A study of the regulation of DNA replication genes of *Plasmodium falciparum.*

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Declaration

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

Paul Daniel Horrocks
May 1996

Abstract of Thesis.

This thesis has focussed on the regulation of the gene encoding the essential DNA replication protein Proliferating Cell Nuclear Antigen (PCNA). PCNA expression in *Plasmodium falciparum* had earlier been shown to be stage specific during development in the intraerythrocytic cycle. Here its expression was examined in more detail. In addition, a preliminary analysis of the regulation of the gene encoding DNA polymerase δ, of which PCNA is an auxiliary factor, was made.

Antisera raised against *P.falciparum* PCNA (PfPCNA) and DNA polymerase δ (PfPolδ) have been used against extracts from synchronised parasites to show that both proteins accumulate in trophozoites and persist in schizonts. The steady-state transcripts from both PfPCNA and PfPolδ also accumulate at the trophozoite stage. However, nuclear run on analysis shows that, whereas PfPolδ promoter activity is absent in rings but present in trophozoites and schizonts, the PfPCNA promoter is active throughout the intraerythrocytic cycle. This suggests that mechanisms regulating the expression of these two genes may be different although their coordinated activity is required for DNA replication.

A major transcription start site 960bp upstream of the translational start of the PfPCNA coding sequence was identified. A second, minor, site is situated a further 40bp upstream. These results were developed from a number of methods to reduce the possibility of ambiguities which may arise due to the extreme AT-richness of *P.falciparum* non-coding DNA. Consensus TATA boxes and an OCT-1 box were identified upstream of the putative transcription start sites. Analysis of cDNA clones identified a putative transcription stop site 250bp downstream of the stop codon.

An attempt was made to develop a transient transfection system using 5′ and 3′ flanking sequences from PfPCNA with the novel reporter gene encoding the green fluorescent protein (GFP) of *Aquorea victoria*. No GFP fluorescence was observed although analysis indicated the construct reached the cytoplasm of the parasite after electroporation. In contrast the intraerythrocytic stages were successfully transfected with constructs

containing a firefly luciferase reporter gene under the transcriptional control of variously modified elements of the PfPCNA 5′ flanking sequence to test the effect on promoter activity. The analysis identified an essential region for promoter activity which contains the physically mapped transcription start sites. In addition, a region approximately 400bp upstream of the transcription start sites is required for efficient promoter activity.

Work from this thesis has been published elsewhere;

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Contents.

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Abbreviations.

amp	ampicillin
ATP	adenosine triphosphate
bp	base pair
BSA	bovine seum albumin
cDNA	complementary DNA
cfu	colony forming unit
CIAP	calf intestinal alkaline phosphatase
cpm	counts per minute
DAPI	4'-6-diamino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddH_20	double distilled water
ddGTP	dideoxyguanosine triphoshate
ddNTP	dideoxynucleoside triphosphate
ddTTP	dideoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	diaminoethanetetra N, N, N', N'-acetic acid (disodium salt)
EGTA	ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid.
EtBr	ethidium bromide
FITC	fluorescein isothiocyanate
g	gram

GFP	green fluorescent protein
HEPES	n-(2-hydroxyethyl) piperazine- N'-(2-ethanesulphonic acid)
ICMB	Institute of Cell and Molecular Biology
IFA	immunofluorescence assay
IPTG	isopropyl-1-thio-β-D-galactoside
kb	kilobase
kDa	kilodalton
kg	kilogram
L	litre
LB	Luria- Bertani medium
M	molar
mCi	millicurie
ml	millilitre
mM	millimolar
MOPS	morpholinepropanesulfonic acid
mRNA	messenger RNA
NP40	nonidet P40
ORF	open reading frame
p	prefix for plasmid DNA
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEG	polyethylene glycol
PfPCNA	*P.falciparum* PCNA
PfPolδ	*P.falciparum* DNA polymerase δ
pfu	plaque forming unit
PMSF	phenylmethylsulphonyl fluoride
RBC	red blood cell
RMPI	Rosewell Memorial Park Institute
RNA	ribonucleic acid
RNase	ribonuclease
sd$H_2$0	sterile distilled water

SDS	sodium dodecyl sulfate
SSC	standard saline citrate
TAE	tris acetate EDTA
TBE	tris borate EDTA
TEMED	N,N,N′,N′- tetraethylmethylenediamine
Tris	tris (hydroxymethyl) aminomethane
Tris-HCl	tris hydrocloride
U	unit
UTR	untranslated region
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
°C	degree celcius
µg	microgram
µl	microlitre
µm	micromolar

Chapter 1.

1.1 Malaria.

Malaria is the disease caused by the parasites of the genus *Plasmodium*. Human malaria is found in the tropics and sub-tropics, and is endemic in over 90 countries. Almost one half of the world's population is at risk of contracting malaria. The exact figures regarding the number of people who develop malaria, and its subsequent mortality, are difficult to assess. Estimates of over 280 million infections, with 110 millions developing disease symptoms leading to 2 million deaths have been made (Brown, 1992, Miller *et al*, 1994). In Africa alone, around 1 million people, mainly infants and children under 5 years of age, die annually from malaria.

References to the symptoms of malaria have been recorded in ancient Egyptian and Chinese writings. From early times malaria has been associated with marshes; *plaudism* is from the French word for swamps or marsh. Initially the "airs" (*mal*: bad, *aria*: air) associated with swamps or evil spirits were blamed for causing the disease. It was not until 1880 that Laveran identified parasites in the blood of patients suffering from malaria (Bruce-Chwatt, 1985, Phillips, 1993).

The protozoan parasites responsible for the disease, of which over 120 species have been identified, are found in a wide range of distinct vertebrate hosts including; birds, man, primates, reptiles and rodents. 4 species of *Plasmodium* infect man, *P.malariae, P.ovale, P.vivax* and the more severe *P.falciparum* (Phillips, 1983).

In 1955 the 8th World Assembly adopted a global programme for the eradication of malaria. This programme was taken over two years later by the World Health Organisation. As part of an 8 year programme insecticides and anti-malarial drugs were used to eradicate the mosquito vector and treat human reservoirs of the disease. After some initial promising progress the disease appeared to returning to many regions from which had been eradicated. In the 1970's malaria was once again endemic in tropical and sub-tropical regions, although malaria had receded from some more temperate regions (Bruce-Chwatt, 1985). Mosquitoes resistant to the insecticide DDT and drug resistant parasites were identified during this period as being responsible for the resurgence.

Human *Plasmodium*, with multiple drug resistance, presents an ever increasing world health problem.

The governments in regions where malaria is endemic have, over the last 10-15 years, faced increasing financial restrictions. The falling levels of income *per capita* have led to a reduced health expenditure during 1980-90s. The financial burden of malaria has been estimated, during 1985 for example, to have accounted for a substantial proportion of the gross domestic product of these developing regions (Evans and Jamison, 1994). This figure was based upon the costs of prevention programmes, clinical treatment and loss of production of goods from people incapacitated by malaria. The burden of the disease at the village and family level is even more disastrous. These governments have had to face choices of how to control malaria and realistically justify the costs to the donor nations of the developed world. Currently, control programmes throughout the world are concentrating on preventative measures such as education, impregnated bed nets and health clinics in the community (Kolberg, 1994).

1.2 *P.falciparum* life cycle.

The life cycle of *P.falciparum* (Phillips, 1983, Bruce-Chwatt, 1985) takes place in two hosts; the asexual phase in the human vertebrate host and a sexual phase in the female *Anopholes* mosquito (Figure 1).

Infection in the human host starts when sporozoites from the saliva of an infected mosquito are introduced into the blood stream during a blood meal. The motile sporozoites move through the skins capillary beds into the main blood stream. When the sporozoites reach the liver they attach to, and invade parenchymal cells. The entire process takes approximately 30 minutes, during which time many of the sporozoites are destroyed by phagocytes. In the parenchymal cells the remaining sporozoites undergo many mitotic divisions, in a process known as exoerythrocytic schizogony, which produces many thousands of merozoites. On completion of schizogony the parenchymal cells rupture releasing the merozoites into the blood stream. The merozoites rapidly recognise,

attach to and invade erythrocytes, a process that takes approximately 20 seconds.

Within the erythrocytes the parasite undergoes intraerythrocytic schizogony, a process that takes 48 hours. The merozoite initially develops into a small circular form known as the ring stage. The ring stage grows and become irregular in shape as it develops into a trophozoite. The trophozoite stage, sometimes referred to as the "feeding form", uses the erythrocyte haemoglobin as a nutrient source. DNA synthesis starts in the mid-trophozoite stage and the nucleus undergoes up to 4 rounds of division. As the trophozoites develop into schizonts the cytoplasm is divided. The nuclei and cytoplasm separate to give up to 16 merozoites which are released when the erythrocyte bursts. The rupture of the infected erythrocytes is synchronised and coincides with a peak in the fever symptoms. The *falciparum* malaria is particularly severe due to the ability of infected erythrocytes to attach to one another, in a process known as rosetting, as well as to the epithelium of capillaries. Attachment to the epithelia of capillaries in the brain, resulting in their subsequent blockage, is thought to be responsible for the fatal coma found in either the young or non-immune victims of *falciparum* malaria (Miller *et al.*, 1994).

The released merozoites may re-invade erythrocytes undergoing a further cycle of intraerythrocytic schizogony or they may become committed to sexual differentiation as gametocytes. The factors that control the switch from the asexual to sexual stages are not completely understood. However, body temperature, host immunity and nutrient levels are all thought to be involved.

A male microgametocyte or a female macrogametocyte are formed, and both types of gametocyte are taken up into the mosquito mid-gut in a subsequent blood meal. The erythrocyte membrane is shed and the parasites complete gametogenesis. The microgametocyte undergoes exflagellation, a process that gives rise to 8 microgametes. A microgamete fertilises a macrogamete to give a zygote. The zygote is the only developmental stage to be diploid as meiosis follows rapidly. Over the following 12-18 hours the zygote develops into an ookinete which moves

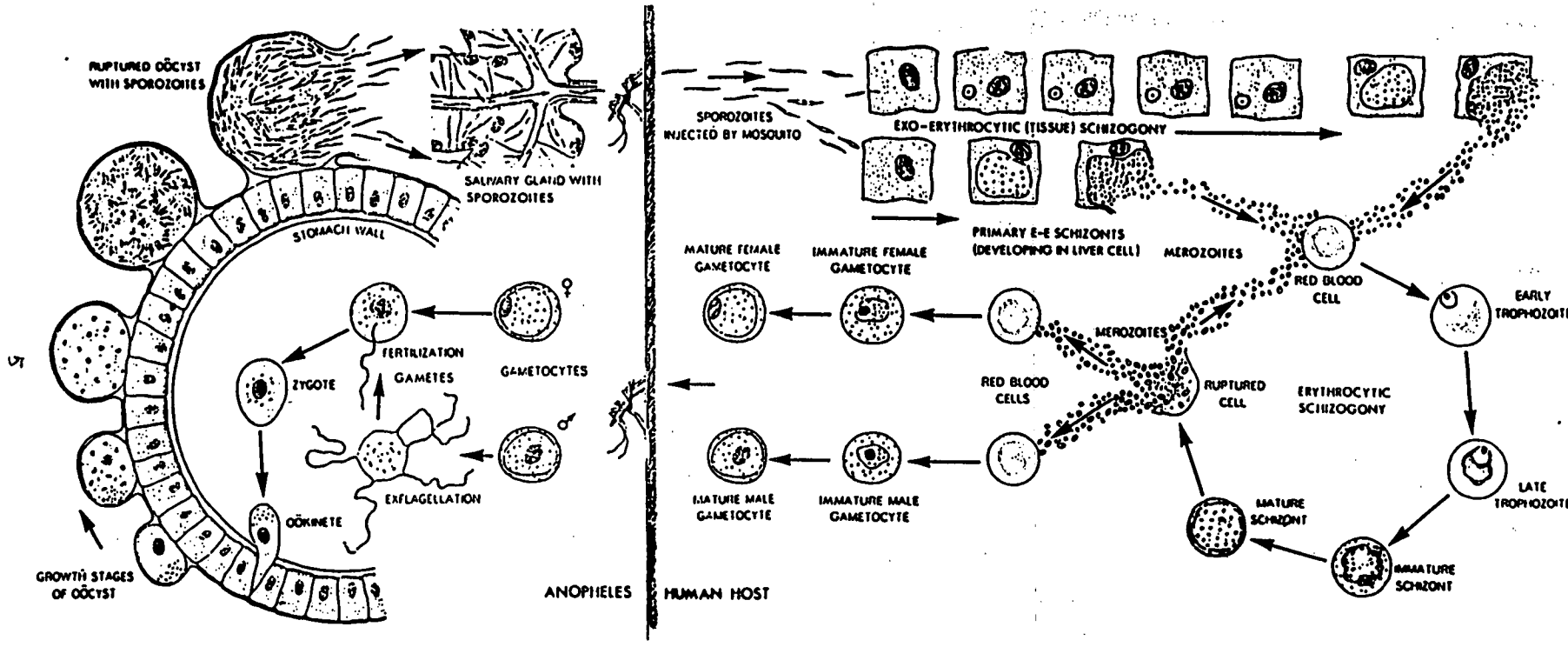


Figure 1.

Plasmodium falciparum life cycle (Bruce-Chwatt, 1985)

through the mid-gut epithelium to rest between the basement cell membrane and the basel lamina of the mid-gut wall. The ookinete undergoes many rounds of mitotic division, over the following 10-12 days, developing into an oocyst in which thousands of sporozoites are formed. When the sporozoites are released from a ruptured oocyst they travel through the haemocoel to the salivary glands. The sporozoites are then introduced into the human host when the next blood meal is taken.

1.3 *P.falciparum* genome.

1.3.1 Genome size and DNA composition.

The *P.falciparum* genome is haploid throughout most of the life-cycle, with zygote formation and meiosis occurring only during the mosquito phase of development (Walliker *et al.*, 1987). Early attempts to study the genome using microscopy did not reveal any information as *P.falciparum* chromosomes do not condense during development. However, the advent of pulsed field gel electrophoresis (PFGE) techniques during the mid-1980s allowed 14 chromosomes, ranging in size between 800kb and 3500kb, to be identified in *P.falciparum* (van der Ploeg *et al.*, 1985, Kemp *et al.*, 1985). Initial estimates of the *P.falciparum* genome size of the order of 1-3X10^7bp were based on the analysis of *P.falciparum* genomic libraries (Goman *et al.*, 1982, Pollack *et al*, 1982, Wellems *et al.*, 1987). More recent genome mapping work has indicated that 3X10^7bp is a more accurate estimation of the size of the genome (Walker-Jonah *et al.*, 1992). The *P.falciparum* genome has an A+T content that averages 69% in coding regions but increases to 90-95% in non-coding regions (Pollack *et al.*, 1982, Weber, 1987).

1.3.2 Molecular karyotype and chromosome plasticity.

The observation from PFGE that *P.falciparum* has 14 chromosomes agrees well with the 14 kinetochores observed using electron microscopy (Sinden and Strong, 1987). A complete genome map is in preparation using the information from mapping of genes and other markers to their respective chromosomes and the subcloning of large regions of these

chromosomes using yeast artificial chromosome technology (Lanzer *et al.*, 1993b, Triglia and Kemp, 1991, Wanatabe and Inselburg, 1994). When gene markers from chromosome 2 were mapped to a complete set of overlapping yeast artificial chromosome clones it appeared that the chromosome was compartmentalised. Gene encoding "housekeeping" proteins were located towards the centre of the chromosome and antigen encoding genes map to positions just within the subtelomeric repeats (Lanzer *et al.*, 1993b, Scherf, 1996).

Variations of up to 20% in chromosome sizes have been observed between isolates and even between clones from the same isolate (Kemp *et al.*, 1985, Sinnis and Wellems, 1988, van der Ploeg *et al.*, 1985). Analysis of the progeny from a genetic cross revealed the presence of size polymorphisms that were not present within the parental clones. Walliker *et al.* (1987) suggested that the size polymorphisms arose during meiosis. However, chromosome size polymorphisms have also been shown to arise during mitotic division when subtelomeric deletions were found in *P.berghei* cultures passaged between murine hosts by syringe rather than through a mosquito host (Janse, 1992).

The molecular mechanisms that result in chromosome size polymorphisms may include reciprocal exchange between heterologous chromosomes, deletions or DNA amplification (Ravetch, 1989, Frontali, 1994). Analysis of shortened chromosomes reveal that the predominant mechanism of shortening was through homologous recombination and deletions. The mechanisms which gave rise to these size polymorphisms were associated with sequences in the subtelomeric regions (Corcoran *et al.*, 1988). The subtelomeric region of all chromosomes appears to contain a series of transcriptionally silent ordered repetitive sequence elements (de Bruin *et al.*, 1994, Lanzer *et al.*, 1993b). The common sequence elements in the subtelomeric regions were proposed to be the preferred sites for chromosomal pairing during the formation of the synaptonemal complex. Cross-overs and gene conversions within the subtelomeric region could give rise to genetically distinct parasites, some possibly with novel antigens (Vernick *et al*, 1988).

1.3.3 Chromatin structure.

Initial evidence suggesting that genomic DNA was packaged in chromatin was obtained from electron microscopy of *P.knowlesi* (Wunderlich *et al.*, 1980). The DNA appeared to have a beaded appearance which suggested that nucleosomes, the first level of chromatin structure, were formed. *P.falciparum* DNA was demonstrated to be organised into nucleosomes when genomic DNA, partially digested with micrococcal nuclease, gave a series of laddered DNA fragments in multiples of 180bp (Cary *et al.*, 1994). The multiples of 180bp represent DNA which is protected from micrococcal nuclease by its association with the nucleosome. The highly conserved histone H2A gene has been cloned from *P.falciparum* (Creedon *et al.*, 1992) and a group of proteins with histone-like properties have been extracted from the nucleus (Cary *et al.*, 1994).

1.3.4 Extrachromosomal DNA.

P.falciparum, along with all other apicomplexan parasites, have 2 extrachromosomal DNAs; multiple tandem repeats of a 6kb element and a single copy of a 35kb circle (Feagin, 1994).

The 6kb element contains 3 open reading frames which encode cytochrome b and the cytocrome c oxidase subunits I and III. Fragments of several genes with extensive homology to ribosomal RNA genes have also been identified (Feagin *et al.*, 1992). Using subcellular fractionation techniques, the 6kb element was shown to probably reside in the mitochondria (Wilson *et al.*, 1992). Uniparental inheritance of the 6kb element provided further evidence to support its mitochondrial origin (Creasey *et al.*, 1993).

The 35kb circle was originally thought to be a second mitochondrial DNA due to its size and circular nature (Feagin, 1994). Recent evidence, however, suggests that the 35kb circle may reside in a plastid rather than a mitochondrion (Palmer, 1992). The 35kb circle encodes rRNA subunits, various tRNAs and several subunits of a RNA polymerase (Feagin, 1994). Mitochondrial genomes, however, do not generally contain RNA polymerase genes. This, and phylogenetical studies of the rRNAs and tRNAs suggest a plastid origin (Feagin *et al.*, 1992, 1994). A gene encoding

a protein with homology to a protein found only in the plastids of a red algae appears to reinforce the idea that there was an early symbiotic relationship between algae and *Plasmodia* (Williamson *et al.*, 1994). "Spherical bodies", which resemble plastid structures, have been found to be closely associated with the mitochondria of *P.gallinaceum* (Wilson, 1991). No function has yet been assigned to this structure. Plastids in some plants contain a porphyrin biosynthetic pathway, this has led to the proposal that "spherical bodies" may play some role in removing the toxic haem, released during haemoglobin digestion, from the parasite's cytoplasm (Wilson *et al.*, 1991).

1.4 *Plasmodium* chromosomal DNA replication.

1.4.1 DNA replication during the Plasmodium life cycle.

Chromosomal DNA replication appears to take place at 5 points during the life cycle: (i) exoerythrocytic schizogony, (ii) intraerythrocytic schizogony, (iii) gametogenesis, (iv) during meiosis in the zygote and (v) sporogony in the oocysts (reviewed in White and Kilbey, 1996). Experimental evidence for DNA replication has not been obtained for the sporogony and exoerythrocytic schizogony stages of development.

The measurement of incorporation of radiolabelled adenosine or hypoxanthine during the intraerythrocytic schizogony determined that DNA synthesis, in *P.falciparum*, starts approximately 30 hours after erythrocyte invasion (Gritzmacher and Reese, 1984, Inselburg and Banyal, 1984). DNA synthesis was found to start in mid-trophozoites and to continue throughout schizont development, stopping only when schizogony is complete (Inselburg and Banyal, 1984). Their observation that DNA synthesis occurs during the latter stages of the intraerythrocytic cycle of *P.falciparum* was similar to the findings from other *Plasmodium* species (Janse *et al.*, 1986a, Conklin *et al.*, 1973, Newbold *et al.*, 1982).

Experiments using microfluorimetry indicates that the DNA contents of both the male and female gametocytes increased during gametogenesis (Janse *et al.*, 1986a, 1988). The DNA content of both *P.berghei* and *P.falciparum* macrogametes almost doubles. The increase in DNA content

is more likely, however, to be the result of DNA amplification rather than DNA replication as Fuelgen staining indicates that no nuclear segregation takes place. Selective amplification of genes encoding ribosomal RNA has been proposed to occur in both macro- and microgametocytes but, as yet, no evidence to support this theory has been found. Microgametocytes are activated in the midgut and undergo exflagellation which leads to an approximate 8-fold increase in the DNA content (Janse *et al.*, 1988, Janse *et al.*, 1986a). In *P.berghei* the 8-fold increase in DNA takes place in 10 minutes; a feat that has been calculated to require at least 1300 origins of replication.

After fertilisation of the macrogamete in *P.berghei* the DNA content of the zygote was observed to double within 3 hours (Janse *et al*, 1986b). This increase is consistent with the duplication of paired chromosomes in the first stage of meiosis. Electron microscopy has shown that synaptonemal complexes are associated with paired chromosomes at this developmental stage. (Sinden *et al.*, 1985).

Various concentrations of the DNA polymerase inhibitor aphidocolin have been shown to inhibit DNA synthesis, in both *P.falciparum* and *P.berghei*, throughout all the developmental stages described (Inselburg and Banyal, 1984, Janse *et al.*, 1986a, 1986b, 1988). The three chromosomal DNA polymerases (α,δ and ε) are all sensitive to aphidocolin (Kornberg and Baker, 1992), indicating that at least one of these polymerases must also replicate *Plasmodium* chromosomal DNA.

Attempts to purify these polymerase activities have been made by separating cell extracts by SDS-PAGE. A stage specific DNA polymerase activity, sensitive to aphidocolin, has been isolated from *P.falciparum* (Abu-Elheiga *et al*, 1990, Choi and Mikkelsen, 1991). Antibodies raised against DNA polymerase α precipitated a 180kDa protein, from the cell extracts, along with several smaller proteins thought to be degradation products. Further analysis indicated that two proteins, of 105kDa and 72kDa, had DNA polymerase activity which were sensitive to aphidocolin but were not precipitated by the antibodies raised against DNA polymerase α. These proteins were tentatively assigned as either full

length and degraded DNA Polymerase δ or degraded DNA Polymerase ε.

1.4.2 Cloning of *P.falciparum* DNA replication protein genes.

The study of chromosomal DNA polymerase activities is complicated by the large numbers of parasites that are required and the presence of contaminating DNA polymerase activities from organelles. A different approach to the study of chromosomal DNA polymerase activity has been taken in Edinburgh, where genes encoding the chromosomal DNA replication proteins are being cloned, heterologously expressed and purified. Chromosomal DNA synthesis may be reconstituted and studied *in vitro* using purified replication proteins. To date, a number of genes have been cloned, including; DNA polymerases α and δ, proliferating cell nuclear antigen (PCNA), topoisomerases I and II (Figure 2). These were identified by screening genomic and cDNA libraries with either oligonucleotides designed to conserved regions or heterologous probing with *S.cerevisiae* gene fragments.

DNA polymerase δ.

DNA polymerase δ is required for the elongation of both the leading and lagging strands (Tsurimoto *et al.*, 1990, Waga and Stillman, 1994). The core enzyme consists of two subunits; a catalytic subunit of 125kDa with polymerase and 3'-5' exonuclease activity and a second subunit of 50kDa with an unknown function (Syruoja *et al.*, 1990).

The catalytic subunit of DNA polymerase δ has been cloned from *P.falciparum* (Fox and Bzik, 1991, Ridley *et al.*, 1991). A single copy of the gene, of 3282bp, is found on chromosome 10. A transcript of approximately 5.2kb encodes a polypeptide of 1094 amino acids with a predicted molecular mass of approximately 120kDa (Figure 2).

The amino acid sequence contains 6 motifs which characterise the polypeptide as a DNA polymerase (I-VI). These motifs, I being the most conserved to VI which is the least conserved, are present in the same order as all other polymerases. Motif I has been implicated in polymerase activity (Dorsky and Crumpacker, 1990) and motif II and III are thought to be required for binding dNTPs (Blasco *et al.*, 1992). A seventh highly

conserved motif (VII) is present in both *P.falciparum* DNA polymerase α and δ (Ridley *et al.*, 1991, White *et al.*, 1993). Although this motif is conserved in other DNA polymerases the literature does not usually describe this motif. One or more of 4 conserved regions (exoI, I', II and III) of DNA polymerase δ are thought to be required for proof reading activity (Bernard *et al.*, 1989, Simon *et al.*, 1991). All four motifs are present in the *P.falciparum* DNA polymerase δ polypeptide. The C terminus contains two putative zinc finger domains (ZnF1 and 2). ZnF2 is more conserved between species than ZnF1 although all the cysteine residues required are still present. Zinc fingers are required for both protein-protein and DNA-protein interactions (Harrison, 1991), of which both types of interaction are required by DNA polymerase δ. The DNA polymerase δ from human, mouse, calf thymus and *S.pombe* (Chung *et al.*, 1990, Cullman *et al.*, 1993, Zhang *et al.*, 1991, Pignede *et al.*, 1991) all contain a pair of conserved motifs towards the N terminus which are thought to be required for either interaction with PCNA or are nuclear targeting signals (Cullman *et al.*, 1993). These motifs, however, are missing from the DNA polymerase δ of *P.falciparum*.

Proliferating Cell Nuclear Antigen.

PCNA was first identified as a human auto-antigen in patients with systematic lupus erythematosus (Miyachi *et al.*, 1978). PCNA was subsequently identified as a protein which accumulated during the S phase (Bravo and Cellis, 1980) and was therefore thought to be a cyclin. PCNA was later shown to be an auxiliary protein required for DNA polymerase δ activity (Tan *et al.*, 1986), where PCNA binds to DNA as a trimer and acts as a toroidal clamp for DNA polymerase δ (Krishna *et al.*, 1994, Kong *et al.*, 1992).

The PCNA gene has been cloned from *P.falciparum* (Kilbey *et al.*, 1993) and found to be present as a single copy on chromosome 13. The gene, of 825bp, encodes a polypeptide of 275 amino acids which has a predicted molecular mass of approximately 30.5kDa (Figure 2). Two transcripts have been reported, a major transcript of approximately 1.6kb and a minor

transcript of approximately 2.2kb. Indirect immunofluorescence assays indicate that the protein accumulates in trophozoites and persists in schizonts (Kilbey *et al.*, 1993).

The polypeptide contains several conserved dispersed amino acids which confirm that it is PCNA, yet the overall identity is weak with the strongest homology to human PCNA (approximately 60%). A conserved region towards the N terminus, between amino acids 61-80, contains an α helix-turn-α helix motif which is a putative DNA binding domain, although no evidence of this interaction has been found. PfPCNA contains 15 more amino acids than other PCNAs, of which 9-10 charged amino acids are present as an insert close to the carboxy terminus. Mutational analysis of human PCNA identified a number of conserved amino acids, found on the surface of the protein, which are presumably required for interactions with DNA polymerase δ, other PCNA subunits and replication factor C (Fukuda *et al.*, 1995, Jonsson *et al.*, 1995). PCNA is discussed in more detail in section 1.7.

DNA polymerase α.

DNA polymerase α is responsible for the initiation of DNA synthesis on both the leading and lagging strand (Stillman, 1989, Waga and Stillman, 1994). The DNA polymerase α catalytic subunit is comprised of a subunit containing the polymerase activity (205kDa), 2 subunits with primase activity (55-60kDa and 48-50kDa) and a 70kDa subunit with an unknown function (Wang, 1991).

The DNA polymerase α gene from *P.falciparum* (White *et al.*, 1993) was shown to be present as a single copy on chromosome 4. The gene of 5.7kb contains a single intron of 204bp. A transcript of approximately 7kb encodes a polypeptide of 1855 amino acids with a predicted molecular mass of approximately 205kDa (Figure 2).

The *P.falciparum* DNA polymerase α polypeptide contains the 7 conserved motifs (I-VII) that characterise it as a DNA polymerase (see above). 4 of the 5 DNA polymerase α specific motifs (A-E) are present in

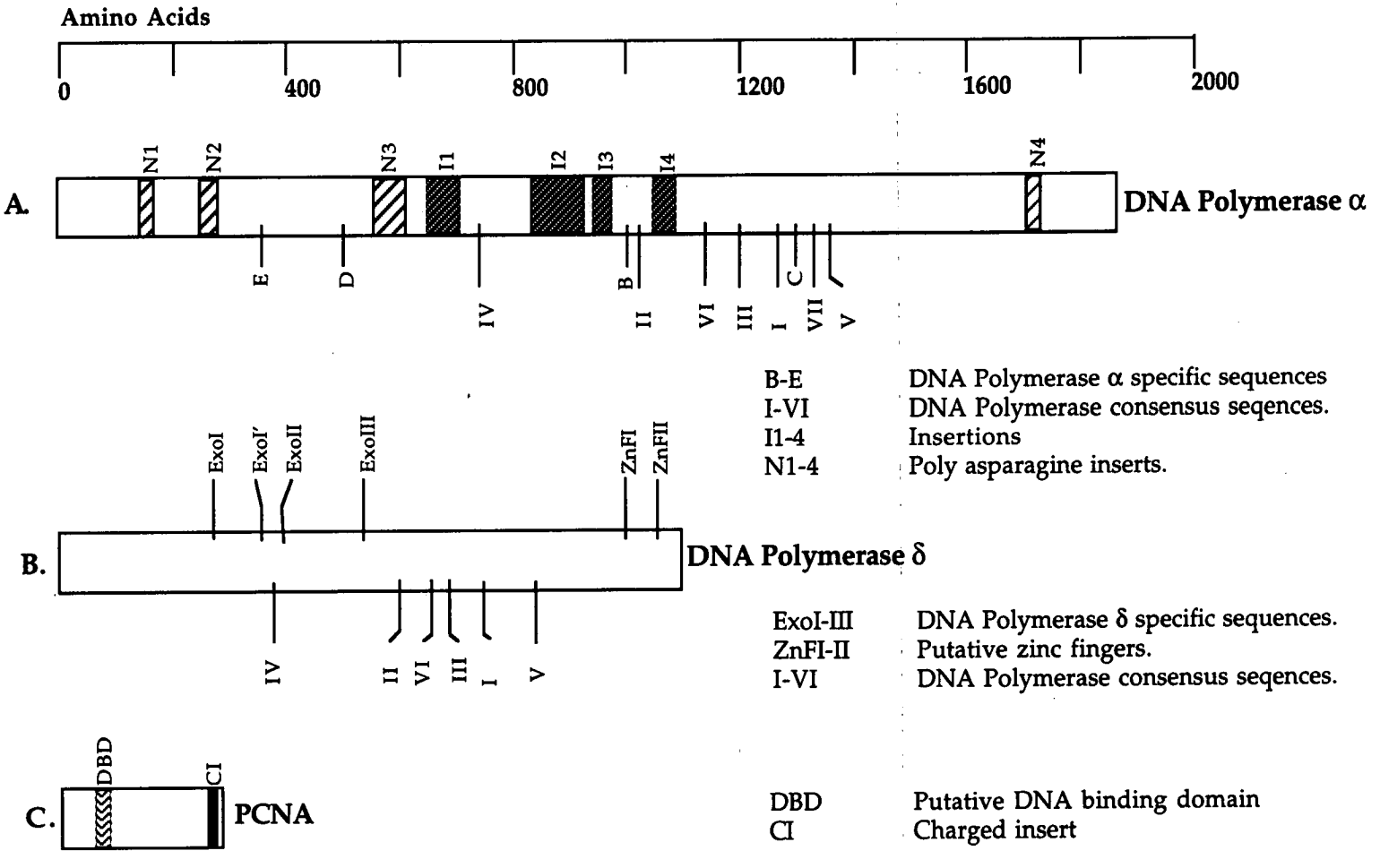
Figure 2.

Schematic representing significant features of the catalytic subunits for DNA polymerases α and δ and PCNA polypeptides from *P.falciparum.*

(A) Catalytic subunit of DNA polymerase α. I-VII, conserved features of all DNA polymerases; B-E, conserved DNA polymerase α features; N1-4, polyasparagine tracts; I1-4, amino acid inserts.

(B) Catalytic subunit of DNA polymerase δ. I-VI, conserved features of all DNA polymerases; exo I′ and I-III, putative proof reading function; ZnF1-2, putative zinc fingers.

(C) PCNA. DBD, putative DNA binding domain; CI, insertion of charged amino inserts.


Amino Acids
0
400
800
1200
1600
2000
A.
N1
N2
N3
I1
I2
I3
I4
N4
DNA Polymerase α
E
D
IV
B
II
VI
III
I
C
VII
V
B-E
DNA Polymerase α specific sequences
I-VI
DNA Polymerase consensus seqences.
I1-4
Insertions
N1-4
Poly asparagine inserts.
ExoI
ExoI'
ExoII
ExoIII
ZnFI
ZnFII
B.
DNA Polymerase δ
IV
II
VI
III
I
V
ExoI-III
DNA Polymerase δ specific sequences.
ZnFI-II
Putative zinc fingers.
I-VI
DNA Polymerase consensus seqences.
DBD
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C.
PCNA
DBD
Putative DNA binding domain
CI
Charged insert

P.falciparum DNA polymerase α. Motif A is missing from the DNA polymerase α of both *P.falciparum* and *T.brucei.* The highly conserved glycine of motif D, which is thought to be required for interaction with the primase subunits (Pizzigalli *et al.*, 1988) is substituted in *P.falciparum* by leucine. *P.falciparum* DNA polymerase α polypeptide contains 4 polyasparagine tracts (N1-4) and 4 further inserts (I1-4) of which 2 (I2 and 4) contain degenerative repeats of short sequences. Reverse transcription-PCR confirmed that these regions were present in the mRNA. The number of degenerative repeats in insert 2 and the polyasparagines in N3 were different in DNA polymerase α from a different *P.falciparum* isolate (White and Kilbey, 1996). Polyasparagine tracts and inserts with polypeptide repeats are immunodominant and are generally cross reactive (Ridley, 1991). The immune system is thought not to be able to mount an effective challenge to the parasite due to the "smokescreen" presented by these regions. Other evidence suggests that these regions may be necessary to facilitate interactions with other macromolecules such as skeletal proteins, receptor molecules and nucleic acids.

1.5 Eukaryotic DNA replication.

1.5.1 Simian virus 40 (SV40) cell free system.

The mechanisms of DNA replication in both *E.coli* and Phage T4 have been characterised extensively (Kornberg and Baker, 1992). A study of eukaryotic DNA replication along the same lines as those of *E.coli* and Phage T4, however, has been hampered by the lack of a suitable biochemical system. Using the SV40 cell free system, however, a valuable insight into the mechanism of eukaryotic DNA replication has been obtained (Stillman, 1989, 1994). Of the six proteins encoded by the SV40 genome, 4 are essential of which 3 are structural proteins (Stillman, 1989). The remaining protein, the T antigen (TAg), plays an essential role in SV40 DNA replication. Extracts from HeLa cells containing the cell's replication apparatus is capable of replicating the SV40 genome when supplemented with the virally encoded TAg (Li and Kelly, 1984, Ishimi *et*

al., 1988, Weinberg *et al.*, 1990, Wobbe *et al.*, 1987). This system offers the opportunity to study the biochemistry of eukaryotic DNA replication with the caveat that the initial step is brought about by a viral protein. The SV40 genome is assembled into a chromatin structure not dissimilar to that of the host genome further lending credence to the study of eukaryotic DNA replication through a SV40 cell free system (Stillman, 1986).

1.5.2 Initiation of DNA synthesis.

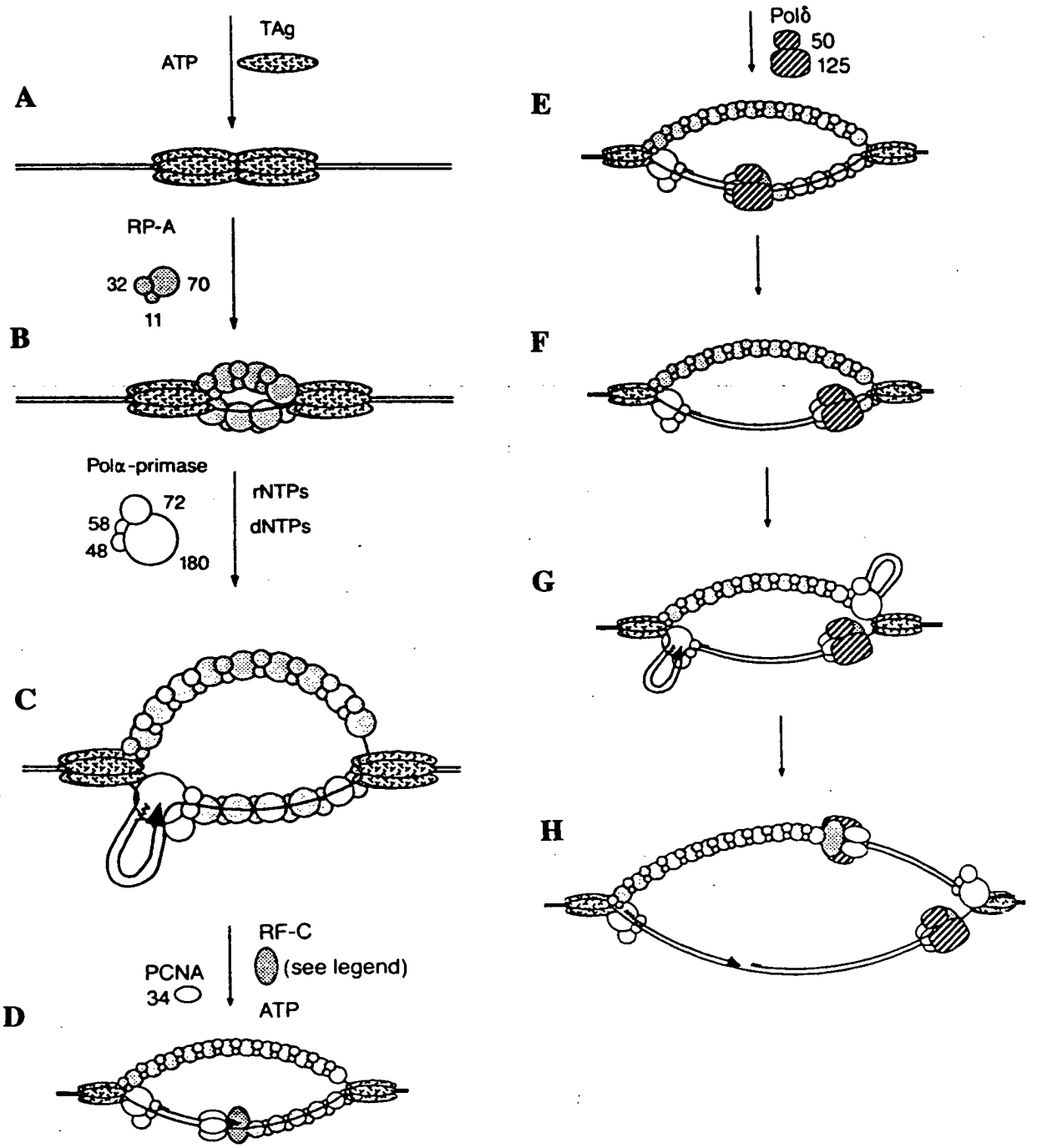
TAg is a large multi-functional protein which recognises and binds to the *ori* site on the SV40 genome (Figure 3). TAg binds to the SV40 *ori* as a complex consisting of a double hexamer wrapped around the DNA (Mastrangelo *et al.*, 1989). The binding of TAg is an ATP dependent process, hydrolysis of ATP, however, is not required for binding (Borowiec and Hurwitz, 1988). The TAg complex undergoes a conformational change which results in a short region of the bound DNA unwinding and the strands separating (Borowiec *et al.*, 1990). The region of separated duplex is further extended by the intrinsic helicase activity present in the TAg (Dean *et al.*, 1987). Topoisomerase I or II is required for the unwinding of the DNA, presumably to release the torsional stress that builds up in the duplex ahead of the separated DNA strands. The single stranded DNA is then stabilised by the binding of a single stranded binding protein, replication factor A (RF-A).

RF-A consists of three subunits (70kDa, 34kDa and 11kDa) and is essential for SV40 DNA replication (Kenny *et al.*, 1990, Tsurimoto and Stillman, 1989). The 70kDa subunit, independent of the other 2 subunits, binds to and stabilises the single stranded DNA (Kenny *et al.*, 1990). RF-A, as well as TAg, recruits the DNA polymerase α-primase complex to the origin of replication (Dornreiter *et al.*, 1990, Melendy and Stillman, 1993). The primase activity of the DNA polymerase α-primase complex is associated with a pair of subunits (58kDa and 48kDa). The short RNA primer is laid down by the primase which is used by the DNA polymerase α to produce a short stretch of DNA (Diffley, 1992). RF-A is required for the efficient

Figure 3.

Figure illustrating the series of events surrounding the initiation of DNA replication at the SV40 *ori* sequence (Diffley, 1992).

The TAg hexamer recognises and binds the *ori* site (A). On strand separation the single stranded DNA is bound by RF-A complex (B). The DNA polymerase α-primase complex is recruited (C). The primase lays down a short RNA primer from which the DNA polymerase α synthesises a short stretch of DNA (D). RF-C recognises and binds to the primer-template junction (E) and recruits PCNA which in turn recruits DNA polymerase δ. (F) The DNA polymerase δ synthesises DNA in a highly processive manner. (G) As more template is exposed, DNA polymerase α-primase complex binds and prepares a primer (H) on the opposite strand of the DNA duplex.


A
TAg
ATP
RP-A
32
70
11
B
Polα-primase
72
58
48
180
rNTPs
dNTPs
C
RF-C
(see legend)
PCNA
34
ATP
D
Polδ
50
125
E
F
G
H

activity of the DNA polymerase α-primase complex (Kenny *et al.*, 1990, Tsurimoto and Stillman, 1989). The short DNA fragment prepared in this way serves as the primer for the assembly of the leading strand replication complex.

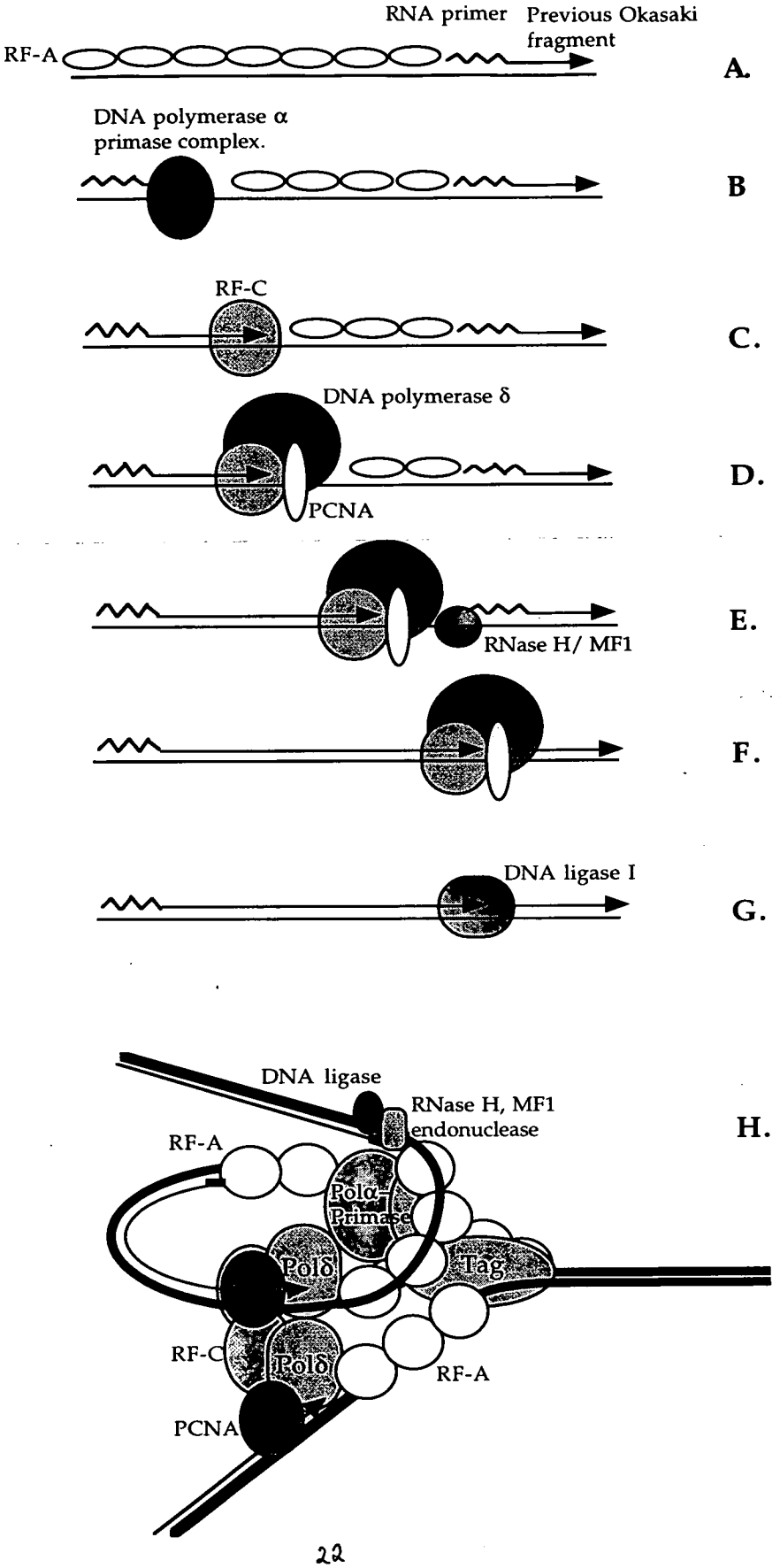
1.5.3 Replication of the leading and lagging strands.

Replication factor C (RF-C) consists of a 140kDa subunit with up to 4 further subunits ranging in size between 30-40kDa (Tsurimoto and Stillman, 1991). The 140kDa subunit recognises and binds to the primer-template junction (Tsurimoto and Stillman, 1991). RF-C then recruits PCNA to the primer template junction (Produst *et al.*, 1995). PCNA binds to DNA as a trimer surrounding the DNA and slides along until it meets the RF-C primer template junction (Prelich *et al.*, 1987). PCNA recruits DNA polymerase δ (Tsurimoto and Stillman, 1991) for which it acts as a sliding clamp, holding the now highly processive DNA polymerase δ to the DNA template during elongation. Leading strand synthesis therefore requires a switch between the DNA polymerase α-primase complex to the DNA polymerase δ complex.

Lagging strand DNA synthesis was presumed to take place by the DNA polymerase α-primase complex synthesising a number of end to end Okazaki fragments which were eventually joined together (Tsurimoto *et al.*, 1990, Diffley, 1992). It was subsequently shown, however, that a switch between the DNA polymerase α-primase complex to the DNA polymerase δ complex was required for the completion of the Okasaki fragments (Waga and Stillman, 1994). When the DNA polymerase δ complex meets the start of another Okazaki fragment it is proposed that the RNA primer and short stretch of DNA, laid down by the DNA polymerase α-primase complex, are removed by the action of RNase H and the 5′-3′ exonuclease MF1 (Figure 4). The region of DNA synthesised by DNA polymerase α would have to be removed as it would be prone to errors as DNA polymerase α does not have a proofreading 3′-5′

Figure 4.

Figure representing a model for the switch between the DNA polymerase α primase complex and DNA polymerase δ on the lagging strand (originally drawn by Waga and Stillman, 1994). As a large enough region of lagging strand template becomes available (A) the DNA polymerase α-primase complex is recruited to prepare a short RNA primer from which DNA polymerase α synthesises a short stretch of DNA (B). RF-C binds, displacing the DNA polymerase α primase complex (C), and the DNA polymerase δ complex is assembled (D). When the DNA polymerase δ complex reaches the tail of an Okazaki fragment the RNA primer and DNA polymerase α synthesised DNA are removed by the RNase H/MF1 complex (E). The remaining gap is filled by DNA polymerase δ (F) and the two DNA fragments are joined by DNA ligase I (G).
A schematic of the multi-protein complex assembled at the replication fork based on this model is represented (H). This figure was originally prepared by J.H. White based on the figure from Stillman and Waga (1994).


RNA primer
Previous Okasaki fragment
RF-A
A.
DNA polymerase α
primase complex.
B
RF-C
C.
DNA polymerase δ
PCNA
D.
RNase H/ MF1
E.
F.
DNA ligase I
G.
DNA ligase
RNase H, MF1
endonuclease
RF-A
Polα-Primase
Polδ
Tag
RF-C
Polδ
RF-A
PCNA
H.

exonuclease activity. DNA ligase I completes the synthesis of the lagging strand by joining the Okazaki fragments together.

1.5.4 DNA polymerase ε: a third polymerase?

Eukaryotic cells have 3 DNA polymerases (α, δ and ε) which take part in chromosomal DNA replication (Hubscher and Spadari, 1994). DNA polymerase ε is a multi-subunit complex with a major subunit of 220kDa which has both DNA polymerase and exonuclease activities, and several smaller subunits between 70kDa and 30kDa of unknown function (Hubscher and Thommes, 1992). DNA polymerase ε is essential for DNA replication in *S.cerevisiae* (Morrison *et al.*, 1990).

DNA polymerases δ and ε were originally confused for one another until DNA polymerase ε was shown to be highly processive in the absence of PCNA (Morrison *et al.*, 1990). Although DNA polymerase ε is highly processive in the absence of PCNA, the presence of PCNA has been shown to be required by DNA polymerase ε to be able to bind to the primer-template junction and for the stimulation of it's activity (Maga and Hubscher, 1995). A model has been proposed whereby DNA polymerases δ synthesises either the leading or lagging strand, and DNA polymerase ε the other, after initiation on both strands by the DNA polymerase α-primase complex (Hubscher and Thommes, 1992).

1.6 Regulation of DNA replication.

1.6.1 Initiation of DNA synthesis and the "licensing" factor.

The events surrounding the initiation and progression of DNA synthesis in higher eukaryotes have been primarily determined from a reconstituted *in vitro* system using the SV40 origin of replication. Using the *in vitro* SV40 system TAg was shown to recognise and bind a specific origin sequence (Waga and Stillman, 1994). In *Saccharomyces cerevisiae* a group of proteins, give rise to an origin recognition complex (ORC) which performs a similar function to the SV40 TAg in the recognition, binding

to and unwinding of the autonomously replicating sequences (ARS). The ORC is made up of a group of 6 proteins of sizes ranging between 50kDa to 120kDa (Bell and Stillman, 1992). Homologues of several of the ORC subunits have been identified in a wide range of eukaryotes including *Schizosaccharomyces pombe, Arabadopsis thaliana, Caenorhabditis elegans, Drosophila melanogaster* and humans which suggests a conserved role for the ORC complex in origin recognition (Benbow *et al.*, 1992). The ORC complex in *S.cerevisiae* was shown to require ATP to bind to the ARS and was found to be continually bound to an ARS throughout the cell cycle. It is presumed that some form of post-translational modification may be responsible for activation of the complex (Diffley *et al.*, 1994).

Sequence specific origins of replication have not been identified in higher eukaryotes despite several attempts. It has been proposed that there are many potential sequences capable of acting as origins of replication and that only a small group are activated during DNA replication (DePamphilis, 1993). Examination of the structure of chromatin in the nuclei of higher eukaryotes has identified a number of foci at which DNA replication is presumed to take place. It has been proposed that only origins of replication associated with these foci would be activated (Cox and Laskey, 1991, Adachi and Laemmli, 1994). The single stranded protein, RF-A, is strongly associated with these foci (Yan and Newport, 1995).

A set of genes encoding proteins structurally related to subunits of the *S.cerevisiae* ORC were identified in a screen of yeast mutants which failed to replicate plasmids containing an ARS (Hennessy *et al.*, 1991). These proteins, including; MCM2, MCM3 and MCM5/CDC46 from *S.cerevisiae* and cdc21 from Schizosaccharomyces *pombe* (Coxon *et al.*, 1992, Maiorano *et al.*, 1996) are conserved throughout eukaryotes with homologues found in *Xenopus* (Madine *et al.*, 1995), human (Hu *et al.*, 1993) and mouse cells (Thommes *et al.*, 1992). Some members of this family of proteins were shown to exhibit cell cycle dependent nuclear localisation. The proteins were present in the nucleus after mitosis and throughout the G1 phase only disappearing when DNA replication starts in the S phase. These proteins have the characteristics of a "licensing" factor proposed to be

responsible for limiting DNA replication to a single round in a single cell cycle (Blow and Laskey, 1988). This factor would be essential for the start of DNA synthesis and would only be able to enter the nucleus when the nuclear envelope forms at the end of mitosis and would be inactivated by DNA synthesis. Some homology to DNA dependent ATPases has led to the proposition that the "licensing" factor may be a DNA helicase responsible for the unwinding of DNA during the initiation of DNA synthesis (Koonin, 1993).

1.6.2 Cyclin dependent kinases.

The role of a cyclin dependent kinase (cdk) in the regulation of the transition between the G1 and S phases has been shown to be a universal mechanism in eukaryotes (for review see Reed, 1992). The progression of the cell cycle of *S.cerevisiae* is determined by a number of extracellular factors such as the level of nutrients and the presence of mating pheromones. The transition through a point during the G1 phase, termed START, was shown to commit a cell to completion of the cell cycle (Hartwell, 1974). Characterisation of temperature sensitive START mutants led to the identification of the *CDC*28 gene. The activation of the gene product of *CDC*28, a 34kDa protein kinase, by association with G1 cyclins (gene products of *CLN*1-3) was shown to be responsible for commitment to START (Reed, 1980). The cyclin activated kinase phosphorylates targets which are responsible for the initiation of a number of cellular activities including DNA replication and budding (Figure 5).

One possible target for the p34 kinase is a transcription factor (MBF, described in more detail later) required for the coordinated expression of DNA synthesis genes (Lowndes *et al.*, 1991). p34 has already been demonstrated to be able to phosphorylate the *SWI4/SWI6* transcription factor complex which shares extensive homology to components of the MBF (Marini and Reed, 1992).

The *S.pombe CDC*28 homologue, called *cdc*2, is able to complement a temperature sensitive *CDC*28 mutant in *S.cerevisiae* at the restrictive temperature (Nurse and Bissett, 1981). The ability to complement

*cdc*2/*CDC*28 temperature sensitive mutants at the restrictive temperature has been used to screen for cdks in a wide range of other eukaryotes.

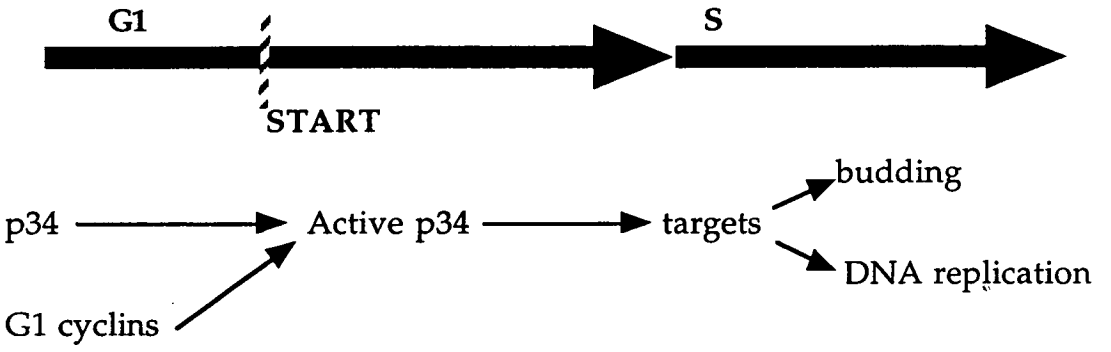


Figure 5. Schematic representing role of the gene product of *CDC*28 during the transition of the G1/S phase boundary in *S.cerevisiae*.

During the G1 phase of higher eukaryotes a point, known as the restriction point, has been identified at which cells are committed to the cell cycle (Pardee *et al.*, 1986, Pardee 1989). This point is analogous to START in *S.cerevisiae*, but not identical. After passing the restriction point it was observed that the cell was no longer sensitive to growth factors or a range of protein synthesis poisons. It was suggested that to pass the restriction point the cell had synthesised enough proteins to carry out a range of activities such as DNA synthesis without any further protein synthesis.

The determination of whether *cdc*2/*CDC*28 homologues play a role in the transition of the G1/S phase boundary in higher eukaryotes is complicated by the presence of several isoforms of the 34kDa protein kinase (reviewed in Pines and Hunter, 1991). *cdc*2/*CDC*28 homologues have been characterised from a wide range of organisms. A putative homologue, Pfcdk5, has been cloned and isolated from *P.falciparum* (Ross-McDonald *et al.*, 1994). Pfcdk5 was identified by sequence homology rather than the ability to complement yeast mutants .

The human cdk2 homologue associates with cyclin throughout the cell cycle. Two G1 cyclins, A and E, associate with cdk2 in a temporal fashion. A cyclin A/cdk2 complex is thought to play a role in the initiation and

elongation of DNA synthesis, possibly by phosphorylation of RF-A (Pagano *et al.*, 1992, Salah-ud-Din *et al.*, 1990). Cyclin E associates with cdk2 early in the G1 phase (Lew *et al.*, 1991). This complex is thought to be responsible for the deactivation of p107-Rb (retinoblastoma gene product), a general negative regulator of cell cycle progression, by its hyperphosphorylation (Bandara and LaThangue, 1991, Shirodkar *et al.*, 1992). p107-Rb is also found during the G1 phase in a complex with cdk2, cyclin A and E2F-DRTF1 (Pagano *et al.*, 1992). This complex may play a role in the cell cycle regulated expression of a number of proteins required for DNA replication which have an E2F-DRTF1 binding site in their promoter (Blake and Azizkhan, 1989, LaThangue, 1994). Another negative regulator of cell cycle progression, p53, is also regulated by the activity of cdk2 (Bischoff *et al.*, 1990). p53 is a transcription factor that represses the activity of a wide range of proteins and plays a role in the response of PCNA to cell damage (Shivakumar *et al.*, 1995). The interactions of cdk2, p107-Rb and p53 are not entirely understood but do suggest a key role for cdk2 in the G1/S phase boundary transition (Figure 6).

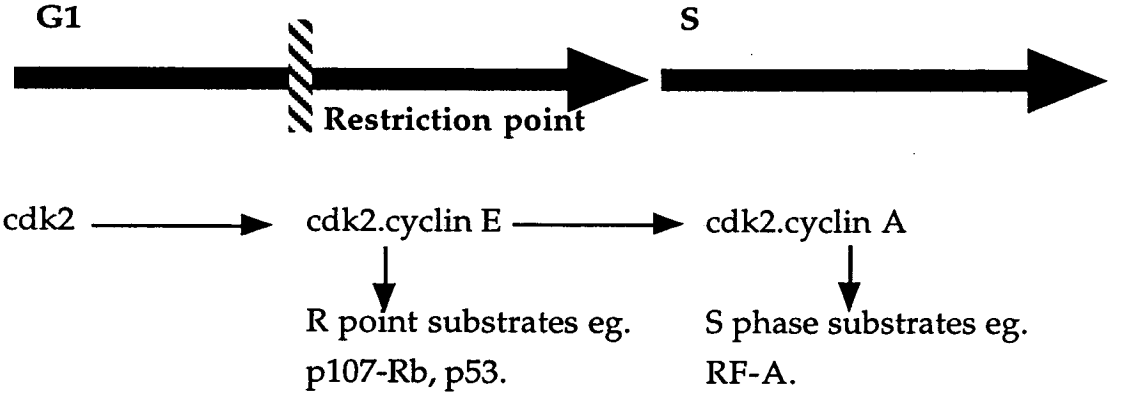


Figure 6. Schematic representing the role of the cdk2 gene product during the transition of the G1/S phase boundary in humans.

1.6.3 Transcriptional regulation of the expression of DNA replication proteins.

The replication of chromosomal DNA is regulated, in part, by the coordinated accumulation of proteins responsible for DNA synthesis at

the start of the S phase. In this section a brief review of the transcriptional mechanisms responsible for the expression of DNA synthesis genes from *S.cerevisiae* and *Drosophila* will be made. The expression of DNA replication proteins from serum starved higher eukaryotic cells after serum stimulation will also be covered.

The genes encoding many of the proteins required for DNA synthesis in *S.cerevisiae* are expressed at the G1/S phase boundary (Johnston and Lowndes, 1992. Table 1). This periodic transcription is mediated by a cis-acting sequence described as the *Mlu*I cell cycle box (McIntosh *et al.*, 1991), or MCB. The MCB binds a transcription factor, the *Mlu*I cell cycle box binding factor (MBF)(Lowndes *et al.*, 1991) which is also known as the DNA synthesis control 1 complex (DSC1). This transcription factor is comprised of the gene product of *SWI6* and a 120kDa protein (Lowndes *et al.*, 1992a, Primig *et al.*, 1992, Verma *et al.*, 1991). The *SWI6* gene product is thought to be regulated by phosphorylation by the *CDC28* gene product (Reed, 1992, Andrews and Mason, 1993). In this way a direct link between cell cycle progression and the induction of proteins responsible for DNA synthesis may be demonstrated (Andrews, 1992).

Gene	Function
POLI	DNA polymerase I (α)
POLII	DNA polymerase II (ε)
POLIII	DNA polymerase III (δ)
POL30	Proliferating Cell Nuclear Antigen
PRI1	DNA primase 1
PRI2	DNA primase 2
RFA1	Replication factor A 70kDa subunit
RFA2	Replication factor A 34Da subunit
RFA3	Replication factor A 14kDa subunit
CDC9	DNA ligase
CDC21	Thymidylate synthase
CDC8	Thymidine kinase

Table 1. *S.cerevisiae* DNA synthesis genes transcribed at the G1/S phase boundary under the control of the MBF/DSC1 transcription factor.

Only the gene product of *cdc*22$^+$, encoding a ribonucleotide reductase subunit, is under periodic control, in *S.pombe*, with a peak of expression at the G1/S phase boundary (Merril *et al.*, 1992). The promoter of this gene contains an MCB element which is recognised by the *S.pombe* MBF/DSC1 homologue. This complex of *cdc*10$^+$ and *res*1$^+$ is phosphorylated by *cdc*2 (Tanaka *et al.*, 1992) providing a link between cell cycle progression and an essential protein required for DNA synthesis.

The role of DSC1/MBF homologues and MCB elements may be a common mechanism of the regulation of the expression of some of the critical DNA synthesis proteins in all yeasts. MCB elements have been found within the promoter of the *Candida albicans* thymidylate synthase and putative DSC1 component homologues have been identified in *Kluyveromyces lactis* (Koch *et al.*, 1993).

Transcripts encoding several DNA synthesis proteins, including: PCNA, DNA Polymerase α and ribonucleotide reductase subunit 2, have been shown to accumulate at the G1/S phase boundary during *Drosophila* embryogenesis (Duronio *et al.*, 1994). A cis-acting sequence in the promoter of PCNA and DNA Polymerase α has been identified to be the binding site for the transcription factor, *Drosophila* DNA replication related factor (DREF, Hirose *et al.*, 1993). This factor, which probably binds as a homodimer, has been strongly implicated in the expression of proteins required for DNA synthesis. Analysis of sequences in databases have identified the DREF binding site in the promoters of a range of genes which have some function relating to cell proliferation (Matsukage *et al.*, 1995).

A mammalian cell starved of serum is in a quiescent state (G_0) in which the levels of DNA replication proteins are extremely low (Moore and Wang, 1994). Stimulation of the cell to proliferate by the addition of

serum leads to the coordinated expression of a range of DNA synthesis proteins which peak at the G1/S phase boundary (Baserga, 1991, Bjorklund *et al.*, 1992, Coverly and Laskey, 1994, Zeng *et al.*, 1994). During subsequent rounds of cell division the levels of many DNA replication proteins remain stable (Baserga, 1991, Wahl *et al.*, 1988, Zeng *et al.*, 1994). There is, however, a small group of proteins whose levels do change throughout the cell cycle peaking at the G1/S phase boundary, these include; thymidine kinase (Gudas *et al.*, 1988), ribonucleotide reductase subunit R2 (Bjorkland *et al.*, 1992) and dihydrofolate reductase (Collins *et al.*, 1984). The genes encoding all these proteins have an E2F-DRTF1 transcription factor binding site in their promoters (Blake and Azizkhan, 1989).

The mechanisms underlying the co-ordinated accumulation of DNA synthesis proteins appears to be conserved throughout the eukaryotic kingdom. A strong sequence similarity exists between the binding sites of the DSC1 complex for both *S.cerevisiae* and *S.pombe* and the E2F/DRTF1 binding site of mammalian (Table 2. Analysis of the secondary structures of the transcription factors that bind these similar sequences reveals a region of homology that is thought to be responsible for the sequence specific interaction with the DNA. A second region, known as the ankyrin motif, has been identified in both *S.cerevisiae* and *S.pombe* (Andrews and Mason, 1993). A role for this motif has not been defined, although it is thought to be required for protein-protein interactions (LaThangue and Taylor, 1993).

DNA motif	organism	DNA sequence	component/ complex bound
MCB	*S.cerevisiae*	TNACGCGT	p120/ DSC1
MCB	*S.pombe*	TNACGCGT	*cdc*10^{+}/ DSC1 homologue
E2F site	Mammalian	TTTCGCGC	E2F/ DRTF1

Table 2. A comparison of the sites to which transcription factors responsible for expression of DNA synthesis proteins bind (LaThangue and Taylor, 1993).

1.7 Proliferating Cell Nuclear Antigen (PCNA).

1.7.1 Structure.

PCNA is the eukaryotic homologue of the β subunit of *E.coli* DNA polymerase III and gp45 of the T4 phage (Kuriyan and O'Donnell, 1993). Although the sequence homology between these three proteins is extremely low, in the order of 5-10%, elucidation of the crystal structures of two of them, PCNA and the β subunit (Kong *et al.*, 1992, Krishna *et al.*, 1994), has shown that the overall structure is strongly conserved. PCNA and the β subunit are ring shaped with a central cavity large enough for the DNA duplex to pass through. The two crystal structures when superimposed are nearly identical (Kelman and O'Donnell, 1995a) even though the β subunit is a dimer and PCNA a trimer. The PCNA polypeptide is 2/3rds the length of the β subunit polypeptide and contains two clearly identifiable domains compared to the three of the β subunit. The organisation of both PCNA and the β subunit suggest that both proteins share a psuedo-6-fold symmetry which surrounds the DNA duplex.

A sequence alignment of PCNA from a range of eukaryotes indicates that relatively few residues are absolutely conserved (Figure 7). Most of the conserved residues are orientated towards the solvent exposed surface and may therefore play a role in interactions with other proteins (Kelman and O'Donnell, 1995a, Krishna *et al.*, 1994).

```
        1                                                        50
Glymax  .......... .......... .......... SSTGFSLQAM DSSHVALVAL
Daucar  MLELRLVQGS LLKKVMDSIK DLVNDANFDC SATGFSLQAM DSSHVALVAV
Catros  MLELRLVQGS LLKKVLESLK DLVTDANFDC SASGFSLQAM DSSHVALVAL
Orysat  MLELRLVQGS LLKKVLEAIR ELVTDANFDC SGTGFSLQAM DSSHVALVAL
Ratnor  MFEARLIQGS ILKKVLEALK DLINEACWDI SSGGVNLQSM DSSHVSLVQL
Homsap  MFEARLVQGS ILKKVLEALK DLINEACWDI SSSGVNLQSM DSSHVSLVQL
Musmus  MFEARLIQGS ILKKVLEALK DLINEACWDV SSGGVNLQSM DSSHVSLVQL
Xenlav  MFEARLVQGS ILKKVLEALK DLIDEACWDI TSSGISLQSM DSSHVSLVQL
Dromel  MFEARLGQAT ILKKILDAIK DLLNEATFDC SDSGIQLQAM DNSHVSLVSL
Schpom  MLEARFQQAA LLKKLLDAIK ELVTDANFDC NDNGISLQAM DSSHVALVSM
Saccer  MLEAKFEEAS LFKRIIDGFK DCVQLVNFQC KEDGIIAQAV DDSRVLLVSL
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Plafal  MLEAKLNNAS ILKKLFECIK DLVNDANVDA DESGLKLQAL DGNHVSLVSL
        * *           *                       *   *   *   * **

        51                                                  100
Glymax  LLRSEGFEHY RCDRNISMGM NLNNMAKMLK CAGNDDIITI KADDGSDTVT
Daucar  LLRSEGFEHY RCDRNISMGM NLGNMAKMLK CAGNDDIITI KADDGSDTVT
Catros  LLRSEGFEHY RCDRNPSMGM NLNNMAKMLK CAGNDDIITL KADDGSDTVT
Orysat  LLRSEGFEHY RCDRNLSMGM NLNNMAKMLR CAGNDDIITI KADDGSDTVT
Ratnor  TLRSEGFDTY RCDRNLAMGV NLTSMSKILK CAGNEDIITL RAEDNADTLA
Homsap  TLRSEGFDTY RCDRNLAMGV NLTSMSKILK CAGNEDIITL RAEDNADTLA
Musmus  TLRSEGFDTY RCDRNLAMGV NLTSMSKILK CAGNEDIITL RAEDNADTLA
Xenlav  TLRSDGFDTY RCDRNQSIGV KMSSMSKILK CAASDDIITL RAEDNADTVT
Dromel  TLRSDGFDKF RCDRNLSMGM NLGSMAKILK CANNEDNVTM KAQDNADTVT
Schpom  LIKSDGFEPY RCDRNIALGI NLNALSKVLR CAQNEDLVTL KAEDTPEVLN
Saccer  EIGVEAFQEY RCDHPVTLGM DLTSLSKILR CGNNTDTLTL IADNTPDSII
Plafal  HLLDSGFSHY RCDRERVLGV NIASLNKVFK LCGANESVVI SSKDDEDNLN
                  * ****    *         *

        101                                                 150
Glymax  FMFESPTQDK ISDFEMKLMD IDSEHLGIPE AE..YHAIVK MPSSEFARIC
Daucar  FMFESPTQDK IADFEMKLMD IDSEHLGIPE AE..YHAIVR MPSAEFARIC
Catros  FMFESPTQDK ISDFEMKLMD IDSEHLGIPE AE..YHAIVR MPSAEFARIC
Orysat  FMFESPNQDK IADFEMKLMD IDSEHLGIPD SE..YQAIVR MPSSEFSRIC
Ratnor  LVFEAPNQEK VSDYEMKLMD LDVEQLGIPE QE..YSCVVK MPSGEFARIC
Homsap  LVFEAPNQEK VSDYEMKLMD LDVEQLGIPE QE..YSCVVK MPSGEFARIC
Musmus  LVFEAPNQEK VSDYEMKLMD LDVEQLGIPE QE..YSCVIK MPSGEFARIC
Xenlav  MVFESPNQEK VSDYEMKLMD LDVEQLGIPE QE..YSCVIK MPSGEFARIC
Dromel  IMFESANQEK VSDYEMKLMN LDQEHLGIPE TD..FSCVVR MPAMEFARIC
Schpom  LVFESEKNDR ISDYDVKLMD IDQEHLGIPD IE..YDATIT MPAAEFQRIT
Saccer  LLFEDTKKDR IAEYSLKLMD IDADFLKIEE LQ..YDSTLS LPSSEFSKIV
Plafal  FVFENNKEDK VTNFSLKLMS IELDSLNIPD CEEGFDAEVE LSSKELTNIF
          **              ***       * *                   *   *

        151                                                 200
Glymax  KDLSSIGDTV VISVTKEGVK FSTKGDIGTA NIVCRQNTSV DKPEEATVIE
Daucar  KDLSSIGDTV VISVTKEGVK FSTRGDIGTA NIVCRQNTTV DKPEEATVIE
Catros  KDLSSIGDTV VISVTKEGVK FSTRGDIGTA NIVCRQNTTV DKPDEATIIE
Orysat  KDLSSIGDTV IISVTKEGVK FSTAgDIGTA NIVCRQNKTV DKPEDATIIE
Ratnor  RDLSHIGDAV VISCAKDGVK FSASGELGNG NIKLSQTSNV DKEEEAVSIE
Homsap  RDLSHIGDAV VISCAKDGVK FSASGELGNG NIKLSQTSNV DKEEEAVTIE
Musmus  RDLSHIGDAV VISCAKNGVK FSASGELGNG NIKLSQTSNV DKEEEAVTIE
Xenlav  RDLSQIGDAV VISCAKDGVK FSASGELGTG NVKLSQTSNV DKEEEAVTIE
Dromel  RDLAQFSESV VICCTKEGVK FSASGDVGTA NIKLAQTGSV DKEEEAVIIE
Schpom  RDLLTLSDSV TINASKEGVR FSCKGDIGNG STTLKQHTDL SDQDQSIEIS
Saccer  RDLSQLSDSI NIMITKETIK FVADGDIGSG SVIIKPFVDM EHPETSIKLE
Plafal  RNLSEFSDTV FIEIDSNCIK FTTKGIVGDA EVALKPRDST SEDDIGVTIK
          *         *         *   *  *

        201                                                 250
Glymax  MNEPVSLTFA LRYMNSFTKA TPLSNTVTIS LSNELPVVVE YKIAE.....
Daucar  MNEPVSLTFA LRYMNSFTKA SPLSSTVTIS LSSELPVVVE YKIAE.....
Catros  MNEPVSLTFA LRYLNSFTKA TPLSNNVTIS LSSELPVVVE YKIAE.....
Orysat  MQEPVSLTFA LRYMNSFTKA SPLSEQVTIS LSSELPVVVE YKIAE.....
Ratnor  MNEPVQLTFA LRYLNFFTKA TPLSPTVTLS MSADVPLVVE YKIAD.....
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Homsap  MNEPVQLTFA LRYLNFFTKA TPLSSTVTLS MSADVPLVVE YKIAD.....
Musmus  MNEPVHLTFA LRYLNFFTKA TPLSPTVTLS MSADVPLVVE YKIAD.....
Xenlav  MNEPVQLTFA LRYLNFFTKA TPLSPTVILS MSADIPLVVE YKIAD.....
Dromel  MQEPVTLTFA CRYLNAFTKA TPLSTQVQLS MCADVPLVVE YAIKD.....
Schpom  LTQAVTLTFS LKYLAQFTKA TPLATRVTLS MSNDVPLLVE YKM.E.....
Saccer  MDQPVDLTFG AKYLLDIIKG SSLSDRVGIR LSSEAPALFQ FDLKS.....
Plafal  SKKKIKQSFA IKYLNLFSKS NILADVVVLG LSDSRPIEFK YEIKDTSPDS
                     *     *    *              *

        251                          279
Glymax  ....MGYVRF YLAPKIEEDE EDTKPQV.. (P22177)
Daucar  ....MGYIRF YLAPKIEEEE DESKP.... (Q00268)
Catros  ....MGYIRF YLAPKIEEDD EEQSLEYQA (P24314)
Orysat  ....MGYIRF YLAPKIEEDE EMKS..... (P17070)
Ratnor  ....MGHLKY YLAPKIEDEE GS....... (P04961)
Homsap  ....MGHLKY YLAPKIEDEE GS....... (P12004)
Musmus  ....MGHLKY YLAPKIEDEE AS....... (P17918)
Xenlav  ....MEHVKY YLAPKIEDEE AS....... (P18248)
Dromel  ....LGHIRY YLAPKIEDNE T........ (P17917)
Schpom  ....SGFLRF YLAPKIGEED EE....... (Q03392)
Saccer  .....GFLQF FLAPKFNDEE ......... (P15873)
Plafal  DTLKIGFVKF FLAPKMDDDM DNKD..... (P31008)
                  ****
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Figure 7. Amino acid sequence pileup of PCNA. Identical amino acids are indicated by a * below the sequence. Inserted amino acids in *P.falciparum* are bolded. Glymax, *Glycine max* (partial sequence, soya bean); Daucar, *Daucus carota* (small form, carrot); Catros, *Catharanthus roseus* (madagascan rosey periwinkle); Orysat, *Oryza sativa* (rice); Ratnor, *Rattus norvegicus* (rat); Homsap, *Homo sapiens* (human); Musmus, *Mus musculus* (mouse); Xenlav, *Xenopus laevis* (african clawed frog); Dromel, *Drosophila melanogaster* (fruit fly); Schpom, *Schizzosaccharomyces pombe* (fission yeast); Saccer, *Saccharomyces cerevisiae* (baker's yeast); Plafal, *Plasmodium falciparum* (K1 isolate). The SwissProt data base accession number is indicated for each organism. The numbering of amino acids corresponds to PfPCNA polypeptide.

Using the co-ordinates from the *S.cerevisiae* crystal structure (Krishna *et al.*, 1994) a computer prediction for the structure of a *P.falciparum* PCNA monomer has been made (Sturrock, S., *pers comm.*). The high degree of conservation between these polypeptides is reflected in the stereogramatic representation of these structures (Figure 8a-b). The insertions (I1-2) do

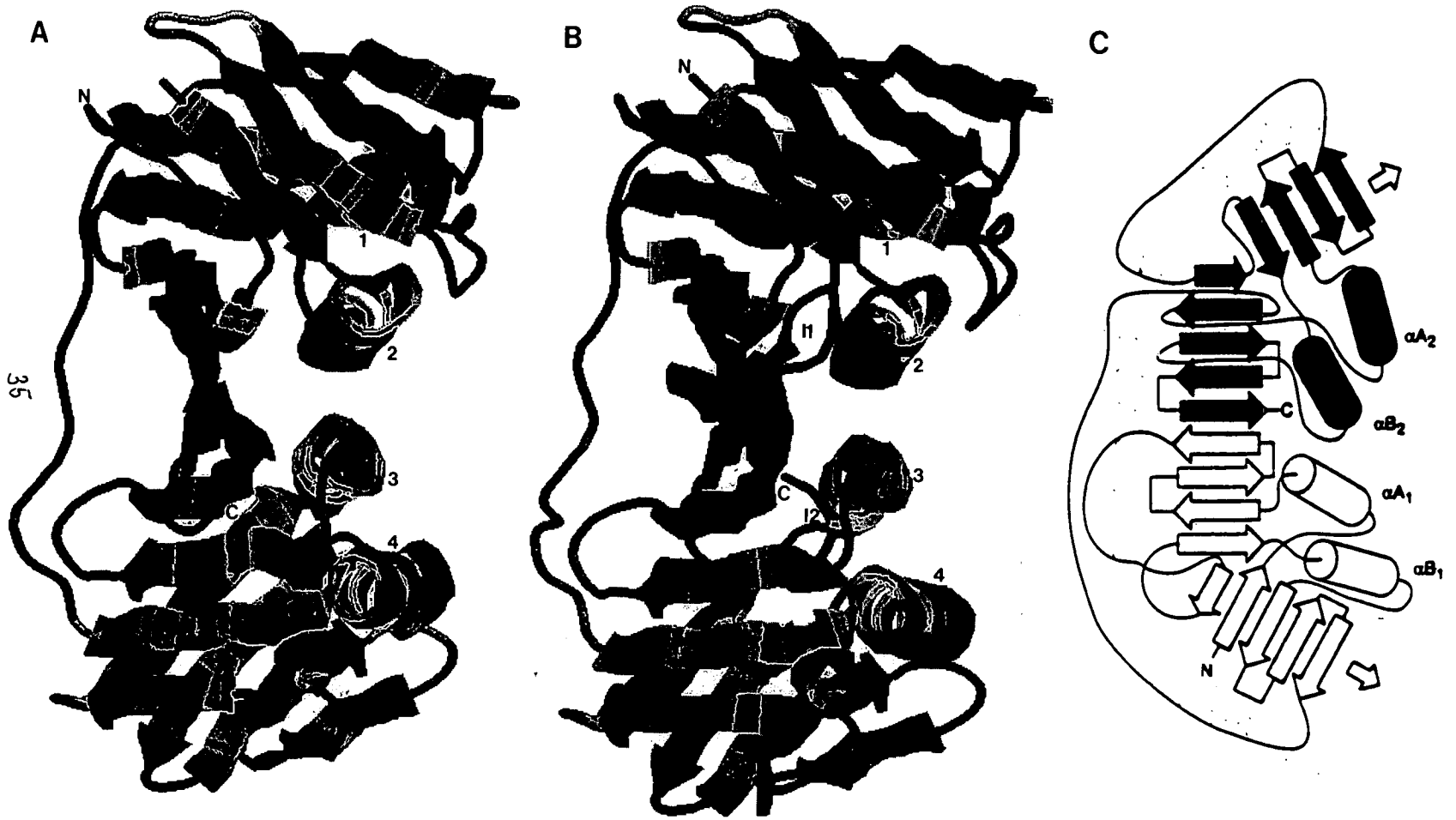
Figure 8.

Three dimensional structure of PCNA.

A. Ribbon schematic of the *S.cerevisiae* PCNA monomer. The position of the N and C terminals of the polypeptide are indicated. The 4 α-helices which interact with DNA are indicated (1-4).

B. Ribbon schematic predicted for the *P.falciparum* PCNA monomer. The position of the N and C terminals of the polypeptide, the 4 α-helices which interact with DNA and the position of the 2 inserts (I1 [133-134] and I2 [246-254]) are indicated.

C. Schematic illustrating the structure of the *S.cerevisiae* PCNA monomer.
The N and C terminals are indicated as are the α-helices. The PCNA monomers interact with their neighbours though the edges of the β-sheet.


A
N
1
2
3
C
4
B
N
1
l1
2
C
l2
3
4
C
αA2
αB2
αA1
αB1
N

not appear to interfere with the positioning of the α-helices or β-sheets.
A schematic representation of the PCNA monomer (Figure 8c) indicates that there are two domains, each consisting of a pair of β–α–β–β motifs. The antiparallel β sheets form an arched backbone which place the α helices on the inside of the arc. The two domains are joined by a large loop which interacts with the antiparallel β sheets. The loop is on the surface of the protein and is highly antigenic (Krishna *et al.*, 1994). PCNA has a strong positive charge arrayed on the inside of the ring which would favour its interaction with DNA. The α helices are positioned so that they may interact non-specifically with the backbone of the DNA rather than a more specific interaction within the major and minor grooves. The central cavity is slightly ovoid with a diameter of approximately 34Å, more than adequate to surround the diameter of DNA (18-21Å).
The interfaces between the monomers are formed principally by the outermost β sheets. On trimerisation the antiparallel β sheets form a continuous ring shaped backbone structure. Hydrogen bonds, hydrophobic forces and ionic pair interactions contribute towards the interaction at the interfaces between the monomers. Charged amino acids at the interface are thought to be required for the correct head-to-tail orientation of the monomers as well as interactions to RF-C and DNA polymerase δ (Kelman and O'Donnell, 1995a).
Site specific mutational analysis has identified a number of regions of PCNA required for its functional activity. The substitution of uncharged for charged residues in the α helices limits the ability of PCNA to stimulate DNA polymerase δ processivity (Fukuda *et al.*, 1995). When the negatively charged residues in the C terminus, which form a loop that projects out of the face of the ring, were substituted the ability of PCNA to stimulate the ATPase activity of RF-C was limited (Fukuda *et al.*, 1995). These observations led to the proposal that PCNA interacts with RF-C and DNA polymerase δ in an orientation specific manner which influences the complexes ability to track along the DNA. Substitution of a tyrosine at the interface between monomers (Tyr^{114}) disrupted the formation of the

trimer (Jonsson *et al.*, 1995). *P.falciparum* and several plant species have a phenylalanine at this position. The opposing face of the monomer from these species use different amino acids which presumably interact with the phenylalanine.

The reason as to why the β subunit and PCNA have an almost identical structure based on a different protomer design is unknown. A protein consisting of a trimer may be more unstable than a dimer and therefore could have some regulatory role based on its stability (Kelman and O'Donnell, 1995a). Interestingly a "long" form of PCNA has been identified from carrot embryos (Hata *et al.*, 1992) which is almost the same size as the β subunit monomer. A PCNA transcript and anti-PCNA cross reacting protein corresponding to a "dimeric" form of PCNA have been identified during *Xenopus* oogenesis (Leibovici *et al*, 1990). These observations have led to some speculation as to whether there is an embryonic form of PCNA which is related to a distant prokaryotic β subunit ancestor (Kelman and O'Donnell, 1995b).

1.7.2 PCNA interacts with a wide range of proteins.

PCNA is essential for both leading and lagging strand synthesis during which it interacts with DNA polymerase δ and RF-C (Waga and Stillman, 1994). PCNA is thought to be loaded onto the DNA in a process which requires the ATPase activity of RF-C (Burgers and Yoder, 1993, Produst *et al.*, 1995). Presumably the energy derived from ATP hydrolysis is required to open the PCNA ring allowing the DNA to pass through to the central cavity. The complex then slides along the DNA until a 3′ hydroxyl group is encountered. PCNA then transforms into a competent clamp which holds DNA polymerase δ onto the template. On completion of an Okasaki fragment the PCNA remains attached to the DNA after the DNA polymerase δ has dissociated (Yao *et al.*, 1996). The ATPase activity of RF-C is also thought to be required to unload PCNA from the DNA to allow it to be recycled to another Okasaki fragment.

The joining of Okasaki fragments requires that a short stretch of DNA synthesised by DNA polymerase α is removed. The 5′-3′ exonucleases,

FEN-1 (humans and mice) and *RTH1* (*S.cerevisiae*), have been implicated in the maturation of the Okasaki fragments (Li *et al.*, 1995). The FEN-1 protein requires PCNA to facilitate its binding to its site of action, this sequestration enhances the exonuclease activity between 10-50 fold (Li *et al.*, 1995).

PCNA is essential for the nucleotide excision repair (NER) pathway (Shivji *et al.*, 1992) which suggests that the gap filling is performed by some of the same components of the chromosomal replication machinery. Both chromosomal replication and NER require the RF-C dependent loading of PCNA to the DNA (Aboussekhra *et al.*, 1995). However, the issue of which DNA polymerase (δ or ε) is required for chromosomal replication or NER, or both, has not been resolved.

In response to DNA damage, higher eukaryotes arrest cell cycle progression at either the G_1 or G_2 checkpoints (Maltzman and Czyzyk, 1984). During G_1 arrest the levels of the transcription factor p53 increase stimulating the expression of a number of cell cycle regulators. One such regulator, p21, is a cyclin dependent kinase inhibitor, which arrests DNA synthesis *in vitro* through its interaction with PCNA (Li *et al.*, 1994). p21 does not, however, interfere with the PCNA dependent NER (Shivji *et al.*, 1994). PCNA also interacts with a second cell cycle regulator, Gadd45, in competition with p21 (Chen *et al.*, 1995). p21 and Gadd45 might play a significant role in the coordination of cell cycle progression and chromosomal DNA replication during DNA repair through their interactions with PCNA.

1.7.3 Regulation of PCNA expression during the cell cycle.

The regulation of the expression of the *S.cerevisiae* (POL30) and *Drosophila* PCNA have been covered elsewhere in the introduction. Since the characterisation of the human cyclin gene which revealed that PCNA was stage specifically regulated (Almendral *et al.*, 1987, Bravo, 1986) the regulation of the PCNA gene in higher eukaryotes has been the focus of intense interest (Baserga, 1991).

When quiescent (G_0) cells are stimulated with serum PCNA accumulates at the G1/S phase boundary (Bravo, 1986). This identified the gene as a

member of a group of late-response genes which includes; histones, dihydrofolate reductase, thymidylate synthase, DNA polymerase α, primase and ligase. The level of the protein remains relatively constant until the G2/M phase boundary is reached when the protein is presumably degraded. The PCNA mRNA is absent in G_0 cells and accumulates after serum stimulation until a peak at around 16-18 hours, a point which coincides with the onset of the S phase (Almendral *et al.*, 1987). This indicated that the regulation of PCNA was principally brought about at the level of transcription. In continually proliferating cells the PCNA mRNA and protein levels do not appear to vary (Morris and Matthews, 1989).

Initial attempts to map the human PCNA promoter showed that 210bp of sequence upstream of the transcription start site was sufficient for the expression of PCNA (Ottavio *et al.*, 1990a). Further analysis revealed that an enhancer-like element was located between 45bp and 73bp upstream of the transcription start site (Ottavio *et al.*, 1990a, Piertzkowski *et al.*, 1991). This region contained a SV40 core enhancer and a CREB (cyclic-AMP binding site) sequence. The introduction of mutations into either sequence abolished PCNA expression and gel retardation assays indicated that a protein was bound to this sequence *in vivo* (Piertzkowski *et al.*, 1991).

During the course of the promoter analysis an attempt to use mini-genes led to PCNA mRNA levels increasing dramatically in G_0 cells. An element within the 4th intron was shown to be responsible for the correct temporal accumulation of transcripts (Ottavio *et al.*, 1990b).

The PCNA promoter is active in both the G_0 cells and serum stimulated cells (Chang *et al.*, 1990). Examination of the relative stability of PCNA mRNA from the two cell types showed that mRNA isolated from proliferating cells was approximately 4 times more stable than in the G_0 cells (Chang *et al.*, 1990). To account for the increase in the mRNA levels observed, however, the serum stimulated mRNA would need to be approximately 40 times more stable. Baserga and co-workers have suggested that the transcriptional regulation of PCNA may be brought

about by processing of the heterologous nuclear RNA (hnRNA). Transcriptional pausing which is released at a particular time point, as observed for murine ribonuclease reductase regulatory 2 subunit (Bjorklund *et al.*, 1992), is one possibility. The human PCNA gene contains five introns (Almendral *et al.*, 1987) presenting the possibility that regulation may be introduced at the level of intron splicing. Polyadenylation and the transport of the mature transcript from the nucleus offer further possibilities. Baserga (1991) has proposed a model for the expression of PCNA in serum-stimulated cells. The PCNA gene is continually transcribed with a basal level regulated by elements within the promoter and the 4th intron. In G_0 cells the transcripts are not completed; only upon serum-stimulation does this occur. Although posttranscriptional phosphorylation of PCNA does not appear to occur (Zeng *et al.*, 1994), the activity of PCNA is likely to be influenced by its interactions with a wide range of proteins.

1.8 Rationale and scope of thesis.

The study of regulation of the expression of DNA replication proteins from *P.falciparum* during intraerythrocytic schizogony has been fragmentary. We therefore proposed to determine when, during intraerythrocytic schizogony, PfPCNA and PfPolδ mRNAs and polypeptides accumulate and to simultaneously determine the activity of their promoters. These experiments would provide us with information regarding the temporal control of the expression of a pair of cofunctional gene products. This analysis would provide evidence of whether there is a coordinated unitary control for these cofunctional proteins, or whether their expression is simultaneous but differently regulated.

In order to identify potential regulatory sequences the intention was to map transcription start sites of both the PfPCNA and PfPolδ genes using both primer extension and RNase protection. The combination of these protocols to map the transcription start sites would allow these sites to be more reliably identified in the extremely AT-rich sequences. Should consensus sequences be found we would try identify whether proteins

bind to these sequences, and whether this binding is stage specific. If this was the case we would try and clone these potential regulatory proteins.
We had set up a collaboration with Dr. Christian Doerig to develop a transfection system for intraerythrocytic stage cultures. We proposed to use the extensive PfPCNA flanking sequences available with a novel reporter gene, the Green Fluorescent Protein. Should a transfection system be developed we hoped to modify the PfPCNA 5′ flanking sequences to facilitate a functional analysis of the PfPCNA promoter.

Chapter 2.

2.1 Materials.

2.1.1 Chemicals.

Unless stated otherwise, chemicals were supplied by Sigma Chemical Co. Ltd., UK or BDH plc., UK, and were all of analytical grade. Cell culture materials were supplied by Gibco-BRL, UK. Solvents were supplied by FSA Laboratory Supplies, UK. Radiolabelled nucleotides were supplied by Amersham International plc., UK.

2.1.2 Equipment.

Benchtop centrifugation was carried out using a Heraeus Biofuge 13 (Eppendorf, Germany) or Juoan CR322 (Saint-Herbain, France). High speed spins were made using either a Sorvall RC5-B high speed centrifuge (DuPont Instruments) or Sorvall OTB50-B ultracentrifuge (DuPont Instruments). PCR were carried out using a PHC-2 thermal cycler (Techne Instruments). Hybridisations were done in HB1/HB2D heated cabinets (Techne Instruments). Plasticware and culture materials were supplied by Beckton Dickinson Labware, UK.

2.1.3 Restriction and modifying enzymes.

Restriction endonucleases and buffers were supplied by Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd., UK. One-phor-all restriction endonuclease buffer was supplied by Pharmacia Biotechnology, UK. Modifying enzymes were supplied by Gibco-BRL, UK, Boehringer Mannheim UK Ltd., UK, and CAMBIO Ltd. through React Scientific, UK.

2.1.4 Imaging.

KODAK X-OMAT AR and X-OMAT LS autoradiography film was supplied by IBI Molecular Biology Products, UK. UV-iradiated ethidium bromide gels were photographed using either HP5 film, ILFORD Ltd., UK,

or by Mitsubishi Video Processor to heat sensitive film.

2.1.5 Microbiology.

2.1.5.1 Bacterial strains.

Strain	Genotype	Reference
DH5α	F'*end*A1 *rec*A1 *hsd*R17(r_k^-, m_k^+) *sup*E44 *thi* -1 *gyr*A96 *rel*A1 ϕ80d*lac*ZΔM15 Δ(*lac* ZYA-*arg*F)U169 *deo*R	Hanahan (1983)
INVα F'	F'*end*A1 *rec*A1 *hsd*R17(r_k^-, m_k^+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 ϕ80*lac*ZΔM15 Δ(*lac* ZYA-*arg*F)U169 *deo*R λ⁻	
Y1090	F' Δ(*lac*U169) *pro*A⁺ Δ(lon) *ara*D139 *str*A *sup* F [*trp*C22::Tn10(tet ^{r})] (pMC9) *hsd*R (r_k^-, m_k^+)	Huynh *et al.* (1985)
NM494	*hfl* Δ(*hsd*MS *mcr*B) *mcr*A	Murray N. (*pers comm.*)
BL21 (DE3)	F⁻ *hsd*D Δ(*lac*U169) gal(λ*c*I857 *ind*1 *Sam*7 *nin* 5 *lac*UV5-T7 gene1) (r_B^-, m_B^-) (+pLysS)	Studier and Moffat (1986) Studier (1991)

2.1.5.2 Bacteriophages.

Bacteriophage	Genotype	Reference
λgt11	λ , *lac* 5 , *nin* 5, *c* I857, *Sam*100	Young and Davis, (1983)

NM1149	λ *imm*^{434}, b538	Murray (1983)

2.1.5.3 *P.falciparum* DNA libraries.

Library	Source	Reference
NM1149, *Hin*dIII restricted K1 genomic DNA.	M. Goman, University of Edinburgh.	Goman *et al.*, (1982)
NM1149, *Eco*RI restricted K1 genomic DNA.	M. Goman, University of Edinburgh.	Goman *et al.*, (1982)
λgt11, partial *Dra*I restricted K1 genomic DNA.	U. Certa, Hoffmann- La-Roche, Basel.	Certa *et al.*, (1988)
λgt11, sheared K1 genomic DNA. *Eco*RI linkers.	U. Certa, Hoffmann- La-Roche, Basel.	Certa *et al.*, (1988)
pJFE14, cDNA from trophozoites, *Xho*I linkers.	A. Craig, IMM, Oxford.	

2.1.6 General stock solutions and media.

BBL bottom agar (supplied by ICMB media service).
1% Baltimore Biological Laboratories trypticase, 0.5% sodium chloride, 1.5% agar.

BBL top agar (supplied by ICMB media service).
As for BBL bottom agar except contains only 0.65% agar.

Denaturation solution.

1.5M sodium chloride and 0.5M sodium hydroxide.

Luria-Bertani medium (LB, supplied by ICMB media service).

1% bacto-tryptone, 0.5% Bacto-yeast extract, 1% sodium chloride adjusted to pH7.2 using 1M sodium hydroxide.

LB agar (supplied by ICMB media service).

LB supplemented with 1.5% agar.

Neutralising solution.

1M ammonium acetate, 0.02M sodium hydroxide.

Oligonucleotide hybridisation buffer.

2XSSC, 0.2% SDS, 0.1% sodium pyrophosphate, 500μgml^{-1} heparin.

Phage buffer (supplied by ICMB media service).

0.3% potassium dihydrogen orthophosphate, 0.7% disodium hydrogen orthophosphate (anhydrous), 0.5% sodium chloride, 1mM magnesium sulphate, 0.1mM calcium chloride, 1% gelatin.

Phosphate buffered saline (PBS).

137mM sodium chloride, 2.7mM potassium chloride, 4.3 mM disodium hydrogen orthophosphate, 1.4mM potassium dihydrogen orthophosphate.

Random-label hybridisation buffer.

0.25M sodium phosphate pH7.2, 7% SDS.

Standard saline citrate (SSC) 20x stock solution.

3M sodium chloride, 100mM trisodium citrate pH7.0.

Tris-acetate-EDTA (TAE) 50x stock solution.

2M Tris-acetate, 2mM EDTA pH 8.0.

Tris-borate-EDTA (TBE) 10x stock solution.
0.9M Tris-borate, 20mM EDTA pH8.0.

Tris-EDTA (TE).
10mM Tris-HCl pH8.0, 1mM EDTA pH8.0.

Tris-EDTA-saline (TES).
20mM Tris-HCl pH7.5, 10mM EDTA, 100mM sodium chloride.

Unless otherwise stated, the methods described in the following text were based on those found in either Sambrook *et al.* (1989), Harlow and Lane (1988) or Ausubel *et al.* (1992). Unless otherwise stated, microcentifugation was for 30 seconds at 12,000rpm.

2.2 DNA Methods.

2.2.1 Isolation of DNA.

2.2.1.1 Preparation of *P.falciparum* DNA.

P.falciparum culture was grown until 10^9 parasites were available. The culture was harvested by centrifugation (2500g, 10 minutes, 4°C) and resuspended in 50ml of PBS. The washed erythrocytes were collected by centrifugation, resuspended in 1xSSC, 0.1% saponin and incubated at room temperature for 5 minutes. The released parasites were collected by centrifugation (3000g, 15 minutes, 4°C) and washed with 1xSSC, 0.1% saponin. The washed parasites were collected by centrifugation (3000g, 15 minutes, 4°C) and resuspended in 9ml of 1xSSC. 2ml of 20% sodium lauryl sarcosine was added, mixed carefully by inversion and the suspension incubated on ice for 5 minutes. The suspension was made up to 21ml by the addition of 1xSSC containing 20.9g of caesium chloride and 0.2ml of ethidium bromide ($10mgml^{-1}$) then divided into 2 heat sealed

tubes (11.5ml tubes for Ti50 rotor). The DNA was banded by caesium chloride gradient ultracentrifugation (120000g, 48 hours, 18°C). A single central band, visible under a UV light, containing the genomic DNA was collected and the ethidium bromide removed by repeated extractions with isoamyl alcohol. The DNA was dialysed against 2L of TE overnight at 4°C, ethanol precipitated (2.2.1.7) and resuspended in 1ml of TE. The DNA was analysed using UV spectroscopy (2.2.1.5).

2.2.1.2 Maxipreparation of plasmid DNA.

Preparation of approximately 1mg of plasmid DNA from *Escherichia coli* was made using either an alkaline lysis/caesium chloride method or Wizard™ Maxipreps. The protocols for both are described below. DNA prepared by both these methods was used primarily for transfection experiments. A comparison of the DNA produced by both methods shows that the DNA is comparable in terms of the results from a transient transfection assay (Brondyk *et al.*, 1994).

Alkaline lysis/caesium chloride method

A single colony was used to inoculate 500ml of LB supplemented with ampicillin (100μgml^{-1}) and grown overnight at 37°C with shaking. The culture was centrifuged (2500g, 10 minutes, 4°C) and the bacterial pellet resuspended in 20ml of solution I (50mM glucose, 25mM Tris-HCl pH8.0 and 10mM EDTA pH8.0). 2ml of lysozyme solution (10$mgml^{-1}$ in 10mM Tris-HCl pH8.0) was added and gently mixed by inversion. 40ml of freshly prepared solution II (0.2M sodium hydroxide, 1% SDS) was added, mixed by inversion and incubated at room temperature for 10 minutes. 20ml of ice-cold solution III (3M potassium acetate pH5.0) was added, mixed by inversion and incubated on ice for 15 minutes with periodic mixing.

The lysate was centrifuged (2500g, 10 minutes, 4°C) to pellet the cell debris. The supernatant was collected, mixed with 0.6 volumes of room temperature isopropanol and incubated at room temperature for 10 minutes. The nucleic acids were pelleted by centrifugation (12000g, 15 minutes, 4°C) and washed with ice-cold 70% and 100% ethanol. The pellet

was allowed to dry for 5 minutes at room temperature before being resuspended in 3ml of TE (pH8.0). The 3ml of DNA solution was added to 18ml of sdH_2O containing 20.9g of caesium chloride and 0.2 ml of ethidium bromide ($10mgml^{-1}$) and divided into 2 heat sealed tubes (11.5ml tubes for Ti50 rotor). The DNA was banded by caesium chloride gradient ultracentrifugation (120000g, 48 hours, 18°C). The lower of the two central bands, visible under a UV light, was collected and the ethidium bromide removed by repeated isoamyl alcohol extractions. The plasmid DNA was dialysed against 2L of TE overnight at 4°C, ethanol precipitated (2.2.1.7) and resuspended in 1ml of TE. The DNA was analysed using UV spectroscopy (2.2.1.5).

Wizard™ Maxiprep protocol

The solutions and protocol were supplied with this commercially available kit (Promega).

A single colony was used to inoculate 300ml of LB supplemented with ampicillin ($100\mu gml^{-1}$) and grown overnight at 37°C with shaking. The culture was pelleted by centrifugation (2500g, 10 minutes, room temperature) and resuspended in 15ml of cell resuspension solution (50mM Tris-HCl pH7.5, 10mM EDTA, $100\mu gml^{-1}$ RNase A). 15ml of cell lysis solution (0.2M sodium hydroxide, 1%SDS) was added and mixed by gentle inversion and left for 20 minutes at room temperature. 15ml of neutralising solution (1.32M potassium acetate pH4.8) was added, mixed completely and incubated at room temperature for 20 minutes.

The bacterial debris was pelleted by centrifugation (3000g, 10 minutes, 4°C) and the supernatant mixed, by inversion, with 0.5 volumes of room temperature isopropanol. The DNA was pelleted by centrifugation (12000g, 15 minutes, room temperature) and resuspended in 2ml of TE (pH7.5).

10ml of Wizard™ Maxiprep DNA purification resin was mixed with the 2ml of DNA solution and transferred into a Wizard™ Maxicolumn. A vacuum pump was used to draw the resin through the column slowly. Two 13ml aliquots of wash solution (100mM sodium chloride, 10mM

Tris-HCl pH7.5, 2.5mM EDTA, 55% ethanol) were added to the column to wash the resin, followed by a final 5ml wash with 80% ethanol. Air was drawn through the column for a further 1 minute after the column was emptied. The column was placed into a 50ml falcon tube and centrifuged (2000g, 5 minutes, room temperature) to dry the resin completely. 1.5ml of sdH_2O, prewarmed to 65°C, was added to the column. After waiting 1 minute the column was centrifuged (2000g, 5 minutes, room temperature) and the eluted DNA analysed using UV spectroscopy (2.2.1.5).

2.2.1.3 Midipreparation of plasmid DNA.

Approximately 100-150µg of plasmid DNA from *Escherichia coli* was prepared using the commercially available Plasmid Midi Kit (Qiagen™).

A single colony was used to inoculate 100ml of LB supplemented with ampicillin ($100\mu gml^{-1}$) and grown overnight at 37°C with shaking. The cells were collected by centrifugation (2500g, 10 minutes, 4°C) and the pellet resuspended in 4ml of buffer P1 (50mM Tris-HCl pH8.0, 10mM EDTA, $100\mu gml^{-1}$ RNase A). 4ml of buffer P2 (200mM sodium hydroxide, 1%SDS) was added, mixed by inversion, and the suspension incubated at room temperature for 20 minutes. The addition of 4ml of buffer P3 (2.55M potassium acetate pH4.8) to the suspension was followed by a 15 minute incubation on ice. Cell debris was pelleted by centrifugation (10000g, 10 minutes, 4°C) and the supernatant filtered through medium-fast Whatman filter paper (90mm circles no. 1) to ensure the bacterial debris was completely removed.

The supernatant was added to a Qiagen™ Tip-100 column, which had been equilibrated using 4ml of buffer QBT (750mM sodium chloride, 50mM MOPS, 15% ethanol pH7.0, 0.15% Triton X-100), and allowed to enter by gravity flow. The column was washed using 20ml of buffer QC (1M sodium chloride, 50mM MOPS, 15% ethanol pH7.0). The DNA was eluted using 5ml of buffer QF (1.25M sodium chloride, 50mM MOPS, 15% ethanol pH8.2). The DNA was pelleted by the addition of 0.7 volumes of room temperature isopropanol and centrifugation (12000g, 20 minutes, 4°C). The DNA pellet was washed with 2ml of ice-cold 70% ethanol and

allowed to air dry before being resuspended in 100µl of TE (pH8.0). The DNA was analysed using UV spectrophotometry (2.2.1.5).

2.2.1.4 Minipreparation of plasmid DNA.

Approximately 10µg of plasmid DNA from *Escherichia coli* was prepared using the commercially available Wizard™ Minipreps kit (Promega).
A single colony was used to inoculate 5ml of LB supplemented with ampicillin (100µgml^{-1}) and grown overnight at 37°C with shaking. 3ml of the cells were pelleted using a microcentrifuge for 20 seconds and resuspended in 200µl of cell resuspension buffer (50mM Tris-HCl pH7.5, 10mM EDTA, 100µgml^{-1} RNase A). The cells were lysed by the addition of 200µl of cell lysis buffer (0.2mM sodium hydroxide, 1% SDS). After the addition of 200µl of neutralisation solution (1.32M potassium acetate pH4.8) the cell debris was pelleted by microcentrifugation for 5 minutes. The supernatant was added to 1ml of DNA purification resin and mixed by inversion. The mixture was pushed into a column, using a 2ml syringe, and washed with 2ml of wash solution (100mM sodium chloride, 10mM Tris-HCl pH7.5, 1mM EDTA, 70% ethanol). The column was dried by microcentrifugation for 2 minutes before elution of the DNA by adding 50µl of TE to the column and microcentrifugation for 20 seconds.

2.2.1.5 UV spectrophotometry.

UV spectrophotometry allows the ready quantification of nucleic acids and a check of their purity. Typically, a sample of nucleic acid was diluted 1:200 in 1ml of sdH_2O. Using the protocol supplied with the Perkin-Elmer λ15 machine a sample of sdH_2O was scanned between 200nm and 300nm to give a base value against which a subsequent scan of the diluted nucleic acid was made. The concentration of nucleic acid (mgml^{-1}) within a sample was calculated using the following formulas:

DNA concentration = absorbtion at 260nm (A^{260}) X 50 X dilution factor.

RNA concentration = absorbtion at 260nm (A^{260}) X 40 X dilution factor.

The purity of the nucleic acid sample, with respect to protein contamination, was determined from the ratio A^{260}/ A^{280}, where a value greater than 1.8 indicates that the sample is free from protein contamination.

2.2.1.6 Phenol/chloroform extraction.

Phenol was saturated and equilibrated with TE (pH7.5) before use in extraction. 20ml of TE (pH7.5) was mixed with 20ml of phenol, mixed thoroughly, and the phases separated by centrifugation (2000g, 5 minutes, room temperature). The upper layer was removed and the pH checked using litmus paper. The process was repeated until the TE removed remained at pH7.5. An equal volume of chloroform (containing 1/25th volume isoamyl alcohol) was then added to the phenol before being stored in the dark at 4°C. Fresh phenol/chloroform was prepared every 4 months.

An equal volume of phenol/chloroform was added to the DNA solution to be treated and vortexed before microcentrifugation for 1 minute. The upper aqueous phase was transferred to a fresh Eppendorf tube and a second phenol/chloroform extraction made on this. The aqueous phase was again collected and the DNA ethanol precipitated (2.2.1.7).

2.2.1.7 Ethanol precipitation.

0.1 volumes of 3M sodium acetate (pH5.0) and 2 volumes of ice-cold 100% ethanol were added to the nucleic acid solution to be precipitated. The nucleic acid was pelleted by microcentrifugation for 20 minutes. The nucleic acid pellet was washed with a small volume of both 70% and 100% ice-cold ethanol before being left to air dry at room temperature. The nucleic acid pellet was resuspended in either TE or sdH_2O.

2.2.2 Endonuclease restriction.

DNA restrictions were typically carried out for 2-3 hours at 37°C using

commercially available restriction endonucleases. 3-10 units of enzyme were usually used to restrict 1μg of DNA in a 20μl volume of the recommended buffer. For larger quantities of DNA the reaction components were scaled up appropriately.

Restriction endonuclease buffers were supplied at 10X the final concentration. The Boehringer Mannheim incubation buffer range (Table 1) was used for single enzyme restrictions. Where more than one restriction endonuclease was required, and the buffer compositions were incompatible, "One-Phor-All" (Pharmacia) was used (100mM Tris-acetate, 100mM magnesium acetate, 500mM potassium acetate pH7.5).

Table 1. Composition of Boehringer restriction endonuclease buffers. (final concentration in mM)

Component	Buffer				
	A	B	L	M	H
Tris-acetate	33	-	-	-	-
Tris-HCl	-	10	10	10	50
Magnesium acetate	10	-	-	-	-
Magnesium chloride	-	5	10	10	10
Potassium acetate	-	100	-	50	100
Dithioerythritol	-	-	1	1	1
Dithiothreitol	0.5	-	-	-	-
β-meracptoetanol	-	1	-	-	-
pH at 37°C	7.9	8.0	7.5	7.5	7.5

- *Sma* I restrictions made at 30°C
- *Kpn* I restrictions supplemented with 100μgml^{-1} BSA.

2.2.3 Agarose gel electrophoresis and DNA fragment isolation.

2.2.3.1 Preparation of agarose gels.

Multipupose agarose was supplied by Boehringer Mannheim and low melting point agarose by Sigma.

Table 2. Range of efficient separation of linear DNA molecules in agarose gels of different concentration. Sambrook *et al.* (1989).

Percentage agarose in the gel.	Efficient range of separation of linear DNA molecules.
0.7	0.8-10kbp
0.9	0.5-7kbp
1.2	0.4-6kbp
1.5	0.2-3kbp

The appropriate amount of agarose as determined using Table 2 was dissolved in 1XTAE (2.1.6) and heated while stirring until the agarose was completely dissolved. For each 50ml of agarose solution 1µl of ethidium bromide ($10mgml^{-1}$) was added. The molten gel was poured into the appropriate casting tray and allowed to set at room temperature. For low melting point agarose the gels were cast at 4°C to aid setting.

2.2.3.2 Running agarose gels.

The cast gel was submerged in 1XTAE (2.1.6). Samples and DNA markers (2.2.3.3) were mixed with a 0.1 volume of loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol). Samples were run in the agarose gel at $15Vcm^{-1}$ until sufficient separation of the DNA bands of interest was achieved.

2.2.3.3 DNA markers.

The DNA markers commonly used were; λ restricted with *Hin*dIII and *Eco*RI (Promega), 1km ladder (Gibco-BRL), ϕx174 restricted with *Hae*III (Promega) and a 100bp ladder (Gibco-BRL). 1µg of the DNA markers were used on each gel.

2.2.3.4 Imaging of the gel.

The DNA was visualised using a short wave UV transilluminator. The image was recorded on either heat sensitive film or a negative was produced on FP5 film (2.1.4). A ruler was photographed beside any gel which was to be subsequently southern blotted (2.2.4).

2.2.3.5 DNA fragment isolation from a low melting point agarose gel.

DNA fragments were routinely isolated from low melting point agarose gels. The resolving capability of this gel, however, is lower than those made using multi-purpose agarose. In cases where the resolution of DNA fragments in low melting point agarose was poor, electroelution from multipurpose agarose gels was used instead (2.2.3.6).

A band of interest was cut from the gel using a clean scalpel blade and added to an equal volume of 1XTAE/0.2M sodium chloride and incubated at 65°C for 15 minutes. 0.75 volumes of phenol saturated with 1XTAE/0.2M sodium chloride was added, vortexed, and microcentrifuged. The upper aqueous layer was transferred to a fresh Eppendorf tube and the phenol extraction repeated. 100µl of butanol was added the the aqueous phase, vortexed, microcentrifuged for 1 minute and the butanol layer removed. This removed any contaminating phenol from the aqueous phase. The DNA in the remaining aqueous phase was ethanol precipitated (2.2.1.7).

2.2.3.6 Electroelution of isolated DNA fragment.

Dialysis tubing was prepared by boiling it in 2% sodium bicarbonate/1mM EDTA for 10 minutes. After washing repeatedly in fresh sdH_2O the dialysis tubing was boiled for a further 10 minutes in 1mM EDTA. The prepared dialysis tubing was then stored at 4°C in 70% ethanol.Before the dialysis tubing was used it was washed repeatedly in sdH_2O.

The agarose gel fragment was placed into a small piece of washed dialysis tubing with 300µl of 1XTAE and the ends closed with clips. The tubing was submerged in 1XTAE and a current passed through for 30 minutes, typically at 90-100mA. The current was reversed for the final 2 minutes of the run to help remove DNA that had attached to the dialysis tubing. Elution of the DNA was checked using a short wave UV

transilluminator. The DNA solution was transferred into a fresh Eppendorf tube and ethanol precipitated (2.2.1.7).

2.2.4 Southern transfer.

2.2.4.1 "Dry" southern transfer.

This was a quick method of capillary transfer of DNA to a nylon membrane when the quantity of DNA in an agarose gel was sufficiently large (>75ng per band). The gel was submerged in 0.2M hydrochloric acid for 10 minutes to depurinate the DNA. The gel was washed several times in sdH_2O before being submerged in denaturing solution (2.1.6) for 10 minutes with gentle shaking. The gel was again washed several times in sdH_2O before being submerged in neutralising solution (2.1.6) for 20 minutes with gentle shaking.

Genescreen *Plus* membrane (DuPont) cut to the size of the gel was wet completely with neutralising solution and placed on the surface of the gel ensuring that all air bubbles were excluded. 4 pieces of Whatman (3MM) paper, cut to size, were placed on top of the membrane. A small stack of paper towels and a light weight were then added.

The transfer of DNA took place over 5-6 hours. The membrane was removed, washed in 2XSSC (2.6.1) for 2 minutes and air dried. It was then ready for prehybridisation (2.2.4.3).

2.2.4.2 "Wet" southern transfer.

A "wet" transfer was used when the DNA of interest is present in small quantities (eg. genomic southern analysis). The initial preparation (depurination, denaturation and neutralisation) of the gel is the same as that described for "dry" southern transfer (2.2.4.1).

The prepared gel was placed on a "wick" consisting of 2 pieces of Whatman (3MM) paper cut to the same width as the gel, but longer, so that the ends could be submerged in a reservoir of neutralising solution (2.1.6). The gel was surrounded with saran wrap to prevent the neutralising solution by-passing the gel into the stack of paper towels. A piece of Genescreen *Plus* membrane (DuPont), cut to the correct size, was

wet completely with neutralising solution and placed on the gel ensuring that all the air bubbles were excluded. 6 pieces of Whatman (3MM) paper were cut to the correct size. 2 of these were wet with neutralising solution and placed on the membrane and then the remaining 4 pieces were added, again eliminating air bubbles. A stack of paper towels approximately 10cm high were added to the blot and a glass plate placed on top. A 300-400g weight was placed on top of the glass plate. The transfer was left overnight.

The next day the blot was carefully taken apart, the membrane washed in 2XSSC (2.6.1) for 2 minutes and air dried. The membrane was ready for prehybridisation (2.2.4.3).

2.2.4.3 Prehybridisation and hybridisation of radiolabelled probes.

Prehybridisation and hybridisation were done using the same buffer, although the composition of the buffer varied depending upon the type of radiolabelled probe used (end-labelled oligonucleotide or random labelled). The membrane was placed in a hybridising tube, with 25ml of the appropriate hybridisation solution (2.6.1), and incubated overnight. The next day the buffer was replaced, the probe added and the membrane incubated overnight.

2.2.4.4 End-labelled oligonucleotide probes.

These were used for membranes where reasonable quantities of DNA were available (>25ng). The prehybridisation and hybridisation were done at 37°C unless an oligonucleotide of greater than 20 bases in length was used, in this case 40°C would be used. The oligonucleotide was end-labelled in the following reaction;

1µl oligonucleotide ($20ng\mu l^{-1}$)

4µl 5X Forward reaction buffer (300mM Tris-HCl pH7.8, 50mM magnesium chloride, 1.65µM ATP, 75mM β-mercaptoethanol)

13µl sdH_2O

1µl [$\gamma.^{32}P$] ATP ($10mCiml^{-1}$)

1µl T4 Kinase ($10U\mu l^{-1}$)

The reaction was incubated at 37°C for 3 hours after which the probe was ready to be added (2.2.4.3).

2.2.4.5 Random labelled probes.

Random labelled probes were prepared using the commercially available Prime-It™II Random Primer Labelling kit (Stratagene). With the exception of the T7 DNA polymerase all materials were supplied with the kit.

100ng DNA in Xμl

10μl random primers (9-mers at 8ngμl^{-1})

26.5 - Xμl sdH_2O

The above were heated to 95-100°C for 5 minutes before being allowed to cool slowly to room temperature. The following were then added;

10μl 5X random label (dCTP) buffer (250mM Tris-HCl pH8.0, 100mM magnesium chloride, 20mM β-mercaptoethanol, 0.5mM each of dGTP, dTTP and dATP)

3μl [α.^{32}P] dCTP (10mCiml^{-1})

0.5μl T7 DNA polymerase (8.5Uμl^{-1})

The reaction was incubated at 37°C for 4 hours before 2μl of stop mix (0.5M EDTA) were added.

To remove the unincorporated dNTPs the commercially available NucTrap™ Probe Purification Columns (Stratagene) were used. 70μl of 1XSTE (100mM sodium chloride, 20mM Tris-HCl pH7.5, 10mM EDTA) was pushed slowly through the column using a 10ml syringe. 20μl of 1XSTE was added to the prepared probe and the 70μl of the reaction pushed through the column. Finally a further 70μl of 1XSTE was added to the column and pushed through. The eluate from the column was collected in a fresh Eppendorf tube and heated to 95-100°C for 5 minutes before being added to the prehybridised membrane (2.2.4.3). Random labelled probes were typically used at a hybridising temperature of 65°C.

2.2.4.6 Washing and exposing a membrane.

End-labelled oligonucleotide probes.

The probes were poured off and stored at -20°C either for reuse or to allow the radiolabel to decay before safe disposal. 25ml washes of 6XSSC/ 0.1%SDS were added to the filter at 37°C. Usually 2-3 washes of 10 minutes were used. Monitoring of the membrane with a β-geiger counter was used to ensure that the probe was still attached.

Random labelled probes.

The probes were stored as described for end-labelled oligonucleotides. Before reuse these probes required heating to 95-100°C for 5 minutes.
25ml washes at 65°C for 10 minutes each were used. Initial washes were made with 6XSSC/0.1% SDS increasing in stringency to 0.1-0.2XSSC/0.1% SDS over a series of twice repeated washes. Monitoring the membrane with a β-geiger counter defined the background noise to signal ratio and indicated whether a more stringent wash was required.

Washed membranes were carefully sealed in a thin plastic bag and placed in an autoradiographic cassette with an image intensifying screen. The position of the membrane was indicated using a Stratagene fluorescent marker strip. Exposure to preflashed film (2.1.4) was made at -70°C. Exposure was usually overnight but longer exposures for weaker signals were sometimes required. After the appropriate length of exposure the film was developed using a X-OGRAPH X1 (IBI Ltd.) automatic X-ray film processor.

2.2.4.7 Stripping a membrane.

The membrane to be stripped was placed in a hybridising tube, at 65°C, containing 100ml of 0.4M sodium hydroxide. After 30 minutes the sodium hydroxide solution was replaced with 100ml of a neutralising solution (0.1XSSC, 0.1%SDS, 200mM Tris-HCl pH7.5). Two 15 minute incubations, at 65°C, in neutralising solution were carried out. The membrane was exposed overnight to ensure all signals had been stripped.

2.2.4.8 Slot-blots.

The sample DNA was denatured by adding 0.1 volumes of 20mM EDTA/ 2M sodium hydroxide and incubated at 37°C for 15 minutes. The DNA

sample was made up to a 100µl volume by adding TE (pH8.0) and placed on ice until blotting.

Slot-blotting was done using apparatus supplied by Bio-Rad. The Genescreen *Plus* membrane was soaked with 2XSSC and placed in the apparatus on 4 pieces of Whatman (3MM) paper. A vacuum source was attached to the apparatus and 100µl of 2XSSC drawn through each well to be used. The denatured DNA was added to a well and drawn through onto the membrane. After blotting all the samples of denatured DNA the apparatus was taken apart and the membrane left to dry before prehybridisation (2.2.4.3).

2.2.5 Ligations.

2.2.5.1 Phosphatase treatment of restricted vector.

To prevent self-ligation of restricted vector the 5′ phosphates were removed using calf intestinal alkaline phosphatase (CIAP). The restriction endonuclease was removed either by phenol/chloroform extraction (2.2.1.6) or DNA fragment purification (2.2.3.5 or 2.2.3.6) and the restricted DNA ethanol precipitated (2.2.1.7) The DNA was resuspended in 20µl of 1XCIAP buffer (50mM Tris-HCl pH8.5, 1mM EDTA, 1mM magnesium chloride, 1mM zinc chloride) with 1µl of CIAP ($1U\mu l^{-1}$) and incubated at 37°C for 30 minutes. To inactivate CIAP, 2µl of 0.5M EGTA (pH8.0) was added and the reaction incubated at 70°C for 10 minutes. The CIAP was removed by phenol/chloroform extraction and the DNA ethanol precipitated before use.

2.2.5.2 Filling of recessed 3′ termini.

To generate a blunt end from a recessed 3′ terminus the 5′ to 3′ polymerase activity of the Klenow fragment of *E.coli* DNA polymerase I was used. The restricted DNA was ethanol precipitated (2.2.1.7) and taken up in 20µl of sdH_2O to which 4µl of 5X klenow buffer was added (0.2mM of each of dCTP, dGTP, dATP and dTTP, 500mM Tris-HCl pH7.8, 50mM magnesium chloride, 100mM β-mercaptoethanol) and 1µl of klenow

fragment ($1U\mu l^{-1}$). The reaction was incubated at 37°C for 1 hour, phenol/chloroform extracted (2.2.1.6) and the DNA ethanol precipitated.

2.2.5.3 Blunting a recessed 5′ terminus.

To generate a blunt end from a recessed 5′ terminus the 3′-5′ exonuclease activity of either the klenow fragment or T4 DNA polymerase were used. In both cases the restriction buffer supplies all the necessary requirements for enzyme activity. The T4 DNA polymerase is preferred as its 3′-5′ exonuclease activity is much more active on single stranded DNA than that of the klenow fragment.

0.1 volume of a 2mM mix of dCTP, dGTP, dATP and dTTP were added to the restriction followed by 1U of either T4 DNA polymerase or klenow fragment. The klenow fragment reactions were incubated at 37°C for 30 minutes and the T4 DNA polymerase for 15 minutes at 14°C (T4 DNA polymerase 3′-5′ exonuclease activity is equivalent to its 5′-3′ polymerase activity at this temperature, thus preventing the enzyme "nibbling" back too far). The reactions were phenol/chloroform extracted (2.2.1.6) and the DNA ethanol precipitated (2.2.1.7).

2.2.5.4 Ligation reaction.

The vector and insert DNA molecules were restricted and the ends modified if necessary (2.2.5.1 to 2.2.5.3. Typically, 50ng of vector was ligated to insert at a range of molar ratios between 3:1 and 10:1 (insert:vector). Vector and insert DNA were added to 10X ligation buffer (500mM Tris-HCl pH7.5, 100mM magnesium chloride, 100mM DTT, 10mM ATP) with 1U of T4 DNA ligase. Blunt ended ligations were done in a total volume of 10µl and ligations with "sticky" ends in 20µl. Reactions were incubated overnight at 16°C.

2.2.5.5 Cloning of PCR products.

PCR fragments were cloned using the TA™ Cloning Kit (Invitrogen).

1µl of a freshly prepared PCR was added to 2µl of TA vector ($25ng\mu l^{-1}$), 1µl of 10X ligation buffer (60mM Tris-HCl pH7.5, 60mM magnesium chloride, 50mM sodium chloride, $1mgml^{-1}$ BSA, 70mM β-mercaptoethanol, 1mM

ATP, 20mM DTT, 10mM spermidine), 5µl of sdH_2O and 1µl of T4 ligase ($4U\mu l^{-1}$) and incubated overnight at 16°C.

2µl of 0.5M β-mercaptoethanol was added to an aliquot of supplied competent cells (INVα F') and gently mixed. 2µl of the ligation reaction was added, mixed gently, and incubated on ice for 30 minutes. The cells were heat shocked at 42°C for 30 seconds before replacing on ice for 2 minutes. 450µl of room temperature SOC (2% tryptone, 0.5% yeast extract, 10mM sodium chloride, 2.5mM potassium chloride, 10mM magnesium chloride, 10mM magnesium sulphate, 20mM glucose) were added before incubation for 1 hour at 37°C while being agitated by rotation.

25µl and 100µl samples of the transformed cells were plated on LB plates supplemented with $25mgml^{-1}$ of kanamycin (2.2.6.2) and 0.004% X-gal. Plates were incubated, inverted, overnight at 37°C. White and light blue colonies were tested for the presence of the required insert.

2.2.6 Preparation of competent *Escherichia coli* and transformation.

2.2.6.1 Preparation of competent *Escherichia coli* .

A single colony of DH5α was picked into 5ml of LB (2.6.1) and incubated, while shaking, overnight at 37°C. 0.5ml of the overnight culture was used to inoculate 50ml of fresh LB which was then incubated for a further 2 hours at 37°C while shaking. The culture was then chilled on ice for 30 minutes before collection by centrifugation (1500g, 10 minutes, 4°C). The pelleted cells were resuspended in 10ml of ice-cold sterile 100mM magnesium chloride and collected by centrifugation (1500g, 10 minutes, 4°C). The washed pellet was resuspended in 10ml of 100mM calcium chloride and incubated on ice for a minimum of 4 hours. The competent cells were collected by centrifugation (1500g, 10 minutes, 4°C) and resuspended in 2ml of 100mM calcium chloride/14% glycerol. 200µl aliquots of competent cells were stored for up to 6 months at -70°C.

2.2.6.2 Preparation of selective media plates.

Ampicillin at a concentration of $100\mu gml^{-1}$ in LB media was used as a

selective medium for all experiments with DH5α cells.
Initial selection of clones from the TA™ Cloning Kit (2.2.5.5) was made using kanamycin (25μgml^{-1}) as the TA vector contains a gene conferring kanamycin resistance where other vectors present in the PCR reaction as a template do not. Subsequent selection was then made using ampicillin, to which the TA vector also confers resistance, at 100μgml^{-1}.

2.2.6.3 Transformation.
Half of the ligation reaction was added to 200μl of competent cells (2.2.6.1), mixed gently, and incubated on ice for 30 minutes. The cells were heat shocked at 42°C for 30 seconds before replacing on ice for 2 minutes. 450μl of room temperature SOC (2% tryptone, 0.5% yeast extract, 10mM sodium chloride, 2.5mM potassium chloride, 10mM magnesium chloride, 10mM magnesium sulphate, 20mM glucose) were added before incubation for 1 hour at 37°C while being agitated by rotation. Typically 100μl and 300μl of transformed cells were spread on selective media and incubated overnight, inverted, at 37°C.

2.2.6.4 Blue/white colour selection.
The insertion of a DNA fragment into the multiple cloning site (MCS) of the commonly used pBluescriptII SK+ (Strategene) vector during the course of this work was tested using a blue/white colour test. Selective plates were supplemented with 200μM IPTG (1ml of 100mM IPTG stock in sdH_2O added to 500ml of LB agar) and 0.004% X-gal (1ml of 2% stock in DMF added to 500ml of LB agar). White colonies or, in the case of small DNA fragments, light blue colonies, were subsequently selected for further analysis.

2.2.7 Colony screening.

2.2.7.1 Colony lifts.
This protocol was adapted from that described by Buluwela *et al.* (1989). Hybond-N (Amersham) membranes, cut to the correct size, were placed

on the surface of the plates and marked for orientation before being carefully lifted off. The membranes were placed colony side up on blotting paper soaked in 2XSSC/5% SDS for 2 minutes. The membranes were microwaved at full power for 45 seconds, this lyses the cells and denatures and fixes the DNA. The membranes were then available for prehybridisation (2.2.4.3).

2.2.7.2 Patching.

Small numbers of colonies were screened using this protocol for ease of subsequently selecting positive clones. Using autoclaved toothpicks individual colonies were scratched across the surface of a Hybond-N (Amersham) membrane which had been stamped with a 100 square matrix and placed on a selective media plate. The same colony was also scratched on a selective media plate at the same position as indicated from a matrix stuck to the underside of the plate. Both plates were incubated overnight at 37°C. The colonies on the membrane was treated as described in 2.2.7.1.

2.2.8 DNA sequencing and analysis.

2.2.8.1 Sequencing of double-stranded DNA template.

DNA sequence determination was made using the chain termination method described by Sanger *et al.* (1977). The commercially available Sequenase® v.II kit (USB) was used. Sequencing primers were supplied by OSWEL DNA service (Chemistry Dept., University of Edinburgh) or Perkin-Elmer.

As double stranded DNA template was always used, the DNA was denatured before annealing to the oligonucleotide primers. 10µg of template was resuspended in a total volume of 30µl of denaturing solution (200mM sodium hydroxide, 2mM EDTA) and incubated for 30 minutes at 37°C. The template was ethanol precipitated (2.2.1.7) and resuspended in 7µl of sdH_2O.

The 7µl of template was quickly mixed with 2µl of reaction buffer (200mM Tris-HCl pH 7.5, 100mM magnesium chloride, 250mM sodium chloride)

and 1µl of oligonucleotide primer (20ngµl^{-1}). The annealing mix was incubated at 37°C for 15 minutes before the sample was allowed to cool to room temperature.

The labelling reaction consisted of 10µl annealed primer/template, 2µl of dGTP labelling mix (1.5µM of dGTP, dTTP and dCTP), 1µl of 100mM DTT, 0.5µl [α^{35}S] dATP (10mCiml^{-1}) and 2µl of Sequenase® DNA polymerase (1.6 Uµl^{-1}). The labelling reaction was incubated at room temperature for 2-5 minutes before 3.5µl aliquots were removed and added to 2.5µl of each termination mix (ddG, ddA, ddT and ddC) prewarmed to 37°C. Each termination mix consisted of the following; 80µM dGTP, dCTP, dATP and dTTP, 50mM sodium chloride and 8µM of the appropriate dideoxynucleoside (ddGTP, ddCTP, ddATP and ddTTP). To compensate for the A-T bias in the *P.falciparum* genome termination mixes containing ddGTP and ddCTP were diluted 1 in 2 compared to 1 in 8 for ddATP and ddTTP. The termination reactions were incubated at 37°C for 2-5 minutes and then stopped by the addition of ice-cold loading buffer (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and placing the reaction on ice.

2.2.8.2 Sequencing gel electrophoresis.

Sequencing reactions were separated on a 6% polyacrylamide gel (20:1 acrylamide:bisacrylamide) containing 7M urea in 1XTBE (2.6.1) available commercially from Scotlab. 40ml of polyacrylamide mix was polymerised by the addition of 240µl of 10% ammonium persulphate and 24µl TEMED. The gel was cast between a pair of plates (380mm X 170mm) separated by spacers (0.3mm).

2.5µl of each termination mix (2.2.8.1) was heated for 5 minutes at 95-100°C before being quickly cooled on ice. The termination mixes were loaded onto a "sharks tooth" comb, in the order; ddGTP, ddATP, ddTTP and ddCTP. Electrophoresis was carried out at a constant 50 Watts. After the appropriate length of run the gel was fixed by placing blotting paper soaked in fix (10% glacial acid, 12% methanol) on the surface for 10 minutes. The gel was lifted onto Whatman 3MM paper and dried under vacuum at 80°C. Gels were exposed to film (2.1.4) overnight at room

temperature.

2.2.8.3 Sequence analysis.

Storage of sequence, mapping of restriction sites and comparison to other database sequences was made using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package v.7 and v.8 (Devereux *et al.* , 1984).

2.2.9 Polymerase Chain Reaction (PCR).

2.2.9.1 Standard PCR.

A typical PCR reaction (Saiki *et al.*, 1988, Innis *et al.*, 1990) was modified as described below to compensate for the extreme A-T richness of the template DNA. PCR reactions were assembled on ice in 500µl Eppendorf tubes. Extreme care was used to ensure that contaminants were not introduced into the reactions.

100ng template DNA
10µl 10X PCR buffer (200mM, Tris-HCl pH8.4, 500mM potassium chloride)
10µl Primer #1 (20ngµl^{-1})
10µl Primer #2 (20ngµl^{-1})
3µl 50mM magnesium chloride
2.5µl 40mM dNTP (10mM each of dATP, dTTP, dCTP and dGTP)
0.5µl Taq DNA Polymerase (5Uµl^{-1})
sdH_2O to a total of 100µl

The Taq DNA polymerase and buffer were supplied by Gibco-BRL. The reaction was overlaid with 100µl of mineral oil and the lid of the tube punctured before being placed on the thermocycler.

A typical programme for the thermocycler for a pair of 18-mer oligonucleotide primers to a genomic DNA template to amplify a 1kb product is described below. Modifications were made to this basic

programme to allow for the optimisation of each reaction.

1.	95°C for 5 minutes 37°C for 1 minute 72°C for 2 minutes	1 cycle
2.	93°C for 3 minutes 37°C for 1 minute 72°C for 2 minutes	1 cycle
3.	93°C for 1 minute 40°C for 1 minute 72°C for 2 minutes	28 cycles
4.	72°C for 5 minutes	1 cycle

The maximum ramp rate ($1°Csec^{-1}$) between each step was used in all PCR reactions.
10-15µl of each reaction were typically analysed by agarose gel electrophoresis (2.2.3).

2.2.9.2 Reverse Transcription-PCR (RT-PCR).
Reverse transcription from an oligonucleotide hybridised to a mRNA of interest was used to give a first-strand cDNA which was used in a subsequent standard PCR reaction (2.2.9.1).
1µl of an oligonucleotide ($20ng\mu l^{-1}$) was added to 5-10µg of total RNA which had been treated with RNase-free DNase to ensure that no genomic DNA was contaminating the sample. The volume was made up to 20µl with DEPC-treated sdH_2O and incubated at 70°C for 10 minutes. The sample was collected by microcentrifugation and the mix allowed to cool slowly to room temperature. 3µl of 10X reverse transcription buffer (200mM Tris-HCl pH8.4, 500mM potassium chloride and 60mM magnesium chloride), 1µl of mixed dNTPs (2.5mM each of dATP, dTTP, dCTP and dGTP), 3µl of 100mM DTT and DEPC-treated sdH_2O to a total of

30µl were added. The reaction was incubated at 42°C for 2 minutes before the addition of 1µl of Superscript II reverse transcriptase (Gibco-BRL, $8U\mu l^{-1}$). The reaction was incubated for a further 90 minutes at 42°C. Typically 2-4µl of the first-strand cDNA reaction mix was used as template for a standard PCR.

2.2.10 Micrococcal nuclease.

The protocol used was adapted from that described by Cary *et al.* (1994). Approximately 10^9 parasites were cultured and then isolated from the erythrocytes by saponin lysis (2.2.1.1). The isolated parasites were collected by centrifugation (2000g, 10 minutes, 4°) and washed in 1XSSC, 0.1% saponin before being collected by centrifugation. The washed parasite pellet was resuspended in 3ml of hypotonic buffer (10mM Tris-HCl pH8.0, 1.5mM magnesium chloride, 15mM potassium chloride, 2mM PMSF, $2\mu gml^{-1}$ leupeptin, $2\mu gml^{-1}$ pepstatin, $2\mu gml^{-1}$ chymotrypsin, $2\mu gml^{-1}$ antipapain). The parasites were lysed using 20 strokes of a prechilled dounce homogeniser B-type pestle. The nuclei were isolated by sucrose gradient centrifugation after the resulting suspension was layered on 3ml of hypotonic buffer containing 0.34M sucrose (12000g, 3 minute, 4°C, no brakes). The nuclei pellet was resuspended in 1ml of hypotonic buffer supplemented with 2mM calcium chloride.

50U of micrococcal nuclease was added to the nuclei suspension and incubated at 30°C. Samples were removed at time points (0, 1, 3, 5 and 20 minutes). EGTA was added to a final concentration of 10mM before incubating the samples on ice. When all the samples were collected proteinase K was added to a final concentration of $100\mu gml^{-1}$ and incubated at 37°C for 30 minutes. For the final 10 minutes of incubation RNase A was added to a final concentration of $100\mu gml^{-1}$. The DNA was phenol/chloroform extracted (2.2.1.6) and ethanol precipitated (2.2.1.7) before analysis on an agarose gel.

2.3 RNA Methods.

2.3.1 Total RNA isolation.

This protocol was based on the acidic guanidinium-phenol-chloroform protocol described by Chomczynski and Sacchi (1987). *P.falciparum* culture was grown until approximately 10^9 parasites were available when the culture was harvested by centrifugation (2500g, 10 minutes, 4°C). The erythrocyte pellet was resuspended in 5 volumes of 1XSSC, 0.1% saponin, mixed by inversion and incubated at room temperature for 5 minutes. The released parasites were collected by centrifugation (3000g, 10 minutes, 4°C), washed in 1XSSC, 0.1% saponin and collected by centrifugation.
The washed parasite pellet was resuspended 7ml of solution D (4M guanidinium isothiocyanate, 25mM sodium citrate, 0.5% sodium lauryl sarcosine, 100mM β-mercaptoethanol) and repeatedly passed through an 18 gauge needle to completely disrupt the parasite pellet. 0.7ml of 2M sodium acetate (pH4.0), 7ml of water saturated phenol (pH5.0) and 1.4ml of chloroform/isoamyl alcohol (24:1 v:v) were sequentially added and mixed by vortex. The suspension was incubated on ice for 20 minutes before centrifugation (10000g, 20 minutes, 4°C) to separate the phases. The upper aqueous phase was collected and 1 volume of isopropanol added before incubation at -20°C for 1 hour. The RNA was pelleted by centrifugation (12000g, 20 minutes, 4°C) and resuspended in 500µl of TE, 0.1%SDS. A phenol/chloroform extraction (pH5.0, 2.2.1.6) was made on the RNA solution after which 1ml of ice-cold 100% ethanol was added to the aqueous phase. The RNA was pelleted by microcentrifugation for 20 minutes. The RNA pellet was resuspended in 300µl of TE, 0.1%SDS and stored, in 100µg aliquots, at -70°C. The RNA was analysed using UV spectrophotometry (2.2.1.5).

2.3.2 Northern analysis.

Total RNA was fractionated on a 1% agarose/1.85% formaldehyde gel prepared and run in 1XMOPS buffer (25mM MOPS, 5mM sodium acetate, 1mM EDTA, pH7.0). Typically 10-20µg of total RNA, or 8µg of 9.5-1.4Kb RNA markers (Gibco-BRL) were added to an equal volume of

formaldehyde sample buffer (2XMOPS, 50% formamide, 25% formaldehyde, 0.25% bromophenol blue, 0.25% xylene cyanol) and incubated at 65°C for 5 minutes. The sample was cooled quickly on ice and 1µl of ethidium bromide (1mgml^{-1}) added before loading on the gel. The gel was run at 100V for 4 hours using a pump to circulate the 1XMOPS buffer.

On completion of separation of the total RNA the gel was washed for 20 minutes in 1XSSC to remove the formaldehyde, soaked in 50mM sodium hydroxide for 10 minutes then washed for 5 minutes with sdH_2O. A blot was assembled essentially as described for a wet southern blot (2.2.4.2) except that the transfer was made in 10XSSC. The following day the Genescreen *Plus* membrane was washed with 10XSSC before prehybridisation. The hybridisation was made using the conditions for a random labelled probe (2.2.3.4, 2.2.4.6) at 60°C.

2.3.3 Primer extension.

Between 5-10ng of the required oligonucleotide was end-labelled (2.2.3.5) overnight to ensure completion of the labelling. The end-labelled oligonucleotides were ethanol precipitated (2.2.1.7) to remove unincorporated radiolabelled nucleotides. The oligonucleotide pellet was resuspended with 5µg of total RNA in a total volume of 18µl. The mixture was incubated at 70°C for 10 minutes and collected by microcentrifugation for 10 seconds. 2.5µl of 10X extension buffer (200mM Tris-HCl pH8.4, 500mM potassium chloride, 60mM magnesium chloride), 1µl of 10mM dNTP mix (2.5mM of each of dATP, dTTP, dCTP, dGTP) and 2.5µl of 100mM DTT were sequentially added. The reaction was incubated at 42°C for 2 minutes before 1µl of Superscript II Reverse Transcriptase (Gibco-BRL, 8Uµl^{-1}) was added. The reaction was incubated for a further 90 minutes at 42°C.

The reaction was stopped by incubation at 70°C for 10 minutes. This was allowed to cool to 50°C when 1µl of RNase H (1Uµl^{-1}) was added and the reaction incubated for 10 minutes at 37°C. The cDNA was ethanol precipitated and resuspended in 4µl of formaldehyde sample buffer

(2.2.1.3).
The primer extension reactions were run on a denaturing polyacrylamide gel (2.2.8.2) with end-labelled 100bp markers (Gibco-BRL) run alongside.

2.3.4 *In vitro* transcription.

2.3.4.1 *In vitro* transcription reaction.

In order to produce large quantities of full length radiolabelled transcript from an AT-rich template the commercially available Ampliscribe™ transcription kit (Epicentre technologies) was used. *In vitro* transcription was made from a plasmid template that has been linearised by endonuclease restriction. The restriction was made at the 3′ end of the transcribed sequence so that all the transcripts terminate at the same point. The restriction site was either blunt ended or had a 5′ protruding end as 3′ protruding ends act as sites for non-specific transcription initiation.
The following components were mixed in the order described; DEPC-treated sdH_2O to give a total volume of 20µl, 2µl 10X transcription buffer (400mM Tris-HCl pH8.0, 80mM magnesium chloride, 20mM spermidine and 500mM sodium chloride), 1.5µl of each of CTP, ATP, GTP (100mM), 2µl of 100mM DTT, 1µg of linearised template, 0.5 µl of 100mM UTP and 3µl of [α^{32}P] UTP (3000Cimmol^{-1}). 2µl of Ampliscribe™ T7 RNA polymerase (contains an RNase inhibitor) was added and the reaction incubated for 2-3 hours at 37°C. 1µl of RNase-free DNaseI was added at this time and the reaction incubated for a further 20 minutes. The reaction was ethanol precipitated (2.2.1.7) to allow unincorporated nucleotides to be removed in the supernatant. The pellet was resuspended in 5µl of loading buffer (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and run on a denaturing polyacrylamide gel, with end-labelled 100bp markers, to isolate the full-length transcripts.

2.3.4.2 Isolation of *in vitro* transcripts from denaturing polyacrylamide gel.

Typically 70-90% of transcripts prepared from *in vitro* transcription were full length. After separating products on a polyacrylamide gel, this was briefly exposed to film to allow the position of the full length transcripts to be identified. A gel slice containing the full length transcripts was cut out using a clean scalpel and placed in 350µl of elution buffer (0.5M ammonium acetate, 1mM EDTA, 0.2% SDS). The gel slice was incubated at room temperature overnight, while rotating, to allow the transcript to be eluted. The gel slice was pelleted by microcentrifugation for 1 minute. The supernatant was removed and 2.5 volumes of ice-cold 100% ethanol added and the transcripts pelleted by microcentrifugation for 25 minutes. The pellet was washed with ice-cold 70% ethanol and stored at -70°C until use.

2.3.5 RNase protection.

RNase protection was carried out using the commercially available RPA II™ kit (Ambion) using a protocol described by M. Lanzer (*pers comm.*). The radiolabelled transcript was prepared using the protocols described above (2.3.4). The RNA used in the assays was treated with RNase-free DNase I to remove any contaminating genomic DNA.

Freshly prepared radiolabelled transcript was resuspended in 25µl of DEPC-treated sdH_2O and a 3µl aliquot added to an Eppendorf for each of the assays to be carried out plus 3 additional control tubes. To each of the assay tubes 10µg of *P.falciparum* total RNA was added and the volume taken to 20µl with DEPC-treated sdH_2O. To two of the control tubes 10µg of yeast total RNA was added and the volume made up to 20µl as described for the assay tubes. 2µl of 5M ammonium acetate was then added to each of the tubes containing total RNA and mixed completely. 2.5 volumes of ice-cold 100% ethanol was added, mixed by inversion and microcentrifuged for 25 minutes to pellet the RNA. After washing the RNA pellets carefully with ice-cold 70% ethanol they were resuspended in 20µl of hybridisation buffer (80%formamide, 100mM sodium citrate pH6.8, 300mM sodium acetate pH6.4, 1mM EDTA). The samples were heated to 90-95°C for 5 minutes and incubated overnight at 30°C.

The incubated samples were microcentrifuged briefly to collect the condensation. 200µl of diluted RNase(s) were added to one tube containing the yeast total RNA and all the assay tubes. Two RNase concentrated mixes were available; RNase A/T1 (250Uml^{-1} RNase A and 10,000 Uml^{-1} RNase T1) or RNase T1 (5000Uml^{-1}). These were diluted appropriately into 200µl of dilution buffer, usually 1:250 or 1:100. The reactions were incubated at 37°C or room temperature for 30 minutes before 300µl of termination mix were added to all tubes containing total RNA. The reactions were incubated at -20°C for 30 minutes before microcentrifugation for 25 minutes to pellet the RNA. The supernatant was removed and the pellet carefully washed with ice-cold 70% ethanol. 5µl of loading buffer (95% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 0.5mM EDTA, 0.025% SDS) were added to each of the 3 control tubes and the *P.falciparum* assay tubes. The samples along with end-labelled 100bp markers (Gibco-BRL) were loaded onto a denaturing polyacrylamide gel and run as previously described (2.2.8.2). The gel was dried under vacuum at 80°C on to a Whatman 3MM blotting paper support and exposed to film overnight.

2.3.6 Nuclear run on analysis.

This protocol was adapted from that described by Sultan (1995) and originally that of Marzluff and Huang (1989). All steps were carried out on ice to help preserve nuclear structure and RNA polymerase activity.
P.falciparum culture was grown until approximately 10^9 parasites were available. The parasite culture was harvested by centrifugation (2000g, 10 minutes, 4°C). The erythrocyte pellet was washed with 5 volumes of PBS and again collected by centrifugation (2000g, 10 minutes, 4°C). 5 volumes of ice-cold 1XSSC, 0.1% saponin were added to the pellets followed by incubation on ice for 10 minutes. The released parasites were collected by centrifugation (2000g, 10 minutes, 4°C) and washed by resuspension in 3ml of buffer E (250mM sucrose, 20mM PIPES 6.3, 0.5mM calcium chloride, 1mM PMSF, 2µgml^{-1} leupeptin, 2µgml^{-1} apotinin, 2µgml^{-1}

pepstatin A, 2μgml^{-1} antipain, 0.1mM benzamidine, 0.1mM sodium metabisulphite). The washed parasites were collected by centrifugation (3000g, 10 minutes, 4°C) and the wash with buffer E was repeated twice.
The washed parasites were finally resuspended in 3ml of buffer E supplemented with 200μl of 10% NP-40 and transferred into a prechilled dounce homogeniser. Using 15-20 strokes of a B-type pestle the parasites were broken open. The intact nuclei were collected by centrifugation (12000g, 10 minutes, 4°C) and then washed three times as previously described using 3ml of buffer E.
The nuclear pellet was resuspended in 600μl of buffer B (50mM HEPES pH7.9, 50mM sodium chloride, 10mM magnesium chloride, 1.2mM DTT, 10mM creatine phosphate, 1mM GTP, 1mM CTP, 4mM ATP, 25% glycerol, 3U RNase inhibitor [Inhibitace], 0.2mgml^{-1} creatine kinase, 1μM [α.^{32}P] UTP [3000 Cimmol^{-1}]) and incubated for 20 minutes at 37°C.
RNA from the nuclei was isolated using the standard total RNA isolation protocol (2.3.1.1). The RNA pellet, containing the nascent radiolabelled transcripts, was resuspended in 5ml of random label hybridisation buffer (2.2.3.4). The probe was added to a prehybridised membrane on which 1μg of denatured gene probe had been immobilised. Hybridisations for nuclear run on analysis were carried out at 55°C overnight. 5μl of 10mgml^{-1} RNase A was added to the first wash in 6XSSC/0.1% SDS (2.2.4.6).

2.4 Protein methods.

2.4.1 SDS-polyacylamide gel electrophoresis (SDS-PAGE).

2.4.1.1 Preparation and running SDS-PAGE.

Protein extracts were fractionated by SDS-PAGE using a discontinuous buffer system. Atto gel rigs (GRI Instruments) 150mm x 150mm x 1mm and Mighty Small II rigs (Hoefer) 80mm x 70mm x 0.75mm were used. The gel mixes were prepared as shown in table 3. Large gels were cast with a 130mm resolving gel and a 20mm stacking gel while small gels were cast

with a 50mm resolving gel and a 10mm stacking gel.
The resolving gel was poured and overlaid with a small volume of water-saturated butanol. The stacking gel was allowed to set for an hour before pouring off the butanol and washing the top with sdH_2O. The stacking gel was poured off and the comb positioned before leaving the stacking gel to set. The comb was removed and the wells rinsed with sdH_2O before use.

Table 3. SDS-PAGE resolving and stacking gel mixes (volumes sufficient for 2 large gels or 5 small gels, in ml).

Component	Resolving gel		Stacking gel
	8%	10%	
Acrylamide/ bis acrylamide (30:2.67)	16	20	2.6
sdH_2O	28.7	24.7	12.3
1.5M Tris-HCl pH8.8	15	15	-
0.5M Tris-HCl pH6.8	-	-	5
20% SDS	0.3	0.3	-
10% Ammonium persulphate	0.3	0.3	0.1
TEMED	0.03	0.03	0.018

Protein samples were added to an equal volume of 2x sample buffer (125mM Tris-HCl pH6.8, 4%SDS, 20% glycerol and 10% β-mercaptoethanol, 0.25% bromophenol blue) and heated to 95-100°C for 3 minutes. Gels were run in running buffer (25mM Tris, 192mM glycine, 1% SDS) at 20mA/gel and approximately 100V. Small gels were run for approximately 40-60 minutes and large gels for 3.5 hours, usually until the blue dye reached the bottom of the gel.

2.4.1.2 Protein molecular weight markers.
Two types of protein markers were used; SDS-6H markers (Sigma) for gels which were stained with coomassie brilliant blue or, if the gels were to be western blotted, prestained broad range markers (New England Biolabs).

10µl of SDS-6H markers in a sample buffer (0.0625M Trizma-HCl pH6.75, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) were loaded on a minigel rig, 20µl were used for the larger Attorigs. The range of markers included were; 205kDa, 116kDa, 97.4kDa, 66kDa, 45kDa and 29kDa.
5µl of the prestained markers were added to 10µl of sample buffer (187.5mM Tris-HCl pH6.8, 50mM sodium chloride, 1mM EDTA, 1mM sodium azide, 125mM dithiothreitol) and heated at 95-100°C for 60 seconds before loading on a minigel. For a larger gel the volumes were doubled. The range of markers included were; 175kDa, 85kDa, 62kDa, 47.5kDa, 32.5kDa, 25kDa, 16.5kDa and 6.5kDa.

2.4.1.3 Coomassie brilliant blue staining (fixing).
In order to visualise the separated proteins a gel was submerged in coomassie fixing stain (0.25% w/v coomassie brilliant blue R-250 in water: methanol: glacial acetic acid, 5:5:1 v:v:v) for 30 minutes at 37°C. The coomassie fixing stain was poured off and replaced with destain (water: methanol: glacial acetic acid, 5:5:1 v:v:v). The destain was replaced every 30 minutes until the background was clear. The gels were dried, under a vacuum, against Whatman 3MM blotting paper at 80°C.

2.4.2 Glutathione-S-transferase (GST) expression and harvesting.

pGEX1, containing the *Schistosoma japonicum* glutathione-S-transferase open reading frame, was transformed (2.2.6.3) into the *E.coli* strain BL21 (DE3) pLysS and grown overnight at 37°C while shaking in 50ml of LB supplemented with 100µgml^{-1} ampicillin. 50ml of pGEX1 culture was added to 500ml of LB supplemented with 100µgml^{-1} ampicillin and grown at 37°C while shaking for 1 hour. A 1ml sample was removed before induction with 0.5ml of 100mM IPTG (final concentration of 0.1mM). The culture was shaken for a further 3 hours, at 37°C, before pelleting the culture by centrifugation (2000g, 10 minutes, 4°C).
The bacterial pellet was resuspended in 5ml of ice-cold PBS. The resuspended culture was lysed, by sonication (5mm diameter probe) for 20

seconds on ice, until the suspension was coloured a dull grey-brown. 0.5ml of 10% Triton X-100 was added to the lysed culture and gently mixed by inversion. The culture was divided into 5 x 1ml aliquots and microcentrifuged (9500rpm) for 5 minutes to pellet the cell debris. The supernatant, containing the GST, was removed and stored in 0.5ml aliquots at -20C.

2.4.3 Preabsorption of immune serum.

2.4.3.1 Preparation of glutathione-sepharose beads.

500mg of glutathione-sepharose beads were washed 4 times in 3.5ml of ice-cold PBS to remove the lactose stabiliser. The beads were allowed to settle between washes rather than pelleting by centrifugation. Brief centrifugation (3500g, 2 minutes, 4°C) was, however, used after the final wash to ensure all the beads were pelleted.

An equal volume (approximately 500µl) of PBS was used to resuspend the beads, giving a 50% slurry. The slurry could be stored, at 4°C, for up to a week before use.

2.4.3.2 Absorption of GST onto glutathione-sepharose beads.

500µl of a 50% slurry of glutathione-sepharose beads will absorb up to 2mg of GST. The concentration of the GST in a sample was estimated on a coomassie stained 10% SDS-PAGE (2.4.1.3) by comparison of various volumes of GST supernatant (2.4.3.1) to known quantities of the 30kDa protein marker.

The appropriate volume of GST supernatant was added to the bead slurry and incubated on ice, with frequent shaking, for 30 minutes. The GST-slurry was microcentrifuged to pellet the beads, and the supernatant removed. The beads were washed 4 times with 0.5ml of ice cold PBS and microcentrifuged in between washes to pellet the beads. The beads were finally resuspended in 0.5ml of ice cold PBS to give a 50% GST-bead slurry.

Samples of beads, supernatant and washes were run on a 10% SDS-PAGE. This ensures that the beads contain GST, the supernatant still contains

GST (indicating the beads were saturated) and the washes eventually contain no GST (as all free GST was washed from the beads).

2.4.3.3 Absorption of anti-GST antibodies from immune serum.

5µl of final bleed antisera was added to 100µl of 50% GST-bead slurry and incubated at room temperature while shaking gently for 30 minutes. The sample was microcentrifuged (9500g) for 5 minutes to completely pellet the GST-beads. The supernatant was carefully removed ensuring that no beads were removed. The anti-GST depleted antiserum (1:10 dilution) was then available for use.

2.4.4 Preparation of total protein from *P.falciparum*.

Approximately 10^9 parasites were cultured and released from erythrocytes by saponin lysis (2.2.1.1). The parasites were collected by centrifugation (2000g, 10 minutes, 4°C) and the pellet washed in 5 volumes of ice cold PBS to remove erythrocyte debris. The washed parasites were collected by centrifugation (3500g, 5 minutes, 4°C). The parasite pellet was lysed by the addition of an equal volume of sdH_2O followed by an equal volume of 2x loading buffer (2.4.1.1). The lysed parasites were passed through a 25 guage needle to break up chromosomal DNA. The parasite preparation was stored until use at -70°C. 15µl of parasite preparation was heated at 95-100°C for 3 to 5 minutes before loading on a polyacrylamide gel (2.4.1).

2.4.5 Indirect immunofluorescence assay (IFA).

Thin blood smears were prepared from culture with a parasitaemia of between 8-12%, dried overnight at room temperature and stored at -20°C in a bag with silica gel.

Slides were fixed in acetone for 5 minutes and allowed to dry at room temperature then divided using nail varnish. On to each section, 40µl of the appropriate first antibodies diluted in PBS was added. The antibodies were incubated, in a moist chamber, for 1 hour. The antibodies were aspirated and the slide washed twice with PBS and once with sdH_2O for 5

minutes each. The slide was then left to dry completely at room temperature. 40µl of a 1:80 dilution of rhodamine isothiocyanate conjugate of immunosorbant-purified anti-rabbit immunoglobin (RITC-anti IgG, Sigma) containing 5µgml^{-1} DAPI in PBS was added to each grid. The slides were incubated, washed and dried as previously described for the first antibody.
The slide was mounted in Citifluor (City University, London) and examined by fluorescence microscopy using a RITC filter set (Leitz).

2.4.6 Western blot analysis.

Western blot were done using the method of Towbin *et al.* (1979) using a Hybond-C membrane (Amersham). The SDS-PAGE gel was assembled into a sandwich in a blotting cassette; Scotchbrite pad, 3 sheets of blotting paper (cut to the size of the gel), the gel, hybond-C membrane (cut to the size of the filter and carefully placed on the gel), 3 sheets of blotting paper and a second Scotchbrite pad. The blotting cassette was submerged in a transfer chamber (Biorad) containing transfer buffer (25mM Tris, 150mM glycine, 20% methanol, 0.1% SDS). The gel was blotted at 40mA and approximately 100V for 4 hours. The filter was carefully removed from the sandwich and submerged in blocking solution (1xTS [150mM sodium chloride, 10mM Tris-HCl pH7.4] and 5% fat-free milk powder) for 1 hour. The filter was allowed to dry completely overnight at room temperature.
The filter was subsequently submerged in blocking solution containing the appropriate dilution of the first antibody while being gently shaken at room temperature for 2 hours. The filter was washed once with 1xTS, twice with 1xTS/0.05% NP-40 and once with 1xTS for 5 minutes each while gently shaking.
The filter was then submerged in blocking solution containing a 1:7500 dilution of alkaline phosphatase conjugated to immunosorbant-purified anti-rabbit immunoglobin (AP-anti IgG, Promega). This was incubated at room temperature for 2 hours while gently shaking. The filter was then washed as described for the first antibody.
5ml of alkaline phosphatase buffer (100mM Tris-HCl pH9.5, 100mM

sodium chloride, 5mM magnesium chloride) containing 33µl of NBT (nitro-blue tetrazolium) and 16.5µl BCIP (5-brom-4-chloro-3-indolyl phosphate) was added to the filter. The filter was shaken gently at room temperature while the signal developed, for a maximum of 5 minutes. When the signal had developed to a suitable extent the reaction was stopped by adding an excess of sdH_2O. The filters were then washed with sdH_2O and left to dry on blotting paper.

2.5 Parasite culture and transfection methods.

2.5.1 Parasite culture.

2.5.1.1 Parasite source.

The K1 isolate of *Plasmodium falciparum* (Thiathong and Beale, 1981) was used during the course of this work. Stocks were either from samples stored at -70°C at ICMB or from the WHO Registry of Standard Strains of Malaria Parasites held at the Centre for Parasite Biology, University of Edinburgh.

2.5.1.2 Human erythrocytes and serum.

The erythrocytes and serum used in this work was obtained from the Edinburgh and Southeast Scotland Blood Transfusion Service. Fresh whole blood, group O Rh +ve and pooled serum was used in parasite culture.

Whole blood was washed 3 times in 4 volumes of incomplete RPMI 1640 medium (Gibco-BRL), to remove the citrate stabiliser, with centrifugation (1500g, 10 minutes, 4°C) between washes. The "buffy coat" of white blood cells was removed after each wash step. The erythrocyte pellet was resuspended in incomplete RPMI1640 medium to give a 66% haematocrit. The washed blood was stored at 4°C for up to 2 weeks.

Human serum packs were pooled, usually 6 to 10 packs, and centrifuged (2000g, 10 minutes, 4°C) to pellet any blood cells. The serum was removed and incubated at 56°C for 1 hour. 50ml aliquots were stored at -20°C for up to 6 months.

2.5.1.3 *P.falciparum* intraerythrocytic stage culture.

This protocol was originally described by Trager and Jensen (1976) and Zolg *et al.* (1982) described a range of conditions which improved parasite yields. Cultures were maintained in 50ml of complete medium (RPMI1640 medium, 10% processed human serum, 50μgml^{-1} gentamycin sulphate, 50μgml^{-1} hypoxanthine) at a 3-5% haematocrit. The average percentage parasitaemia, as determined using methanol fixed thin blood smears stained with 10% giemsa, was usually kept at 1-2%. Cultures were maintained at 37°C and gassed with 3%O_2, 2CO_2 and 95% N_2.

When large numbers of parasites were required the average percentage parasitaemia would be allowed to increase up to 10%. To maintain a healthy culture the numbers of flasks were increased rather than increase the haematocrit within a small number of flasks.

2.5.1.4 Synchronisation of intraerythrocytic stage cultures.

Sorbitol treatment of intraerythrocytic stages of the parasite to obtain a synchronous culture of ring forms was made using a protocol adapted from that described by Lambros and Vandenberg (1979).

A culture with an average percentage parasitaemia of 5% containing predominantly ring forms was used as the starting material. The culture was centrifuged (2000g, 10 minutes, 4°C) and the erythrocyte pellet resuspended in 5 volumes of sterile 5% sorbitol. The culture was incubated at room temperature for 5 minutes and the culture collected by centrifugation (2000g, 10 minutes, 4°C). The erythrocyte pellet was washed in 5 volumes of complete medium (2.4.1.3), collected by centrifugation (2000g, 10 minutes, 4°c) and resuspended in complete medium at a 5% haematocrit. The culture was gassed then incubated at 37°C. The culture was examined after 48 hours, and if necessary the protocol repeated to ensure tight synchrony.

2.5.2 Transfection of intraerythrocytic stages.

2.5.2.1 Electroporation of intraerythrocytic stage cultures.

Electroporation of intraerythrocytic stage cultures was first described by Wu *et al.* (1995). This protocol has been adapted from her original method.

Either synchronised or asynchronous cultures were used, which were typically at a 5% haematocrit and with an 8-12% parasitaemia. The culture was harvested by centrifugation (2000g, 10 minutes, 4°C) and washed in 5 volumes of ice cold PBS. The culture was collected by centrifugation (2000g, 10 minutes, 4°C) and 200µl of pelleted blood resuspended in 800µl of cytomix (van den Hoff *et al.* (1992) 120mM potassium chloride, 0.15mM calcium chloride, 2mM EGTA, 5mM magnesium chloride, 25mM HEPES, 10mM potassium phosphate pH7.6.) containing 60µg of plasmid to be transfected.

Electroporations were made using a Bio-Rad Gene Pulser with a pulse control unit. Conditions were typically set at 2.1kVcm^{-1}, 200Ω serial resistance and 25µF capacitance using 0.4cm cuvettes. These conditions gave time constants of 0.9 +/- 0.1 sec^{-1}. Immediately after electroporation samples were pipetted into 25ml flasks containing 10ml of prewarmed complete medium (2.4.1.3). Samples were gassed (2.4.1.3) before incubation at 37°C. The medium was replaced after 24 hours.

2.5.2.2 Luciferase Assay.

The commercially available Luciferase Assay System kit (Promega) was used. All solutions were supplied with the kit and the protocol was adapted from that described by Goonewardene *et al.* (1993).

Luciferase assays were done 48 hours after the electroporation using the parasites from two flasks which had been electroporated with the same construct. The cultures from the flasks were pelleted by centrifugation (2000g, 10 minutes, 4°C) and the erythrocytes lysed in 5 volumes of 1XSSC, 0.1% saponin (2.2.1.1). The released parasites were collected by centrifugation (2000g, 10 minutes, 4°C) and washed in 5 volumes of PBS before collection by centrifugation (2000g, 10 minutes, 4°C). The washed parasite pellet was resuspended in room temperature lysis buffer (25mM Tris-phosphate pH7.8, 2mM DTT, 2mM 1,2-diaminocyclohexane-N, N, N', N'-tetracetic acid, 10% glycerol, 1% Triton X-100). Where indicated the

lysis buffer was supplemented with a 1/25 volume of protease inhibitor cocktail in 1XPBS (Complete™, Boehringer Mannheim). The parasites were disrupted using 3 cycles of freezing on dry ice and thawing at 37°C before pelleting the cell debris by microcentrifugation for 1 minute. The lysate was quickly added to 100µl of luciferase assay reagent (20mM tricine, 1.07mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 magnesium sulphate, 0.1mM EDTA, 33.3mM DTT, 270µM coenzyme A, 470µM luciferin, 530µM ATP, pH7.8). The light produced by the samples was measured at 25°C using a Hamamatsu Argus-50 Image Processor (Hamamatsu Photonics K.K.) controlled by the Argus Photon Counter Programme v3.0. Sampling was for 10 minutes at the photon camera's maximum sensitivity.

Chapter 3.

Summary.

Antisera raised against PfPCNA and PfDNA polymerase δ (PfPolδ) have been used against extracts from synchronised parasites to show that both proteins accumulate in trophozoites and persist in schizonts. The steady-state transcripts from both PfPCNA and PfPolδ accumulate in the trophozoite stage. In contrast to these results, nuclear run on analysis suggests that, whereas PfPolδ promoter activity is absent in rings but present in trophozoites and schizonts, the PfPCNA promoter is active throughout the intraerythrocytic cycle. This suggests that mechanisms regulating the expression of these two genes must be different although their coordinated activity is required for DNA synthesis.

The construction of the pGEX1/δ6 construct and raising of anti-PfPolδ serum was done by Mandy Jackson supervised by Sandie Cheesman and John White. The anti-PfPolδ serum was raised by Jennifer Daub and John White.

3.1 Introduction.

Chromosomal DNA replication in *Plasmodium* takes place at 5 points during the parasite's life cycle (White and Kilbey, 1996). During the intraerythrocytic cycle of *P.falciparum* DNA synthesis was shown to start in mid-trophozoites and continue into the schizont stages. In *P.falciparum* there are up to four rounds of nuclear division which result in a schizont containing 16 nuclei (Tilney and Tilney, 1996). Cytokinesis divides the cytoplasm up between the 16 nuclei which then undergo merogony forming merozoites which are released when the erythrocyte bursts .

A number of genes encoding replication proteins of *P.falciparum* have now been isolated and characterised, including the two major DNA polymerases and PfPCNA (White *et al.*, 1993, Ridley *et al.*, 1991, Fox and Bzik, 1991, Kilbey *et al.*, 1993). Little is known about the expression of these genes during the complex life cycle of this parasite, what work has

been done has focussed on the cultured intraeythrocytic stages of development. Jaikaria *et al.* (1993) have shown that PfDNA polymerase α transcripts are absent in ring stage parasites but accumulate in trophozoites and schizonts. There is no evidence concerning the levels of the enzyme itself. Indirect immunofluorescence assays with antisera raised against PfPCNA show that the protein starts to accumulate in the mid-trophozoite stage and persists in schizonts, but there is no data available regarding stage specific transcript accumulation (Kilbey *et al.*, 1993). There is no reliable data on PfPolδ expression. Here we report a more complete picture of the regulation of PfPCNA and PfPolδ which, by analogy to the SV40 system, are assumed to cooperate during DNA synthesis in the intraerythrocytic cycle.

3.2 PfPCNA and PfPolδ expression is stage specific during the intraerythrocytic cycle.

3.2.1 Initial screening of anti-PfPolδ serum.

A 31 amino acid region from the murine DNA polymerase δ sequence has been shown to be highly immunogenic (Cullman *et al.*, 1993). The nucleotides encoding this region in the PfPolδ sequence were identified, amplified by PCR and expressed as a 58.5kDa GST fusion protein (pGEX1/δ6). The antiserum obtained following immunisation with this fusion protein detected both the antigenic fusion protein and the 120kDa full length recombinant PfPolδ after the GST moiety had been removed from the fusion protein using factor Xa (Figure 1). An expected band of 120kDa was also detected when the anti-PfPolδ antiserum was used with parasite extract from an asynchronous culture (Figure 1). The pre-immune serum failed to detect a band of this size from either western blot.

3.2.2 Indirect immunofluorescence assays (IFA) with anti-DNA Polδ serum were inconclusive.

IFA (2.4.5) were made on thin film blood smears from an asynchronous culture at a parasitaemia of approximately 12%. The anti-PfPolδ serum was used at dilutions of 1:80 and 1:200. The results of the analysis proved inconclusive as the intensity of the fluorescence from neither dilution of anti-PfPolδ was significantly greater than that from a 1:80 dilution of the pre-bleed antiserum.

As IFA with the anti-Polδ serum was unable to give any indication of the stage specific nature of PfPolδ expression it was decided to use western analysis with extracts from synchronised intraerythrocytic culture.

3.2.3 Stage specific western analysis.

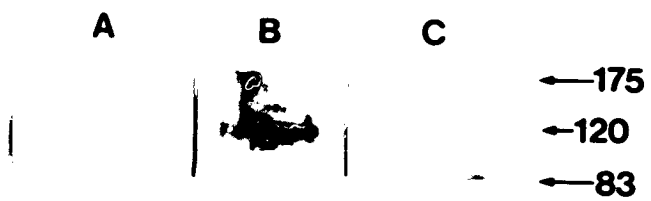
Intraerythrocytic cultures were synchronised by sorbitol treatment (2.5.1.4), an initial treatment was followed 48 hours later by a second to ensure synchrony. The cultures were divided into 3 parts (each of $2X10^9$ parasites) and harvested 4h (rings), 20h (trophozoites) and 28h (schizonts) after the second sorbitol treatment (thin blood smears, stained with giemsa, were taken before each harvest to determine the degree of synchronisation). Equal amounts of each parasite extract (2.4.4) were size fractionated together with prestained size markers (Biolabs) on two 8% acrylamide gels by SDS-PAGE (2.4.1). The gels were then western blotted (2.4.6) to cellulose membranes. The membranes were probed with a 1:1000 dilution of either anti-PfPCNA (Kilbey *et al.*, 1993) or anti-PfPolδ serum (Figure 1).

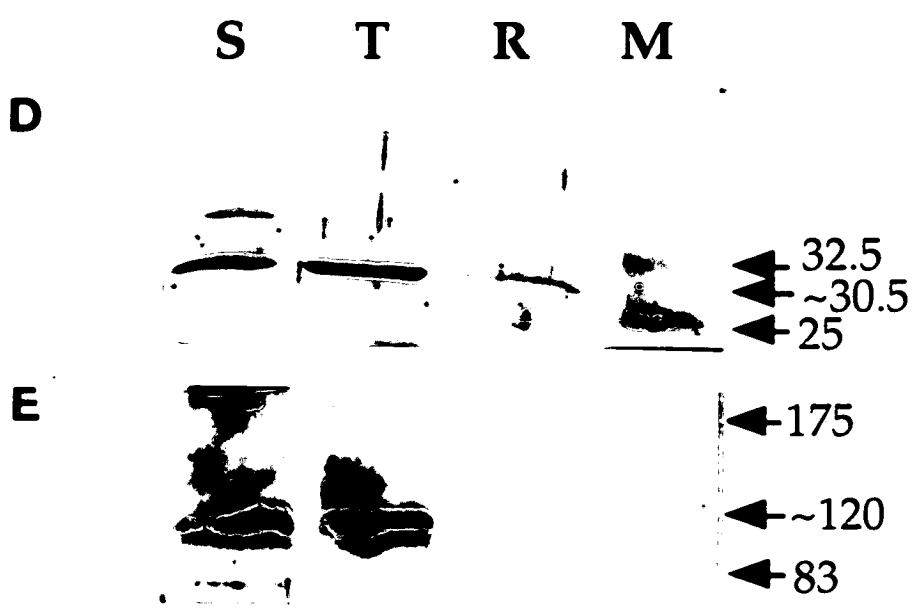
The results from the anti-PfPCNA serum agree with the data previously obtained by indirect immunofluorescence assays. A band of the predicted size, of approximately 30.5kDa, can be just detected in rings but protein levels increase dramatically in trophozoites and schizonts. The 120kDa band detected by the anti-PfPolδ serum follows a similar pattern of accumulation to PfPCNA.

The faint signals observed with both antisera in ring stage extracts are probably the result of trophozoite contamination of this stage (12%) or perhaps a low level of expression of both these proteins.

Figure 1.

Analysis of the anti-PfPolδ serum. Western blots of (A) total parasite extract with pre-bleed serum, (B) total parasite extract with anti-PfPolδ serum and (C) recombinant PfPolδ with anti-PfPolδ serum.
Western analysis of parasite extract from synchronised intraerythrocytic stage cultures using (D) anti-PfPCNA serum and (E) anti-PfDNA Polδ serum. The percentage of each stage (ring: trophozoite: schizont) in a sample were; R, rings (88:12:0), T, trophozoites (5:87:8) and S, schizonts (3:26:71). M, prestained markers (Biolabs) indicted in kDa.





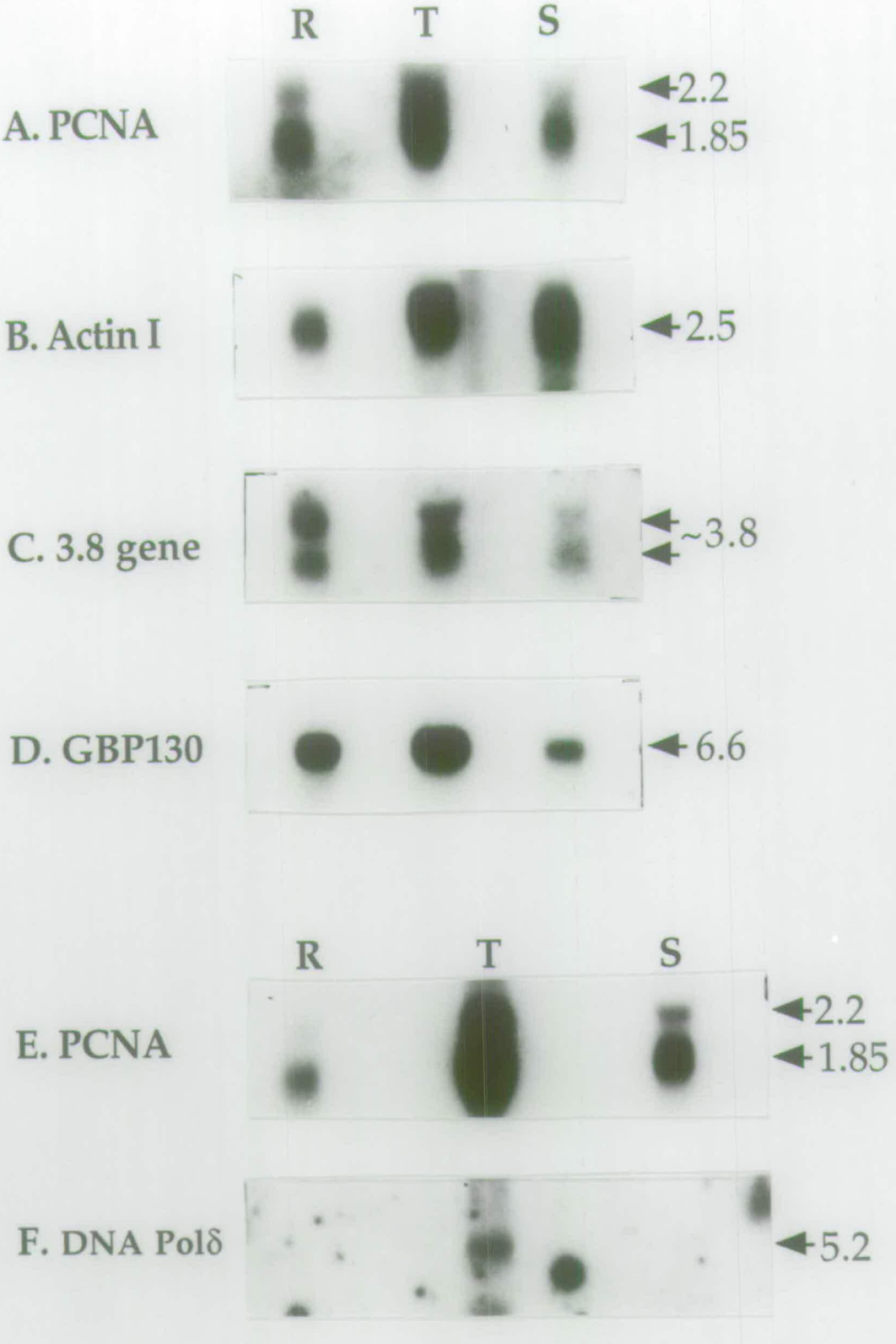
3.3 PfPolδ and PfPCNA transcripts accumulate in trophozoites.

Intraerythrocytic cultures were synchronised by sorbitol treatment and harvested as described above. The synchrony of the harvested samples was checked on giemsa stained thin blood smears. 15μg of total RNA from each major developmental stage was isolated (2.3.1) and size fractionated on a formaldehyde agarose gel together with RNA markers supplied by Gibco-BRL (2.3.2). The gel was blotted to Genescreen plus membrane and probed with gene specific probes (Figure2).

The PfPCNA transcripts, sized at 1.85kb and 2.2kb, are apparently present in all stages but accumulate significantly in the trophozoite stage. Microscopic examination of the samples reveal a contamination of both the ring and schizont stages by trophozoites, this is the result of the difficulty in achieving complete synchrony with large volumes of culture. The membrane was probed using a series of probes, specific to different stages of the intraerythrocytic cycle, to indicate whether the signals obtained from the PfPCNA probe in the ring and schizont samples were due to trophozoite contamination. The Actin I transcript, 2.5kb, accumulates only in the trophozoite and schizont samples (Wesseling *et al.*, 1989). The 3.8 gene transcript, 3.8kb, accumulates in rings and trophozoites (Lanzer *et al.*, 1992b). The GBP130 gene probe a 6.6kb transcript, which only accumulates in trophozoites (Lanzer *et al.*, 1992b), is seen to follow the same pattern of accumulation as PfPCNA. Using a second northern blot where the trophozoite contamination of the ring sample was substantially reduced the PfPCNA signal was also proportionately reduced. The data suggest that the PfPCNA transcript accumulates mainly in the trophozoite stage, although low levels of transcripts may be present in rings and schizonts. The PfDNA Polδ transcript of 5.2kb only accumulates in the trophozoite stage.

Figure 2.

Northern analysis of *P.falciparum* synchronised intraerythrocytic stage culture. The transcript detected, and its size in kb, are indicated. The percentage of each stage (ring: trophozoite: schizont), for A-D, sampled were: R, rings (79:20:1), T, trophozoites (5:81:14) and S, schizonts (4:12:84). The percentage of each stage (ring: trophozoite: schizont) for E and F: R, rings (87:13:0), T, trophozoites (6:85:9) and S, schizonts (0:28:72).


R
T
S
A. PCNA
2.2
1.85
B. Actin I
2.5
C. 3.8 gene
~3.8
D. GBP130
6.6
R
T
S
E. PCNA
2.2
1.85
F. DNA Polδ
5.2

3.4 PfPCNA and PfPolδ promoter activity during the intraerythrocytic cycle differ.

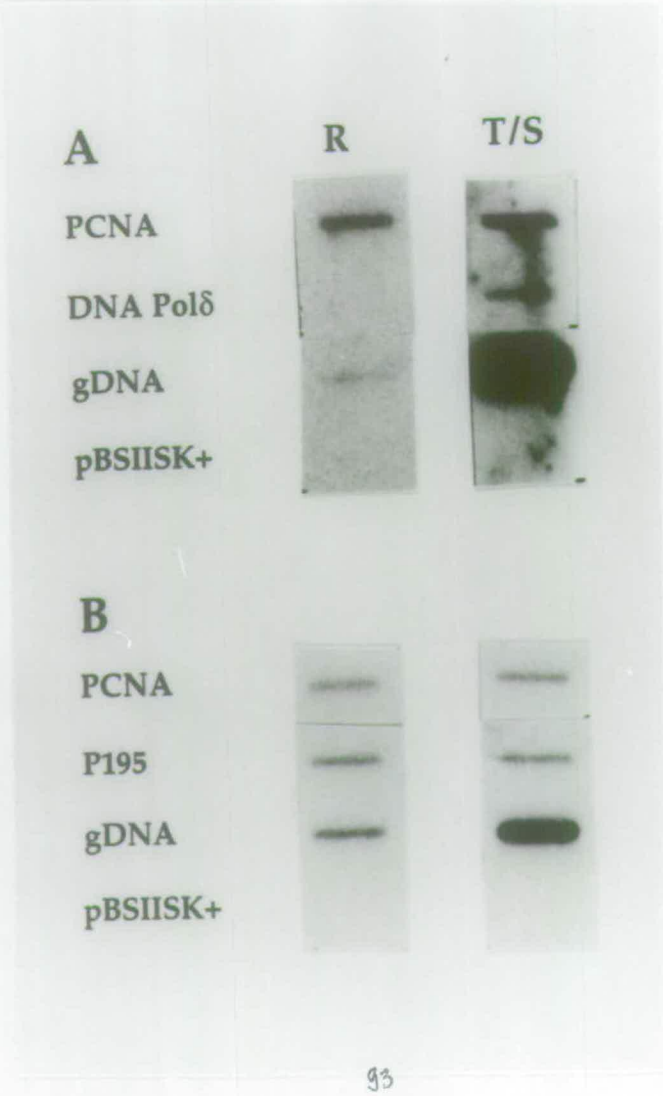
Intraerythrocytic cultures were synchronised as described in 3.2.3. The cultures were divided into 2, the first was harvested 6h (ring) and the second 24h (trophozoites and schizonts) after the second sorbitol treatment. Thin blood smears, stained with giemsa, were taken before each harvest to determine the degree of synchronisation. Nuclei harvested ($5X10^9$) from the cultures at either time point were allowed to continue transcription in the presence of radiolabelled UTP. The radiolabelled nascent RNAs were isolated and hybridised to a membrane on to which approximately 1μg of single stranded PCR products of genes of interest had been immobilised. The PCR products were all approximately 1kb in size (Figure 3).

The PfPolδ promoter did not appear to be active in the ring stages but activity was observed in the trophozoite and schizont nuclei preparation. In contrast the PfPCNA promoter appeared to be active throughout the intraerythrocytic cycle. Although equal amounts of nuclear RNA from the synchronised cultures were used in hybridisations, the experiment was repeated with a 1.2kb MSP-1 probe (P195) as an internal control. The promoter of the MSP-1 gene appears to be active throughout the intraerythrocytic cycle (Holder *et al.*, 1985, Lanzer *et al.*, 1992a). The results confirm that the PfPCNA promoter is active throughout the intraerythrocytic cycle.

1μg of *P.falciparum* genomic DNA had been immobilised to the membranes to demonstrate that the overall transcriptional activity of the latter stages of the intraerythrocytic cycle are greater than that of rings. 1μg of the cloning vector, pBluescriptII SK+ (Stratagene), was used as a negative control.

Figure 3.

Nuclear run on analysis of *P.falciparum* synchronised intraerythrocytic stage culture. (A) Initial comparison of PfPCNA and Pf DNA Polδ. (B) Confirmation of PfPCNA with MSP-1 (P195). The percentage of each stage (ring: trophozoite: schizont) sampled were; R, rings (91:9:0), T/S, trophozoites/ schizonts (2:62:34). pBSII SK+, pBluescriptII SK+.



3.5 Discussion.

We have shown that PfPCNA and PfPolδ protein levels increase from very low levels in rings to high levels in trophozoites and schizonts. The transcript levels of both PfPCNA and PfPolδ show a similar increase as the parasite develops into a trophozoite, but these levels substantially decrease in the schizonts. In contrast to this, promoter activity for these two genes behaves differently. There is no detectable PfPolδ promoter activity in the ring sample but a dramatic increase is observed in the trophozoites/schizont sample. The promoter for PfPCNA, in contrast, remains active in both the ring and trophozoite/schizont samples. This suggests that although transcript and protein levels increase in parallel in preparation for DNA synthesis, the mechanism by which this is brought about is different for PfPCNA and PfPolδ. The parallel increase in transcript level and promoter activity for PfPolδ suggests that message levels are regulated at the level of transcription initiation, whereas PfPCNA transcript levels must be regulated post-transcriptionally. How the transcript is post-transcriptionally regulated will be examined further. One interesting observation from these studies is that the stage specific western data indicates that both the PfPCNA and PfPolδ protein are found at approximately the same level in trophozoites and schizonts even though the transcripts are probably only present in trophozoites. Presumably sufficient levels of polypeptide must be translated, while the transcripts are present in trophozoites, to complete schizogony. The mechanisms which regulate the supply of DNA replication proteins are probably distinct from those which control the initiation of DNA replication. How DNA synthesis is regulated during the individual rounds of replication during schizogony is yet to be investigated. In higher eukaryotes the levels of PCNA (Baserga, 1991), DNA polymerase δ (Zeng *et al.*, 1994) and DNA polymerase α (Wahl *et al.*, 1988), in cells in a proliferative state, remain constant. The activity of DNA polymerase α (Nasheuer *et al.*, 1991), and possibly DNA polymerase δ (Zeng *et al.*, 1994),

are regulated post-translationally by phosphorylation. The investigation of DNA synthesis during schizogony in *P.falciparum* may reveal some form of post-translational regulation of the proteins required for DNA synthesis during the multiple rounds of DNA synthesis. However, investigation of this part of the life cycle may prove to be technically very demanding.

Chapter 4.

Summary.

The transcription start site of the PCNA gene from *Plasmodium falciparum* was mapped using a number of methods. These experiments indicated that there is a major start site approximately 960bp upstream of the ORF and a second, minor, transcription start site approximately 40bp further upstream. Sequence analysis revealed the presence of consensus transcription factor binding sequences.

Comparison between the sequence at the 3' end of a cDNA clone with the genomic sequence identified a putative transcription stop site approximately 250bp downstream of the ORF. Northern analysis of the two PfPCNA transcripts indicates that the size difference may be due to differences in the sizes of the 3' UTRs.

4.1 Introduction.

Transcripts from *P.falciparum* are eukaryotic in nature; capping, intron-splicing and polyadenylation all take place to some degree (Levitt, 1993). One striking feature of *Plasmodium* transcripts is their extremely long 5' untranslated regions (5'UTR). The transcription start sites mapped to date have an average 5'UTR of approximately 400 to 500bp (Table 1) compared to approximately 100bp for most eukaryotes. mRNA from the related apicomplexan,*Toxoplasma gondii*, also have long 5'UTRs (Nagel and Boothroyd, 1988, Roos, D., *pers comm.*). A role for the large 5'UTR has not yet been determined but suggestions include a role in the regulation of transcript stability, stage specific expression or even the targeting of the message.

The enzymes responsible for transcription in eukaryotes are large multisubunit structures consisting typically of 2 large subunits with up to 11 associated smaller subunits (Bzik, 1991). The large subunits for RNA polymerases I, II and III have been cloned and characterised for *P.falciparum* (Li *et al.*, 1989, Li *et al.*, 1991 and Fox *et al.*, 1993) The *P.falciparum* RNA polymerases appear to have similar sensitivities to α-

amanitin (Lanzer *et al.*, 1992a, 1992b) as other eukaryotic RNA polymerases which suggests that they may have the same transcriptional specificities. RNA polymerase I typically transcribes ribosomal RNAs, RNA polymerase III the transfer RNAs and small nuclear RNAs and RNA polymerase II the heterogeneous nuclear RNAs which are then processed to give messenger RNA.

Genes transcribed by RNA polymerase II have regulatory sequences upstream from their transcription start site. The highly conserved TATA box (or Hogness box) is the binding site for the TFIID transcription factor. After recognition and binding to the TATA box by TFIID the RNA polymerase II complex is sequentially assembled. The TFIID homologue from *P.falciparum*, PfTBP, has been cloned and characterised (McAndrew *et al.*, 1993). Although the primary sequence of PfTBP is divergent from that of other eukaryotes, the architecture of the protein is very similar. It is possible that these differences may help to facilitate recognition of a TATA box in the extremely AT-rich intergenic sequences found in *Plasmodium* genomic DNA.

Other common transcription factor binding sites have been identified upstream of transcription start sites in *Plasmodium* including CAAT boxes, octomer immunoglobin factor (OCT1) and SP1 sequences (Table 1). Characterisation of the promoters from *Plasmodium* has not led to the identification of a common structure or of common transcription factor binding sites. Transcription start sites have been identified in a number of *Plasmodium* species, from genes encoding both house-keeping and antigenic proteins. To date it has only been possible to speculate on nature of putative transcription factor binding sites due to the lack of a functional assay. Only in the case of the SV40 element, found upstream of the KHARP gene (Lanzer *et al.*, 1992a), has a protein actually been demonstrated to bind to the DNA.

Transcription termination in *Plasmodium* takes place at a consensus sequence similar to that of mammals (Figure 1). Single transcription stop sites have been determined for *P.falciparum* actin I and II (Wesseling *et al.*, 1989) and *HSP*90 (Su and Wellems, 1994), but multiple sites have been described downstream of the *P.falciparum* 3.8 gene (Lanzer *et al.*, 1992b)

and *P.berghei* circumsporozoite antigen gene (Ruvolo *et al.*, 1993). Too few transcription stop sites have been characterised to speculate on whether transcription terminates at single or multiple sites, or both. The serine repeat antigen (SERA) of *P.falciparum* does not have a consensus polyadenylation site at the 3′ end of the cDNA clone characterised (Fox and Bzik, 1994).

AATAA.....$(N)_{0-23}$.....**A**.....$(N)_{6-54}$.....G+T rich *Plasmodium*

AATAAA....$(N)_{10-30}$....**A**.....$(N)_{0-10}$.....G+T rich mammalian

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Figure 1. A comparison of the consensus sequences for putative polyadenylation signals of *Plasmodium* and mammalian transcription units (Lanzer *et al.*, 1993). The suggested *Plasmodium* consensus polyadenylation signal is AATAA compared to that of AATAAA for mammals. In addition to these sequences Lanzer *et al.* suggest that long genomic stretches of poly d(A) and poly d(T) flank the consensus site in *Plasmodium*. **A** indicates the adenosine from which polyadenylation takes place. N indicates any nucleotide and the subscript figure indicates the number of nucleotides.

Table 1.

Comparison of the transcription start sites mapped in *Plasmodium*. The position of the transcription start site (relative to the A of the translational start site), the method(s) of mapping employed, any potential regulatory sequences identified and a reference are indicated in the table.

5'RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription- PCR.

Plasmodiaum Species.	Gene product.	Transcription Start Site (TSS).	Sequences around TSS.	Methods of Experimental Determination.	Reference.
falciparum	**Heat Shock Protein Family 90, HSP90 (*pfhsp86*)**	Major at -655 Minor at -659	CAAT box, TATA box, SP1 binding site	Primer extension	Su and Wellems (1994)
falciparum	**Serine Repeat Antigen (SERA)**	Major at -146 and -234 Minor at -330 and -600	None described	Primer extension	Fox and Bzik (1994)
falciparum	**Knob Associated Histidine Rich Protein (KHARP)**	Major at -849	Palindromic motif	Primer extension RNase protection	Lanzer *et al.* (1992a)
falciparum	**Glycophorin Binding Protein (GBP130)**	Major at -985	SV40 element	S1 nuclease protection Primer extension	Lanzer *et al.* (1992b)
falciparum	**Calmodulin**	Major at -62	TATA box, CAAT box	Primer extension	Robson and Jennings (1991)
falciparum	**Aldolase**	Site at -322	None described	Primer extension	Knapp *et al.* (1990)
falciparum	**Merozoite Major Surface Antigen**	Several sites between -250 to -550	TATA box, CAAT box	Primer extension RNase protection	Myler (1989)
knowlesi	**Circumsporozoite Antigen (CS)**	App. 10 sites between -200 to -340	Several TATA boxes, SV40 element, CACCCCTC boxes, TGCATGCA boxes, OCT1 binding site	S1 nuclease protection Primer extension	Ruiz i Altaba *et al.* (1987)
yoelii	**230KDa Merozoite Antigen (Py230)**	Major at -381 and -417 Minor at -377 and -378 and -402	TATA boxes, OCT1 binding site	Primer extension	Lewis (1990)
berghei	**Circumsporozoite Antigen (CS)**	Multiple sites between -246 to -343	TATA boxes, CAAT box, TGCATGCA boxes, OCT1 binding site	5' RACE	Levitt *et al.*, (1993)

4.2 Approximate mapping of the PfPCNA transcription start site.

In view of the fact that the transcription start may be up to 1kb from the translational start site two methods, RT-PCR and transcriptional mapping, were employed to provide an approximate position.

4.2.1 Reverse Transcription - Polymerase Chain Reaction (RT-PCR).

First strand cDNA synthesis was primed from an antisense oligonucleotide (366T) designed to hybridise to total RNA at the start of the PfPCNA open reading frame (2.2.9.2). The cDNA synthesis is presumed to continue to the end of the template, the extreme 5′ end of the PfPCNA transcript, where cDNA synthesis stops. All RNA was removed by RNase digestion. The cDNA was then used as a template for PCR reactions between 366T and a series of sense-strand oligonucleotides located progressively further upstream of the translational start. An indication of the transcriptional start site is obtained by determining which sense-strand oligonucleotides give, and which do not give, a product.

Genomic DNA (gDNA) contamination would give PCR products of the expected size with every oligonucleotide. To guard against gDNA contamination total RNA was treated with RNase free DNase before first stand cDNA synthesis. The total RNA was tested for contamination with gDNA by RT-PCR across the *P.falciparum* DNA polymerase α intron. Reverse transcription from oligonucleotide 973F (5′ GAAGCATCTTCA CTCGT 3′) was followed by a PCR reaction between 973F and 413E (5′ TTT ATGGTTGTTTAGGA 3′). PCR using a gDNA template should give a product of approximately 940bp, whereas the cDNA template product should be 730bp. Both PCR products can be hybridised to with the internal oligonucleotide 456H (Figure 2, lanes 1-2).

First strand cDNA synthesis from total RNA, tested as described above, was made from anti-sense oligonucleotide 366T (5′ GCATTATTTAAT TTGGCC 3′). Subsequent PCR reactions were made between 366T and G6589 (5′ TTTGCAAAATGTTAGAATG 3′), 117V (5′ GAATTACACATA AGTTATAC 3′), H0919 (5′ ATTCCCACATATATTGTG 3′), and 949T (5′

Figure 2.

A. Schematic representation of the position of oligonucleotides, across the PfDNA polymerases α intron, used to check for gDNA contamination of total RNA. Both the cDNA and gDNA PCR products hybridise to the end-labelled oligonucleotide 456H (5′ AACTTTTGCGCATGCAT 3′).

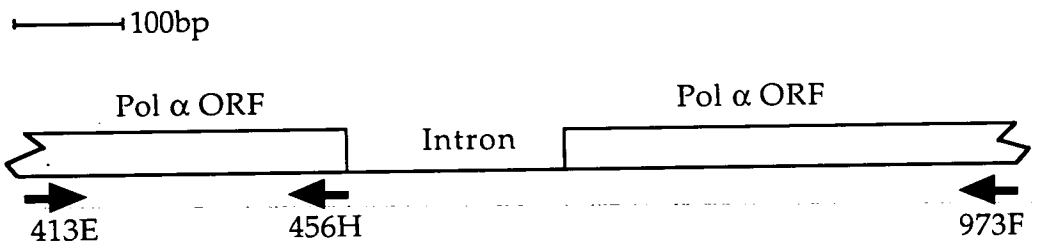
B. Schematic representation of the position of oligonucleotides used for the approximate mapping of the PfPCNA transcription start site. Both the cDNA and gDNA PCR products hybridise to the end-labelled oligonucleotide 543T (5′ TGACAATTTTCTAATAGC 3′).

C. Autoradiograph of the RT-PCR products hybridised to with the respective end-labeled oligonucleotide probe (see above). The sizes of the PCR products are indicated below, and on the figure, in bp .

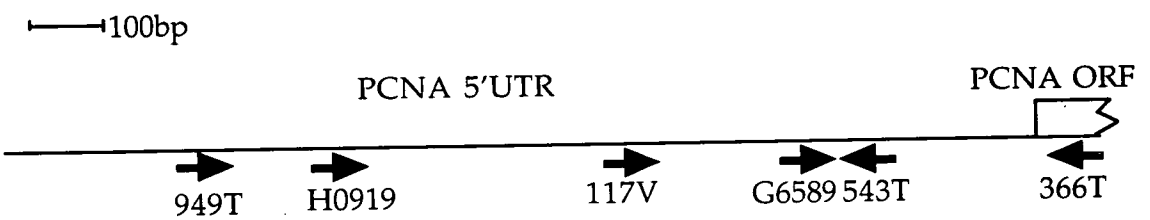
Lane 1. gDNA, DNA polymerase α control (940bp).
Lane 2. cDNA, DNA polymerase α control (730bp).

Lane 3. gDNA, 366T-G6589 (380bp).
Lane 4. cDNA, 366T-G6589 (380bp).
Lane 5. gDNA, 366T-117V (740bp).
Lane 6. cDNA, 366T-117V (740bp).
Lane 7. gDNA, 366T-H0919 (1060bp).
Lane 8. cDNA, 366T-H0919 (1060bp).
Lane 9. gDNA, 366T-949T (1440bp).
Lane 10. cDNA, 366T-949T(1440bp).

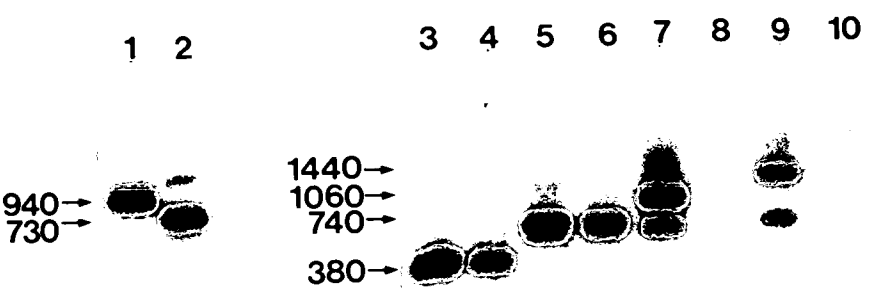
A. RT-PCR control for gDNA contamination.



B. PfPCNA 5′ flanking sequence.



C. Autoradiographs.



CTATAGAGGAGCCAAAT 3') in turn (Figure 2). The results indicate that there may be a major transcription start site upstream of 117V but down stream of H0919, a region between 690 and 1000bp upstream of the ORF. A longer exposure of the autoradiograph shows a weak band in the H0919 cDNA track (lane 8) that indicates that there may be a minor transcription start site upstream of 1000bp from the ORF. The absence of products from the PCR using cDNA as a template with 949T was expected. The 949T oligonucleotide binding site is too far upstream of the translational start for a transcriptional start site to be expected to be found upstream of this site.

4.2.2 Transcriptional mapping of the PfPCNA locus.

Using transcriptional mapping a second approach was available to approximately map the transcription start site. Nuclei from trophozoite parasites were isolated and allowed to continue transcription in a buffer containing radiolabelled UTP (see Nuclear Run On Analysis 2.3.1.7). The radiolabelled nascent RNA was isolated from the nuclei (2.3.1.1) and used to probe a membrane to which were fixed PCR products derived from around the PCNA locus (Figure 3). The PCR products were checked on an ethidium bromide agarose gel to estimate the concentration of the DNA and approximately 100ng of each was loaded onto an agarose gel and subsequently transferred to Genescreen *Plus* membrane (DuPont). The probed and washed membrane was exposed to a phosphoimager cassette before autoradiography at -70°C.

The results from the phosphoimager were used to quantify the counts after an overnight exposure and is shown in Figure 3. The autoradiograph indicates that the transcription start site is located in the region represented by the product B. This region extends from 690bp to 1000bp upstream of the translational start and agrees with that identified by RT-PCR (4.2.1).

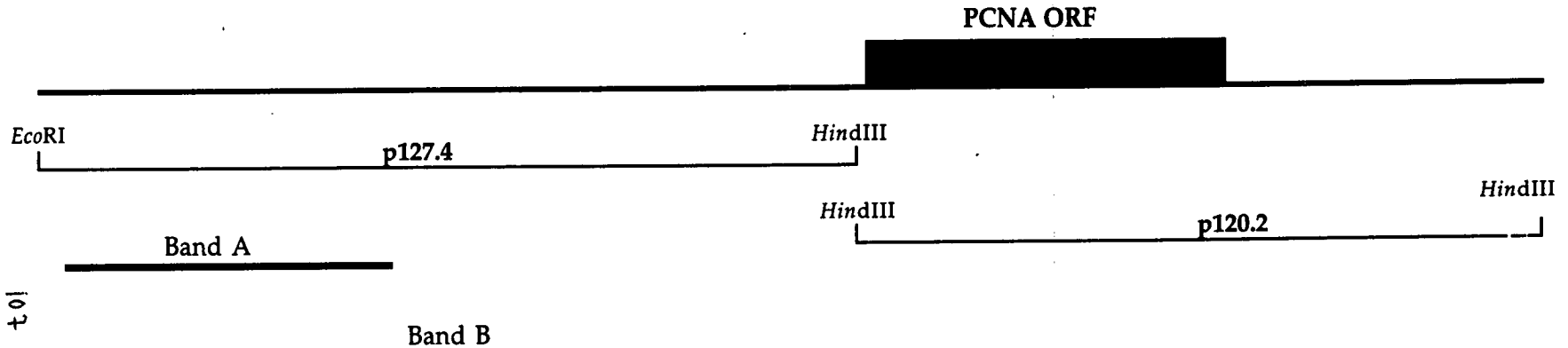
The intensity of the signals from the PCR products D to F decreases in intensity. This is probably due to the lack of incorporation of radiolabelled UTP during transcription through these regions *in vitro*. A subsequent check of the concentration of the DNA from product C on an ethidium

Figure 3.

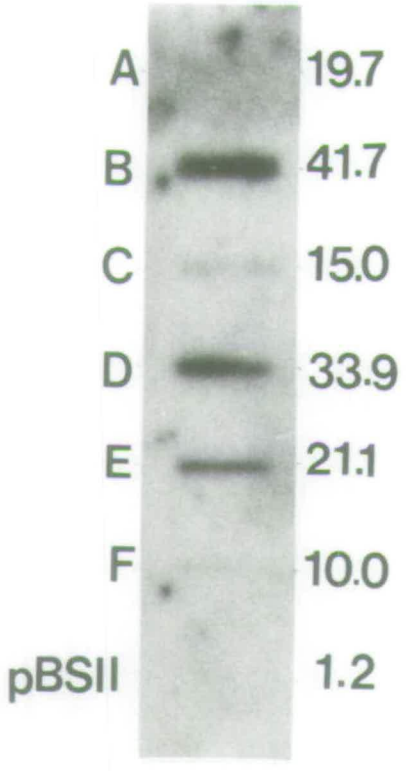
A. Schematic indicating the location of the PCR products used for the transcriptional mapping of the PCNA locus. The oligonucleotides used and the size of the PCR product are indicated. Bands A to C were amplified using clone p127.4 as a template, bands D to F used clone p120.2 (Kilbey *et al.*, 1993). The oligonucleotide sequences are;
Uni (5′ GTAAAACGACGGCCAGT 3′), 948T (5′ CACAATATATGTGGG AAT 3′), H0919 (5′ ATTCCCACATATATGTG 3′), 828T (GTATAACTT ATGTGTAATTC 3′), 117V (5′ GAATTACACATAAGTTATAC 3′), 543T (5′ TGACAATTTTCTAATAGC 3′), 534S (5′ GTCTATTGAATTGGACTC 3′), 322M (5′ CATTTTGGGAGCTAAGAA 3′), 302S (5′ ACAATCCTTTGCCAT CAA 3′), H0666 (5′ GAGGGTGAAAGGTGTTTT C 3′) and H0665 (5′ TGT ATGATGATTTATCACC 3′).

B. Transciptional mapping autoradiograph. The membrane was also exposed overnight in a phosphoimager cassette and the counts indicated beside the appropriate band. pBluescriptII SK+ (pBSII) restricted with *Hin*dIII was used as a negative control.

Figure 3A. PCNA locus



Band.	Oligonucleotides.		App. Size (bp)
A	UNI	948T	640
B	H0919	828T	380
C	117V	543T	500
D	534S	332M	380
E	302S	H0666	400
F	302S	H0665	540


A
B
C
D
E
F
pBSII
19.7
41.7
15.0
33.9
21.1
10.0
1.2

bromide agarose gel indicated that the DNA concentration was approximately 1/3rd of that estimated which would account for the lower than expected intensity of this signal.
The high background on the filter in the region where PCR product A is fixed may hide a signal from a weak transcription start site. The background is not high enough, however, to hide the signal from a major transcription start site .

4.3 Primer extension analysis.

The results from RT-PCR and transcriptional mapping define a region which contains the transcription start site. Two, more precise, approaches were used, the first of which was primer extension analysis. End-labelled oligonucleotides were hybridised to total RNA which was shown to be free of gDNA contamination, and first strand cDNA synthesis performed. The extension products were separated on denaturing polyacrylamide gel with radiolabelled markers.
Two oligonucleotides, 694T (5' GTGTAAAAATTTCTCTTC 3') and H0216 (5' CTATGGAGAAAAAAACATATAATATATATATG 3'), were selected for primer extension (Figure 4A). The end-labelled oligonucleotides were hybridised to 5µg of *P.falciparum* total RNA and used as a primer for reverse transcription (2.3.3).
The results of the primer extension are shown in Figure 4B. The products of primer extension from 694T indicate a single transcription start site at a position 960bp upstream from the ORF. The products from H0216 indicate a pair of transcription start sites, of apparently equal intensity, at sites approximately 970bp and 1005bp upstream of the ORF. Other faint bands present are probably signals from premature interruption of the reverse transcriptase on the AT-rich template.
Controls were used to eliminate the possibility of aberrant signals (Figure 4C). Primer extension from 694T using 5µg of *S.cerevisiae* total RNA as template gave no signals indicating that oligonucleotide 694T did not cross-hybridise at random to RNA. A second control in which 694T was hybridised to 5µg of *P.falciparum* total RNA but no reverse transcriptase

Figure 4.

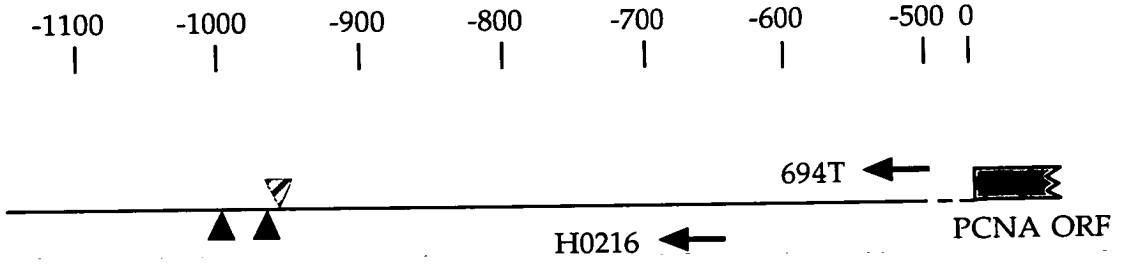
A. Schematic representing the relative positions of the oligonucleotides used for primer extension of PfPCNA. The triangles indicate the position of the transcriptional start site(s). The transcription start site identified by 694T uses a hatched triangle, those identified using H0216 are identified by full triangles.

B & C. Autoradiographs of the primer extension products;

B. 1. 694T hybridised to *P.falciparum* total RNA.
2. H0216 hybridised to *P.falciparum* total RNA.

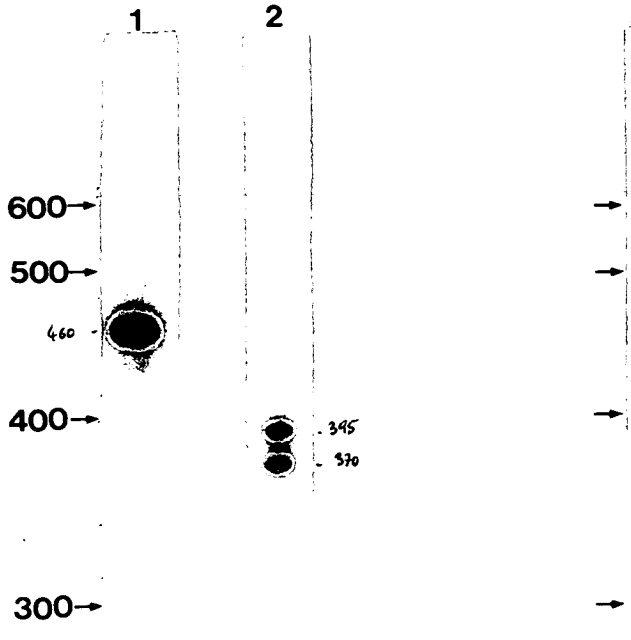
C. 3. 694T hybridised to *S.cerevisiae* total RNA.
4. 694T hybridised to *P.falciparum* total RNA without reverse transcriptase.

A. PfPCNA 5′ flanking sequence.



B. Primer Extension.

C. Negative Controls.



added again gave no signal indicating that the products observed were due to the action of reverse transcriptase.

4.4 RNase protection analysis.

Since reverse transcriptase can terminate prematurely on AT-rich templates, possibly accounting for the multiple transcription start sites reported (Table 1), RNase protection analysis was used to check the results of the primer extension analysis.
A construct containing a 565bp fragment of the 5′ flanking sequence, including the putative transcription start site, was cloned into pBluescriptII SK+. *In vitro* transcription from the T7 RNA polymerase promoter gave a radiolabelled antisense transcript which was hybridised to total RNA free of gDNA contamination. Single stranded RNA was digested using RNases, the protected RNA duplex products were isolated and fractionated on a denaturing polyacrylamide gel (Figure 5A-C).

4.4.1 Construction of pRNase vector.

Using gDNA as template for a PCR reaction, oligonucleotides 828T (5′ GTATAACTTATGTGTAATTC 3′) and PH1 (5′ GCTCTAGAGGGTGCT TTTAAAAGAATGG 3′) were used to give a 565bp product. The product was cloned into the TA cloning kit vector (2.2.5.5) and sequenced to check for errors during amplification.
The 565bp fragment was excised from the TA vector by restriction with *Xba*I and *Eco*RI and ligated into pBluescriptII SK+ vector (Stratagene) restricted with the same enzymes (Figure 5b). The pRNase construct junctions were checked by sequencing. Subsequent restriction and PCR analysis confirmed the correct orientation of the insert in the pRNase construct.

4.4.2 Preparation of the riboprobe.

The pRNase construct was linearised by restriction with *Xba*I and isolated

by gel electrophoresis (2.2.3.6). The linearised plasmid was used as a template for *in vitro* transcription (2.3.4.1) using the commercial T7 Ampliscribe kit (Epicentre Technologies). The T7 Ampliscribe kit allows for the preparation of large quantities of RNA transcript of full length (approximately 630 bases) incorporating radiolabelled UTP.
The products of *in vitro* transcription were fractionated on a denaturing polyacrylamide gel and full length transcripts isolated (2.3.4.2).

4.4.3 Hybridisation of riboprobe to total RNA and RNase digestion.

The full length riboprobe was used for RNase protection assays using the commercially available RPA II™ kit (Ambion, Lanzer *et al.*, 1992a). Protected fragments (Figure 5c) of approximately 270 bases and 310 bases were observed only when the hybridisation was treated with RNase T1, a protocol recommended by the manufacturer when AT-rich genomic sequences are to be analysed. The major protected fragment, from digestion with a 1:100 dilution of the supplied RNase T1, indicates a major transcription start site at 960bp upstream of the translational start. A second minor product, at 310 bases, indicates a transcription start site located approximately 1000bp upstream of the translational start (Figure 5c). The same products were observed more clearly when a 1:250 dilution of RNase T1 was used. The other products in this lane are probably due to incomplete digestion of the riboprobe.
Digestion with either a 1:100 or 1:250 dilution of RNase A/T1 completely digested the riboprobe, even when associated with the complementary PfPCNA transcript (Figure 5c). The riboprobe contains a large proportion of adenosine and uracil nucleosides which would lower the melting point of the RNA duplex, the essentially single stranded RNA is then available as a substrate for RNase A digestion.
Control experiments using yeast RNA were used. Attempted hybridisation of the riboprobe to yeast RNA did not protect it from either RNase A/T1 or RNase T1 only. The riboprobe when mixed with yeast RNA, but not subjected to RNase digestion, is not degraded (Figure 5c).

Figure 5A.

Schematic to describe the principle of RNase Protection. From the T7 RNA polymerase promoter, of a linearised plasmid containing the genomic sequence of interest, a radiolabelled transcript antisene to PCNA mRNA is produced (Riboprobe). The riboprobe extends past the putative transcription start site to give antisense RNA to which no complementary sequence is available in *P.falciparum* total RNA.
After hybridisation the RNA-RNA duplex is treated with RNase which digest single stranded RNA. The protected radiolabelled fragment is separated on a denaturing polyacylamide gel with markers.

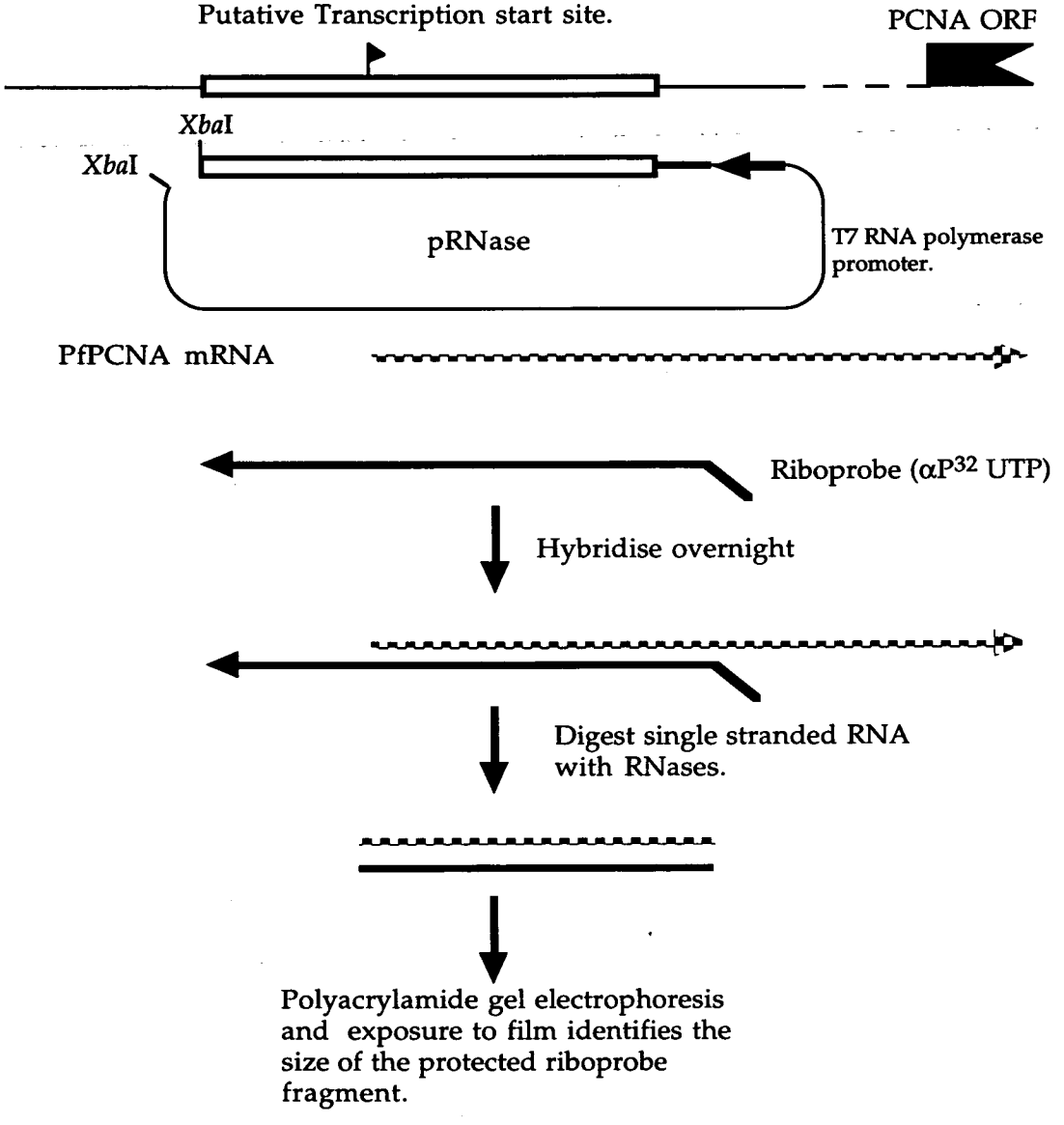
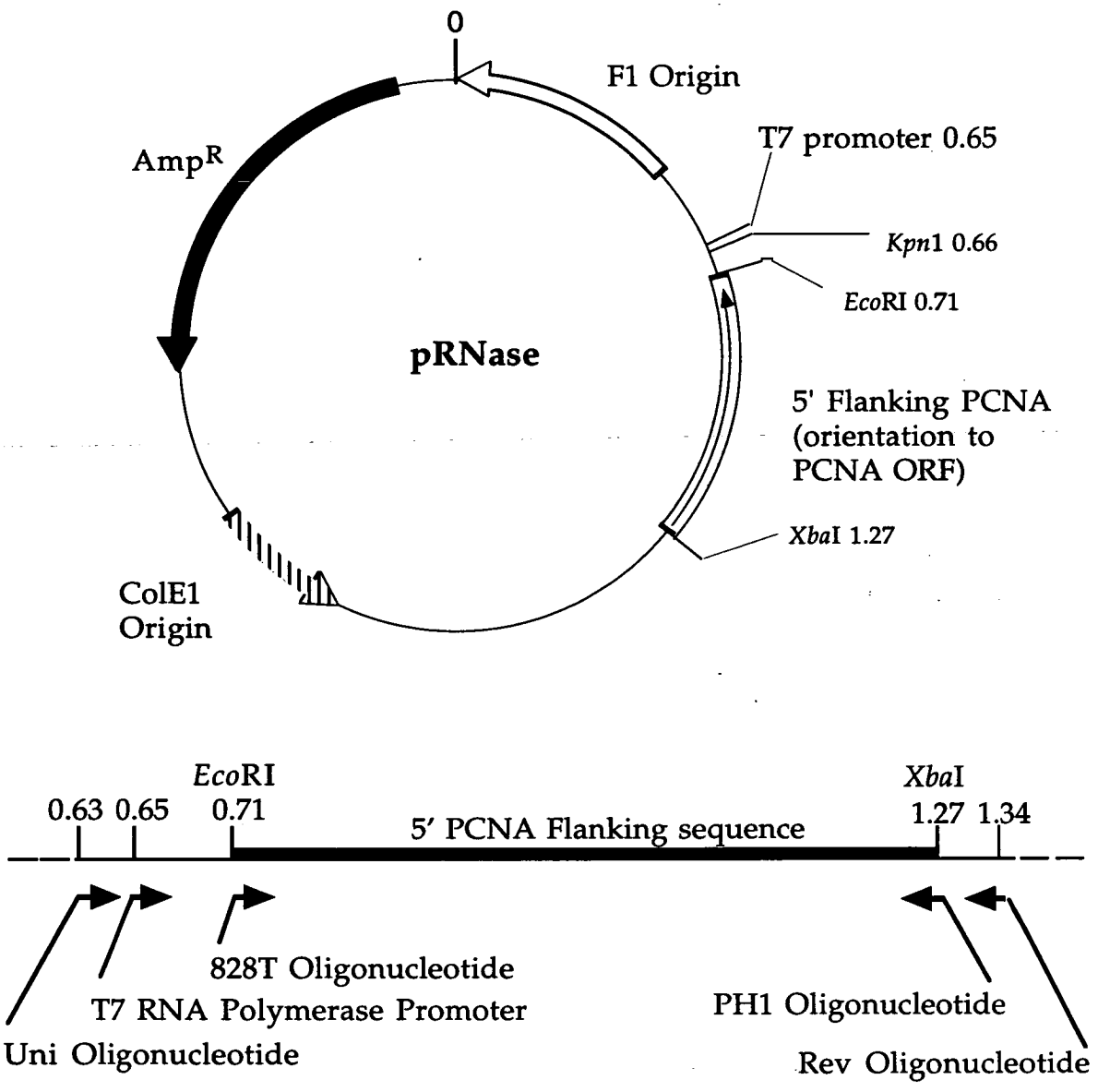


Figure 5b.



Schematic of the pRNase construct (approximately 3.5kb). All positions are indicated in kb from the point 0 marked on the vector map. Amp^R (ampicillin resistance gene). The PCNA flanking sequence orientation with respect to the ORF is indicated, note that transcription from the T7 promoter will give an antisense transcript.

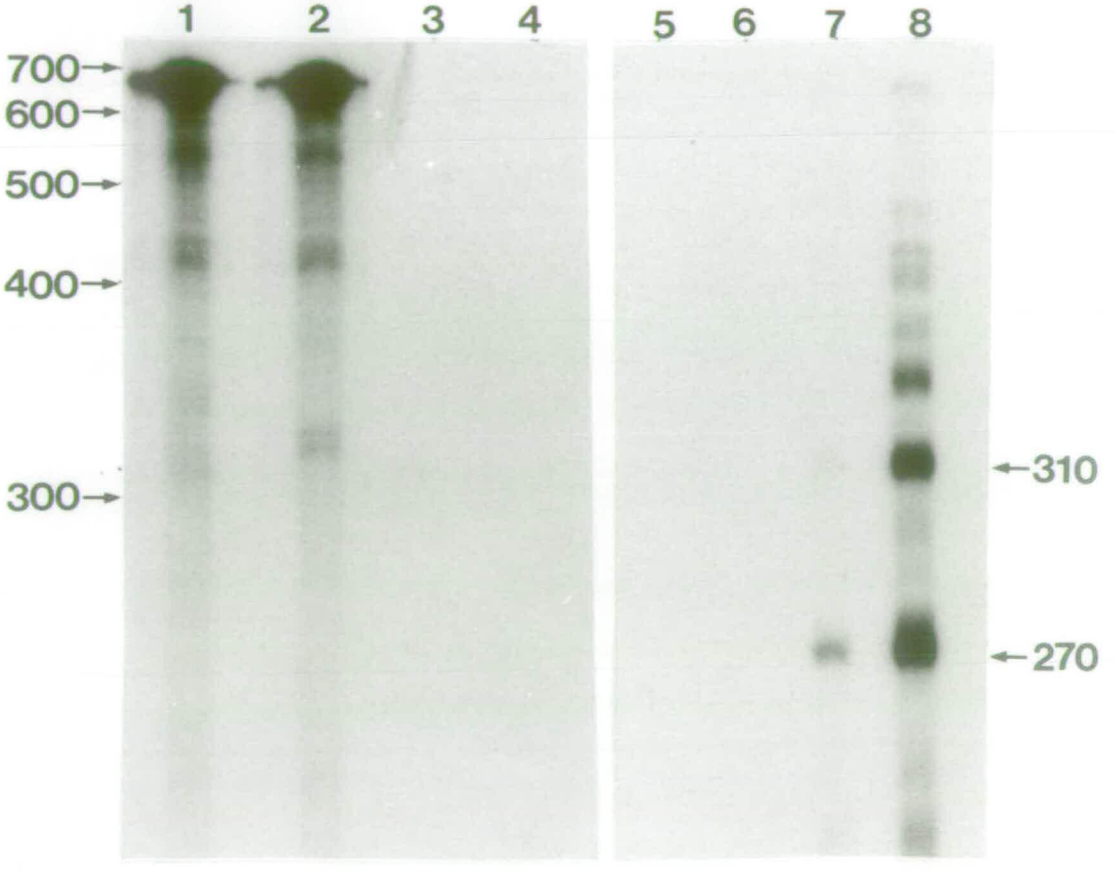
The multiple cloning site has been enlarged to indicate the relative positions of the T7 RNA polymerase promoter and relevant oligonucleotides (see text). Uni (5′ GTA AAA CGA CGG CCA GT 3′), Rev (5′ AAC AGC TAT GAC CAT G 3′).

Figure 5c.

Autoradiograph of results of the RNase protection analysis. The position of the 270 bases and 310 bases products and the 100bp markers are indicated.

1. riboprobe alone.
2. riboprobe hybridised to *S.cerevisiae* total RNA.
3. riboprobe hybridised to *S.cerevisiae* total RNA, digested with 1:100 dilution of RNase A/T1.
4. riboprobe hybridised to *S.cerevisiae* total RNA, digested with 1:100 dilution of RNase T1.
5. riboprobe hybridised to *P.falciparum* total RNA, digested with 1:100 dilution of RNase A/T1.
6. riboprobe hybridised to *P.falciparum* total RNA, digested with 1:250 dilution of RNase A/T1.
7. riboprobe hybridised to *P.falciparum* total RNA, digested with 1:100 dilution of RNase T1.
8. riboprobe hybridised to *P.falciparum* total RNA, digested with 1:250 dilution of RNase T1.

Lanes 1-4 are the result of a 30 minute exposure to film and lanes 5-8 of a 2 hour exposure to film.


1
2
3
4
5
6
7
8
700
600
500
400
300
310
270

4.5 Analysis of genomic sequence around putative transcription start sites.

Analysis of sequences around the *Plasmodium* transcription start sites mapped to date revealed little homology (Table 1). A typical eukaryotic RNA polymerase II transcription start site, as characterised from work with the SV40 and yeast systems, has a TATA box around 25bp upstream with other sequences such as CCAAT boxes, GC boxes, AP1-AP5 boxes, then OCT1 and OCT2 boxes (Singer and Berg, 1991) upstream of this. Approximately 600bp of genomic sequence upstream of the PCNA putative transcription start site is available for analysis (Figure 6).

Consensus TATA boxes have been identified approximately 35bp upstream of the major transcription start site (nt 590) and 35-40bp upstream of the minor transcription start site (nt 545).

Two sites with good homology to OCT1 boxes (ATGCAAAT) have been identified. These oppositely orientated OCT1 boxes are approximately 235bp and 275bp upstream of the major transcription start site. OCT1 is a 90kDa protein that has been found in a variety of organisms and cell types. It is proposed to be a general enhancer of transcription required for the formation of a stable RNA polymerase complex. OCT1 boxes have been identified in a number of other *Plasmodium* promoters (Ruiz i Altaba *et al*, 1987, Lewis, 1990, Levitt *et al.*, 1993)

No CCAAT boxes, typically found up to 100bp upstream of the transcription start site, were identified. A weak homology at nt526 (CAAT) is present, approximately 100bp upstream of the transcription start site. The same sequence is found upstream of the *P.knowlesi* circumsporozoite antigen transcription site (Ruiz i Altaba *et al*, 1987).

The AT content of the PfPCNA 5′ flanking is in excess of 85% with large poly d(A), poly d(T) and poly d(AT) tracts. Analysis of the sequence has identified a number of "GC rich patches". These are sequences where 3 guanosine or cytidine are clustered within 5 nucleotides (italiced on figure 6). Interestingly these "GC rich patches" occur on or around the transcription start site or approximately 400-500bp upstream of the transcription start site.

Figure 6.

Sequence of the 5′ flanking DNA of the PCNA gene of *P.falciparum.*

The start of the ORF (1581) is indicated by a bold enlarged ATG. The restriction sites *Hin*dIII and *Eco*RI are overlined. Putative TATA boxes (nt550 and nt590) are in bold and enlarged. Oppositely orientated putative OCT1 boxes (nt350 and nt390) are over and underlined. "GC rich patches" (see above) positioned upstream and around the putative transcription start sites are italicised.

The position of transcription start sites are indicated by large arrows; **a**, transcription start site mapped using primer extension (694T) and RNase protection, **b**, transcription start site mapped using RNase protection only, **c**, transcription start site mapped using primer extension (H0216) and **d**, transcription start site mapped using primer extension (H0216).

The position and orientation of oligonucleotides important in the mapping of the PCNA transcription start site are indicated by thin arrows under the sequence.

*Eco*RI

1 GAATTCTTTA TTTTTACACA TATATATATA TATATATATA TATATAATAT

51 ACATATCAAA TTGTTATGGT TTTTA*CTAGC* *C*ATTACAAAT ATGTATAAAT

101 TTCTATATAT ATATATATAT ATATATATAA ACATA*CCGC*A AATATTAGTT

151 *GCA*CTTTTTA TATATACAAA AACTATAGAG A*GAGCC*AAAT AATAAAAAAA

949T ⟶

201 AAATTAATTA AATGATTTGA AATGGTTATA TATATTAAAA CATTTTATAA

251 GACAAAATTC ATAAGATATT AATAATTTTT ATATTTTATT TTCCTATTCA

301 TTTTTAATAA TATATCATAT ATAAAAA*GGG* *TGC*TTTTAAA AGAATGGATT

PH1 ⟶

351 GAGATTCAAA TATAATATTA TAAATATATA AACATAATTA TATATGCATC

401 TTATATTAAA AACTCATAGA ATAATATATA TAATAATATA TAGCTTAATT

451 ATATATAATC TTTCTTAAAA AACAAACAAA CAAACAAACA AACAAACAAA

501 AAAAAATTTT TTTTTTTATT AATTACAAAT AATTTATATA TATATA**TATA**

d ⟶ b ⟶

551 ATATT*CCCAC* ATATATTGTG ATAATTCTTA TATGATTCAA **TATA**AAAAAT

H0919 ⟶ c ⟶ a ⟶

601 TGAAAAATAA AATATTATCT TTATATAT*GT* *GC*ATATATTA AAAATTAAAC

651 A*CCTAGGC*AT ATATTACTCA TTATTTTATT CAAGATAGGA TAAAAAAAAA

701 AAAAAAAAAA AAGAGAAAAG AGAATATTAA AATTAT*CATG* *ACC*TTTTATC

751 ATTTTGATTT TTCTTCATCC TTATATTTTA TAAATAAAAT TAATATAATT

801 CATAAAATAT TTAATATATT TATATATATA TTTTTTTTTT TCATATGCTA

851 ATATAATAAA CAATATATAT AATGAATTAC ACATAAGTTA TACATTAAGA

117V ⟶

901 AACATTTATA AATAAATATA TATATATATA TATATATATA TATATACATA

⟵

951 TATATATTAT ATGTTTTTTT CTCCATAGTG TTAAAAAAAT AAACTAATAA

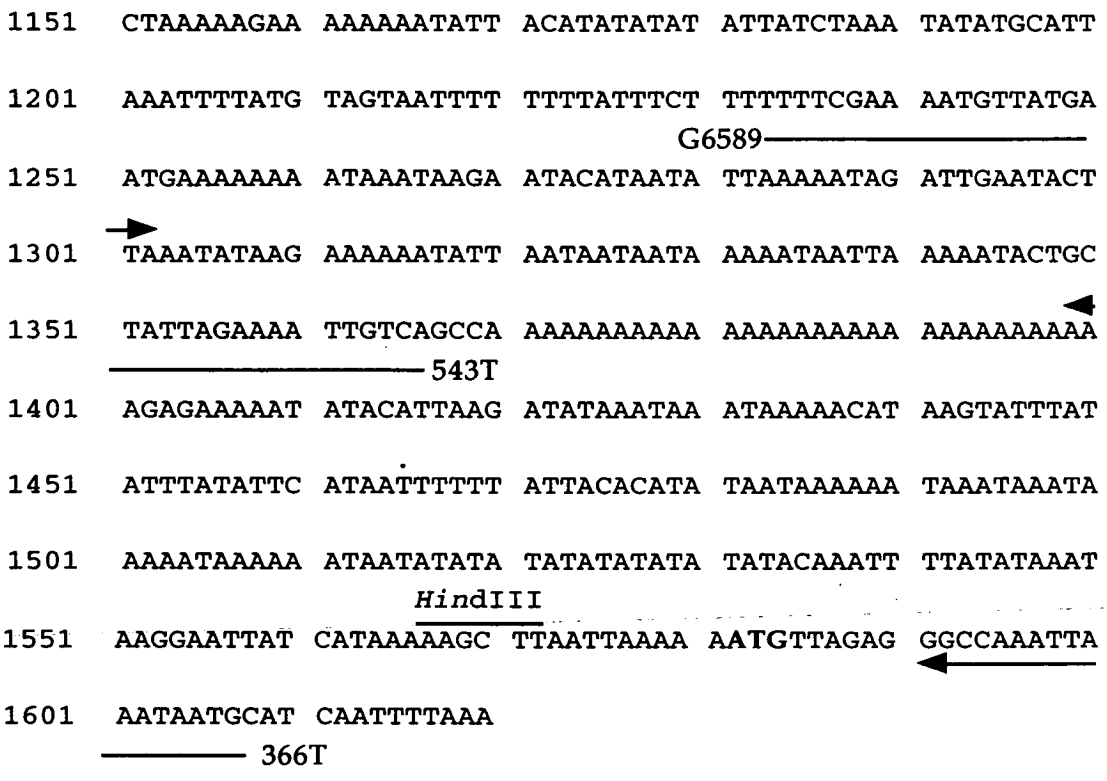
H0216 ⟶

1001 ATATATATAT AATATTAATA TATATACAAT ATATATAATA TGCACATTAT

1051 CATTTATATC TTTAAAAATA ACTTTTGAAG AGAAATTTTT ACACAATAGT

⟵ 694T

1101 ATTTTTAAGG TATAAGTTTT TTTTTTTTTT TTTTTTCTCT TCTTTTCTAA


1151 CTAAAAAGAA AAAAAATATT ACATATATAT ATTATCTAAA TATATGCATT
1201 AAATTTTATG TAGTAATTTT TTTTATTTCT TTTTTTCGAA AATGTTATGA
G6589
1251 ATGAAAAAAA ATAAATAAGA ATACATAATA TTAAAAATAG ATTGAATACT
1301 TAAATATAAG AAAAAATATT AATAATAATA AAAATAATTA AAAATACTGC
1351 TATTAGAAAA TTGTCAGCCA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
543T
1401 AGAGAAAAAT ATACATTAAG ATATAAATAA ATAAAAACAT AAGTATTTAT
1451 ATTTATATTC ATAATTTTTT ATTACACATA TAATAAAAAA TAAATAAATA
1501 AAAATAAAAA ATAATATATA TATATATATA TATACAAATT TTATATAAAT
HindIII
1551 AAGGAATTAT CATAAAAAGC TTAATTAAAA AATGTTAGAG GGCCAAATTA
1601 AATAATGCAT CAATTTTAAA
366T

4.6 Initial analysis of the 3′ end of the PfPCNA transcription unit.

An initial analysis of the 3′ end of the transcription unit was made by screening a plasmid cDNA library for PfPCNA clones. The cDNA library (constructed by P.Alano) was prepared by reverse transcription from an oligo d(T) primer with trophozoite total RNA template, linkering the products with a *Xho*I fragment and subcloning into the expression vector pJFE14.

The library was used to transform the DH5α strain of *E.coli* and transformants were lifted on to nitrocellulose membranes (2.2.6.5). The filters were screened using a random radiolabelled *Hin*dIII-*Eco*RI DNA fragment containing 725bp of the PCNA ORF. Positive clones were purified and replated until individual clones were obtained.

Three individual clones were identified and subsequently characterised by restriction and sequence analysis. The restriction analysis showed that each of the three clones contained a single *Hin*dIII and *Eco*RI site. A single 1350bp fragment was released from *Xho*I restriction of all the clones. Sequence analysis at both ends of the insertion from oligonucleotides hybridising to the multiple cloning site confirmed that all three clones are identical (Figure 7a).

The sequence at the extreme 3′ end of the cDNA clones was compared to sequence available from the genomic clone p120.2 (Kilbey *et al.*, 1993). The sequences are identical until a polyadenosine tract is reached approximately 250bp past the stop codon of the ORF. The genomic clone has a tract of 12 adenosines and the cDNA clone a tract of 14 adenosines followed by a *Xho*I linker (Figure 7b). The genomic clone sequence in this vicinity indicates that the sequence at the 3′ end of the cDNA clone may correspond to a consensus *Plasmodium* transcription stop site (Lanzer *et al.*, 1993). The genomic sequence contains the consensus AATAA sequence, several potential sites for polyadenylation and GT-rich sequences downstream of the AATAA site (Figure 7c). However, the precise point in the poly d(A) tract at which polyadenylation occurs cannot be identified from this analysis alone.

Further screening of the cDNA library for other clones corresponding to

Figure 7a. Schematic representing position and size of PfPCNA cDNA clones isolated.

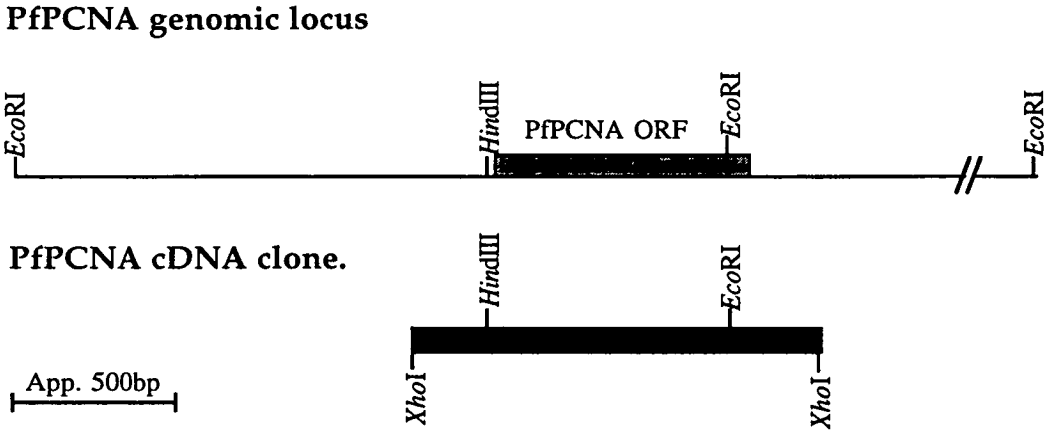


Figure 7b. Comparison of the genomic and cDNA sequence at the extreme 3′ end of the PfPCNA cDNA clone. The putative transcription stop site (Figure 1) is underlined. The cDNA linker is italicised with the *Xho*I site in bold. The distance in bp from the PfPCNA stop codon is indicated on the gDNA sequence.

```
241 gDNA ttttgctctc attttgaatt ttctaataaa aaaaaaaaat
    cDNA ttttgctctc attttgaatt ttctaataaa aaaaaaaaaa

281 gDNA ttttatatgt acacatatgt ataaaaacaa tatataacca
    cDNA aactttccag cacaattgga tctcgtg

321 gDNA tattatatac tcatatataa tagttcctat aggttaaaaa

361 gDNA atttatggtg ataaatcatc
```

Figure 7c.

PfPCNA ORF sequence is represented in capital letters and stops at nt 8-10 (TAA). The putative transcription stop site (from nt255) is indicated in bold characters with potential polyadenylation sites underlined. Sequence analysis indicates a second consensus polyadenylation site starting at around nt120 (italicised).

1 TAAAGATTAA tctcaataaa atattatata tatatatatg tacatatttt

51 catatatata tttatcgtca tctatatatt gaccttacat attatataca

101 tatatatata tatatgcca*a ataa*gaaaat tat*gagatct ttt*taataac

151 aaaatatttt aaacataaaa acaaaaaaaa tatgaaaaca cctttcaccc

201 tcaaaatgaa aactgtcata taaatcaaac ttttgctctc attttgaatt

251 ttct**aataa**a aaaaaaaaat ttttatatgt acactatgta taaaaacaat

301 atataaccat attatatact catatataat a**gttcctata ggtt**aaaaaa

351 **tttatggtg**a taaatcatca tacattataa aatataaaaa aatctatatc

401 ccgaaaaaaa atgaatgtat ccatatttgt tcacacacac acatatttat

451 atatatatat atatatatat atatatatat atttgttata attccacttt

501 atttcaaagt taatacatat ttataaaatt tcaatttatt attttataat

551 gtttttttta aatatctata tttagtagtt cttatatttg ataaaagaac

601 acaacaaaat aatcgtcgta tatgaaaata aaaataaaaa atacgaatgt

651 atctaaatat ttaacactct tagagaaaaa agaaacaaaa atataaacaa

701 ttaacaaaca tgcaaatttt aaatgtgtat gaataaaact taaaaatgta

751 gatgcttcta cttttatatg aatacacaca ggaatatata tatgtatata

801 tata

other transcription stop sites yielded no new clones and although the genomic sequence was extended 500bp to approximately 800bp past the stop site of the ORF, analysis of the sequence revealed only one other potential transcription stop site approximately 120bp past the stop site (Figure 7c).

4.7 Northern analysis of the 3′ end of the PfPCNA transcript.

The sizes of the PfPCNA transcripts were originally reported at 1.6kb and 2.2kb (Kilbey *et al.*, 1993). At the same time it was commented that the significance of two PCNA transcripts was unknown. Further northern analysis has revealed that the 1.6kb transcript is nearer 1.85kb in size and has confirmed the approximate size of the minor transcript.
As described above, a pair of putative transcriptional start sites have been mapped between 960bp-1000bp upstream of the translational start, and a putative transcriptional stop site identified approximately 250bp downstream of the stop codon. These figures would predict a transcript of approximately 2035bp. If this transcript has a long polyadenylated tail it could represent the observed 2.2kb transcript. There does not appear to be a second transcription start site less than 960bp from the translational start. The RT-PCR analysis did not indicate whether regions in the 5′UTR were removed during processing, and the 5′UTR cDNA sequence available was colinear with gDNA sequence. All these results indicate that differences in the sizes of transcripts would be most likely the result of different sizes of the 3′UTRs. A second putative transcription stop site was identified approximately 120bp downstream of the translational stop site. The termination of transcription at this site would result in a transcript of 1895bp, a possibility for the observed 1.85kb transcript, especially if the transcript is not polyadenylated. The cDNA library was screened several times but only the clones corresponding to what may be the longer transcript were isolated. Clones representing the shorter transcript derived from the potential transcription stop site are unlikely to have been lost from the library during amplification but may have been excluded if the transcript is not polyadenylated.

In order to assess whether other transcription stop sites exist 3′ RACE (Froham *et al.*, 1988) from an oligo d(T) to mRNA is generally used. This is very difficult in *Plasmodium* as extensive poly(A) tracts are common. In the case of the potential consensus stop site (120bp downstream of the PfPCNA ORF) a poly(A) tract exists immediately 3′ of the site. Under these circumstances it would be difficult to decide whether a clone is amplified from a *bone fide* polyadenylated tail or from this fortuitous poly(A) tract. Ruvolo *et al.* (1993) circumvented a similar problem by tailing all the RNA with a poly (C) tail using polynucleotide phosphorylase (PNPase). Subsequent cDNA synthesis and PCR using a G-rich oligonucleotide can be used to amplify a region of interest including the poly(A) tail. Unfortunately PNPase is no longer commercially available and the method could not be tried here.

We tried to investigate whether the difference in the transcript size was due to differently sized 3′UTRs using northern analysis. Two probes of approximately the same size were prepared. One from the region between the potential transcription stop site and the putative transcription stop site described (130bp, 3′ probe) and the second from the PCNA ORF (140bp, ORF probe) to act as a control (Figure 8). Two samples of 15µg of *P.falciparum* total RNA from an asynchronous culture were northern blotted (2.3.2) and each probed with one of the probes at 60°C using the same hybridising and washing conditions. The filters were exposed for 5 days to film at -70°C against X-ray film (Figure 8).

The film was scanned using a densitometer and the transmission values of the two transcripts compared. They were as follows;

	1.85kb transcript	2.2kb transcript
ORF probe	1.96	0.45
3′ probe	5.40	3.04

The results show that although identical conditions were used to prepare the two probes, the specific activity of the 3′ probe was greater than that of

Figure 8.

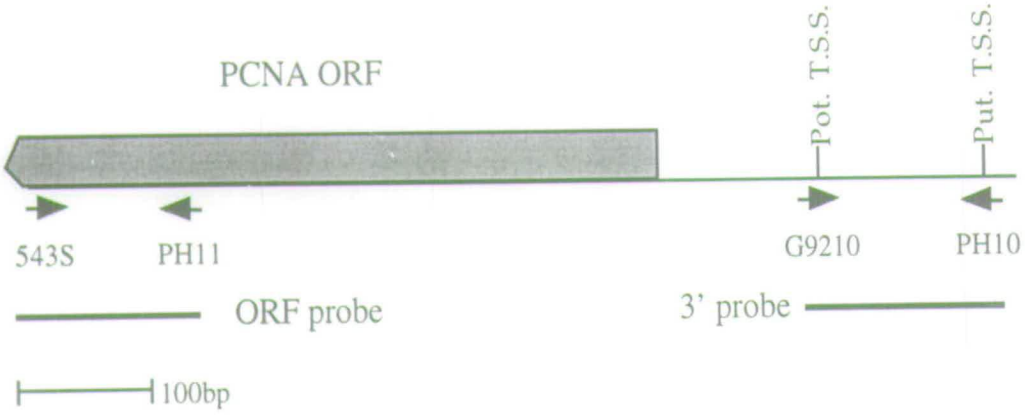
Northern analysis of the two PfPCNA transcripts using probes derived from different parts of the PfPCNA locus.

A. Schematic indicating the position of the ORF probe and 3′ probe used in the northern analysis. The position of the potential (pot. T.S.S) and putative (put. T.S.S) transcription stop sites are indicated. The probes were randomly labelled PCR products using the following oligonucleotides with the cDNA clone as template; 534S (5′ GTCTATTGAATTGGACTC 3′), PH11 (5′ GAATCAATTTCAATAAAAACGG 3′), G9210 (5′ GCCAAA TAAGAAAATTATGAG 3′) and PH10 (5′ GAAAATTCAAAATGAGAGC 3′).

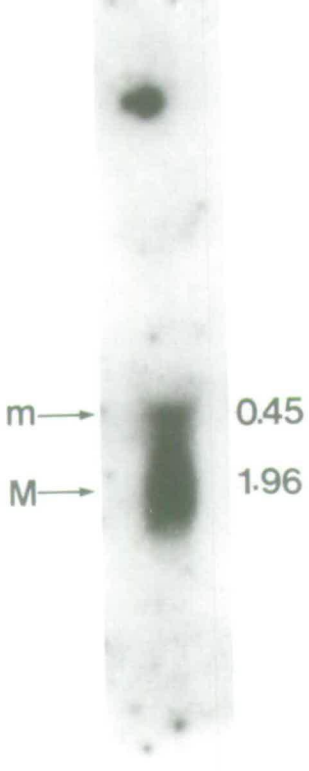
B. Northern blot using 15µg of *P.falciparum* total RNA from an asynchronous culture hybridised to with the ORF probe. The position and transmission values of the major (M) 1.85kb and minor (m) 2.2kb transcripts are indicated.

C. Northern blot using 15µg of *P.falciparum* total RNA from an asynchronous culture hybridised to with the 3′ probe. The position and transmission values of the major (M) 1.85kb and minor (m) 2.2kb transcripts are indicated.

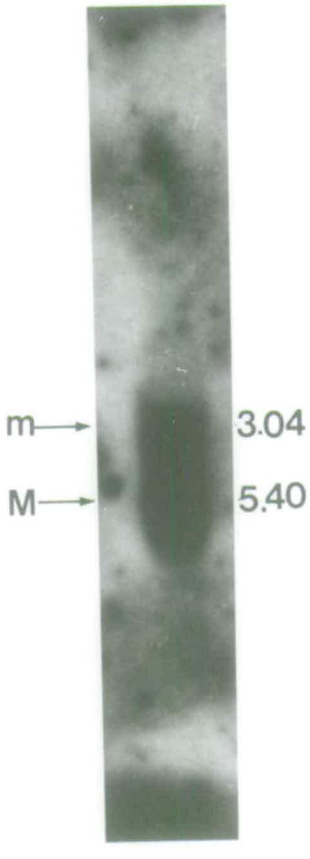
A. Schematic of PCNA locus



B. ORF probe



C. 3' probe



the ORF probe. The 2.2kb transcript should contain the hybridisation sites for both the ORF and 3′ probe allowing the relative specific activities of the probes to be compared. The specific activity of the 3′ probe is 6.75 times (3.04/0.45) greater than the ORF probe. When this is taken into account the data suggest that the 3′ ORF probe hybridises to the 1.85kb transcript only about 40% ([5.4/6.75]/ 1.96) as strongly as does the ORF probe. This might be the case if the 1.85kb transcript has a shorter 3′UTR than the 2.2kb transcript and only hybridises to part of the 3′ probe.

4.8 Discussion.

Combining the data from the mapping experiments we show that there appears to be a major transcription start site 960bp upstream of the translational start site. A second transcription start site may be present a further 40bp upstream. Using more than one method to map the transcription start site allowed us to counter potential errors that may arise when dealing with extremely AT-rich DNA. Where only one method is used to map a transcription start site multiple sites are generally reported (Table 1).

Analysis of the sequence upstream of the PfPCNA transcription start sites has identified consensus transcription factor binding sites. TATA boxes and OCT1 boxes are sequences commonly found in other eukaryotic promoters for genes transcribed by RNA polymerase II. DNase I protection analysis is required to demonstrate that these sequences are actually protected by transcription factors binding to them. DNase I protection with extracts from nuclei isolated from the different major stages of the asexual cycle may give some indications of a stage specific transcription factor protection. This, however, may not occur in the case of PfPCNA as the promoter for this gene appears to be active throughout the asexual cycle (Chapter 3).

Isolation of a cDNA clone and the subsequent comparison of the cDNA and gDNA sequences identified a consensus transcription stop site approximately 250bp downstream of the end of the ORF. Sequence analysis alone indicates that a second transcription stop site may be found

120bp downstream of the end of the ORF.

Two PfPCNA transcripts of 1.85kb and 2.2kb have been identified, with the 1.85kb transcript predominating. Assignation of transcription start and stop sites for both these transcripts is difficult. The major transcription start site and the putative transcription stop site 250bp downstream of the PfPCNA ORF can be tentatively assigned to the larger, but minor, transcript of 2.2kb. However, it is not possible to assign a transcription stop site for the 1.85kb transcript. No cDNA clone has been cloned which use the potential transcription stop site 120bp downstream of the PfPCNA ORF. Although it is possible that amplification of the cDNA library has excluded clones which correspond to this potential site, this seems rather unlikely, it is also possible that the 1.85kb transcript is not polyadenylated. Transcripts lacking a polyadenylated tail can be translated by *Plasmodium* (Levitt, 1993) although in practice only a small group circumsporozoite antigen transcripts of *P.berghei* have been demonstrated to lack a poly(A) tail (Ruvulo *et al.*, 1993). The possibility that even these transcripts are precursor transcripts or the products of degradation was not fully investigated.

Indications that there may be a transcription stop site before the 250bp site came from the finding that a probe that recognises the extreme 3' end of the cDNA clone hybridises less strongly to the 1.85bp transcript than to the 2.2kb transcript. This would suggest that the probe has less overlap with sequences in the 1.85kb transcript than the 2.2kb transcript. To investigate this more completely further northern analysis, RNase protection and transient transfection analysis will be required. That PfPCNA is regulated at the post-transcriptional level and is apparently encoded by two transcripts, the prominent of which has no defined transcription stop site, presents us with an intriguing problem.

Chapter 5.

Summary.

An attempt was made to transiently transfect and express in *Plasmodium falciparum* a novel marker gene, the Green Fluorescent Protein (GFP). A vector containing the cDNA of the GFP gene from *Aequoria victoria* flanked by 5′ and 3′ PfPCNA non-coding sequences was constructed. Double pulse electroporation of intraerythrocytic stage culture was attempted using a wide range of experimental conditions. Although no heterologous expression of GFP was observed, experiments indicated that the plasmid DNA enters the parasite and appears to reach the nucleus.
The electroporations and screening for GFP fluorescence was made with the assistance of Dr. Christian Doerig and Caroline Doerig.

5.1 Introduction.

A green light is produced at the fringe of the umbrella of the jellyfish *A.victoria*. The fluorescence is due to the action of two proteins, aequorin and GFP (Figure 1). Calcium activated aequorin produces a blue light in the absence of GFP, however, when GFP is present, the energy is transferred from aequorin to GFP by an unknown mechanism. The reason why two proteins are required for fluorescence is not understood although it has been suggested that efficiency of light transfer is greater through these two proteins. GFP will fluoresce green in the absence of aequorin if illuminated with a blue light. An absorption spectrum shows a maximum at around 395nm with a smaller peak at 490nm. Absorption at 395nm, however, leads to rapid photobleaching of the GFP but a stable fluorescence at 509nm can be obtained by excitation at 490nm (Inouye and Tsuji, 1994).
The gene for GFP from *A.victoria* has been cloned (Prasher *et al.*, 1992) and shown to encode a protein of 238 amino acids which is present as a monomer of 27kDa. The chromophore is contained completely within the coding sequence of the protein as a modified tripeptide. Cody *et al.* (1993) demonstrated that a ser-tyr-gly tripeptide is cyclised and oxidised to give rise to the para-hydroxybenzylidene-imidazolidonone chromophore

(Figure 2).

The key to GFP's usefulness is that the protein can be heterologously expressed in a wide range of organisms, as diverse as *Escherichia coli* and *Caenorhabditis elegans* (Chalfie *et al.*, 1994), and retain its fluorescent properties. The chromophore formation does not require any cofactors or exogenous substrates. This led to the conclusion that the chromophore is formed either by autocatalysis or as a by-product of a cellular pathway common to the organisms in which GFP has been expressed. Heim *et al.* (1994, 1995) report that chromophore formation in *E.coli* required oxygen and proceeded with a time constant of approximately 2 to 4 hours depending upon culture conditions. The time constant was unchanged when cell lysates were diluted implying that there were no requirements for enzymes or cofactors and that the formation of the chromophore was more likely to be autocatalytic.

After the initial demonstration that GFP could be used as an indicator of promoter activity in the nematode *C.elegans*, GFP has been used for the same purpose in a wide variety of other organisms including; *Drosophila* (Yeh *et al.*, 1995), Potato Virus X (Baulcombe *et al.*, 1995), Zebrafish (Peters *et al.*, 1995), *Xenopus* (Tannahill *et al.*, 1995), mouse (Moss and Rosenthal, 1994) and human (Rizzuto *et al.*, 1995). GFP expression allows transformants to be identified and isolated by means of their fluorescence under UV light. More recently use has been made of GFP fused to proteins of interest to study their function *in vivo*. Previously, large quantities of relatively pure protein were conjugated to a fluorophore before being introduced into cells by some invasive technique such as reverse permeabilisation or microinjection. These techniques are not only difficult to control but the results from the disrupted cells may be suspect. GFP is a small protein and its fusion to another protein may not necessarily disrupt the function of the protein of interest nor the GFPs fluorescence. Wang and Hazelrigg (1994) demonstrated that fusion of GFP to either the N or C terminus of the *Drosophila* Exu protein did not affect the spatial and temporal pattern of GFP-Exu expression compared to the native Exu. Marshall *et al.*(1995) also showed that a GFP fusion to NMDAR1, a component of ion channels in human kidney cells, would

facilitate studies on NMDAR1 expression, localisation and processing. The function of the GFP-NMDAR1 protein did not alter the properties of the ion channel.

GFPs have been found in several classes of *Cnidaria* (Prasher, 1995). The GFP of the sea pansy (*Renilla reniformis*) is thought to have a similar chromophore to that of *A.victoria* although it has a different absorption spectrum (Prasher, 1995). Heim *et al.* (1994) and Delagrave *et al.* (1995) both report altered emission and absorption spectra in mutated *A.victoria* GFP genes. These mutations include red shifted absorption spectra which are similar to that of the *R.reniformis* GFP, and mutants with increased fluorescence. Given a range of different *Cnidaria* GFPs, possibly with mutations introduced, a variety of GFP fusion proteins may be used within individual cells permitting gene expression cascades and the effects of drugs, toxins and hormones on protein expression and function to be more closely examined.

Figure 1.

Schematic illustrating the mechanism of bioluminescence from the jellyfish *Aquorea victoria*. * indicates an activated form of coelenterazine. (a) is also described as the Blue Fluorescent Protein, in the absence of Green Fluorescent Protein a blue fluorescence at $hv\lambda_{max470nm}$ is observed. (b) is an unknown mechanism of energy transfer from the Blue Fluorescent Protein to the Green Fluorescent Protein.

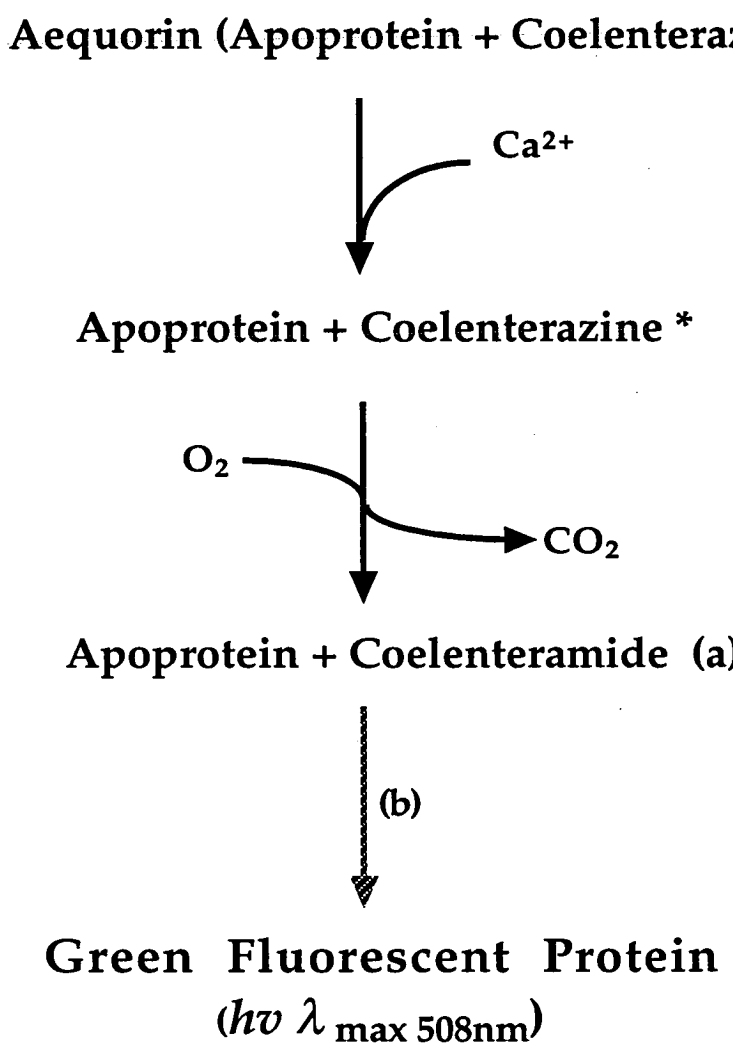
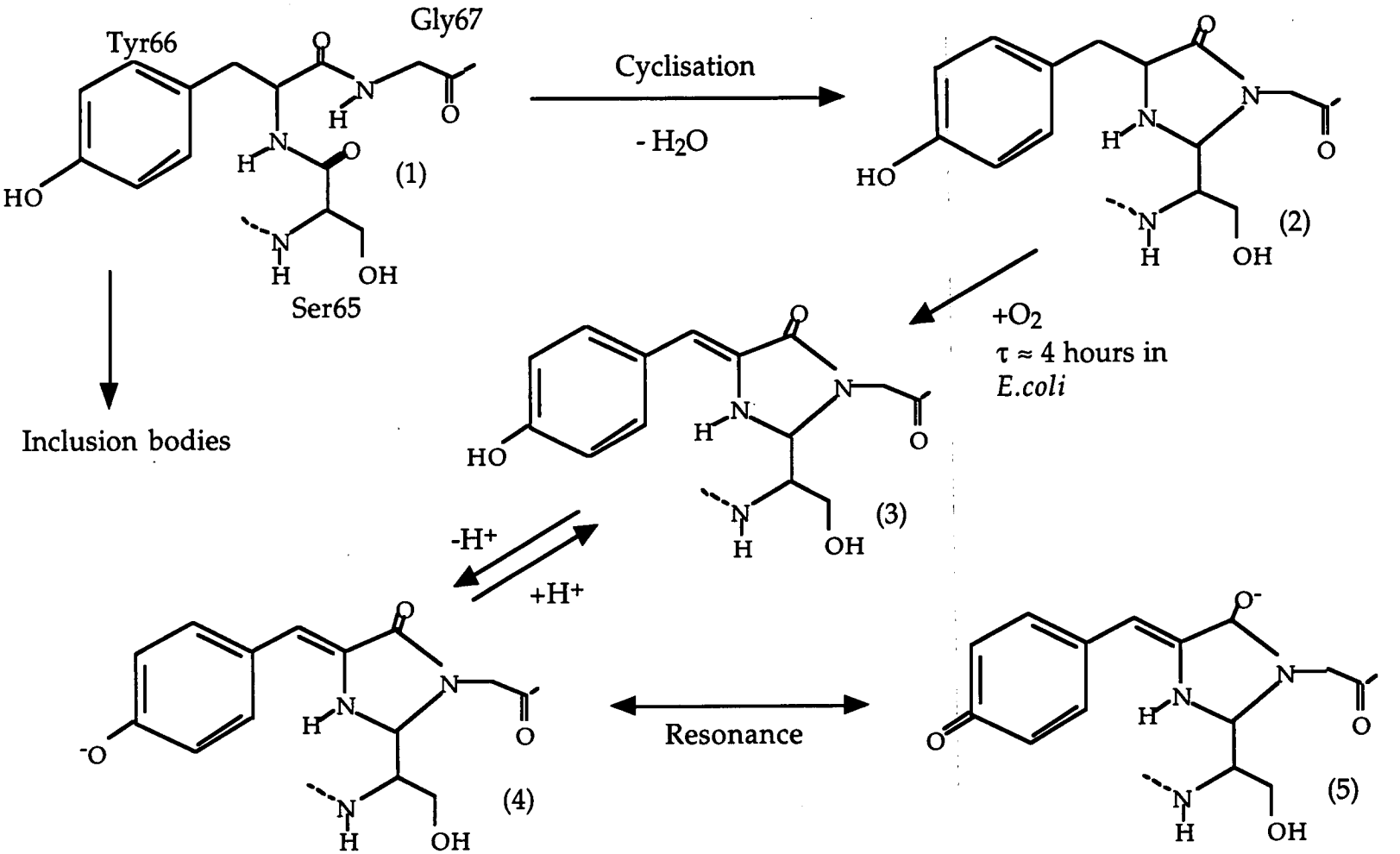


Figure 2.

Illustration of the proposed biosynthetic formation of the GFP chromophore from *A.victoria* (Heim *et al.*, 1994).

Newly translated GFP (1) is either precipitated into inclusion bodies or remains soluble. Cyclisation of the chromophore within soluble GFP takes place when the amino group of glycine 67 forms a Schiff base linkage onto the carbonyl group of serine 65 to form an imidazolidine-5-one (2). The new N=C bond promotes dehydrogenaton of the adjacent carbon bond of the tyrosine 66 in the presence of oxygen. The autoxidation results in the formation of the parahyroxybenzylidene-imidazolidonone moiety which acts as a chromophore (3).

Deprotonation gives the acidic forms (4 and 5) which are stabilised by resonance. As the chromophore can exist in a number of states it is thought that this is the reason why GFP absorbs light at 390, 470 and 475nm. Excited phenols are more stable when acidic so emission is only thought to be from the deprotonated forms.


Tyr66
Gly67
Ser65
(1)
Cyclisation
- H_2O
(2)
$+O_2$
$\tau \approx 4$ hours in
E.coli
Inclusion bodies
(3)
$-H^+$
$+H^+$
(4)
Resonance
(5)

5.2 Construction of the GFP vector.

Enough DNA sequences that flank the PfPCNA gene were placed on either side of the GFP gene so we could make the assumption that all the necessary 5′ and 3′ controlling elements for PfPCNA transcription were present. Initially 1.58kb of PfPCNA 5′ flanking sequence was placed upstream of the GFP gene to give the pGFP1 construct, subsequently 1.5kb of 3′ PfPCNA flanking sequence was added downstream of the GFP gene to give the pGFP2 construct.

5.2.1 Construction of pGFP1.

The GFP open reading frame was amplified by PCR (2.2.9.1) from the plasmid pGFP10.1 (Chalfie *et al.*, 1994, supplied by Douglas Prasher) which contains the cDNA of the GFP gene from *A.victoria*. The following oligonucleotide primers were used; GFP/F (5′ CCCAAGCTTAATTAA AAAATGAGTAAAGGAGAAGAA 3′) and GFP/R (5′ CCCAAGCTTGAA TTCTTATTTGTATAGTTCATCCAT 3′) which introduce *Hin*dIII restriction endonuclease sites either side of the GFP gene (Figure 3a). The 760bp DNA product was isolated, purified (2.2.3.6) and restricted (2.2.2) with *Hin*dIII.

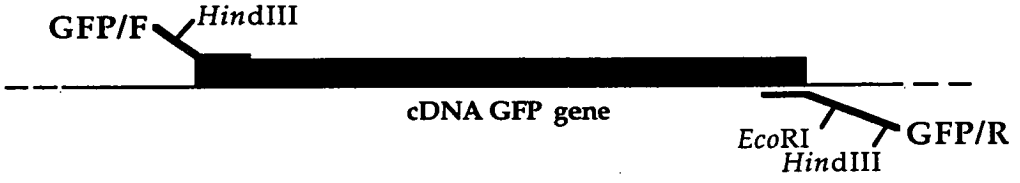
The PfPCNA gene was available as a pair of clones, p127.4 and p120 (Figure 3b), from a genomic library (Kilbey *et al.*, 1993). The PfPCNA 5′ flanking sequence had subsequently been subcloned as an *Eco*RI/*Hin*dIII DNA fragment into pBluescript II SK+ (Stratagene) to give pPCNA15. pPCNA15 was restricted using *Hin*dIII and subsequently treated with phosphatase (2.2.5.1). The *Hin*dIII DNA fragment containing the GFP ORF was ligated (2.2.5.4) into the phosphatased *Hin*dIII restricted pPCNA15.

The correct clone was selected by means of a PCR test. PCR between the oligonucleotides 117V (5′ GAATTTCACATAAGTTATAC 3′) and GFP/R give a product of a predicted size of approximately 1.4kb which would indicate that the GFP ORF had been placed in the correct orientation downstream of the PfPCNA 5′ flanking sequence (Figure 3c).

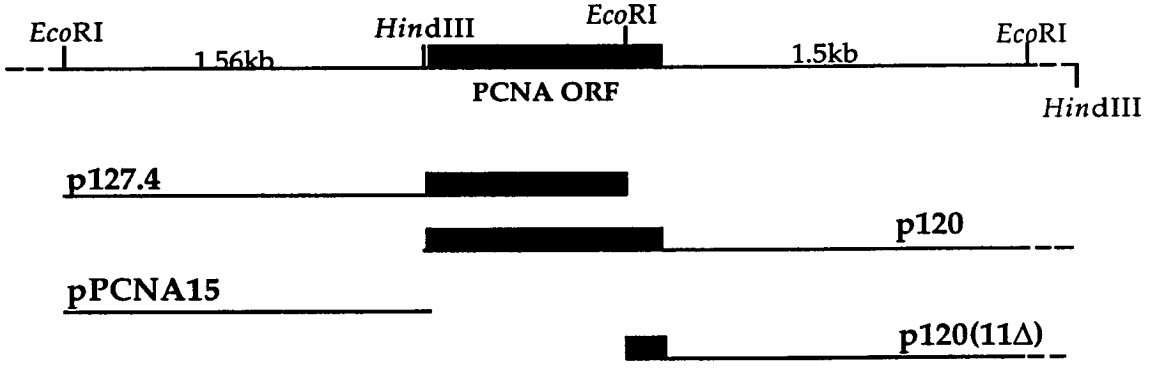
The *Hin*dIII junctions and the entire GFP ORF were sequenced (2.2.8). The only departure from the reported sequence of wild type GFP was that

Figure 3.

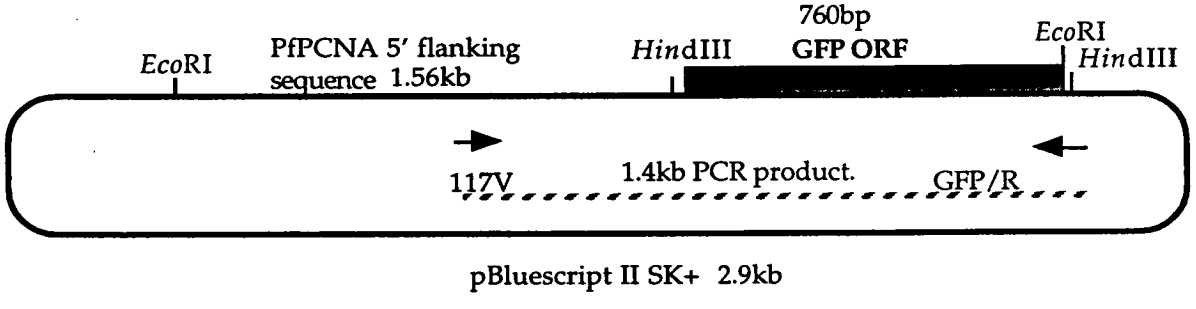
A. Schematic representing the PCR amplification of the cDNA GFP gene from the pGFP10.1 plasmid.



B. Schematic representing the PCNA genomic clones isolated by Kilbey *et al.* (1993) and the modified clones used to construct pGFP1 and pGFP2.



C. Endonuclease restriction site and oligonucleotide binding site map of pGFP1 (not to scale).



pBluescript II SK+ 2.9kb

described by Chalfie *et al.* (1994) where codon 80 of the GFP ORF is changed from CAG to CGG. The substitution of arginine for glutamine was known to have no detectable effect on the spectral properties of GFP and was known to be present in the pGFP10.1 plasmid template. Further confirmation of the construct was provided using a series of restrictions; *Hin*dIII, *Eco*RI and *Eco*RI/*Hin*dIII. All the DNA fragments of the expected sizes were obtained; 4.4kb and 780bp using *Hin*dIII, 2.9kb and 2.35kb using *Eco*RI and 2.9kb, 1.56kb and 760bp from *Eco*RI/*Hin*dIII (Figure 3c).

5.2.2 Construction of pGFP2.

pGFP1 was partially restricted using *Eco*RI for 1, 3 and 5 minutes at both 25°C and 37°C. DNA fragments of 4-4.5kb were isolated, purified (2.2.3.6) and phosphatase treated (2.2.5.1).

A 1.5kb DNA fragment was isolated and purified from an *Eco*RI restriction of p120(11Δ). The 1.5kb DNA fragment, containing the extreme 3′ of the PfPCNA ORF and the following 1.4kb of 3′ flanking sequence, was ligated into the isolated *Eco*RI restricted and phosphotased pGFP1.

The correct clone (Figure 4) was isolated using a series of tests ;

a) An *Eco*RI restriction which resulted in DNA fragments of 2.9kb, 2.3kb and 1.5kb indicated that all the necessary DNA fragments were present in the clone.

b) A *Kpn*I/*Hin*dIII restriction which resulted in DNA fragments of 4.4kb and 2.3kb indicated the PfPCNA 3′ flanking sequence was inserted into the *Eco*RI site towards the 3′ of the GFP ORF.

c) A PCR between the oligonucleotides GFP/F and H0666 (5′ GAGGGT GAAAGGTGTTTTC 3′) which gave a product of 1kb indicated that the PfPCNA 3′ flanking sequence was inserted in the correct orientation.

d) The *Eco*RI junction between the PfPCNA 3′ flanking sequence and the pGFP1 clone was sequenced.

The pGFP2 plasmid was completely checked by restriction with a variety of restriction enzymes (2.2.2), southern blotted (2.2.4.2) and hybridised (2.2.4.3-4) to a series of end labelled oligonucleotides. The results of this analysis (Figure 5) indicate that the plasmid was correctly constructed.

Figure 4.

Schematic representing the pGFP2 plasmid with endonuclease restriction sites and approximate oligonucleotide binding sites indicated. The position of the restriction sites are marked in kb from the arbitrary point 0 at the top of the plasmid map.

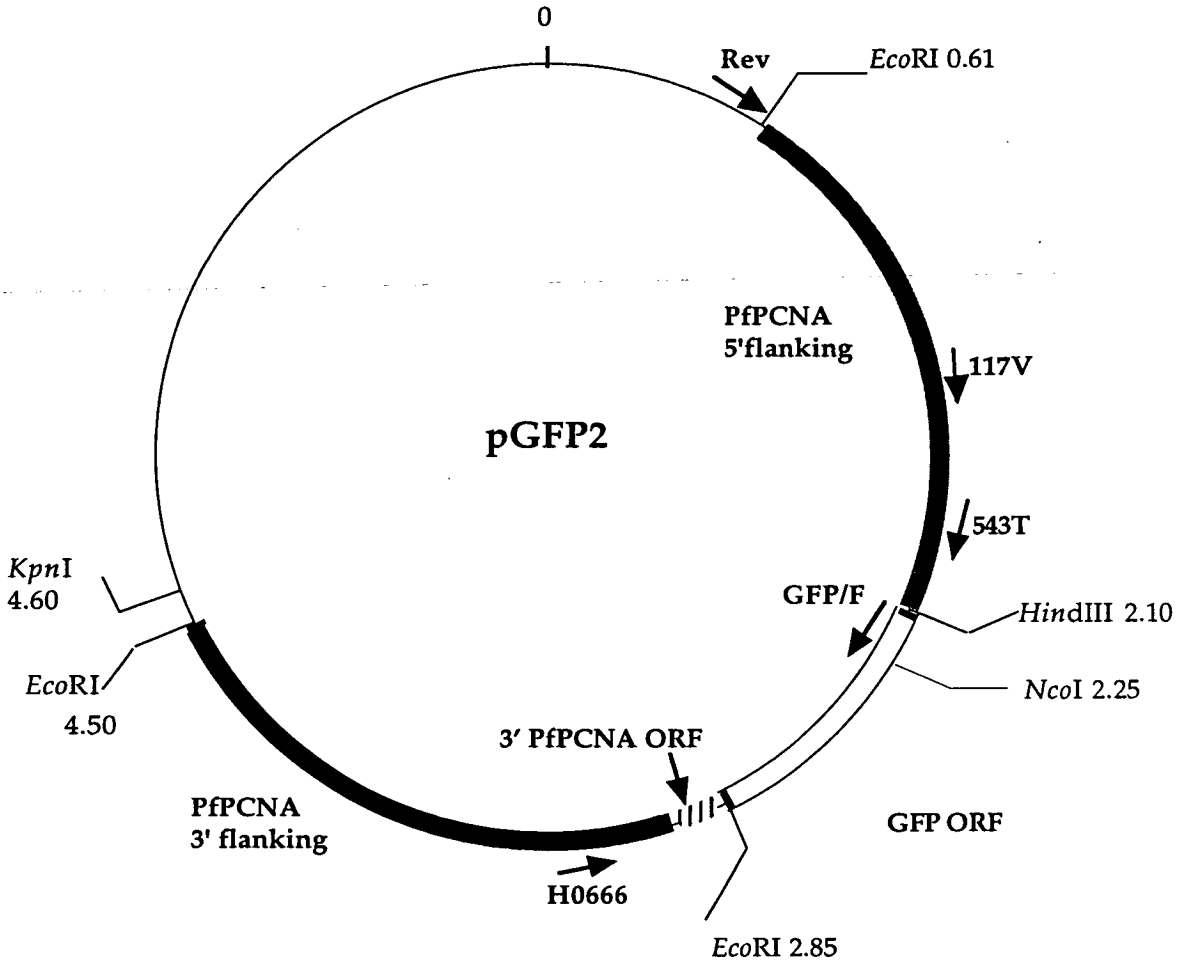


Figure 5.

Results of the restriction endonuclease and southern analysis confirm the correct construction of pGFP2. The restricted pGFP2 DNA was separated by agarose gel electrophoresis, blotted and hybridised to using a range of end-labelled oligonucleotide probes. The position of the 2.02kb marker of λ *Hin*dIII /*Eco*RI markers is indicated by an arrow. The position of 1, 2 and 3kb is indicated next to the autoradiographs.

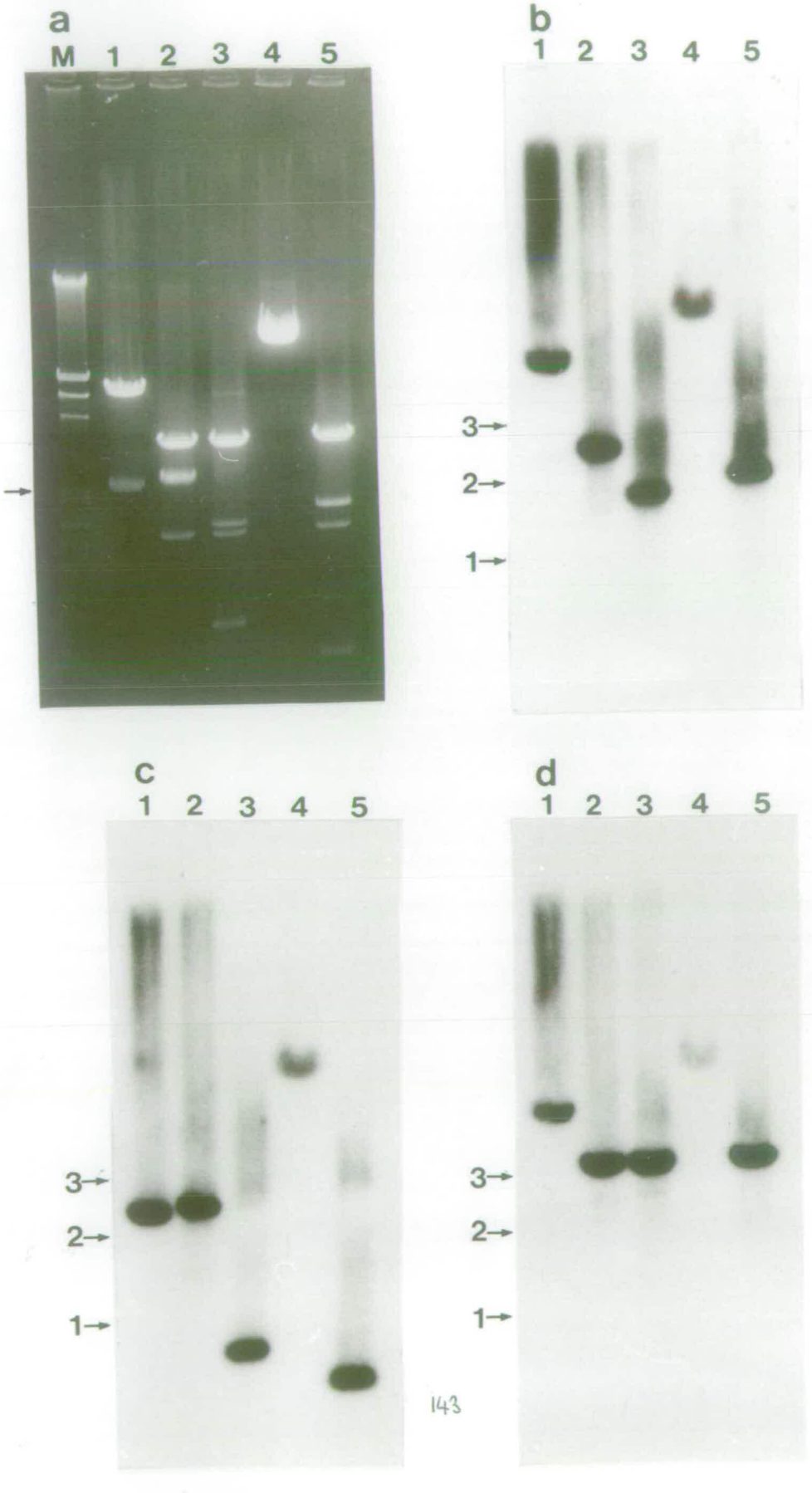
1. λ *Hin*dIII /*Eco*RI markers
2. *Hin*dIII
3. *Eco*RI
4. *Hin*dIII /*Eco*RI
5. *Nco*I
6. *Nco*I /*Eco*RI

a) Ethidium bromide stained agarose gel.

b) Southern blot hybridised with the end-labelled oligonucleotide 543T (5′ TGACAATTTTCTAATAGC 3′) probe.

c) Southern blot hybridised with the end-labelled oligonucleotide GFP/I (5′ CTTATGGTGTTCAATGC 3′) probe.

d) Southern blot hybridised with the end-labelled oligonucleotide REV (5′ GGAAACAGCTATGACCATG 3′) probe.


a
M 1 2 3 4 5
b
1 2 3 4 5
3
2
1
c
1 2 3 4 5
3
2
1
d
1 2 3 4 5
3
2
1

5.3 Establishing a transfection protocol for the GFP reporter gene.

When this work commenced there was no established protocol for the electroporation of plasmid DNA into cultured *P.falciparum*. A series of experiments were required to establish the parameters for use in the electroporation experiments and the subsequent detection of the GFP luminescence.

5.3.1 Effect of double pulse electroporation on *P.falciparum* survival.

A set of parameters for the first and second pulse were supplied by the manufacturer of the electroporator (Flowgen Inc.). Using these conditions a series of electroporations on intraerythrocytic stage cultures were carried out. The parasitaemia of the culture before and 4 hours after the electroporation was measured by examination of giemsa stained thin smear slides in order to assess the effect of the pulse on parasite survival. The conditions suggested by the manufacturer to be initially assessed were; a first pulse 600V-1200V for 2.77msec at 25μF and 99Ω, and a second pulse of 150V-300V for 150msec-2000msec, at 25μF and 156Ω.

An intraerythrocytic stage culture was grown until the average parasitaemia reached 8-12% in a 5% haematocrit. 0.5ml of culture was microcentrifuged briefly and the supernatant removed. The erythrocyte pellet was resuspended in 0.5ml incomplete medium (RPMI1640 supplemented with 50μgml^{-1} gentamycin sulphate and hypoxanthine) and electroporated in a 1cm cuvette. This was immediately mixed with 1ml of complete medium prewarmed to 37°C. 0.1ml aliquots were made into a microtitre plate and incubated at 37°C in a gas chamber (3%O_2, 2% CO_2, 95% N_2). Samples were periodically removed and thin smear slides examined.

A kill curve was produced and an arbitrary choice of a 50% kill was chosen to be the standard conditions for the experiments that followed. This was achieved by a first pulse of 700V for 2.77msec at 25μF and 99Ω, and a second pulse of 150V for 234msec at 25μF and 156Ω. These conditions appeared to consistently give a parasite survival of 50-55%. Although these determinations were made without plasmid DNA the

addition of plasmid DNA to the system was subsequently seen not to affect the parasite survival.

5.3.2 Detection of GFP fluorescence.

Chalfie *et al.*(1994) describe the detection of GFP fluorescence using a fluorescent microscope set up to detect fluorescein isothiocyanate (FITC) epifluorescence. To detect GFP fluorescence we used a Leitz Instruments microscope with a filter set for excitation at 450 - 490nm and emission at 520nm which was illuminated using a mercury lamp.

To establish that the microscope was able to detect GFP fluorescence *E.coli* were transformed with a TU#58 plasmid where GFP is under control of a T7 promoter (kindly made available by Douglas Prasher). TU#58 was transformed into the BL21 (DE3) pLysS strain of *E.coli* (2.2.6.3) Transformants were selected based on their ampicillin resistance (2.2.6.2) and grown overnight at 37°C in 5ml of LB supplemented with ampicillin to a concentration of 100μgml^{-1} (LB-amp). 2 samples of 200μl of the overnight culture were added to 5ml of LB-amp, of which 1 contained an inducer of T7 polymerase expression (0.1mM IPTG), and were grown for 4 hours at 37°C. 50μl of each culture was removed and placed on a slide under a coverslip and examined for GFP fluorescence, using the equipment described above. The GFP could only be observed in bacteria transformed with TU#58 and induced with IPTG (Figure 6).

Any attempts to fix the *E.coli* culture with methanol or acetone abolished the GFP luminescence. Chalfie *et al.* (1994) also note that some fixing agents abolish GFP luminescence. Gluteraldehyde or formaldehyde were recommended as possible alternatives to fix slides. However, in all experiments with pGFP2 the intraerythrocytic stage cultures were not fixed to maximise the chances of detecting GFP fluorescence.

The functionality of the GFP gene was tested by transforming pGFP2 into BL21 (DE3) pLysS, where, without an inducer, GFP fluorescence is detected (data not shown). This test was recommended by T.Wellems (*pers comm.*) who detected chloramphenicol acetyl transferase (CAT) activity in *E.coli* transformed with the CAT gene under the control of the *P.falciparum* HRP3 promoter.

Figure 6.

Confirmation of the ability to detect GFP fluorescence. 0.1mM IPTG induced or non induced TU#58 transformed *E.coli* (BL21 [DE3] pLysS) were visualised at a 100X magnification (see above).

a) IPTG induced *E.coli* visualised without the FITC filter set.

b) IPTG induced *E.coli* visualised with the FITC filter set.

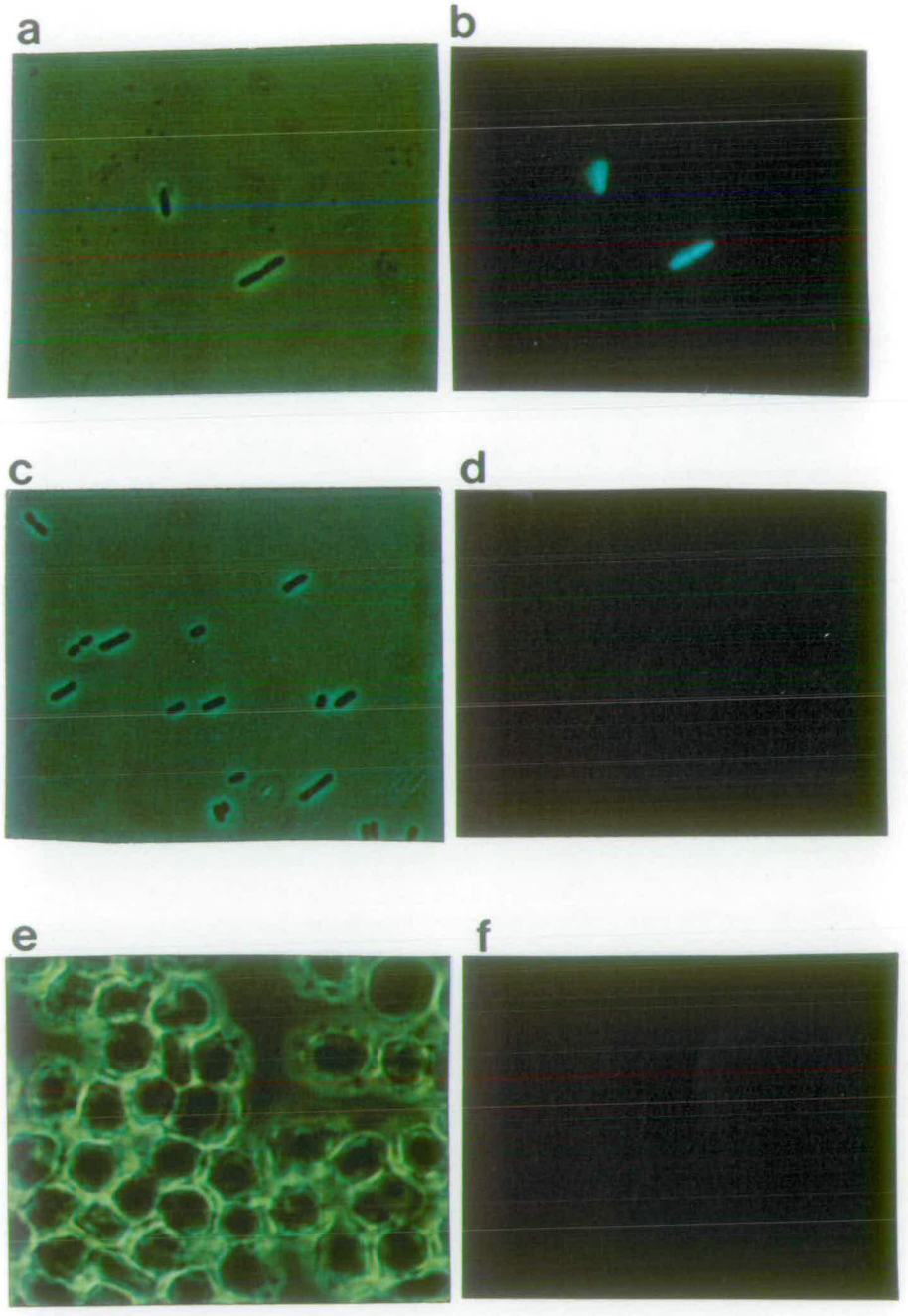
c) Uninduced *E.coli* visualised without the FITC filter set.

d) Uninduced *E.coli* visualised with the FITC filter set.

Intraerythrocytic stages of *P.falciparum* transfected with the pGFP2 construct 48 hours earlier.

e) visualised without the FITC filter set.

f) visualised with the FITC filter set.


a
b
c
d
e
f

5.4 Transfection of *P.falciparum* with pGFP2.

Using the standard conditions for double pulse electroporation and the protocol described above (5.3.1) an initial series of experiments was carried out with different amounts of plasmid DNA concentrations (0.1, 0.5, 1, 5, 20, 50 and 100μg). Samples were taken at a number of time points after electroporation (4, 8, 16, 24, 36 and 48 hours) and examined for GFP luminescence. In the absence of information regarding the transfection efficiency, between $4X10^3$ and $6X10^3$ parasites, per time point, were examined at a 100X magnification.

There was no clear evidence of GFP fluorescence, although some luminescence was observed. Using the fluorescein filter set, all fluorescence was green whether derived from GFP fluorescence, background autofluorescence from erythrocytes or parasites, or from debris. Possible cases of GFP fluorescence, characterised by a region of more intense fluorescence or fluorescence appearing to occupy all or part of an erythrocyte, were more carefully examined. In all cases the luminescence appeared to be associated with cell debris.

The manufacturer of the electroporation equipment recommended a variety of pulse parameters which may need to be optimised for the introduction of plasmid DNA. These variations in experimental conditions and other changes in the protocol were made.

a) *Variation in the intensity of the pulses.* Where the first pulse intensity was altered (500V-900V) a standard 150V second pulse was used. First pulses in excess of 900V were observed to be too high. Giemsa stained thin smears indicated not only a very low parasite survival rate but large numbers of lysed parasites. A standard first pulse of 700V was used while varying the second pulse intensity between 150V-300V. Variation of the second pulse did not appear to effect the parasite survival or erythrocyte viability.

b) *Increasing the length of the second pulse* between 150msec-2000msec. This did not appear to effect the parasite survival or erythrocyte viability.

c) *Changing the cuvette size* from a 1cm gap to a 0.4cm gap. This had the effect of increasing the intensity of the pulse as well as changing the

electric field within the cuvette. Using the same conditions for the two cuvette sizes it was observed that the parasite survival was much lower in the 0.4cm cuvette.

d) Using parasite cultures synchronised for rings, trophozoites or schizonts. These experiments indicated that trophozoites or schizonts were less likely to survive electroporation than the ring stage.

e) Increasing the frequency and overall time of parasite sampling. Time point samples were removed every 4 hours for the first 36 hours (except for an overnight time point) and every 8 hours until 76 hours.

f) Increasing the number of fields observed.

The modifications described above did not lead to the observation of GFP fluorescence, an example is shown in Figure 6.

There are several possible reasons why GFP fluorescence was not observed. These will be considered in more detail later, however, one obvious reason could be that the plasmid DNA does not reach the parasite, even though the changes in parameters a to c were made with this intention of minimising this problem. The parasite is protected by several plasma membranes (erythrocyte, parasitophorus vacuole and parasite membranes) through which the plasmid DNA must pass. A series of experiments was carried out to investigate how far the plasmid DNA did penetrate.

5.5 Plasmid DNA penetrates to the parasite.

5.5.1 Plasmid DNA passes through an uninfected erythrocyte plasma membrane on electroporation.

Before examining the question of how many of the membrane barriers plasmid DNA may cross, we considered the first membrane barrier, that of the erythrocyte itself. We wanted to know if plasmid DNA when electroporated would pass through an erythrocyte membrane or whether electrostatic forces, for example, would attach the plasmid DNA to the membrane. Uninfected erythrocytes were used in these experiments as we were interested in the behaviour of plasmid DNA when confronted by a single plasma membrane. We recognise that the plasma membrane of an

infected erythrocyte is modified and that this may alter the ability of plasmid DNA to pass through. The conclusions from this work were used to plan experiments with infected erythrocytes. However, we acknowledge that infected erythrocyte membranes, or any of the other membranes, may behave differently. In the final analysis, the questions we asked here had less importance than was anticipated.

a) Plasmid DNA doesn't adhere to the plasma membrane and can be easily removed by washing

20μg of plasmid DNA was added to 0.5ml of erythrocytes at a 5% haematocrit and incubated for 10 minutes at room temperature. The erythrocytes were washed 4 times with 10 volumes of ice-cold PBS. They were then subjected to phenol-chloroform extraction (2.2.1.6) and the aqueous phase used in a PCR (2.9.1) using oligonucleotides from the GFP ORF. The aqueous extract was also southern blotted (2.2.4) and a radiolabelled *Hin*dIII-*Eco*RI DNA fragment of the GFP gene used as a probe. Both the PCR and southern blot failed to indicate (Figure 7, lanes 1) any evidence of plasmid DNA either attached to or in the erythrocyte.

A second experiment with the same quantity of plasmid DNA and erythrocytes was prepared except that the mix was electroporated using the standard conditions described (5.3.1). The erythrocytes were again washed as described above to ensure that plasmid DNA surrounding the erythrocytes had been removed. The erythrocytes were then disrupted using a Potter homogeniser with a B type pestle. After 20 strokes the plasma membranes were isolated by centrifugation at 1000g for 20 minutes at 4°C. The pelleted plasma membranes were washed to ensure that DNA from the cytoplasm had been removed. The washed plasma membrane was then phenol-chloroform extracted and the aqueous phase analysed by PCR and southern blotting as previously described. This analysis (Figure 7, lanes 2) showed that plasmid DNA did not remain attached to the plasma membrane after electroporation.

b) 50μg of plasmid DNA should be used in an electroporation to facilitate its subsequent detection.

To demonstrate that the plasmid DNA is present within the cytoplasm of the erythrocyte and to have some indication of the optimal quantity of

plasmid DNA to use for transfection a series of electroporations with increasing amounts of plasmid DNA were carried out. The electroporations were made using 0, 1, 5, 20 and 50µg of plasmid DNA with 0.5ml of uninfected erythrocytes at a 5% haematocrit. The erythrocytes were washed and then subjected to phenol-chloroform extraction. The aqueous phase was subjected to PCR and southern analysis (Figure 7, lanes 3-7). The results of this analysis indicate that upwards of 50µg of plasmid DNA should be used to facilitate the detection of transfected plasmid DNA.

5.5.2 Electroporated plasmid DNA may enter the intracellular parasite.

Since it was established that plasmid DNA can pass through the plasma membrane of uninfected erythrocytes a second series of electroporations were used to establish whether the parasite contains plasmid DNA after electroporation.

Three experiments were carried using both uninfected and infected erythrocytes. 0.5ml of erythrocytes at a 5% haematocrit were used in all experiments. An asynchronous culture at approximately 10% parasitaemia was used for the infected erythrocytes. After the experiment the erythrocytes were extracted with phenol-chloroform and the aqueous extract used for PCR and southern analysis (5.5.1). All results are shown in Figure 8.

a) 50µg of pGFP2 was added but not electroporated (lanes 1 and 4).

b) no plasmid DNA was added but the erythrocytes were electroporated (lanes 2 and 5).

c) 50µg of pGFP2 was added and the erythrocytes were electroporated (lanes 3 and 6).

Two samples of infected erythrocytes were subjected to conditions c. The parasites were subsequently isolated from one sample by saponin lysis (2.2.1.1). They were washed to ensure that plasmid DNA derived from the erythrocyte cytoplasm had been removed and an aqueous extract from a phenol-chloroform extraction was subjected to PCR and southern analysis (lanes 7).

The results of this analysis show that only when plasmid DNA is added

Figure 7.

Southern blot and PCR analysis of nucleic acids isolated from electroporated uninfected erythrocytes. DNA markers sizes are indicated in kb.

PCR analysis results.

Isolated nucleic acids were used in a PCR with oligonucleotides GFP/F and GFP/R (expected product of 760bp indicated by an arrow). The PCR products were separated by agarose gel electrophoresis, blotted, and the membrane hybridised to with an end-labelled oligonucleotide GFP/I probe.

a) Ethidium bromide stained agarose gel.

b) Autoradiograph of the southern blot.

M. Φx174 *Hae*III DNA markers.

1. 20 μg pGFP2 added but not electroporated.
2. 20 μg pGFP2 added and electroporated, erythrocyte plasma membrane isolated.
3. 0μg pGFP2 added and electroporated.
4. 1μg pGFP2 added and electroporated.
5. 5μg pGFP2 added and electroporated.
6. 20μg pGFP2 added and electroporated.
7. 50μg pGFP2 added and electroporated.

Southern blotting analysis.

The nucleic acids isolated were separated by agarose gel electrophoresis, blotted, and hybridised to with an end labelled oligonucleotide GFP/I probe. The position of the 2.02 marker of λ *Hin*dIII/*Eco*RI is indicated by an arrow.

c) Ethidium bromide stained agarose gel.

d) Autoradiograph of the southern blot.

M. λ *Hin*dIII/*Eco*RI DNA markers.

Lanes 1 to 7 as described above.

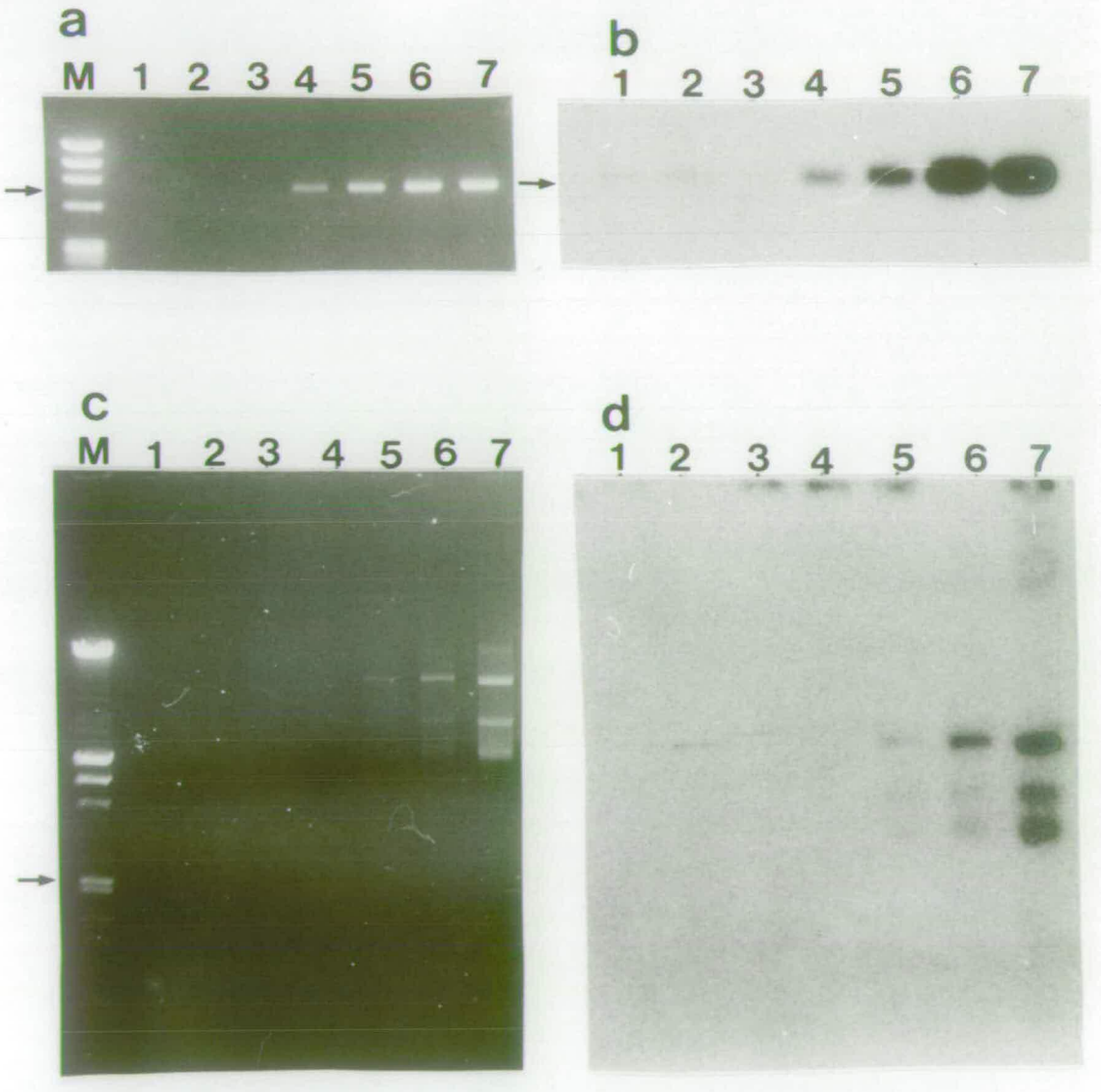

a
M 1 2 3 4 5 6 7
b
1 2 3 4 5 6 7
c
M 1 2 3 4 5 6 7
d
1 2 3 4 5 6 7

Figure 8.

PCR and southern blot analysis of the nucleic acids isolated from electroporated uninfected and infected erythrocytes. DNA marker sizes are indicated in kb.

PCR analysis results.

The isolated nucleic acid from each of the electroporations was used as a template in two PCRs (2.2.9.1). Oligonucleotides to the PfPCNA ORF; 533S (5' GGATTT AATTGTAACTCC 3') and 534S (5' GTCTATTGAATTGGACTC 3'), would give an expected product of 250bp (indicated by an arrow) which confirms the presence of *P.falciparum* gDNA. Oligonucleotides GFP/F and GFP/R (the expected PCR product of 760bp, indicated by an arrow) confirms the presence of pGFP2. The PCR products were separated by agarose gel electrophoresis, blotted and hybridised to an end-labelled oligonucleotide GFP/I probe.

a) Ethidium bromide stained agarose gel of PfPCNA PCR.

b) Ethidium bromide stained agarose gel of GFP PCR.

c) Autoradiograph of the southern blot of GFP PCR.

M. 100bp DNA markers.
1. 50μg pGFP2 added to uninfected erythrocytes but not electroporated.
2. No pGFP2 added to uninfected erythrocytes and electroporated.
3. 50μg pGFP2 added to uninfected erythrocytes and electroporated.
4. 50μg pGFP2 added to infected erythrocytes but not electroporated.
5. No pGFP2 added to infected erythrocytes and electroporated.
6. 50μg pGFP2 added to infected erythrocytes and electroporated.
7. 50μg pGFP2 added to infected erythrocytes and electroporated, the parasites were subsequently isolated.

Southern blotting analysis.

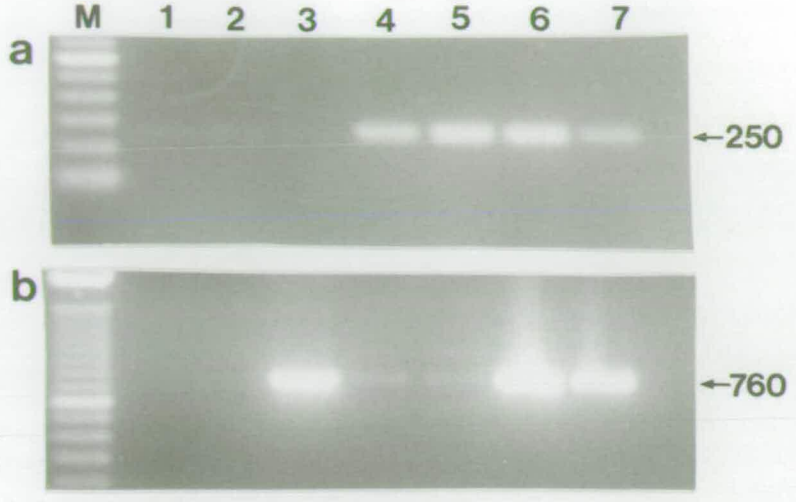
The nucleic acids isolated were separated by agarose gel electrophoresis, blotted and hybridised to with an end-labelled oligonucleotide GFP/I probe. The position of the 2.02kb marker of is λ *Hin*dIII /*Eco*RI indicated by an arrow.

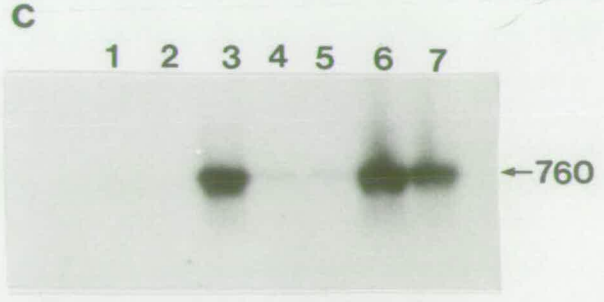
d) Ethidium bromide stained agarose gel.

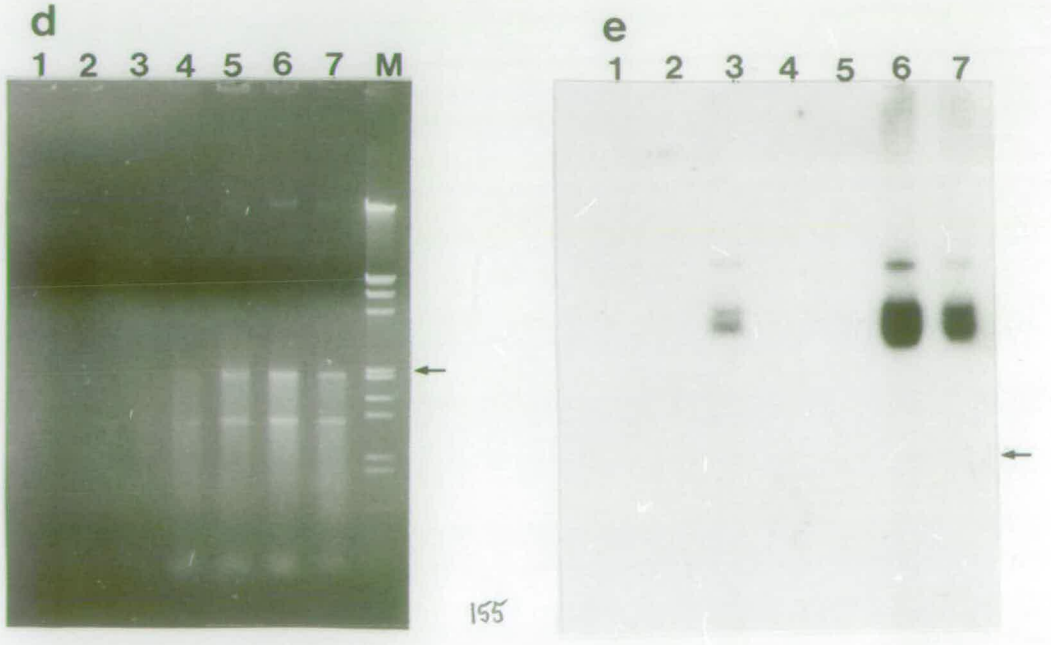
e) Autoradiograph of the southern blot.

M. λ *Hin*dIII /*Eco*RI DNA markers.

Lanes 1-7 as described above.


M 1 2 3 4 5 6 7
a
←250
b
←760


c
1 2 3 4 5 6 7
←760


d
1 2 3 4 5 6 7 M
e
1 2 3 4 5 6 7

and electroporated can plasmid DNA be subsequently detected at least within the erythrocyte cytoplasm. The isolated parasite also contains plasmid DNA indicating that plasmid DNA must at least reach the surface of the parasite.

5.5.3 Micrococcal nuclease treatment of isolated parasite nuclei indicates that plasmid DNA penetrates into the parasite.

Micrococcal nuclease can enter intact nuclei and digest DNA not protected by its association with nucleosomes. Chromosomal DNA is wrapped around a succession of nucleosomes so that when digested incompletely by micrococcal nuclease a characteristic ladder pattern can be identified. Cary *et al.* (1994) showed that the chromosomal DNA of the asexual stage of *P.falciparum* is associated with nucleosomes using this method. Using micrococcal nuclease on nuclei isolated from electroporated parasites we asked whether the plasmid DNA was present in the nucleus, and if so, was it associated with nucleosomes?

50μg of pGFP2 was electroporated in to 5 samples of 0.5ml of infected erythrocytes (5% haematocrit) at approximately 10% parasitaemia. The cultures were incubated for 6 hours at 37°C to allow any nucleosomes to associate with the plasmid DNA (Mertz, 1982). The parasite nuclei were isolated and the nuclei treated with micrococcal nuclease (2.2.10) for either 0, 1, 2, 5 and 15 minutes. The isolated DNA was separated by agarose gel electrophoresis, blotted and probed using a random-labelled *Hin*dIII/*Eco*RI DNA fragment containing the GFP ORF (Figure 9). The ethidium bromide stained agarose gel shows a faint pattern characteristic of nucleosome bound chromosomal DNA. The southern blot analysis shows that, although the plasmid DNA must, at least, reach the surface of the parasite's nucleus after electroporation, there is no evidence that it enters to become associated with nucleosomes.

5.6 Single pulse electroporation.

During the course of this work a protocol describing the introduction of plasmid DNA into the asexual stages of *P.falciparum* was described by Wu *et al.* (1995). The introduction of a chloramphenicol acetyl transferase

Figure 9.

Results of the micrococcal nuclease treatment of nuclei isolated from pGFP2 transfected infected erythrocytes. The isolated nuclei from infected erythrocytes electroporated with 50μg of pGFP2 were treated with micrococcal nuclease for 0, 1, 2, 5 and 15 minutes. The isolated DNA was separated by agarose gel electrophoresis, blotted and hybridised to with a random-labelled *Eco*RI/*Hin*dIII DNA fragment containing the GFP ORF. The position of the 2.02kb marker (λ *Hin*dIII/*Eco*RI) and the 1.35kb marker (Φx174 *Hae*III) are indicated by arrows.

a) Ethidium bromide stained agarose gel.
b) Autoradiograph of the southern blot.

M. λ *Hin*dIII/*Eco*RI DNA markers.
1. 0 minutes of micrococcal nuclease digestion.
2. 1 minute of micrococcal nuclease digestion.
3. 2 minutes of micrococcal nuclease digestion.
4. 5 minutes of micrococcal nuclease digestion.
5. 15 minutes of micrococcal nuclease digestion.
m. Φx174 *Hae*III DNA markers.

reporter gene was made using a single pulse electroporator. Using an adaptation of her original protocol (2.4.2) 60μg of pGFP2 was electroporated into approximately $2X10^8$ parasites. The electroporated parasites were harvested at 24h and 48h after electroporation and examined for GFP fluorescence, no GFP fluorescence was observed.

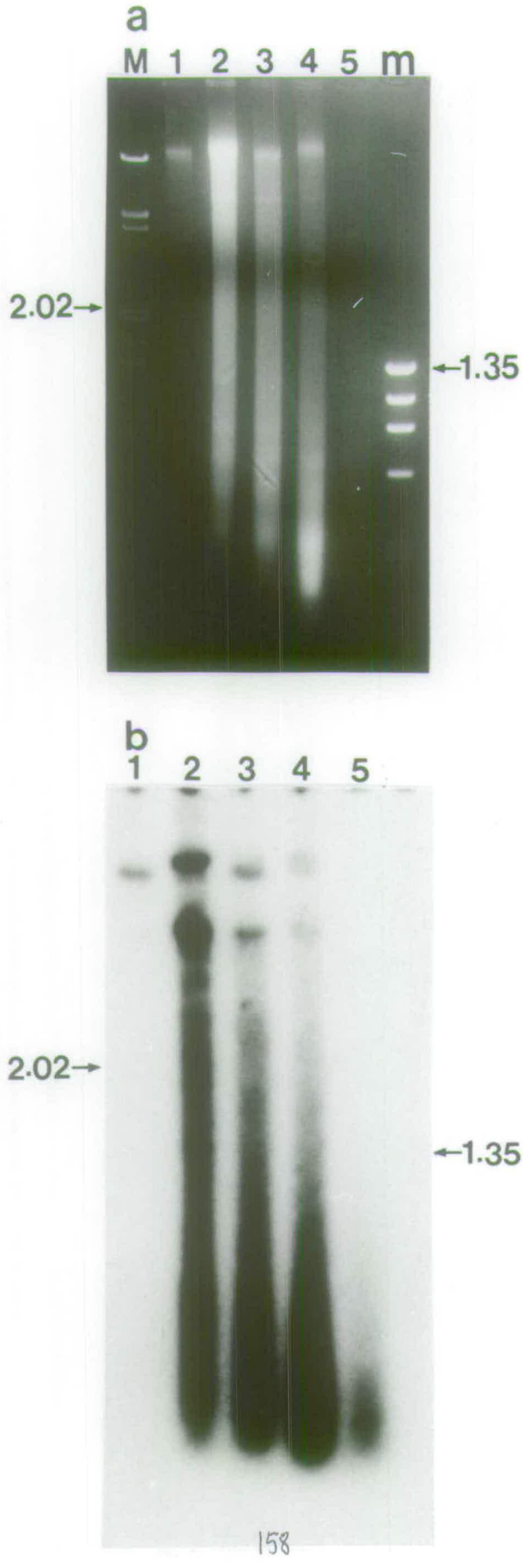
5.7 Discussion.

The possibility that the failure to observe GFP fluorescence resulted from a failure of the plasmid DNA to penetrate the parasite has been examined. These experiments show that, after electroporation, plasmid DNA penetrates at least to the parasite cytoplasm. Other work in this thesis and work carried out elsewhere (Wu *et al*, 1995, 1996) confirms that the introduction of plasmid DNA into intraerythrocytic stage cultures can be reliably carried out and failure to detect GFP fluorescence here seems unlikely to be the consequence of failure of transfection.

The functional integrity of the construct was tested by demonstrating that, even without an *E.coli* promoter, sufficient expression of GFP occurs in *E.coli* for activity to be detected. A similar observation has been made by T. Wellems (*pers comm.*) using a construct with a CAT reporter gene. This expression could be due to "leaky" expression of T7 RNA polymerase in the BL21 (DE3) pLysS strain or a cryptic bacterial promoter in either the PfPCNA 5′ flanking sequence or plasmid backbone.

No attempt was made to ascertain whether the GFP polypeptide or transcript were being produced in the transfected parasites. Techniques to detect transcripts, such as northern analysis or RT-PCR, would be hampered by the small quantities of transfected culture, and the large quantities of plasmid DNA that would contaminate the RT-PCR. GFP has not been detected, using commercially available anti-GFP antibodies, from *P.falciparum* transfected with a GFP construct known to be capable of expressing a reporter gene (Goodyeri, I and Taraschi, T., *pers comm.*).

The 5′ flanking DNA from PfPCNA used in the construct has been shown elsewhere in this thesis to be sufficient for promoter activity. The level of activity of *Plasmodium* promoters in intraerythroctyic stage cultures


a
M 1 2 3 4 5 m
2.02
1.35
b
1 2 3 4 5
2.02
1.35

appears to be up to 1000 fold less than their activity in zygotes and ookinetes (Wirth, D., *pers comm.*). It is possible that the levels of GFP expressed are so low that they are undetectable using the fluorescence microscope.

The regulation of expression of a protein may be brought about by variation in the stability, and therefore the turnover rates, of either the transcripts or polypeptides, or even both. To date neither issue appears to have been properly addressed for GFP. Since the PfPCNA 5′ sequence flanking GFP has been subsequently successfully utilised to transiently express firefly luciferase (Chapter 6) it seems unlikely that a failure to detect GFP luminescence in transfected parasites is the result of transcript instability brought about by a signal in this region. It is possible, however, that there may be elements within either the GFP ORF or the PfPCNA 3′ flanking sequence which are responsible for degradation.

Codon usage bias have been proposed as a reason for the low levels of expression of *Plasmodium* genes in heterologous systems. The reverse may also be true; heterologous genes may be difficult to express in *Plasmodium*. A comparison of the codon usage in *Plasmodium* (Saul and Battistutta, 1988) to that of the ORFs of firefly luciferase (X65325) and GFP (M62653) was made. This comparison revealed that there were regions in both ORFs where several rare codons are clustered close together. However, there appeared to be more of them in the luciferase ORF. Although a case for codon usage problem could not be made based on this quick visual comparison, it would only require a single "problem" codon in the GFP ORF to prevent expression.

Molecular oxygen is required for the formation of the GFP chromophore (Hiem *et al.*, 1994). The position regarding the oxygen tension in *Plasmodium* is the subject of debate as it is uncertain whether the parasite is a facultative anaeobe or a microaerophile. The asexual culture conditions originally described by Trager and Jensen (1979) establish a low O_2 concentration (3%) for optimal growth, with levels as low as 0.5% being tolerated. Parasites survive sequestration in sites of low oxygen tension (eg. post capillary venuoles) surrounded by large quantities of haemoglobin. Attempts to force up the molecular oxygen level, by

increasing the O_2 concentration in culture, does not facilitate GFP luminescence (Goodyeri, I. and Tarasche, T., *pers comm.*). The possibility that low oxygen tension within the parasite is responsible for the lack of GFP luminescence remains, at present, unresolved. As it was, work with other transient transfection reporter systems overtook the work with GFP.

Chapter 6.

Summary.

The intraerythrocytic stages of *P.falciparum* were transiently transfected with constructs containing a firefly luciferase reporter gene under the transcriptional control of variously modified elements of the PfPCNA 5′ flanking sequence to test for transient promoter activity. This analysis identified an essential region for promoter activity which contains the physically mapped transcription start sites. In addition, a region between 290bp-620bp upstream of the major transcription start site, containing a number of short GC-rich sequences, is required for efficient promoter activity.

6.1 Introduction.

The introduction of novel DNA into the genome facilitates an in depth analysis of gene function and expression. Over recent years several attempts to develop a transfection system for *Plasmodium* have failed. To ensure that enough flanking sequences are available to flank a reporter gene long tracts of AT-rich DNA have to be used which make the plasmid unstable in *Escherichia coli*. Extracellular *Plasmodium* parasites are not readily available in the large quantities required for transfection. The only stages of *P.falciparum* that can be cultured in reasonable volumes are protected by multiple membrane barriers within an erythrocyte. Attempts to introduce plasmid DNA into *Plasmodium* have included the use of the biolistic gun, lipofectin and the electroporator (Hyde, 1996, Roberts, 1994).
The first successful report of the transfection of *Plasmodium* was made when a firefly luciferase reporter gene was introduced into the gametes and zygotes of *P.gallinaceum* by electroporation (Goonewardene *et al.*, 1993). The luciferase open reading frame was inserted into the sexual stage specific pgs28 gene flanked by 2.5kb of endogenous DNA. Ookinetes harvested 24 hours later contained readily detectable levels of luciferase.
The introduction of plasmid DNA into *P.falciparum* was first described by Wu *et al.* (1995). Plasmid DNA, containing the chloramphenicol acetyl transferase (CAT) reporter gene, was introduced by electroporation into

the intraerythrocytic stages. In these experiments the expression of the CAT reporter gene was under the control of either a *HRP3* or *hsp86* 5′ flanking sequence and *HRP2* 3′ flanking sequence. The firefly luciferase reporter gene has also been succesfully expressed in *P.falciparum* intrerythrocytic stages under the control of the *HRP3* 5′ flanking sequence (Wu *et al.*, 1995).

Stable transfection of *Plasmodium* was achieved using a mutant dihydrofolate reductase-thymidylate synthase gene (*dhfr-ts*) which confers resistance to the drug, pyrimethamine (van Dijk *et al.*, 1995, 1996, Wu *et al.*, 1996, Crabb and Cowman, 1996). The first report of stable transfection of *Plasmodium* was made when a *dhfr-ts* gene under the control of 3.5kb of endogenous flanking DNA was electroporated into *P.berghei* merozoites (van Dijk *et al*, 1995). It was subsequently shown that resistance to pyrimethamine, in cloned intraerythrocytic stage parasites, was due to up to 15 copies of the episomally maintained plasmid. When 2.2kb of a 2.3kb sub-telomeric repeat was added to the transfection construct the plasmid integrates within the sub-telomeric region and is stably maintained for at least 70 generations (van Dijk *et al.*, 1996).

Using mutant *dhfr-ts* genes from both *P.falciparum* and *Toxoplasma gondii*, under the control of 5′ flanking sequences from both *P.falciparum* and *P.chabaudi* genes, stable transformations of *P.falciparum* intraerythrocytic stages have been made (Wu *et al.*, 1996, Crabb and Cowman, 1996). Analysis indicates that the plasmids are initially maintained episomally, but after several months of drug selection the plasmid DNA integrates into the genome. Integration appears to be site specific and requires as little as 600bp of homologous sequence (Wu *et al.*, 1996).

Analysis of gene expression in *Plasmodium* is already underway. Wu *et al.* (1995) demonstrated that the deletion of a 560bp region most proximal to the ORF of the *HRP3* 5′ flanking sequence decreased but did not abolish the promoter activity. Crabb and Cowman (1996) using a series of deletions have identified enhancer regions in the 5′ flanking sequences of the *P.falciparum dhfr-ts* and calmodulin genes and the P.*chabaudi* dhfr-ts gene. Analysis of the 5′ flanking sequences of the pgs28 gene of

P.gallinaceum has identified a region of approximately 250bp which is essential for promoter activity (Wirth, D. *pers comm.*). Deletion of sequences upstream of this essential region appears to disrupt the stage specific expression of the pgs28 gene. This data lends credence to the idea that *Plasmodium* promoters consist of a core promoter proximal to the transcription start site with stage- and sexual- specific elements further upstream (Wirth, 1995).

Here we report the analysis of the PfPCNA 5′ flanking sequence using the firefly luciferase reporter gene. Firefly (*Photinus pyralis*) luciferase is a 61kDa monomeric protein that does not require any post-translational modification for enzymatic activity (de Wet *et al.*, 1985, Wood *et al.*, 1984). The relatively short half life of luciferase and the ease of detection of the protein's activity makes it a useful indicator of promoter activity.

6.2 Construction of vectors used in transient transfection analysis.

The firefly luciferase (*luc*) open reading frame was supplied in the construct, pHLH1 (Figure 1a), originally described by Wu *et al.* (1995). The reporter gene's expression is under the control of *HRP*3 5′ flanking sequence and *HRP*2 3′ flanking sequence. In the construction of the various vectors used for transient transfection analysis the *luc* gene and *HRP*2 3′ flanking sequence were unmodified.

All constructs were checked by restriction and southern analysis. A complete set of autoradiographs are presented for pPLH1. However, for the remaining constructs, except for the example of pPLH1ΔB, the autoradiographs have been omitted with only the photograph of the restricted DNA presented.

6.2.1 Replacement of pHLH1 *HRP*3 5′ flanking sequence with PfPCNA 5′ flanking sequence to give pPLH1.

PCR with the primers 5′-CCCGGTACCGAATTCTTTATTTTTACAC-3′ (L) and 5′-CCCCCATGCATTTTTTAATTAAGCTTTTTATG-3′ (N7687, R) was used to amplify the 1.58kbp PfPCNA flanking sequence from the plasmid 127-4 (Kilbey *et al.*, 1993). The PCR product contained 2 further *Nsi*I

restriction sites to that introduced by oligonucleotide N7687. The PCR product was initially restricted with *Kpn*I for 1 hour in "1-phor-all" buffer (2.2.2) then restricted with *Nsi*I for 1, 3 and 5 minutes. The products of the restriction were fractionated using agarose gel electrophoresis and products of approximately 1.58kb purified by electroelution (2.2.3.6). The *Kpn*I-*Nsi*I DNA fragment containing the *HRP*3 5′ untranslated region from the plasmid pHLH1 was replaced with *Kpn*I-*Nsi*I restricted PCR product to give pPLH1 (Figure 1b). The PfPCNA 5′ flanking sequence was completely sequenced (2.2.8) to ensure no mutations had been introduced during amplification. The construction of pPLH1 was confirmed by restriction and southern analysis (Figures 2a & 3). The addition of the *Nsi*I site at the PfPCNA 5′ flanking-*luc* gene boundary introduces a histidine encoding codon immediately after the start codon, this has no effect on the enzymatic activity of luciferase (Wu *et al.*, 1995).

6.2.2 Partial *Nsi*I restriction of pPLH1 to give the deletion constructs pPLH1ΔB, pPLH1ΔC and pPLH1ΔBC.

Restriction of the 5′PfPCNA 5′ flanking sequence with *Nsi*I was used to remove either, or both, of the restriction fragments delineated by these sites (Figure 2a & j). pPLH1 was restricted with *Nsi*I under conditions which produced incomplete restriction. The length of restriction time, limitation of *Nsi*I concentration and restriction at 25°C were all conditions used. The products of restriction were size fractionated by agarose gel electrophoresis and DNA fragments greater than 5kb in size purified by electroelution (2.2.3.6). The purified fragments were ligated (2.2.5) and transformed into *E.coli* (2.2.6). The DNA from transformants was restricted with *Nsi*I to identify clones of interest. Clones lacking either, or both, the 800bp or 400bp *Nsi*I restriction fragments were selected for a more complete restriction analysis (Figures 2 b-d, j & 4). The *Nsi*I restriction site junctions were checked by sequencing (2.2.8).

6.2.3 Inversion of the central 800bp *Nsi*I restriction fragment.

Inversion of the 800bp *Nsi*I restriction fragment (B) was complicated by the presence of the *Nsi*I site at the 3′ end of the PfPCNA flanking sequence

Figure 1a.

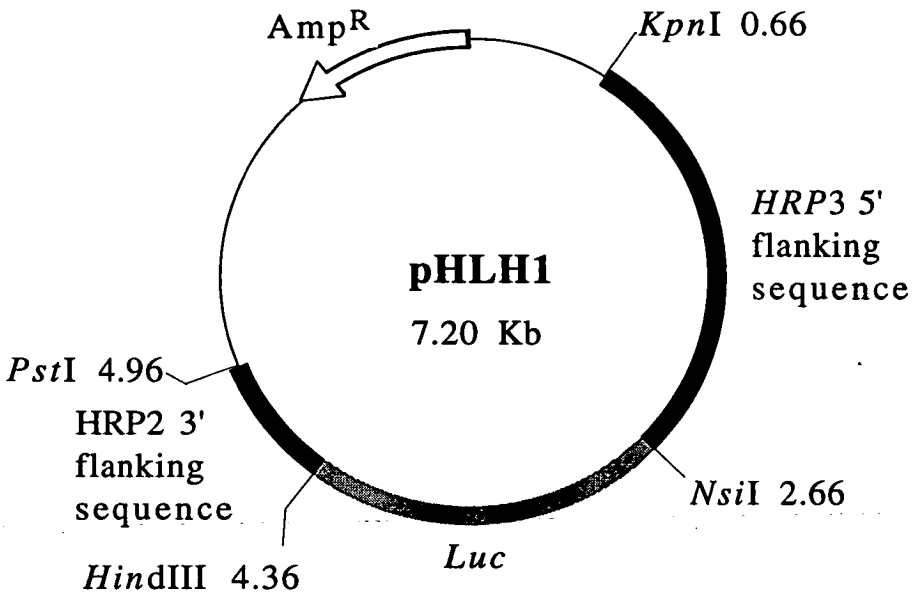
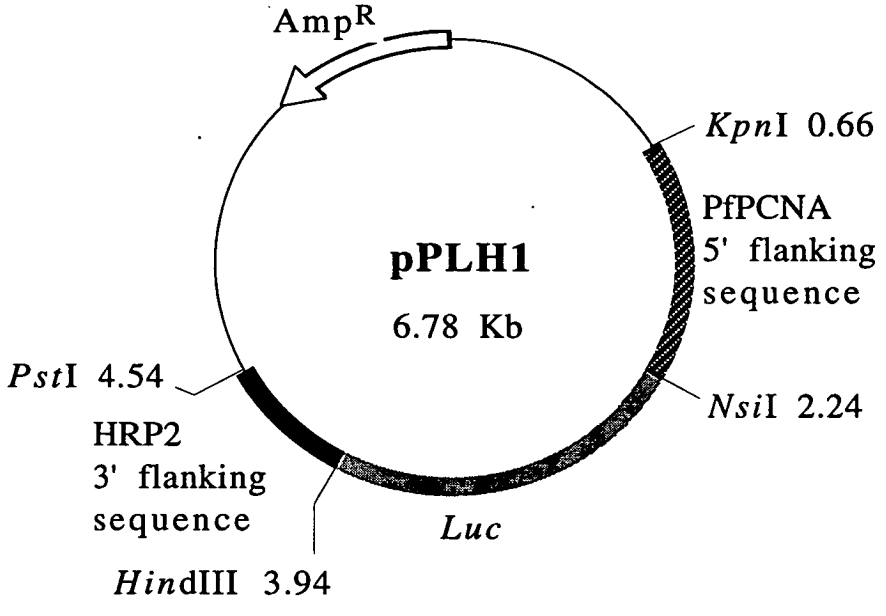


Figure 1b.



Sizes are indicated in kb from an arbitrary point 0 on the pBluescript II SK+ vector (Stratagene). Amp^R, ampicillin resistance gene; *Luc*, luciferase open reading frame.

(Figures 2a & j). *Hind*III restriction was therefore used to remove a 1.7kb DNA fragment containing *luc* to give an intermediate construct, pINT (Figure 2h), removing the *Nsi*I site at the start of the *luc* gene. Complete *Nsi*I restriction was used to remove an 800bp DNA fragment from the centre of the PfPCNA 5′ flanking sequence to give pINTΔB (Figure 2i). The 800bp DNA fragment was reinserted into pINTΔB and screened for the inverted orientation by PCR analysis (Figure 5) and checked by sequencing across the *Nsi*I junctions. pINTΔB was restricted with *Hind*III and the 1.7kb *Hind*III DNA fragment, containing the *luc* gene, reinserted in the correct orientation. The PfPCNA 5′ flanking sequence-*luc* boundary of pPLH1(R) was checked by restriction (Figures 2e, j & 4) and sequence analysis.

6.2.4 Deletion from the extreme 5′ end of the PfPCNA 5′ flanking sequence.

We wished to obtain a number of deletions, of increasing size, at the 5′ end of the PfPCNA 5′ flanking sequence. Initial attempts with a method using *Bal*31 (Sambrook *et al.*, 1989) were unsuccessful as the digested products could not be recovered in the transfection vector. Attempts to engineer pPLH1 to facilitate *Exo*III deletions were also unsuccessful, only rearranged constructs were recovered after transformation of the ligations into *E.coli*.

We decided to use PCR amplification of the PfPCNA 5′ flanking sequences of interest. Oligonucleotides containing suitable restrictions sites for insertion into pINT (6.2.3) were used to overcome any potential problems in cloning regions containing multiple *Nsi*I sites. PCR with the primers 5′-CCCGGTACCGGGTGCTTTTAAAAGAATGG-3′ (L) or 5′-CCGGTACCG AATTACACATAAGTTATAC-3′ (L) and N7687 (R) with the p127-4 plasmid gave products of 1.25kb and 710bp, respectively. The *Kpn*I-*Hind*III PfPCNA 5′ flanking sequence from pINT was replaced with *Kpn*I-*Hind*III restricted PCR products. The 1.7kb DNA fragment containing the *luc* gene was reinserted, in the correct orientation, to give the constructs pPLH1Δ330 and pPLH1Δ870 (Figures 2 e-f & j). The PfPCNA 5′ flanking

Figure 2a-i.

Schematics representing the pPLH1 series of transfection constructs and their derivatives used in their construction . All figures are drawn to scale and indicate the positions of restriction enzyme restriction sites used in either the construction of the plasmid or the confirmation of its construction. Oligonucleotide binding sites, used in southern analysis of constructs, are also indicated. 949T, 5′ CTATACAGAGAGCCAAAT 3′; 117V, 5′ GAATTACACATAAGTTATAC 3′; 543T, 5′ TGACAATTT TCTAATAGC 3′; P0901, 5′ GCAGTTGCTCTCCAGCGG 3′; Uni, 5′ TGAAAACGACGGCCATG 3′.

a. pPLH1
b. pPLH1ΔBC
c. pPLH1ΔB
d. pPLH1ΔC
e. pPLH1 (R)
f. pPLH1Δ330
g. pPLH1Δ870
h. pINT
i. pINTΔ

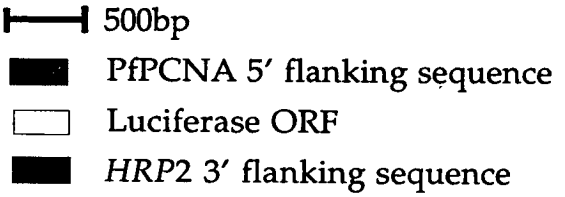
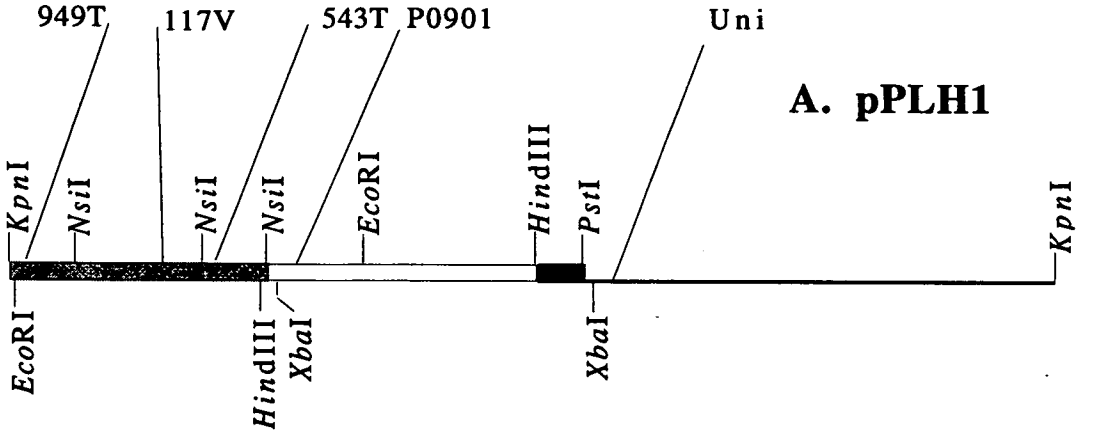
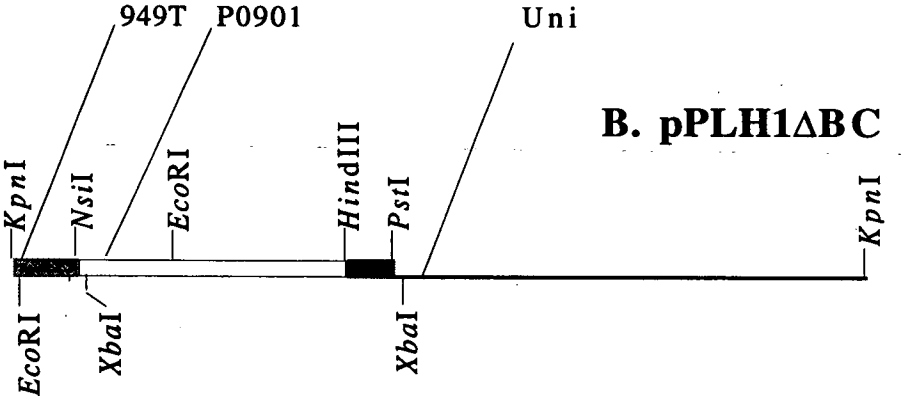
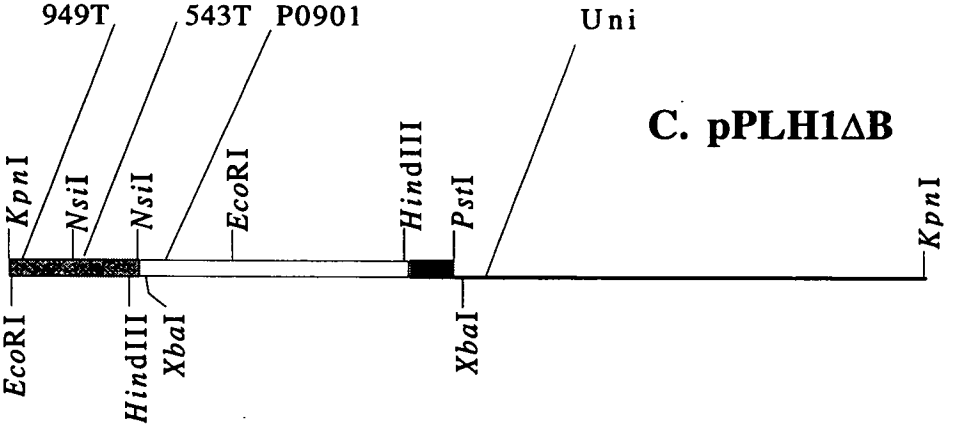


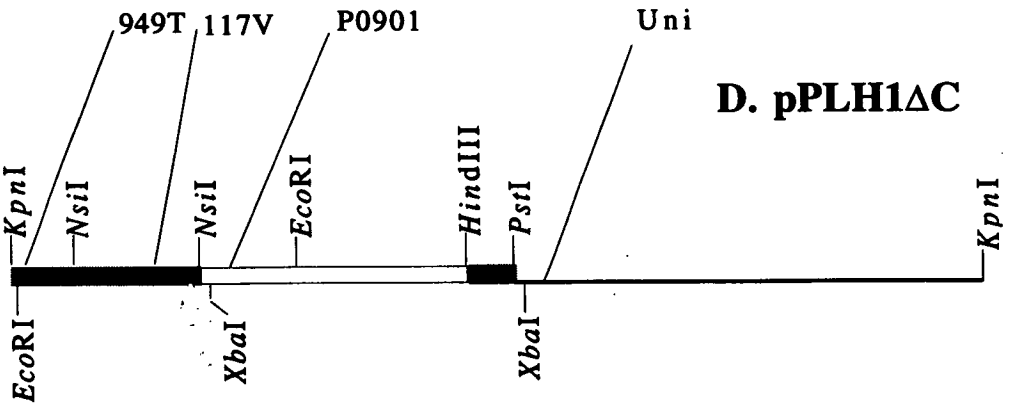
Figure 2j.

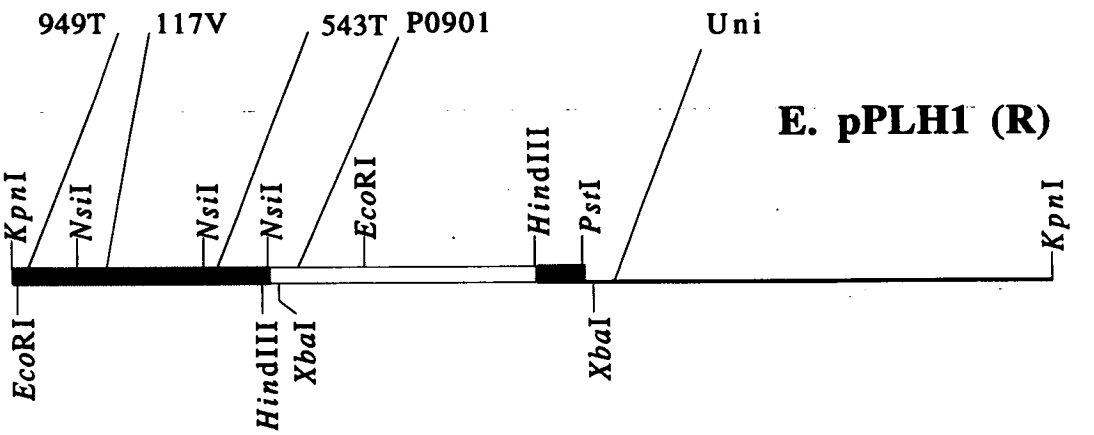
Schematic indicating the modifications made to the PfPCNA 5′ flanking regions. The positions of the endonuclease restriction sites discussed in the text are indicated.

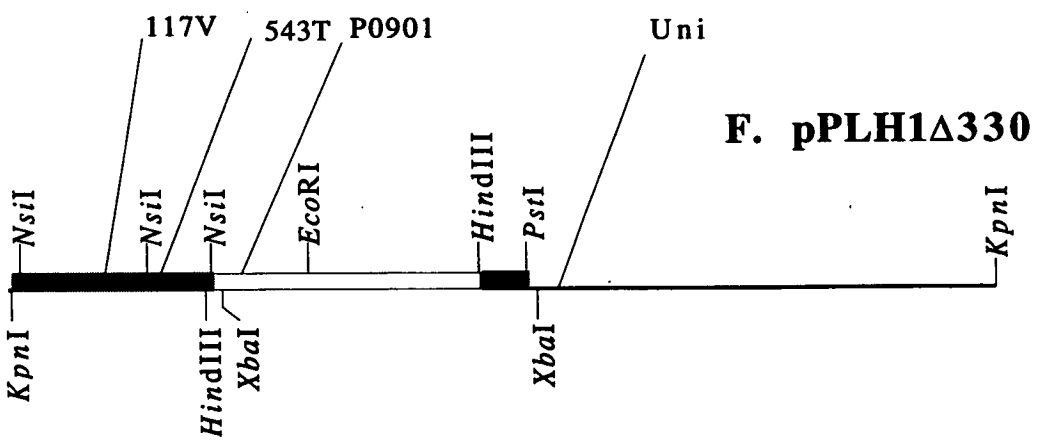

949T
117V
543T
P0901
Uni
A. pPLH1
KpnI
NsiI
NsiI
NsiI
EcoRI
HindIII
PstI
KpnI
EcoRI
HindIII
XbaI
XbaI

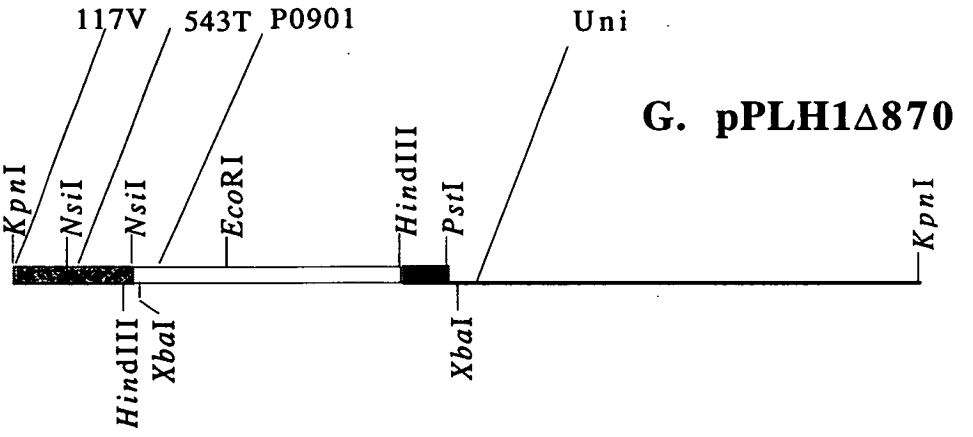

949T
P0901
Uni
B. pPLH1ΔBC
KpnI
NsiI
EcoRI
HindIII
PstI
KpnI
EcoRI
XbaI
XbaI


949T
543T
P0901
Uni
C. pPLH1ΔB
KpnI
NsiI
NsiI
EcoRI
HindIII
PstI
KpnI
EcoRI
HindIII
XbaI
XbaI

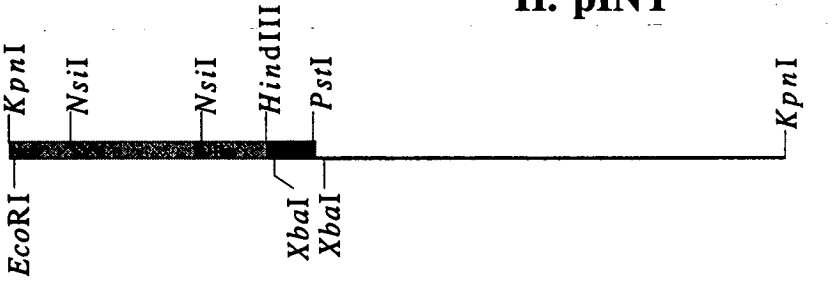

949T
117V
P0901
Uni
D. pPLH1ΔC
KpnI
NsiI
NsiI
EcoRI
HindIII
PstI
KpnI
EcoRI
XbaI
XbaI


949T
117V
543T
P0901
Uni
E. pPLH1 (R)
KpnI
NsiI
NsiI
NsiI
EcoRI
HindIII
PstI
KpnI
EcoRI
HindIII
XbaI
XbaI

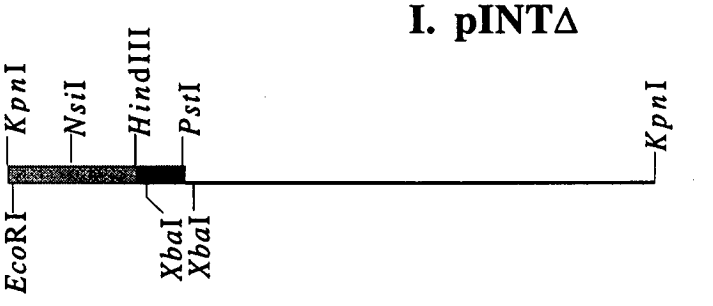

117V
543T
P0901
Uni
F. pPLH1Δ330
NsiI
NsiI
NsiI
EcoRI
HindIII
PstI
KpnI
KpnI
HindIII
XbaI
XbaI



G. pPLH1Δ870



H. pINT



I. pINTΔ

Figure 2j.

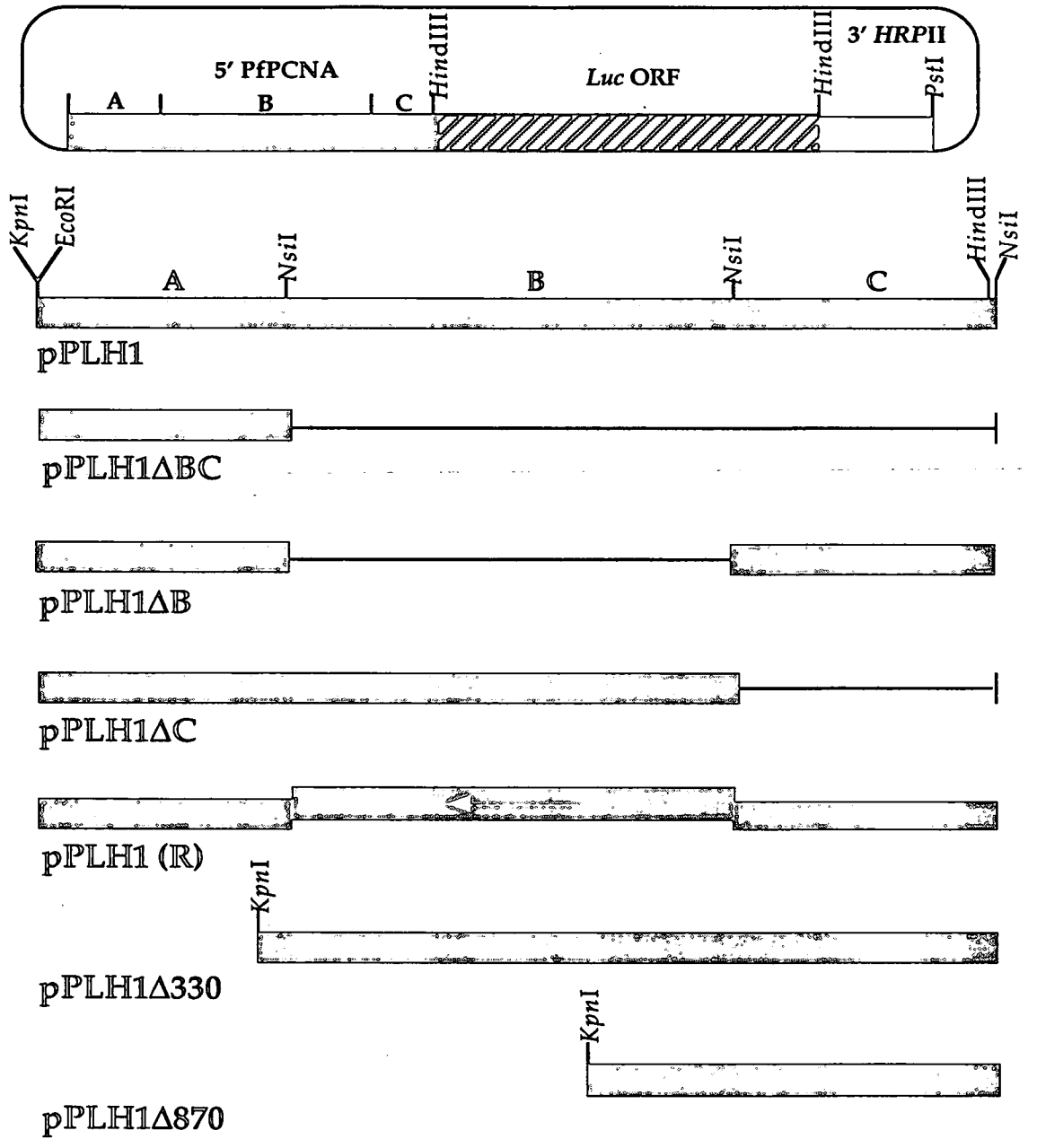

5′ PfPCNA
A
B
C
HindIII
Luc ORF
HindIII
3′ HRPII
PstI
KpnI
EcoRI
NsiI
A
NsiI
B
NsiI
C
HindIII
NsiI
pPLH1
pPLH1ΔBC
pPLH1ΔB
pPLH1ΔC
pPLH1 (R)
KpnI
pPLH1Δ330
KpnI
pPLH1Δ870

Figure 3.

Restriction and southern analysis of pPLH1. The restriction map indicating the positions of the oligonucleotide binding sites are indicated in figure 2a. The restriction fragments were size fractionated by agarose gel electrophoresis, blotted and hybridised to a range of end-labelled oligonucleotide probes.

Restriction analysis.

The 1.6kb marker of the 1kb marker (Gibco-BRL) range is indicated by an arrow.

a) ethidium bromide stained agarose gel. The 1.6kb DNA fragment of the 1kb marker range (Gibco-BRL) is indicated by an arrow.

Southern analysis.

The positions of the 1, 2 and 3kb DNA fragments are indicated.

b) autoradiograph using the 949T end-labelled oligonucleotide probe.

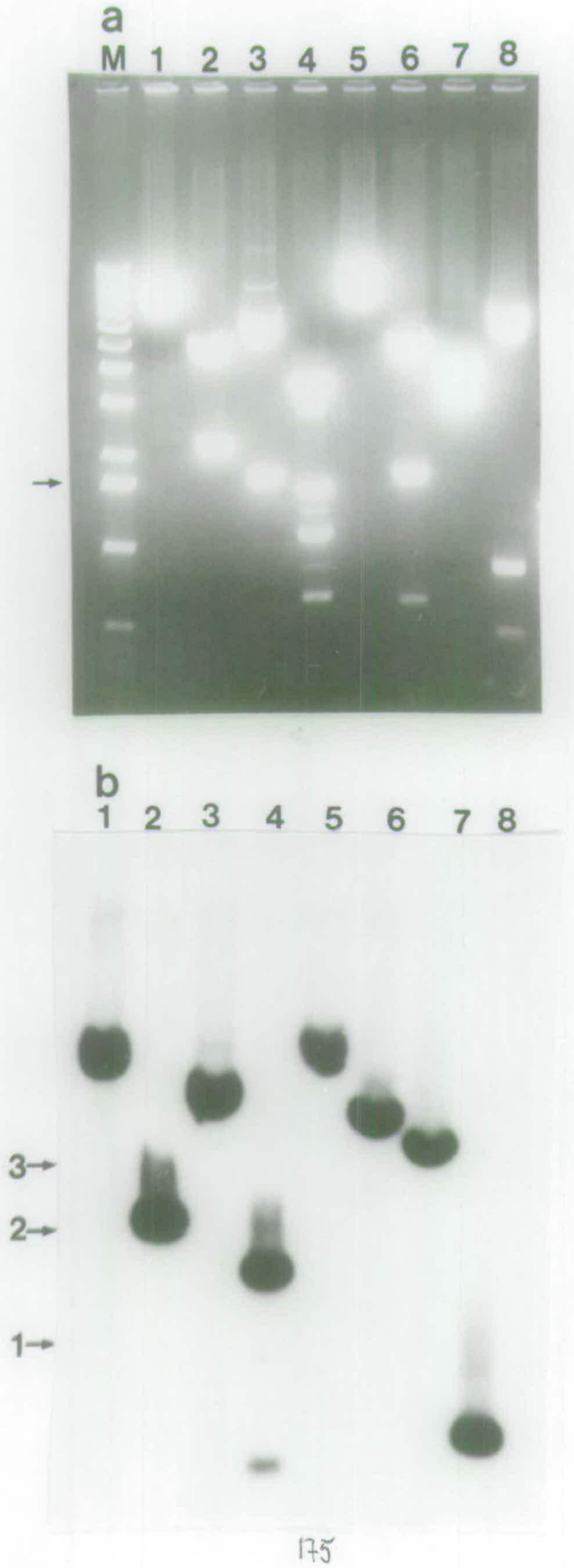
c) autoradiograph using the 117V end-labelled oligonucleotide probe.

d) autoradiograph using the 543T end-labelled oligonucleotide probe.

e) autoradiograph using the P0901 end-labelled oligonucleotide probe.

f) autoradiograph using the Uni end-labelled oligonucleotide probe.

M. 1kb markers
1. *Kpn*I
2. *Eco*RI
3. *Hin*dIII
4. *Eco*RI/*Hin*dIII
5. *Pst*I
6. *Pst*I/*Hin*dIII
7. *Kpn*I/*Hin*dIII
8. *Kpn*I/*Nsi*I


a
M 1 2 3 4 5 6 7 8
b
1 2 3 4 5 6 7 8
3
2
1

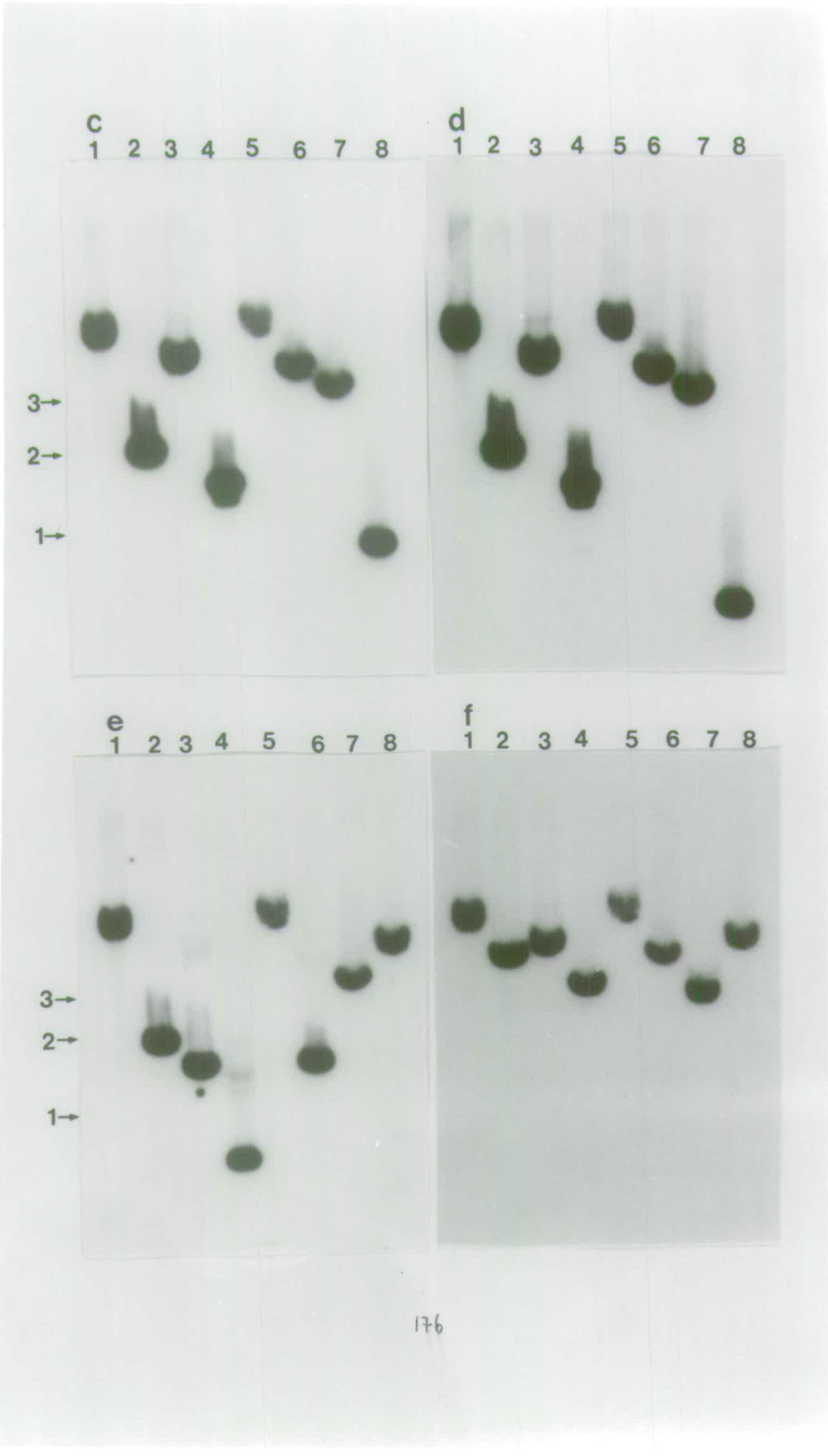

c
1 2 3 4 5 6 7 8
d
1 2 3 4 5 6 7 8
3→
2→
1→
e
1 2 3 4 5 6 7 8
f
1 2 3 4 5 6 7 8
3→
2→
1→

Figure 4.

Restriction and southern analysis of the pPLH1 derivatives; pPLH1ΔBC, pPLH1ΔB, pPLH1ΔC, pPLH1 (R), pPLH1Δ330 and pPLH1Δ870. The restriction map indicating the positions of the oligonucleotide binding sites are indicated in figure 2b-g. For each construct the restriction fragments were sizw fractionated by agarose gel electrophoresis, blotted and hybridised to a range of end-labelled oligonucleotide probes. In this figure the data from the restriction analysis is presented with a representative set of autoradiographs from the southern analysis of the restricted construct pPLH1ΔB.

Restriction analysis.

All photographs have the position of the 1.6kb marker, from the 1kb marker range (Gibco-BRL), indicated.

a) pPLH1ΔBC

b) pPLH1ΔB

c) pPLH1ΔC

d) pPLH1 (R)

e) pPLH1Δ330

f) pPLH1Δ870

Southern analysis of pPLH1ΔBC.

The position of the 1, 2 and 3kb markers are indicated.

g) autoradiograph using the 117 end-labelled oligonucleotide probe.

h) autoradiograph using the 949T end-labelled oligonucleotide probe.

i) autoradiograph using the 543T end-labelled oligonucleotide probe.

j) autoradiograph using the P0901 end-labelled oligonucleotide probe.

M. 1kb markers.

1. *Kpn*I

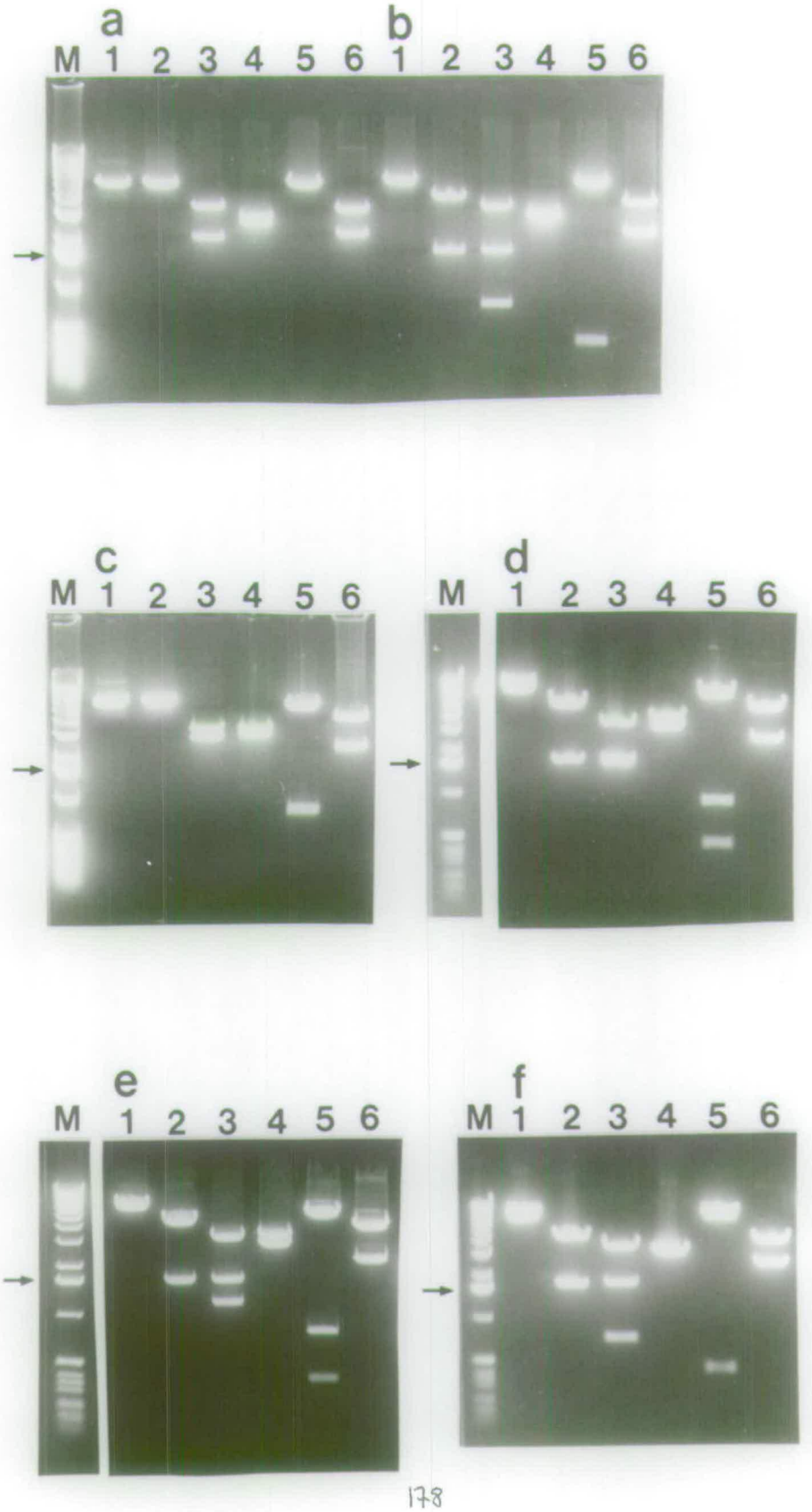
2. *Hin*dIII

3. *Kpn*I/*Hin*dIII

4. *Kpn*I/*Pst*I

5. *Nsi*I

6. *Xho*I


a
M 1 2 3 4 5 6
b
1 2 3 4 5 6
c
M 1 2 3 4 5 6
M
d
1 2 3 4 5 6
e
M 1 2 3 4 5 6
M
f
1 2 3 4 5 6

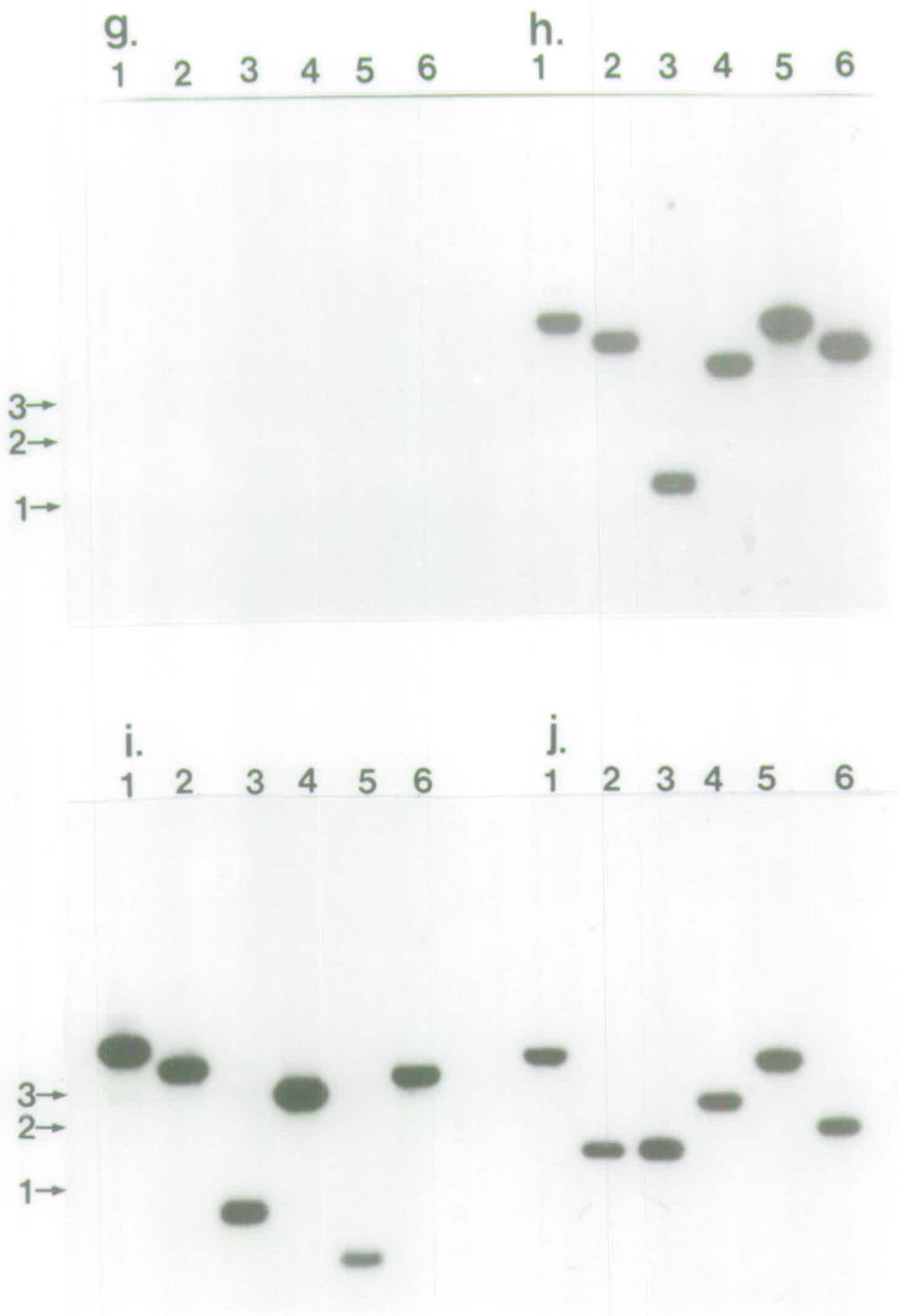

g.
1 2 3 4 5 6
h.
1 2 3 4 5 6
3→
2→
1→
i.
1 2 3 4 5 6
j.
1 2 3 4 5 6
3→
2→
1→

Figure 5.

PCR analysis of the PfPCNA 5′ flanking sequence to demonstrate the inversion of the central 800bp *Nsi*I DNA restriction fragment (Figure 2e). Schematics indicating the positions of the oligonucleotides 949T, P0901 and 828T (5′GTATAACTTATGTGTAATTC3′) and their relative orientation in pPLH1 (**A**) and pPLH1(R) (**B**) are shown on the facing page. The sizes of the expected products are indicated in bp.

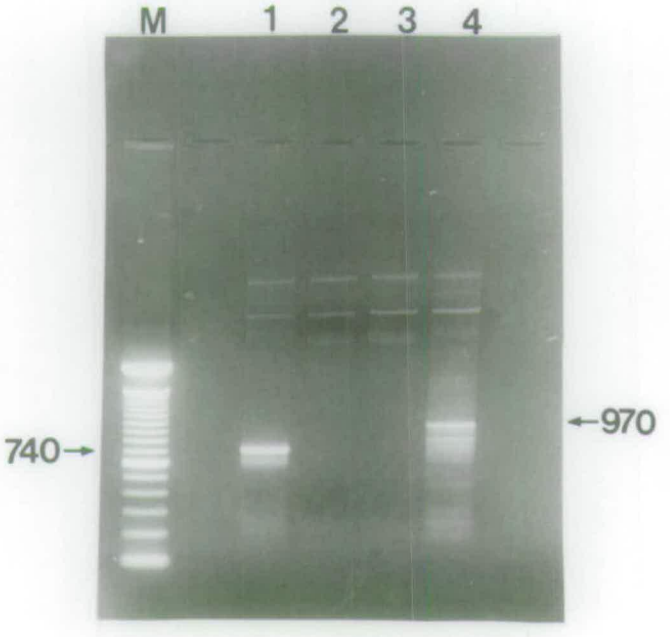
C. An ethidium bromide stained agarose gel on which the results of a PCR between the oligonucleotides indicated below are size fractionated. The sizes of the principle products are indicated in bp.

M. 100bp markers.
1. pPLH1, 949T and 828T.
2. pPLH1, 828T and P0901.
3. pPLH1 (R), 949T and 828T.
4. pPLH1 (R), 828T and P0901.

A. pPLH1

B. pPLH1 (R)

C.



sequence was completely sequenced to ensure no mutations were introduced during amplification, the constructs were analysed by restriction analysis (Figure 4) and the PfPCNA 5′ flanking sequence-*luc* boundary checked by sequencing.

6.3 Transfection of *P.falciparum.*

6.3.1 Tests of the ability to detect luciferase expression from the pHLH1 and pPLH1 constructs.

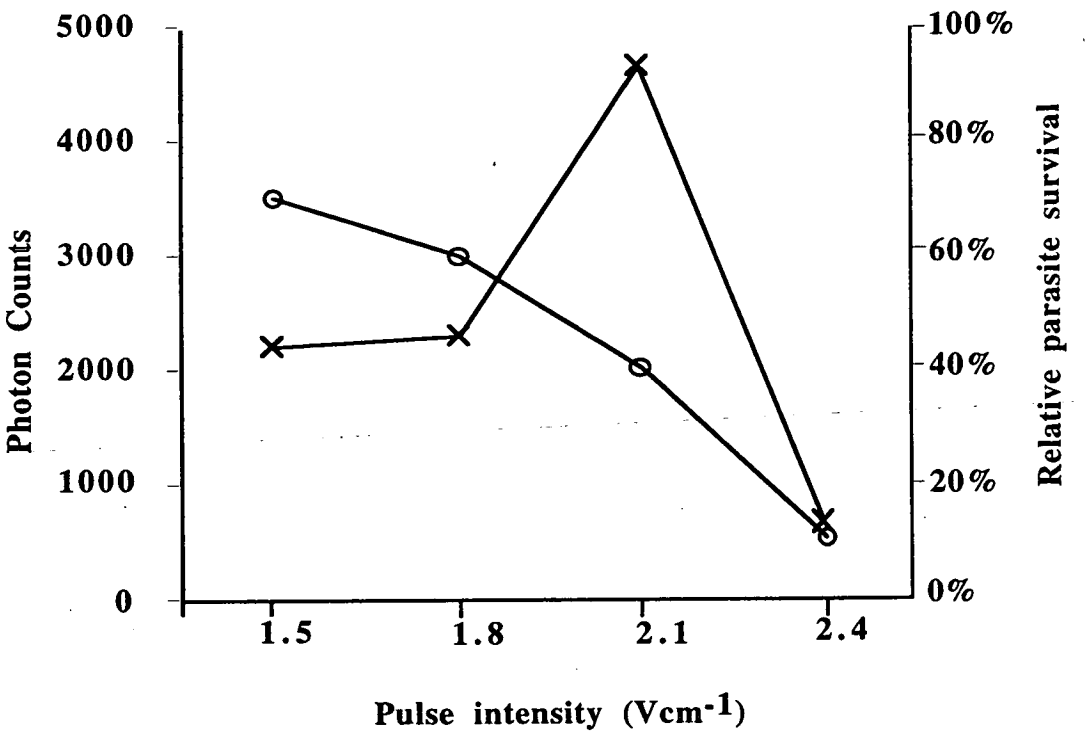
On receiving the pHLH1 plasmid we demonstrated that, in our hands, luciferase was expressed in *P.falciparum*. An asynchronous culture was divided to give samples each containing approximately $2X10^8$ parasites. Each sample was electroporated with 60µg of pHLH1 using a range of pulse intensities (2.5.2). After 48 hours the parasites were harvested and the luciferase activity measured in terms of the photons counts measured. The results of this analysis indicated that not only could we detect luciferase activity but that the levels of luciferase expressed varied with the pulse intensity used (Figure 6). The maximum activity was measured when approximately 50% of the parasites were killed. The same effect was noted with constructs containing the CAT reporter gene (Wu, Y., *pers comm.*).

The *HRP*3 5′ flanking sequence of pHLH1 was replaced with 1.58kb of PfPCNA 5′ flanking sequence to give pPLH1 (6.2.1). Luciferase was expressed after electroporation of *P.falciparum* with 60µg of this new construct (Figure 7a). Control experiments in which either no pPLH1 (Figure 7j) was added or pPLH1 was added but not electroporated both gave no detectable levels of luciferase activity.

6.3.2 pPLH1 recovered from the parasite 48 hours after electroporation is unrearranged.

During preparation of the various constructs, rearrangement of the plasmids in *E.coli* was a considerable problem. To ensure that there were no rearrangements of plasmids in the parasite during the several rounds

Figure 6.

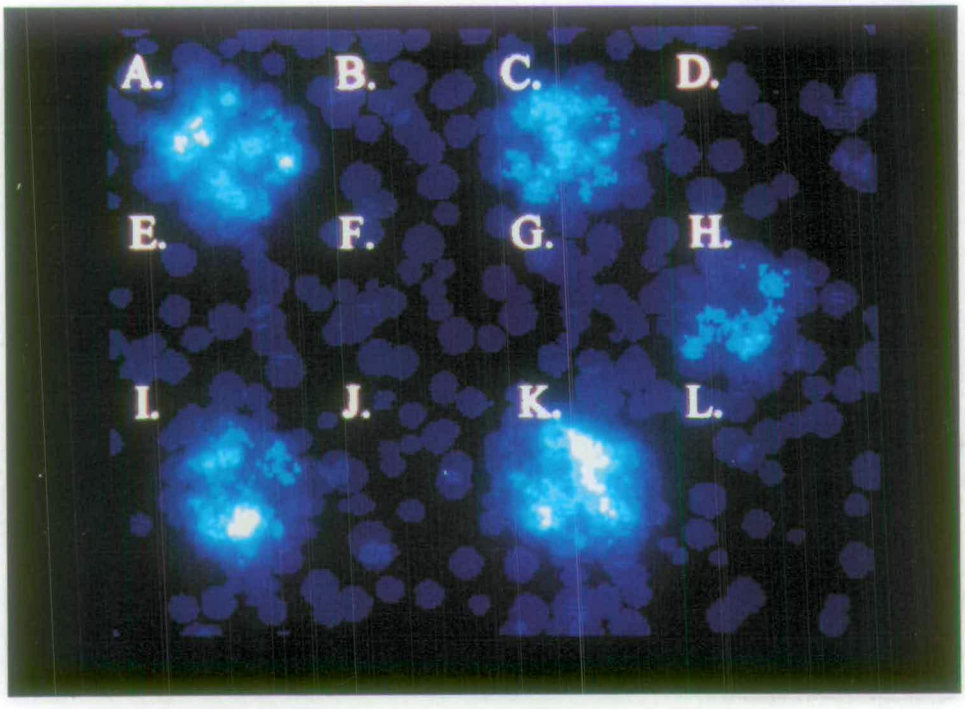


The photon count from a luciferase assay where 60µg of pHLH1 was electroporated into $2x10^8$ parasites at a range of pulse intensities. The parasite survival relative to a group of parasites to which 60µg of pHLH1 had been added but not electroporated is also indicated.

Figure 7.

Colour representation of the image detected using the Argus-50 image processor (Hamamatsu Photonics K.K.). The image represents the photons detected over 10 minutes using the camera's most sensitive setting. Brighter regions of the image indicate a higher fequency of photon strikes. Extracts of parasites electroporated with 60μg of the following constructs were used in the assay;

A. pPLH1	B. pPLH1ΔBC
C. pPLH1ΔC	D. pPLH1ΔB
E. pBluescriptIISK+	F. pPLH1 (R)
G. pPLH1Δ870	H. pPLH1Δ330
I. pPLH1	J. no DNA
K. pHLH1	L. assay buffer alone.



of DNA replication which occurs during the 48 hours following electroporation, we rescued the plasmid for restriction analysis. 6μg or 60μg of pPLH1 was electroporated into ring synchronised cultures, each containing $2X10^8$ parasites, and incubated for 48 hours. One further sample had 60μg of pPLH1 added but was not electroporated. The erythrocytes were harvested by centrifugation (3000g, 10 minutes, 4°C) and washed 4 times in 10 volumes of ice-cold PBS. These washes had previously been demonstrated to be sufficient to remove any DNA not inside the erythrocytes (5.5.1). The erythrocytes were lysed by saponin treatment (2.2.1.1.) and the washes repeated on the released parasites. The parasites were resuspended in 1ml of ice-cold hypotonic buffer (2.2.10) without the protease inhibitors, and broken open using a Potters homogeniser with a B-type pestle. 10μl of each suspension recovered was used to transform 200μl of *E.coli* (2.2.6). Various volumes of transformed *E.coli* were plated on LB plates supplemented with $100\mu gml^{-1}$ ampicillin (2.2.6.2) and incubated overnight at 37°C. The results of the plating (Table 1) indicate that the plasmid recovered originated from the protected environment of the parasite. 5 colonies were selected, at random, and along with a pPLH1 control restricted with *Eco*RI. This analysis showed that all the recovered plasmid appears to be the same as the original pPLH1 (data not shown), one recovered plasmid was restricted with a range of restriction enzymes and compared to pPLH1 (Figure 8).

Table 1. Results of the transformation with plasmid isolated from transfected parasites. The numbers of cfu recovered on LB-Amp plates are indicated with the dilution of the parasite extract.

Dilution	60μg pPLH1	6μg pPLH1	no DNA
10^{-3}	380	200	0
10^{-4}	60	29	0
10^{-5}	7	5	0
10^{-6}	1	0	0

Figure 8.

Restriction analysis of pPLH1 recovered from *P.falciparum* 48 hours after electroporation. pPLH1 was rescued by transformation of *E.coli* with the extract of transfected parasites. Sizes are indicated in bp.

a) pPLH1 that has not been electroporated into *P.falciparum*.

b) pPLH1 rescued from transfected parasites .

M. 1kb markers
1. *Kpn*I
2. *Eco*RI
3. *Hin*dIII
4. *Eco*RI/*Hin*dIII
5. *Pst*I
6. *Kpn*I/*Nsi*I
7. *Kpn*I/*Pst*I
8. *Hin*dIII/*Pst*I
m. 100bp markers

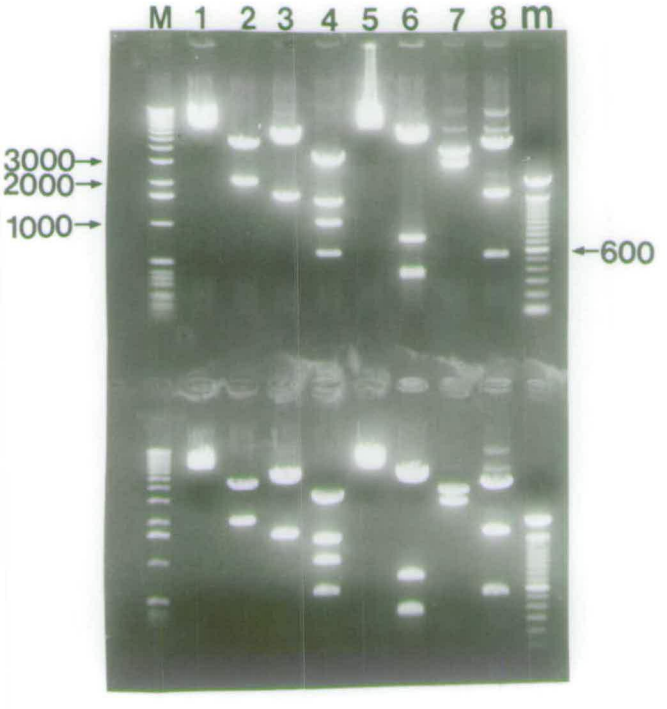


Table 2.

The photon counts for each construct indicated were corrected for the parasitaemia measured when the parasites were harvested at the mononuclear ring stage 48 hours later. The mean average and the standard deviation are also presented for each construct. **A**. Represents data collected from experiments without the addition of proteinase inhibitors. **B**. Represents data collected from experiments where protease inhibitors have been added.
pBSII SK+, pBluescriptII SK+; negative, 60µg of pPLH1 added but not electroporated.

Table 3.

The corrected photon counts within the transient transfection experiments were normalised relative to the value obtained for pPLH1. The mean average and the standard deviation for these relative activities of the promoter are presented. The constructs pBSII SK+ and pHLH1 are not displayed as these constructs are not part of the pPLH1 series of transfection constructs.
pBSII SK+, pBluescriptII SK+; negative, 60µg of pPLH1 added but not electroporated.

Table 2A: Photon counts/%parasitaemia from transient transfection assays (without protease inhibitors).

Construct	Experiment						Mean Average	Standard Deviation
	1	2	3	4	5	6		
pPLH1	345	338	410	289	241	347	328	57.5
pPLH1ΔBC	28.7	22.1			14.2		21.7	7.26
pPLH1ΔB	45.2	20.4	36.8		12.8	16.3	26.3	14.0
pPLH1ΔC		223	320	237	183	281	249	53.0
pPLH1 (R)					16.6	12.9	14.8	2.66
pPLH1 Δ330								
pPLH1 Δ870								
pBSII SK+	31.6	19.8	15.6	17.5			21.1	7.20
negative	22.9	14.9		6.35	6.07	9.86	12.0	7.05
pHLH1	433		616	376	286	409	424	53.0

Table 2B: Photon counts/%parasitaemia from transient transfection assays (with protease inhibitors).

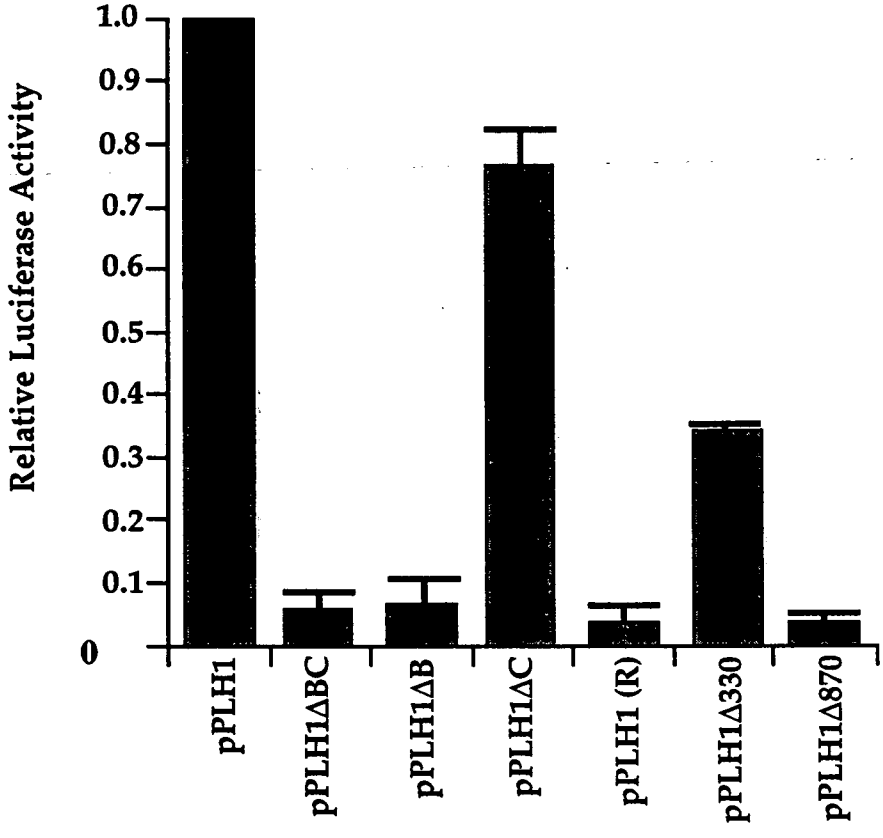
Construct	Experiment			Mean Average	Standard Deviation
	1	2	3		
pPLH1	726	786	983	831	134
pPLH1ΔBC			14.5	14.5	
pPLH1ΔB			8.70	8.70	
pPLH1ΔC			782	782	
pPLH1 (R)			22.6	22.6	
pPLH1 Δ330	260	269	344	291	46.1
pPLH1 Δ870	38.7	39.4	24.1	34.1	8.64
pBSII SK+			40	40	
negative	28.2	12.3	24.0	21.5	8.24
pHLH1	1059	989	1323	1123	176

Table 3: Relative photon counts/%parasitaemia of transient transfection assays.

Construct	Experiment									Mean Av.	Standard Dev.
	1	2	3	4	5	6	7	8	9		
pPLH1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
pPLH1ΔBC	0.08	0.07			0.06				0.01	0.06	0.03
pPLH1ΔB	0.13	0.06	0.09		0.05	0.05			0.01	0.07	0.04
pPLH1ΔC		0.66	0.78	0.82	0.76	0.81			0.80	0.77	0.06
pPLH1 (R)					0.07	0.04			0.02	0.04	0.03
pPLH1 Δ330							0.36	0.34	0.35	0.35	0.01
pPLH1 Δ870							0.05	0.05	0.02	0.04	0.02
pBSII SK+											
negative	0.07	0.04		0.02	0.03	0.04	0.04	0.02	0.02	0.03	0.02
pHLH1											

Figure 9.

Graphical representation of the relative promoter activities of the PfPCNA 5′ flanking sequences in the pPLH1 transfection constructs.



flanking sequence was deleted (Figure 2j and Table 3). Deletion of 870bp to give pPLH1Δ870, effectively abolished promoter activity (relative promoter activity of 0.04+/-0.02) indicating that a region of 470bp at the 5′ end of segment B contains elements essential for the activity of the PfPCNA promoter. It is this region which contains the putative transcription start sites described earlier (Chapter 4).

6.5 A region of 330bp (between 1250-1580bp upstream of the translational start site) in the PfPCNA 5′ flanking sequence is required for efficient promoter activity.

The deletion of 330bp from the extreme 5′ end of the PfPCNA 5′flanking sequence, in pPLH1Δ330, has a substantially reduced promoter activity (relative promoter activity of 0.35+/-0.01). This region lies between 290 and 620bp upstream of the putative major transcription start sites and contains the "GC patches" referred to earlier (Chapter 4). At present we do not know if these have any functional significance.

6.6 A region of 380bp (between 1-380bp upstream of the translational start site) in the PfPCNA 5′ flanking sequence can be removed without abolishing promoter activity.

The deletion of sequences proximal to the translational start site in the PfPCNA 5′ flanking sequence appears to decrease, but not to abolish promoter activity. The deletion of *Nsi*I DNA fragment C, in pPLH1ΔC (Figure 3), gives a relatively high promoter activity compared to the full length 5′ UTR (relative promoter activity of 0.77+/-0.06). This region lies between 580bp and 960bp downstream of the transcription start sites. The approximately 20% reduction in promoter activity may be the result of moving the transcription start sites relative to the translational start rather than the removal of sequences important to the activity of the promoter.

6.7 Protease inhibitors increase the efficiency of the luciferase assay.

Lysis of the parasite during the assay procedure (2.5.2) releases a range of proteases from organelles within the parasite such as the food vacuole. To help preserve the luciferase during the subsequent assay the lysis buffer in experiments 7-9 (Table 2b) was supplemented by the addition of a protease inhibitor cocktail. The data from pPLH1 and pHLH1 suggest that the sensitivity of the luciferase assay was increased 2-3 fold (taking the mean values before and after addition of the proteases in Table 2).

6.7 Discussion.

Firefly luciferase expression in *Plasmodium* had previously been demonstrated in both the intraerythrocytic and ookinete stages of development (Goonewardene *et al.*, 1993, Wu *et al.*, 1995). The ease of detection of the activity of this protein makes it a useful marker of the promoter activity. In these experiments we were assaying the cumulative expression of this protein over the first 48 hours following introduction of the constructs. We tried to eliminate inaccuracies in measuring the promoter activities by determining the photon counts of the samples immediately after extraction and calculating an activity related to the percentage parasitaemia. In spite of these precautions precise reproduction of the assays is difficult and differences are found between the absolute photon counts from experiment to experiment. These we assume to be attributable to minor differences in culture conditions and other experimental variables which will be constant for the samples in a given experiment but will probably vary across the different experiments carried out. When the data are expressed relative to the activity of the full length construct, a common control for every experiment, a striking consistency is found (Table 3). This was maintained even when the experimental conditions were knowingly changed, as was demonstrated by the addition of protease inhibitors (experiments 7-9).

The analysis of the promoter activity of a number of constructs identified a region of PfPCNA 5′ flanking sequence approximately 710-1180bp upstream of the translational start which is essential for promoter activity

(Figure 2j). This region contains the putative transcriptional start sites identified by physical mapping methods (Chapter 4). A second region, between 1250-1580bp upstream of the translational start site, has also been shown to be required for efficient promoter activity. However whether this region can be described as an enhancer has not been settled since we have not yet tested the effect of inverting it on promoter activity. Enhancers have recently been identified from the 5′ flanking sequences of *P. falciparum* calmodulin and *dhfr-ts* and *P.chabaudi dhfr-ts* encoding genes (Crabb and Cowman, 1996). These enhancers share neither sequence similarity to each other nor with the region required for PfPCNA promoter efficiency.

The apparent lack of a significant effect on the level of the promoter activity due to the deletion of sequences proximal to the open reading frame prompts us to question the role of such extensive 5'UTRs so frequently found for genes of *Plasmodium*. Suggestions of probable functions have included the targeting of the transcript, control of transcript stability or perhaps the presence of elements which determine stage specific translation. At present we do not know which, if any, of these suggestions, apply. The substitution of these sequences, rather than their deletion, may give a clearer indication of whether these sequences are required for promoter activity.

As far as we are aware this is the first time that transient transfection in *P.falciparum* has been used to confirm the essential nature of a region which contains putative transcription start sites. At present the analysis is limited and future studies will be directed towards a more detailed study of the PfPCNA promoter region including a mutational analysis of the various sequences identified in this work; the transcription start site(s), TATA boxes, OCT1 binding sites and the "GC patches". It is also possible that other regions which influence PfPCNA promoter expression lie beyond the limits of the 5′ flanking region that has been studied here.

Chapter 7.

This study has provided a start to a more complete analysis of the regulation of the expression of DNA replication proteins of *P.falciparum*. The pattern of accumulation of PfPCNA and PfPolδ mRNA and polypeptide levels were the same. However, the promoter activities of these genes were different dismissing the possibility that their expression is through a unitary control. PfPCNA appears to be regulated at the post-transcriptional level whereas PfPolδ apperars to be regulated at the level of transcript initiation. PfPCNA and PfPolδ mRNA and polypeptide levels increase in trophozoites yet only the polypeptide persists in schizonts, the mRNA presumably degrades. These results suggest that enough polypeptide is translated in trophozoites to complete DNA synthesis. Although we now have some ideas regarding the regulation of expression of PfPCNA and PfPolδ at the onset of DNA replication, the regulation of the activity of these proteins during the subsequent rounds of DNA replication is unknown, although this is presumably through some posttranslational mechanism. The activity of both DNA polymerase α and δ in proliferating human cells appears to be regulated by phosphorylation (Nasheuer *et al.*, 1991, Zeng *et al.*, 1994), PCNA does not appear to be phosphorylated at any point in the cell cycle (Zeng *et al.*, 1994). To date, no studies have been made regarding such posttranslational mechanisms in the regulation of the activity of DNA polymerase δ and PCNA in *Plasmodium*. The *P.falciparum* DNA polymerase α major subunit, however, does not appear to be phosphorylated at any point during intraerythrocytic schizogony (Choi and Mikkelson, 1991).

Initial attempts to compare the 5′ flanking sequences from PfPCNA and PfPolδ were thwarted when we were unable to isolate an overlapping genomic clone containing PfPolδ 5′ flanking sequence. In mapping the PfPCNA transcription start sites we employed a strategy which involved the use of 4 methods. The transcription start sites were first mapped approximately to identify a region that we could concentrate on. This region was then analysed more closely using both primer extension

analysis and RNase protection assays. Two transcription start sites at 960bp and 1000bp upstream of the translational start site were identified. Associated with these sites were good consensus-matching TATA boxes between 30-35bp upstream of both transcription start sites and two oppositely orientated OCT1 boxes placed between 235-275bp upstream of the major transcription start site. We also identified a number of "GC patches", short regions of DNA unusually rich in guanosine and cytosine nucleosides.

Northern analysis indicates that there are two PfPCNA transcripts; a predominant 1.85kb transcript and a minor 2.2kb transcript. We found no evidence for additional transcription start sites apart from the two sites already mapped. Analysis of the 3' flanking sequence identified a good consensus transcription stop site at the extreme 3' end of a cDNA clone. The distance between the mapped transcription start and the putative stop site, 250bp downstream of the PfPCNA ORF, would correspond to the 2.2kb transcript. Repeated screening of the cDNA library indicated that no other PfPCNA cDNA clones were present even though there is a weak consensus transcription stop site 120bp downstream of the PfPCNA ORF. Evidence from RT-PCR experiments across the 5' flanking sequence did not suggest that there were deletions present in the 5'UTR of the transcripts. The 5'UTR sequence available from the cDNA clone was colinear with the genomic sequence. Both observations would suggest that any differences in transcript size would be a result of different sized 3'UTRs. Northern analysis using a probe from the extreme 3' of the cDNA clone indicated that the 1.85kb transcript may lack sequences from this region. To date we have no explanation for the presence of two PfPCNA transcripts, we hope that a combination of RNase protection and transient transfection assays may provide some clues. That two different transcripts arise from a gene that is regulated at the post-transcriptional level is intriguing.

We were disappointed with the lack of expression of the GFP reporter gene. The use of GFP as either a reporter of expression or a tag for a protein of interest has flourished since the first report of heterologous expression by Chalfie *et al.* (1994). However, it now seems apparant that

expression of "reasonable" quantities of the protein is required to visualise the fluorescence. The expression of firefly luciferase in the intraerythrocytic stages of parasites is approximately 1000 fold less than in the sexual stages (Wirth, D., *pers comm.*). Given the apparantly greater efficiency of the sexual stages to express heterologous proteins we would suggest that an attempt to express GFP in these stages be tried.

Transient transfection analysis identified 3 regions of interest in the 5' flanking sequence of PfPCNA. (1) An essential region, containing the putative transcription start sites, was characterised when deletion or inversion of a region between 810bp-1180bp upstream of the translation start abolished promoter activity. (2) A region essential for the efficiency of the promoter was characterised between 295bp-625bp upstream of the major transcription start site. (3) The deletion of sequences proximal to the ORF did not appear to significantly lower the activity of the promoter. Unfortunately these studies did not help in identifying a role for the extremely long 5'UTRs.

This preliminary analysis of the 5' flanking sequence identified gross regions of interest. A new range of constructs will enable a more accurate mapping of the regions already identified as well as hopefully finding new regions to study more closely. More specifically; the transcription start sites, TATA boxes, and OCT1 boxes should be replaced or deleted to assess their effect on the promoter activity. The region required for promoter efficiency will be replaced in the opposite orientation to determine whether this is an enhancer region. Sequences proximal to the reporter gene will be replaced with other sequences and the effect on both the level and periodicity of promoter activity monitored.

The ability to transfect *Plasmodium* has, in recent years especially, been regarded as the next step towards the successful development of rationally designed drugs. With the system now available within our laboratory we should be able to start to address questions regarding the biology of the parasite in general and of DNA replication in particular.

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