily of small GTPases therefore rests primarily on sequence homology data and not as yet on functional data.

The *pfran* gene appears to be present as a single copy as inferred indirectly by the observation that in genomic DNA blots the cDNA probe hybridised to bands whose intensity on autoradiographs was within the range expected for a single copy gene. No further hybridising bands appeared after longer exposures with less stringently washed filters. Using standard calculations for the melting temperatures of mismatched DNA hybrids at these salt concentrations and temperatures it can be estimated that it is unlikely that a gene with nucleotide sequence homology to *pfran* of more than 80% exists within the *P. falciparum* genome. In other organisms where Ran/TC4 has been isolated, the gene is present in a single copy (Matsumoto and Beach, 1991).

The size of the *pfran* mRNA supports the evidence obtained from cDNA and genomic DNA sequences that a much larger transcript is produced than would be required to encode the protein. It is becoming clear that many *Plasmodium* transcripts are considerably longer than required for coding purposes alone, and that this appears to be mainly a consequence of having exceptionally long 5' untranslated regions (Levitt, 1993; Chakrabarti *et al.*, 1993 and Delves *et al.*, 1990). The function of these long 5'UTR remains to be determined and the structure of ribosome binding sites in these organisms is very unclear.

A Northern blot of total RNA prepared from different developmental stages of *P. falciparum* probed with clone C4.1 hybridised to a diffuse band of approximately 1.5-1.6 kb. Transcripts produced from sexual and asexual stage parasites appear to be similar in size. Thus, it is likely that clone C4.1 represents a full-length (or nearly full-length) cDNA of the mature *pfran* transcript. Signals were strongest on the schizont and trophozoite lanes indicating progressively accumulating mRNA as the parasite prepares for and then undergoes active nuclear division. Very low levels were observed in early asexual stages and in gametocytes. It cannot be excluded that these low signals may result from contamination of rings or gametocytes preparations with

small amounts of trophozoites or schizonts. Similar amounts of RNA were present in all lanes, as indicated by ethidium bromide staining of the gel prior to transfer.

The results indicate that the steady-state *pfran* mRNA levels are developmentally regulated, in that mRNA levels are high during schizogony and barely detectable in the gametocyte stages. In *S. cerevisiae,* the Gsp/SRM-1 complex (homologous to the mammalian Ran/RCC1 complex) is involved in mating type differentiation (Clark & Sprague 1989), a process which requires cell cycle arrest at the phase preceding DNA synthesis (the G1/S boundary). In mammalian systems expression of a mutant Ran/TC4 allele interferes with DNA synthesis and therefore disrupts cell cycle progression (Ren *et al.*, 1993). It is tempting to speculate that low levels of *pfran* mRNA in gametocytes may play a role in cell cycle arrest of these differentiating cells. Testing this hypothesis will require information on pfRan protein levels and activation state throughout the parasite life cycle. An antibody reagent would also permit temporal and spatial localisation of the protein within the parasitised cell and a search for associated proteins such as a homologue of the RCC1 protein.

CHAPTER 4

FURTHER CHARACTERISATION OF *Pfran*.

4.1 Introduction

The identification and initial characterisation of the *P. falciparum pfran* gene has been described in Chapter 3. To characterise further the *pfran* gene three experiments were carried out. Firstly, preliminary cytological localisation studies on the Ran protein using antisera raised in rabbits were done. Secondly, in order to assign a role for the *pfran* gene in the cell cycle of *P. falciparum* functional complementation tests using the *Schizosaccharomyces pombe* temperature sensitive mutant were carried out. Finally, a series of experiments were carried out on nuclei isolated from synchronous cultures of the parasite to study the stage specific expression of the *pfran* gene.

4.2 Antibody characterisation of *Plasmodium falciparum pfran* gene product

4.2.1 Introduction

Antibodies were raised to the non-conserved (variable) region of *pfran* to avoid potential cross-reactions with host GTPases. A DNA fragment was first subcloned into the plasmid expression vector pGEX and the expressed polypeptide was then purified and used to immunise rabbits. The resulting sera were then tested by Western blotting, immunoprecipitation and immunofluorescence assay (IFA).

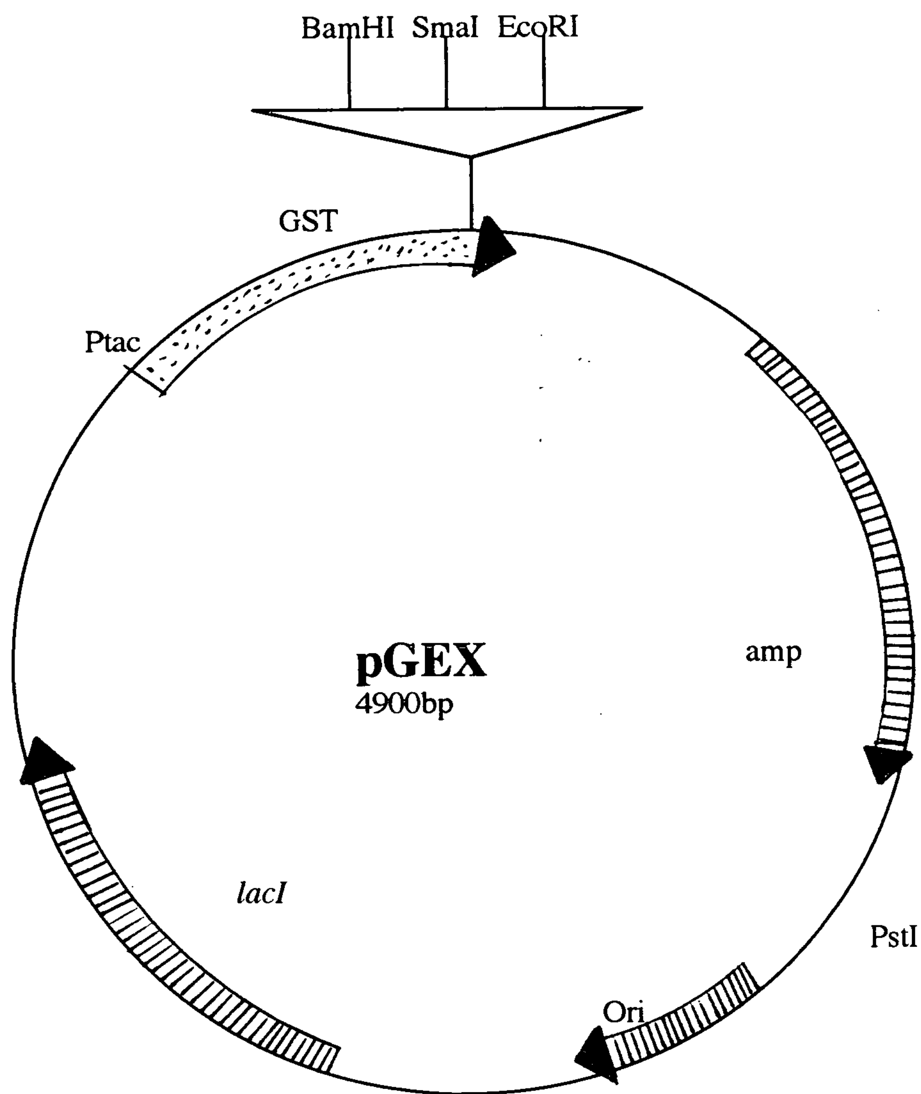
4.2.2 pGEX expression vector

The structure of the pGEX plasmid expression vector is shown in Figure (4.1). pGEX is a plasmid that allows expression of foreign polypeptides as fusions to the C-terminus of glutathione-*S*-transferase (GST), a 26kDa cytoplasmic enzyme that is capable of detoxifying a variety of xenobiotics by the conjugation of reduced glutathione to electrophilic centres in such molecules (Mannervik, 1985; Smith and Johnson, 1988). The plasmid carries a *$lacI^q$* allele which produces high levels of the *lac* repressor (10x more than the wild-type *lacI*). The *lac* repressor binds to the P_{tac} promoter, repressing the expression of the GST fusion protein. This allows control over expression, since the synthesis of potentially toxic fusion proteins is minimised when the system is not induced. The P_{tac} promoter contains consensus sequences of -35 and -10 promoter regions and is a hybrid between the *trp* and *lac*UV5 promoters (Amann *et al.*, 1983). Expression of fusion protein can be induced by isopropyl β-D-thiogalactopyranoside (IPTG) which inactivates the *lac* repressor. Fusion proteins expressed in this system often remain soluble within bacteria and can be easily purified

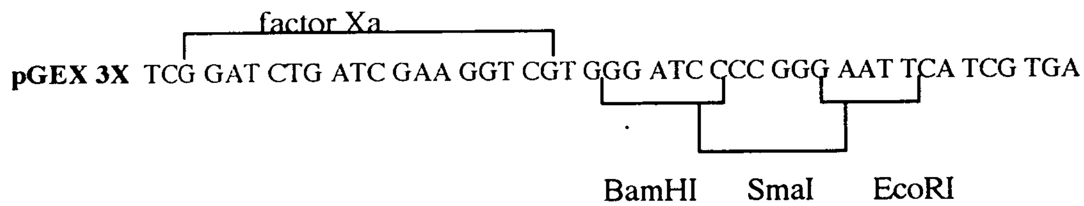
Figure 4.1 : The pGEX-3X expression vector map.

pGEX-3X is a fusion-protein expression vector that expresses a cloned gene as a fusion protein to glutathione S-transferase (GST). The *lac* repressor (product of the *lacI* gene) binds to the Ptac promotor, repressing expression of GST fusion protein. Upon induction with IPTG, derepression occurs and GST fusion protein is expressed.

The gene of interest can be inserted into the polylinker located at the end of the GST gene. The polylinker sequences are shown below the map of the vector where the restriction endonuclease cloning sites are bracketed. The polylinker of pGEX-3X contains protease (factor Xa) cleavage site so the cloned protein can be released from the GST carrier. Modified from Smith and Jonhson, (1988) *Gene* 67,, 31-40.



The pGEX poylinker sequences



under non-denaturing conditions because of the affinity of the GST moiety for glutathione.

DNA of interest can be inserted into a polylinker located at the 3' end of the GST gene. The DNA sequence of the polylinker of pGEX-3X encodes an amino acid sequence which contains protease cleavage sites so that the expressed fusion protein can be cleaved between the GST and the foreign moieties.

4.2.3 Strategies used for subcloning a fragment of *pfran* into pGEX vector

A strategy was devised to generate antibodies to a unique Ran-specific, non-conserved, region of the protein encoded by the *pfran* gene. For this, it was necessary to identify a region which was suitable for subcloning and expression in a pGEX vector, and against which antiserum could be raised. In deciding which unique region to subclone, it was thought unwise to generate antibodies to portions of conserved regions because it seemed possible that such antibodies would cross-react with mammalian Ran proteins. A *pfran*-specific fragment was therefore identified near the 3' end of the *pfran* gene. This region is not very conserved among the different Ran/TC4 genes.

The main determinant for successful purification of foreign polypeptides using the pGEX system is the solubility of the fusion protein. The fusion protein is likely to be insoluble if it is much larger than 50kDa or if the protein contains regions that are strongly hydrophobic or charged (Smith and Corcoran, 1990). Since the size of the GST moiety is 26kDa the size of the foreign polypeptide should be no more than 15-25kDa and thus encoded by an ORF of approximately 450-750bp.

A DNA fragment which would yield approximately the ideal size from GST fusion was identified in the *pfran* gene. The chosen construct is illustrated in (Fig 4.2). A 157bp fragment (nucleotides 1164-1321 of clone C4.1 which encodes the whole *pfran* gene Fig. 3.4, Chapter 3) from the *pfran* plasmid was amplified using oligonucleotide primers containing a *BamHI* restriction site for the forward primer and an *EcoRI* restriction site for the reverse primer. This fragment, which contained the *pfran* stop codon was ligated with pGEX-3X at the *BamHI/EcoRI* sites. Positive clones were identified by the use of these restriction enzymes to analyse the DNA prepared from putative clones and cloning junctions were confirmed further by sequencing.

Figure 4.2 : The sequence of the *pfran* construct used for cloning into pGEX-3 for the pfRan antibody production
Two oligonucleotides primers (primer G1701, forward and primer G1702, reverse) contain *BamHI* and *EcoRI* were used to amplify a 157bp fragment from the *pfran* gene. The amplified product was ligated into the *BamHI/EcoRI* site of the pGEX-3 expression vector and transformed with TG-1. The site of the primers are underlined.

5' G(A/C)(A/T)G(C/T)(A/T)GG(A/T)GG(A/T)ATGCA 3'

primer G6037

---------->

```
Human     QA | EAGGMH | TVCLSKSGQVY-------T | LGCGEQGQLGR | VPELFANRG
Hamster   QA | EAGGMH | TVCLNQSGQVY-------T | LGCGEQGQLGR | VPELFANRG
Xenopus   QA | AAGGMH | TVCLGASGSIY-------T | SGCGEQGQLGR | VPERFINRG
Droso.    DI | SAGGMH | NLVLTKSGDIY-------T | VGCAEQGQLGR | LSERSISGE
S.cerv.   SF | AVGGMH | TLALDEESNVW-------A | WGNGQQNQLGR | --KVMRFRL
S.pombe   DL | AVGGMH | SAALLHDGRVY-------T | WGNGQQFQLGR | --RMLERRR
           |                                                          |
          56                                                        201
```

<-------------------

primer G5146

3' ACCCC(T/A)TT(A/G)CC(T/A)GT(T/C)GT(T/C)(T/A)(T/A)AGT(T/C)(G/A)A(T/A)CC(T/A)GC 5'

4.2.4 Results

4.2.4.1 Expression and Purification of GST fusion proteins

The pfRan construct was transformed into *E. coli* strain TG1 and induced with IPTG. The proteins extracts from the bacterial cells were separated on an SDS-PAGE gel (Fig. 4.3) As shown in the figure, upon induction with IPTG the two clones, pGEX-4 and pGEX-7, were found to express a GST fusion protein of the expected size (33 kDa).

The GST system has been designed so that soluble fusion proteins can be easily purified using glutathione attached to agarose beads (Pharmacia). In order to assess whether the expressed fusion protein was soluble, first small scale purification was carried out on construct pGEX-4 and pGEX-7 as described in method 2.2.22.4.2. The results shown in figure (4.4), indicated that at least some of the fusion protein was soluble. Large scale purification was next carried out, as described in chapter 2 section 2.2.22.2, from clone pGEX-7 to obtain sufficient fusion protein to inject into rabbits for production of antiserum. The protein was purified on S-linked glutathione-agarose beads.

4.2.4.2 Production of antiserum to fusion protein

In total two 5 month old male New Zealand rabbits were immunised. One rabbit was immunised with GST fusion protein expressing the *pfran* fragment (construct pGEX-7). The other rabbit was immunised with the GST protein only as a control, since the GST moiety was not separated from the expressed fusion protein.

The rabbits were bled before the injection of the foreign protein. Approximately 500μg of the soluble fusion protein in 1ml of complete Freund's adjuvant were used per immunisation for each rabbit. The rabbits were boosted at three and six weeks after the initial immunisation with the protein in incomplete adjuvant.

4.2.4.2 Western blotting analysis

To identify which proteins were recognised by the immune sera, and to determine if the *pfran* construct was expressed, Western blots prepared from total parasite extract were incubated with antisera from the above experiments as described in method 2.2.22.6.

Initially, the anti-pfran sera used to probe a Western blot prepared from the total protein extracts prepared from the 3D7A parasite strain were separated by SDS-PAGE under reducing conditions and electrophoretically transferred onto

Figure 4.3 :

Coomassie-stained SDS-PAGE pattern of *E. coli* protein extracts. Proteins from an approximately equal number of cells were electrophoresed on each gel (SDS-PAGE) lane.

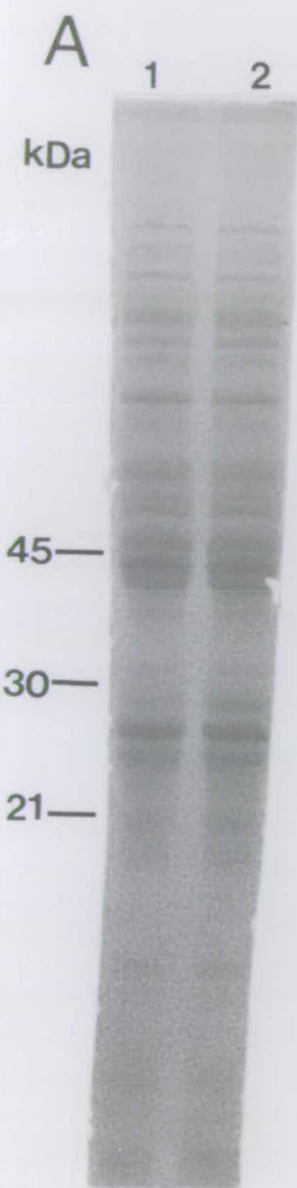
A.

Lanes 1 and 2 of panel (A) are uninduced protein extracts from *E. coli* containing the plasmid pGEX-3 harbouring the *pfran* insert, insert No.4 and insert No.7 respectively,

B.

Lanes 1 and 2 of panel (B) are protein extracts from *E. coli* containing the plasmid pGEX-3 with the *pfran* insert, (insert No. 4 and No. 7), both induced with IPTG (final concentration 0.1mM). Lane 3 and 4 of panel (B) are protein extracts from two *E. coli* colonies containing the plasmid pGEX-3 without the *pfran* insert induced with IPTG.

The heavily staining band in the IPTG-induced tracks (double arrow), which is larger than glutathione-*S*- transferase (single arrow), is the fusion protein.
The molecular weights of size standards are indicated in kDa.


A
1
2
kDa
45
30
21

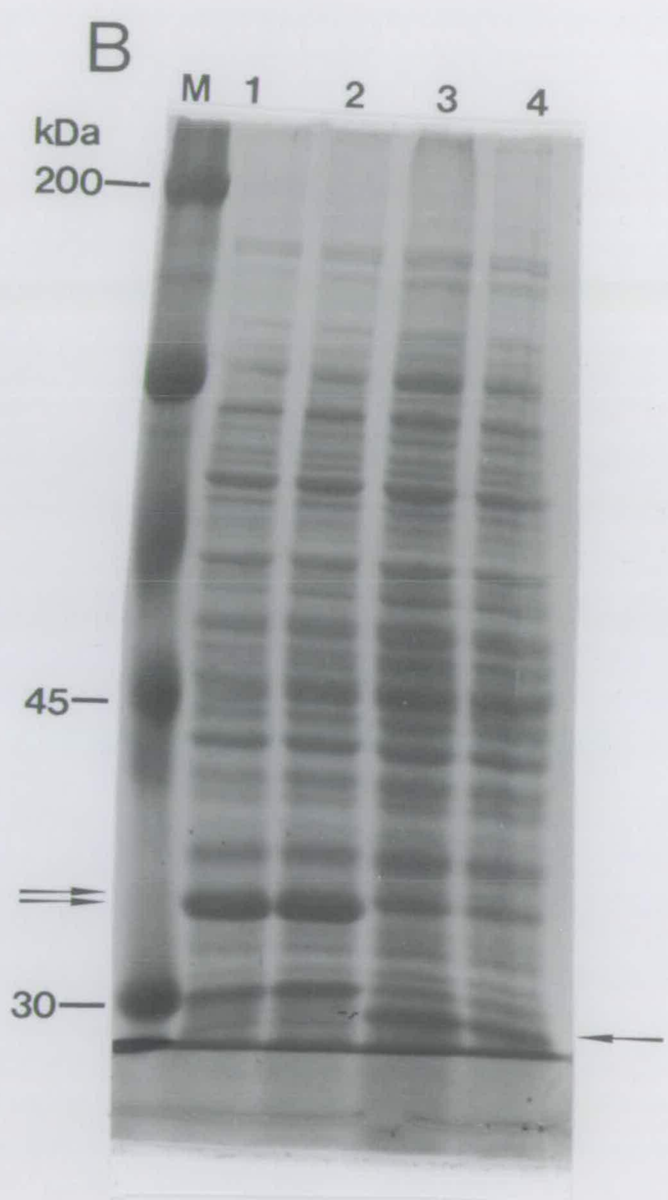

B
M
1
2
3
4
kDa
200
45
30

Figure 4.4 :

Purification of clone No. 7 which expressed pfRan fusion protein and the GST moiety protein from the plasmid pGEX-3 without the construct. The purified proteins were used for immunisation of rabbits for production of pfRan antibodies.

Protein extracts from the above two clones were first sonicated and treated with mild detergent (Triton X-100) and then pelleted. The pellet was kept for analysis and the supernatant was purified on S-linked glutathione agarose beads and eluted in reduced glutathione. For description of the purification technique used see method 2.2. 22.4.2.

Lane 1. Protein product of clone no.7 purified by agarose beads and eluted in reduced glutathione.

Lane 2. Protein product of the pGEX-3 (without an insert) plasmid purified by agarose beads and eluted in reduced glutathione.

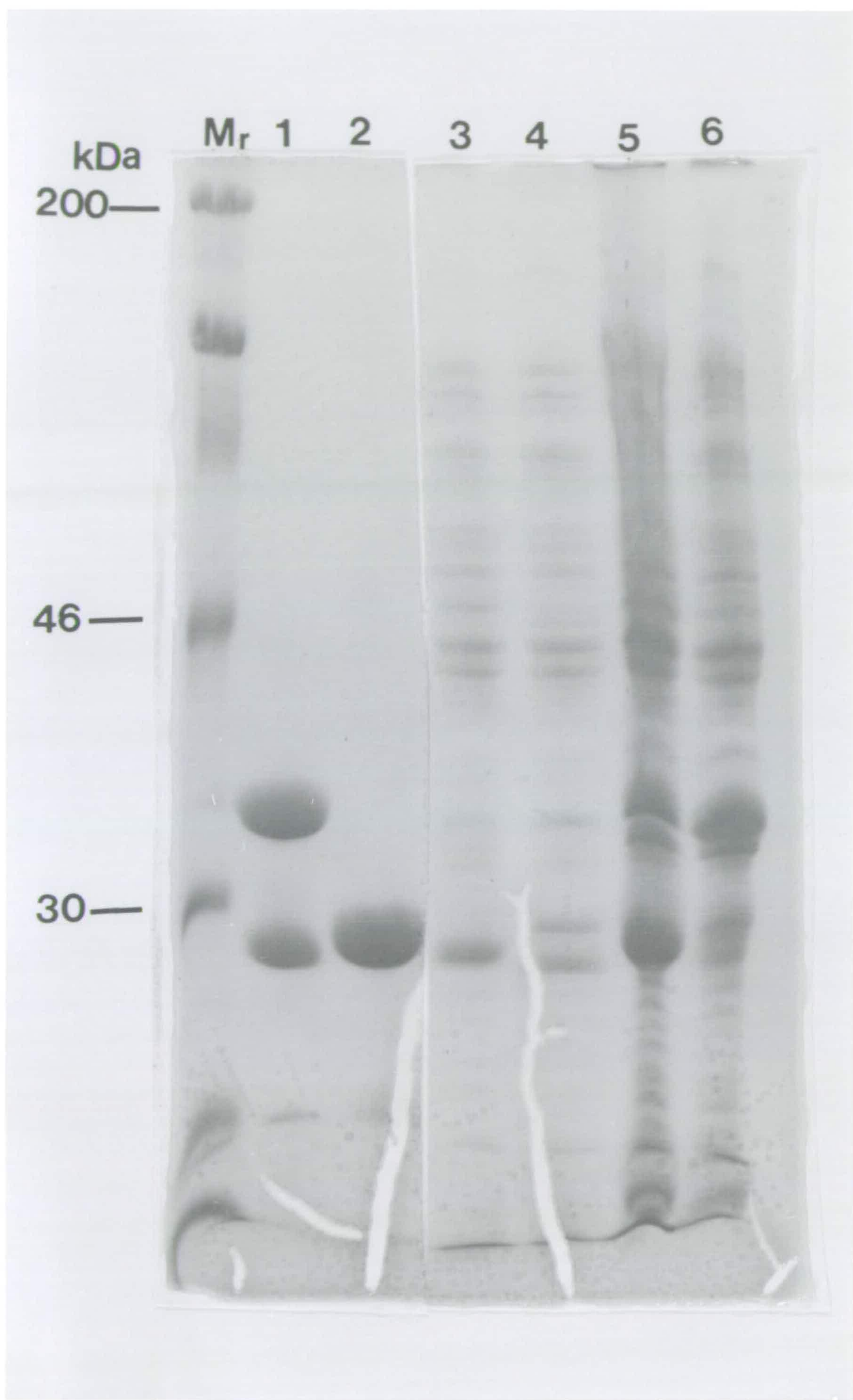
Lane 3. The agarose beads after elution of the protein product of clone 7 in reduced glutathione.

Lane 4. The agarose beads after elution of the pGEX-3 protein in reduced glutathione.

Lane 5. the pellet kept after the detergent treatment of the pGEX-3 protein product.

Lane 6. the pellet kept after the detergent treatment of the protein product of clone 7.

N.B. the molecular weight (kDa) of size standards are indicated.



kDa
Mr
1
2
3
4
5
6
200
46
30

Figure 4.5 : Western blot analysis of pfRan srea.

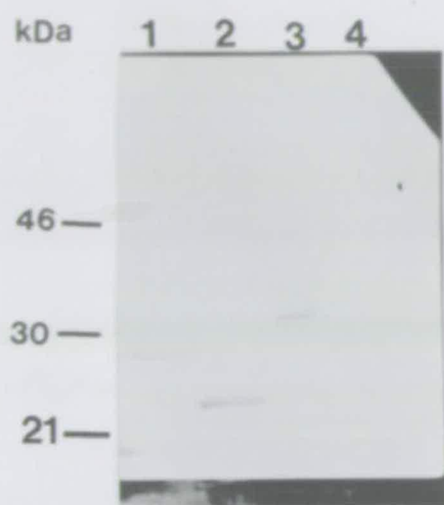
A. Extracts from the original protein from clone no.7 and the protein containing the GST moiety were used to immunise rabbits for production of antibodies against pfRan. Total protein extracts from *P. falciparum* cultures and from uninfected red blood cells were separated on SDS-PAGE and electrophoretically transferred onto nitocellulose filters. The blot was probed with sera from the hyper-immune rabbit injected with protein from clone no. 7, containing the *pfran* insert.

Lane 1. total parasite protein from *P. falciparum* 3D7 clone.
Lane 2. the protein product of GST fragment.
Lane 3. protein product of clone 7.
Lane 4. protein extract from uninfected RBCs.

B. Total protein extracts prepared from asexual and sexual 3D7 parasite were separated on SDS-PAGE and electrophoretically transferred onto nitocellulose filters. Strips of the blot were incubated with anti-pfRan and anti-PCNA sera.

Lane 1. asexual parasite protein extracts incubated with anti-pfRan antisera.
Lane 2. gametocyte protein incubated with anti-pfRan antisera.
Lane 3. asexual parasite protein extracts incubated with anti-PCNA antiserum.
Molecular weights of protein standards are indicated.

a



b

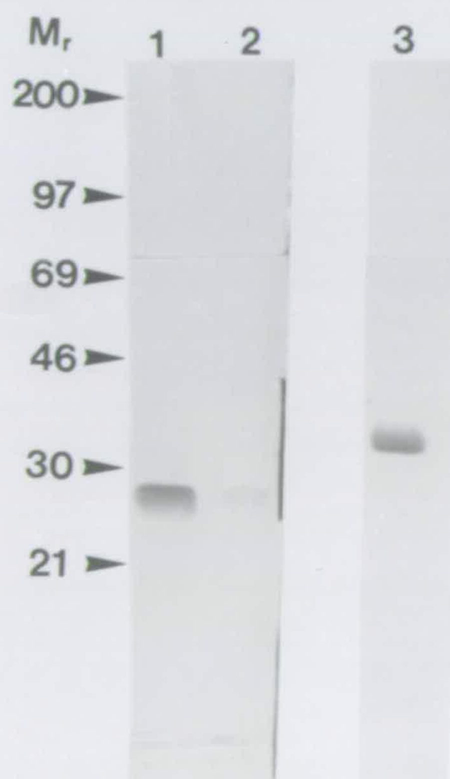
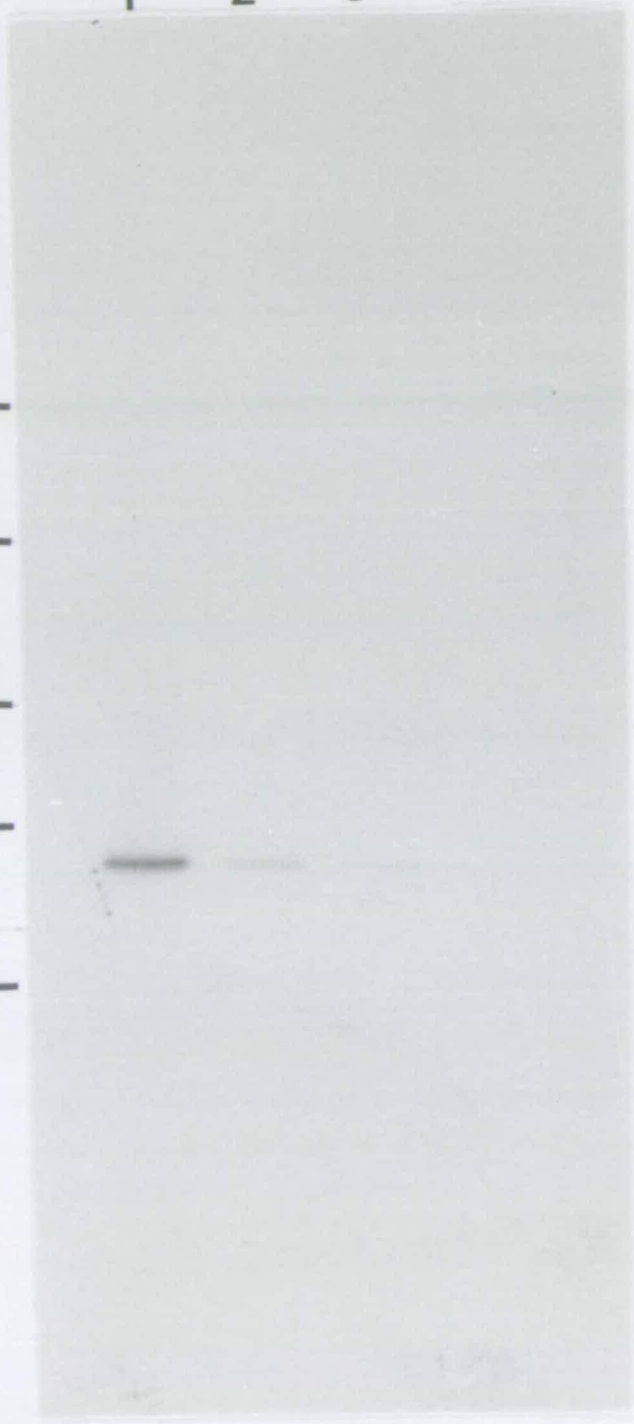


Figure 4.6: Immunoprecipitation of *P. falciparum* total protein extract.

^{125}I radiolabelled total parasite (clone 3D7) protein extracts precipitated with pfRan antibodies. Different dilutions of the sera were used : lane 1, 1:50; lane 2, 1:100 and lane 3 1:500 dilutions. total proteins from uninfected RBCs were also used (lane 4).


kDa
1
2
3
4
97
67
46
30
21

nitrocellulose filters (method 2.2.22.6). Strips of these blots were probed with the pre-immune as well as the immune sera. The immune sera recognise a band of 34 kDa in size in the original protein extracts used to immunise the rabbit, which corresponds to the expected size of the fusion protein (Fig.4.5A, lane 3). When the rabbit hyper-immune serum was incubated with total parasite proteins extract a band of 27 kDa in size was observed (Fig. 4.5A , lane 1). Sera obtained by immunisation of a rabbit with the GST moiety only recognised a band of 25 kDa (Fig.4.5A, lane 2) The pre-immune sera did not recognise any *Plasmodium* proteins (data not shown).

When protein from synchronous culture transferred to nitrocelluose membrane and and incubated with antiserum raised to the pfRan-construct, one major band of 27 kDa predominantly in the asexual stage extracts (lane 1, Fig. 4.5B) was seen, although a very weak band in the protein extracts from gametocytes can be seen also of the same size (lane 2, Fig.4.5B). Anti-PCNA serum (Kilbey *et al.*, 1993) was used as a positive control in order to distinguish any bands that were due to the pGEX expression system and the immunisation protocol used. The PCNA serum recognised a band migrating at 32 kDa in the asexual extract only (lane 3, Fig. 4.5B). These results indicated that pfRan is present among the parasite proteins, predominantly in the asexual stages.

4.2.4.3 Immunoprecipitation

Iodinated parasite proteins were precipitated with the pfRan polyclonal serum (method 2.2.22.7), and were then separated on SDS-PAGE under non-reducing conditions. Different dilutions of the serum (1:50, 1:100, and 1:500 dilutions) were used to precipitate the parasite proteins. Proteins extracted from uninfected RBCs were precipitated with the pfRan sera also as a control. After electrophoresis the gel was air-dried and exposed to X-ray film stored in a -70°C freezer. The film was developed after two weeks. Results are shown in Fig. 4.6. The serum recognised a 27 kDa protein band even at the lowest concentration of the serum used (Fig. 4.6, lane 3). No signals were seen with the control.

4.2.4.4 Immunolocalisation of pfRan

To attempt to localise the pfRan protein in the parasitised red blood cell and to determine further when it appears during the intra-erythrocytic cycle, immunofluorescence assays (IFA) were performed on acetone fixed thin smears of synchronous and asynchronous *P. falciparum* strain 3D7A infected RBCs as described in method 2.2.22.8.

The parasite nuclei were counter-stained with DAPI (at 1:40 dilution). The rabbit polyclonal immune serum made against the pfRan construct was used at a dilution of 1:100 and 1:200 (method 2.2.22.8). Antibodies bound to the parasite preparation were detected using fluorescein conjugated anti-rabbit IgG antibodies, which give a green fluorescence under U/V illumination. Results are shown in Fig 4.8.

A series of IFA experiments were carried out which consistently gave the same ffluorescence patterns. Appropriate control experiments, using the (9.8) merozoite-specific monoclonal antibody (a gift from Dr. J. McBride) and 12.F.10, gametocyte-specific monoclonal antibody (provided by Dr. R. Carter), were performed. No staining of infected or non-infected red blood cells was observed when the pre-immune serum was used (Fig.4.7) or when the fluorescin-conjugated antibody was used alone.

When immune serum was used, parasites were found to be stained with fluorescein. The fluorecein cells were also positive for DAPI stain, indicating that antibodies recognised parasitised cells, and showed the parasitic origin of the protein recognised by the rabbit serum. A diffuse pattern of staining of the ring, trophozoite and early schizont of the asexual forms was seen (Fig. 4.8 and 4.9). The sexual gametocytic forms also gave a diffuse pattern of staining although the pattern of fluorescence appeared to be localised in the intracellular parasite (Fig. 4.10) . In the late schizont or segmenter stage of the parasite, the fluorescence appeared to be localised in the nuclei and the gaps between the developing merozoites (Fig 4.9).

4.2.5 Discussion

From the deduced pfRan open reading frame (Chapter 3), the predicted size of the expressed protein is likely to be around 25 kDa. Using Western blot analysis and immunoprecipitation, the rabbit antiserum raised against pfRan recognised a parasite protein approximately of 27 kDa in size. Western blot analysis also showed that the pfRan protein was mainly present in the asexual stage of the parasite which was consistent with the Northern blot analysis.

The IFA indicated that pfRan is expressed mainly in the asexual parasite with some evidence of its presence in gametocytes. In addition, the IFA results indicated that pfRan is diffusely distributed in the parasitised cell localised both in the nuclei and the cytoplasm of the cell. Ideally these results need to be confirmed by ultra- structural studies using immuno-gold localisation, in order not to over-interpret them. However, some interpretations of these findings will be offered in the general discussion section.

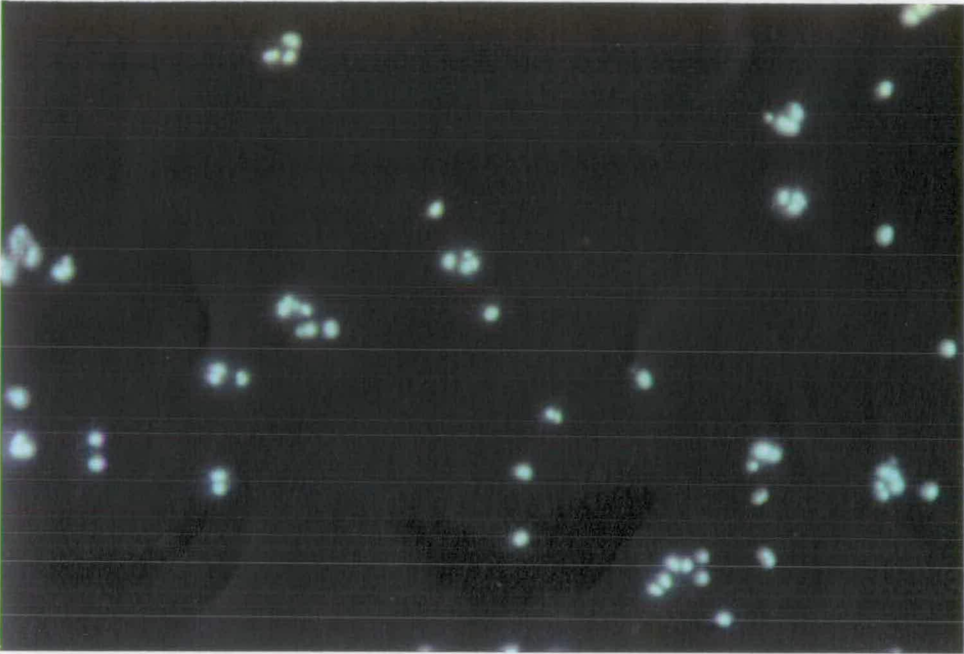
Figure 4.7 :

Pattern of indirect fluorescence assay (IFA) staining produced by rabbit pre-immune sera on blood films of *P. falciparum* asynchronous cultures (clone 3D7). Acetone fixed smears of *P. falciparum* infected RBCs were incubated with the pre-immune sera, followed by FITC-conjugated goat anti-rabbit IgG second antibodies. Parasite nuclei were counter-stained with DAPI.

a) Infected cells analysed for presence of DAPI under U.V. illumination (x40).

b) The same infected cells were analysed for presence of fluorescein. No specific pattern of staining was seen.

a



b

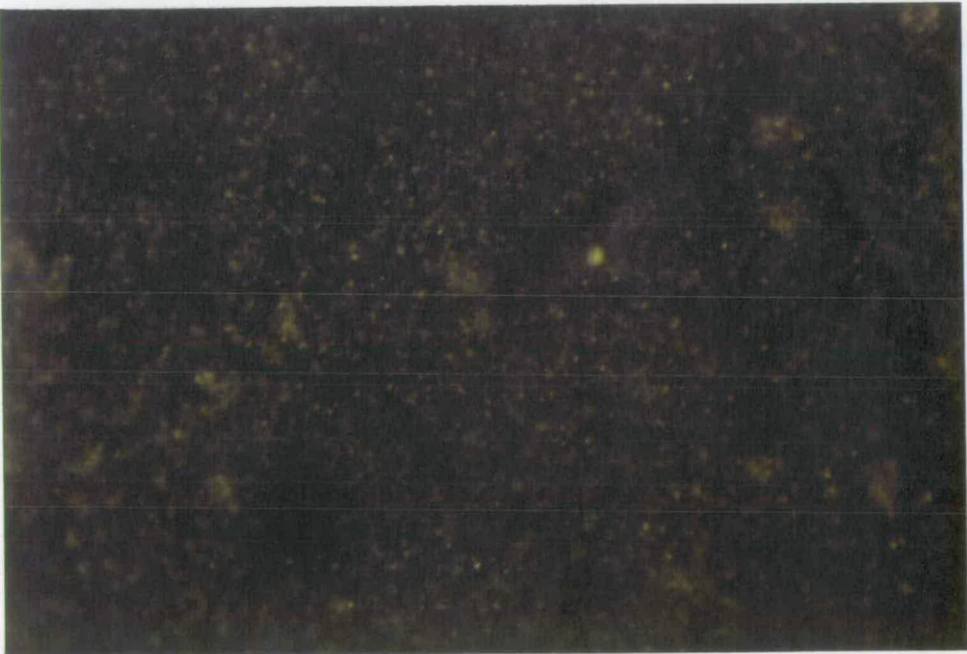
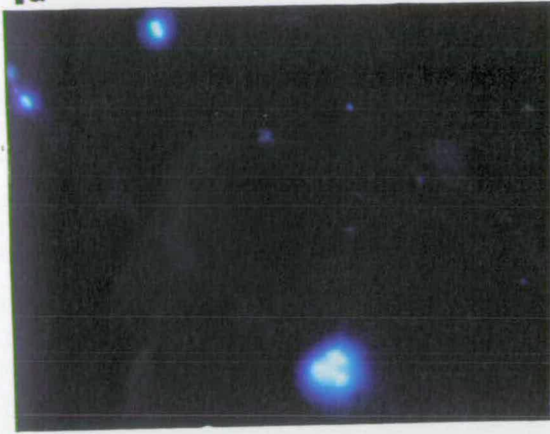


Figure 4.8 :

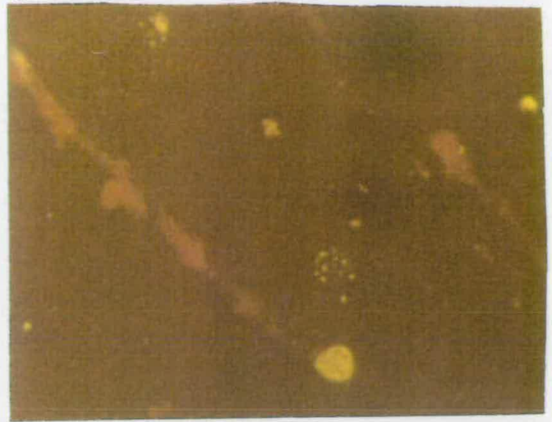
Indirect immunofluorescence assay (IFA) of 3D7 infected erythrocytes with anti-pfRan fusion protein. The slide containing acetone-fixed smears prepared from asynchronous cultures. The cells were fixed with acetone and reacted with a rabbit anti-pfRan fusion protein (dilution 1/100) followed by FITC-conjugated goat anti-rabbit IgG. Parasite nuclei were counter-stained with DAPI. The same infected cell was analysed for presence of DAPI (Panel 1a-3a) and fluorescein (1b-3b).

Panel 1b, 2b, and 3b are fluorescent micrographs of pfRan antibody reacted with rings (R), trophozoites (T) and schizonts (S).

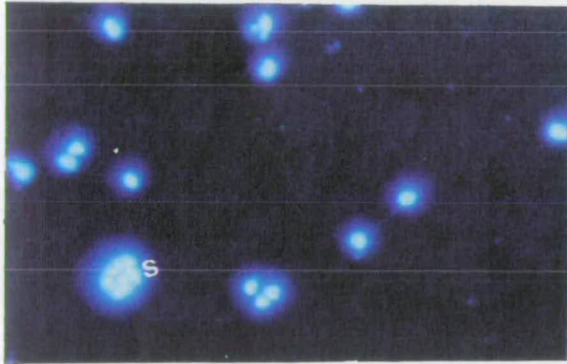
1a



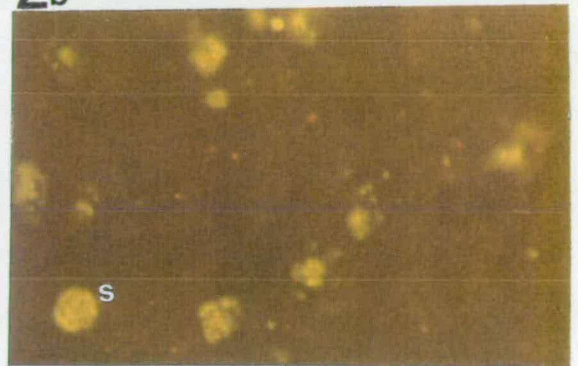
1b



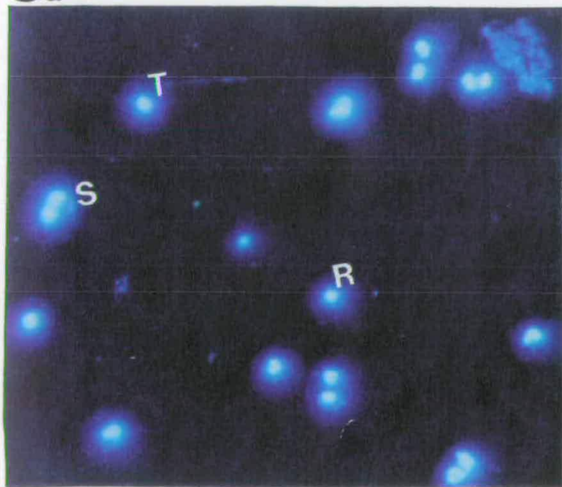
2a



2b



3a



3b

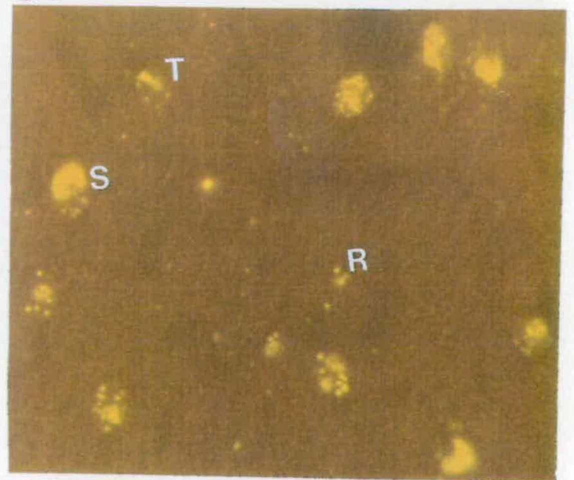
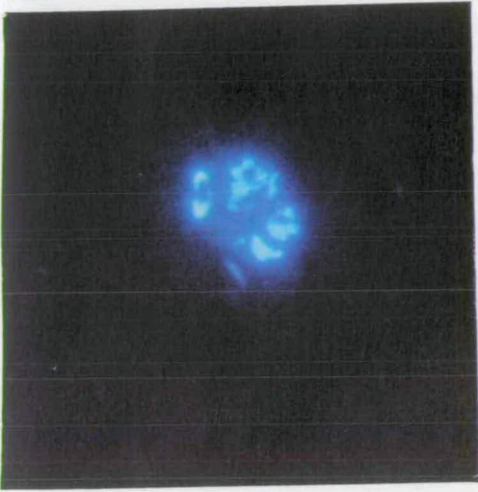


Figure 4. 9:

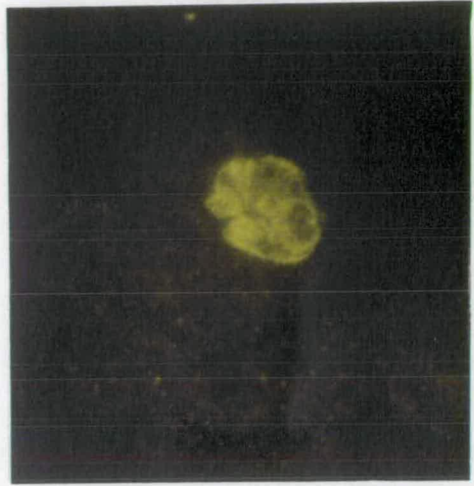
Indirect immunofluorescence assay (IFA) of 3D7 infected erythrocytes with anti-pfRan fusion protein. The slide contains acetone-fixed smears prepared from synchronised 3D7 cultures, as described in materials and methods section. The cells were fixed with acetone and reacted with a rabbit anti-pfRan fusion protein (dilution 1/100) followed by FITC-conjugated goat anti-rabbit IgG. Parasite nuclei were counter-stained with DAPI. The same infected cell was analysed for presence of DAPI (Panel 1a and 2a) and fluorescein (1b and 2b).

Panel 1b, and 2b are fluorescent micrographs of pfRan antibody reacted with trophozoites and schizonts respectively.

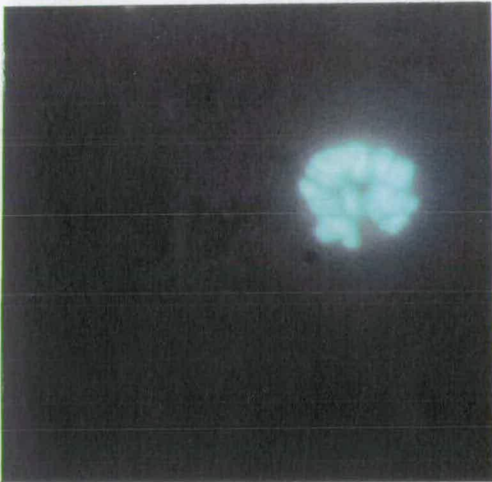
1a



1b



2a



2b

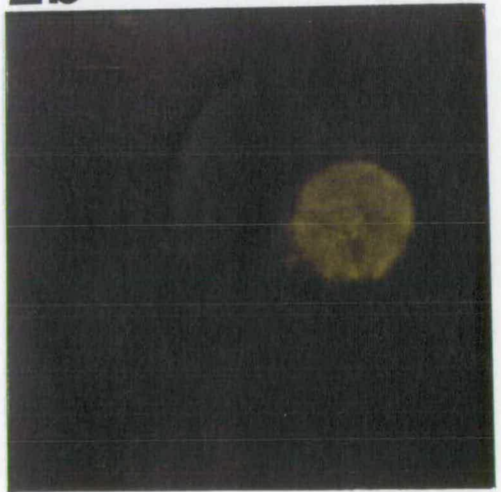


Figure 4.10 :

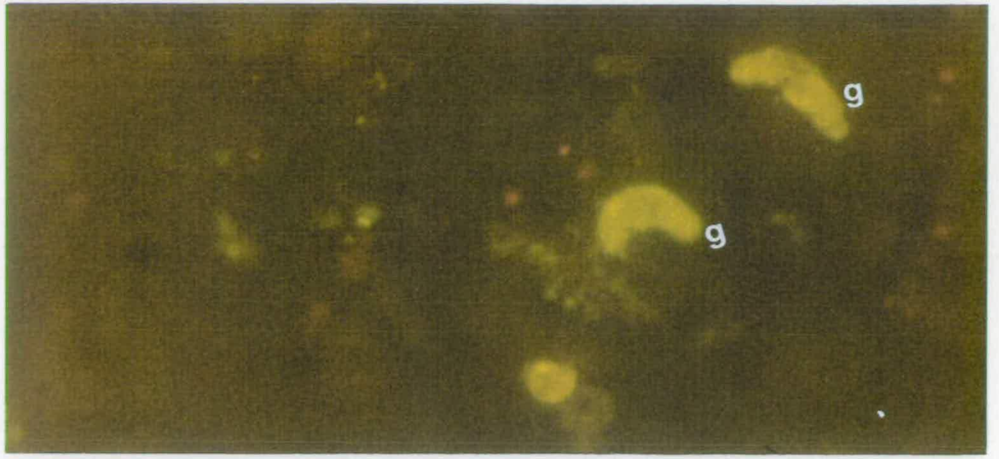
Indirect immunofluorescence assay (IFA) of 3D7 infected erythrocytes with anti-*pfran* fusion protein. The slides containing acetone-fixed smears were prepared from synchronised 3D7 gametocyte cultures. The cells were fixed with acetone and reacted with a rabbit anti-pfran fusion protein (dilution 1/100) followed by FITC-conjugated goat anti-rabbit IgG. Parasite nuclei were counter-stained with DAPI. The same infected cells were analysed for the presence of DAPI (not shown) and fluorescein (1a and 2 a and b).

Panel (1) shows a fluorescent micrograph of pfRan antibody reacted with gametocytes (g).

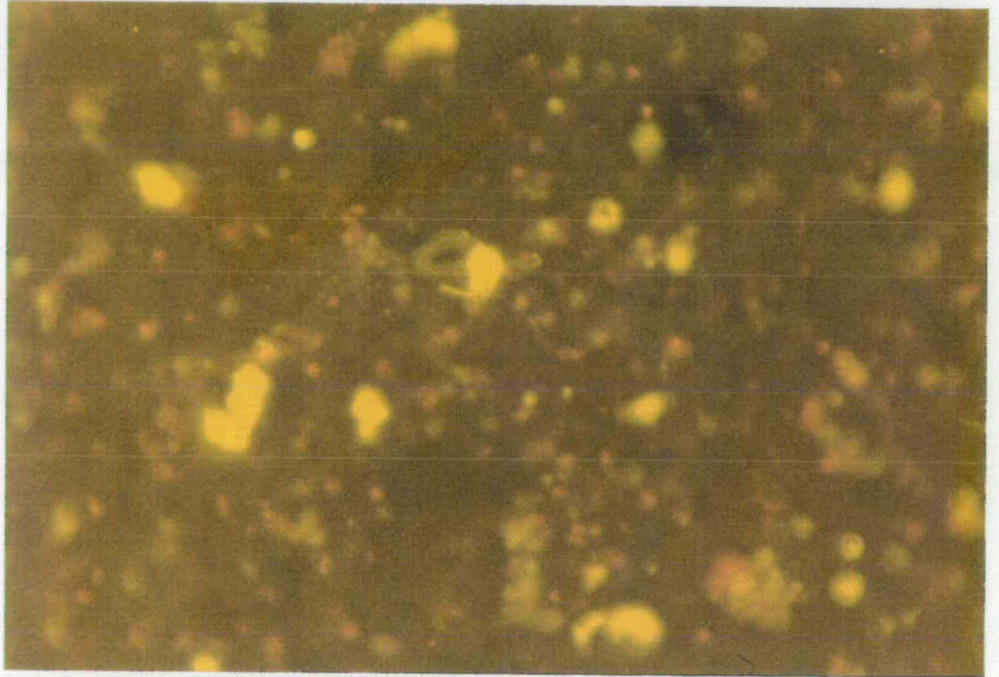
Panel (2a) shows a fluorescent micrograph of pfRan antibody reacted with gametocytes and gametes.

Panel (2b) is the same micrograph as (2a) but with x100 magnification to illustrate the morphology of the male gamete (indicated with arrows).

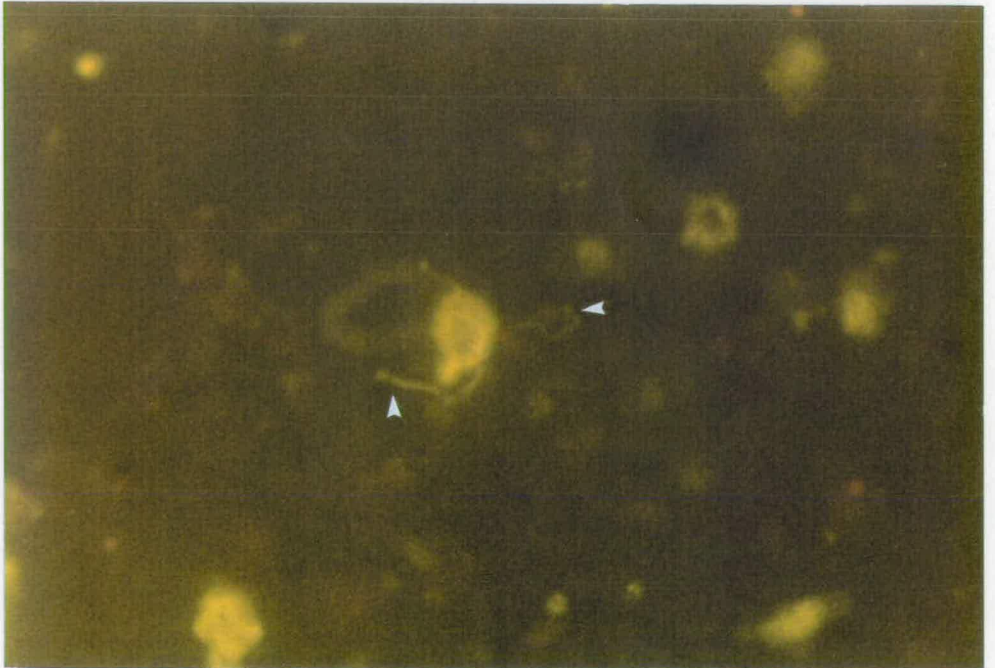
1



2a



2b



4.3 Test for functional complementation of *Schizosaccharomyces pombe pim1* mutant.

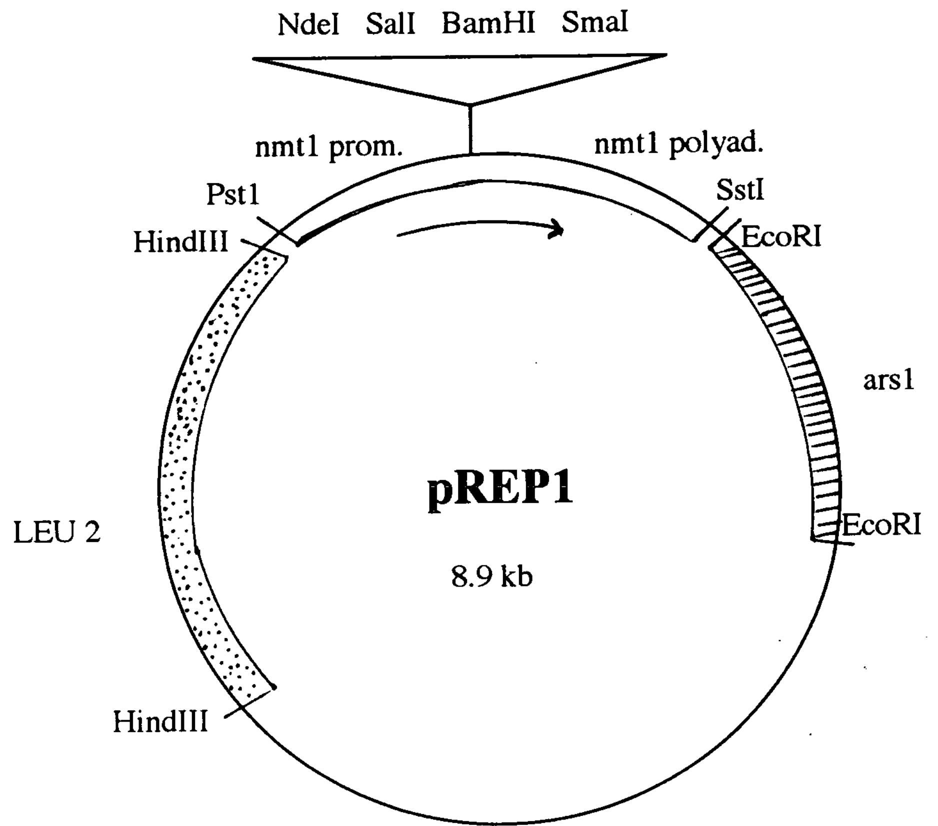
4.3.1 Introduction

A fission yeast mutant *pim1*(premature initiation of mitosis) has been isolated by Matsumoto and Beach (1991) in which the onset of mitosis is uncoupled from the completion of DNA replication. *Pim1* cells can undergo mitotic chromosome condensation and mitotic spindle formation without completion of S-phase. Overexpression of the wild-type allele of a second gene, *spi1*(suppressor of *pim*) whose predicted protein product shares 81% identity with human Ran/TC4, has been found to suppress the *pim1* mutant phenotype (Matsumoto and Beach, 1991). It was therefore considered that it might be possible to test the function of *P. falciparum pfran* gene by assessing whether it can complement the pim genetic defect in the same way that the *spi1* mutants do.

4.3.2 Strategy used for the yeast complementation test

To test for complementation, a PCR fragment containing the whole coding region of *pfran* gene was obtained by amplification of the *pfran* plasmid (clone C4.1) which encodes the complete *Pfran* gene. Primer G5793 containing *NdeI* (forward) and primer G579 containing *BamHI* (reverse) were used for amplification of the *pfran* construct as described in method 2.2.16. The PCR product was cloned into the *NdeI/BamHI* site of a *Schizosaccharomyces pombe* thiamine-repressible pREP1 expression vector, Figure 4.11, (Maundrell, 1993), and the resulting plasmid was used to transform the temperature sensitive mutant of *S. pombe pim1*1-46, (Matsumoto and Beach 1991).

The cells were transformed by electroporation, as described in method 2.2.23.3, and then selected on minimal medium plates containing thymine by incubation at 28°C for a period of 4-6 days until single isolated colonies were seen. The colonies were replicated in duplicate on minimal medium plates and grown at 28°C, and after 24 hours, one set of plates was shifted to 36°C to test for rescue of the *pim1* phenotype. Vector pREP1 and plasmid containing the *S. pombe pim* gene (Matsumoto and Beach 1991) were used as negative and positive controls respectively. The final construct was sequenced on both strands to ensure that the correct reading frame was present in the construct and that no mutations had occurred during manipulation.



Sequence of plylinker

C A T A T G T C G A C T C T A G A G G A T C C C C G G G

NdeI SalI XbaI BamHI SmaI

Figure 4.11: pREP-1 *S. pombe* expression vector.

The thiamine-repressible expression vector of *S. pombe* used for complementation test. The nmt-1promotor and polyadenalation signal have been indicated. This plasmid permit thiamine-mediated control of transcription to be applied to cloned genes. Modified from Maundrell, 1993, Gene 123,127-130.

4.3.3 Results and discussion

In an attempt to assign a role for the *pfran* gene in the control of the malaria parasite life cycle, tests for complementation of *Schizosaccharomyces pombe* temperature sensitive *pim1* mutant were carried out. Although the *pfran* fragment was successfully cloned into the yeast expression vector pREP1, the *pfran* construct was not expressed in yeast mutant, and this prevented testing for possible functional complementation of appropriate mutant.

Rescue of temperature sensitive *pim1* as described above failed at both at 33°C and 36°C. However, Western blot analysis of lysates from both transformed and untransformed yeasts probed with anti-pfRan serum showed that the protein was not expressed at a detectable level in any of the transformants, even though a protein band of 27 kDa was clearly visible in the control lysate from *P. falciparum* (data not shown). Thus the failure of complementation may have been due to lack of expression.

This result does not of course rule out a regulatory role for *pfran* in the cell cycle of *P. falciparum* since cross-species complementation tests may be inappropriate for organisms which are phylogenetically distant. Functional complememtation using yeast mutants have been carried out for the *cdc2*-homologue of *P. falciparum* and *L. mexicana* but without any reported success (Mottram *et al.*, 1993; Ross-MacDonald *et al.*, 1994).

4.4 Transcription of *pfran* in *Plasmodium falciparum* RNA isolated nuclei

4.4.1 Introduction

The technique of nuclear run-on is a sensitive *in vitro* method for studying the mechanism of transcription of specific genes and the processing of primary transcripts in eukaryotic cells. This approach starts with isolated nuclei and uses endogenous chromatin as the template for transcription. The advantage of this method is that, the activity measured reflects the activity of that nucleus in the cell, that is, the same genes are being expressed in the same relative amounts in the isolated nucleus as in the intact cell.

The level of expression of *pfran* RNA during the intra-erythocytic stages was examined by Northern blot analysis and was found to be expressed in a stage specific manner as described in Chapter 3. To test whether this was due to transcriptional activity nuclear run-on experiments were carried out.

4.5.2 Strategy for transcriptional analysis

The following genes used in this experiment: *pfran* gene (Sultan *et al.*, 1994), *pfrcc1* (this study, see Chapter 5), *msp-1* gene encoding the major merozoite surface antigen, (Holder *et al.*, 1985), *Pfg25/27* and *Pfs230* genes expressed during the gametocyte stage (Alano *et al.*, 1991; Williamson *et al.*, 1993), and *CS* gene encoding the circumsporozoite antigen expressed during the insect and hepatocytic stages (Enea *et al.*, 1984). Since most of the genes used in this experiment were cloned either into plasmid or phage vectors, a PCR amplifiction was performed using specific primers against these genes. The PCR products were purified (method 2.2.3) before aliquots were spotted on Hybond-N+ membrane using the dot-blot apparatus (method 2.2.10) For comparison other genes of known stage specific expression were used. These were Pfcrk-1 (Doerig *et al.*, in preparation), β tubulin (Delves *et al.*, 1990), Topoisomerase II (Cheesman *et al.*, 1994), PCNA (Kilbey *et al.*, 1993), Polymerase α (Ridley *et al.*, 1991) and Polymerase δ (White *et al.*, 1993).

Nuclei were isolated from synchronised cultures of (clone 3D7) at different stages during the life cycle as described in method 2.2.21. Transcription was performed using labelled ^{32}P-UTP, and the labelled nascent RNA was used as a probe for DNA fragments specific for the above mentioned genes. After hybridisation was completed the blots were exposed to X-ray film for variable periods of time.

4.4.3 Results and discussion

The results are shown in figure 4.12. It was observed that the *pfran* and *pfrcc1* were mainly transcribed during the schizont stage (Fig.4.12A lanes 1 and 2 respectively). No transcriptional signals were seen in gametocyte (Fig.4.12B lane 1 and 2). Nuclear run-on for nuclei isolated from ring and trophozoite stages gave very faint signals even with longer exposures (data not shown). Signals were observed for the genomic DNA, *msp-1* during schizont stage, and *Pfg25/27, Pfs230* during gametocytic stages. No signals were seen in the negative control lanes (pUC18, λ phage DNA and the TE buffer) and for the CS gene; figure 4.12. No signals were observed during the schizont stage for Pol α, Pol δ, and PCNA genes, although RNA studies have shown that these genes are expressed during the asexual stages. However, with longer exposure weak signals were seen (data not shown). The experiment on these genes is not conclusive and needs to be optimised further.

The experiment is difficult to optimise because of the technical difficulty in obtaining reasonable amounts of intact nuclei from synchronous cultures of the parasite. However, the results indicate that both *pfran* and *pfrcc1* are transcribed just before and during the S-phase of the *P. falciparum* cell cycle in accordance with the observations made on the presence of the proteins themselves.

Figure 4.12 :

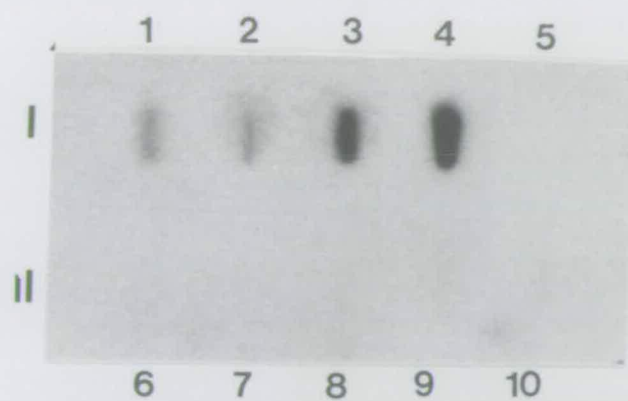
Autoradiographs of nuclear run-on experiments showing stage specific transcriptional activity of *pfran* gene. Nuclei were isolated from schizont (panel A) and gametocyte (panel B) parasite stages. Nascent, ^{32}P dUTP labelled RNA was used to probe equal concentration of DNA fragments containing *pfran*, *pfrcc1*, *msp-1*, 3D7 genomic DNA, *pfg27/25, pfs230,* and pf-circumsporozoite genes. DNA from pUC18 and λphage and TE buffer were used as internal controls.

A. Nuclei isolated from schizont stage of synchronous culture of clone 3D7

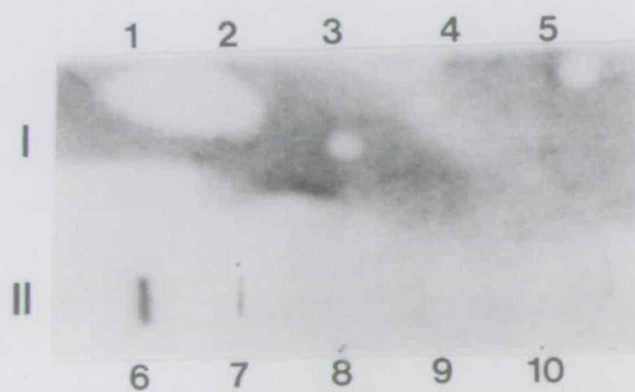
1. pfran.
2. pfrcc1.
3. msp-1.
4. *P. falciparum* genomic DNA.
5. TE buffer.
6. pfg27/25.
7. pfs230.
8. circumsporozoite gene.
9. pUC 18 plasmid DNA
10. λ phage DNA.

B. Nuclei isolated fromthe gametocyte stages of a synchronous culture of clone 3D7.

1. pfran.
2. pfrcc1.
3. msp-1.
4. *P. falciparum* genomic DNA.
5. TE buffer.
6. pfg27/25.
7. pfs230.
8. circumsporozoite gene.
9. pUC 18 plasmid DNA.
10. λ phage DNA.



a



b

CHAPTER 5

CLONING AND CHARATERISATION OF THE *P. falciparum* RCC1 HOMOLOGUE.

5.1 Summary

In the previous chapter the molecular cloning and characterisation of the *pfran* gene, a member of the small GTPase superfamily (the *P. falciparum* homologue of Ran/TC4) was described. Ran/TC4 encodes a protein which has been shown in yeast and mammalian systems to interact with RCC1 (regulator of chromosome condensation), a protein proposed to be required for the normal coupling of completion of DNA synthesis with initiation of mitosis. Genetic and biochemical experiments suggest that Ran/TC4 and RCC1 are key components of a feedback-checkpoint control which monitor the progress of DNA replication throughout the eukaryotic cell cycle.

In order to investigate the role of RCC1 and its interaction with *pfran* in the life cycle of *P. falciparum*, it is necessary to isolate and characterise the RCC1 homologue of *P. falciparum*. This chapter describes the cloning and partial sequencing of the *P. falciparum* RCC1 homologue (*pfrcc1*). DNA sequence analysis carried out is partial, although quite extensive. This partial sequence codes for a protein of 925 amino acids, and although incomplete is almost twice as long as any previously sequenced RCC1 molecule. The N-terminal portion of the predicted protein, from amino acids 55-725, has around 20-30% homology to the RCC1 proteins of human, hamster, *Xenopus*, *Drosophila*, *S. pombe*, and *S. cerevisiae*. The characteristic feature of the RCC1 proteins, the seven 60 amino-acid blocks of conserved repeats, are also found in the *P. falciparum* gene. Unlike the yeast and vertebrate RCC1 proteins, pfRCC1 has a C-terminal extension of 290 amino acids (amino acids 727-1017) with no homology to RCC1 repeats, but containing clusters of glutamic acid residues balanced by a lysine-rich sequence. The *pfrcc1* has been mapped to chromosome 5 and is present as a single copy in the *P. falciparum* genome. The RCC1 transcript is approximately 8.5 kb in size. Northern blot analysis of total RNA from different stages of the parasite indicates that *pfrcc1* is expressed mainly in the trophozoites.

5.2 Introduction

Genetic and biochemical analyses suggest that Ran/TC4 may play a key role in the regulation of cell cycle progression in eukaryotes, and that its function depends on its interaction with the product of a second gene, called RCC1 (regulator of

chromosome condensation) (Matsumoto and Beach, 1991; Bischoff and Ponstingl, 1991a).

An RCC1 mutant was first isolated in a temperature-sensitive cell line (*ts*BN2 cells) derived from baby hamster kidney (BHK) cells (Nishimoto *et al.*, 1978). It has been observed that *ts*BN2 cells which are shifted to higher, restrictive temperatures at different points in the cell cycle display different defects: they are unable to pass the G1/S transition if shifted during G1 phase, and subject to premature chromosome condensation (PCC) and entry into mitosis if shifted during S phase (Nishimoto *et al.*, 1978). Early investigations showed that the difference between the phenotypes of cells shifted in G1 and S phases was due to a requirement for mitosis-inducing factors that are not synthesised until cells undergo G1/S transition (Nishimoto *et al.*, 1981).

Using DNA-mediated gene transfer to rescue the *ts*BN2 cell line, the human gene and cDNA encoding RCC1 were isolated and found to be located on chromosome 1 (Ohtsubo *et al.*, 1987). Subsequent analysis of the mammalian RCC1 protein has shown that it is a nuclear localised DNA-binding protein and that it is lost from the DNA during mitosis lead to activation of $p34^{cdc2}$ and entry into mitosis (Ohtsubo *et al.*, 1989). Later, it was shown that the *ts*BN2 defect in the hamster RCC1 gene is due to a single point mutation (Uchida *et al.*, 1990). Because of this mutation, the RCC1 protein disappears in the *ts*BN2 cell line following a shift to a restrictive temperature. Upon loss of RCC1 function, the cdc2/histone H1 kinase is activated and the *ts*BN2 cells enter mitosis prematurely (Nishitani *et al.*, 1991). The factors controlling the apparent degradation of RCC1 are not understood.

Fission yeast mutants, in which the onset of mitosis is uncoupled from the completion of DNA replication have been isolated by Matsumoto and Beach (1991). These mutants, known as *pim* mutants, (for premature initiation of mitosis), undergo mitotic chromosome condensation and mitotic spindle formation without completion of S phase and without the *cdc25* mitotic inducer, a gene product that activates the cdc2/cyclin B complex. The *pim1* gene product was later found to share approximately 30% homology with the human RCC1 protein, and the cells display a phenotype that has certain similarities to the *ts*BN2 cells. In addition it has been shown that both yeast and mammalian mutants enter mitosis from S phase, i.e. omitting the normal G2 interval (Matsumoto and Beach 1991). The most distinct difference between the yeast and mammalian mutants is that a mammalian *ts*BN2 line transferred to a restrictive temperature in the G1 phase of the cell cycle becomes arrested in G1 and does not proceed to S-phase; in contrast, the *pim1* mutant appears to complete DNA replication and then arrests in mitosis (Matsumoto and Beach, 1991). Matsumoto and Beach (1991), in the process of cloning *pim1*, isolated a

multicopy plasmid suppressor which they have designated *spi1* (for suppressor of *pim1*). *spi1* is the *S. pombe* homologue of Ran/TC4 and *pfran*. Overexpression of the wild-type allele of *spi1* suppresses the *pim1* mutant phenotype, although *spi1* overexpression cannot rescue null mutants of *pim1*, indicating that *spi1* cannot bypass the requirement for *pim1* and that both proteins are required for efficient regulation of the cell cycle with respect to completion of DNA replication (Matsumoto and Beach, 1991).

RCC1 homologous genes have been isolated from *Xenopus*, *Drosophila* and yeast (Nishitani *et al.*, 1990; Frasch, 1991; Aebi *et al.*, 1990; and Matsumoto & Beach, 1991). The isolation of RCC1 homologues from other organisms has confirmed a requirement for RCC1 in the cell cycle and suggested that RCC1 might have a number of other roles in nuclear function. It is interesting that a very diverse collection of screens have yielded mutants in RCC1 homologues and the phenotypes associated with these mutations are highly pleiotropic (see Table 5.1).

5.3. Strategy used for isolation of *P. falciparum* RCC1 homologue

In order to design oligonucleotide primers which permitted successful amplification of a genomic fragment encoding a possible RCC1 homologue in *P. falciparum*, a sequence alignment comparison was carried out between all RCC1 genes across species which are deposited in the databank.

Two stretches of amino acids in the RCC1 gene from other species gene *pim1* were found to be most conserved, one between positions 58-63 and another between positions 182-192 (see Fig.5.1). Two oligonucleotides were synthesised to code for these peptides. (i) G6037,+ a redundant 17 mer coding for AVGGMH amino acid residues and (ii) G5146,- an antisense 32 mer coding for WGNGQQFQLGR, designed according to the codon usage bias of *P. falciparum* (Weber, 1987; Saul and Battistutta 1988).

Amplification of *P. falciparum* RCC1 was carried out (method 2.2.16) using these primers mentioned above. The template was genomic DNA from clone 3D7A. Amplification was performed for 30 cycles (each cycle consisted of 30 seconds at 94°C, 60 seconds at 45°C and 90 seconds at 72°C. Human DNA was used simultaneously, as an internal control in the PCR reaction.

Table 5.1 Homologs of RCC1: isolation methods and properties (After Dasso, 1993)

Species	Name	Isolation/ genetic screen	Implied role
Hamster (BHK cell)	RCC1	DNA-mutant (*ts*BN2 cells)	Required for G1/S phase transition to prevent PCC. Chromatin-binding protein. Transcription and translation inhibited in RCC1's absence
Human	RCC1	Complement *ts*BN2 cells	As above
Human (HeLa cells)	RCC1	Recognised by anti-kinetochore antibody	Chromatin-binding protein. Mediates GTP exchange for Ran (*spi*).
Xenopus laevis	RCC1	Sequence homology to human RCC1	Chromatin-binding protein. Required for DNA replication *in vitro*.
Drosophila melanogaster	BJ1	Chromatin-binding protein	Maternally-expressed. Chromatin-binding protein.
Schizosacchro-myces pombe	pim1	premature mitosis mutant	Required for PCC. Interacts with small *ras*-like GTP-binding protein *spi1*.
Saccharomyces cerevisiae	SRM1	Mating rescue of a-factor receptorless mutant	Regulates mating pathway. Required for G1/S phase transition. Required for plasmid and chromosome stability.
S. cerevisiae	PRP20	Mutant defective in pre-mRNA processing	Required for pre-mRNA splicing &3'end formation. Required for structural integrity of the nucleus. Required for correct trans-cription initiation and termination.
S.cerevisiae	MTR1	Mutant defective in mRNA export	Required for mRNA export from nucleus.

PCC= Premature Chromosome Condensation

Figure 5.1:

Testing for RCC1 conserved regions in the DNA of *P. falciparum.* Amino acids 56-201 of human (Ohtsubo *et al.*, 1987), have been aligned with RCC1 homologs from hamster (Uchida *et al.*, 1990), *Xenopus* (Nishitani *et al.*, 1990), *D. melanogaster* (Frasch, 1991), *S. cerevisiae* (Clark and Sprague, 1989; Aebi *et al.*, 1990) and *S. pombe* (Matsumoto and Beach, 1991). The highly conserved undecapeptide and hexapeptide (boxed in) were used to design oligonucleotide primers for PCR amplification of the corresponding region of *P. falciparum* RCC1 (see text). These peptides are lying within the conserved repeat number 2 of RCC1 proteins. The position, sequence and redundancy of oligonucleotides G6037 and G5146 are shown.

5' G(A/C)(A/T)G(C/T)(A/T)GG(A/T)GG(A/T)ATGCA 3'

primer G6037

--------->

Human	QA	EAGGMH	TVCLSKSGQVY--------T	LGCGEQGQLGR	VPELFANRG
Hamster	QA	EAGGMH	TVCLNQSGQVY--------T	LGCGEQGQLGR	VPELFANRG
Xenopus	QA	AAGGMH	TVCLGASGSIY--------T	SGCGEQGQLGR	VPERFINRG
Droso.	DI	SAGGMH	NLVLTKSGDIY--------T	VGCAEQGQLGR	LSERSISGE
S.cerv.	SF	AVGGMH	TLALDEESNVW--------A	WGNGQQNQLGR	--KVMRFRL
S.pombe	DL	AVGGMH	SAALLHDGRVY--------T	WGNGQQFQLGR	--RMLERRR

56 <-------------------- primer G5146 201

3' ACCCC(T/A)TT(A/G)CC(T/A)GT(T/C)GT(T/C)(T/A)(T/A)AGT(T/C)(G/A)A(T/A)CC(T/A)GC 5'

5.4 Results

5.4.1 A fragment encoding a sequence homologous to the RCC1 detected by PCR

A DNA fragment of 480bp was detected using the above mentioned primers. No amplified band was detected from the control (human DNA) (Fig. 5.2). The amplified PCR fragment was subcloned directly into the pCRII cloning vector and plasmid DNA minipreps prepared from 2 positive transformants. These clones were sequenced, on both strands, by the dideoxy chain termination method (method 2.2.18), using either pJFE14 vector specific primers or oligonucleotides homologous to specific parts of the gene.

Sequencing revealed that the PCR fragment had an 84% AT content. The predicted protein encoded by this insert matched the human RCC1 protein only in the amino acid repeats; there was no homology outside these repeats. This finding ruled out the possibility that amplification had taken place on a template of human DNA origin, and suggested strongly that the PCR amplified fragment truly consisted of a portion of *P. falciparum* RCC1 gene.

5.4.2 Partial sequencing of the *P. falciparum* RCC1 gene

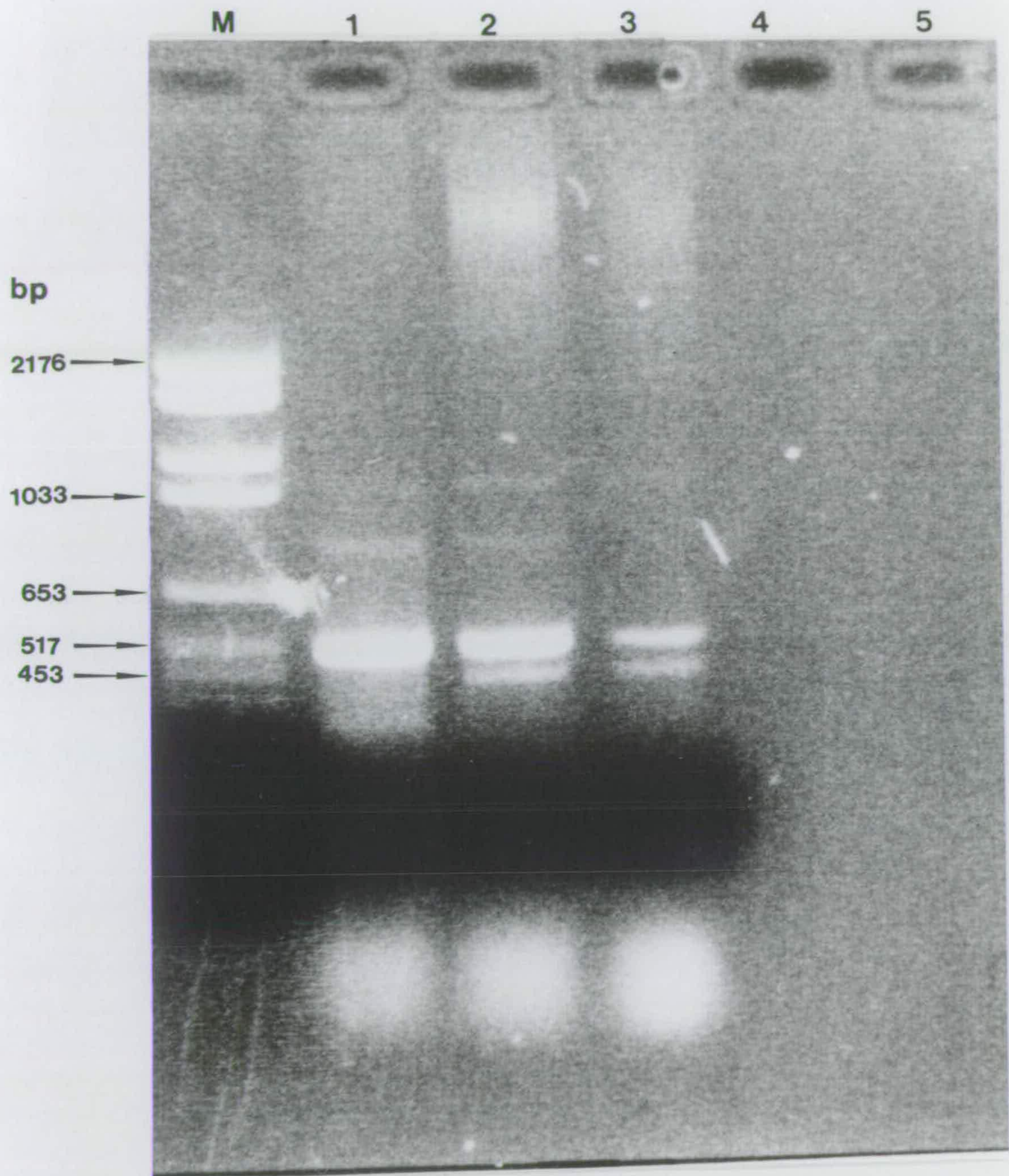
The 480bp fragment was then used to screen a cDNA asexual library in order to obtain further sequences of the gene. Three positive cDNA clones were isolated, the longest of which (clone C10) was about 3.0 kb long. Upon sequencing, this fragment was found to contain one major open reading frame of 2776 nucleotides which codes for a protein of 925 amino acids. The A+T content of the sequence is 83% (Fig. 5.3).

The clone C10 lacked the 5' and 3' termini of the gene, because of the absence of stop codons. The criterion for completion of the gene was to find at the N-terminus of the gene a methionine codon (presumably the initiation codon) preceded by blocked reading frames, and to identify a stop codon at the 3' prime end of the gene. In order to obtain the remaining part of the *P. falciparum* RCC1 gene three rounds of screening of the cDNA library with unique sequence probes from clone C10 were carried out. However, this approach was unsuccessful. Genomic DNA libraries (*HindIII* digested K1 DNA and *DraI* digested K1 libraries) were also screened, but again failed to produce clones with sequences beyond these identified in the clone C10. Two oligonucleotides primers were designed in order to amplify a genomic DNA by inverse PCR (method 2.2.17). However, after several attempts this approach also failed to extend the clone beyond the previously identified sequence.

Figure 5.2 :

Amplification of *P. falciparum* genomic DNA of clone 3D7 using two degenerate primers (primers G6037 and G5146) in order to obtain the *P. falciparum* homologue. Different annealing temperature were tried until a single band was obtained. dH_2O and Human DNA were used as controls. 5μl of the amplification products were loaded on a 1.5% agarose gel in 1xTAE buffer, run at 70V for 30 minutes, stained with ethidium bromide and photographed on a U.V. trans-illuminator.

M. DNA molecular weight marker.
Lane 1. PCR product from clone 3D7 genomic DNA with RCC1 primers, annealing temperature 45°C.
Lane 2. PCR product from clone 3D7 genomic DNA with RCC1 primers, annealing temperature 40°C.
Lane 3. PCR product from clone 3D7 genomic DNA with RCC1 primers, annealing temperature 37°C.
Lane 4. PCR product of amplification of human DNA with RCC1 degenerate primers
Lane 5. dH_2O (control).


M
1
2
3
4
5
bp
2176
1033
653
517
453

5.4.3 Comparison of the *P. falciparum* RCC1 protein sequence with other RCC1 protein homologues

Alignment between the *P. falciparum* RCC1 predicted partial protein sequence and the human, *Drosophila* (BJ1), *S. cerevisiae* (SRM1), and *S. pombe* (Pim1) protein sequences is shown in figure 5.4. The protein data-bases Swissprot (Devereaux *et al.*, 1984) was screened for protein with homology to the *P. falciparum* RCC1(hereafter known as *pfrcc1* gene). In the conserved portion of the molecule, the seven repeats (Fig. 5.5), *P. falciparum* RCC1 has an amino acid identity of 69%, 71%, and 74% with the RCC1 molecules from human, *D. melanogaster*, and yeast (*S. pombe* and *S. cerevisiae*) respectively (Fig. 5.4 and Table 5.2). PfRCC1 has overall amino acid identity around 10-15% with the human, *Drosophila* and yeast protein sequences.

5.4.4 Structural analysis of the *pfrcc1*

A single open reading frame was found in the 3.0 kb cDNA clone C10, consisting of 2776 base pairs, encoding 925 amino acids (Fig.5.3). The estimated molecular weight of this incomplete but probably near full size protein is 118 kDa, which is twice as big as other previously identified RCC1 proteins. Southern blot analysis of restriction enzyme digests of genomic DNA of *P. falciparum* hybridised with the C10 probes (Fig. 5.6) was consistent with the presence of a single copy of the gene. The data were also consistent with the restriction enzyme map obtained by computer analysis of the clone C10 sequence.

To investigate whether polymorphism occurred within the *pfrcc1* gene, genomic DNA prepared from four, geographically different, isolates (102/1, T9/94, T9/96 and 7G8) was digested with three different restriction endonuclease enzymes (*AluI*, *MspI* and*TaqI*) separated on an agarose gel, transferred onto Hybond N$^+$ membrane and probed with clone C1. No fragment size polymorphisms were observed among these four isolates (Fig.5.7).

In order to determine the location of the *pfrcc1* gene in the *P. falciparum* genome, a blot of chromosomal DNA gel fractionated by pulsed-field gradient electrophoresis (method 2.2.7), was hybridised with *pfrcc1* specific probe. In the all *P. falciparum* clones used (102/1, 105/1, 106/1, 3D7A, Dd2, and T9/94), the *pfrcc1* gene was found to give a hybridisation signal in the position corresponding to chromosome 5 (Fig.5.8). As a further confirmation, the same blots were stripped and probed with *pfmdr-1* probe, a known chromosome 5 marker (Foote *et al.*, 1989). This gave a signal in the same position (data not shown).

Figure 5.3 :

The nucleotides sequence of the partial *pfrcc1* gene. The entire sequence of the cDNA clone C10 is shown. The open reading frame of the clone is indicated in the single-letter code below the DNA sequence. The primers used in sequencing the clone are underlined. The nucleotide sequence data shown in this figure has been submitted to Genbank and DDBJ nucleotide sequence Databases under the accession number X80476

Primer H4254 ----->

AAGGAAAAATTATATTCAAATATAAAAGAAAATGATAATAAACATGACTGGTTTACTCCA 60

K E K L Y S N I K E N D N K H D W F T P

TTCCCTATGAAAATAGTTTTCCCTAAAAAAAATACAAAAATAAAATTTATTTCTTGTGGT 120

F P M K I V F P K K N T K I K F I S C G

<------ Primer G9161

GATATGCACACATTGGCTATCTGCACAGGTGGTGTTTTATATTCCTGGGGTTATAACAAT 180

D M H T L A I C T G G V L Y S W G Y N N

TTTGGTTGTATAGGAAATGGGACAAATCAAAATGTATATGAACAAAACCCGATATTTTTA 240

F G C I G N G T N Q N V Y E P T P I F L

GAGCCTCATTCGGTTGATAATAATGTTGATAATAATGATGATGATTATTATGATGATAAT 300

E P H S V D N N G D N N D D D Y D D D N

AATAATAATAGTACATACAAATTTCATAAAAAGGAAGATCTCATAAAATGGGATAAAAAA 360

N N N S T Y K F H K K E D S I K W D K K

AAAAATGTAGTCATACATTGCTCAGCAGGGAGTAAGCATAGTCTTGCTTGTAACCTACAC 420

K N V V I H C S A G S K H S L A C N L H

GGAGATATTTATAGTTGGGGTTATGGAGGTAATGTTAGATTGGGTCTTGGGAATATAAAA 480

G D I Y S W G Y G G N G R L G L G N I K

Primer G8696 ---->

AGTTATAATAAGCCCCAATTAATTAAAGGATTAAGAAATAAAAGAATTCTTTATGTATGT 540

S Y N K P Q L I K G L R N K R I L Y V C

GCAGGAAACTCTCATTCAGGATGTATAGATTCTAATTATTATGTATATCCGTGGGGAAAT 600

A G T S H S G S I D S N Y Y V Y P W G N

GGGAAATTTTACAAATTAGGTCATGGTAATGATGATATAGATATATATTATCCTAAATTA 660

G K F Y K L G H G N D D I D I Y Y P K L

GTAGAATGTTTAAATCATAATAAGAAAGTATGTATGTTAAGTTTTGGGTGTTTTAATTCT 720

V E C L N H N K K V C M L S F G C F N S

TTGGCATTAAATGTTAAAGGAGATGTATATGTATGGGGTACATTTAATATAACAAATAAT 780

L A L N V K G D V Y V W G T F N I T N N

TATGTAAATTATTATATTTCTAAATTACCTAAGCAAATTAATACAAATTATAAATGTATA 840

Y V N Y Y I S K L P K Q I N T N Y K C I

TCTATACATGCTTCTACATATGTTTGCTTTGGTATTACGTTAGTAGGTGATTTAATTAGT 900

S I H A S T Y V C F G I T L V G D L I S

TTTGGGAATTATAAAAACTATTCCTATGAAAAAGAAATAAATGATAATGATTCAGATGAC 960

F G N Y K N Y S Y E K E I N D N D S D D

GATTTAATCAAATATTTAATAAATAAAGAAGTTGAAGAGCAACATTATAATGGGGATAAT 1020

D L I K Y L I N K E V E E Q H Y N G D N

AAAAGTGTTCACACAAATAAGAATAACATCATATCATCATATATTGTAAAAAAGAAGGAT 1080

K S V H T N K N N I I S S Y I D K K K D

GTACATTTTGATATACATTATATAAAAGAAATGAGAGGCAAAATGTATATAAAAGATATT 1140

V H F D I H Y I K E M R G K M Y I K D I

GTAACACATTTTTATAATTTAGATACTATGACATATTATATGAATAAGATAAACAAGCAC 1200

V T H F Y N L D T M T Y Y M N K I N K H

ATTGATAATGTTACCTTAAATAATAATGATAATTACTATACGTATGATAATATAAATGGT 1260

I D N V T L N N N D N Y Y T Y D N I N G

TCCCATGGAAACATTTTTGAAAAGAACAAACTTGTCTTGAAGTCTAAAGTTAAAATAATA 1320

S H G N I F E K N K L V L K S K V K I I

GATGGAAGTGATCATTTTAGTGTATTTTTATTAGAAAGCGGGAAAGTATATACTGCTGGA 1380

D G S D H F S V F L L E S G K V Y T A G

TATAATAAGAATGGAGAATTGGGAAATGGAGAATTTAATTTAAAGAAAAAATTTGGAGTG 1440

Y N K N G E L G N G E F N L K K K F G V

GGTATATTATTAAACATATGTGTAAACAAGATAATAAAAATTGCATGTGGATATAATTAT 1500

P I L L N I C V N K I I K I I C G Y N Y

GTATTATGTGTAAGTGATGTGGGTTTAGTATATGGCTGGGGAAAAAATGATAAGAGCCAA 1560

V L C V S D V G L V Y G W G K N D K S Q

Primer H1790 ---->

TTGGGAATTGGGGTAATAAAAGATTTTTATGAACCTGTTCATTGTAAGAATTTGACAAAT 1620

L G I G V I K D F Y E P V H V K N L T N

GTGATAAATATATTTGCTGGATATGACCATAGTGCTTGTATTATAAATAAGTTATTAGAT 1680

V I N I F A G Y D H S A C Y Y N K L L D

AAGGGTAACACGAATGGTGTGAATTATTATAGTTCAGACGAATGTGGAGATTTATATATT 1740

K G N T N G V N Y Y S S D E C G D L Y I

TGGGGTAATGCCGAAAGCGGAAAGGTCGGGTTAGGTGTTGATTATACCCAAGGATGTATA 1800

W G N A E S G K V G L G V D Y T Q G C I

TTATTACCTAGAAAAATTAATATAATAAATAAAATATATAAATGTTCTTTAGGTAATAGT 1860

L L P R K I N I I N K I Y K C S L G N S

CATAGTTTATTTTTAACGAATACTAACGAATTATACGTTTGTGGAAGTAATAATAATGGT 1920

H S L F L T N T N E L Y V C G S N N N G

AGGTTAGGTTTATTTGAAAAATCTAAAATGGTTTGTAAATTTGTTAGTGTACCTACTAAA 1980

R L G L F E K S K M V C N I V S V P T K

GTTATATTACATAATAATATATATATTAAGGATATCCTAGCAGGTAATACATATAGTATA 2040

V I L H N N I Y I K D I L A G N T Y S I

ATATTATCTGTTGATGGGTTTATATATATTTTTGGAGAATTCATAAAAGGTAGTAATGGT 2100

I L S V D G F I Y I F G E F I K G S N G

AATAGTAATAATATGTCATGTAGGGTCATAAGCTTATATAATGAAATTACCAATGTTAAA 2160

N S N N M S C E V I S L Y N E I T N V K

TATATGAATGGGAAATATGAACATCTTTTTTTTTTTCTTACATATGATAATAAATTATTT 2220

Y M N G K Y E H L F F F L T Y D N K L F

GGTATAGGTAATAATAAGAATTGTCAAATATTATGTGATAATGAAAAAGAGAAGAATTAT 2280

G I G N N K N C Q I L C D N E K E K N Y

ATAAAAAAACCTAAATTAATAACATATTTTTTAAAAGAAAATAATAAAATAATTGAATTC 2340

I K K P K L I T Y F L K E N N K I I E F

Primer H3738 ---->

ATTTTTTCTTTTCATAATGTTACATTTATTCTTTTCAATAATTCAGAAATATTTGTATGG 2400

I F S H F N V T F I Q F N N S E I F A W

GGTTATACTAATAATTATCATTTATGTATAGGAATACCTAATAATATAAAATATTTAAAA 2460

G Y T N N Y H L C I G I P N N I K Y L K

CATCCTACGAAAATTATAAAAACATGGTTAACATATGATCAGAAAGATTTAGATTATAAT 2520

H P T K I I K T W L T Y D Q K D L D Y N

Primer H4253 ----->

GGAAGTTGTAGTGATATGGAATTGAATGAATATAATACTACTAATAATATGTCTTATAAT 2580

G S C S D M E L N E Y N T T N N M S Y N

AATTATATAAAATATATTAATGAAATTACTCTTGTTAAAAAAAATATAAATACAACAGAA 2640

N Y I K Y I N E I T L V K K N I N T T E

CTGTTTCAAAATGAATATACTTATTTTGGTTATTATGAAGAAGAAATCGAAAATTTTATA 2700

L F Q N E Y T Y F G Y Y E E E I E N F I

<----- Primer H4252

TATAATATAGTAATAATGAATAATATTATAAATTGGTGTCATTTACAAAATTTATTAAAA 2760

Y N I V I M N N I I N W C H L Q N L L K

AAAAAAAAAAACTTT 2775

K K K N F

Figure 5.4 : Amino acids comparisons of the *P. falciparum* pfRCC1 protein with the other eukaryotic RCC1 proteins. Sequence alignment of pfRCC1 with human RCC1 (Ohtsubo *et al*, 1987), *Drosophila* BJ1 (Frasch, 1991), *S. cerevisiae* SRMI (Aebi *et al.*, 1990) and *S. pombe* Pim1 (Matsumoto and Beach, 1991). The figure is derived from an analysis performed with the PILEUP alogrithm (Feng and Doolittle, 1987). Dots indicate gaps introduced to maximise the alignment. The amino acids within the seven conserved repeats are boxed in and their positions are numbered I-VII. Numbers to the right of the sequence refer to residue position of the pfRCC1 protein (Clone C10).

```
                                     1                                              40
Rcc1    ..................................................MSPKRI AKRRSPPADA
BJ1     .......... .......... .......... .......... .......... .....MPRRK ALTNNNNAGE
SRM1    .......... .......... .......... .......... .......... ....MVKRTV ATNGDASGAH
pim1    .......... .......... .........M TSNRSTRSST KREEVSKNGV EKRELDESDV MKNGK.KPVK
RccPf                                                          KEKLYSNIKE NDNKHDWFTT

        41                                  I                                          110
Rcc1    I....PKSKK VKVSHR.... SH....STEP GLVL|TLG|QGD VGQ|LG|LGENV ME......RK KPALVSIPED
BJ1     AEQQPPKAKR ARIAFHLELP KR....RTVL GNVL|VCG|NGD VGQ|LG|LGEDI LE......RK RLSPVAGIPD
SRM1    RAKKMSKTHA SHIIN.AQED YKHMYLSVQP LDIF|CWG|TGS MCE|LG|LGPLA KNKEVKRPRL NPFLPRDEAK
pim1    RAKVSSLPKP VRVPGSAKRI NKIPELPTER LNVY|VFG|SGS MNE|LG|MGEEE MD.VVYRPRL NPILSTDKVG
Pf      FPMKIVFPKK NTKIKFISCG DMHTLAICTG GVLY|SWG|YNN FGC|IG|NGTNQ NVYEPTPIFL EPHSVDNNGD

        111                                                          II                180
Rcc1    VVQAEA.... .......... .......... .........|G GMH|TVCLSKS |GQVY|SFG|CND EGA|LG|RDTS.
BJ1     AVDISA.... .......... .......... .........|G GMH|NLVLTKS |GDIY|SFG|CND EGA|LG|RDTS.
SRM1    IISFAV.... .......... .......... .........|G GMH|TLALDEE |SNVW|SWG|CND VGA|LG|RDTSN
pim1    VVDLAV.... .......... .......... .........|G GMH|SAALLHD |GRVY|TWG|VND DYA|LG|RLTKD
Pf      NNDDDYDDDN NNNSTYKFHK KEDSIKWDKK KNVVIHCSA|G SKH|SLACNLH |GDIY|SWG|YGG NGR|LG|LGN..

        181                                                          III 250
Rcc1    .......... .VEGSEMVPG KVELQE.... .......... ...KVVQVSA |GDSH|TAALTD D|GRVF|LWG|SF
BJ1     .......... .EDGSESKPD LIDLPG.... .......... ...KALCISA |GDSH|SACLLE D|GRVF|AWG|SF
SRM1    AKEQLKDMDA DDSSDDEDGD LNELESTPAK IPRESFPPLA EGHKVVQLAA |TDNM|SCALFS N|GEVY|AWG|TF
pim1    QK........ DENGDKVDND L..LEGTPSK VE.GALSHL. ...RVTKVIC |SDNL|TAAITD N|GCCF|TWG|TF
Pf      .......... .......... .......IKS YNKPQLIKGL RNKRILYVCA |GTSH|SGCIDS N|YYVY|PWG|NG

        251                                                          IV                320
Rcc1    RDNNGV|IG|LL EPMKKSMVPV QVQLDV.... PVVKVAS|GND H|LVMLTAD|GD LY|TLG|CGEQG Q.........
BJ1     RDSHGN|MG|LT IDGNK.RTPI DLMEGT.... VCCSIAS|GAD H|LVILTTA|GK VF|TVG|CAEQG Q.........
SRM1    RCNEGI|LG|FY Q.DKIKIQKT PWKVPTFSKY NIVQLAP|GKD H|ILFLDEE|GM VF|AWG|NGQQN Q.........
pim1    RCSDGV|LG|YS DSQKRTAEPT QMRLP..... EICQLAT|GTD H|IIALTTT|GK VY|TWG|NGQQF Q.........
Pf      KFYK..|LG|HG NDDIDIYYPK LVECLNHNKK VCMLSF.|GCF N|SLALNVK|GD VY|VWG|TFNIT NNYVNYYISK
```

```
       321                                                                   390
Rccl   .......... .......... .......|LG|R VPELFANRGG RQGLERLLVP KCVMLKSRGS ..........
BJ1    .......... .......... .......|LG|R LSERSISGEG RRGKRDLLRP TQLIITR... ..........
SRM1   .......... .......... .......|LG|R .......... .....KVMER FRLKTLDPRP ..........
piml   .......... .......... .......|LG|R .......... .....RMLER RRLQGLTPQP ..........
Pf     LPKQINTNYK CISIHASTYV CFGITL.|VG|D LISFGNYKNY SYEKEINDND SDDDLIKYLI NKEVEEQHYN

       391                                                                   460
Rccl   .......... .......... .......... .......... .......... .......... ..........
BJ1    .......... .......... .......... .......... .......... .......... ..........
SRM1   .......... .......... .......... .......... .......... .......... ..........
piml   .......... .......... .......... .......... .......... .......... ..........
Pf     GDNKSVHTNK NNIISSYIVK KKDVHFDIHY IKEMRGKMYI KDIVTHFNYL DTMTYYMNKI NKHIDNVTLN

       461                                                  V                     530
Rccl   .......... .......... ...RGHVRFQ DAFC|GAYF|TF A.ISHE|GHVY| |GFG|LSNYHQ|L G|...TPGTES
BJ1    .......... .......... .....AKPFE AIWA|TNYC|TF MRESQT|QVIW| |ATG|LNNFKQ|L A|...HETKGK
SRM1   .......... .......... ...FGLRHVK YIAS|GENH|CF A.LTKD|NKLV| |SWG|LNQFGQ|C G|VS.EDVEDG
piml   .......... .......... ...LALKNII SVGA|GSYH|SF A.IDNK|GRVY| |AWG|LNITRQ|C G|IEVEDEEEG
Pf     NNDNYYTYDN INGSHGNIFE KNKLVLKSKV KIID|GSDH|FS VFLLES|GKVY| |TAG|YNKNGE|L G|NGEFNLKKK

       531                                          VI                                  600
Rccl   CFIPQNLTSF KNSTKSWVGF SG|GQHH|TVCM DSE|GKAY|SLG| RAEYGR|LG|LG E..G...... .......AEE
BJ1    EF...ALTPI KTELKDIRHI AG|GQHH|TVIL TTD|LKCS|VVG| RPEYGR|LG|LG D..V...... .......KDV
SRM1   ALVTKPKRLA LPDNVVIRSI AA|GEHH|SLIL SQD|DDLY|SCG| RLDMFE|VG|IP K..DNLPEYT YKDVHGKARA
piml   AVITKPTLVD ALEGYNVKSI TG|GEHH|TLAL LED|GRVL|AWG| RDDRHQ|LG|IP D..NALPETV VKDEKGNNYY
Pf     FGVPILLNIC VNKIIKIAC. ..|GYNY|VLCV SDV|GLVY|GWG| KNDKSQ|LG|IG VIKDFYEPVH VKNLTNVINI

       601                                          VII                                 670
Rccl   KSIPTLISRL PA.VSSVAC|G ASV|GYAVTKD ...|GRVF|AWG| MGTNY..Q|LG| TGQDEDAWS. PVEMMGKQLE
BJ1    VEKPTIVKKL TEKIVSVGC|G EVC|SYAVTID ...|GKLY|SWG| SGVNN..Q|LG| VGDGDDELE. PIVVVSKNTQ
SRM1   VPLPTKLNNV P.KFKSVAA|G SHH|SVAVAQN ...|GIAY|SWG| FGETY..A|VG| LGPFEDDTEV PTRIKNTATQ
piml   LSTPTIIPGL T.NVIQVVC|G THH|NLAVTSD ...|GKVY|SWG| SAENY..E|VG| QGDNDEDVAV PTLVRSKAIK
Pf     FAGYDHSACI INKLLDK..|G NTN|GVNYYSS DEC|GDLY|IWG| NAESGKVG|LG| VDYTQGCILL PRKINIINKI
```

```
      671                                                                      740
Rcc1  NRVVLSVSS|G GQH|TVLLVKD KEQS...... .......... .......... .......... ..........
BJ1   GKHMLLASG|G GQH|AIFLVKA DKQDQKENVP VKVSGSSSIS KKDKTPPQDN VDKEAENVDK QEQKENLPAK
SRM1  DHNIILVGS|G GQF|SVSGGVK LSDEDAEKRA DEMDD..... .......... .......... ..........
pim1  EVAIRVAGA|G GQF|SIIAGIP NASEEPVANG IKSEPENEKK LKTEETSKTD DSPVTDAKPD VTSNGEPSTA
Pf    YKCSL....|G NSH|SLFLTNT NELYVCGSNN NGRLGLFEKS KNVCNIVSVP TKVILHNNIY IKDILAGNTY

      741                                                                      810
Rcc1  .......... .......... .......... .......... .......... .......... ..........
BJ1   ASTSSKKNKT PPQDNADKEA ENADKQEQKE NLPAKASTSS KKIKTPPQDD AAEEVEEESA QEPTPKKAKK
SRM1  .......... .......... .......... .......... .......... .......... ..........
pim1  TSDSKDSDLE PSSTTA.... .......... .......... .......... .......... ..........
Pf    SIILSVDGFI YIFGEFIKGS NGNSNNMSCE VISLYNEITN VKYMNGKYEH LFFFLTYDNK LFGIGNNKNC

      811                                                                      880
Rcc1  .......... .......... .......... .......... .......... .......... ..........
BJ1   PAAKRGGKKT .......... .......... .......... .......... .......... ..........
SRM1  .......... .......... .......... .......... .......... .......... ..........
pim1  .......... .......... .......... .......... .......... .......... ..........
Pf    QILCDNEKEK NYIKKPKLIT YFLXENNKII EFIFSFHNVT FIQFNNSEIF AWGYTNNYTL CIGIPNNIKY

      881                                                                      950
Rcc1  .......... .......... .......... .......... .......... .......... ..........
BJ1   .......... .......... .......... .......... .......... .......... ..........
SRM1  .......... .......... .......... .......... .......... .......... ..........
pim1  .......... .......... .......... .......... .......... .......... ..........
Pf    LKHPTKIIKT WLTYDQKDLD YNGSCSDMEL NEYNTTNNMS YNNYIKYINE ITLVKKNINT TELFQNEYTY

      951                                987
Rcc1  .......... .......... .......... .......
BJ1   .......... .......... .......... .......
SRM1  .......... .......... .......... .......
pim1  .......... .......... .......... .......
Pf    FGYYEEEIEN FIYNIVIMNN IINWCHLQNL LKKKKNF
```

Table 5.2. Sequence homology of RCC1 proteins.

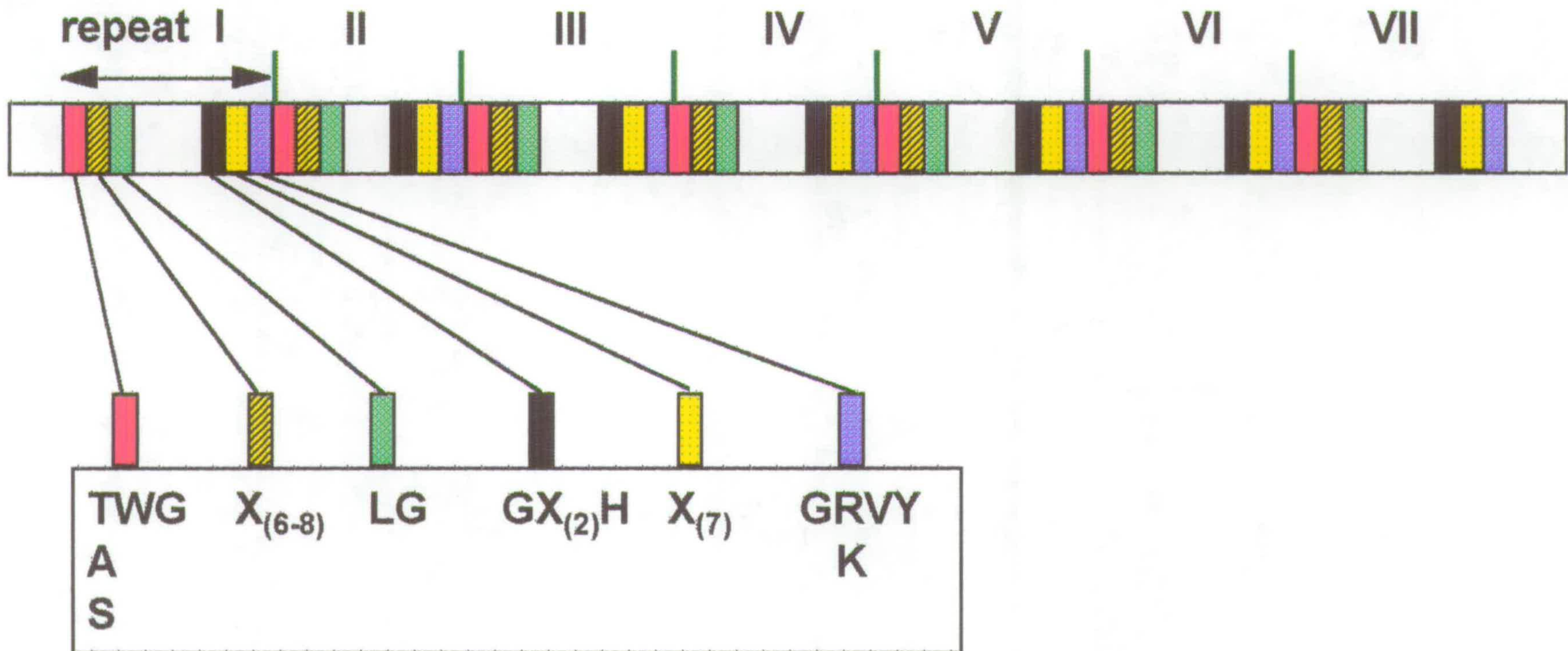
The figure shown are the percent identities from pairwise comparison between the following organisms: *P. falciparum* (this study), human (Ohtsubo *et al*, 1987), *D. melanogaster* (Frasch, 1991), *S. cerevisiae* (Aebi *et al.*, 1990) and *S. pombe* (Matsumoto and Beach, 1991).

N.B. The figures were calculated by expressing the number of identities as a percentage of the total number of amino acids in the non-*P. falciparum* RCC1 in each case.

	P. falciparum	Human	*D. melanogaster*	*S. cerevisiae*	*S. pombe*
P. falciparum	100	10	11	12	13
Human		100	26	16	16
D. melanogaster			100	15	17
S. cerevisiae				100	26
S. pombe					100

Figure 5.5 :

Schematic diagram showing the conserved repeats within the RCC1 proteins. Consensus amino acids sequences at the start and end of each repeat are shown below the diagram.



repeat I
II
III
IV
V
VI
VII
TWG
A
S
X(6-8)
LG
GX(2)H
X(7)
GRVY
K

5.4.5 RNA expression of the *P. falciparum* RCC1 gene

In order to determine the size of the message and level of expression of *pfrcc1* during the life cycle, Northern blots were probed with a specific probe from the *pfrcc1* gene. Total RNA was isolated from synchronous cultures of different stages of the parasite using the method of Chomczynski and Sacchi (1987), as described in method 2.2.20.1. The RNA was separated by gel electrophoresis (see method 2.2.20.2), blotted onto Hybond-N+ (method 2.2.20.3) and probed with a 1.2 kb fragment from clone C10, washed and autoradiographed. As an internal control the same filter was stripped and probed with the *P. falciparum* actin specific probe (Wesseling *et al.*, 1989).

The *pfrcc1* was found to hybridise to one major band of approximately 8.5kb. The strongest signal was observed in the early trophozoite stage RNA lane (Fig. 5.9). The hybridisation signal was very weak in the schizont stage, and no signal seen in the ring and gametocyte stages lanes even longer exposures.

5.4.6 Expression of pfRCC1 into pGEX vector

In an attempt to raised polyclonal antibodies against the pfRCC1 protein, a 450bp *pfrcc1* fragment was cloned into the *BamHI/EcoRI* digested pGEX-3 expression plasmid as described in method (2.2.22.4). After transformation with the appropriate *E. coli* strain, three colonies were sequenced to confirm that constructs were in frame with the pGEX vector. However, after several rounds of induction these constructs failed to induced the expected protein even though the insert was in frame with the fusion protein sequence.

5.5 Discussion

P. falciparum, in common with probably all eukaryotic cells, does have a RCC1 gene homologue. The most striking feature emerging from the analysis of the cDNA clone (C10) is that it predicts a size of the protein much larger (more than 117 kDa) than that of any previously described RCC1 species. The seven conserved repeats which are constant feature of the RCC1 molecule are all found in the putative *P. falciparum* RCC1 (*pfrcc1*) gene product and their order is also conserved. Each repeat is about 50-60 amino acids long, except for the fourth domain which appears to be longer (about 120 amino acids). Increased size of the fourth repeat has also been observed in other RCC1 proteins such as the *Drosophila* homologue (Dasso, 1993).

Figure 5.6

Southern analysis of *pfrcc1* gene.

A. An autoradiograph demonstrating that *pfrrc1* is a single copy gene. Genomic DNA from *P. falciparum* was digested with various enzymes, resolved, blotted and hybridised with *pfrcc1* specific probe (cDNA clone C10). The enzymes used were : (Lanes 1-12): *AccI, Bgl II, Sau3A, MspI, XhoI, DraI, HincII, HindIII, AluI, HinfI, XbaI* and *NheI.*

The DNA molecular weight marker is indicated in kb.

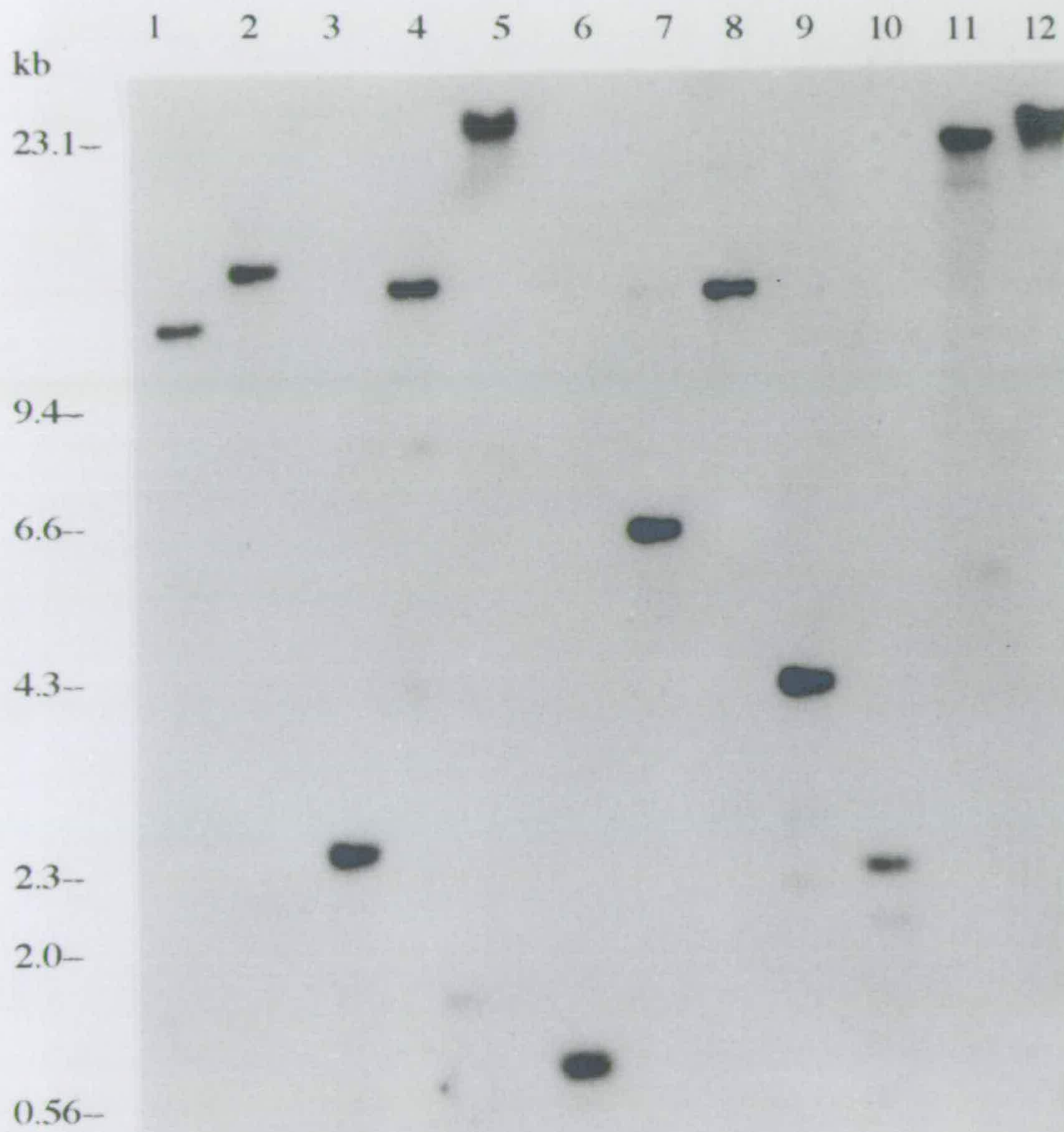
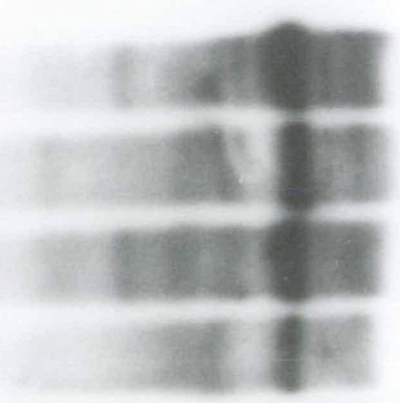

1
2
3
4
5
6
7
8
9
10
11
12
kb
23.1–
9.4–
6.6–
4.3–
2.3–
2.0–
0.56–

Figure 5.7 :

Restriction digestion analysis of *pfrcc1* gene for polymorphism among different *P. falciparum* isolates.

DNA prepared from 4 isolates (clone 102/1 a Sudanese isolate, T9/94 and T9/96 Thai clones, and 7G8 a Brazilian isolate) were digested with four restriction endonucleases, resolved on 1.2% agarose gel, blotted and probed with *pfrcc1* specific sequence.

7G8
126/1
a
T9/96
T9/94


7G8
126/1
b
T9/96
T9/94

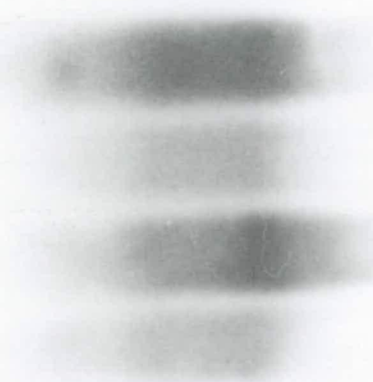
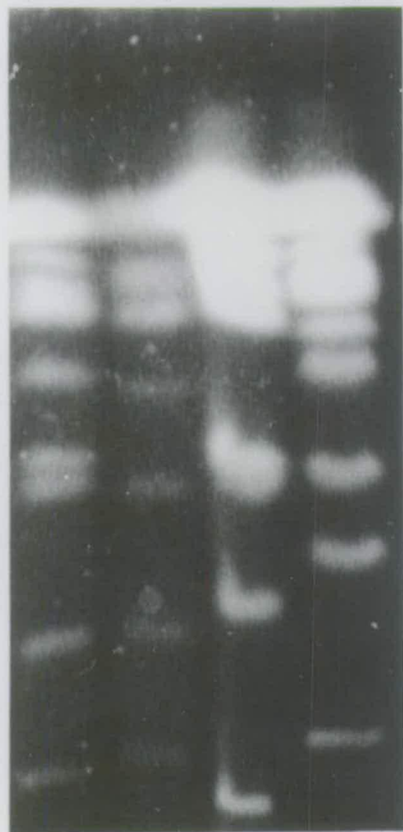

7G8
126/1
c
T9/96
T9/94

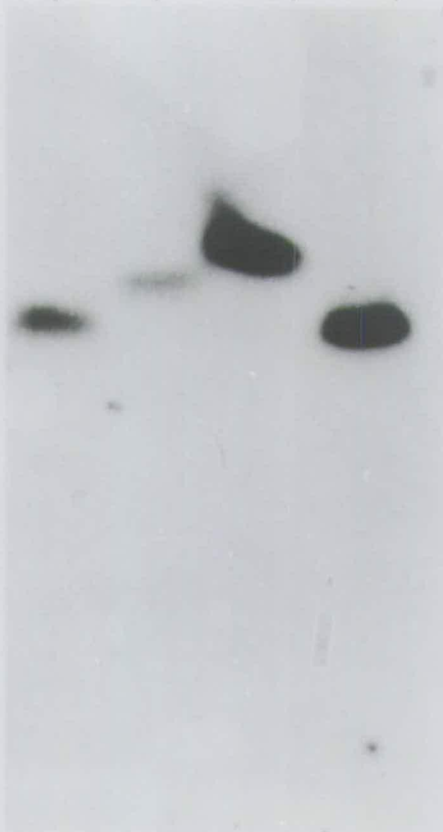
Figure 5.8 : Chromosomal location of *P. falciparum pfrcc1* gene.

A. Left panel is ethidium bromide-stained chromosomes from different *P. falciparum* clones (clones: 3D7, Dd2, T9/94, and 102/1, 105/1, 106/1) separated on 0.8% agarose gel, blotted and probed with *pfrcc1* specific probe (right panel). The signals obtained from all these clones were localised to chromosome 5. Size polymorphisms between different isolates can be seen in this chromosomal blot.

B. Panel (I). Ethidium bromide-stained gel of chromosome from *P. falciprum* clone 105/1 and clone 106/1 separated on 0.8% agarose gel, blotted and probed with *pfrcc1* fragment C10. Panel (II) is an autoradiograph of the gel. The gene was found to be mapped to chromosome 5. As a further confirmation, the same blot was stripped and probed with *pfmdr-1* probe, a known chromosome 5 marker (Foote *et al.*, 1989). This gave a signal in the same position (data not shown).

A





B

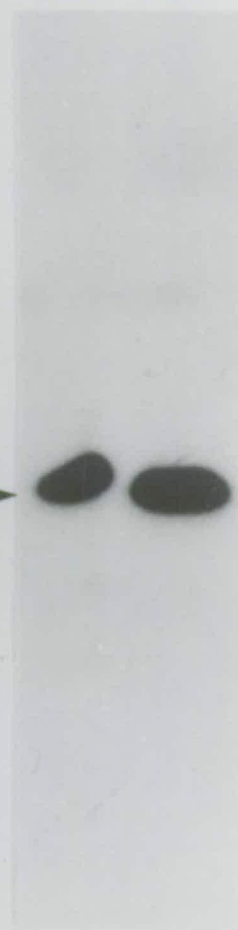
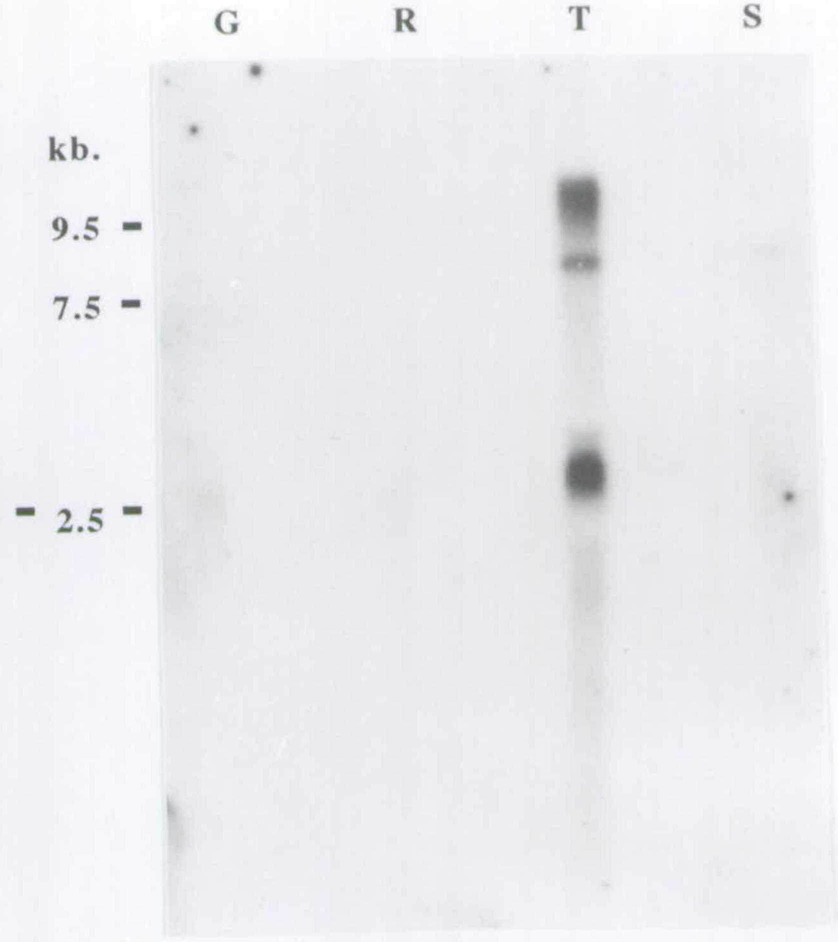


Figure 5.9 : Northern blot analysis of *pfrcc1.*

Right panel is an autoradiograph of total RNA from synchronised culture of gametocytes (lane G), rings (lane R), trophozoites (lane T) and schizonts separated on 1.2% denaturing formaldehyde gel, transferred to Nylon membrane and hybridised to *pfrcc1* specific probe. The same filter was stripped and reprobed with *P. falciparum* actin gene as a control. The sizes of RNA markers are indicated in kb.

G
R
T
S
ACTIN


G
R
T
S
kb.
9.5
7.5
2.5
pfRCC1

In all RCC1 proteins so far identified, the N-terminal region outside the conserved repeats consists of positively charged amino acids. Similar amino acid sequences have been observed for the *P. falciparum* RCC1 homologue. Deletion mutagenesis has demonstrated that the basic N-terminal region outside the repeats is necessary for the human RCC1 protein to bind to DNA directly and that this region is also important for efficient transport in the nucleus (Seino *et al.*, 1992). However, it has been shown that even in the absence of these residues, RCC1 retains a partial function in preventing premature chromosome condensation (PCC) in *ts*BN2 cells and in restoring DNA replication in RCC1-depleted *Xenopus* extracts (Dasso and Newport, 1990; Dasso *et al.*,1991).

Approaches to obtain the full coding length of the RCC1 malarial homologue have not been successful. However, the sequence homology of clone C10 to the gene products of RCC1 from human, *Drosophila* and yeast containing the seven repeated motifs domain characteristic of the pfRCC1 polypeptide is highly significant and strongly suggest a conserved function for these genes. Immediately following the repeated domains, Clone C10 has a C-terminal extension which is 290 amino acids long. C-terminal extensions have been reported in other *P. falciparum* proteins such as DNA polymerase α (White *et al.*, 1993). The *Drosophila* homologue, BJ1, also contains a C-terminal extension of 130 amino acids which is composed of a cluster of glutamic acids, followed by a lysine-rich sequence (Frasch, 1991). Although RCC1 proteins from vertebrates and yeast lack the C-terminus extension, monoclonal antibodies binding to the BJ1 C-terminus react with other RCC1 proteins from these species. It has been suggested that the glutamine-rich stretch near the C-terminus of BJ1 might be a histone-binding region because motifs with similar sequences have been found in nuclear proteins from *Xenopus*, particularly in the histone H1-like protein B4 and the histone-binding proteins N1/N2 and nucleoplasmin (Frasch, 1991). However, the C-terminus extension of the pfRCC1 partial sequence has no homology to any such proteins in the data-bases. Raising specific antibodies to the C-terminal region of pfRCC1 can be used for various biochemical studies such as histone-binding activity and this will help to elucidate the function of such long C-extensions in this molecule.

It has been suggested that BJ1, the *Drosophila* homologue, has evolved from two ancestral sequences by a duplication event, with the C-terminal repeats of more recent origin. The larger size of clone C10 and presence of the C-terminal extension might also suggest the same for the *pfrcc1* homologue. Gene duplication is a known phenomenon in malaria parasites. A good example of an ancient duplication that resulted in the evolution of two gene products performing different but related

functions is the ribosomal RNA genes (Gunderson *et al.*, 1987; McCutchan *et al.*, 1988). Another example of duplicated genes is the histidine rich proteins (HRPII and HRPIII) (Wellems *et al.*, 1987). It has been suggested that the duplication of genes allows the organism to evolve into a more complex state to deal with environmental pressure or to develop an advantage in survival (MaConkey *et al.*, 1990). For example, it has been suggested that gene duplication may arise in *Plasmodium* as a response to selective pressures e.g. the duplication of gene encoding dihydrofolate reductase-thymidylate synthase DHFR-TS in some pyrimethamine resistant strains (Inselburg *et al.*, 1987).

The size of the transcript of the *pfrcc1* gene is found from Northern blot analysis to be approximately 8.5 kb. This is three times larger than the size of sequenced clone C10 which possibly encodes the bulk of the *P. falciparum* RCC1 sequence. This large transcript of the *pfrcc1* gene might be explained by the possibility of the existence of a long 5' untranslated region (5'UTR) in the gene which has been observed in many *P. falciparum* transcripts (Levitt, 1993, Sultan *et al.*, 1994). In addition, identification of such a long C-terminus extension indicated that there might be more coding sequence in this region. Work to obtain the full coding sequence of the *P. falciparum* RCC1 homologue is under way in our laboratory. From the Northern blot analysis carried out on the stage specific RNA of the parasite, it was observed that the level of expression is high at the trophozoite stage. There were no signals observed for RNA from ring and gametocyte forms. This result indicates that the steady state expression of *pfrcc1* mRNA is developmentally regulated, and that RCC1 protein is probably required during nuclear division of the parasites. This result is consistent with the data from *Xenopus* and *Drosophila* . In *Xenopus* oogenesis and early development, RCC1 protein and mRNA are both very abundant, with the mRNA declining during the adult stages, a result which indicates a requirement for the protein in the nuclei of the early embryo (Nishitani *et al.*, 1990). In *Drosophila*, BJ1 mRNA levels are high in oocytes and the maternally expressed mRNA is abundant through the first two hours of development, after which total BJ1 mRNA levels decline rapidly (Frasch, 1991)

To understand better the function of the *pfrcc1* gene product in *P. falciparum* antibodies raised against the protein are needed. This will help to localise the protein in the parasite and see whether the pfRCC1 antibodies co-precipitate the pfRan protein, as it has been the case with the human antibodies against the RCC1 protein. In addition, obtaining the full coding sequence of *pfrcc1* gene will help in understanding the structure of the protein.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The aim of the work presented in this thesis has been to attempt to begin to understand the molecular mechanisms that regulate the parasite cell cycle during the intra-erythrocytic proliferation of the human malaria species *Plasmodium falciparum*. In this study two genes known to be involved in cell cycle regulation in other eukaryotes have been cloned and partially characterised. It was hoped that studying the functions of the proteins encoded by these two genes would help elucidate the cell-cycle pathways which the parasite undergoes in infected erythrocytes. Although it is perhaps more often said than done, such pathways could present alternative targets for anti-parasite therapeutics.

6.1 *pfran* and *pfrcc1* are the homologues of their namesakes.

In an attempt to identify and study proteins that are known to be important regulators of the cell cycle in better studied, higher eukaryotes, the *P. falciparum* homologue of the Ras-related nuclear protein Ran/TC4 has been isolated and sequenced (Sultan *et al.*, 1994). The predicted protein sequence of pfRan is 214 amino acids long and contains the GTP-binding motifs found in all the Ras superfamily of GTPases. PfRan has around 70% amino acid identity with other Ran/TC4 proteins. These two features are significant enough to admit pfRan as a member of the small GTPase protein superfamily and almost certainly the Ran/TC4-type subfamily. It has been shown recently that the *P. falciparum* recombinant PfRan binds GTP in a similar way to other Ras-like proteins (Dontfraid and Chakrabarti, 1994). This further strengthens the argument that pfRan is a GTPase. A second protein that has been isolated as a complex with Ran/TC4 and shown to stimulate its rate of nucleotide exchange *in vitro* , is the RCC1 protein (Bischoff and Ponstingl, 1991a). I have isolated and sequenced a 3.0kb cDNA clone encoding a protein 925 amino acid in length. Sequence comparison with other RCC1 proteins has shown that the clone encoded a protein with significant homology to the other family members especially in regard to the organisation of the seven conserved, approximately 60 amino acids, repeats. Although the ends of the gene have not yet identified, the high level of sequence and repeat organisation conservation make the argument that this gene is the *Plasmodium falciparum* homologue of the RCC1 gene quite convincing.

A striking feature of the *Plasmodium falciparum* RCC1 gene is its long C-terminus extension following the conserved repeats. Although this region has no significant homology to other protein so far found, this could change as the complete sequence emerges. The BJ1 *Drosophila* homologue has a similar extension at the C-terminus, which has some homology with histone 1β and the *Xenopus* nucleoplasmin

proteins B4 and N1/N2. This has led to a suggestion that it might be the histone-binding region (Frasch, 1991).

6.2 What do Ran and RCC1 do in eukaryotic cells?

All the diverse RCC1 homologues are abundant nucleosome bound proteins found within the nucleus during interphase (Frasch 1991) yet disappearing or degraded at mitosis. Although different genetic screens were used to isolate the different types of RCC1 mutant in different organisms, the mutations appear similar. The mutants show chromosomal instability, DNA synthesis defects and often a decrease in overall protein and RNA synthesis and nuclear transport. *In vitro* mutagenesis studies have proved that the basic amino terminal region is essential for human RCC1 to directly bind DNA (Seino *et al*, 1992). Although RCC1 is expressed in all dividing mammalian cells its expression is not tightly coordinated with the cell cycle and it is possible that post translational modifications regulate RCC1 activity during the cell cycle.

The interaction between RCC1 and Ran has been confirmed genetically by Matsumoto and Beach (1991). They first isolated RCC1 as a premature mitosis mutant and then isolated Ran as a multicopy plasmid supressor of these premature mitosis mutants. However, Bischhoff and Ponstingl (1991a) noted that while auto-immune serum co-precipitates RCC1 and Ran there is a 25 fold exess of Ran over the (quite abundant) RCC1. Ran is in fact extremely abundant (0.36% of total HeLa cell protein, 100 x more abundant than Ras protein), and this high concentration appears to be strictly required in that *S. pombe* mutants with one half normal amount of Ran have severe defects in nuclear structure and cell cycle regulation.

Additional functions accounting for this puzzling excess of Ran have been proposed by Moore and Blobel (1994). They showed that two cytosolic fractions (fraction A and fraction B) from *Xenopus* oocytes were sufficient to support protein import into nuclei of digitonin-permeablised cells. Fraction A was necessary for recognition of the nuclear localisation sequences (NLS), whereas fraction B mediated the subsequent passage of bound substrate into the nucleus. After purification, two interacting components were found to be required for full activity of fraction B, and one of these components was the 25kDa *Xenopus* homologue of Ran/TC4. This strongly supports a role for Ran in nuclear import and micro-injection studies have indicated that molecules such as Ran are capable of diffusing through nuclear pores followed by nuclear binding (Forbes, 1992). There is a known intra-nuclear binding site for Ran i.e. RCC1. However, Ran does not contain consensus nuclear localisation sequences (NLS). Such sequences are composed of either a short stretch

of basic amino acids or two basic domains separated by about ten amino acids (Dingwall and Laskey, 1991). The large excess of Ran over RCC1 still seem to leave the question of why Ran is primarily intra-nuclear with an unsatisfactory answer.

Ran and RCC1 seem to be involved in every aspect of nuclear structure and function that is examined in detail and it would be possible to quote recent hypothetical models for their role in eukaryoyic cell biology at even greater length. However , one view, from a leading cell biology laboratory, is that the extremely pleiotropic effects of Ran or RCC1 disruption stem from their key role in intra-cellular import/export pathways (Moore and Blobel 1994). The observed effects on the cell cycle are then explained by the disruption of transport of critical substrates that coordinate and catalyse cytoplasmic and intra-nuclear events. However, in the absence of any real evidence of what kind of cell cycle *Plasmodium falciparum* has, it is wise to curtail further speculation and complete a discussion of the actual data gathered here which might help understand pfRan and pfRCC1 function.

6.3 When and where are pfRan and PfRcc1 expressed in *Plasmodium falciparum*?

Transcription of the pfRan gene is strongly up regulated around the period of trophozoite development (i.e. around 24 hours after invasion). The parasites spend between 6-10 hours in the trophozoite stage then move into schizogeny around 30-34 hours post invasion. Northern blots indicated that steady-state levels of *pfran* were low in rings, high in trophozoites and schizonts and very low or absent in gametocytes. Assays of actual transcriptional activity (nuclear run-on) tend to confirm that these steady-state levels also represent ongoing transcription since *pfran* RNA was only found in the nuclei from trophozoite and schizont stages. The steady state levels of *pfran* RNA reach a peak at the trophozoite and schizont stages that is, when DNA synthesis occurs during development of the parasite in the host cells. This is consistent with a requirement for Ran protein during S-phase of the cell cycle as established in other eukaryotes (Bischoff and Bonstingl, 1991b; Matsumoto and Beach, 1991).

In contrast to other GTPases of the Ras superfamily which play a wide variety of roles in the cytoplasm, Ran/TC4 has been shown to be the only member of the Ras-superfamily of the small-GTP binding proteins to be localised primarily inside the nucleus (Bischoff and Ponstingl, 1991a). Ran/TC4 represents 0.36% of the total protein in HeLa cells and about 80% of this is nuclear-localised (Bischoff and Ponstingl, 1991b). Western blotting with anti-Ran antibody was not carried out on protein extracts from synchronised cultures. The relative accumulation of pfRan

protein during the intra-erythrocytic cycle was not therefore confirmed in this way. However, immunofluorescence staining of the morphologically distinct forms with anti-pfRan antibody revealed a somewhat surprising result. Distinct and specific fluorescence indicate the presence of pfRan protein in both the nucleus and cytoplasm of ring forms. Similar fluorescence pattern indicates the presence of pfRan in both early trophozoites, late trophozoites, and schizonts. In all of these stages fluorescence is not confined to the nucleus but distinctly present in both nucleus and cytoplasm. These observations are consistent with function of pfRan in 'trafficking' RNA between nucleus and cytoplasm (Moore and Blobel, 1994).

In addition, the IFA results has shown that pfRan protein is present in all stages (sexual and asexual) of the parasite in approximately equal amounts. However, the Northern blot analysis has shown that the highest amounts of *pfran* mRNA are present during the trophozoite and schizont stages with much lower message levels present during the gametocyte and ring stages. Antiserum raised against pfRan recognised a major band of 27 kDa predominantly in the asexual stage extracts although a very weak band in the protein extracts from gametocytes was detected in Western blots.

Less work has been carried out on pfRcc1 and I do not yet have an antibody against this protein. RNA expression studies have shown that the *pfrcc1* is expressed mainly during the trophozoite stage. This might suggest that this gene is required, in association with the *pfran* gene, somehow during the DNA replication which peaks at the late trophozoite of the life cycle of the malaria parasite.

The apparent abundant quantity of pfRan in gametocytes, probably in both sexes but certainly abundant in male gametes (Chapter 4, Fig.4.10) are slightly puzzling in view of the low or absent levels of *pfran* gene transcribed in these stages. The conclusion must therefore be that the protein was accumulated in the earlier stages of gametocytic development before morphologically distinct gametocytes appear. The protein could be present in both the sexual and asexual stages but performing different functions during the different developmental stages of the parasite. Since the IFA showed that the pfRan protein is present throughout the intra-erythrocytic cycle of the parasite its GTP versus GDP bound status or possible post-translational modification may be the mechanism that regulate the *in vivo* activity of the pfRan protein. If pfRan serves a true cell cycle-regulatory role in malaria parasite, one would expect such changes to occur in association with DNA replication and /or mitosis. Chromosomal DNA replication in *P. falciparum* starts in trophozoite stage (Gritzmacher and Reese, 1984; Inselburg and Banyal, 1984) and is followed in schizogony by four or five rapid rounds of mitosis and DNA synthesis, finally yielding a schizont containing 16-

32 merozoite, each with a single nucleus. However, even individual schizonts, despite their syncytial nature, rapidly lose synchrony of division (Read *et al.*, 1993), so analysis of the role of any postulated cell cycle regulatory molecules in the actively dividing schizont will probably require an expanded panel of antibodies against proteins known to play key roles in the eukaryotic cell cycle.

6.4 Future work

Two evolutionarily conserved genes, *pfran* and *pfrcc1* that are proposed to function in regulation of the cell cycle in eukaryotes, have been isolated and characterised in *P. falciparum* during the course of this study. Work is underway to complete the missing part of the *Pfrcc1* gene sequence and to raise antibodies to this protein for further analysis. The RCC1 protein has been shown to bind chromatin in other eukaryotic cells probably with a strict one RCC1 molecule to one nucleosome ratio. Therefore, antibodies against the *pfrcc1* would be very helpful in understanding the precise events during mitosis. In addition, functional complementation experiment might be performed on *S. pombe* pim1 mutants to elucidate its role in the cell cycle.

The aim of cell cycle control studies in malaria parasites should be try to understand the mechanisms that regulate parasite development. This will be without any doubt a useful approach to a rational chemotherapy, perhaps by identifying key *P. falciparum* specific cell cycle events.

REFERENCES

Aebi, M., Clark, M.W., Vijayraghavan, U., and Abelson, J. (1990). A yeast mutant, PRP20, altered in mRNA metabolism and maintenance of the nuclear structure, is defective in a gene homologous to the human gene RCC1 which is involved in the control of chromosome condensation. *Mol. Gen. Genet.* 224, 72-80.

Aikawa, M. and Seed, T.M. (1980). Morphology of *Plasmodia.* In: Malaria (edited by Kreier, J.P.), Vol. 1. pp. 285-345. Academic Press, New York.

Alano, P. and Carter, R. (1990). Sexual differentiation in malaria parasites. *Annu. Rev. Microbiol.* 44: 429-449.

Alano, P., Premawansa, S., Bruce, M.C., Carter, R. (1991). A stage specific gene expressed at the onset of gametocytogenesis in *Plasmodium falciparum. Mol. Biochem. Parasitol.*49: 81-88.

Alano, P., Cartwright, L.C. and Carter, R. (1993). In: Protocols in Molecular Parasitology, pp. edited by John Hyde: Humana Press, New Jersey.

Amann, E., Brosius, J. and Ptashne, M. (1983). Vectors bearing a hybrid *rp-lac* promotor useful for regulated expression of cloned genes in *Escherichia coli. Gene* 25,167-178.

Arion, D., Meijier, L., Brizuela, L., and Beach, D. (1988). Cdc2 is a component of the M-phase-specific histone H1 kinase: evidence for identity with MPF. *Cell* 55: 371-378.

Aslund, L., Franzen, L., Weshin, G., Persson, T., Wigzell, H. and Pettersson, U. (1985). Highly reiterated non-coding sequence in the genome of *Plasmodium falciparum* is composed of 21-base pair tandem repeats. *J. Mol. Biol.* 185: 509-516.

Bahr, G.F. and Mikel, U. (1972). The arrangement of DNA in the nucleus of rodent malaria parasites. *Proc.Helminth.Soc.Washington*, 39: 361-372.

Barbacid, M. (1987). *ras* genes. *Annu. Rev. Biochem.* 56, 779-827.

Beach, D.H., Durkacz, B., Nurse, P.M. (1982). Functionally homologous cell cycle control genes in budding and fission yeast. *Nature* 300, 706-709.

Belhumeur, P., Lee, A., Tam, R., DiPaolo, T., Fortin, N.and Clark, M.W. (1993). GSP1 and GSP2, genetic suppressors of the *prp20-1* mutant in *Saccharomyces cerevisiae*: GTP-binding proteins involved in the maintenance of nuclear organisation. *Mol. Cell. Biol* 13: 2152-2165.

Bhasin, V.K., Clayton, C., Trager, W. and Cross, G.A.M. (1985). Variations in the organisation of repetitive DNA sequence in the the genome of *Plasmodium falciparum* clones. *Mol. Biochem. Parasitol.* 15, 149-158.

Birago, C., Bucci, A., Dore, E., Frontali, C. and Zenobi, P. (1982). Mosquito infectivity is directly related to the proportion of repetitive DNA in *Plasmodium berghei. Mol. Biochem. Parasitol.* 6, 1-12.

Bischoff, F.R., Maier, G. Tilz, G. and Ponstingl, H. (1990). A 47-kDa human nuclear protein recognised by antikinetochore autoimmune sera is homologous with protein encoded by RCC1, a gene implicated in the onset of chromosomal condensation. *Proc. Natl. Acad. Sci. USA.* 87, 8617-8621.

Bischoff, F.R. and Ponstingl, H. (1991a). Mitotic regulator protein RCC1 is complexed with a nuclear ras-related polypeptide. *Proc. Natl. Acad. Sci. USA.* 88, 10830-10834.

Bischoff, F.R. and Ponstingl, H. (1991b). Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. *Nature.* 354: 80-82.

Boguski, M.S. and McCormick, F. (1993). Proteins regulating Ras and its relatives. *Nature.* 366:643-654.

Booher, R. and Beach, D. (1986). Site specific mutagenesis of *cdc2*$^+$, a cell cycle control gene of the fission yeast *Schizosaccharomyces pombe. Mol. Cell. Bioll* 6, 3523-3530.

Bourne, H.R., Sanders, D.A. and McCormick, F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. *Nature.* 348:125-132.

Bourne, H.R., Sanders, D.A. and McCormick,F. (1991). The GTPase superfamily: Conserved structure and molecular mechanism. *Nature.*349:117-127.

Bruce-Chwatt, L.J. (1985). Essential Malariology. 2nd Edition, William Heinemann Medical Books Ltd, London.

Bruce, M., Alano, P., Duthie, S. and Carter, R. (1990). Commitment of the malaria parasite *P. falciparum* to asexual and sexual development. *Parasitology* 100, 191-200.

Buluwela, L., Forster, A., Boehm, T., and Rabbitts, T.H. (1989). A rapid procedure for colony screening using nylon filters. *Nucl. Acids Res.*17: 542.

Bush, J. and Cardelli, J. (1993). Molecular cloning and DNA sequence of a *Dictyostelium* cDNA encoding a Ran/TC4 related GTP-binding protein belonging to the Ras superfamily. *Nucl.Acids Res.* 21: 1675.

Carter, R. and Miller, L.H. (1979). Evidence for environmental modulation of gametocytogenesis in *Plasmodium falciparum* in continuous culture. Bulletin of the World Health Organisation. 57, 37-52.

Cary, C., Lamont, D., Dalton, J.P. and Doerig, C. (1994). *Plasmodium falciparum* chromatin: nucleosomal organisation and histone-like proteins. *Parasitol. Res.* 80, 255-258.

Casaglia, O., Dore, E., Frontali, C., Ramoni, C. and Zenobi, P. (1985a). Cytofluorometric analysis of DNA content in different life stages of *Plasmodium yoelii. Annali dell'Istituto Superiore di Sanita.* 21, 401-406.

Casaglia, O., Dore, E., Frontali, C., Zenobi, P. and Walliker, D. (1985b). Re-examination of earlier work on repetitive DNA and mosquito infectivity in rodent malaria. *Mol. Biochem. Parasitol.* 16, 35-42.

Cerami, C., Travert, V., Sinnis, P., Tackacs, B., Clavijo, P., Santos, M.J. and Nussenzweig, V. (1992). The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of *Plasmodium falciparum* sporozoites. *Cell* 70, 1021-1033.

Chakrabarti, D., Schuster, S.M. and Chakrabarti, R. (1993). Cloning and characterisation of subunit genes of ribonucleotide reductase, a cell-cycle-regulated enzyme, from *Plasmodium falciparum. Proc. Natl. Acad. Aci (USA)* 90, 12020-12024.

Chavier, P., Simons, K. and Zerial, M. (1992). The complexity of the Rab and Rho GTP-binding protein subfamilies revealed by a PCR cloning approach. *Gene* 112, 261-264.

Cheesman, S., McAleese, S., Goman, M., Johnson, D., Horrocks, P., Ridley, R.G., Kilbey, B.J. (1994). The gene encoding topoisomerase II from *Plasmodium falciparum. Nucl. Acids Res.* 22, 2547-2551.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18, 5294-5299.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate phenol chloroform extraction. *Analyt. Biochem.* 162: 156-159.

Clark, K. and Sprague, G. (1989). Yeast pheromone response pathway: characterisation of a suppressor that restores mating to receptorless mutants. *Mol. Cell. Biol.* 9, 2682-

Colasanti, J, Tyres, M., and Sundaresan, V. (1991). Isolation and identification of cDNA clones encoding a functional $p34^{cdc2}$ homologue from *Zea mays. Proc. Natl. Acad. Sci. (USA)* 88, 3377-3381.

Corcoran, L.M., Forsyth, K.P., Bianco, A.E., Brown, G.V., and Kemp, D.J. (1986). Chromosome size poymorphisms in *Plasmodium falciparum* can involve deletions and are frequent in natural parasite populations. *Cell* 44, 87-95.

Cornelissen, A.W.C.A., Overdulve, J.P. and Van der Ploeg, M. (1984). Determination of nuclear DNA of five eucoccidian parasites, I*sospora (Toxoplasma)*

gondii, Sarcocystis cruzi, Eimera tenella, E. acervulina and Plasmodium berghei, with special reference to gametogenesis and meiosis in *I.(T) gondii. Parasitology* 88, 531-553.

Cornelissen, A.W.C.A., Langsley, G., Walliker, D.and Scaife, J.G. (1985). Gametocytogenesis and ribosomal RNA gene organisation in rodent malarias *Plasmodium chabaudi* and *Plasmodium berghei. Mol. Biochem. Parasitol.*, 14:165-174.

Cornelissen, A.W.C.A. (1988). Sex determination and sex differentiation in malaria parasites. *Biol. Rev.* 63, 379-394.

Creasey, A.M., Ranford-Cartwright L., Moore, D.J., Williamson, D.H., Wilson, R.J., Walliker, D. and Carter, R. (1993). Uniparental inheritance of mitochondrial gene cytochrome b in *Plasmodium falciparum. Curr. Genet.* 23, 360-364.

Creedon, K.A., Kaslow, D.C.,, Rathod, P.K., Wellem, T.E. (1992). Identification of a *Plasmodium falciparum* histone H2A gene. *Mol. Biochem. Parasitol.* 54, 113-116.

Crowe, J.S., Cooper, H.J., Smith, M.A., Sims, M.J., Parker, D., and Gewert, D. (1991). Improved cloning efficiency of polymerase chain reaction (PCR) products after proteinase K digestion. *Nucl. Acids Res.* 19 (1), 184.

Dasso, M. and Newport, J.W. (1990). Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis *in vitro*: studies in *Xenopus. Cell* 61, 811-823.

Dasso, M., Nishitani, H., Kornbluth, S., Nishimoto, T. and Newport, J.W. (1992). RCC1, a regulator of mitosis, is essential for DNA replication. *Mol. Cell. Biol.* 12: 3337-3345.

Dasso, M. (1993). RCC1 in the cell cycle: the regulator of the chromosome condensation takes on new roles. *Trends Biochem.Sci.* 18: 96-101.

Delves, C.J., Ridley, R.G., Goman, M., Holloway, S.P., Hyde, J.E. and Scaife, J.G. (1989). Cloning of a β-tubulin gene from *Plasmodium falciparum. Mol. Microbiol.* 11, 1511-1519.

Delves, C.J., Alano, P., Ridley, R.G., Goman, M., Holloway, S.P., Hyde, J. E. and Scaife, J.G. (1990). Expression of α and β tubulin genes during the asexual and sexual blood stages of *Plasmodium falciparum.. Mol. Biochem. Parasitol.* 43: 271-278.

Devereux, J., Haeberli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.*12: 387-395.

DeVries, E., Stam, J.G., Franssen, F.F.J., Van der Vliet, P.C. and Overdulve, J.P. (1991). Purification and characterisation of DNA polymerases from *Plasmodium berghei. Mol. Biochem. Parasitol.* 45, 223-232.

Dingwall, C. and Laskey, R.A. (1991). Nuclear targetting sequence; a consensus. *Trends Biochem.Sci.* 16, 478-481.

Dissanayake, S., Xu, M. and Piessens, W.F. (1992). Filarial parasites contain a Ras homolog of the TC4/Ran/Spi family. *Mol. Biochem.Parasitol.* 56: 259-268.

Dontfraid, F.F. and Chakrabarti, D. (1994). Cloning and expression of a cDNA encoding the homologue of Ran/TC4 GTP-binding protein from *Plasmodium falciparum. Biochem. Biophsic. Res. Commun.* 201, 423-429.

Dore, E. , Birago, C., Frontali, C., and Battaglia, P.A. (1980).Kinetic complexity and repetitivity of *Plasmodium berghei* DNA. *Mol. Biochem. Parasitol.*, 1: 199-208.

Dore, E., Pace, T., Ponzi M., Scotti, R. and Frontali, C. (1986). Homologous telomeric sequences are present in different species of the genus *Plasmodium. Mol. Biochem. Parasitol.* 21: 121-127.

Doree, M. (1990). Control of M-phase by maturation-promoting factor. *Curr. Opin. Cell Biol.* 2, 269-273.

Downward, J. (1992). Rac and Rho in tune. Nature 359, 273-274.

Draetta, G., Brizuela, L., Potashkin, J. and Beach, D. (1987). Identification of p34 and p13, human homologs of the cell regulators of fission yeast encoded by *cdc2*$^+$ and *suc1*$^+$. *Cell* 50, 319-325.

Draetta, G. and Beach, D. (1989). The mammalian cdc2 protein kinase: mechanisms of regulation during the cell cycle. *Journal of Cell Science Supplement* 12, 21-27.

Drivas, G.T., Shih, A., Coutavas, E.E., Rush, M.G. and D'Eustachio, P. (1990). Characterisation of four novel ras-like genes expresssed in a human teratocarcinoma cell line. *Mol.Cell.Biol.* 10: 1793-1798.

Drivas, G.T., Palmieri, S., D'Eustachio, P., and Rush, M.G. (1991). Evolutionary grouping of the Ras-protein family. *Biochem. Biophys. Comm.* 176, 1130-1133.

Dulzewski, A.R., Ling, I.T., Rangachari, Bates, P.A., and Wilson, R.J.M., (1984) A simple method for isolating viable mature parasites of *Plasmodium falciparum* from cultures. *Trans. Roy. Soc. Trop. Med. Hyg.* 78, 622-624.

Elliott, J.F., Albrecht, G.R., Gildadoga, A., Haundunnett, S.M., Neequaye, J., Lallinger, G., Minjas, J.N., Howard, R.J., (1990). Genes for *Plasmodium falciparum* surface antigens cloned by expression in COS cells. *Proc. Natl. Acad. Sci. (U.S.A.)* 87: 6363-6367.

Enea, V., Ellis, J., Zavala, F., Arnot, D. E., Asavanich, A., Masuda, A., Quakyi, I. and Nussenzweig, R.S.(1984). DNA cloning of *Plasmodium falciparum* circumsporzoite gene: Amino acid sequence of repetitive epitope. *Science* 225, 628-630.

Enoch, T. and Nurse, P. (1990). Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. *Cell* 60, 665-673.

Enoch, T., Carr, A.M. and Nurse, P. (1992). Fission yeast genes involved in coupling mitosis to completion of DNA replication. *Genes Develop.* 6, 2035-2046.

Feinberg, A.P. and Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analy. Biochem.* 132: 6-13.

Feng, D-F and Doolittle, R.F. (1987). Progressive sequence alignment as a prerequisite to correct phylogenetic trees. *J. Mol. Evolu.* 35, 351-360.

Fenton, B. and Walliker, D. (1990). Genetic analysis of polymorphic proteins of the human malaria parasite *Plasomdium falciparum. Genet. Res. Cambridge* 55, 81-86.

Ferone, R., Burchall, J.J. and Hitchings, G.H. (1969). *Plasmodium berghei* dihydrofolate reductase. isolation, properties and inhibition by antifolates. *Molec. Pharmac.* 5, 49-59.

Foote, S.J. and Kemp, D.J. (1989). Chromosomes of malaria parasites. *Trends in Genetics* . 5: 337-342.

Foote, S.J., Thompson, J.K., Cowman, A.F. and Kemp, D.J. (1989). Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum. Cell* 57:921-930.

Forbes, D.J. (1992). Structure and function of the nuclear pore complex. *Annu. Rev. Cell Biol.* 8, 495-527.

Forsburg, S. and Nurse, P. (1991). Cell cycle regulation in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe. Annu Rev. Microbiol* 7:227-256.

Francoeur, A.M., Gritzmacher, C.A., Peebles, C.L., Reese, R.T. and Tan, E.M. (1985). Synthesis of small nuclear ribonucleoprotein particles by the malarial parasite *Plasmodium falciparum. Proc. Natl. Acad Sci. (USA).*82: 3635-3639.

Frasch, M. (1991). The maternally expressed *Drosophila* gene encoding the chromatin-binding protein BJ1 is a homolog of the vertebrate gene regulator of chromatin condensation, RCC1. *EMBO. J.* 10, 1225-1230.

Freeman, R.S. and Donoghue, D.J. (1991). Protein kinases and proto-oncogenes: biochemical regulator of the eukaryotic cell cycle. *Biochemistry* 30, 2293-3302.

Gautier, J., Norbury, D., Lohka, M., Nurse, P. and Maller. J. (1988). Purified maturation promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene *cdc2*+. *Cell* 54, 433-439.

Gilman, A.G. (1984). G-proteins and dual control of adenylate cyclase. *Cell* 37, 577-579.

Gilman, A.G. (1987). G-proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* 56, 615-649.

Goldfarb, D.S. (1994). GTPase cycle for nuclear transport. *Curr.Biol.* 4, 57-60.

Goman, M., Langsley, G., Hyde, J.E., Yankovsky, N.K., Zolg, J.W. and Scaife, J.G. (1982). The establishment of genomic DNA libraries for the human malaria parasite *Plasmodium falciparum* and identification of individual clones by hybridisation. *Mol. Biochem. Parasitol.* 5: 391-403.

Gritzmacher, C.A. and Reese, R.T. (1984). Protein and nucleic acid synthesis during synchronized growth of *Plasmodium falciparum. J. Bacteriol.* 5, 391-400.

Gunderson, J.H., Sogin, M.L., Wollett, G., Hollingdale, M., de la Cruz, V. (1987). Structurally distinct, stage specific ribosomes occur in *Plasomdium. Science* 238, 933-937.

Guntaka, R.V., Gowda, S., Rao, A.S. and Green, T.J. (1985). Organisation of *Plasmodium falciparum* genome: I. Evidence for a highly repeated DNA sequence. *Nucl. Acids Res.* 13, 1965-1975.

Gutteridge, W. E., Trigg, P.I. and Williamson, D.H. (1971). Properties of DNA from some malarial parasites. *Parasitology* 62, 209-219.

Hadley, T.J., Koltz, F.W. and Miller, L.H. (1986). Invasion of erythrocytes by malaria parasites: a celluar and molecular overview. *Annu. Rev. Microbiol.* 40, 451-477.

Hall, A. (1990). The cellular function of small GTP-binding protein. *Science* 249: 635-640.

Hanahan, D. and Meselson, M. (1983). Plasmid screening at high colony density. *Methods. Enzymol.* 100: 333.

Hanahan, D. (1983). Studies in transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166: 557-580.

Hartwell, L.H. and Weinert, T.D. (1989). Checkpoints : control that ensure the order of cell cycle events. *Science* 246, 629-634.

Hawking, F., Wilson, M.E., and Gammage, K. (1971). Evidence for cyclic development and short lived maturity in the gametocytes of *Plasmodium falciparum. Trans. Roy. Soc. Trop. Med. Hyg.* 65: 549-559.

Heidrich, H.G. (1988). Isolation and purification of malaria parasites and their antigens, in *Malaria: Principles and Practice of Malariology* (Wernsdorfer, W.H. and McGregor, I, eds), Churchill Livingstone, Edinburgh, UK, 349-377.

Holder, A.A., Lockyer, M.J., Odink, K.G., Sandhu, J.S., Riveros-Moreno, V., Nicholls, S.C., Hillman, Y., Davey, L.S., Tizard, M.L., Schwartz, R.T. and Freeman, R.R. (1985). Primary sequence of the precursor to the three major surface antigens of *P. falciparum* merozoite. *Nature* 317, 270-273.

Homewood, C.A. and Neame, K.D. (1980). Biochemistry of malarial parasites. In: Malaria (edited by Kreier, J.P.), Vol. 1. pp. 346-406. Academic Press, New York.

Hough-Evans, B.R. and Howard, J. (1982). Genome size and DNA complexity of *Plasmodium falciparum. Biochemica et Biophysica Acta.* 698, 56-61.

Howard, A., and Pelc, S.R. (1953). Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. *Heredity (London) supplement* 16, 261-273.

Howard, R. J., Kaushal, D.C. and Carter, R. (1982). Radio-iodination of parasite antigen with 1,3,4,6-tetrachloro-3α-6α-diphenylglycoluril (IODOGEN): studies with zygotes of *Plasmodium gallinaceum. J. Protozol.* 29, 114-117.

Hyde, J.E., Goman, M., Hall, R., Osland, A., Hope, I.A., Langsley, G., Zolg, J.W. and Scaife, G.J. (1984). Characterisation and translation studies of messenger RNA from the human malaria parasite *Plasmodium falciparum* and construction of a cDNA library. *Mol. Biochem. Parasitol.* 10: 269-285. **Hyde, J.E.** (1993). In: Protocols in Molecular Parasitology, first edition. Humana Press, Totowa, New Jersey.

Ifediba, T. and Vandenberg, J. P. (1981). Complete *in vitro* maturation of *Plasmodium falciparum* gametocytes. *Nature.* 294:364-366.

Ikegami, S., Taguchi, T., Ohashi, M., Ogura, M., Nagono, A. and Mano, Y. (1978). Aphidicolin prevents mitotic cell division by interfering with the activity of DNA polymerase-α. *Nature* 275, 458-460.

Inselburg, J. and Banyal, H.S. (1984). Synthesis of DNA during the asexual cycle of *Plasmodium falciparum* in culture. *Mol. Biochem. Parasitol.* 10: 79-87.

Inselburg, J., Bzik, D.J. and Horii, T. (1987). Pyrimethamine resistant *Plasmodium falciparum*: overexpression of dihydrofolate reductase by a gene duplication. *Mol. Biochem. Parasitol.* 26, 121-134.

Janse, C., Van der Klooster, P.F.J., Van der Kaay, H.J., Van der Ploeg, M., and Overdulve, J.P. (1986a). Mitomycin C is an unreliable inhibitor of DNA synthesis in *Plasmodium. Mol. Biochem. Parasitol.* 20: 33-36.

Janse, C., Van der Klooster, P.F.J., Van der Kaay, H.J., Van der Ploeg, M., and Overdulve, J.P. (1986b). DNA synthesis in *P.berghei* during asexual and sexual development. *Mol. Biochem. Parasitol.* 20: 173-182.

Janse, C.J., Van der Klooster, P.F.J., Van der Kaay, H.J., Van der Ploeg, M. and Overdulve, J.P. (1986c). Rapid repeated DNA replications during microgametogenesis and DNA synthesis in young zygotes of *P. berghei. Trans. Roy. Soc. Trop. Med. Hyg.* 80: 154-157.

Janse, C., Ponnudurai, T., Lensen, A.H.W., Meuwissen, J.H.E.Th., Ramesar, J., Van der Ploeg, M., and Overdulve, J.P. (1988). DNA synthesis in gametocytes of *Plasmodium falciparum.. Parasitology* 96: 1-7.

Janse, C.J., Boorsma, E.G., Ramesar, J., Van Vianen, Ph., Van der Meer, R. (1989). *Plasmodium berghei*: Gametocyte production, DNA content and chromosome size polymorphisms during asexual multiplication *in vivo. Exp. Parasitol.* 68: 274-282.

Janse, C.J. (1992). Chromosome size polymorphism and DNA rearrangements in *Plasomdium. Parasitol. Today* 9, 19-22.

Jensen, J.B. (1978). Concentration from continuous culture of erythrocytes infected with trophozoites and schizonts of *Plasmodium falciparum. Am. J. Trop. Med. Hyg.* 27, 1274-1276.

Kadowaki, P., Goldfarb, D.S., Spitz, L., Ohno, M. and Tartakoff, A. (1993). Regulation of RNA processing and transport by a nuclear guanine nucleotide release protein and members of the Ras superfamily. *EMBO. J.* 12, 2929-2937.

Kemp, D.J., Corcoran, L.M., Coppel, R.L., Stahl, H.D., Bianco, A.E., Brown, G.V. and Anders, R.F. (1985). Size variation in chromosomes from independent cultured isolates of *Plasmodium falciparum. Nature* 315: 347-350.

Kemp, D.J., Coppel, R.L., Stahl, H.D., Bianco, A.E., Corcoran, L.M., McIntyre, P., Lanford, C.J., Favaloro, J.M., Crewther, P.E., Brown, G.V., Mitchel, G.E., Culveror, J.G. and Andres, R.F. (1986). Genes for antigens of *Plasmodium falciparum. Parasitology.* 91, 83-108.

Kemp, D.J., Coppel, R.L. and Anders, R.F. (1987a). Repetitive proteins and genes of malaria. *Ann. Rev. Microbiol.* 41:181-208.

Kemp, D.J., Thompson, J.K., Walliker, D. and Corcoran, L.M. (1987b). Molecular karyotype of *Plasmodium falciparum*: conserved linkage groups and expendable rich protein genes. *Proc. Natl. Acad. Sci. (U.S.A.)* 84:7672-7676.

Kemp, D.J., Cowman, A. and Walliker, D. (1990). Genetic diversity in *Plasmodium falciparum. Adv. Parasitol.* 29, 75-149.

Kerr, P.J., Randford-Cartwright, L.C., and Walliker, D. (1994). Proof of intragenic recombination in *Plasmodium falciparum. Mol. Biochem. Parasitol.* 66, 241-248.

Kilbey, B.J., Fraser, I., McAleese, S., Goman, M., Ridley, R.G. (1993). Molecular characterisation and stage-specific expression of proliferating cell nuclear antigen (PCNA) from the malaria parasite, *Plasmodium falciparum. Nucl. Acids Res.* 21, 239-243.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 277: 680-685.

Lambros, C. and Vanderberg, J.P. (1979). Synchronisation of *Plasmodium falciparum* erythrocytic stages in culture. *J. Parasitol.* 65: 418-420.

Langsley, G., Sibilli, L., Mattei, D., Falanga, P. and Mercereau-Puijalon, O. (1987). Karyotype comparison between *P. chabaudi and P. falciparum*: Analysis of a *P. chabaudi* cDNA containing sequences highly repetitive in *P. falciparum. Nucl. Acid Res.* 15, 2203-2211.

Lee, M.G. and Nurse, P. (1987). Complementation used to clone a human homologue of fission yeast cell cycle gene *cdc2. Nature* 327, 31-35.

Lehner, C.F. and O'Farrel, P.H. (1990). *Drosophila cdc2* homologs: a functional homologue is expressed with a cognate variant. *EMBO J.* 9, 3573-3581.

Levitt, A. (1993). RNA processing in malarial parasites. *Parasitol. Today.* 9, 465-468.

Mannervik, B. (1985). The isoenzymes of Glutathione S-transferase. *Adv. Enzymol.* 357-417.

Manser, E., Leung, T., Salihuddin, H., Zhao, Z. and Lim, L. (1994). A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* 367, 40-46.

Marsh, L. (1991). Signal transduction during phermone response in yeast. *Annu. Rev. Cell. Biol.* 7, 699-728.

Marzluff, W.F. and Huang, R.C. (1989) Transcription of RNA in isolated nuclei. pp.89-129. In: Transcrition and translation. edited by Hames, B.D. and Higgins, S.J. BRL Press.

Masui, Y. and Marker, C.L. (1971). Cytoplasmic control of nuclear behaviour during meiotic maturation of frog oocytes. *J. Exp. Zool.* 177, 129-146.

Matsumoto, T. and Beach, D. (1991). Premature initiation of mitosis in yeast lacking RCC1 or an interacting GTPase. *Cell.* 66, 247-360.

Matsumoto, T. and Beach, D. (1993). Interaction of the piml/spil mitotic checkpoint with a protein phosphatase. *Mol. Biol. Cell.* 4, 337-345.

Maundrell, K. (1993). Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene* 123, 127-130.

McCutchan, T.F., Dame, J.B., Miller, L.H. and Barnwell, J. (1984). Evolutionary relatedness of *Plasmodium* species as determined by the structure of the DNA. *Science* 225, 808-811.

McCutchan, T.F., de la Cruz, V., F., Lal, A.A., Gunderson, J.G., Elwood, H.J. (1988). Primary sequences of two small subunit ribosomal RNA genes from *Plasomdium falciparum. Mol. Biochem. Parasitol.* 28, 63-68.

McConkey, G.A., Waters, A.P. and McCutchan, T.F. (1990). The generation of genetic diversity in malaria parasites. *Annu. Rev. Microbiol.* 44, 479-498.

Mead, D.A., Kristy Pey, N., Herrnstadt, C., Marcil, R.A., and Smith, L.M. (1991). A universal method for the direct cloning of PCR amplified nucleic acid. *Biotechnology* 9, 657-663.

Meijer, L., Arion, D., Golsteyn, R., Pines, J., Brizuela, L., Hunt, T. and Beach, D. (1989). Cyclin is a component of the sea urchin egg M-phase specific histone H1 kinase. *EMBO J.* 8, 2275-2282.

Miao, G-H., Hong, Z. and Verma, D.P.S. (1993). Two functional soybean genes encoding p34^{cdc2} protein kinases are regulated by different plant development pathways. *Proc. Natl. Acad. Sci. (USA).* 90, 943-947.

Millar, J.B.A. and Russell, P. (1992). The *cdc2* M-phase inducer: an unconventional protein phosphatase. *Cell* 68, 407-410.

Milligan, G. (1988). Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine binding proteins. *Biohem. J.* 255, 1-13.

Milligan, G. (1989). Guanine nucleotide-binding proteins and cellular control. *Curr. Opin. Cell Biol.* 1, 196-200.

Mitchison, J.M. (1971). The biology of the cell cycle. pp58-114. Cambridge University Press. Cambridge, U.K.

Moore, M.S. and Blobel, G. (1993). The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature* 365, 661-663.

Moore, M.S. and Blobel, G. (1994). A G-protein involved in nucleocytoplasmic transport: the role of Ran. *TIBS* 19, 211-216.

Mottram, J.C., Kinnaird, J.H., Shiel, B.R., Tait, A. and Barry, D.J. (1993). A novel CDC2-related protein kinase from *Leishmania mexicana*, LmmCRK1, is post-transcriptionally regulated during the life cycle. *J. Biol. Chem.* 268, 21044-21052.

Murray, A.W. and Hunt, T. (1993) The Cell Cycle, an introduction. pp 3-12. W.H. Freeman, New York.

Newport, J.W. and Kirschner, M.W. (1984). Regulation of the cell cycle during early *Xenopus* development. *Cell.* 37, 731-742.

Nishimoto, T., Eilen, E. and Basilico, C. (1978). Premature chromosome condensation in ts DNA$^-$ mutant of BHK cells. *Cell* 15, 475-483.

Nishimoto,T., Ishida, R., Ajiro, K.,Yamamoto, S. and Takahashi,T. (1981). The synthesis of protein(s) for chromosome condensation may be regulated by a post-transcriptional mechanism. *J. Cell Physiol.* 109, 299-308.

Nishitani, H., Kobayashi, H., Ohtsubo, M and Nishimoto, T. (1990). Cloning of *Xenopus* RCC1 cDNA, a homolog of the human RCC1 gene: complementation of tsBN2 mutation and identification of the product. *J. Biochem.* 107, 228-235.

Nishitani, H., Ohtsubo, M., Yamashita, K., Iida, H., Pines, J., Yasudo, H., Shibata, Y., Hunter, T. and Nishimoto, T. (1991). Loss of RCC1, a nuclear DNA binding protein, uncouples the completion of DNA replication from the acctivaattion of cdc2 protein kinase and mitosis. *EMBO.J.* 10, 1555-1564.

Norbury, C. and Nurse, P. (1992). Animal cell cycles and their control. *Ann. Rev. Biochem.* 61, 441-470.

Nurse, P. and Thuriaux, P. (1980). Regulatory genes controlling mitosis in the fission yeast *Saccharomyces cerevisiae. Genetics* 96,627-637.

Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. Nature 344, 503-508.

Ohtsubo, M., Kai, R., Furuno, N., Sekiguchi, T., Sekiguchi, M., Hayashida, H., Kuma, K., Miyata, T., Fukushige, S., Murotu, T. Matsubara, K. and Nishimoto,T. (1987). Isolation and characterisation of the active cDNA of the human cell cycle gene (RCC1) involved in the regulation of onset of chromosome condensation. *Genes Develop.* 1, 585-593.

Ohtsubo, M., Okazaki, H. and Nishimoto, T. (1989). The RCC1 protein, a regulator for the onset of chromosome condensation locates in the nucleus and binds to DNA. *J. Cell. Biol.* 109, 1389-1397.

Ohtsubo, M., Yoshida ,T., Seino,H., Nishitani ,H., Clark , K.L., Spragu, F., Frasch, M. and Nishimoto, T. (1991). Mutation of the hamster cell cycle gene RCC1 is complemented by the homologous genes of *Drosophila* and *S.cerevisiae*. *EMBO J.* 10, 1265-1273.

Oquendo, P., Goman, M., MacKay, M., Langsley, G., Walliker, D.and Scaife, J.G. (1986). Characterisation of a repetitive DNA sequence from the malaria parasite, *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 18, 89-101.

Pace, T., Ponzi, M., Dore, E., Janse, C.J., Mons, B. and Frontali, C. (1991). Long insertions within telomeres contribute to chromosome-size polymorphism in *Plasmodium berghei*. *Mol. Cell. Biol.* 10, 6759-6764.

Peter, M. and Herskowitz, I. (1994). Joining the complex: cyclin-dependent kinase inhibitory proteins and the cell cycle. *Cell* 79, 181-184.

Peters, W. (1987). In: Chemotherapy and drug resistance in malaria. Academic Press. London.

Pollack, Y., Katzen, A.L., Spira, D.T. and Golenzer, J. (1982). The genome of *Plasmodium falciparum*. I: DNA base composition. *Nucl. Acids Res.* 10, 539-546.

Ponzi, M., Pace, T., Dore, E. and Frontalli, C. (1985). Identification of a telomeric DNA sequence in *Plasmodium berghei*. *EMBO J.* 4, 2991-1995.

Prensier, G. and Slomianny, C. (1986). The karyotype of *Plasmodium falciparum* determined by ultrastructural serial sectioning and 3D reconstruction. *J. Parasitol.* 72, 731-736.

Pryer, N.K., Wuestehube, L.J. and Schekman, R. (1992). Vesicle-mediated protein sorting. *Annu. Rev. Biochem.* 61,471-561.

Rao, A.S., Green, T.J. and Guntaka, R.V. (1986). Organisation of *Plasmodium falciparum* genome: II. Sequence analysis of *falci*. element. *Nucl. Acid Res.* 14, 9542.

Read, M., Sherwin,T., Holloway, S.P., Gull, K. and Hyde, J.E. (1993). Microtubular organisation visualised by immunofluorescence microscopy during erythrocytic schizogony in *Plasmodium falciparum* and investigation of post-transcriptional modification of parasite tubulin. *Parasitology* 106, 223-232.

Reed, S.I. (1980). The selection of *S. cerevisiae* mutants defective in the start event of cell division. *Genetics* 95, 561-577.

Ren, M., Drivas, G., D'Eustachio, P., and Rush, M.G. (1993). Ran/TC4: a small nuclear GTP-binding protein that regulates DNA synthesis. *J. Cell. Biol.* 120, 313-323.

Ridley, R.G., White, J.H., McAleese, S.M., Goman, M., Alano, P., Vries, E. and Kilbey, B.J. (1991). DNA polymerase δ: gene sequences from *Plasmodium falciparum* indicate that this enzyme is more highly conserved than DNA polymerase α. *Nucl. Acids Res.* 19, 6731-6736.

Robbins, E. and Scharff, M. (1967). The absence of detectable G1 phase in a cultured strain of Chinese hamster lung cell. *J. Cell Biol.* 34, 684-685.

Roberge, M. (1992). Checkpoint controls that couple mitosis to completion of DNA replication. *Trends Cell Biol.* 2, 277-281.

Ross-MacDonald, P.B., Graeser, R., Kappes, B., Franklin, R. and Williamson, D.H. (1994). Isolation and expression of a gene specifying a *cdc2*-like protein kinase from the human malaria parasite *Plasmodium falciparum*. *Europ. J. Biochem.* 220, 693-701.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Elrich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487-491.

Sambrook, J., Fritch, E.F. and Maniatis, T. (1989). In: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain termination inhibitors. *Proc. Natl. Acad. Sci. (U.S.A.)* 74, 5463-5467.

Saul, A. and Battisutta, D. (1988). Codon usage in *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 27, 35-42.

Schwartz, D.C. and Cantor, C.R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* 37, 67-75

Scovassi, A.I., Pelvani, P. and Bertazzoni, V. (1980) Eukaryotic DNA polymerases. *Trends iBiochem. Sci.* 5, 335-337.

Seino, H., Hisamoto, N., Uzawa, S., Sekiguchi, T. and Nishimoto, T. (1992). DNA-binding domain of RCC1 protein is not essential for coupling mitosis with DNA replication. *J. Cell Sci.* 102, 393-400.

Seki, T., Yamashita, K., Nishitani, H., Takagi, T., Russell, P. and Nishimoto, T. (1992). Chromosome condensation caused by loss of RCC1 function requires the cdc25 protein that is located in the cytoplasm. *Mol. Biol. Cell* 3, 1373-1379.

Sharkey, A., Langsley, G., Paturapotikul, J., Mercereau-Puijalon, O., Maclean, A.P. and Walliker, D. (1988). Chromosome-size variation in the malaria parasite of rodent, *Plasmodium chabaudi*. *Mol. Biochem. Parasitol.* 28, 48-54.

Segal, A.W. and Abo, A. (1993). The biochemical basis of the NADPH oxidase of phagocytes. *Trends Biochem. Sci.* 18, 43-47.

Sheppard, M., Thompson, J. K., Anders, R. F., Kemp, D.J. and Lew, A.M.(1989). Molecular karyotyping of rodent malarias ; *Plasmodium chabaudi, P. berghei* and *P. vinkei. Mol. Biochem. Parasitol.* 34, 45-52.

Sherman, I.W. (1979). Biochemistry of *Plasmodium* (malaria parasites) *Microbiological Reviews* 43, 445-495.

Simon, M.I., Strathmann, M.P. and Gautam, N. (1991). Diversity of G-Proteins in signal transduction. *Science* 252, 802-807.

Sinden, R.E., Canning, E.U. and Spain, B. (1976). Gametocytogenesis and fertilisation in *Plasmodium yoelii nigeriensis*: a transmission electron microscope study. *Proc. Roy. Soc.* (London) B193, 55-76.

Sinden, R.E. (1978). Cell biology pp- 85-186. In: rodent malaria. Edits. Killick-Kendrick, R. Peters, W.A. Academic Press. New York.

Sinden, R. E. and Smalley, M. E. (1979). Gametocytogenesis of *Plasmodium falciparum* in vitro: the cell cycle. *ParasitoLogy* 79, 277-296.

Sinden, R.E. (1982). Gametocytogenesis of *Plasmodium falciparum in vitro*: an electron microscopic study. *Parasitology.* 84, 1-11.

Sinden, R.E. (1983). The cell biology of sexual development in *Plasmodium.. Parasitology* 86, 7-28.

Sinden, R.E. and Hartley, R.H. (1985). Identification of the meiotic division of malarial parasites. *J. Protozool.* 32,742-744.

Sinden, R.E. and Strong, K. (1987). An ultrastructural study of the sporogenic development of *Plasmodium falciparum* in *Anopheles gambiae. Trans. Roy. Soc. Trop. Med. and Hyg.*, 72: 477-491.

Sinden, R.E. (1991). Mitosis and meiosis in malarial parasites. *Acta Leidensa.* 60, 19-27.

Sinha, S.N., Hellwig, R.J., Allison, D.P.and Niyogi, S.K. (1982). Conversion of simian virus 40 DNA to ordered nucleoprotein structures by extracts that direct accurate initiation by eukaryotic RNA polymerase II. *Nucl. Acids Res.* 10, 5533-5552.

Sinnis, P. and Wellems, T.E. (1988). Long-range restriction maps of *Plasmodium falciparum*: crossing over and size variation among geographically distant isolates. *Genomics* 3, 287-295.

Smith, D.B. and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67:31-40.

Smith, D.B. and Corcoran, L.M. (1990). Expression and purification of glutathione-S-transferase fusion proteins. In: *Curr. Prol Mol. Biol.*, Unit 16.7.

Smythe, C. and Newport, J.W. (1992). Coupling of mitosis to the completion of S-phase in *Xenopus* occurs via modulation of the tyrosine kinase that phosphorylates *p34*^{cdc2}. *Cell* 68, 787-797.

Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98: 503-517.

Spithill, T.W. and Samaras, N. (1987). Genomic organisation , chromosomal location and transcription of dispersed and repeated genes in *Leishmania major*. *Mol. Biochem. Parasitol.* 24, 23-38.

Stanley, H.A., Langreth, S.G., Reese, R.T., and Trager, W. (1982). *Plasmodium falciparum* merozoites: isolation by density gradient centrifugation using Percoll and antigenic analysis. *J. Parasitol.* 68, 1059-1067.

Sultan, A.A., Richardson, W.A., Alano, P., Arnot, D.E., Doerig, C. (1994). Cloning and characterisation of a *Plasmodium falciparum* homologue of the Ran/TC4 signal transducing GTPase involved in the cell cycle control. *Mol. Biochem. Parasitol.* 65, 331-338

Szarfman, A., Walliker, D., McBride, J.S., Lyon, J.A., Quakyi, I.A. and Carter, R. (1988). Allelic forms of gp195, a major blood-stage antigen of *Plasmodium falciparum*, are expressed in liver stages. *J. Exp. Med.* 167: 231-236.

Towbin, H., Staehlin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some application. *Proc. Natl. Acad.Sci. (U.S.A.)* 76: 4350-4354.

Toye', P.J., Sinden, R.E., Canning, E.U. (1977). The action of metabolic inhibitors on microgametogenesis in *Plasmodium yoelii nigeriensis*. *Z. Parasitenkd.* 53: 133-141.

Trager, W. and Jensen, J.B. (1976). Human malarial parasites in continuous culture. *Science* 193:673-675.

Triglia, T., Peterson, M.G. and Kemp, D.J. (1988). A procedure for *in vitro* amplification of DNA segments that lie outside the boundaries of known sequences. *Nucl. Acids Res.* 16: 8186.

Triglia, T. and Kemp, D.J. (1991). Large fragments of *Plasmodium falciparum* DNA can be stable when cloned in yeast artificial chromosomes. *Mol. Biochem. Parasitol.* 44, 207-212.

Triglia, T., Wellem., T.E. and Kemp, D.J. (1992). Towards a high resolution map of the *Plasmodium falciparum* genome. *Parasitol. Today.*8, 225-229.

Uchida, S., Sekiguchi, T., Nishitani, H., Miyauchi, K., Ohtsubo, M. and Nishimoto, T. (1990). Premature chromosome condensation is induced by a point mutation in the hamster RCC1 gene. *Mol. Cell. Biol.* 10, 577-584.

Valencia, A., Chardin, P., Wittinghofer, A. and Sander, C. (1991). The Ras protein family: Evolutionary tree and role of conserved amino acids. *Biochemistry.* 30(19): 4637-4648.

Van der Ploeg, L.H.T., Schwartz, D.C., Cantor, C.R. and Borst, P. (1984). Antigenic variation in *Trypanosomabrucei* analysed by electrophoretic separation of chromosomal-sized DNA molecules. *Cell* 37, 77-84.

Van der Ploeg, L.H.T. and Cornelissen, A.W.C.A. (1984). The contribution of chromosomal translocation to antigenic variation in *Trypanosma brucei. Plil. Trans. Roy. Soc.*(London). B 307, 13-26.

Van der Ploeg, L.H.T., Smits, M., Ponnudurai, T., Vermeulen, A., Meuwissen, J.H.E.TH. and Langsley, G. (1985). Chromosome-sized DNA molecules of *Plasmodium falciparum. Science,* 229: 658-661.

Waki, S., Yonome, I. and Suzuki, M. (1985). X-ray sensitivity and DNA synthesis in synchronous culture of *Plasmodium falciparum. Zeitschrift fur Parasitenkunde* 71, 213-218.

Walker-Jonah, A., Dolan, S.A., Gwadz, R.W., Panton, L.J. and Wellems T.E. (1992). An RFLP map of the *Plasmodium falciparum* genome, recombination rates and favored linkage groups in a genetic cross. *Mol. Biochem. Parasitol.* 51, 313-320.

Walliker ,D., Carter, R. and Morgan, S. (1973). Genetic recombination in *Plasmodium berghei. Parasitology.* 66, 309-320.

Walliker, D., Carter, R. and Sanderson, A. (1975). Genetic studies on *Plasmodium chabaudi*: recombination between enzyme markers. *Parasitol.* 70, 19-24.

Walliker, D.(1983). The genetic basis of diversity in malaria parasites. *Adv. Parasitol.,* 22: 217-259.

Walliker, D., Quakyi, I.A., Wellems, T.E., McCutchan, T.F., Szarfman, A., London, M.T., Corcoran, L.M., Burkot, T.R. and Carter, R. (1987). Genetic analysis of the human malaria parasite *Plasmodium falciparum. Science* 236:1661-1666.

Watanabe, J. and Inselburg, J. (1994). Establishing a physical map of chromosome No.4 of *Plasmodium falciparum. Mol. Biochem. Parasitol.* 65, 189-200.

Weber, J.L. (1987). Analysis of sequences from the extremely A+T- rich genome of *Plasmodium falciparum. Gene* 52:103-109.

Wellems, T.E., Walliker, D., Smith, C.L., DoRosario, V.E., Maloy, W. L., Howard, R.J., Carter, R. and MacCutchan, T.F. (1987). A histidine-rich protein gene marks a linkage group favoured strongly in a genetic cross of *Plasmodium falciparum. Cell*, 49: 633-642.

Wesseling, J.G., Snijders, P.J.F., Someren, P., Jansen, J., Smits, M.A. and Schoenmahers, G.G. (1989). Stage specific expression and genomic organisation of the actin genes of the malaria parasite *Plasmodium falciparum. Mol. Biochem. parasitol.* 35, 167-176.

White, J.H., Kilbey, B.J., Vries, E., Goman, M. Alano, P., Cheesman, S., MacAleese, S., and Ridley, R.G. (1993). The gene encoding DNA polymerase α from *Plasmodium falciparum. Nucl. Acids Res.* 21, 3643-3646.

W.H.O. (1992). Tropical Disease Research . 11th programme report. World Health Organisation, Geneva, Swizerland.

Williamson, K.C., Criscio, M.D. and Kaslow, D.C. (1993). Cloning and expression of the gene for *Plasmodium falciparum* transmission-blocking target antigen pfs230. *Mol. Biochem. Parasitol.* 58, 355-358.

Wilson, R.J.M., Gardner, M.J., Feagan, J.E. and Williamson, D.H. (1991). Have malaria parasites three genomes?. *Parasitol.Today* 7, 134-146.

Winship, R.R. (1989). An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. *Nucl. Acid Res.* 17: 1266.

Wunderlich, F. (1980). Nucleosomal organisation of chromatin in *Plasmodium Knowlesi. J. Parasitol.* 66(6): 1063-1065.

Young, R.A. and Davis, R.W. (1983a). Efficient isolation of genes by using antibody probes. *Proc. Natl. Acad. Sci. (U.S.A.).* 80: 1194-1198.

Young, R.A. and Davis, R.W. (1983b). Yeast RNA polymerase II genes: isolation with antibody probes. *Science* 222, 778-782.

Zolg, J.W., Andrae, L.E. and Scott, E.D. (1987). Deletion of *Plasmodium falciparum* DNA using repetitive DNA clones as species specific probes. *Mol. Biochem. Parasitol.* 22, 145-151.

Zolg, J.W., Mcleod, A.J., Dickson, I.H. and Scaife, J.G. (1982). *Plasmodium falciparum:* modifications on the *in vitro* culture conditions improving parasite yields. *J. Parasitol.* 68, 1072-1080.

APPENDIX

Contains 4 publications on which the candidate is coauthor :

Bayoumi *et al.*, 1993. Trans. Roy. Soc. Trop. Med. Hyg. 87,454-458.

Babiker *et al.*, 1993. Trans. Roy. Soc. Trop. Med. Hyg. 88, 328-331.

Arnot *et al.*, 1994. Parasitology Today 10, 324-327.

Sultan *et al.*, 1994. Mol. Biochem. Parasitol. 65,331-338.

threat to health than Edge Hill virus itself. Two monoclonal antibodies, 1B7 and 4G2, which react with epitopes common to Edge Hill and dengue viruses, enhanced infection of a human monocytic cell line (U-937) by dengue virus (HENCHAL *et al.*, 1985). Most isolations of Edge Hill virus have been made in the north-east region of Australia (DOHERTY, 1972) where dengue 1 appears to have become endemic and the first cases of dengue 2 infection for almost 50 years were diagnosed in 1992.

Apart from drawing attention to the possibility of clinical infections with Edge Hill virus, this report raises 2 other significant issues. Diagnostic laboratories which do not routinely test for antibody against Edge Hill virus should be aware of the close serological relationship between it and dengue 2, even in IgM assays which are often virus specific (SCOTT *et al.*, 1972), and consideration may need to be given to the possibility of antibody against Edge Hill virus enhancing subsequent dengue infections in residents of areas where only a single dengue serotype is in circulation.

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References

Aaskov, J. G., Ross, P., Davies, C. E. A., Innis, M. D., Guard, R. W., Stallman, N. D. & Tucker, M. (1981). Epidemic polyarthritis in north-eastern Australia, 1978–1979. *Medical Journal of Australia*, **ii**, 17–19.

Blok, J., Henchal, E. A. & Gorman, B. M. (1984). Comparison of dengue viruses and some other flaviviruses by cDNA-RNA hybridisation analysis and detection of a close relationship between dengue virus serotype 2 and Edge Hill virus. *Journal of General Virology*, **65**, 2173–2181.

Doherty, R. L. (1972). Arboviruses of Australia. *Australian Veterinary Journal*, **48**, 172–180.

Doherty, R. L., Marks, E. M., Carley, J. G. & Mackerras, M. J. (1963). Studies of arthropod-borne virus infections in Queensland. III. Isolation and characterisation of virus strains from wild caught mosquitoes in north Queensland. *Australian Journal of Experimental Biology and Medical Science*, **41**, 17–40.

Doherty, R. L., Carley, J. G., Kay, B. H., Filippich, C., Marks, E. N. & Frazier, C. L. (1979). Isolation of virus strains from mosquitoes collected in Queensland, 1972–1976. *Australian Journal of Experimental Biology and Medical Science*, **57**, 509–520.

Francis, T. (1955). The current status of the control of influenza. *Annals of Internal Medicine*, **43**, 534–538.

Hawkes, R. A., Boughton, C. R., Naim, H. M., Wild, J. & Chapman, B. (1985). Arbovirus infections of humans in New South Wales. Sero-epidemiology of the flavivirus group of togaviruses. *Medical Journal of Australia*, **143**, 555–561.

Henchal, E. A., McCown, J. M., Burke, D. S., Sequin, M. C. & Brandt, W. E. (1985). Epitope analysis of antigenic determinants on the surface of dengue 2 virions using monoclonal antibodies. *American Journal of Tropical Medicine and Hygiene*, **34**, 162–169.

Hunder, G. G. & Hazleman, B. L. (1985). Giant cell arteritis and polymyalgia rheumatica. In: *Textbook of Rheumatology*, Kelly, W. N., Harris, E. D., Ruddy, S. & Sledge, C. B. (editors), 2nd edition. London: W. B. Saunders, pp. 1166–1173.

Lumley, G. F. & Taylor, F. H. (1943). *Dengue*. Sydney: School of Public Health and Tropical Medicine, Commonwealth of Australia, Service Publication no. 3.

Marshall, I. D. & Woodroofe, G. M. (1975). Epidemiology of arboviruses. *Report of the John Curtin School of Medical Research*, p. 2.

Scott, R. M., McCown, J. M. & Russell, P. K. (1972). Human immunoglobulin specificity after group B arbovirus infections. *Infection and Immunity*, **6**, 277–281.

Spradbrow, P. B. (1972). Arbovirus infections of domestic animals in Australia. *Australian Veterinary Journal*, **48**, 181–185.

Wiemers, M. A. & Stallman, N. D. (1975). Immunoglobulin M in Murray Valley encephalitis. *Pathology*, **7**, 187–191.

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Announcement

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TRANSACTIONS OF THE ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE (1993) 87, 454–458

Drug response and genetic characterization of *Plasmodium falciparum* clones recently isolated from a Sudanese village

Riad A. L. Bayoumi[2], Alison M. Creasey[1], Hamza A. Babiker[1,4], Jane M.-R. Carlton[1], Ali A. Sultan[1,3], Gwiria Satti[3], Awinder K. Sohal[1], David Walliker[1], James B. Jensen[5] and David E. Arnot[1] [1]*Institute of Cell, Animal and Population Biology, University of Edinburgh, Scotland, UK;* [2]*Biochemistry Department, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al-Ain, United Arab Emirates;* [3]*Faculty of Medicine, University of Khartoum, Khartoum, Sudan;* [4]*Malaria Administration, Ministry of Health, Khartoum, Sudan;* [5]*Brigham Young University, Provo, Utah, USA*

Abstract

We have isolated 20 clones of *Plasmodium falciparum* from isolates from patients attending a village clinic in Sudan during 10 d in October–November 1989. The clones were genetically diverse, having highly variable molecular karyotypes and a wide range of drug responses. Chloroquine-sensitive (50% inhibitory concentration [IC_{50}] in the 4–15 nM range) and chloroquine-resistant clones (IC_{50} in the 40–95 nM range) co-existed in the population, but no obvious amplification of the P-glycoprotein homologue gene, *Pgh*1 (previously known as the multi-drug resistance gene, *mdr*1) marked the chloroquine-resistant clones. Chloroquine resistance was reversible by verapamil in these clones, although they varied in their susceptibility to verapamil alone. These observations indicate that the biochemical characteristics of the Sudanese chloroquine-resistant *P. falciparum* are similar to those reported from south-east Asian and Latin American isolates, which is consistent with there being a similar molecular basis for this phenomenon.

Introduction

Chloroquine is the only antimalarial drug available at a cost accessible to even a minority of the Sudanese people and as such constitutes the major element of malaria control in Sudan and similar African countries (PETERS, 1987). The emergence of chloroquine-resistant *Plasmodium falciparum* in the Eastern Province since around 1986 (BAYOUMI *et al.*, 1989) is causing acute problems as the episodic, post-rainy season malaria epidemics characteristic of sub-Sahelian Sudan cease to be controlled by this drug.

The mode of action of chloroquine and the mechanism of resistance to the drug are unclear, and several opposing viewpoints exist (HOMEWOOD *et al.*, 1972; KROGSTAD *et al.*, 1987; GINSBURG, 1988; WARHURST, 1988; FOOTE *et al.*, 1990; WELLEMS *et al.*, 1990). Studies *in vitro* on chloroquine resistance in different laboratories have made use of different long-term cultured lines of *P. falciparum* originating mainly from south-east Asia or the Amazon basin. Conclusions drawn from the analysis of such isolates may not accurately reflect the present situation in an area such as Sudan. Although currently spreading rapidly, chloroquine-resistant *P. falciparum* was not reported in Sudan until 25 years after its appearance in south-east Asia and South America, and several years after its appearance in East Africa (AL TAWIL & AKOOD, 1983). Chloroquine is essentially the only antimalarial drug available in most of Sudan, and for economic and climatic reasons usage has not been as heavy as in more prosperous areas with more stable malaria transmission.

In order to study the genetic basis for the spread of chloroquine-resistant *P. falciparum*, we have recently characterized 29 isolates from a single village in eastern Sudan with a developing problem of chloroquine-resistant malaria (BABIKER *et al.*, 1991a, 1991b). Since most of these isolates proved to be mixtures of genetically different parasites, we have now obtained cloned lines from some of the isolates, in order to define parasite genotypes and their biochemical profiles of drug resistance. We showed that the clones had highly variable molecular karyotypes and wide ranges of drug responses. Chloroquine resistance is reversible by verapamil, but no obvious amplification of the P-glycoprotein homologue gene, *Pgh*1 (previously known as the multi-drug resistance gene, *mdr*1) marked the chloroquine-resistant clones.

Address for correspondence: R. A. Bayoumi, Biochemistry Department, Faculty of Medicine and Health Sciences, U.A.E. University, Al-Ain, P.O. Box 17666, United Arab Emirates.

Materials and Methods

Study area

The study area in Asar village, 20 km from Gedaref in the Eastern Province of Sudan, has been described elsewhere (BABIKER *et al.*, 1991b). Malaria transmission is seasonal and reaches a peak in October or November following the rainy season. The main *Plasmodium* species present is *P. falciparum*. Chloroquine resistance was first reported in this region in 1986 (BAYOUMI *et al.*, 1989).

Isolation and characterization of P. falciparum *clones*

The initial isolates were obtained with informed consent from villagers attending a local clinic during the October–November malaria season of 1989. Clones were obtained using the limiting dilution method (ROSARIO, 1981), from a selection of isolates known to exhibit a range of sensitivity to chloroquine and to pyrimethamine. Certain of these isolates were known to be mixed infections by their possession of more than one allele of genes for antigens and other proteins (BABIKER *et al.*, 1991a). Following the cloning procedure, the resulting cultures were shown to be pure clones by ensuring that each haploid clone was monoallelic for each of 2 highly polymorphic antigens (merozoite surface protein [MSP]-1 and MSP-2) when tested in immunofluorescence assays with a panel of allele-specific monoclonal antibodies (CONWAY & MCBRIDE, 1991).

Pulsed field gels

P. falciparum chromosomes were separated by pulsed field gradient gel electrophoresis (PFG) as described previously (BABIKER *et al.*, 1991b), with a basic regime of 22 h, 120s pulses, 140 V followed by 22 h, 180s pulses, 140 V, and finally 24 h, 300s pulses, 120 V. Southern blotting, dot-blotting and hybridization were performed using standard techniques (SAMBROOK *et al.*, 1989).

Measurement of drug sensitivity

Hypoxanthine incorporation assay. Chloroquine-induced inhibition of uptake of [^{3}H]hypoxanthine by the clones was measured using the methods of DESJARDINS *et al.* (1979) and GEARY *et al.* (1983). Tests were carried out on unsynchronized cultures, diluted in RPMI medium containing 10% human serum to 1% parasitaemia and 1% haematocrit, in a final volume of 0·2 mL in microtitre plate wells. [^{3}H]hypoxanthine was added to a final concentration of 5μCi/mL. Chloroquine sulphate was added to final concentrations ranging from 10 to 160 nM. After 40–44 h incubation, cells were lysed, washed and harvested on to fibre glass filters. Filters were then baked

and counted in a PPO/POPOP*/toluene scintillation fluid supplemented with 30% Triton X-100®. Results were expressed as percentage inhibition compared to the incorporation of label in control wells without drug. The concentration of chloroquine which produced 50% inhibition of parasite growth (IC_{50}) was obtained from the regression line of dose/response curves of parasite inhibition plotted against the logarithm of drug concentration.

Microscopical determinations. Clones were cultured in microtitre plates in serial dilutions of chloroquine, mefloquine and pyrimethamine in complete RPMI medium for 72 h, essentially according to the method of THAITHONG *et al.* (1983). Each well contained 100 μL, at 1% parasitaemia and 5% haematocrit. After 72 h, thin blood films were made from each well, and the viability assessed by microscopical examination. The results were expressed as the minimum inhibitory concentration (MIC) which killed all, or nearly all, of the parasites.

Effect of verapamil

Drug sensitivity of the parasites to chloroquine, mefloquine and pyrimethamine was assessed with and without the addition of 1 μM verapamil to the cultures. The effect of verapamil alone over a wider range of concentrations (0–5 μM) was also tested on some clones.

Chemicals

[^{3}H]hypoxanthine (40 Ci/mmol) was obtained from Amersham International Ltd, UK; chloroquine sulphate (Nivaquine®) from May and Baker Ltd, UK; pyrimethamine from Wellcome, UK; mefloquine from Hoffman La Roche, Switzerland; and verapamil from Sigma Chemical Company. Glass fibre filters were obtained from Titertek.

of results were obtained.

(i) All the clones from a single isolate possessed chromosomes of identical size, as observed in isolate SUD 111. This suggests that, at the time of cloning, the parasites in these samples were of a clonal type, due either to a clonal-type infection in the patient or to selection of a predominant clone during initial culturing of the uncloned isolate.

(ii) The clones of a single isolate varied in the size of only one or 2 chromosomes. This was most probably due to deletions or accretions in the chromosomes concerned during asexual growth in culture of a clonal-type parasite (WELLEMS *et al.*, 1988). This was seen in isolate SUD 105, in which the 4 clones obtained were karyotypically identical, except that clone 7 had a 300–400 kilobase (kb) deletion of chromosome 10, and clones 9 and 11 both had 200 kb accretions in chromosome 4. These related clones had identical drug sensitivities and possessed the same MSP-1 and MSP-2 alleles.

(iii) Several quite different karyotypes were obtained from a single isolate. This result can be presumed to have been due to the patient's harbouring a mixed infection at the time the parasites were obtained. The clones derived from patients SUD 106 and 124 illustrate this situation. While SUD 106/7 and 106/10 differed from other clones of the same isolate, they were identical to each other, except in the size of 2 chromosomes.

Identical clones have never been isolated from different individuals, a result consistent with the extremely diverse genetic profiles of malaria isolates in general (CREASEY, 1990) and in particular the uncloned Asar isolates from which these clones were derived (BABIKER *et al.*, 1991a, 1991b).

An autoradiograph of a Southern blot of gel 1A probed

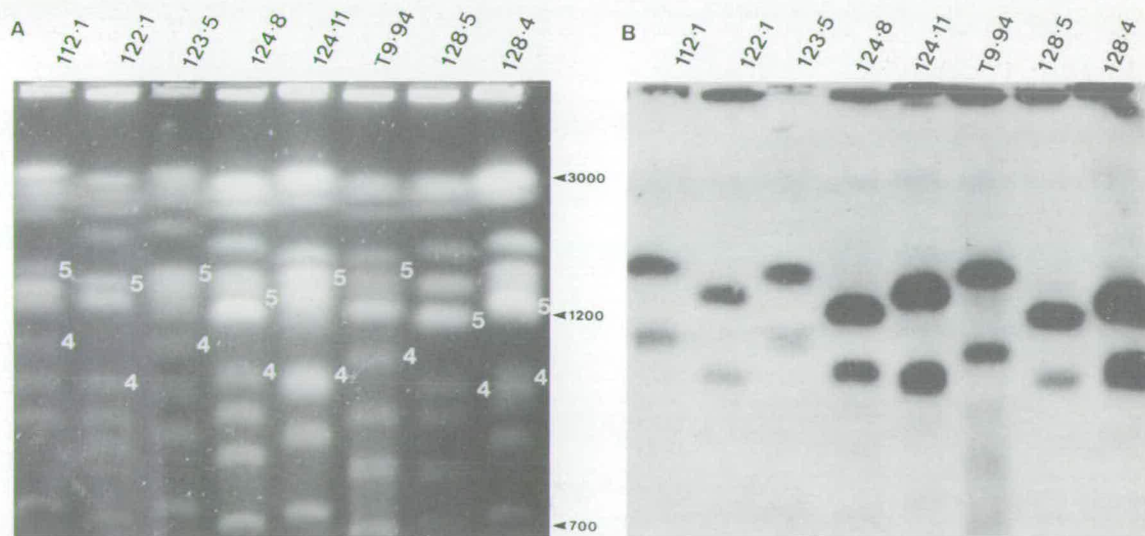

Fig. 1. Karyotypic diversity in some examples of *P. falciparum* clones from Asar village, eastern Sudan. A. Ethidium bromide stained chromosomes separated on a 0·8% agarose gel using a CHEF® electrophoresis apparatus. Seven of the parasites tested are Asar clones, 2 of which, 112/1 and 124/11, were not included in the study (see the Table for the drug sensitivity phenotypes). T9/94 is a Thai clone. Chromosomes 4 and 5 are marked, and approximate chromosome sizes in kilobases are indicated on the right. B. After Southern blotting, gel A was successively probed with gene markers for chromosomes 4 and 5, the *Pgh*1 gene and the *DHFR* gene respectively. The 2 autoradiographs from the same blot have been superimposed before photography for comparison and signal intensities are not proportional to gene copy number.

Results

Clone characterization

All the clones were confirmed as pure by their possession of single alleles of MSP-1 and MSP-2, using monoclonal antibody typing (results not shown).

The clones were examined for chromosomes by pulsed field electrophoresis. Results for a representative sample of clones are shown in Fig. 1A. When clones from individual isolates were compared, the following 3 categories

with the genes encoding the *P. falciparum* dihydrofolate reductase (*DHFR* gene) located on chromosome 4, and *Pgh*1 located on chromosome 5 is shown in Fig. 1B. In each clone the *DHFR* probe always hybridized to the fourth smallest chromosome, which, however, differed in size among the clones. The *Pgh*1 probe hybridized to the fifth smallest chromosome in only 2 clones (SUD 128/5 and SUD 128/4). These results illustrate clearly the considerable size polymorphism of these 2 chromosomes, especially of chromosome 5, among this group of isolates.

*2,5-Diphenyloxazole/1,4-bis[5-phenyl-2-oxazolyl]benzene.

Although there were differences in the relative intensity of hybridization of the probes shown in Fig. 1, deoxyribonucleic acid (DNA) transfer from pulsed field gels is not reliably quantitative and the amount of DNA per lane in this gel was not constant. Using more quantitative dot-blots and Southern blots of DNA digested with restriction enzymes, we have not been able to detect *DHFR* or *Pgh*1 gene amplification in DNA samples of any of the clones, whether drug-resistant or drug-sensitive.

Chloroquine sensitivity

Drug resistance measurements, even with identical clones, may show considerable inter-laboratory variation. To increase confidence in assessing the drug response of a given clone, we have, therefore, employed and compared 2 different assays in this work. IC_{50} values above 35 nM, as assessed by [^{3}H]hypoxanthine incorporation, and MIC values of 16×10^{-7}M, as assessed microscopically, were taken to indicate clear chloroquine resistance. The drug responses of the 20 clones are presented in the Table. They are listed in ascending order of IC_{50} values,

Table. Drug sensitivity phenotypes of twenty *P. falciparum* clones derived from patient blood samples collected in the village of Asar, Sudan, during October and November 1989

	Chloroquine[a] IC_{50}[b] ($\times10^{-9}$M)		Chloroquine[a] MIC[c] ($\times10^{-7}$M)		Mefloquine[a] MIC[c] ($\times10^{-7}$M)		Pyrimethamine[a] MIC[c] (M)	
Verapamil	No	Yes	No	Yes	No	Yes	No	Yes
Experimental clones[d]								
SUD 105/1	4	3	8	4	4	4	10^{-7}	10^{-7}[f]
SUD 105/9	6	5	8	4	4	4	10^{-7}	10^{-7}[f]
SUD 105/11	7	4	8	4	4	4	10^{-7}	10^{-7}[f]
SUD 106/10	7	4	2	2	4	4	10^{-6}[e]	10^{-6}
SUD 105/7	8	4	8	4	4	4	10^{-7}	10^{-7}[f]
SUD 106/9	9	8	4	4	4	4	10^{-6}	10^{-6}
SUD 106/7	13	4	4	2	4	4	10^{-6}[e]	10^{-6}
SUD 106/11	11	5	4	4	4	4	10^{-6}	10^{-6}
SUD 106/1	15	6	2	2	4	4	10^{-6}[e]	10^{-6}
SUD 128/5	43	10	8	4	4	2	10^{-8}	10^{-8}[f]
SUD 128/4	45	11	8	4	4	2	10^{-8}	10^{-8}[f]
SUD 124/8	58	19	16[e]	4	2	2	10^{-5}	10^{-5}[f]
SUD 128/1	64	18	8	4	4	2	10^{-8}	10^{-9}
SUD 123/5	64	18	16	8	8	8[f]	10^{-5}[e]	10^{-5}
SUD 124/5	65	28	16	4	2	2	10^{-5}	10^{-5}
SUD 124/1	73	27	16	8	2	2	10^{-5}	10^{-6}[f]
SUD 126/1	83	18	16[f]	1	2	2	10^{-7}	10^{-7}
SUD 102/1	85	12	16	8	2	2	10^{-8}	10^{-9}
SUD 122/1	92	23	16	4	2	2	10^{-6}	10^{-6}[f]
SUD 111/1	95	21	16	8	2	2	10^{-5}[e]	10^{-5}
Control clones								
3D7[g]	8	3	4	2	4	2	10^{-8}	10^{-9}
Dd2[g]	57	15	16	8	16	8	10^{-6}	10^{-6}[f]

[a]All values are means of 3 or more separate experiments, with ('yes') and without ('no') verapamil (1 μM).
[b]Concentration giving 50% inhibition of parasite growth.
[c]Concentration killing all parasites within 72 h.
[d]Clones with the same 'one hundred' number before the solidus were isolated from a single patient sample.
[e]A few parasites were still viable at the indicated concentrations.
[f]Reduced parasitaemia at the indicated concentrations.
[g]'Control' chloroquine-sensitive and chloroquine-resistant clones, respectively.

with the chloroquine-resistant Dd2 and chloroquine-sensitive 3D7 laboratory-adapted clones as reference controls.

On the whole, the 2 tests of sensitivity *in vitro* to chloroquine gave comparable results. No clone with an MIC of 16×10^{-7}M chloroquine had an IC_{50} <50 nM. Similarly, no clone with an IC_{50} value <35 nM has an MIC >8×10^{-7}M. Certain ambiguities remain, in that some clones of isolate SUD 105 which appeared very chloroquine-sensitive in the IC_{50} test, showed intermediate sensitivity in the MIC test. Apart from this, the combination of both tests appeared to allow differentiation of the parasites into a sensitive group comprising all the SUD 105 and SUD 106 clones, and a resistant group consisting of all the other clones. No clone had chloroquine sensitivity in the IC_{50} range 15–43 nM.

Resistance to mefloquine and pyrimethamine

Most of the clones were sensitive to mefloquine (Table), in agreement with the results of tests on the original 29 uncloned isolates from Asar village (BABIKER *et al.*, 1991b). Again in accordance with our earlier results, 5 clones (SUD 124/1, SUD 124/5, SUD 124/8, SUD 123/5 and SUD 111/1) were highly resistant to pyrimethamine.

One clone, SUD 123/5, was resistant to both chloroquine and pyrimethamine and showed a slight decrease in susceptibility to mefloquine. However, none of the other clones was resistant to all 3 drugs. Mefloquine resistance is clearly not linked to chloroquine resistance. While high level pyrimethamine resistance was found only in chloroquine-resistant clones, many of the latter were sensitive to pyrimethamine.

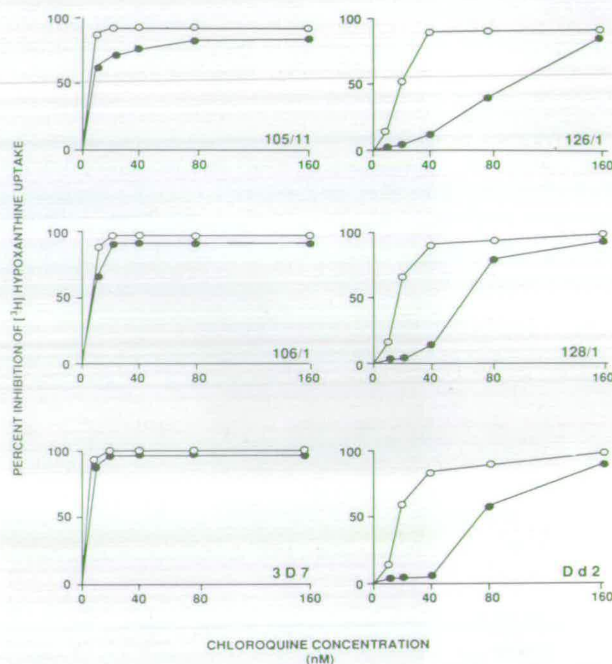

Fig. 2. The effect of 1 μM verapamil on the response of 4 Sudanese and 2 standard laboratory-adapted *P. falciparum* clones to chloroquine. Closed circles (●), without verapamil; open circles (○), with verapamil. SUD 105/11, SUD 106/1 and 3D7 were chloroquine sensitive, SUD 126/1, SUD 128/1 and Dd2 were chloroquine resistant. Points represent the average of simultaneous duplicate experiments. Similar curves have been obtained for all clones tested.

Effect of verapamil

Verapamil, a calcium channel blocker, has been reported to reverse chloroquine resistance in some isolates of *P. falciparum* (MARTIN *et al.*, 1987). We tested the effects of verapamil on our clones (Table) to ascertain whether verapamil reversibility was also characteristic of the chloroquine-resistant parasites studied here. Examples of drug tests where the capacity of increasing concentrations of chloroquine to inhibit [^{3}H]hypoxanthine uptake has been measured, with and without the addition of 1 μM verapamil, are shown in Fig. 2. Clones SUD 105/11, SUD 106/1 and 3D7 were chloroquine-sensitive. Verapamil did not shift the inhibition curve of the 3D7 reference control, although it slightly increased the toxicity of chloroquine to the drug-sensitive Asar (SUD) clones. Clones SUD 126/1, SUD 128/1 and Dd2 were chloroquine-resistant, and verapamil clearly increased their chloroquine sensitivity, although not to the levels of the naturally sensitive clones.

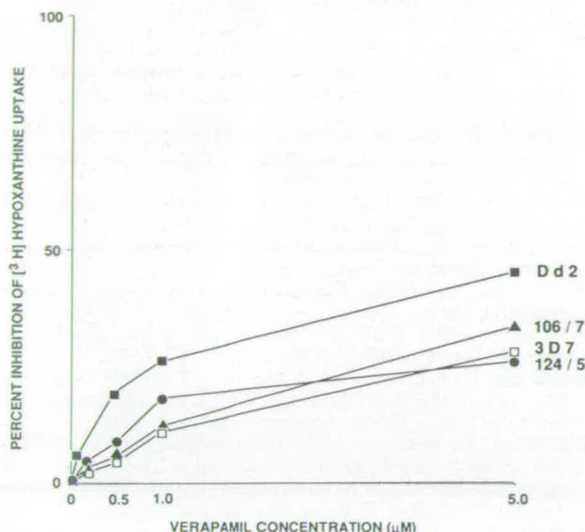

Fig. 3. The effect of verapamil alone on [^{3}H]hypoxanthine uptake over 40–44 h by 2 Sudanese and 2 standard laboratory-adapted *P. falciparum* clones. Dd2 and SUD 124/5 were chloroquine resistant and 3D7 and SUD 106/7 were chloroquine sensitive. Points represent the average of duplicate experiments carried out simultaneously on the same microtitre plate. Three separate experiments gave the same rank order of drug sensitivities.

It has been reported (MARTIN *et al.*, 1987) that verapamil itself has intrinsic antimalarial activity. An example of the effect of verapamil alone on the capacity of parasites to incorporate [^{3}H]hypoxanthine is shown in Fig. 3. Dd2 and SUD 124/5 were chloroquine-resistant, and SUD 106/7 and 3D7 were chloroquine-sensitive. There were large intrinsic differences in their susceptibility to the antimalarial effect of verapamil, Dd2 being particularly sensitive.

The sensitivity of some of the Sudanese clones to mefloquine and pyrimethamine also appeared to increase on addition of verapamil (Table), although to only a limited extent.

Discussion

In our initial survey of uncloned *P. falciparum* isolates from Asar village, we found that no 2 isolates were genetically identical and that it was possible to detect several obviously mixed isolates (BABIKER *et al.*, 1991a, 1991b). The molecular karyotypes of the clones derived from these isolates have further confirmed the highly diverse nature of this small parasite population. Mapping of *DHFR* and *Pgh*1 markers on to chromosomes demonstrates that comparisons of chromosome separations stained with ethidium bromide underestimate the true extent of karyotypic diversity. More detailed genome maps would undoubtedly reveal more radical differences in genome organization between these clones which, it should be emphasized, represent only a small sample of the total population of *P. falciparum* in this small community.

In general, our results indicated that the biochemical characteristics of the Sudanese chloroquine-resistant *P. falciparum* are similar to those reported for south-east Asian and Latin American isolates, and are consistent with there being a similar molecular basis for the phenomenon. The use of genetically pure clones from the 1989 transmission peak permitted a clearer differentiation of some of the characteristics of the drug resistant *P. falciparum* in this village, as follows.

(i) Clones of *P. falciparum* exhibiting low, intermediate and high level chloroquine resistance co-existed with highly sensitive parasites in the population of this village.

(ii) The reversibility of chloroquine resistance of *P. falciparum* by verapamil (MARTIN *et al.*, 1987) also appeared to be characteristic of the chloroquine resistant malaria currently spreading in Asar. However, verapamil has an antimalarial effect of its own, as shown by MARTIN *et al.* (1987) and in our work. The addition of verapamil increased the sensitivity of these clones not only to chloroquine but also, in some instances to mefloquine and pyrimethamine.

(iii) The *Pgh*1 gene has been reported to be amplified in some, but not all, chloroquine-resistant *P. falciparum* isolates (FOOTE *et al.*, 1989). However, we have not been able to detect *Pgh*1 gene amplification in DNA samples from either chloroquine-resistant or chloroquine-sensitive Sudanese *P. falciparum* clones.

(iv) In accordance with our earlier findings with the uncloned isolates, 5 clones were highly resistant to pyrimethamine. This result is of interest, since pyrimethamine was not widely used in Sudan before 1986. Since then, however, Fansidar® (pyrimethamine/sulfadoxine) became available through relief agencies. This may explain the appearance and selection of mutants resistant to this drug. It may also be relevant that clinical pyrimethamine resistance was noted in Sudan as early as 1954 (PHILLIPS, 1954) and, more recently, in the Sennar region (IBRAHIM *et al.*, 1991). Only a few parasites in this and our previous survey (BABIKER *et al.*, 1991b) showed any degree of resistance to mefloquine.

(v) In the survey of the uncloned isolates, some were found to exhibit resistance to all 3 drugs. However, none of the clones examined in this work was multi-drug resistant, with the possible exception of clone SUD 123/5. Since clones resistant to both chloroquine and, at a high level, to pyrimethamine coexisted in this community, and many patients were infected with more than one clone, it is certainly possible that genetic recombination during mosquito transmission could increase the frequency of multi-drug resistant clones.

These results suggest that chloroquine-resistant *P. falciparum* in this small village (and presumably the rest of Sudan) is not a distinct parasite 'strain'. Rather, it appears that genes conferring resistance to this drug are increasing in frequency in the parasite population, probably due to the continuing use of chloroquine in the area. While clinical chloroquine resistance has increased in frequency among patients attending the nearby Gadaref District Hospital during 1986–1990 (unpublished data), most patients in the village appeared to be cured by standard chloroquine therapy. Whether the situation is slowly deteriorating and chloroquine will soon be clinically useless remains to be seen.

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References

Al Tawil, N. & Akood, M. A. (1983). *Response of falciparum malaria to a standard regimen of chloroquine in Khartoum Province, Sudan*. Geneva: World Health Organization, mimeographed document no. WHO/MAL/83.991.

Babiker, H. A., Creasey, A. M., Fenton, B., Bayoumi, R. A., Arnot, D. E. & Walliker, D. (1991a). Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 1. Diversity of enzymes, 2D-PAGE proteins and antigens. *Transactions*

of the Royal Society of Tropical Medicine and Hygiene, **85**, 572–577.

Babiker, H. A., Creasey, A. M., Bayoumi, R. A., Walliker, D. & Arnot, D. E. (1991b). Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 2. Drug resistance, molecular karyotypes and the *mdr*1 genotype of recent isolates. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **85**, 578–583.

Bayoumi, R. A., Babiker, H. A., Ibrahim, S. M., Galib, H. W., Saeed, B. O., Khider, S. & Karim, E. A. (1989). Spread of chloroquine resistant *Plasmodium falciparum* in Sudan. *Acta Tropica*, **46**, 157–165.

Conway, D. J. & McBride, J. S. (1991). Population genetics of *Plasmodium falciparum* within a malaria hyperendemic area. *Parasitology*, **103**, 7–16.

Creasey, A., Fenton, B., Walker, A., Thaithong, S., Oliveria, S., Mutambu, S. & Walliker, D. (1990). Genetic diversity of *Plasmodium falciparum* shows geographical variation. *American Journal of Tropical Medicine and Hygiene*, **42**, 403–413.

Desjardins, R. E., Canfield, C. J., Hyanes, J. D. & Chulay, J. D. (1979). Quantitative assessment of antimalarial activity *in vitro* by a semiautomated technique. *Antimicrobial Agents and Chemotherapy*, **16**, 710–718.

Foote, S. J., Thompson, J. K., Cowman, A. F. & Kemp, D. J. (1989). Amplification of the multidrug resistance gene in some chloroquine resistant isolates of *Plasmodium falciparum*. *Cell*, **57**, 921–930.

Foote, S. J., Kyle, D. E., Martin, R. K., Oduola, A. M., Forsyth, K., Kemp, D. J. & Cowman, A. F. (1990). Several alleles of the multidrug resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature*, **345**, 255–258.

Geary, T. G., Divo, A. A. & Jensen, J. B. (1983). An *in vitro* assay system for the identification of potential antimalarial drugs. *Journal of Parasitology*, **69**, 577–583.

Ginsberg, H. (1988). Effect of calcium antagonists on malaria susceptibility to chloroquine. *Parasitology Today*, **4**, 209–211.

Homewood, C. A., Warhurst, D. C., Peters, W. & Babbaley, V. C. (1972). Lysosomes, pH and the antimalarial action of chloroquine. *Nature*, **235**, 50–52.

Ibrahim, M. E., Awad-El-Kariem, E. M., El Hassan, I. M. & El Mubarak, E. R. M. (1991). A case of *Plasmodium falciparum* malaria sensitive to chloroquine but resistant to pyrimethamine/sulphadoxine in Sennar, Sudan. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **85**, 446.

Krogstad, D. J., Gluzman, I. Y., Kyle, D. E., Oduola, A. M. J., Martin, S. K., Milhous, W. K. & Schlesinger, P. H. (1987). Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. *Science*, **238**, 1283–1285.

Martin, S. K., Oduola, A. M. J. & Milhous, W. K. (1987). Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. *Science*, **235**, 899–901.

Meshnik, S. (1990). Chloroquine as intercalator: a hypothesis revived. *Parasitology Today*, **6**, 77–79.

Peters, W. (1987). *Chemotherapy and Drug Resistance in Malaria*. London: Academic Press.

Phillips, M. G. (1954). Malaria prophylaxis. *British Medical Journal*, **i**, 155.

Rosario, V. (1981). Cloning of naturally occurring mixed infections of malaria parasites. *Science*, **212**, 1037–1038.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edition. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Thaithong, S., Beale, G. H. & Chutmongkonkul, M. (1983). Susceptibility of *Plasmodium falciparum* to five drugs: an *in vitro* study of isolates mainly from Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **77**, 228–231.

Warhurst, D. C. (1988). Mechanism of chloroquine resistance in malaria. *Parasitology Today*, **4**, 211–213.

Wellems, T. E., Oduola, A. M. J., Fenton, B., Desjardins, R., Panton, L. J. & Do Rosario, V. E. (1988). Chromosome size variation occurs in cloned *Plasmodium falciparum* on *in vitro* cultivation. *Revista Brasileira de Genetica*, **11**, 813–825.

Wellems, T. E., Panton, L. J., Gluzman, I. Y., Rosario, V. E., Gwadz, R. W., Walker-Jonah, A. & Krogstad, D. J. (1990). Chloroquine resistance not linked to *mdr*-like genes in *Plasmodium falciparum* cross. *Nature*, **345**, 253–255.

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Announcements

The Tropical Health and Education Trust

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similar to those reported previously (WARRELL *et al.*, 1982; WHO, 1990). However, a considerably larger study would be needed to detect anything other than a major increase in mortality.

Use of a quinine loading dose regimen in severe malaria was not associated with serious quinine toxicity. In particular there was no residual blindness or deafness and no serious cardiovascular adverse effect. There is no evidence that these regimens are associated with toxicity more than other parenteral regimens that do not include a loading dose. On the other hand, there is evidence that rapidly achieving therapeutic blood concentrations of quinine produces faster clinical and parasitological recovery (WHITE *et al.*, 1983a; FARGIER *et al.*, 1991; PASVOL *et al.*, 1991). For these reasons, the quinine loading dose regimen has become standard practice in Thailand and many other countries during the past decade. The lack of toxicity of quinine regimens for severe malaria which consistently produce total plasma concentrations over 10 mg/L and often produce free concentrations over 2 mg/L (WHITE *et al.*, 1982; DAVIS *et al.*, 1988) contrasts with experience in self poisoning, when such quinine concentrations regularly produce toxicity (BOLAND *et al.*, 1985; DYSON *et al.*, 1985). The pharmacodynamic effects in the 2 situations are clearly different. However, further increase in the dose of quinine in severe malaria to combat resistance would risk producing major toxicity, and it is unlikely that this would be tolerated.

The results of this study show that quinine can still be relied upon in severe multi-drug resistant falciparum malaria, but this will probably change as resistance continues to increase. New antimalarial drugs for severe malaria are therefore needed urgently.

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References

Boland, M. E., Brennand-Roper, S. M. & Henry, J. A. (1985). Complications of quinine poisoning. *Lancet*, **i**, 384–385.

Chongsuphajaisiddhi, T., Sabcharoen, A. & Attanath, P. (1981). *In vivo* and *in vitro* sensitivity of falciparum malaria to quinine in Thai children. *Annals of Tropical Paediatrics*, **1**, 21–26.

Davis, T. M. E., White, N. J., Looareesuwan, S., Silamut, K. & Warrell, D. A. (1988). Quinine pharmacokinetics in cerebral malaria: predicted plasma concentrations after rapid intravenous loading using a two-compartment model. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **82**, 542–547.

Davis, T. M. E., Supanaranond, W., Pukrittayakamee, S., Karbwang, J., Molunto, P., Mekthon, S. & White, N. J. (1990). A safe and effective consecutive-infusion regimen for rapid quinine loading in severe falciparum malaria. *Journal of Infectious Diseases*, **161**, 1305–1308.

Dyson, E. H., Proudfoot, A. T., Prescott, L. F. & Heyworth, R. (1985). Death and blindness due to overdose of quinine. *British Medical Journal*, **291**, 31–33.

Fargier, J. J., Louis, F. J., Cot, M., Maubert, B., Hounsinou, C., Louis, J.-P., LeBras, J. & Tauze, J. E. (1991). Reduction of coma by quinine loading dose in falciparum cerebral malaria. *Lancet*, **338**, 896–897.

Harinasuta, T. & Bunnag, D. (1984). Drug resistant malaria with special reference to chemotherapy. *Mosquito-Borne Diseases Bulletin*, **1**, 23–30.

Harinasuta, T. & Bunnag, D. (1988). Management of malaria with special reference to drug resistance. *Japanese Journal of Tropical Medicine and Hygiene*, **16**, 121–130.

Harinasuta, T., Suntharasami, P. & Viravan, C. (1965). Chloroquine-resistant falciparum malaria in Thailand. *Lancet*, **ii**, 657–660.

Hien, T. T. & White, N. J. (1993). Qinghaosu. *Lancet*, **341**, 603–608.

Hien, T. T., Phu, N. H., Mai, N. T. H., Chau, T. T. H., Trang, T. T. M., Loc, P. P., Cuong, B. M., Dung, N. T., Vinh, H., Waller, D. J. & White, N. J. (1992). An open randomized comparison of intravenous and intramuscular artesunate in severe falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **86**, 584–585.

Looareesuwan, S., Wilairatana, P., Vanijanonta, S., Kyle, D. & Webster, K. (1992). Efficacy of quinine–tetracycline for acute uncomplicated falciparum malaria in Thailand. *Lancet*, **339**, 369.

Nosten, F., ter Kuile, F., Chongsuphajaisiddhi, T., Luxemburger, C., Webster, H. K., Edstein, M., Phaipun, L., Thew, K. L. T. & White, N. J. (1991). Mefloquine-resistant falciparum malaria on the Thai–Burmese border. *Lancet*, **337**, 1140–1143.

Pasvol, G., Newton, C. R. J. C., Winstanley, P. A., Watkins, W. M., Peshu, N. M., Were, J. B. O., Marsh, K. & Warrell, D. A. (1991). Quinine treatment of severe falciparum malaria in African children: a randomized comparison of three regimens. *American Journal of Tropical Medicine and Hygiene*, **45**, 713.

Reacher, M., Campbell, C. C., Freeman, J., Doberstyn, E. B. & Brandling-Bennett, A. D. (1981). Drug therapy for *Plasmodium falciparum* malaria resistant to pyrimethamine–sulphadoxine (Fansidar®). A study of alternate regimens in eastern Thailand, 1980. *Lancet*, **ii**, 1066–1069.

Rieckmann, K. H., McNamara, J. V., Frischer, H., Stockert, T. A., Carson, P. E. & Powell, R. D. (1968). Effects of chloroquine, quinine, and cycloguanil upon the maturation of asexual erythrocytic forms of two strains of *Plasmodium falciparum in vitro*. *American Journal of Tropical Medicine and Hygiene*, **17**, 661–671.

Sabchareon, A., Chongsuphajaisiddhi, T., Attanath, P., Kanjanapipatkul, K., Doberstyn, E. B. & Suebsaeng, L. (1985). *In vitro* sensitivity of *Plasmodium falciparum* collected from pyrimethamine–sulphadoxine sensitive and resistant areas in Thailand. *Bulletin of the World Health Organization*, **63**, 597–602.

Smrkovski, L. L., Buck, R. L., Alcantara, A. K., Rodriguez, C. S. & Uylangco, C. V. (1985). Studies of resistance to chloroquine, quinine, amodiaquine and mefloquine among Philippine strains of *Plasmodium falciparum*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **79**, 37–41.

Warrell, D. A., Looareesuwan, S., Warrell, M. J., Kasemsarn, P., Intraprasert, R., Bunnag, D. & Harinasuta, T. (1982). Dexamethasone proves deleterious in cerebral malaria: a double-blind trial in 100 comatose patients. *New England Journal of Medicine*, **306**, 313–319.

White, N. J. (1992). Antimalarial drug resistance. The pace quickens. *Journal of Antimicrobial Chemotherapy*, **30**, 571–585.

White, N. J. & Krishna, S. (1989). Treatment of malaria: some considerations and limitations of the current methods of assessment. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **83**, 767–777.

White, N. J. & Warrell, D. A. (1983). Clinical management of chloroquine-resistant *Plasmodium falciparum* malaria in southeast Asia. *Tropical Doctor*, **13**, 153–158.

White, N. J., Looareesuwan, S., Warrell, D. A., Warrell, M. J., Bunnag, D. & Harinasuta, T. (1982). Quinine pharmacokinetics and toxicity in cerebral and uncomplicated falciparum malaria. *American Journal of Medicine*, **73**, 564–572.

White, N. J., Looareesuwan, S., Warrell, D. A., Warrell, M. J., Chanthavanich, P., Bunnag, D. & Harinasuta, T. (1983aa). Quinine loading dose in cerebral malaria. *American Journal of Tropical Medicine and Hygiene*, **32**, 1–5.

White, N. J., Warrell, D. A., Chanthavanich, P., Looareesuwan, S., Warrell, M. J., Krishna, S., Williamson, D. H. & Turner, R. C. (1983b). Severe hypoglycaemia and hyperinsulinaemia in falciparum malaria. *New England Journal of Medicine*, **309**, 61–66.

White, N. J., Miller, K. D., Marsh, K., Berry, C. D., Turner, R. C., Williamson, D. H. & Brown, J. (1987). Hypoglycaemia in African children with severe malaria. *Lancet*, **i**, 708–711.

White, N. J., Krishna, S., Waller, D., Craddock, C., Kwiatkowski, D. & Brewster, D. (1989). Open comparison of intramuscular chloroquine and quinine in children with severe chloroquine-sensitive falciparum malaria. *Lancet*, **i**, 1313–1316.

WHO (1990). Severe and complicated malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **84**, supplement 2.

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TRANSACTIONS OF THE ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE (1994) **88**, 328–331

Genetic evidence that RI chloroquine resistance of *Plasmodium falciparum* is caused by recrudescence of resistant parasites

Hamza Babiker[1,2], **Lisa Ranford-Cartwright**[1], **Ali Sultan**[1,3], **Gwiria Satti**[3] and **David Walliker**[1] [1]*Institute of Cell, Animal and Population Biology, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JN, Scotland, UK;* [2] *Department of Malaria Administration, Ministry of Health, Khartoum, Sudan;* [3]*Department of Biochemistry, Faculty of Medicine, University of Khartoum, Sudan*

Abstract

Isolates of *Plasmodium falciparum* from patients in a Sudanese village exhibiting RI resistance to chloroquine have been typed for allelic variants of 2 merozoite surface antigens, *MSP1* and *MSP2*. Blood forms were taken from each patient before chloroquine was administered, and after parasites had reappeared following treatment. Each patient was found to be infected with genetically different parasites. However, in each patient the parasites of the recrudescent infections possessed the same alleles of each gene as those of the primary infection. The results show that the parasites which reappeared after chloroquine were a genuine recrudescence of the primary forms, and not derived from a new infection.

Introduction

Chloroquine resistance in *Plasmodium falciparum* is conventionally defined as RI (low), RII (intermediate) or RIII (high), depending on the clinical response of patients to a standard course of treatment with the drug (BRUCE-CHWATT, 1986). In RI resistance, parasites are temporarily cleared from the blood following chloroquine treatment, but reappear several days after the treatment is terminated. These parasites are usually considered to represent a recrudescence of the initial parasites which are resistant. Alternatively, they could be due to reinfection of the patient with fresh parasites. Such parasites might exhibit a different drug response from those of the primary infection, since it is well known that mixtures of resistant and sensitive parasites occur within a single region (see, e.g., BABIKER *et al.*, 1991b).

During surveys of *P. falciparum* among inhabitants of a village in eastern Sudan in 1989 and 1990, it was found that many isolates exhibited resistance *in vitro* to chloroquine (BABIKER *et al.*, 1991b). Some patients who had been treated with chloroquine following diagnosis were found to exhibit parasites as well as febrile symptoms after periods of one to 4 weeks following treatment. In the study reported here, we typed parasites of the recrudescent and primary infections of these patients for 2 highly polymorphic antigen genes, using the polymerase chain reaction (PCR) combined with the use of non-radiolabelled chemiluminescent gene probes. We showed that, in each patient, the recrudescent parasites possessed the same alleles of these genes as the primary parasites. The work thus showed unequivocally that the secondary parasites were a genuine recrudescence, and not the products of a new infection.

Subjects, Materials and Methods

Study area and patients

Details of the study area, Asar Village in eastern Sudan, have been given previously (BABIKER *et al.*, 1991a, 1991b). In this area, malaria transmission occurs from September to November, following seasonal rains. The principal parasite is *P. falciparum*, causing more than 90% of the malaria cases (EL-GADDAL, 1986). Drug tests *in vitro* have shown that the parasite population of Asar contains a diversity of chloroquine-resistant and chloroquine-sensitive forms (BABIKER *et al.*, 1991b).

Blood films were prepared from patients attending a daily clinic in Asar throughout the transmission seasons of 1989 and 1990. On diagnosis of *P. falciparum*, venous blood samples were immediately taken from each patient, with their consent, and cryopreserved in liquid nitrogen (BABIKER *et al.*, 1991a). Each patient was then treated with a standard dose of chloroquine of 25 mg/kg over 3 d and examined daily for clearance of parasitaemia, which occurred by day 7 or earlier if the parasites were sensitive to the drug. Six patients returned to the clinic with febrile symptoms after periods ranging from 10 to 30 d, all of whom proved to have *P. falciparum* in their blood. A further sample of blood was taken and cryopreserved. The patients were then treated with Fansidar® (sulfadoxine/pyrimethamine), to take account of the possibility that the parasites could be resistant to chloroquine.

Tests in vitro *for chloroquine resistance*

The isolates which caused the primary infections in the 6 patients who later returned to the clinic were established in culture *in vitro* and tested for chloroquine response, using graded doses of the drug in microtitre plates (BABIKER *et al.*, 1991b). The minimum inhibitory concentration (MIC) which killed all, or nearly all, the parasites was estimated by microscopical examination of Giemsa-stained blood smears.

Genotyping parasites

Parasites were characterized for alleles of genes determining 2 polymorphic merozoite surface proteins, denoted *MSP1* and *MSP2*. Both these genes contain regions encoding tandemly repeated amino acids, which may vary in both number and sequence in different alleles (TANABE *et al.*, 1987; SMYTHE *et al.*, 1991). Variations in the length of these regions can be readily identified as size differences on agarose gels of fragments amplified by PCR, using primers recognizing conserved sequences flanking the repeats (FOLEY *et al.*, 1992; RANFORD-CARTWRIGHT *et al.*, 1993). The sequence variations in this region of *MSP1* can be grouped into 3 types, denoted K1, MAD20 and RO33 after the isolates from which they were originally described (KIMURA *et al.*, 1990). For *MSP2*, 2 types of sequence have been found, denoted IC1 and FC27 (SNEWIN *et al.*, 1991).

PCR amplification of MSP1 *and* MSP2 *genes*. *P. falciparum* deoxyribonucleic acid (DNA) was first isolated from each sample of parasitized blood using the method of FOLEY *et al.* (1992). PCR was used to amplify the repeat region of *MSP1*, denoted block 2 by TANABE *et al.* (1987), and the central region of *MSP2* which includes repeat sequences (FENTON *et al.*, 1991; SMYTHE *et al.*, 1991). Details of the primers used, the PCR conditions, and electrophoresis of the amplified products have been given by RANFORD-CARTWRIGHT *et al.* (1993). Southern blots of the gels were made on to Genescreen® nylon membranes (SAMBROOK *et al.*, 1989).

Probing of PCR-amplified products. Oligonucleotide probes were made which recognized K1-, MAD20- and RO33-type sequences of *MSP1*, and IC1- and FC27-type sequences of *MSP2*. The sequences of the *MSP1* probes have been given by SNEWIN *et al.* (1991). The sequences of the *MSP2* probes were IC1: 5'-GCAGAAGCATC-TACCAGTACC-3' and FC27: 5'-CACCTTCACCACC-CATCAC-3'. The oligonucleotides were labelled using the Amersham Enhanced Chemiluminescence® (ECL) 3' oligolabelling kit. 1×10^{-10} moles of each oligonucleotide

were labelled with fluorescein-11-d-uridine triphosphate by incubation with terminal transferase for 60–90 min at 37°C, according to the manufacturer's instructions.

Hybridization of the labelled oligonucleotides with Southern blots was carried out according to the manufacturer's instructions, with the following modifications. Hybridization was for 1 h at temperatures of 56°C, 68°C and 74°C for *MSP1* probes MAD20, RO33 and K1 respectively, and 56°C and 59°C for *MSP2* probes FC27 and IC1 respectively. The probes were used at concentrations of 5–10 ng/mL of hybridization solution. The blots were first washed with standard saline citrate (1×SSC)*/0·1% sodium dodecyl sulphate (SDS) at room temperature, followed by higher stringency washes with 0·1×SSC/0·1%SDS for 30 min at the same hybridization temperatures. Hybridized oligonucleotides were detected using the Amersham ECL detection kit. Each blot was exposed wet, wrapped in Saranwrap®, to ECL-Hyperfilm® (Amersham) for 1–2 min before being developed. Following each hybridization, the probes were stripped from the membranes by boiling in 0·1×SSC/1%SDS for 15 min, followed by rinsing in 5×SSC for 5 min with continuous agitation (SAMBROOK *et al.*, 1989), and stored in Saranwrap® at -20°C for eventual hybridization with other probes.

Table 1. Chloroquine responses *in vitro* of *P. falciparum* parasites from initial infections from six patients, taken before commencement of drug treatment

Parasites	MIC[a] ($\times 10^{-6}$M)
Patient isolates	
107/89	0·4
123/89	0·8
101/90	0·8
103/90	1·6
120/90	1·6
124/90	1·6
Control clones	
3D7[b]	0·2
Dd2[c]	1·6

[a]Minimum inhibitory concentration (which kills all, or nearly all, parasites.
[b]Chloroquine sensitive.
[c]Chloroquine resistant.

Results

Tests for chloroquine response

Tests in vivo. All the 6 patients who exhibited recrudescences showed an RI type of chloroquine resistance response. In 2 patients, parasitaemias were cleared, but reappeared within 7 d. The other 4 patients showed late recrudescences, 2–3 weeks after completion of treatment.

Tests in vitro. The chloroquine response of parasites from the primary infections of each of the 6 patients was tested *in vitro* (Table 1). The MICs ranged from $0{\cdot}4\times10^{-6}$M to $1{\cdot}6\times10^{-6}$M, compared to $0{\cdot}2\times10^{-6}$M for the drug-sensitive control clone 3D7.

MSP1 *and* MSP2 *characterization or parasites*

PCR followed by hybridization of blots with non-radiolabelled probes was used to identify *MSP1* and *MSP2* alleles of the parasites from the 6 patients who had recrudescences of *P. falciparum* following chloroquine treatment, as well as from 64 further patients in the village.

Parasites from chloroquine-treated patients. The *MSP1* and *MSP2* alleles of the parasites from these patients are shown in Table 2. The Figure illustrates the *MSP2* alleles of primary and recrudescent isolates from 3 of these patients. Each patient was found to contain parasites with different combinations of alleles of the 2 genes. The recrudescent parasites in each patient possessed alleles identical to those of the parasites of the primary infections.

In one patient (no. 123/89), 2 alleles of *MSP1* were seen in both primary and recrudescent samples, showing that this infection contained a mixture of at least 2 genetically distinct chloroquine-resistant parasite clones. In the other 5 patients, only single alleles of each gene were detectable, suggesting clonal-type infections in each case; however, it cannot be entirely excluded that these patients contained mixtures of parasites which by chance were identical at the *MSP1* and *MSP2* loci, but differed at other loci not examined in this work.

Parasites of other patients in the village. Seventy patients from Asar, including 6 showing recrudescent infections, were examined for *MSP1* and *MSP2* alleles. Eleven alleles of *MSP1* were found among these isolates, characterized by PCR products ranging in length from 470 to 580 base pairs (bp), as well as by K1-, MAD20-, or RO33-type sequences. Sixteen alleles of *MSP2* were found, with PCR products ranging in size from 470 to 700 bp and containing either IC1 or FC27 sequences.

Table 2. Alleles of *MSP1* and *MSP2* genes of *P. falciparum* from primary and recrudescent (R) infections in patients treated with chloroquine

Patient isolate no.	Age (years)	Date sampled	*MSP1* alleles Size[a]	*MSP1* alleles Sequence[b]	*MSP2* alleles Size[a]	*MSP2* alleles Sequence[b]
107/89	30	25.10.89	470	RO33	650	IC1
107/89-R		24.11.89	470	RO33	650	IC1
123/89	17	31.10.89	540	MAD20	540	IC1
			470	RO33		
123/89-R		23.11.89	540	MAD20	540	IC1
			470	RO33		
101/90	8	7.10.90	530	K1	580	FC27
101/90-R		26.10.90	530	K1	580	FC27
103/90	15	20.10.90	470	RO33	540	FC27
103/90-R		9.11.90	470	RO33	540	FC27
120/90	50	29.10.90	470	K1	n.d.	n.d.
120/90-R		13.11.90	470	K1	580	FC27
124/90	20	1.11.90	470	RO33	n.d.	n.d.
124/90-R		10.11.90	470	RO33	540	IC1

[a]Estimated number of base pairs of DNA fragments of each allele amplified by the polymerase chain reaction on agarose gels; n.d.=not determined.
[b]The probe which hybridized with each amplified fragment; n.d.=not determined.

*SSC was prepared at 20× normal strength as sodium chloride 3M and trisodium citrate 0·3M, pH 7·0, and then diluted to 5×, 1× and 0·1× as required.

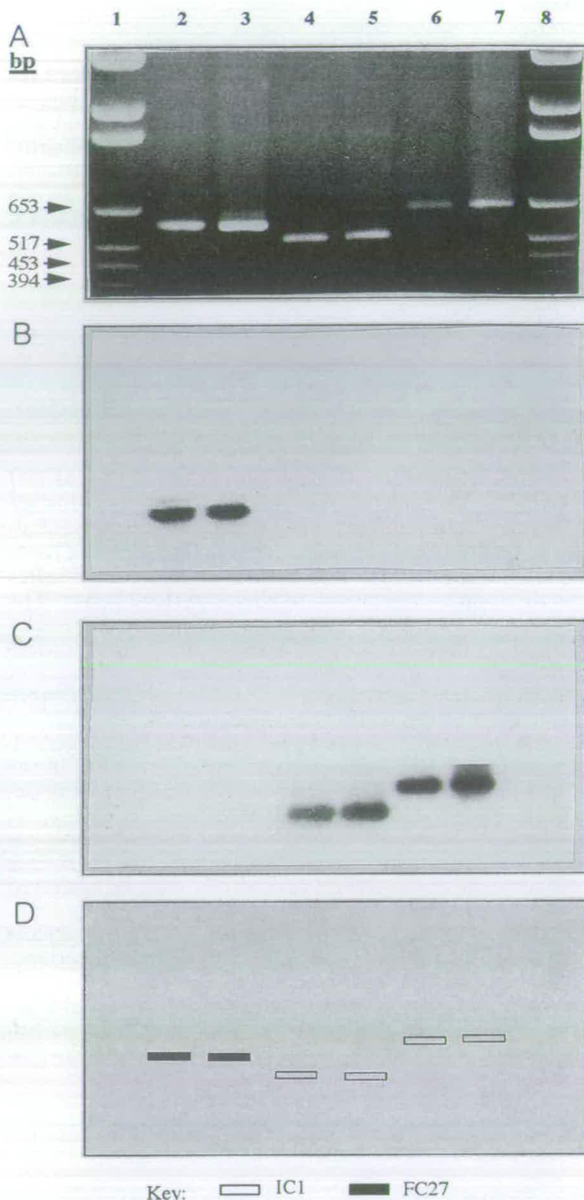

Figure. *MSP2* alleles in isolates from 3 patients with *P. falciparum* malaria, classified by size and sequence. Primary and recrudescent (R) isolates from each patient were run side by side. Tracks 1 and 8, size markers; 2, 101/90; 3, 101/90-R; 4, 123/89; 5, 123/89-R; 6, 107/89; 7, 107/89-R. A. Polymerase chain reaction fragments separated on 1·6% agarose gel and stained with ethidium bromide (bp=base pairs); the fragments were blotted on to a nylon membrane and hybridized separately with the allele-specific IC1 and FC27 probes. B. Film exposed to blot hybridized with FC27 probe. C. Film exposed to blot hybridized with IC1 probe. D. Schematic representation of the results.

Only 2 of the 70 patients examined contained parasites with an identical combination of *MSP1* and *MSP2* alleles; these isolates were from 2 children (sisters) from the same household, who presented at the clinic on the same day.

Discussion

This work has demonstrated that parasites reappearing in the blood of patients suspected of having chloroquine resistant (RI) *P. falciparum* possess the same alleles of *MSP1* and *MSP2* as the parasites of the corresponding primary infections. Tests *in vitro* have confirmed that the primary parasites were slightly or moderately chloroquine resistant when compared to a control drug sensitive clone. We conclude that the recrudescing forms were derived directly from those of the resistant primary parasites, and not from a reinfection with new parasites.

Extensive polymorphisms of the *MSP1* and *MSP2* genes occur among the parasites of Asar village. In our previous study, 8 alleles of *MSP1* and 4 of *MSP2* were detected in the samples collected in 1989 (BABIKER *et al.*, 1991a), using a panel of monoclonal antibodies specific for each antigen. In the current work, 11 alleles of *MSP1* and 16 of *MSP2* have been identified by the PCR/hybridization technique in samples collected in the 2 years 1989 and 1990. Most of the epitopes recognized by the monoclonal antibodies were not in the repeat regions of the antigens, and so no clear correlation could be made between the polymorphisms revealed by each technique. However, the combined results of the 2 studies show that the number of allelic variants of these antigens in the parasites of Asar is very high.

The existence of 11 alleles of *MSP1* and 16 of *MSP2* means that 176 (11×16) possible combinations of the alleles of each gene could occur among the parasites in the village. Although some alleles of each gene are more frequent than others (unpublished observations), the probability that 2 parasite clones taken at random from the inhabitants will have identical alleles at these loci is very low. Among the 70 isolates examined, only 2 had the same allele combinations, and these were from 2 sisters sleeping in the same house who had most probably been infected by the same mosquito on the same day. The probability that a single person will become reinfected from mosquitoes with parasites with *MSP1* and *MSP2* alleles identical to those of the primary infection is also exceedingly low. For these reasons, we conclude that the parasites reappearing in the blood of the 6 chloroquine-treated patients discussed in this paper were a genuine recrudescence of the primary parasites, and not a reinfection.

It was also of interest that the parasites in the 6 patients caused febrile episodes on each occasion they presented at the clinic. The causes of a new episode of malaria illness in a single patient are not understood, although one possible explanation is infection with a novel 'strain' of *P. falciparum*, to which the patient has not been previously exposed. (LINES & ARMSTRONG, 1992). It seems clear that this was not the case with the patients examined in the present study.

The method described here of genotyping *P. falciparum* for *MSP1* and *MSP2* alleles is similar to some of those previously described (e.g., by KIMURA *et al.*, 1990 and MERCEREAU-PUIJALON *et al.*, 1991), except that we have made use of fluorescein-labelled probes in the hybridization work. As discussed elsewhere (e.g., by FOLEY *et al.*, 1992), the PCR technique is particularly sensitive and useful for this type of work, since it can be carried out with low numbers of parasites without the necessity of culturing them. Fluorescein-labelled probes, as used here, have obvious advantages over radio-labelled material. They are easy to make, and probably cheaper in the long term because they can be stored for long periods without loss of activity. They are particularly suitable for use in countries where radioactivity is difficult to obtain, and they have none of the hazards associated with the handling and disposing of radioactivity. The method is thus of great potential value in field surveys of genes in populations of parasites such as *Plasmodium*.

The prevalence of chloroquine resistance in Asar was found to be high in 1989 (BABIKER *et al.*, 1991b), resistant *P. falciparum* having been detected in this area since 1986 (BAYOUMI *et al.*, 1989). RII and RIII types of chloroquine resistance are easy to diagnose and alternative antimalarial drugs can be prescribed immediately. However, symptoms caused by recrudescent RI parasitaemias may frequently be confused by clinicians with other conditions, for which quite different treatments may be described. Thus, patients infected with RI chloroquine-resistant *P. falciparum* may harbour their parasites for a long time, with obvious epidemiological significance, es-

pecially it they have gametocytaemia. Correct diagnosis of recrudescent RI parasites is therefore important, and has a practical impact on management of *P. falciparum* malaria in areas where resistance occurs. The techniques described here for characterizing parasites are at present expensive and need well-trained personnel; however, PCR technology is already finding a place in the diagnosis of malaria (see, e.g., JAUREGUIBERRY *et al.*, 1990), and we envisage that the methods used here might eventually be developed for field use.

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References

Babiker, H. A., Creasey, A. M., Fenton, B., Bayoumi, R. A. L., Arnot, D. E. & Walliker, D. (1991a). Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 1. Diversity of enzymes, 2D-PAGE proteins and antigens. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **85**, 572–577.

Babiker, H. A., Creasey, A. M., Bayoumi, R. A. L., Walliker, D. & Arnot, D. E. (1991b). Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 2. Drug resistance, molecular karyotypes and the *mdr1* genotype of recent isolates. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **85**, 578–583.

Bayoumi, R. A., Babiker, H. A., Ibrahim, S. M., Galib, H. W., Saeed, B. O., Khider, S. & Karim, E. A. (1989). Spread of chloroquine resistant *Plasmodium falciparum* in Sudan. *Acta Tropica*, **46**, 157–165.

Bruce-Chwatt, L. J., editor. (1986). *Chemotherapy of Malaria*, 2nd edition. Geneva: World Health Organization.

El Gaddal, A. (1986). Malaria in the Sudan. In: *Proceedings of the Conference on Malaria in Africa*, Buck, A. A. (editor). Washington: American Institute of Biological Sciences, pp. 156–159.

Fenton, B., Clark, J. T., Anjam Khan, C. M., Robinson, J. V., Walliker, D., Ridley, R., Scaife, J. G. & McBride, J. S. (1991). Structural and antigenic polymorphism of the 35- to 48-kilodalton merozoite surface antigen (MSA-2) of the malaria parasite *Plasmodium falciparum*. *Molecular and Cellular Biology*, **11**, 963–971.

Foley, M., Ranford-Cartwright, L. C. & Babiker, H. A. (1992). Rapid and simple method for isolating malarial DNA from fingerprick samples of blood. *Molecular and Biochemical Parsitology*, **53**, 241-244.

Jaureguiberry, G., Hatin, I., d'Auriol, L. & Galibert, G. (1990). PCR detection of *Plasmodium falciparum* by oligonucleotide probes. *Molecular and Cellular Probes*, **4**, 409–414.

Kimura, E., Mattei, D., Di Santi, S. M. & Scherf, A. (1990). Genetic diversity in the major merozoite surface antigen of *Plasmodium falciparum*: high prevalence of a third polymorphic form detected in strains derived from malaria patients. *Gene*, **91**, 57–62.

Lines, J. & Armstrong, J. R. M. (1992). For a few parasites more: inoculum size, vector control and strain-specific immunity to malaria. *Parasitology Today*, **8**, 381-383.

Mercereau-Puijalon, O., Fandeur, T., Bonnefoy, S., Jacquemot, C. & Sarthou, J.-L. (1991). A study of the genomic diversity of *Plasmodium falciparum* in Senegal. 2. Typing by the use of the polymerase chain reaction. *Acta Tropica*, **49**, 293–304.

Ranford-Cartwright, L. C., Balfe, P., Carter, R. & Walliker, D. (1993). Frequency of cross-fertilization in the human malaria parasite *Plasmodium falciparum*. *Parasitology*, **107**, 11–19.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Smythe, J. A., Coppel, R. L., Day, K. P., Martin, R. K., Oduola, A. M. J., Kemp, D. J. & Anders, R. F. (1991). Structural diversity in the *Plasmodium falciparum* merozoite surface antigen MSA-2. *Proceedings of the National Academy of Sciences of the USA*, **88**, 1751–1755.

Snewin, V. A., Herrera, M., Sanchez, G., Scherf, A., Langsley, G. & Herrera, S. (1991). Polymorphism of the alleles of the merozoite surface antigens MSA1 and MSA2 in *Plasmodium falciparum* wild isolates from Colombia. *Molecular and Biochemical Parasitology*, **49**, 265–276.

Tanabe, K., Mackay, M., Goman, M. & Scaife, J. G. (1987). Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*. *Journal of Molecular Biology*, **195**, 273–287.

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TRANSACTIONS OF THE ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE (1994) **88**, 332–333

Long term exposure of *Trypanosoma brucei gambiense* to pentamidine *in vitro*

Tanoé W. Miézan[1], **Ulf Bronner**[2], **Félix Doua**[1], **Pierre Cattand**[3] and **Lars Rombo**[2] [1]*Projet de Recherches Cliniques sur la Trypanosomiase (PRCT), Daloa, Côte d'Ivoire;* [2]*Unit of Tropical Pharmacology, Division of Infectious Diseases and Division of Clinical Pharmacology, Karolinska Institute, Huddinge University Hospital, Huddinge, Sweden;* [3]*Division of Control of Tropical Diseases, World Health Organization, Geneva, Switzerland*

Abstract

In order to study the sensitivity *in vitro* of *Trypanosoma brucei gambiense* to pentamidine, 5×10^4 parasites were exposed to 0, 0·1, 1·0, 2·0, 10, 100, 1000 and 10 000 μg/L of pentamidine isethionate for up to 10 d. The viability of parasites was determined each day by microscopy. Multiplication was retarded during continuous exposure to 2 μg/L. After 4 d no further multiplication took place, although the trypanosomes remained alive for another 3 d. The parasiticidal effect was more pronounced when higher concentrations were used; when exposed to 10 and 100 μg/L, all parasites were dead after 4 and 3 d, respectively. Despite exposure to 1000 μg/L, 74% of the parasites were still alive the next day. 10 000 μg/L killed all parasites within 24 h of exposure. Our results show that the time period of exposure to pentamidine plays a major role in determining the sensitivity *in vitro* of *T. b. gambiense*, and we suggest that prolonged exposure *in vivo* may be more important than attaining high but brief peak concentrations.

Introduction

Pentamidine has been used for many years in the treatment of *Trypanosoma brucei gambiense* sleeping sickness without involvement of the central nervous system. Nevertheless, no uniform recommendation for treatment exists. We are in the process of evaluating dosage regimens, to be based on proper pharmacokinetic characterization of the drug. During the course of this work we have found little information on the minimum concentration and time required to eliminate parasites. Without such knowledge it is not possible to utilize pharmacokinetic data properly. We now report on the effect of duration of exposure and concentration of pentamidine on the response *in vitro* of *T. b. gambiense*.

Materials and Methods

Parasites

The trypanosomes were isolated in 1990 from a patient with *T. b. gambiense* infection and involvement of the central nervous system (isolate MHOM/CI/90 Dal 1442). After cryopreservation they were kept in Hepes-buffered minimum essential medium (MEM) with Earle's salts (Gibco, Paisley, Scotland, UK) in 24-well tissue culture plates (Costar, Cambridge, Massachusetts, USA). The medium was supplemented with 0·4% MEM non-essential amino acids (Gibco), 3·5% foetal calf serum (Gibco), 15·5% horse serum (Gibco), 2 mM L-glutamine, 2 mM sodium pyruvate, 10 mM glucose, 400 μg/mL of kanamycine and 400 units/mL of nystatine. In addition, 0·1 mM hypoxanthine, 0·016 mM thymidine and 0·2 mM 2-mercaptoethanol were added (BALTZ *et al.*, 1985). Parasites were adapted to culture conditions for one month before the study but during this period 10% foetal calf serum and 10% horse serum were used. During the first week of adaptation the trypanosomes were grown in the presence of *Microtus montanus* fibroblast feeder layer cells (BRUN *et al.*, 1981). Each well contained 1 mL of medium. The cultures were incubated at 37°C in 4% CO_2 in air.

Drug

200 mg of pentamidine isethionate powder (Pentacarinat®, May & Baker, Dagenham, UK) were dissolved in 5 mL of distilled water. This stock solution was further diluted with medium to the required concentrations, the dilution factor being calculated on the assumption that all the pentamidine remained in solution. All concentrations were calculated in terms of pentamidine isethionate.

Study design

5×10^4 parasites were exposed to 0, 0·1, 1·0, 2·0, 10, 100, 1000 or 10 000 μg/L of pentamidine for up to 10 d. The viability of the parasites was determined each day by microscopy and the number of live parasites in each well was calculated using a haemacytometer (Neubauer). Dividing parasites were counted as one individual. Whenever the number of live parasites exceeded 1×10^5, the corresponding well was diluted with medium containing the same concentration of pentamidine in order to reduce the number to 5×10^4 parasites. Each drug concentration was tested in triplicate.

Results

The response of live trypanosomes to prolonged exposure to different concentrations of pentamidine is illustrated in the Figure. When no drug was added to the medium, the trypanosomes continued to multiply and the population increased approximately 9-fold over each period of 24 h, indicating at least 3 divisions per day.

Pentamidine at 0·1 μg/L had no effect during the study period. When parasites were exposed to 1·0 μg/L, multiplication was retarded after a few days but continued for 8 d. These parasites were examined for 20 d and remained viable even if no multiplication was occurring, as determined both by visual inspection and calculation of parasite numbers.

During exposure to 2·0 μg/L, multiplication was retarded and after 4 d no further multiplication took place, although parasites remained alive for another 3 d. When higher concentrations were used, killing was more rapid and no live parasite was seen after 4 d at 10 μg/L or 3 d at 100 μg/L. Even at the latter concentration, parasites continued to multiply to some extent during the first 24 h. At 1000 μg/L, 74% were viable on the next day, but at 10 000 μg/L no live parasite was seen on the next day.

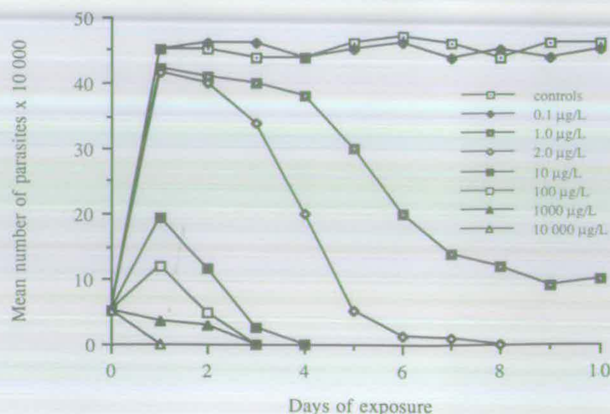

Figure. Activity *in vitro* of pentamidine on *T. b. gambiense*. Parasite numbers are means of 3 experiments. Whenever multiplication occurred, parasites were diluted to 5×10^4 each day.

Address for correspondence: Dr Ulf Bronner, Division of Infectious Diseases, Huddinge University Hospital, S-141 86 Huddinge, Sweden.

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Techniques

MVR-PCR Analysis of Hypervariable DNA Sequence Variation

D.E. Arnot, C. Roper and A.A. Sultan

Techniques for accurate marking of infectious microbial agents circulating in populations would be very useful to epidemiologists. In this article, David Arnot, Cally Roper and Ali Sultan review recent progress in transferring MVR-PCR DNA fingerprinting techniques from human forensic medicine to parasitology.

A common feature among eukaryotic genomes is the occurrence of mini-satellite repeats comprising short, tandemly reiterated DNA sequences, sometimes referred to as VNTR (variable number of tandem repeats) loci. High levels of inter- and intra-specific variation in the number of tandem repeats can occur at such loci. Where DNA probes exist that hybridize to many dispersed mini-satellite sequences simultaneously, a genome 'fingerprint' can be obtained for use as a taxonomic characteristic. Such techniques have been widely used in parasitology, especially in species where morphological differentiation is difficult (eg. in *Leishmania*[1]).

The Development of MVR-PCR

Despite the usefulness of genomic fingerprinting, the method depends on the length variation of target sequences and such variation can be limited. Length variation is also quasi-continuous, making definitive allele identification difficult. This can limit population genetic analysis, since real (but unquantifiable) differences in allelic size usually have to be handled by effectively degrading the information content of the data ('binning')[2]. Mini-satellite variant repeat analysis using a specialized polymerase chain reaction (MVR-PCR, also known as the Jeffreys' method) is a recently developed technique that solves these problems by detecting allelic sequence polymorphism without the effort required by DNA sequencing[3]. The original version of the method exploits the hypervariable mini-satellite tandem repeats at a human interstitial locus on chromosome 1 (the D1S8 locus). There are two types of 29 bp repeat at this locus that differ by a single G–A transition mutation, these are referred to as a-type repeats and t-type repeats. The a-type and t-type repeats show highly diverse interspersion patterns within different alleles. Oligonucleotide primers can be designed that specifically hybridize to each of the variants. Combining such variant repeat specific primers and a primer located at a monomorphic site in the DNA flanking the repeats, it is possible to generate PCR products extending from the flanking primer to each a-type or t-type repeat. Interspersion patterns can then be 'read' from autoradiographs of the separation gel (which have a certain resemblance to supermarket product barcodes) and converted into allele-specific digital codes.

Advantages and Flexibility

Digitized codes derived from the sequences of individual mini-satellite loci reveal more polymorphism than is detectable by allelic size comparisons and, therefore, more precise identification of individual alleles is achieved. This allows accurate allele-frequency estimation and simplifies standard population-genetic analysis. The technique is relatively simple, rapid and internally well controlled, in that the PCR fragment sizes are predictably spaced in the gel and are thus themselves accurate size markers. This permits accurate comparison of the migration of different lanes, and makes intergel comparisons more reliable. Digital codes are also easier to compile and compare using computers. The main obstacle to the adoption of a MVR-PCR system is that of finding suitable MVR-PCR loci. Repeated DNA sequences flanked by conserved regions exist in most eukaryotes but few have been conveniently characterized. An exception is *Plasmodium falciparum*, which has many genes containing tandemly repeated DNA encoding conserved, repetitive amino acid sequences. We have used these features of the *P. falciparum* circumsporozoite (CS) gene to develop an MVR-PCR assay that can reveal very high levels of DNA sequence polymorphism at this locus[4]. Box 1 illustrates the principles of the technique as applied to an ideal target locus in a haploid organism such as *P. falciparum*. MVR-PCR methodology must be adapted according to the ploidy of the organism, the size of the tandemly repeated unit and the extent of conservation of the flanking sequences. The *P. falciparum* system is adapted to the CS gene tandem-repeat unit of 12 bp and, since primers have to be around 20 bp, our CS gene variant-specific primers overlap two units. In CS genes inter-repeat variation creates 'null' positions that do not cross-hybridize with either primer and these positions are interspersed in the repeat array and contribute to allelic polymorphism. The results of MVR-PCR reactions on DNA extracted from blood

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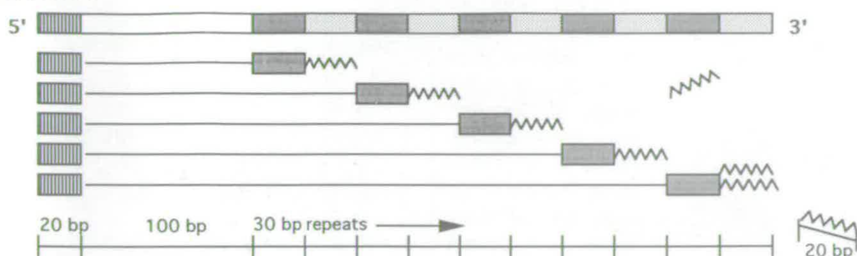
Box 1. The Principle of Single-allele MVR-PCR

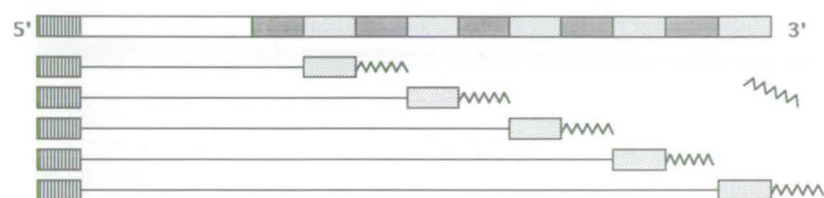
The target locus is shown as the 5′–3′ bar with two alternating variants of a 30 bp repeat. A conserved sequence is situated 100 bp upstream and is the binding site for a 20 bp flanking primer (vertical hatch). Variant specific primers (VSPs) corresponding to the Type 1 (dark grey) and the Type 2 (light grey) repeats are 30 bp oligonucleotides, extended by a further 20 bp TAG. (The tagging strategy is necessary because high concentrations of primers complementary to the repeats will themselves result in a progressive shortening of fragment size at each PCR cycle through internal priming within the PCR products.) Addition of a sequence TAG onto the VSP and amplifying with low concentrations of TAGGED VSP and high concentrations of flanking primer and an oligonucleotide identical to the TAG sequence (the TAG primer) effectively uncouples the initial annealing reaction from the subsequent cycles of amplification.

Thermostable DNA polymerase then catalyses the following reactions (see Fig. below):

Reaction 1



Reaction 2

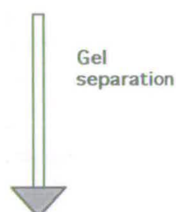


Key

Flanking primer

Variant specific primers with TAG sequence

TAG Primer



Reaction 1	Reaction 2	Fragment size (bp)
	▬	380
▬		350
	▬	320
▬		290
	▬	260
▬		230
	▬	200
▬		170

(1) The low concentration of TAGGED Type 1 VSP anneals to approximately one Type 1 repeat per target locus and extends in a 5′→3′ direction into the flanking DNA (Reaction 1).
(2) The flanking primer primes from the conserved upstream sequence until ending in a sequence complementary to the TAG addition.
(3) Fragments terminating at the flanking sequence and the TAG complement will now be amplified preferentially (because of the high concentrations of the flanking and TAG primers) to create bands whose size corresponds to the distance between the flanking sequence and the position of each Type 1 repeat unit. A separate reaction tube containing Type 2 VSP will generate a complementary set of fragments terminating at the position of Type 2 repeat units (Reaction 2). Infrequent internal priming events using the VSP primers will generate authentic products but with a bias towards shorter fragments.

Electrophoretic separation of the products of Reaction 1 and Reaction 2 is shown in diagrammatic form. Either agarose or acrylamide gels could be used, depending on the size of products (which largely depends on the size of the unit repeat). We use 6% acrylamide gels to separate fragments radioactively labelled by including a ^{32}P-labelled dNTP during the polymerization reaction. The first PCR product will be 170 bp long, that is, the distance from the 5′ end of the flanking primer to the 3′ end of the fragment created by annealing of the VSP to the closest variant repeat unit. Thereafter, PCR products from Reactions 1 and 2 occur every 30 bp, the unit step of the tandem repeat.

samples obtained from eight individuals infected with *P. falciparum* are illustrated in Fig. 1. Extensive allelic variation in the interspersion pattern of variant DNA repeats encoding the well-conserved repetitive amino acids (the famous NANP sequence) is displayed. Individual alleles are readily identified and encoded according to the position of PCR bands. For example, sample 7 with Type 1 and 2 variants in Lanes a and b, respectively, is numerically encoded

10000010122222222000

for the first 20 repeats in the array. As with all genetic typing of clinical isolates, parasite mixtures can cause problems of interpretation, giving rise to occasional bands in both lanes of the MVR-PCR reaction when two or more genotypes are present in roughly equimolar concentrations. However, not all isolates are multiclonal and information can be extracted from mixed isolates, particularly where one clone clearly predominates.

Hypervariable sequences are the result of elevated mutation rates in tandem repeats and mutation rates as high as 15% per gamete occur at some human

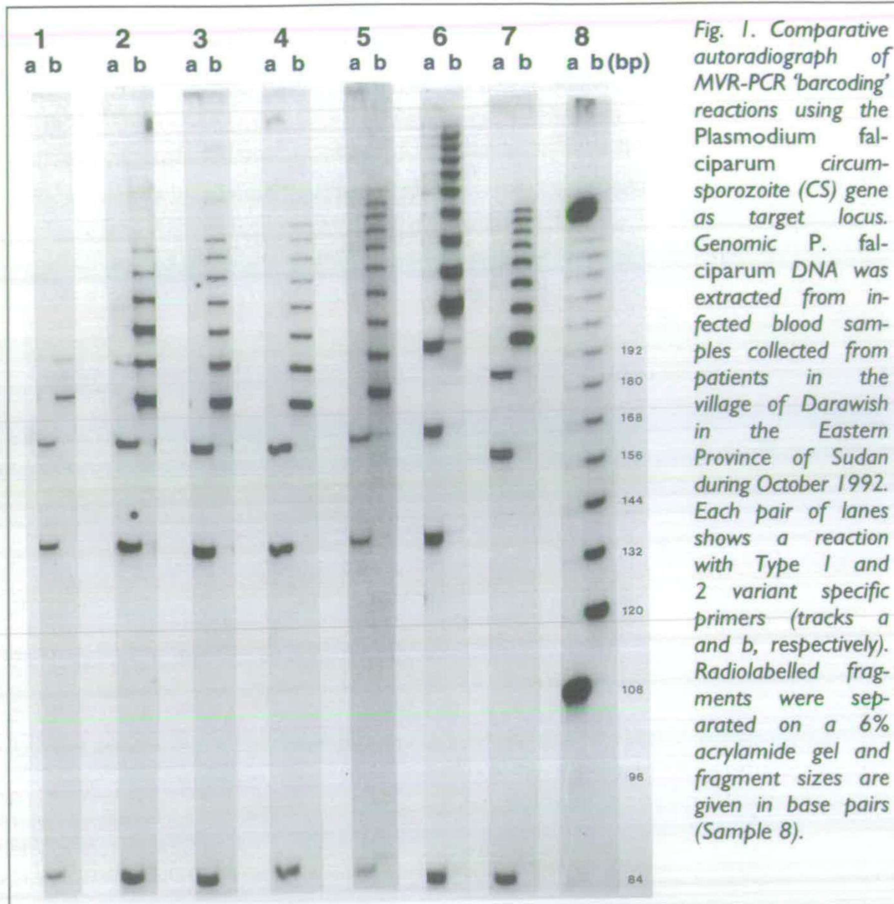


Fig. 1. Comparative autoradiograph of MVR-PCR 'barcoding' reactions using the Plasmodium falciparum *circumsporozoite (CS) gene as target locus. Genomic P. falciparum DNA was extracted from infected blood samples collected from patients in the village of Darawish in the Eastern Province of Sudan during October 1992. Each pair of lanes shows a reaction with Type 1 and 2 variant specific primers (tracks a and b, respectively). Radiolabelled fragments were separated on a 6% acrylamide gel and fragment sizes are given in base pairs (Sample 8).*

minisatellite loci[5] although pedigree analysis of VNTR segregation[3] generally shows somewhat lower rates (0.8–1.0%). The mutation rate in sperm DNA appears to be around 20 times higher than in blood DNA samples, which may suggest that most mutation is germ-line specific, perhaps meiotic (A.J. Jeffreys, pers. commun.). The CS repeats are evolving faster than the rest of the gene[6,7] and, although we do not know the mutation rate, the MVR-PCR code of clone 3D7 has not perceptibly changed over a four-year period of mitotic division in continuous culture, nor has the HB3 code been affected by meiosis and mosquito passage.

Microepidemiological Studies

Epidemiology and forensic scientific detection have a lot in common. Each seeks the identity and mode of operation of some undesirable agent with a view to halting its activities. MVR-PCR was developed as a means of exploiting hypervariable DNA sequences to identify humans unambiguously from small (and often poorly preserved) samples of cellular tissue such as blood found at the

Fig. 2. (left) Global variation in the MVR-PCR codes from Plasmodium falciparum *CS gene tandem repeats. The numerical coding of interspersion patterns has been converted into symbolic codes for ease of visual comparison. The first ten isolates are derived by encoding published sequences and the complete repeat array is shown, the remaining six are experimentally derived and are, therefore, incomplete. NF54, 3D7 and CVD1 are related; 3D7 and CVD1 being clones derived from NF54, an uncloned isolate whose country of origin is unknown. Wel is known to be West African, LE5 Liberian, B11 Ugandan and 7G8 Brazilian. T4, T9/98, T9101 and K1 are all Thai, MAD/20 is from Papua New Guinea, Dd2 is from Vietnam, HB3 is from Honduras, PA17 is Ugandan and the origin of R is uncertain, possibly Brazilian. Type 1 repeat, closed box; Type 2 repeat, hatched box; neither Type 1 nor Type 2, open box.*

Fig. 3. (right) Local variation in the MVR-PCR codes of the Plasmodium falciparum *CS gene in isolates from the Gedaref region in the Eastern Province of Sudan. The SUD clones are all from Asar village (population 4000) isolates of the 1989 transmission season, whereas the Dara isolates were from patients who became ill in the smaller village of Darawish (population 385, 1.5 km from Asar) during October 1992. Type 1 repeat, closed box; Type 2 repeat, hatched box; neither Type 1 nor Type 2, open box.*

scene of a crime. Malariological MVR-PCR is intended to identify *P. falciparum* isolates unambiguously in patient blood samples in order to investigate the population structure and transmission of individual parasite genotypes.

The potential of MVR-PCR polymorphism in the CS gene for the differentiation of parasites at a local level is illustrated in Fig. 2 and Fig. 3. The extent of variation in a sample of *P. falciparum* isolates collected worldwide is shown in Fig. 2. Of 16 isolates, 14 have different MVR-PCR codes (it should be noted that 3D7 and CVD1 are both clones derived from the NF54 isolate). The variation present in two neighbouring Sudanese villages is shown in Fig. 2. In 23 patient isolates, 21 MVR-PCR codes are detectable. Dara 1 has two bands at the same position, indicating that this isolate contains more than one genotype. There is apparently as much variation present at the local level as there is in the global sample. Given that both village data sets were collected over short periods within the brief Eastern Sudanese transmission season (late August – mid-October), there is no indication that expanded clones dominate the population. This is in agreement with other published studies from The Gambia[8] and Sudan[9,10].

Two of the 1992 Darawish isolates (Dara 5 and Dara 7) were derived from sisters, living in the same hut, who became ill on the same day, and these isolates were identical. Our interpretation (confirmed with additional polymorphic markers) is that they were infected by the same parasite, perhaps acquired from the same mosquito during interrupted feeding. We are currently extending these trial studies to monitor the temporal and geographic spread of MVR-PCR marked parasite genotypes through each malaria patient within Darawish village during the 1993 transmission season. It may thus become possible to estimate the basic reproductive rate of infection (*Ro*), the number of secondary cases generated by a primary case, for each parasite lineage being transmitted in the village[11].

Acknowledgements

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References

1 Macedo, A.M. *et al.* (1992) *Mol. Biochem. Parasitol.* 53, 63–70
2 Lander, E.S. (1989) *Nature* 339, 501–505
3 Jeffreys, A.J. *et al.* (1991) *Nature* 354, 204–209
4 Arnot, D.E., Roper, C. and Bayoumi, R.A.L. (1993) *Mol. Biochem. Parasitol.* 61, 15–24
5 Jeffreys, A.J. *et al.* (1988) *Nature* 332, 278–281
6 Galinski, M.R. *et al.* (1987) *Cell* 48, 311–319
7 Arnot, D.E., Stewart, M.J. and Barnwell, J.W. (1990) *Mol. Biochem. Parasitol.* 43, 147–150
8 Conway, D.J. and McBride, J.S. (1991) *Parasitology* 103, 7–16
9 Babiker, H.A. *et al.* (1991) *Trans. R. Soc. Trop. Med. Hyg.* 85, 572–577
10 Bayoumi, R.A. *et al.* (1993) *Trans. R. Soc. Trop. Med. Hyg.* 87, 454–458
11 Gupta, S. *et al.* (1994) *Science* 263, 961–963

David E. Arnot, Cally Roper and Ali A. Sultan are at the Institute of Cell, Animal and Population Biology, Division of Biology, Edinburgh University, West Mains Road, Edinburgh, UK EH9 3JT. Ali A. Sultan is on leave from the Department of Biochemistry, Faculty of Medicine, University of Khartoum, PO Box 102, Khartoum, Sudan.

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MOLECULAR AND BIOCHEMICAL PARASITOLOGY

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Cloning and characterisation of a *Plasmodium falciparum* homologue of the *Ran/TC4* signal transducing GTPase involved in cell cycle control

Ali A. Sultan*, William A. Richardson, Pietro Alano[1], David E. Arnot and Christian Doerig

Institute of Cell, Animal and Population Biology, University of Edinburgh, Ashworth Laboratories, West Mains Road, Edinburgh EH9 3JT, Scotland, UK

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MOLECULAR AND BIOCHEMICAL PARASITOLOGY

Aims and Scope

The journal provides a medium for the rapid publication of investigations of the molecular biology, molecular immunology and biochemistry of parasitic protozoa and helminths and their interactions with both the definitive and intermediate host. The main subject areas covered are: chemical structure, biosynthesis, degradation, properties and function of small molecular weight substances, DNA, RNA, proteins, lipids and carbohydrates – intermediary metabolism and bioenergetics – molecular and biochemical studies on the mode of action of antiparasitic drugs – molecular and biochemical aspects of membrane structure and function – molecular and biochemical aspects of host–parasite relationships including analysis of parasitic escape mechanisms – characterisation of parasite antigen and parasite and host cell surface receptors – characterisation of genes by biophysical and biochemical methods, including recombinant DNA technology – analysis of gene structure, function and expression – mechanisms of genetic recombination.

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Department of Medical
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Fax: 071 636 8739

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National Instute of
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Tel.: 081 959 3666 ext. 2175
Fax: 081 913 8593

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Cloning and characterisation of a *Plasmodium falciparum* homologue of the *Ran/TC4* signal transducing GTPase involved in cell cycle control

Ali A. Sultan*, William A. Richardson, Pietro Alano[1], David E. Arnot and Christian Doerig

Institute of Cell, Animal and Population Biology, University of Edinburgh, Ashworth Laboratories, West Mains Road, Edinburgh EH9 3JT, Scotland, UK

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Abstract

On the basis of conserved sequences characteristic of the Ran/TC4 subfamily of the GTPase superfamily, a fragment of the gene encoding a *Plasmodium falciparum* Ran/TC4 homologue was amplified in the polymerase chain reaction. The fragment was used to screen a cDNA library to obtain clones which allowed determination of the complete gene sequence. The gene, designated *pfran* (*Plasmodium falciparum ras*-like nuclear protein), has around 70% amino acid identity with previously characterised Ran/TC4 proteins. Like other malarial mRNAs, the *pfran* mRNA contains a long (at least 679 bp) 5′ untranslated region. Southern blotting experiments show that *pfran* is a single copy gene located on chromosome 11. RNA hybridisation experiments indicate that *pfran* mRNA is abundant in late trophozoite and schizont stages, but present at very low levels in gametocytes and early asexual stages.

Key words: *Plasmodium falciparum*; *pfran*; GTPase; Cell cycle

1. Introduction

Invasion of red blood cells by *Plasmodium falciparum* merozoites leads to entry into one of 2 developmental pathways. Either the invading merozoite enters the asexual cycle during which several mitotic divisions result in production of 8–32 merozoites followed by haemolysis, or sexual differentiation into gametocytes occurs [1]. Gametocytogenesis requires the arrest of cell cycle progression, suggesting that sexual differentiation and cell cycle regulatory circuits may share some

*Corresponding author. Tel: 031-650-8656; Fax: 031-668-3210; e-mail: EANV02@ uk.ac.ed.ercvax

Abbreviations: PCR, polymerase chain reaction; UTR, untranslated region; ORF, open reading frame.

Note: Nucleotide sequence data reported in this paper have been submitted to the EMBL, Genbank^{TM} and DDBJ data bases under the accession number X73954.

[1]*Present address:* Istituto Superiore Di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

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common features. Neither the signals triggering sexual development nor the pathways of signal transduction during regulation of the cell cycle are understood in the malaria parasite.

The molecular processes underlying these aspects of the *Plasmodium* life cycle are likely to include some forms of the signal transduction and regulatory pathways essential to all living cells. For example, in mammalian systems, small cytosolic enzymes of the Ras subgroup of the GTPase superfamily [2], are known to play a key role in growth factor receptor-mediated signal transduction [3], and are also implicated in the control of normal cell differentiation and mitotic regulation [4]. Mutant forms of the *ras* gene can also function as oncogenes capable of transforming normal cells [5,6]. Ran/TC4, a small *ras*-related GTPase originally identified in a human teratocarcinoma cDNA library [7], appears to function in signal transduction in the context of cell cycle regulation. Homologues have been reported from *Dictyostelium* [8], fission and budding yeast [9,10] and filarial worms [11].

Ran/TC4 protein is abundant (0.36% of total HeLa cell protein [12]), and forms a complex with another abundant nuclear protein called RCCI (regulator of chromosomal condensation-1), which binds to chromatin and catalyses the exchange of guanine nucleotides on Ran/TC4 [13]. Functional heterodimers of the Ran/TC4 and RCC1 proteins are required for several major cellular processes, including protein import into the nucleus [12], regulation of mitosis [14], RNA transcription, processing and export [15], regulation of DNA synthesis [16] and regulation of the *Saccharomyces cerevisiae* mating pathway [17].

We report the identification of a putative Ran/TC4 homologue in *P. falciparum, pfran*. The gene encodes a 214 amino acids protein containing the five conserved GTP-binding sites characteristic of the GTPase superfamily, and is interrupted by a single intron. In common with several other *P. falciparum* messenger species [18], the coding sequence is preceded by a long 5′ untranslated region. Northern blotting experiments indicate that mRNA levels vary significantly during the erythrocytic development of the parasite.

2. Materials and methods

Parasite culture, DNA isolation and characterisation. Parasites of cloned *P. falciparum* lines 3D7A, C10 and 1776 were grown under standard culture conditions. Gametocyte cultures were harvested at stages 2/3 (young) and 4/5 (mature) [19]. Genomic DNA extraction and endonuclease digestion were performed following standard protocols. Filters were prehybridised for 1 h at 65°C in 5× SSC (1× SSC = 0.15 M NaCl/ 0.015 M Na_3 citrate · $2H_2O$, pH 7.0/ 5× Denhardt's solution/ 0.1% SDS/ 50 mM sodium phosphate buffer pH 7/ 100 μg ml^{-1} denatured salmon sperm DNA. Hybridisation was carried out in the same buffer with 10% dextran sulphate added, at 65°C overnight. Filters were extensively washed in 2× SSC at room temperature and then washed in 0.5× SSC/ 0.1% SDS at 65°C for 1 h.

PCR amplification of genomic DNA. Two stretches of amino acids that are highly conserved among members of the Ran/TC4 family (residues 32–38 [GEFEKKY] and residues 154–160 [YNFEKPE] [9]) were used to design degenerate oligonucleotides biased towards *P. falciparum* codon usage [20]. The oligonucleotides were then used to prime the polymerase chain reaction (PCR) with *P. falciparum* genomic DNA (clone 3D7A) as template. The oligonucleotide sequences were: the forward primer, 5′GG(A/T)GAATT(A/C)GAAAA-(A/G)AA(A/G)TA 3′ and the reverse primer, 5′GG(C/T)TT(T/C)TC(T/G) AA(A/G)TT(A/G)-TA(A/G)TT 3′.

PCR amplification was carried out in 100 μl PCR reaction mixtures containing 0.5 μg of *P. falciparum* genomic DNA/1.0 μM each primer/ 200 μM each dNTP/10 mM Tris-HCl, pH 8.8/50 mM KCl/2.5 mM $MgCl_2$/0.02% (w/v) gelatin/0.5 units of Taq DNA polymerase. PCR amplification was carried out in a Hybaid™ thermal reactor as follows: 30 s at 94°C, 60 s at 55°C and 120 s at 72°C for 30 cycles.

Library screening and sequencing. The PCR fragment was radiolabelled by random priming and used as a hybridisation probe to screen a *P. falciparum* asexual stage cDNA library constructed in

the plasmid vector pJFE14 (a gift from A. Craig). 50 000 colonies were screened, and both strands of the positive cDNA inserts were sequenced by the dideoxynucleotide chain termination method.

Pulsed field gel electrophoresis. *P. falciparum* chromosomal DNA blocks were prepared as previously described [21]. Pulsed field gradient gel electrophoresis (PFGE) was performed using a Bio-Rad™ CHEF DRII gel apparatus and 1% Fastlane agarose gels. The chamber buffer was 10 mM Tris HCl, 20 mM acetic acid and 2 mM EDTA circulated at 14°C. Gels were ramped from 360–800 s pulse times at a constant 100 V for 96 h.

Northern blot analysis. Cultures were synchronised by the method of Lambros and Vanderberg [22]. Total RNA from synchronous cultures of rings, trophozoites, schizonts and gametocytes was prepared as described [23]. For Northern blotting, 2.5 μg of total RNA were separated by electrophoresis in a 1.2% agarose gel containing 0.74% formaldehyde in MOPS buffer, and transferred to Hybond-N^+ membranes (Amersham) by alkaline transfer. Prehybridisation, hybridisation and washing of the membranes were performed according to the conditions described above for Southern blotting.

3. Results and discussion

Isolation and sequencing of the pfran gene. A 552 bp DNA fragment was obtained by amplifying *P. falciparum* genomic DNA using 2 degenerate primers based on conserved regions of the Ran/TC4 protein. Sequencing of the PCR product confirmed that the amplified fragment represented part of a gene encoding an open reading frame (ORF) with homology to the published Ran/TC4 sequences. The ORF was interrupted by a 165 bp intron (see below). This fragment was used as a hybridisation probe to screen a *P. falciparum* cDNA library made with RNA extracted from asexual parasites. Screening of 50 000 clones yielded 3 strongly hybridising clones. One clone with a 1.4 kb insert, C4.1, was selected for sequence analysis.

The sequence of the cDNA clone C4.1 is shown

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A.
                                                        1
                                                        AAGTTTTTTTTTTCATATTATTTAATAATCCTTTTTTTTCTTTCCCCCTTTTTCTGAGATTATAAATTAATATATATAT  79
ATATATATATATAATTATTAAATTAATTAAAATTAAAATATATTTTTTTTATTTCCATTATTTTTATTTTTGCTCTAATTTCTTTCCAACTTTGCTTATTTTTCTAAAAAAAAAAATTTT  199
ATTTTTATTTGTTTAAAATATAATATAAGGAAAAAAAAAAAAAAAAAAAAAAAAAAGGCCTTCCTTTTTTAAACCTTGGAATTCCAATTAAAAAAAAAAAATTAAAACCATTAATTTTAGGT  319
TATTATTAATTAAAAAAAAAAAAAAAAATTAAACCATTAATTTTATTGGTTGGAATTAATTTATTTTTTTTATATTAATTAACCTTAAATTATATATTATATAAATTCCTTTAAAAAGAATAAC  439
CGTTTTTTACATTAAATCTAGTGCAGTTTTAAAACACACTTAAAAGAAAAATTTGCAACCTTTTAAAGCTCTGAGTACATCATTTAAGAATATAGTTTTCTATTATATATACGTATATTT  559
TTGTATATAACACATAAGCAATATGTCAGTTGATATATAAATTAAATATATATTTACAACATAGTAGTCTTATTTTAATATATCATTTTTTTTTTTCTTTTTATTAGTACAATTTAAAAA  679
                                                                                                                      ▼
ATGGATTCACAAGAATATATTCCACAATATAAATTAATCTTAGTCGGTGATGGTGGTGTTGGCAAAACAACCTTTGTGAAAAGACACTTGACTGGAGAATTCGAAAAAAAATATATAGCT  799
 M  D  S  Q  E  Y  I  P  Q  Y  K  L  I  L  V  G  D  G  G  V  G  K  T  T  F  V  K  R  H  L  T  G  E  F  E  K  K  Y  I  A
ACTCTTGGTGTGGAAGTTCACCCCTTAAAATTTCAAACAAACTTTGGAAAAACTCAATTTAACGTATGGGATACTGCAGGACAAGAAAAGTTTGGTGGTTTAAGAGATGGATATTATATA  919
 T  L  G  V  E  V  H  P  L  K  F  Q  T  N  F  G  K  T  Q  F  N  V  W  P  T  A  G  Q  E  K  F  G  G  L  R  D  G  Y  Y  I
AAAAGTGATTGTGCTATAATTATGTTTGATGTATCTTCTCGTATTACTTACAAGAACGTTCCAAATTGGTATAGAGATATTACAAGAGTGTGTGAAACAATTCCTATGGTTTTAGTTGGA  1039
 K  S  D  C  A  I  I  M  F  D  V  S  S  R  I  T  Y  K  N  V  P  N  W  Y  R  D  I  T  R  V  C  E  T  I  P  M  V  L  V  G
AACAAAGTTGATGTTAAAGACAGACAAGTAAAATCAAGGCAAATTCAATTTCACAGAAAAAGGAATTTACAATACTACGATCTATCTGCAAGATCAAACTACAATTTCGAAAAACCTTTC  1159
 N  K  V  D  V  K  D  R  Q  V  K  S  R  Q  I  Q  F  H  R  K  R  N  L  Q  Y  Y  D  L  S  A  R  S  N  Y  N  F  E  K  P  F
TTATGGTTAGCTAGGAGATTGTCCAACCAACACCATCTTGTTTTCGTAGGAGAACATGCTAAAGCACCAGAGTTCCAAATTGATCTAAATATTGTAAGAGAAGCTGGAAAAGAATTAGAG  1279
 L  W  L  A  R  R  L  S  N  Q  P  N  L  V  F  V  G  E  H  A  K  A  P  E  F  Q  I  D  L  N  I  V  R  E  A  G  K  E  L  E
CAAGCAGCAGCTGTAGCTATTGATGAAGAAGATATTGAAAATTAAATAAATAGAAAAAAAAAAAAAAAAAAAAAAAAAAGGGAAACAGCCAAAATGGAAGTAAAAAAAAAAAAAAAAAAAGAAA  1398
 Q  A  A  A  V  A  I  D  E  E  D  I  E  N  *

B.         796
             |
GAAAAAAAATATATA gtaagtgaaaaggagaaataataaaatgggaacattaatatatatatatatatatatatatatatatttatttatttatttatatgtttacatatatatt
 E  K  K  Y  I                                                     797
                                                                     |
tatatttatttttatattttgtatttatttattttattataattttttttttttttttttag GCTACTCTTGGTGTG
                                                                A  T  L  G  V
```

Fig. 1. (A) The complete sequence of cDNA clone 4.1 containing the large 5′ UTR and complete coding sequence of *pfran* gene of *P. falciparum*. The predicted amino acid sequence for the coding region is shown below the nucleotide sequence in the single letter code and the amino acid sequences used to design the oligonucleotide primers are underlined. The site of the intron is indicated by an arrow. (B) Nucleotide sequence of the intron in the *P. falciparum pfran* gene obtained from genomic DNA. Intron sequence is in lower case typescript. Numbering as in (A).

in Fig. 1A. The insert contained a single ORF extending from nucleotide 677 to a stop codon 77 bases upstream from the 3′ terminus. The deduced amino acid sequence had a methionine codon at nucleotide 680 which permitted a homologous alignment of the sequence with available sequences of Ran/TC4 proteins. The C4.1 cDNA contained a long (679 bp) 5′ untranslated region (UTR), and 77 bp of non-coding sequences at the 3′ end. Sequences of genomic DNA adjacent to the original 552 bp PCR product were obtained by utilising the inverse PCR procedure [24]. The coding sequence, the intron-exon boundaries, and 300 bp of 5′ UTR sequence proximal to the start codon were confirmed as identical in both the genomic DNA and the cDNA clone.

The ORF is interrupted by an intron between nucleotides 796 and 797 (Fig. 1B). The intron is 165 nucleotides long and is 90% A + T which compares with 67% A + T for the coding sequence and 85% A + T for the 5′ and 3′ UTRs. The sequences at the ends of the intron have the universally conserved GT and AG dinucleotides marking the intron-exon boundaries, and the 3′ AG splice acceptor site is preceded by a stretch of 17 thymidine residues, an exaggeration of the pyrimidine nucleotide motif generally found at the 3′ intron boundary of eukaryotic genes [25]. The sequence at the 5′ intron boundary (GTAAGTGAA) is also well conserved among *Plasmodium* introns and similar to the consensus eukaryotic intron boundary sequence.

pfran is a single copy gene located on chromosome 11. Genomic *P. falciparum* DNA was digested with restriction enzymes and analysed by Southern blotting, using the C4.1 insert as a probe (Fig. 2A). All lanes containing DNA digested with restriction enzymes that do not cleave within the probe (*Acc*I, *Bgl*II, *Hinc*II, *Hind*III, *Msp*II, *Nde*I, *Xba*I or *Xho*I) show hybridisation to a single fragment. DNA digested with *Alu*I, *Dra*I, *Hin*fI and

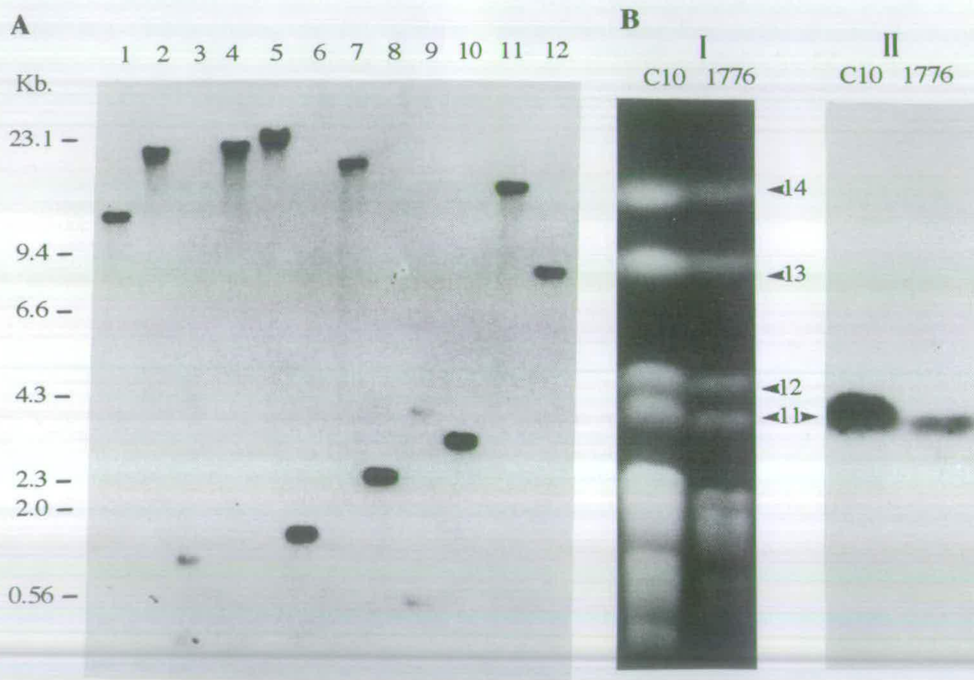

Fig. 2. (A) Southern blot analysis of the *pfran* gene. Genomic DNA from *P. falciparum* was digested with various enzymes, resolved, blotted and hybridised with *pfran* probes. The enzymes used were: (lanes 1–12): *Acc*I, *Bgl*II, *Sau*3A, *Msp*II, *Xho*I, *Dra*I, *Hinc*II, *Hind*III, *Alu*I, *Hin*fI, *Xba*I and *Nde*I. (B) Chromosome assignment of the *pfran* gene. (I): Ethidium bromide-stained pulsed field gradient gel of clones C10 and 1776, with chromosomes 11, 12, 13 and 14 marked; (II): autoradiograph of the blot from the gel in panel (I) probed with the radiolabelled *pfran* cDNA.

*Sau*3A, enzymes which cleave within the probe, yields more than one hybridising fragment, whose sizes correspond to the sizes predicted from the sequence. No further hybridising bands appeared after longer exposures with less stringently washed filters (final washing in 2× SSC/ 0.1% SDS at 55°C), indicating that a single copy of *pfran* exists in the genome of *P. falciparum*. Using standard calculations for the melting temperatures of mismatched DNA hybrids at these salt concentrations and temperatures it can be estimated that it is unlikely that a gene with nucleotide sequence homology to *pfran* of more than 80% exists within the *P. falciparum* genome.

P. falciparum chromosomes were resolved on the CHEF gel system and the blots were hybridised to the C4.1 cDNA fragment. The *pfran* gene was found to be located on chromosome 11 (Fig. 2B), according to the definition of chromosome numbers proposed by Kemp et al. [21]. This was confirmed by probing the same filters with chromosome 5 and 9 specific probes (*pfmdr-1* and *msp-1*) respectively (data not shown).

Sequence homologies between pfran and other members of the Ran/TC4 GTPase subfamily. Comparison of the amino acid sequence deduced from clone C4.1 with the sequences contained in the GenBank database showed that pfRan was clearly related to proteins of the small GTPase superfamily (Fig. 3). The highest similarity scores were with the members of the Ran/TC4 subgroup of this family; 72% and 70% with *S. cerevisae* Gspl and Gsp2 respectively [10], 69% with *Schizosaccharomyces pombe* Spil [9], 68% with the filarial worm Ran/TC4 proteins [11], 67% with the human Ran/TC4 [7], and 66% with *Dictyostelium* Dd.TC4 [8].

The argument that the *pfran* gene is a *P. falciparum* homologue of the Ran/TC4 subfamily of small GTPases rests on sequence homology data and not as yet on functional data. Predicted molecular weight and possession of the 5 conserved guanine nucleotide binding regions clearly indicate that *pfran* is a member of the small GTPase superfamily. However, outside these conserved regions, there are no obvious homologies between the pfRan sequence and those of the other three

```
                               G-1                              G-2
TC4      MAAQGEPQVQ FKLVLVGDGG TGKTTFVKRH LTGEFEKKYV ATLGVEVHPL 50
PfRan     MDSQ.YIP. Y..I...... V......... .........I ..........
Dd.TC4        MAKKE. I......... V......Q.. ......PR.I P....S....
Spil      M..PQNVPT .......... .......... .........I ..........
                         G-3
TC4      VFHTNRGPIK FNVWDTAGQE KFGGLRDGYY IQAQCAIIMF DVTSRVTYKN 100
PfRan    K.Q..F.KTQ .......... .......... .KSD...... ..S..I....
Dd.TC4   I.Y..F.K.H .......... .......... ..GN...... .....IS...
Spil     H....F.E.C .......... .L........ ..G..G.... .....I....
                                    G-4                   G-5
TC4      VPNWHRDLVR VCENIPIVLC GNKVDIKDSK VKAKSIVFHR KKNLQYYDIS 150
PfRan    ....Y..IT. ...T..M..V .....V..RQ ..SRQ.Q... .R......L.
Dd.TC4   .....S..T. .......... .....V..R. ..PSQ..... RY..S...V.
Spil     ..H.W..... .......... .....V.E.. ....A.T... ..........

TC4      AKSNYNFEKP FLWLARKLIG DPNLEFVAMP ALAPPEVVMD PALAAQYEHD 200
PfRan    .R........ ......R.SN Q...V..GEH .K..-.FQI. LNIVREAGKE
Dd.TC4   .......... .V..TS..L. NKAVTL.QQ. T.KL..T.L. SN.MSL..KE
Spil     .......... ........V. N.......S. .......QV. QQ.L...QQE

TC4      LEVAQNPALP DEDD-DL 216
PfRan    ..Q.AAV.I- ..E.IEN
Dd.TC4   VAD.AALP.. EDN.-DL
Spil     MNE.AAMP.. ....ADL
```

Fig. 3. Comparison of protein sequences from 3 members of the Ran/TC4 subfamily with the *Pfran* amino acid sequence. Sequences are aligned relative to the human Ran/TC4 protein, the first member of the subfamily to be described [7]. Dd.TC4 is the *D. discoideum* sequence [8] and Spil is the *S. pombe* sequence [9]. Dots indicate identical residues, dashes indicate gaps introduced to maximise similarity within the alignment. The five regions involved in guanine nucleotide binding (G1 to G5), which are highly conserved between all members of the small GTPase superfamily, are underlined.

main subfamilies of this group, the *ras*-like proto-oncogenes, the *ypt/rab* genes and the *rho* type genes [2]. pfRan does not have the carboxyterminal cysteine motif that mediates isoprenylation and membrane association in Ras family members, and like the Ran/TC4 proteins, has a stretch of acidic amino acid residues at this position [14]. pfRan shares 66–72% amino acid identity with all members the fourth small GTPase subfamily so far characterised, the Ran/TC4 type proteins. Although the homology between mammalian representatives of this subfamily is at least 95%, this drops to around 80% amino acid identity between human and yeast homologues and 68% identity between human and *Dictyostelium* Ran/TC4 proteins. The sequence homology argument for provisionally accepting pfRan as the *P. falciparum* representative of the Ran/TC4 small GTPases is therefore strong.

mRNA size and expression during parasite development. To obtain some information on how *pfran* mRNA levels are regulated during intraerythrocytic development, we performed a Northern blot analysis of RNA isolated from synchronised asex-

ual and gametocyte cultures. Total RNA from ring, trophozoite, schizont and gametocyte stages was probed with cDNA clone C4.1 (Fig. 4). The probe hybridised to a diffuse band of approximately 1.3–1.5 kb. T. and it is likely that clone C4.1 represents a full-length (or nearly full-length) cDNA. Transcripts produced from sexual and asexual stage parasites appear to be similar in size. Signals were strongest on the schizont and trophozoite lanes (Fig. 4B, lanes 1 and 2), indicating progressively accumulating mRNA as the parasite prepares for and then undergoes nuclear division. Very low levels were observed in early asexual stages and in gametocytes (Fig. 4B, lanes 3, 4 and 5). It cannot be excluded that these low signals may result from contamination of rings or gametocytes preparations with small amounts of trophozoites or schizonts. Similar amounts of RNA were present in all lanes, as indicated by ethidium bromide staining of the gel prior to transfer (Fig. 4A). As an internal control for hybridisation to gametocyte mRNA, the same filter was hybridised to sequences from the gametocyte-specific Pfg25/27 gene [26]. As expected, we observed an intense signal only in lanes containing RNA from gametocytes (Fig. 4C).

The size of the *pfran* mRNA supports the evidence obtained from cDNA and genomic DNA sequences that a much larger transcript is produced than would be required to encode the protein. It is becoming clear that many *Plasmodium* transcripts are considerably longer than required for coding purposes alone, and that this appears to be mainly a consequence of having exceptionally long 5′ untranslated regions [18]. The function of these long 5′UTR remains to be determined.

In both higher and lower eukaryotic cells it is known that GTPase activity of the Ran/TC4 protein requires the association of both a Ran specific GTPase activating protein [27] and an abundant DNA binding nuclear protein known as RCC1 in human [15] and Pim1 (premature initiation of mitosis) in *S. pombe*. These three molecules have been postulated to have an important, although

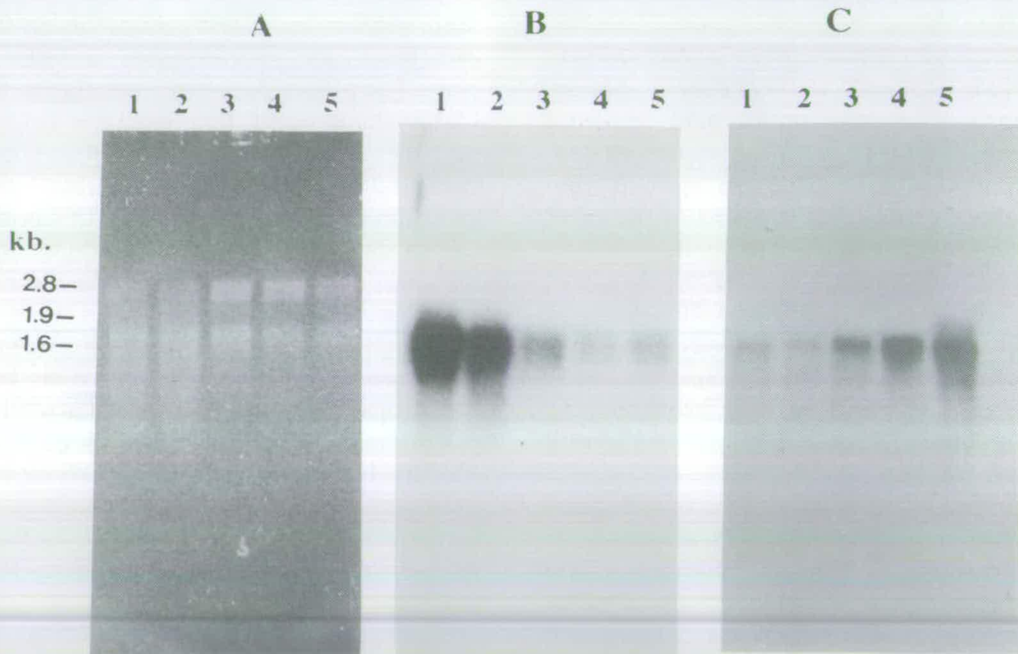

Fig. 4. Northern blot analysis. Total *P. falciparum* RNA obtained from different developmental stages of clone 3D7A was fractionated in a denaturing formaldehyde gel, blotted and hybridised to the *pfran* gene probe and *Pfg27/25* gametocyte specific gene. A; Ethidium bromide stained gel, B and C; autoradiographs of the above gel probed with *pfran* and *pfg27/25* genes respectively. Lane 1: schizonts, lane 2: trophozoites, lane 3: rings, lane 4: mature gametocytes and lane 5: young gametocytes.

as yet poorly understood, role in the regulation of the eukaryotic cell cycle. Various possibilities have been suggested including a Ran mediated signal transduction inhibiting mitosis when chromosome replication is incomplete [13] and involvement of the Ran/RCC1 complex in the maintainance of interphase chromosome structure [15]. DNA synthesis in erythrocytic *P. falciparum* starts during the latter part of trophozoite development [28]. Our results indicate that the steady-state *pfran* mRNA levels are developmentally regulated, in that mRNA levels are high during schizogony and low in the ring and gametocyte stages. Low levels of *pfran*mRNA in these developmental stages may reflect the fact that little or no DNA synthesis is occurring in these cells [29]. Increased synthesis of *pfran*mRNA may be required from mid-trophozoite stage onwards to produce a protein involved in the regulation of subsequent nuclear division and it is known that Ran/TC4 mutants interfere with DNA synthesis and therefore disrupt cell cycle progression [16]. An anti-pfRan antiserum would permit temporal and spatial localisation of the protein within the parasite and facilitate a search for associated proteins such as homologues of the RCC1 protein [13] and the Ran-specific GTPase activating protein [27].

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References

[1] Alano, P. and Carter, R. (1990) Sexual differentation of malaria parasites. Annu. Rev. Microbiol. 44, 429–449.

[2] Bourne, H.R., Sanders, D.A. and McCormick, F. (1991) The GTPase superfamily: Conserved structure and molecular mechanism. Nature, 349, 117–127

[3] Burgering, B.M., Medema, R.H., Maassen, J.A., van der Watering, M.L., van der Eb, A.J., McCormick, F. and Bos, J.L. (1991) Insulin stimulation of gene expression mediated $p21^{ras}$ activation. EMBO J. 10, 1103–1109.

[4] Hall, A. (1990) The cellular function of small GTP binding proteins. Science 249, 635–640.

[5] Mulcachy, L.S., Smith, M.R. and Stacey, D.W. (1985) Requirement for *ras* oncogene function during stimulated growth of NIH-3T3 cells. Nature 313, 241–243.

[6] Bar Sagi, P. and Feramisco, J.R. (1985) Microinjection of the Ras oncogene protein into PC12 cells induces morphological differentiation. Cell 42, 841–848.

[7] Drivas, G.T., Shih, A., Coutavas, E.E., Rush, M.G., and D'Eustachio, P. (1990) Characterisation of four novel *ras*-like genes expressed in a human teratocarcinoma cell line. Mol. Cell. Biol. 10, 1793–1798.

[8] Bush, J. and Cardelli, J. (1993) Molecular cloning and DNA sequence of a *Dictyostelium* cDNA encoding a Ran/TC4 related GTP-binding protein belonging to the Ras superfamily. Nucleic Acids Res. 21, 1675.

[9] Matsumoto, T. and Beach, D. (1991) Premature initiation of mitosis in yeast lacking RCC1 or an interacting GTPase. Cell 66, 347–360.

[10] Belhumeur, P., Lee, A., Tam, R., DiPaolo, T., Fortin, N., and Clark, M.W. (1993) GSP1 and GSP2, genetic suppressors of the *prp20-1* mutant in *Saccharomyces cerevisiae*: GTP-binding proteins involved in the maintenance of nuclear organisation. Mol. Cell. Biol. 13, 2152–2161.

[11] Dissanayake, S., Xu, M., Piessens, W.F. (1992) Filarial parasites contain a Ras homolog of the TC4/Ran/Spil family. Mol. Biochem. Parasitol 56, 259–268.

[12] Shannon, M. and Blobel, G. (1993) The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature 365, 661–663

[13] Bischof, F.R. and Ponstingl, H. (1991) Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. Nature, 354, 80–82.

[14] Dasso, M., Nishitani, H., Kornbluth, S., Nishimoto, T. and Newport, J.W., (1992) RCC1, a regulator of mitosis, is essential for DNA replication. Mol. Cell. Biol. 12, 3337–3345.

[15] Dasso, M. (1993). RCC1 in the cell cycle: the regulator of chromosome condensation takes on new roles. Trend. Biochem. Sci. 18, 96–101.

[16] Ren, M., Drivas, G., D'Eustachio, P., and Rush, M.G. (1993). Ran/TC4: A small nuclear GTP-binding protein that regulates DNA synthesis. J. Cell Biol. 120, 313–323.

[17] Clark, K.L. and Sprague, G.F. (1989) Yeast phermone response pathway: Characterization of a suppressor that

restores mating to receptorless mutants. Mol. Cell. Biol. 9, 2682–2694.

[18] Levitt, A. (1993) RNA processing in malarial parasites. Parasitology Today, 9, 465–468.

[19] Graves, P.M., Carter, R., and McNeill, K.M. (1984) Gametocyte production in cloned lines of *Plasmodium falciparum* Am. J. Trop. Med. Hyg. 33, 1045–1050.

[20] Weber, J.L. (1987) Analysis of sequences from the extremely A-T rich genome of *Plasmodium falciparum*. Gene 52, 103–109.

[21] Kemp, D.J., Thompson, J.K., Walliker, D. and Corcoran, L.M. (1987) Molecular karyotype of *Plasmodium falciparum*: Conserved linkage groups and expendable histidine-rich protein genes. Proc. Natl. Acad. Sci. USA 84, 7672–7676.

[22] Lambros, C. and Vanderberg, J.P. (1979) Synchronisation of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol. 65, 418–420.

[23] Delves, C.J., Alano, P., Ridley, R.G., Goman, M., Holloway, S.P., Hyde, J.E. and Scaife, J.G. (1990) Expression of alpha and beta-tubulin genes during the asexual and sexual blood stages of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 43, 271–278.

[24] Triglia, T., Peterson, M.G. and Kemp, D.J. (1988) A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acids Res. 16, 8186.

[25] Weber, J.L. (1988) Molecular biology of malaria parasites. Exp. Parasitol. 66, 143–170.

[26] Alano, P., Premawansa, S., Bruce, M.C. and Carter, R., (1991) A stage specific gene expressed at the onset of gametocytogenesis in *Plasmodium falciparum*. Mol. Biochem. Parasitol. 46, 81–88

[27] Coutavas, E., Mindong, R., Oppenheim J.D., D'Eustachio, P. and Rush, M. (1993) Characterization of proteins that interact with the cell-cycle regulatory protein Ran/TC4. Nature 366, 585–587.

[28] Inselburg, J. and Banyal, H.S. (1984) Synthesis of DNA during the asexual cycle of *Plasmodium falciparum* in culture. Mol. Biochem. Parasitol. 10, 79–87.

[29] Janse, C.J. Ponnudurai, T., Lenson, A.H.W., Meuwissen, J.H.E., Ramesar, J., Van Der Ploeg, M. and Overdulve, J.P. (1988) DNA synthesis in gametocytes of *Plasmodium falciparum*. Parasitology 96, 1–7.

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