Identification and analysis of a novel scavenger receptor-like protein conserved in *Plasmodium* species

Thesis

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Identification and analysis of a novel Scavenger Receptor-like protein conserved in *Plasmodium* species

Cecilia Caroline Eriksdotter Waller, MSc

Division of Parasitology
National Institute for Medical Research
London, United Kingdom

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Abstract

In October 2002, the complete sequence of the genome of the human malaria parasite *Plasmodium falciparum* was published. The data showed that as many as 60% of all identified open reading frames were unique to the parasite, with no known homologues in other species. Identification and characterization of novel molecules that may provide targets for drug and vaccine design is crucial to fight malaria.

The aim of this project has been to analyse a novel gene and protein in *Plasmodium*. A short nucleotide fragment was originally identified in *Plasmodium chabaudi chabaudi* (AS) and the complete sequence subsequently obtained using Vectorette PCR. A single-exon gene was identified and Southern analysis showed the presence of a single copy in the *P. chabaudi* genome. Analysis of the protein secondary structure revealed a multi-domain molecule consisting of an N-terminal secretion signal, two Scavenger Receptor Cysteine-Rich (SRCR) domains, three *Limulus* factor C, Coch-5b2 and Lgl1 (LCCL) modules and an LH2/PLAT domain. These motifs all have adhesive properties, and the protein was subsequently named *Plasmodium* SRCR LCCL Adhesive-like Protein (*P*SLAP).

Multiple sequence alignment established that *P*SLAP is highly conserved within *Plasmodium* and also identified a paralogue in *Toxoplasma gondii*, suggesting it may play a specific and vital role in apicomplexan biology. Results from indirect immunofluoresence assays showed a punctate expression pattern contained within the parasite, indicating that the protein is distributed in vesicles. Northern analysis and immunofluorescence co-localisation studies showed transcription and expression in blood-stage gametocytes, demonstrating that *P*SLAP is a sexual stage protein.

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Abbreviations

°C	degrees Celsius
α-	Anti-
A	Ampere
ALCAM	Activated Leukocyte Cell Adhesion Molecule
AMA-1	Apical Membrane Antigen 1
AP	Alkaline Phosphatase
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine Serum Albumin
CBD	Chitin-binding type 2 domain
CD	Cluster of Differentiation
cDNA	Complementary DNA
cm	Centimeters
CSA	Chondroitin sulphate A
CSP	Circumsporozoite Protein
CTRP	CSP- and TRAP-related protein
Da	Dalton
DAPI	4,6-diamidino-2-phenylindole
dATP	Deoxyadenosine triphosphate
DBL	Duffy Binding-Like
DDT	Dichlorodiphenyltrichloroethane
dhfr-ts	Dihydrofolate reductase-thymidylate synthase
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EBA-175	Erythrocyte-binding Antigen 175
EBP	Erythrocyte Binding Protein
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked ImmunoSorbent Assay

ER	Endoplasmic Reticulum
EST	Expression Sequence Tag
F	Farad
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
g	Gram
x g	Relative centrifugal force (gravities)
GalNAc	N-acetyl-D-galactoseamine
gDNA	Genomic DNA
GlcNAc	N-acetyl-D-glucoseamine
GLURP	Glutamate-rich protein
GNBP	Gram-negative bacteria-binding protein
GPI	Glycosylphosphatidylinositol
HIP	Hyperimmune plasma
ICAM-1	Intracellular Adhesion Molecule-1
IFA	Indirect immunofluorecence assay
IFN-γ	Interferon gamma
Ig	Immunoglobulin
ip	Intraperitoneal
IPTG	Isopropyl-β-D-thiogalactopyranoside
iv	Intravenous
k	Kilo
KO	Knock-out
L	Liter
LAP	LCCL/Lectin Adhesive-like Protein
LB	Luria–Bertani
LCCL	*Limulus* factor C, Coch-5b2 and Lgl1
LDLRA	Low-density lipoprotein receptor class A
Lgl1	Late gestation lung protein-1
LH2/PLAT	Lipoxygenase Homology 2/Polycystin-1, Lipoxygenase and α-Toxin

LPS	Lipopolysaccharide
LSA-1	Liver-Stage Antigen-1
M	Molar
MCS	Multiple cloning cassette
µ	Micro
m	Milli
MHC	Major Histocompatibility Complex
MIC2	Micronemal Protein 2
MSP-1 / 2	Merozoite surface protein-1 / -2
MudPIT	Multidimensional Protein Identification Technology
NADH	nicotinamide adenine dinucleotide
n	Nano
Ni-NTA	Nickel-nitrilotriacetic acid
NK cells	Natural killer cells
NO	Nitric oxide
NOS	Nitric oxide synthase
NP	Normal plasma
OD	Optimal density
ORF	Open reading frame
PATS	Prediction of Apicoplast Targeted Sequences
PBMC	Peripheral mononuclear cells
PBS	Phosphate-buffered saline
PCE	Proclotting enzyme
PCR	Polymerase chain reaction
PfEMP-1	Plasmodium falciparum Erythrocyte Membrane Protein 1
PNPP	p-Nitrophenylphosphate
PRBC	Parasitised red blood cells
PVM	Parasitophorous vacuole membrane
RAP-1	Rhoptry-associated protein-1
RBC	Red blood cells
rif	repetitive interspersed family
RNA	Ribonucleic acid

ROS	Reactive oxygen species
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT	Room temperature
RT-PCR	Reverse Transcriptase PCR
s	Second
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SERA	Serine-rich Antigen
SMART	Simple Modular Architecture Research Tool
SRCR	Scavenger Receptor Cysteine-Rich
SSC	NaCl sodium citrate (buffer)
SSPE	Phosphate-buffered NaCl sodium citrate EDTA (buffer)
stevor	*sub-telomeric variable open reading frame*
TBE	Tris-borate electrophoresis (buffer)
TE	Tris-EDTA
TgSR1	Toxoplasma gondii scavenger receptor 1
Th	T helper
TNF	Tumor necrosis factor
TRAP	Thrombospondin-related adhesion protein
TRITC	Tetramethylrhodamine isothiocyanate
UTR	Untranslated region
var	*variant*
V	Volt
w/v	weight over volume

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Chapter One

Introduction

1.1 Malaria – The current situation

Today approximately 40% of the world's population is at risk of contracting malaria. Malaria is found throughout tropical and sub-tropical regions and is endemic in Africa, parts of Asia, Central and South America, Oceania, and certain Caribbean islands (Figure 1.1). It causes an estimated 500 million cases of acute illness a year, and over one million deaths, 90% of which occur in sub-Saharan Africa (Breman, 2001). It has been estimated that 76% more productive life years are lost from malaria than from all cancers in all developed countries in the world taken together. Still, funding for malaria research is at best a tenth of that for cancer research (Malaria Foundation International). To make matters worse malaria is on the rise (WHO, 1998). Several factors have contributed to this: the emergence of drug resistance in the parasite and insecticide resistance in the mosquito, the creation of new mosquito breeding grounds due to road building, deforestation, mining, irrigation projects and new agricultural practices, and movements of non-immune populations to malarious areas and malaria-infected individuals to malaria-free areas.

Apart from the obvious human pain and suffering caused by a life-threatening disease, malaria has been shown to be a major constraint to economic development. Economists believe that malaria is responsible for a growth penalty of up to 1.3% per year in some African countries (Gallup and Sachs, 2001). Lost productivity and income, as well as direct public and personal costs for prevention and treatment, have a significant effect on the economy of countries heavily burdened by malaria.

1.2 *Plasmodium* taxonomy

Plasmodium belongs to the kingdom Protista, and the phylum Apicomplexa, a group of approximately 5000 species, all of which are parasitic. Apicomplexan parasites have a vast medical and economic impact as they infect both humans and domestic animals.

Seven genera within *Apicomplexa* cause disease in man: *Plasmodium, Toxoplasma, Cryptosporidium, Babesia, Isospora, Cyclospora* and *Sarcocystis*. Host cell invasion in Apicomplexans is mediated by the apical complex, a set of structural and secretory organelles including the rhoptries and micronemes, present at the anterior end of the sporozoite, merozoite and ookinete stages (Section 1.3). The complex is believed to be involved in attachment to, and penetration of, host cell plasma membranes. Most Apicomplexans are intracellular, hence there is little need for movement and there are no specialized locomotor organelles (except for the microgamete flagella), although some species, including *Plasmodium*, possess gliding motility.

Over 100 species of *Plasmodium* infect organisms as diverse as mammals, birds and reptiles (reviewed by Garnham, 1966). Four species infect humans: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae* (Section 1.5). Species used in experimental animal models, and therefore of scientific interest, include the simian malarias *Plasmodium cynomolgi* and *Plasmodium knowlesi*, the rodent parasites *Plasmodium chabaudi, Plasmodium berghei* and *Plasmodium yoelii* (Section 1.6), and the avian parasite *Plasmodium gallinaceum.*

Although the details vary greatly between species, all Apicomplexa exhibit a similar general life cycle that includes both sexual and asexual reproduction phases.

1.3 The *Plasmodium* life cycle

Plasmodium parasites have a complex life cycle, which involves passage through both a vertebrate host and an insect vector. The development includes asexual multiplication in the warm-blooded host, as well as sexual and asexual reproduction in the vector (Figure 1.2).

Infection is initiated when sporozoites are injected with the saliva of an infected, feeding female mosquito. Within 45 minutes the sporozoites have been carried by the circulatory system to the liver, where they invade hepatocytes. The intra-cellular parasite undergoes growth and asexual reproduction (exo-erythrocytic schizogony) within the hepatocyte, resulting in the production of thousands of merozoites. The merozoites are released back into the bloodstream where they infect erythrocytes, initiating the asexual blood stages that are associated with the characteristic symptoms and pathology of malaria. A period of growth, accompanied by an active metabolism that includes ingestion of host

cytoplasm and proteolysis of hemoglobin into amino acids, then follows: the immature "ring-stage" parasite develops into a trophozoite, followed by nuclear and cytoplasmic division resulting in an erythrocytic schizont that contains four to 24 individual merozoites (depending on the species). The schizont finally ruptures and the merozoites are released into the blood, rapidly invading new erythrocytes and repeating the cycle.

However, as the infection develops, conditions within the host become less favorable for parasite survival (fewer non-infected erythrocytes available, an increase in the host immune response). Some trophozoites will therefore instead of maturing into multi-nucleated schizonts, proceed to become sexual forms, developing into uni-nuclear male and female gametocytes (micro- and macro-gametocytes, respectively). Further development of these into gametes, gametogenesis, is dependent upon uptake by another mosquito. Gametogenesis is triggered by the environment in the mosquito gut: a drop in temperature, an increase in carbon dioxide and exposure to gametocyte-activating factor (GAF), a mosquito-derived substance recently identified as xanthurenic acid (Billker *et al.*, 1998).

Macrogametocytes develop into macrogametes and ex-flagellation is induced in the microgametocyte, generating eight haploid, motile microgametes. Each microgamete subsequently fertalises a macrogamete, producing a zygote. Within 24 hours the zygote has developed into a motile ookinete that penetrates the mosquito midgut wall and lodges between the basal lamina and the epithelial cells, where it matures into an oocyst. Each oocyst undergoes multiple rounds of asexual replication forming up to 8000 sporozoites, which upon rupture of the oocyst are released and migrate to the salivary glands of the mosquito, awaiting injection into another warm-blooded host (Fujioka and Aikawa *et al.*, 2002).

The liver-stage development does not elicit any symptoms in the vertebrate host, rather, clinical disease is caused by the exponential expansion of parasites during the erythrocytic stages.

1.4 Clinical manifestations of malaria

Symptoms of malaria appear six to 40 days (depending on the species) after an infectious mosquito bite and are almost exclusively caused by the asexual erythrocytic stage parasite. The infection starts off with flu-like symptoms such as fever, headaches, nausea,

chills and vomiting. This is followed by periodic febrile attacks known as malarial paroxysms. Without medication the condition worsens and can become life threatening (WHO web site). The severity of disease is dependent on the infecting parasite species but in the case of *P. falciparum,* complications include renal failure (also in *P. malariae* infections), severe anemia, acidosis (excessive acids in the body fluids), liver dysfunction, hypoglycemia (low glucose levels), acute pulmonary edema and cerebral malaria (CM; White, 1998). CM is infection of the nervous system and accounts for a substantial proportion of the mortality associated with *P. falciparum* malaria. CM is mainly seen in young children (one to four years of age), pregnant women and non-immune travelers. Symptoms include convulsions, impaired conciousness, delirium and coma, and once diagnosed has a mortality frequency of 30%, even with treatment (English and Newton, 2002). Patho-physiological characteristics include breakdown of the blood brain barrier, cerebral edema and cytoadhesion of parasitised erythrocytes in brain capillaries (Adams *et al.*, 2002). Cytoadherence, also termed sequestration, causes obstruction of blood vessels in the microvasculature, leading to a reduced oxygen and substrate supply, with anaerobic glycolysis and lactic acidosis as a consequence (White and Ho, 1992). Sequestration is the direct cause of the multi-organ complications seen in *P. falciparum* malaria, however, it is not a feature of infections with the other human parasites. The overall severity of the disease depends on the *Plasmodium* species that is causing the infection.

1.5 Human malaria

Four species of *Plasmodium* cause malaria in man: *P. falciparum, P. vivax, P. malariae* and *P. ovale.* Although many general characteristics are shared, there are a number of significant differences between the species. These include blood-stage morphology (parasite and erythrocyte size and shape, number of merozoites), minor life cycle variations, ability to sequester in the host, hypnozoite formation, host erythrocyte preference and geographical distribution. These differences cause variations in disease and clinical manifestations, and ultimately in outcome of the infection.

Of the four human Plasmodia, *P. falciparum* causes most morbidity and almost all mortality associated with malaria. This is due to the higher parasitemias seen in *P. falciparum* infections as compared with the other species, and the complications arising

from sequestration of infected erythrocytes in various organs of the host (Section 1.4). Factors that contribute to the elevated parasite loads include a lack of erythrocyte preference, a greater number of merozoites produced per schizont and the parasite's ability to sequester, resulting in fewer infected erythrocytes being cleared by the spleen. Complications in *P. falciparum* malaria are very serious and most notably include severe anemia, multi-organ failure and cerebral malaria (Section 1.4). The latter two are both associated with sequestration of infected erythrocytes in the host's circulatory system. *P. falciparum* is widespread in tropical and subtropical areas but is most prevalent in sub-Saharan Africa and south-east Asia where it has a great impact on economy and development.

P. vivax is the most widespread malaria parasite worldwide and is found throughout most of South America. It causes a chronic disease due to the persistence of hypnozites - dormant forms of the parasite produced within hepatocytes during development in the liver (also seen in *P. malariae*). Although it does not have as high a mortality rate as *P. falciparum*, *P. vivax* inflicts debilitating morbidity and frequent relapses of disease, with a consequent negative impact on endemic communities due to the loss of productivity.

P. malariae causes the third most prevalent malaria infection, with a broad worldwide, but spotty, distribution. Because this species prefers senescent erythrocytes, which are relatively scarce in normal blood, parasitemias are generally low. However, *P. malariae* is the most chronic of the human malarias and recrudescences have been documented several decades after the initial infection. This chronicity is sometimes associated with renal complications, which are probably due to depositions of antigen-antibody complexes in the glomeruli of the kidneys (Eiam-Ong, 2003).

P. ovale is the rarest of the four malaria species that infect humans, and is only common in the West African countries of Ghana, Liberia and Nigeria. Disease symptoms are mild and infections rarely cause complications.

1.6 Rodent models for studying malaria

For ethical reasons research involving malaria in the human host is very limited. Several species of non-human primates are partly susceptible to human Plasmodia but issues concerning ethics, methodology and cost make research in these species controversial and difficult. These problems have led to extensive use of other experimental model systems

to study *Plasmodium* parasites, mainly rodent and avian malarias. Despite some similarities with human parasites, certain aspects of avian parasite biology are fundamentally different from the human situation. Avian parasites (*P. gallinaceum* and *P. relictum*) develop in culicine vectors (*Aedes egypti*) rather than anopheline mosquitos like mammalian parasites, and when entering a warm-blooded host the sporozoites invade macrophages instead of hepatocytes (Section 1.3). The immune system of birds also differs significantly from that of mammals, and has not been characterised in great detail. The availability of inbred and congenic strains of mice with defined MHC haplotypes and targeted genetic modifications allows for a controlled approach to studying interactions between host and parasite, and offers a great advantage over the avian parasite species. Thus, rodents have become the preferred model system for studying experimental malaria.

A major criticism for using animal models has been their uncertain phylogenetic relationship with human parasites. However, there is a substantial degree of similarity between the rodent and human parasite species. On a basic genetic level, chromosome numbers (14 nuclear chromosomes) are conserved, as is gene synteny (the chromosomal location of different genes) to a large degree, as shown by studies comparing *P. falciparum* with the rodent parasites *P. chabaudi* and *P. yoelii* (Carlton *et al.*, 1998; Carlton *et al.*, 2002). A comparative analysis of the recently completed *P. falciparum* genome and the five-fold covered *P. yoelii* genome found that 63% of all proteins identified in *P. falciparum* had orthologs in the rodent parasite (Carlton *et al.*, 2002).

Naturally there are differences between the human and rodent parasites. However, a great deal of variation can also be seen between the different species that infect man. Morphology, erythrocyte preference, length of the erythrocytic replication cycle, virulence and pathology are all characteristics that differ between the human malaria parasites *P. falciparum, P vivax, P. malariae* and *P. ovale* (Section 1.5). Studies on the phylogeny of *Plasmodium* based on comparisons of small subunit ribosomal RNA sequences have found that *P. falciparum* is more closely related to the chimpanzee parasite *P. reichenowi* than to *P. vivax* and *P. malariae* (*P. ovale* was not included in the study; Escalante and Ayala, 1994). Because the divergence of the human parasites predates the origin of hominids, the evolution of the different parasite species was

initially host independent. This is consistent with the diversity of physiological and epidemiological characteristics of infections by these species (Section 1.5). Due to this plasticity of the parasite genome no experimental animal model is identical to the human disease, instead different rodent parasites are useful for studies of different aspects of malaria in man.

Four species of rodent parasites are used for experimental malaria: *Plasmodium chabaudi, Plasmodium berghei, Plasmodium yoelii* and *Plasmodium vinckei.* Originally isolated from African thicket rats (*Thamnomys rutilans*), they have all been adapted to grow in laboratory rodents (Killick-Kendrick and Peters, 1978). In the natural host, infections are chronic but with low virulence and parasitemias do not usually exceed a few percent. Infections in laboratory mice, on the other hand, are usually acute and either lethal or self-curing, depending on the parasite species and the mouse strain. Differences due to genetic polymorphisms in the rodent parasites exist both between and within species. Inter-species diversity includes variations in host cell preference, chronobiology (biological rhythm), virulence and morphology. For example, *P. berghei* ANKA causes a lethal infection in BALB/c and C57BL/6 mice, but death rarely occurs with *P. chabaudi chabaudi* (AS) infections in the same mouse strains (Neill and Hunt, 1992; Cross and Langhorne, 1998). Phenotypical differences between strains or clones are generally subtler, although there is a substantial degree of variation at the genomic level. A study locating parasite genetic markers to distinguish *Plasmodium chabaudi chabaudi* cloned isolates AS and AJ found over 800 loci present only in one of the two clones (Grech *et al.*, 2001).

Maintenance of rodent parasites *in vivo* is relatively simple and follows well-established protocols. The parasites can be cultured either through the full life cycle using laboratory bred *Anopheles stephensi* for mosquito transmission, or exclusively at the blood stage phase. In the latter case blood from an infected animal is syringe passaged into a healthy one, usually via the intraperitoneal route. Blood stage infections in laboratory mice produces an initial, acute, primary parasitemia that increases exponentially. The parasite population is either synchronous (*P. chabaudi* and *P. vinckei*), with the majority of parasites at the same stage of development, or asynchronous (*P. berghei* and *P. yoelii*),

where all developmental stages are present at all times. The parasitemia subsequently reaches a peak, referred to as crisis, at which point animals either die (susceptible strains), or start recovering as the parasitemia suddenly begins decreasing and after a few days reaches sub-patent levels (resistant strains). The post-crisis period is characterised by hemolysis, damaged parasites and asynchronous schizogony, also in synchronous species. In *P. chabaudi chabaudi* (AS) parasites later re-appear after a short period of sub-patency, a phenomenon referred to as recrudescence. This secondary infection has a shorter duration and results in a parasitemia of only a few percent (Langhorne *et al.*, 2002).

The disease symptoms in mice are similar to those in humans and are partly parasite species dependent (Section 1.5). General features include anemia, hypoglycemia, a decrease in body temperature, loss of body weight and in some infections, cerebral malaria (reviewed in Langhorne *et al.*, 2002). Differences between the rodent parasite species have made them suitable for investigating different aspects of human malaria. *Plasmodium chabaudi* has been used for studying the host immune response during the erythrocytic stages of the life cycle (reviewed in Langhorne *et al.*, 2002), genetic determinants for disease susceptibility (Fortin *et al.*, 2002), drug resistance (reviewed in Carlton *et al.*, 2001) and antigenic variation (Phillips *et al.*, 1997; Brannan *et al.*, 1993; Gilks *et al.*, 1990). *Plasmodium berghei* has been used extensively to investigate the causes of cerebral malaria (reviewed in Lou *et al*, 2001), and *Plasmodium yoelii* for *in vivo* drug testing and work on pre-erythrocytic immunity and vaccines (reviews in Hollingdale and Krzych, 2002, and Ballou *et al.*, 2002). Many experimental procedures now used in *P. falciparum* were initially developed in rodent systems, such as stable genetic transformation in *P. berghei* (van Dijk *et al.*, 1995). Targeting of specific genes has been extensively and successfully applied in *P. berghei*, and recently also in *P. yoelii*, in order to study the function and significance of specific proteins (Menard *et al.*, 1997; *et al.*, 2000; Tewari *et al.*, 2002; Mota *et al.*, 2001). As a result of the ongoing rodent malaria sequencing projects, functional genomics and proteomics studies on rodent malaria species are now also underway, similar to those carried out in *P. falciparum* (Carlton and Carucci, 2002; Section 1.10.1).

1.7 Parasite – Host Interactions

The malaria parasite is a complex organism with the ability not only to survive, but also to exploit and modify several different habitats in its two hosts. Its life cycle involves developmental stages in vertebrate hepatocytes and erythrocytes, as well as in the insect gut, circulatory system and salivary glands (Section 1.3). With this in mind it is perhaps not surprising that, compared to other eukaryotes, such a small part of the parasite genome encodes enzymes and transporter proteins, and a large proportion is devoted to proteins involved in parasite-host interactions. At least 1.3% of identified *P. falciparum* genes code for surface molecules involved in either cell-cell adhesion or immune evasion (Gardner *et al.*, 2002; Section 1.9).

In the human host growth and multiplication takes place under the surveillance of a fully functional immune system, hence the parasite has developed sophisticated mechanisms to survive, such as sequestration and antigenic variation. Proliferation in the mosquito is equally challenging due to a vigorous innate immune response, resulting in large numbers of parasites being lost at each step of the developmental process.

1.7.1 Survival and development in the vertebrate host

It is estimated that between 10 and 100 sporozoites are injected with the bite of a malaria-infected mosquito (Rosenberg *et al.*, 1990; Ponnudurai *et al.*, 1991). Specific interactions between the parasite and host, including the binding of sulphated glucosaminoglycans on hepatocytes to circumsporozoite protein (CSP) and thrombospondin-related adhesion protein (TRAP), guide the sporozoite to the liver (Müller *et al.*, 1993; Frevert *et al.*, 1993). After invasion the parasite increases in size and finger-like projections develop from the parasitophorous vacuole membrane (PVM) into the hepatocyte cytoplasm, presumably to increase nutrient uptake from the host cell (Aikawa *et al.*, 1984). Parasite antigens, such as liver-stage antigen-1 (LSA-1), glutamate-rich protein (GLURP) and merozoite surface protein-1 (MSP-1), are subsequently expressed and inserted into the parasite membranes or exported to the hepatocyte cytoplasm (Hollingdale & Krzych, 2002). Rapid nuclear division ensues, giving rise to up to 20,000 merozoites, which after a few days (five to six days in humans) are released into the liver sinusoid where they invade red blood cells (Wolf *et al.*, 1986).

The invasion of red blood cells is a complex, multi-step process involving erythrocyte attachment, re-orientation and binding of the apical end of the merozoite to the erythrocyte, junction formation, invagination of the erythrocyte membrane and formation of the parasitophorous vacuole. MSP-1 is thought to be the major constituent on the merozoite and is bound to the surface via a glycosylphosphatidylinositol (GPI) anchor. A significant role for *P. falciparum* MSP-1 in merozoite invasion was indicated by the finding that antibodies which inhibit proteolytic cleavage of the C-terminal of the molecule also inhibited parasite invasion (Blackman *et al.*, 1990). Monoclonal antibodies to several other *P. falciparum* merozoite antigens, including the surface-expressed merozoite surface protein-2 (MSP-2), serine repeat antigen (SERA) in the parasitophorous vacuole and rhoptry-associated protein-1 (RAP-1) have also been shown to inhibit erythrocyte invasion *in vitro* (Epping *et al.*, 1988; Clark *et al.*, 1989; Banyal and Inselburg, 1985; Perrin and Dayal, 1982; Harnyuttanakorn *et al.*, 1992; Howard *et al.*, 1998).

During the erythrocytic phase of the parasite life cycle, any parasite-encoded proteins that are to interact with the environment have to be expressed on the surface of the infected red cell. However, the erythrocyte is essentially empty, containing hemoglobin but no organelles or protein machinery. Targeting of proteins to different locations is an especially complex process in *Plasmodium*. As a eukaryote the parasite cytoplasm is compartmentalized, with a nucleus and a number of membrane-bound organelles. However, at least a dozen additional parasite protein destinations are known, including the food vacuole, the micronemes, the rhoptries, the apicoplast, the parasite plasma membrane, the parasitophorous vacuole membrane, the erythrocyte cytosol and the erythrocyte plasma membrane. To reach beyond its own boundaries and into the host cell, the parasite has to transport the proteins past its plasma membrane, through the parasitophorous vacuole, and across the parasitophorous vacuole membrane. Transportation through the red cell cytoplasm then takes place through an extensive tubovesicular network, a structural system of internal membranes induced by parasite invasion (Elford *et al.*, 1995). The mechanisms of trafficking of parasite-encoded molecules in infected erythrocytes are not yet fully understood. Studies have shown both vesicles budding from the PVM and transport in the form of protein aggregates (Trelka *et al.*, 2000; Kara *et al.*, 1988). In addition, there is evidence suggesting that the parasite

encodes classical transport machinery molecules known from other eukaryotes to be involved in vesicle-mediated protein trafficking (Albano *et al.*, 1999).

Although the parasite develops intra-cellularly and essentially is "hidden" from the host immune system, infected erythrocytes appear structurally different from un-infected ones and so are recognized as defective and destroyed as the blood passes through the spleen. To avoid this the parasite expresses receptors on the erythrocyte surface that bind to the endothelium of postcapillary venules in the host. This phenomenon is termed sequestration and is most pronounced in deep vascular tissues such as the kidneys, lungs, liver, brain and placenta (Section 1.4). Sequestration is a major cause of pathology in malaria and can result in obstruction, and ultimately failure, of major organs (Raventos-Suarez *et al.*, 1985). Adhesion of parasitised red blood cells (pRBC) in the brain is believed to be involved in the development of cerebral malaria (CM), a severe complication in *P. falciparum* infections with a bad prognosis and high mortality rate. The sequestering phenotype seems to be critical for parasite virulence, as isolates that have lost the ability to bind cause milder disease in animal models (Langreth and Peterson, 1985). In addition, infections by the other human Plasmodia do not cause sequestration of asexual parasites or CM, and are also less severe with almost no mortality.

The most well-characterised parasite receptor involved in sequestration is *Plasmodium falciparum* Erythrocyte Membrane Protein-1(PfEMP-1), a multi-domain protein expressed on the surface of the infected red cell. PfEMP-1 is coded for by the *var* multi-gene family, comprised of 40-50 gene copies (Kyes *et al.*, 2001). Although a number of host cell receptors have been identified as binding partners for PfEMP-1, their relevance *in vivo* has not been clearly established. The most studied host molecules are CD36 and Intracellular Adhesion Molecule-1 (ICAM-1; Berendt *et al.*, 1989; Baruch *et al.*, 1996; Oquendo *et al.*, 1989). The genes coding for both of these show polymorphisms in African individuals, which may influence adhesion and disease severity, and may partly explain the variation in disease outcome (Fernandez-Reyes *et al.*, 1997; Omi *et al.*, 2003). Sequestration also causes complications in pregnancies, due to binding of infected erythrocytes to the placenta. A different set of host receptors appear to be involved in

these circumstances, with chondroitin sulphate A (CSA) and hyaluronic acid being the most important binding partners in the host (Duffy and Fried, 2003).

1.7.2 Survival and development in the mosquito host

As the parasite population expands within the vertebrate host the environment successively becomes less permissive for continuing growth, as un-infected erythrocytes become more scarce and the anti-parasitic immune response develops. Transmission into a new host becomes crucial. This is dependent on sexual-stage parasites, and thus a subset of parasites start developing into gametocytes. Sexual commitment is determined already in the merozoite, before erythrocyte invasion, and transcription of early sexual-stage specific genes takes place before any morphological sexual characteristics are evident. In *P. falciparum* expression of gametocyte-specific proteins is seen from 24 hours post-invasion and onwards (Figure 1.3; Bruce *et al.*, 1994). The earliest event in the sexual differentiation process described to date is the transcription of the gametocyte-specific gene *pfs16* (Dechering *et al.*, 1997). The *Pf*s16 protein is expressed 24 hours post invasion in sexually committed stage I gametocytes and localised in the parasitophourous vacuole membrane. Its function is not yet known. As the gametocyte matures, the genes coding for *Pf*s230 and *Pf*s48/45 are activated. *Pf*s230 and *Pf*s48/45 belong to a family of sexual-specific molecules that all share unique structural motifs named six-cysteine domains. Transcripts for *Pf*s230 and *Pf*s48/45 can be detected immediately after erythrocyte invasion of a committed merozoite, with protein expression taking place in stage III gametocytes and onwards (Figure 1.3; Niederwieser *et al.*, 2000; Kocken *et al.*, 1993). Biochemical studies have shown that as the parasite emerges from the erythrocyte in the mosquito gut, *Pf*s230 undergoes proteolytic cleavage producing two polypeptides that stay associated with the newly formed gamete. It has been suggested that removal of the immunodominant region of the protein is an immune evasion strategy, protecting the parasite from antibodies carried over from the vertebrate host in the blood meal (Williamson *et al.*, 1996). However, the function of *Pf*s230 remains to be discovered. Within ten to 20 minutes of gametocyte uptake, fertilization and zygote formation commence in the mosquito midgut. *Pf*s48/45 plays a central role in male gamete fertility, as shown by gene disruption studies where greatly reduced numbers of ookinetes were produced due to impaired zygote formation (van Dijk *et al.*, 2001). *Pf*s48/45 is expressed

as a complex together with *Pfs*230 on gametocytes, the surface of male and female gametes and on zygotes (Kaslow *et al.*, 2002).

The *pfs25* gene is induced following transmission to the mosquito and the protein is detectable on zygotes and ookinetes (Figure 1.3; Niederwieser *et al.*, 2000; Kumar and Carter, 1985). Gene disruption studies in *P. berghei* recently showed that *Pbs*25 and another late sexual stage protein, *Pbs*28, have partly redundant functions. Single knock-out parasites (either *pbs25* or *pbs28* disrupted) showed only mildly compromised development in the mosquito, whereas double knock-outs (both genes disrupted) demonstrated a reduction in number of ookinetes, defects in gut penetration and oocyst formation, as well as impaired protection against proteases (Tomas *et al.*, 2001). Also expressed in ookinates is CSP- and TRAP-related protein (CTRP; Figure 1.3). Immunofluorescence studies have shown that CTRP is located in the micronemes and disruption of the *ctrp* gene demonstrated that the protein is required for invasion of the midgut epithelium (Yuda *et al.*, 1999; Dessens *et al.*, 1999).

In oocysts, curcumsporozoite surface protein (CSP) is the major surface marker on developing sporozoites (Figure 1.3). CSP contains extensive internal repeats and is attached to the membrane via a GPI anchor (Godson *et al.*, 1983; Moran and Caras, 1994). It has been known for some time that CSP is essential for initiation of the liver stages in the vertebrate host, as it is involved in sporozoite attachment to hepatocytes through the binding of heparan sulphate (Frevert *et al.*, 1993; Section 1.3). However, recent gene disruption studies have shown that CSP also is important for development during the insect stages, as CSP-negative parasites were able to infect mosquitoes but unable to produce sporozoites in developing oocysts (Menard *et al.*, 1997).

1.8 The host immune response to *Plasmodium* infection

In its vertebrate host the parasite is exposed to the immune system only for very short periods of time: when sporozoites are first injected into the blood stream by a mosquito, when exoerythrocytic merozoites are released from the liver, and during the erythrocytic cycle when the mature schizont ruptures and merozoites are searching for new erythrocytes to invade (Section 1.3). The rest of the time, apart from a short period in the liver, the parasite resides inside red blood cells. Erythrocytes are often described as a site of immunological privilege since they lack expression of MHC molecules and a protein-

processing machinery, preventing any direct antigen presentation to the immune system. However, after parasite invasion, red blood cells acquire a new set of properties, including expression of novel surface molecules and a more rigid cell structure. These changes are recognized by the host and elicit both innate and adaptive immune responses.

1.8.1 Innate resistance in the vertebrate host

Innate resistance in humans against *Plasmodium* parasites includes genetic polymorphisms, natural killer (NK) cells and macrophages and monocytes. Human populations are genotypically very diverse and although innate resistance is rarely absolute, certain genetic variabilities may affect susceptibility and severity of disease. Thus, in malaria-endemic areas where certain alleles provide a survival advantage, they have been maintained and selected for. The sickle-cell trait is such an example, where the disadvantage of having a defective hemoglobin gene is out-weighed by the improved survival rate for the carrier, and the allele therefore exists in a state of balanced polymorphism (Lell *et al.*, 1999).

NK cells and mononuclear phagocytes seem to be most important early in malaria infections. NK cells increase in numbers and have been shown to lyse *P. falciparum* infected erythrocytes *in vitro* (Orago and Facer *et al.*, 1991). However, studies with *P. chabaudi chabaudi* (AS) suggest that their ability to produce large amounts of interferon-γ (IFN-γ), a potent activator of macrophages, may be of greater importance, as mice carrying NK cells with defective cytotoxic function but normal IFN-γ production, control infection as well as wild-type mice (Mohan *et al.*, 1997). Macrophages and monocytes have the ability to phagocytose whole infected erythrocytes. Studies on opsonin-independent phagocytosis in acute phase infections with *P. chabaudi* showed a four-fold increase in phagocytic activity on day seven post-infection in resistant mice (C57Bl/6), but no change in susceptible mice (A/J). In addition, there was an inverse correlation between phagocytic activity and parasitemia, indicating that opsonin-independent mechanisms of parasite killing is a crucial component of the innate immunity that controls parasite load during the early stages of infection (Su *et al.*, 2002).

1.8.2 Acquired immune responses to liver stage parasites

Although individuals in endemic areas with repeated parasite exposure develop a certain degree of immunity, it is rarely clinical and infections usually result in low-grade parasitemias and episodes of disease throughout life.

Because injected sporozoites are extracellular, they are targets for antibody-mediated immune responses. It has been shown that immunization with high numbers (>1000) of gamma-irradiated sporozoites induces protective immunity in humans, mice and monkeys (Nussenzweig *et al.*, 1967; Clyde *et al.*, 1973). However, transfer of immune spleen cells, but not sera, from immunized animals confers protection, suggesting that this is a cellular-mediated mechanism (Sinnis and Nardin, 2002). This is initiated when the parasite invades hepatocytes and becomes intra-cellular and in rodent systems has been shown to be MHC class I restricted and dependent on cluster of differentiation (CD) 8^+ T cells (Nardin *et al.*, 1999). In humans, several studies have linked protection against *P. falciparum* with immune responses to Liver-Stage Antigen-1 (LSA-1), a molecule expressed exclusively during the liver-stages (Connelly *et al.*, 1997; May *et al.*, 2001; Kurtis *et al.*, 1999; Hollingdale *et al.*, 1990). *In vitro* studies have also shown perforin-mediated killing by cytotoxic T lymphocytes that recognise infected hepatocytes (Weiss *et al.*, 1990).

1.8.3 Acquired immune responses to blood stage parasites

After hepatic schizogony the parasite enters the circulation and begins the disease-causing cycle of infection and re-infection of erythrocytes. The spleen is the main site for filtration and recognition of soluble antigens and initiation of T cell responses take place in the white pulp. Because erythrocytes are devoid of MHC molecules, generation of an immune response must proceed through antigen presenting cells, ie macrophages/monocytes, B lymphocytes or dendritic cells. These cells phagocytose whole infected cells as well as free parasites, resulting in processing and presentation of parasite-derived peptides to immune effector cells (Kuby, 1997).

The role of $CD8^+$ T cells in protection during blood stage infections is not clear but the regulatory and effector functions of different subsets of $CD4^+$ T cells are relatively well established in both experimental and human malaria. In *P. chabaudi* infections, mice lacking or depleted of $CD4^+$ T cells, and B cell knock out mice, are unable to clear blood

stage infections (Langhorne *et al.*, 1990). These animals were shown to retain a predominant $CD4^+$ T helper (Th)1-like response throughout infection, unlike control mice where a switch in $CD4^+$ cells to a Th2-type response resulted in recovery, demonstrating that B cells play an important role in the regulation of $CD4^+$ T cell responses (Langhorne *et al.*, 1998). In humans, *in vitro* stimulation of $CD4^+$ T lymphocytes with malaria antigens results in proliferation and/or secretion of IFN-γ and interleukin (IL)-4, although correlation with protection is poor (Troye-Blomberg, 2002).

As described above, the course of a malaria infection is highly dependent on the balance of pro- and anti-inflammatory cytokines secreted by activated immune cells. Tumor necrosis factor (TNF)-α, induced in macrophages and monocytes by IFN-γ, a Th1-type cytokine, plays a central role in both protection and pathology. In human malaria TNF-α levels are positively correlated with disease severity and malarial fever (Grau *et al.*, 1989; Kwiatkowski *et al.*, 1993). Although TNF-α does not kill parasites directly, it induces production of nitric oxide (NO) and reactive oxygen species (ROS) in other cell types such as leukocytes and endothelial cells (Burgner *et al.*, 1999; Anstey *et al.*, 1996). Production of NO is catalysed by nitric oxide synthase (NOS) and has been shown to be inhibitory to sporozoites and gametocytes (Mellouk *et al.*, 1994; Naotunne *et al.*, 1993). Two studies of children recovering from malaria showed higher levels of NOS in peripheral blood mononuclear cells from those with prior mild malaria than those with prior severe disease (Perkins *et al.*, 1999; Chiwakata *et al.*, 2000). However, several findings indicate involvement of NO in the pathogenesis of human cerebral malaria, and a recent report showed an association of elevated levels of NO with severe malarial anemia in African children (Taylor-Robinson and Smith, 1999; Troye-Blomberg, 2002; Gyan *et al.*, 2002).

1.8.4 Anti-parasitic responses in the mosquito

Insect immune responses rely entirely on innate immunity since no adaptive immune system exists in invertebrates. Major components of the mosquito defense system include the hemocoel – an open circulatory system filled with hemolymph, hemocytes – circulating immune cells involved in pathogen destruction through phagocytosis and encapsulation, and the fat body – equivalent of the liver in vertebrates and main source of immune-related components secreted into the hemolymph (Figure 1.4).

The specific interaction between *Anopheles* and *Plasmodium* has been extensively investigated and it has been shown that malaria infection induces a strong innate immune response in the mosquito. The parasite must survive for at least ten days inside the mosquito to complete its development, during which time it suffers significant losses, likely to be at least in part due to the immune response of the mosquito (Dimopoulos *et al.*, 2001). *In vitro* culture systems using *P. falciparum* and *A. gambiae* have shown between 40-fold and 300-fold reductions as the parasite develops from one stage to the next (gametocyte to ookinete, ookinete to oocyst, oocyst to sporozoite; Vaughan *et al.*, 1992; Vaughan *et al.*, 1993; Beier, 1998; Ghosh *et al.*, 2000). The response to a malaria infection results in activation of immune-related genes in a temporal and spatial pattern that follows the parasites life cycle in the mosquito, with a robust response around 24 hours post-infection as the ookinete traverses the midgut epithelium, followed by a prolonged late phase between days 10 and 25 when sporozoites are released from the midgut (Dimopoulos *et al.*, 1998).

1.8.4.1 Pathogen-recognition

The mosquito is capable of mounting a range of defense reactions, including both humoral and cellular responses. Triggering of these mechanisms requires a pathogen-specific recognition system. Several genes encoding proteins with domains known to be involved in microbial binding have been cloned in *Anopheles*, and are likely to function as pattern-recognition receptors (Figure 1.4, c). Lectins are molecules that bind various sugar residues and are widely used as innate immune receptors since the pattern of surface carbohydrate expression on microbes generally differs from that on higher organisms. *IGALE20*, a putative galactose binding lectin, is highly expressed in the mosquito midgut and has been shown to be up-regulated upon malaria infection (Dimopoulos *et al.*, 1998). The gene coding for another potential carbohydrate-binding protein, gram-negative bacteria-binding protein (GNBP), is also activated upon malaria infection, systemically as well as locally in the mosquito midgut. Maximal transcription of this gene was seen around 24 hours post parasite infection, at the time when the ookinete traverses the gut epithelium (Section 1.3; Dimopoulos *et al.*, 1997; Dimopoulos *et al.*, 1998).

1.8.4.2 Serine proteases as immune response mediators

Recognition of a pathogen subsequently activates immune signaling pathways in the mosquito, many of which are initiated by the proteolytic activities of serine proteases. Serine proteases play a crucial role in invertebrate immunity by regulating a number of immune reactions leading to central anti-pathogenic defense mechanisms such as melanotic encapsulation, hemolymph coagulation and antimicrobial peptide synthesis (Figure 1.4, a and c; Gorman and Paskewitz, 2001). The proenzymes are usually monomers with a C-terminal catalytic domain, and one or more N-terminal domains. The enzymes are activated by cleavage at the amino-terminal end of the protease domain and the catalytic function of serine proteases is executed through the activity of a catalytic triad (reactive serine, histidine and aspartic acid). The type of substrate and degree of specificity is determined by the residues lining the binding-site (Perona and Craik, 1995). Although a number of serine proteases in *A. gambie* have been identified recently, few have a demonstrated function in anti-parasitic defenses. Transcriptional activation, systemically as well as locally in the midgut, of two serine protease genes, *ISP13* and *ISPL5,* occurs around 24 hours after infection with *Plasmodium* (Dimopoulos *et al.*, 1997 & 1998). Sp14A (Serine protease from chromosomal division 14A), another serine protease expressed in the mosquito midgut, showed specific up-regulation upon bacterial but not parasite challenge, with transcription also peaking 24 hours post *Plasmodium* infection. It has been suggested that Sp14A may activate prophenoloxidases, a family of proteins that upon proteolytic cleavage catalyses an enzymatic cascade resulting in melanotic encapsulation of invasive pathogens (Gorman and Paskewitz, 2001). Melanisation is the best-studied mosquito defense mechanism and is vital in mosquito strains refractory to parasite infection (Collins *et al.*, 1986). A proteinacious layer of mainly melanin is deposited around the ookinete, killing it through either starvation or toxic by-products, such as free radicals, from the enzymatic reactions catalyzing capsule formation (Paskewitz *et al.*, 1998). Another mechanism of refractoriness was recently observed in a selected strain of resistant *Anopheles*, based on lysis of ookinetes (Vernick *et al.*, 1995).

Activation of serine proteases also triggers transient transcription of antimicrobial peptides. These act through a detergent-like mechanism, disrupting the integrity of the pathogen cell membrane. Four such peptides have been isolated from *A. gambiae*: one

defensin, two cecropins and a novel molecule named gambicin. Defensin has been shown to have activity against sporozoites and enhanced transcription of the *defensin* gene takes place approximately 10 days post infection (Dimopoulos *et al.*, 1997). Gambicin has been shown to cause a 2-fold increase in ookinete death in *in vitro* anti-parasitic assays (Vizioli *et al.*, 2001).

1.8.4.3 Immunity-related serine proteases in other invertebrates

Serine proteases are widely used as immune activators in invertebrate immune defense against microbes. Hemolymph coagulation is a central mechanism of protection in many invertebrates. In insects this response is not well understood, however, horse shoe crab (*Limulus*) hemolymph coagulation is so far the most well-understood immune response in invertebrates. The cascade that leads to pathogen destruction is initiated by Limulus Factor C, a protein with both a serine protease catalytic domain and a lipopolysaccharide (LPS)-binding region. Binding of bacterial LPS to Factor C induces a conformational change in the enzyme, resulting in autoactivation. Activated Factor C cleaves another serine protease, Factor B, which in turn cleaves proclotting enzyme (PCE), a third serine protease. PCE then catalyses the last step in the pathway, the processing of coagulogen to coagulin, which subsequently self aggregates and traps the pathogens in a gel-like clot (Iwanaga *et al.*, 1998).

Another vital invertebrate mechanism of protection against pathogens is the production of antimicrobial peptides. Fungal infection in *Drosophila melanogaster* induces transcriptional activation of the gene encoding drosomycin, an anti-fungal agent. The pathway is induced by the activation of an extracellular serine protease, which subsequently cleaves the protein Spätzle. Spätzle dimerises and becomes a ligand for the membrane receptor Toll. Activation of Toll then results in a signal transduction cascade, ending with the up-regulation of the *drosomycin* gene (Hoffman *et al.*, 1999; Levashina *et al.*, 1999).

1.8.4.4 Nitric oxide in *Anopheles* immune defense

It was recently reported that nitric oxide production is used as an additional anti-parasitic defense mechanism in mosquitoes (Figure 1.4, b). Transcriptional induction of nitric oxide synthase (NOS) in the gut was shown to rise to twice the normal level at 24 hours

post infection (Luckhart *et al.*, 1998). NOS catalyses the production of the free radical nitric oxide (NO), demonstrated in mice and humans to inactivate sporozoites and gametocytes (Mellouk *et al.*, 1994; Naotunne *et al.*, 1993). Interestingly, a dietary NOS inhibitor increased oocyst formation whereas the NO precursor L-arginine depressed oocyst numbers (Luckhart *et al.*, 1998). Enhanced NOS gene expression and enzyme activity has also been detected in the salivary glands on day ten post-infection, as has transcription of the anti-microbial peptide defensin (Figure 1.4, d; Dimopoulos *et al.*, 1997; Luckhart *et al.*, 2003). This would be coinciding with the onset of sporozoite release into the hemolymph, suggesting that *Anopheles* NO may also interfere with the later stages of parasite development in the mosquito.

Immunity to malaria is complex, and very different from many viral and bacterial infections, where a single infection often results in protection to re-infection throughout life. The difference may be attributed to the more complex life cycle and genome of *Plasmodium* parasites. The parasite develops through several different stages and consequently expresses a number of different antigens. Many of these molecules have been shown to elicit strong immune responses, hence the parasite has evolved a number of evasion mechanisms to avoid and counteract these.

1.9 Avoiding the host immune response

1.9.1 Antigenic variation

In malaria, symptomatic disease is caused by development and expansion of the parasite population during the blood stages. Development of immunity to this part of the life cycle is thought to involve responses to parasite-encoded proteins expressed at the surface of infected erythrocytes. Because of their exposed location these proteins are targets for humoral host immune responses. To avoid these responses the parasite uses a strategy termed antigenic variation, whereby different variants of a protein is expressed at different times, allowing changes in antigenic identity at rates higher than random mutations. Variant antigens are encoded by multi-copy gene families. In *P. falciparum* three multi-gene families have been identified: the *variant* (*var*) family, the *repetitive interspersed family* (*rif*) and the *sub-telomeric variable open reading frame* (*stevor*) family (Su *et al.*, 1995; Kyes *et al.*, 1997; Cheng *et al.*, 1998). The genes within these

families are expressed in a successive fashion, and the proteins they encode show little immunological cross-reactivity (Newbold *et al.*, 1992; Bull *et al.*, 1999). This enables the parasite to evade immune responses that are specifically targeting these proteins, thereby allowing it to persist in the host for longer. It also results in a prolonged acquisition of immunity in the host, and allows the parasite to re-infect previously exposed individuals.

The most studied variant antigen is *Plasmodium falciparum* Erythrocyte Membrane Protein-1 (PfEMP-1), encoded by the *var* genes. These are, along with the other multi-gene family genes, clustered towards the telomeres and *in vitro* studies have shown an antigen switching rate of approximately 2% per generation (Gardner *et al.*, 2002; Biggs *et al.*, 1992; Roberts *et al.*, 1992). Even though there is some controversy as to what the primary function of PfEMP-1 is, it is well established that it mediates adhesion of infected erythrocytes to the host endothelium (Sections 1.4 and 1.7.1). PfEMP-1 is located on the surface of infected red blood cells and studies have shown that it is a target for the vertebrate host immune response and, more importantly, that antibodies to it are associated with protection against disease. In an extensive study using sera from children in Kenya, Bull *et al.*, showed that the PfEMP-1 variant expressed during clinical disease was less likely to be recognized by the corresponding child's own antibodies, than by those from other children of the same age and in the same community (Bull *et al.*, 1998). This suggests that responses against variant antigens are significant components of protective immunity in malaria, and that accumulation of variant-specific immunity against PfEMP-1 in particular, plays a key role in controlling *P. falciparum* infections.

1.9.2 Immunosuppression

Immunity to malaria involves activation of several different types of immune cells (Section 1.8). However, evidence is accumulating showing that *Plasmodium* parasites have the ability to extensively modulate the host response by suppressing and/or altering the functions of certain cells.

Macrophages and monocytes play crucial roles in both innate and acquired immune responses (Section 1.8). As phagocytic cells, they ingest hemozoin, the end product of parasite hemoglobin degradation, both as precipitates inside infected erythrocytes and as isolated pigment. However, recent reports have shown that hemozoin, causes inhibition of crucial immune functions. The crystals are insoluble and persist unmodified within the

cells for long periods, suppressing normal monocyte functions such as MHC class II expression, IL-2 production and antigen processing (Schwarzer *et al.*, 1998; Scorza *et al.*, 1999). Furthermore, other studies have shown that macrophage chemotaxis and generation of superoxide radicals for parasite killing, are perturbed in malaria infections (Nielsen *et al.*, 1986; Schwarzer and Arese, 1996).

For the acquired response antigen-presenting cells are crucial, perhaps most importantly dendritic cells (DC) because of their ability to activate both memory and naïve T lymphocytes, and hence initiate immune responses (Banchereau and Steinman, 1998; Aystyn, 1998). However, recently it was shown that *P. falciparum*-infected erythrocytes are able to bind to the CD36 and CD51 surface receptors on myeloid DCs *in vitro,* inhibiting the normal upregulation of MHC class II molecules, co-stimulatory molecules and adhesion molecules. The DCs also had a profoundly reduced ability to stimulate antigen-specific primary and secondary T cell responses, as well as allogenic T cell responses (Urban *et al.*, 1999).

1.9.3 Molecular mimicry

In many micro-organisms, including bacteria (*Salmonella* spp., *Shigella* spp, *Listeria monocytogenes,* reviewed in Stebbins and Galán, 2001), viruses (poxviruses, herpesviruses and retroviruses, reviewed in Bernet *et al.*, 2003) and parasites (*Schistosoma mansonii,* Damian, 1964; Capron *et al.*, 1965*; Trypanosoma cruzi,* Hudson and Hindmarsh, 1985; *Leishmania major*, Handman and Goding, 1985) structural and functional mimicry is a common strategy for immune evasion. Mimicry is achieved either by acquisition of virulence factors from the host by horizontal gene transfer, or through convergent evolution that results in "re-modelling" of molecules from the pathogens own protein repertoire to perform a new function. The latter strategy can produce a protein that has a three-dimensional structure distinct from that of the molecule it mimics, but has evolved to imitate the chemical groups and characteristics of its functional homologue (Stebbins and Galán, 2001). Functional mimicry has previously been demonstrated in *Plasmodium falciparum* during the blood-stages in the human host: anti-sera against *Plasmodium falciparum* blood-stage antigen Pf11-1 was shown to cross-react with the human protein thymosin-alpha1, and parasite GPI moieties have been shown to substitute for endogenous host GPI signal transduction pathways (Dubois *et al.*, 1988; Schofield

and Hackett, 1993). Although not yet shown, it does not seem unlikely that the parasite also uses mimicry to avoid the immune response in the mosquito.

1.9.4 Avoiding the mosquito immune response

As the parasite leaves its vertebrate host and enters the mosquito it escapes from the erythrocyte, leaving it exposed to attack by the mosquito immune system. The parasite consequently suffer enormous losses, however, it still succeeds in establishing an infection. Very little is known about *Plasmodium* survival strategies in its insect vector and how it counteracts the mosquito immune response. A field study looking at resistance in *A. gambiae* to *P. falciparum* found a high frequency of naturally occurring resistance alleles in the mosquito, suggesting that the parasite exerts a significant selective pressure on its insect host in the form of adaptive responses, perhaps analogous to those found in asexual-stage parasites (Niaré *et al.*, 2002).

1.10 Parasite and mosquito genome sequencing projects

1.10.1 The *Plasmodium falciparum* genome project

October 2002 saw the official release and publication of the complete sequence of the genome of *Plasmodium falciparum* (strain 3D7; Nature, 3 Oct 2002). The project was initiated in 1996 as a combined effort between the Sanger Center, Cambridge, UK, The Institute for Genomic Research (TIGR), Maryland, USA, and Stanford University, California, USA. Behind the program lay the overall objective to provide a foundation that would facilitate identification of new potential drug- and vaccine targets, and assist in gaining a better understanding of the unique biology of the *Plasmodium* parasite.

The sequencing project identified a total of 5,268 open reading frames distributed over 14 chromosomes, with a total genome size of 22.8 megabases. The data revealed a number of unusual features of the parasite genome. Several genes coding for molecules that in other species are vital for energy metabolism appeared to be absent - two genes coding for components of the ATP synthase complex were missing, as well as all genes for the subunits of a conventional NADH dehydrogenase (Wirth *et al.*, 2002). A comparison with free-living eukaryotic microbes showed that *P. falciparum* encodes fewer enzymes and transporter proteins, but a substantially larger repertoire of genes involved in immunity and host–parasite interactions. As much as 1.3% of the entire genome codes for

molecules involved in cell adhesion or host cell invasion (Gardner *et al.*, 2002). Interestingly, out of almost 5,300 predicted proteins, 60% (3,208 hypothetical proteins) have no as yet identified homologues in sequence databases of other organisms (Gardner *et al.*, 2002). This may be due to the greater evolutionary distance between *Plasmodium* and other sequenced eukaryotes, and provides a substantial source of unique molecules that may have potential to be used as targets for medical intervention without harmful side effects to the human host.

In addition to the identification of the full repertoire of *P. falciparum* genes, the genome project also provided a basis for large-scale, comprehensive analysis of transcription and protein expression in relation to parasite development (Li *et al.*, 2003, Mamoun *et al.*, 2001; Spielmann *et al.*, 2003). Novel techniques, such as large-scale microarray analysis and high throughput mass spectrometry, as well as the development of more advanced computer algorithms (reviewed in Carucci, 2002, and Carucci *et al.*, 2002) now allow a global approach to studying messenger RNA and protein expression profiles that cover the whole organism, rather than just individual genes or molecules. A high-accuracy mass spectrometric study of selected stages of *P. falciparum* examined 1,289 proteins in asexual and sexual-stage parasites. 714 of these were associated with asexual parasites, 931 with gametocytes, and 645 were found in gametes. Between 15 and 30% of proteins expressed in each set were found to be unique to that particular developmental stage (Lasonder *et al.*, 2002).

1.10.2 Rodent malaria sequencing projects

At the same time as the *Plasmodium falciparum* sequencing project was released, a five-fold coverage of the genome of another malaria species was completed, the rodent species *Plasmodium yoelii yoelii* (17XNL strain; Carlton *et al.*, 2002). Rodent malaria models reproduce many biological characteristics of the human malaria parasite and have been used widely to complement research on *P. falciparum* (Section 1.6). The *P. yoelii* genome was found to be slightly larger than the human parasite, with 5,878 predicted genes distributed over 23.1 megabases. A comparative analysis with the *P. falciparum* genome (5,268 predicted genes) identified 3,310 orthologs in *P. yoelii*. However, two gene families completely absent in *P. falciparum* were also reported: the *yir* genes (Janssen *et al.*, 2002), which have homologs in *P. chabaudi* (*cir*; Janssen *et al.*, 2002) and

P. vivax (*vir*; del Portillo *et al.*, 2001), and the *Py235* family (Preiser *et al.*, 1999). These multi-gene families are thought to be involved in antigenic variation and erythrocyte invasion, respectively, and indicate a divergence between the rodent and human malaria species, with species-specific mechanisms for these particular processes.

Sequencing of the *P. chabaudi* genome is currently being undertaken by the Sanger Center in Cambridge, UK, with a three-fold coverage to be released "as soon as possible" from a partial genome shotgun library (Sanger website).

1.10.3 The *Anopheles gambie* sequencing project

Concomitantly with the release of the sequence data from the *Plasmodium* projects, a ten-fold shotgun sequence coverage of its mosquito vector, *Anopheles gambiae* (PEST strain), was also published (Science, October 2002). The project estimated a total of 13,683 genes distributed over five chromosome arms, with almost half not previously reported (Holt *et al.*, 2002). In order to relate the findings of the mosquito genome, a comparative analysis with *Anopheles* closest sequenced evolutionary neighbor, *Drosophila melanogaster*, was carried out (divergence estimated approximately 250 million years ago). Over the entire genome, almost half of the genes identified in *Anopheles* have orthologs in *Drosophila* (Zdobnov *et al.*, 2002). However, average sequence identity between these two species is approximately 56%, a number lower than that for the same comparison between human (*Homo sapiens*) and pufferfish (*Fugu rubripes*; diverged approximately 450 million years ago) orthologs. This indicates a divergence rate that is higher than in most vertebrates, and may reflect the specialized and intimate relationships of *Anopheles* with both vertebrates and parasites (Zdobnov *et al.*, 2002).

As part of the analysis of the *Anopheles* genome, an extensive study was carried out focusing on genes involved in mosquito immunity. 242 *Anopheles* genes from 18 gene families were identified (Christophides *et al.*, 2002). Comparisons with the fruitfly showed prominent expansions of protein families involved in cell adhesion and immunity in the mosquito. Serine proteases, central mediators of invertebrate innate immunity and involved in processes such as hemolymph coagulation, melanin production and anti-microbial peptide synthesis were well represented in both genomes. However, *Anopheles*

has nearly 100 additional protease members. Again, this is probably, at least in part, due to the mosquito's interactions with both parasites and vertebrates (Holt *et al.*, 2002).

The identification of additional molecules involved in anti-parasitic responses and an improved overall understanding of *Anopheles* biology and immunity is essential in the fight against malaria, and it is now the hope of many that the availability of the genome sequence of *Anopheles gambiae,* in combination with the data from the fully sequenced genomes of *Plasmodium falcipaum* and man, will facilitate the development of new vaccines and drugs.

1.11 Disease intervention strategies

1.11.1 Vector control and drug development

It is believed that *Plasmodium* parasites originated in Africa and were brought across the Pacific to the New World by early travelers. It was not until the 17th century, however, that treatment became available to the Western world in the form of "Jesuit powder". This had been given to missionaries by native Peruvian Indians and was the bark of the cinchona tree, with the bitter substance quinine as the active ingredient. Chloroquine, a derivative of quinine was developed in the mid-1930ies and became a success, as it was both safe and very cheap.

In the 1940s the discovery of the insecticide DDT inspired a global eradication program aiming to completely eliminate malaria by reducing the level of mosquito transmission. The effort was initially successful, resulting in a dramatic decrease of the disease in many countries and almost eliminating the disease in the subtropics (Figure 1.1; Utzinger *et al.*, 2001). However, the program was problematic due to social and political barriers. In addition, mosquito resistance to DDT started appearing. This was soon followed by reports of emerging *P. falciparum* chloroquine resistance in South America and Southeast Asia, which by 1985 also had spread across the entire African continent (Bjorkman and Phillips-Howard, 1990; Wernsdorfer, 1991). By now, chloroquine resistance has been observed everywhere where malaria is endemic and the drug is considered ineffective unless coupled with antibiotics. Today it is agreed that the only realistic way to fight malaria is with the development of a vaccine.

1.11.2 Vaccine development

The highly complex life cycle of *Plasmodium* includes several developmental stages that offer candidate proteins for vaccine and drug development. Antigens on the sporozoite, the asexual blood-stage parasite and the sexual stage parasite are all being considered as targets. However, the ability of *Plasmodium* to adapt to the human immune system and to misdirect and suppress it presents the main difficulty in developing a successful vaccine (Section 1.9; Wakelin, 1989).

Three main types of vaccines are currently being developed: pre-erythrocytic vaccines, vaccines against asexual blood-stage antigens, and transmission-blocking vaccines. A vaccine targeted at antigens expressed during the erythrocytic stages would mimic the natural, partial immunity acquired by persistent infection found in individuals living in endemic areas, and reduce the symptoms of the illness. Merozoite Surface Protein 1 (MSP-1), a complex of polypeptides on the surface of the merozoite involved in erythrocyte invasion, is one of the main candidates considered for this type of vaccine. Immunisation experiments with recombinant polypeptide ($MSP\text{-}1_{19}$) and native protein in *Aotus* monkeys have been successful, making this antigen a promising candidate for a blood stage vaccine (Kumar *et al.*, 1995). Other potential targets include Apical Membrane Antigen 1 (AMA-1) found in the micronemes and on the surface of the merozoite (Deans *et al.*, 1988; Collins *et al.*, 1994). A study using squirrel monkeys immunized with *Plasmodium fragile* AMA-1, and subsequently challenged with *P. fragile* trophozoites showed reduced levels of parasitemia, or delays in accumulated parasite counts in four out of five animals, suggesting some degree of protection (Collins *et al.*, 1994). Further candidates include the microneme protein Erythrocyte-binding Antigen 175 (EBA-175) and Serine-rich Antigen (SERA), a molecule secreted into the parasitophorous vacuole (Jones *et al.*, 2001).

Although a blood-stage vaccine would prevent serious disease and pathology, it would not protect a previously unexposed individual, such as a traveler, who would require protection aimed at the very early stages of infection, ie the sporozoite or liver-stage parasite in order not to fall ill (Phillips 2001). Following experiments showing that immunisation with whole, irradiated sporozoites induced complete protection against *P. falciparum* and *P. vivax* infections, Circumsporozoite Protein (CSP) was identified as the

main antigen on the sporozoite surface (Clyde, 1990; Nussenzweig and Nussenzweig, 1989). More recently, in limited trials on malaria-naive volunteers, RTS,S, a hybrid of the C-terminal of CSP and the Hepatitis B surface antigen HbsAg, generated protection in six out of seven subjects (Stoute et al., 1997). Moreover, in a Phase I study with semi-immune individuals in The Gambia, RTS,S in combination with the adjuvant AS02 generated strong T cell responses, and when preceeded by a PfCSP DNA vaccine in a sequential immunisation regime, also induced PfCSP-specific antibodies and Th1 $CD4^+$ T cells, and $CD8^+$ cytotoxic and Tc1 cells (Pinder *et al.*, 2004, Wang *et al.*, 2004).

Finally, in order to limit the spread of disease, the parasite life cycle would have to be interrupted. A vaccine targeting the parasite's sexual reproduction within the mosquito would not protect against disease but would stop other individuals being infected by a mosquito carrying parasites, thereby reducing transmission. This type of immunity is mediated by antibodies against surface antigens on gametocytes and later developmental stages. Antibodies induced in the human host would be carried over to the mosquito, where they would act on parasites in the midgut. Antigens for these transmission-blocking vaccines fall into three groups depending on their time of expression: (i) prefertilisation antigens - expressed on gametocytes, (ii) postfertilisation antigens - from zygotes or ookinetes, and (iii) late-midgut-stage antigens - expressed in late ookinetes. One advantage of using pre-fertilisation antigens is that antibody responses may be boosted by natural infection, since these molecules are expressed by the parasite as it is developing within the vertebrate host. Post-fertilisation antigens, on the other hand, would not have been subjected to selection by immune evasion mechanisms, and may therefore be more immunogenic (Phillips, 2001).

Several prefertilisation antigens, including *Pfs*230, *Pfs*48/45 and *Pf*11.1 have been shown to have transmission-blocking activity *in vitro*. Two postfertilisation antigens, *Pfs*25 and *Pfs*28, have been considered as vaccine candidates. *Pfs*25 shows little antigenic diversity and has been shown to induce transmission-blocking antibodies in mice and monkeys following immunisation with recombinant protein (Barr *et al.*, 1991; Kaslow and Shiloach, 1994). *Pfs*25 has recently gone into Phase I trials (Kaslow, 1997). Of the late

Table 1.1. Antigens considered for a malaria vaccine

Vaccine type	Candidate antigen
Pre-erythrocytic	Circumsporozoite Protein (CSP)
Blood-stage	Merozoite Surface Protein 1 (MSP-1) Apical Membrane Antigen 1 (AMA-1) Erythrocyte-binding Antigen 175 (EBA-175) Serine-rich Antigen (SERA)
Transmission-blocking	*Pfs*230, *Pfs*48/45, *Pf*11.1 (pre-fertilisation antigens) *Pfs*25, *Pfs*28 (post-fertilisation antigens) Chitinase (late-midgut-stage antigen)

midgut-stage antigens chitinase has received some interest. As the parasite is ingested along with a blood meal, a layer of chitin, the peritrophic membrane matrix, forms around the mosquito midgut. This acts as a barrier as the ookinete tries to pass through the midgut wall, making penetration more difficult (Section 1.3). However, there is evidence suggesting that the parasite secretes a chitinase that breaks down the peritrophic membrane and chitinase inhibitors have been shown to block transmission (Huber *et al.*, 1991; Duffy *et al.*, 1993; Shahabuddin *et al.*, 1993).

Major potential vaccine candidates are summarized in Table 1.1.

Because the expression of most parasite antigens is restricted to a certain stage of the life-cycle, a fully effective vaccine would ideally include molecules from all developmental stages. Moreover, as most immunogenic blood-stage antigens are highly polymorphic it is also likely that inclusion of more than one allelic variant of an antigen would increase protection (Richie and Saul, 2002). These multi-stage, or combination vaccines, are currently being developed and some have gone into, or are awaiting, clinical trials. SPf66, a synthetic polyvalent vaccine comprised of *P. falciparum* sequences from three putative blood-stage antigens and the four amino acid repeat of the pre-erythrocytic CS protein has undergone the most extensive field testing of any malaria vaccine. Early trials in South America and Tanzania showed moderate efficacy, however, later testing in Thailand, Brazil and The Gambia showed no significant protection and at present no further trials are planned (Valero *et al.*, 1993; Valero *et al.*, 1996; Alonso *et al.*, 1994; Nosten *et al.*, 1996; Urdaneta *et al.*, 1998; D'Alessandro *et al.*, 1995).

DNA based vaccine technology may also provide a useful approach to induce protection against malaria. Animal studies have shown that both $CD8^+$ T cell and Th1-type immune responses can be induced with DNA vaccines, something that has been difficult to achieve with protein-based methodology. The American Navy research group is currently working on a candidate vaccine, MuStDo, that will include sequences encoding chosen epitopes from five pre-erythrocytic and ten blood-stage antigens (Doolan and Hoffman, 2002).

However, so far no malaria vaccine has gone into routine use.

1.12 Aims of project

One of the greatest obstacles in the development of a vaccine against malaria is the parasite's ability to avoid the host immune response through antigenic variation. The variant proteins involved are encoded by large gene families in the parasite genome. A better understanding of how these genes are regulated and how the molecules they encode affect pathogenesis and survival of the parasite, is key in the progress towards a vaccine and more effective therapeutics. A rodent animal model would allow investigations into the molecular biology and immunology of this phenomenon that for ethical and practical reasons are impossible in infected humans or primates.

Previously in the division where the present work was carried out had identified a short sequence of an unknown gene that appeared to be a member of a multi-gene family in *Plasmodium chabaudi chabaudi* (AS). However, experiments performed early on in the present project demonstrated that this was not the case. Instead it was discovered that the gene encoded the first scavenger receptor-like protein in *Plasmodium*. Thus, the overall objective became to analyse this novel gene and protein, with the following aims:

i) To identify the full length sequence the *P. c. chabaudi* (AS) gene

ii) To analyse the protein sequence for structural and functional domains, as well as other sequence motifs, such as targeting signals

iii) To determinate sub-cellular localisation of the protein and stage-specificity of expression

iv) To investigate protein function by targeted gene disruption

Figure 1.1

Global distribution of areas with a high risk of malaria

Geographical map showing the changes in global distribution of areas with a high risk of malaria between the years of 1946 and 1994. The burden of disease is increasingly confined to the tropics.

Modified from Sachs and Malaney, 2002.

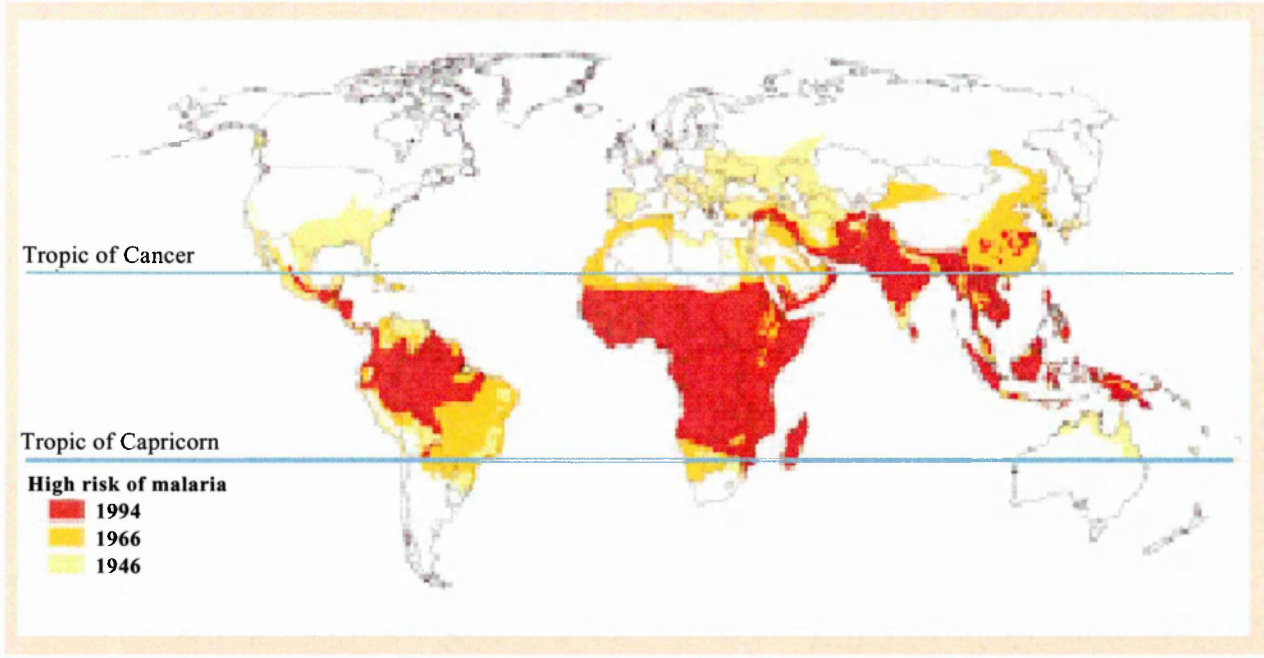

Tropic of Cancer
Tropic of Capricorn
High risk of malaria
1994
1966
1946

Figure 1.2

The *Plasmodium* life cycle

The *Plasmodium* parasite has a complex life cycle that involves a series of developmental transitions in both the vertebrate host and the insect vector.

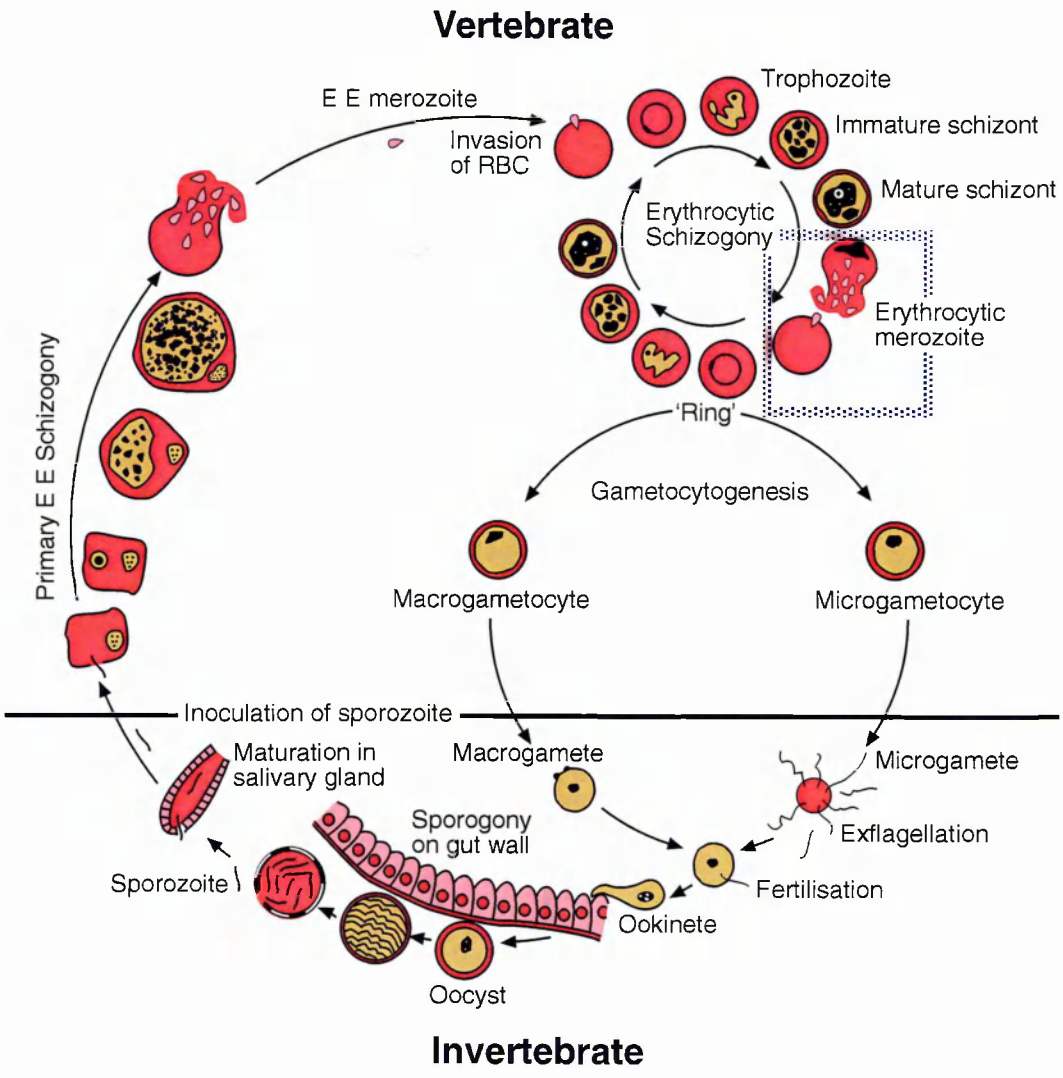

Vertebrate
E E merozoite
Invasion of RBC
Trophozoite
Immature schizont
Mature schizont
Erythrocytic Schizogony
Erythrocytic merozoite
'Ring'
Gametocytogenesis
Macrogametocyte
Microgametocyte
Primary E E Schizogony
Inoculation of sporozoite
Maturation in salivary gland
Macrogamete
Microgamete
Exflagellation
Sporogony on gut wall
Sporozoite
Fertilisation
Ookinete
Oocyst
Invertebrate

Figure 1.3

A selection of *P. falciparum* proteins expressed in sexual- and sporogonic stages of parasite development

The sexual and sporogonic stages of the *Plasmodium* life cycle start with gametocytogenesis in the vertebrate host and are completed in *Anopheles* with the final maturation of sporozoites in the mosquito salivary glands. Differentiation through these stages involves a series of morphological changes, driven and defined by a developmentally regulated expression of specific antigens.

The diagram illustrates the expression of proteins at different life cycle stages, with continuous arrows signifying high levels of expression, and hatched lines low expression.

Modified from Sauerwein and Eling, 2002.

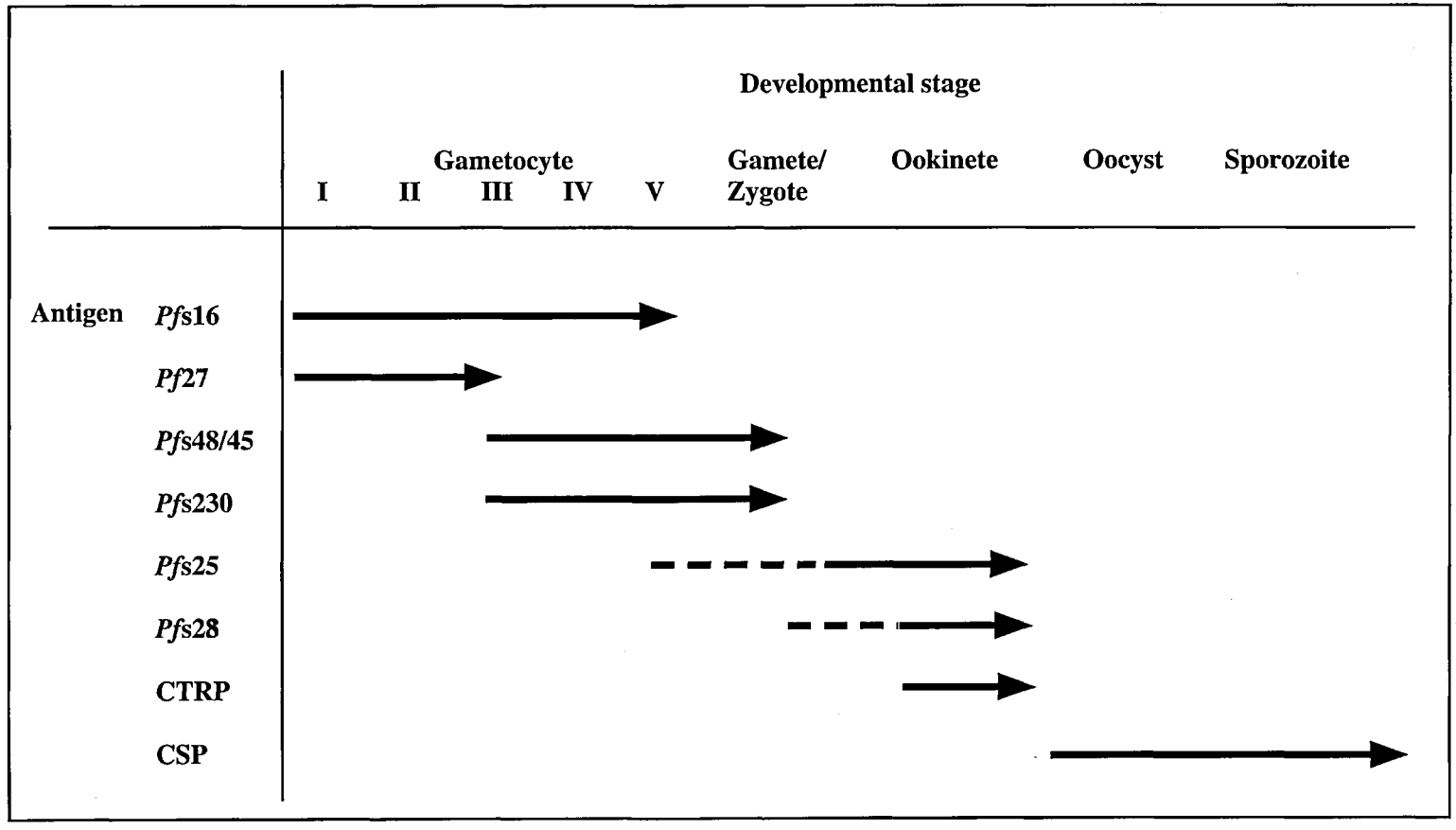

Developmental stage
Gametocyte
I
II
III
IV
V
Gamete/
Zygote
Ookinete
Oocyst
Sporozoite
Antigen
Pfs16
Pf27
Pfs48/45
Pfs230
Pfs25
Pfs28
CTRP
CSP

Figure 1.4
Mosquito defense reactions against malaria parasites

The parasite's development in the mosquito (yellow arrows) starts as ingested gametocytes fuse and produce a zygote, which soon develops into a motile ookinate. The ookinate traverses the epithelial cells and, once on the basal side, transforms into an oocyst. The oocyst matures and undergoes nuclear division, resulting in the production of thousands of sporozoites. The sporozoites are released into the mosquito hemocoel and travel to, and invade, the salivary gland.

Mosquito immune responses against the parasite (red arrows) have been documented in the midgut epithelium, the hemocoel and in the salivary glands. In the epithelium melanotic encapsulation (a) and lysis have been observed as anti-parasitic defense mechanisms in two separate refractory mosquito strains. Nitric oxide (NO) has also been shown to restrict parasite development in the midgut (b). In the hemocoel, the insect's circulatory system, the parasite is exposed to hemocytes, antimicrobial peptides and other humoral factors. Binding of soluble or surface-bound pattern-recognition factors (green) can trigger a cascade of serine proteases, which in turn activate various immune-related molecules, such as the cleaving of PPO to PO, an enzyme involved in melanisation (c). Antimicrobial peptides and NO are also found in the salivary gland (d).

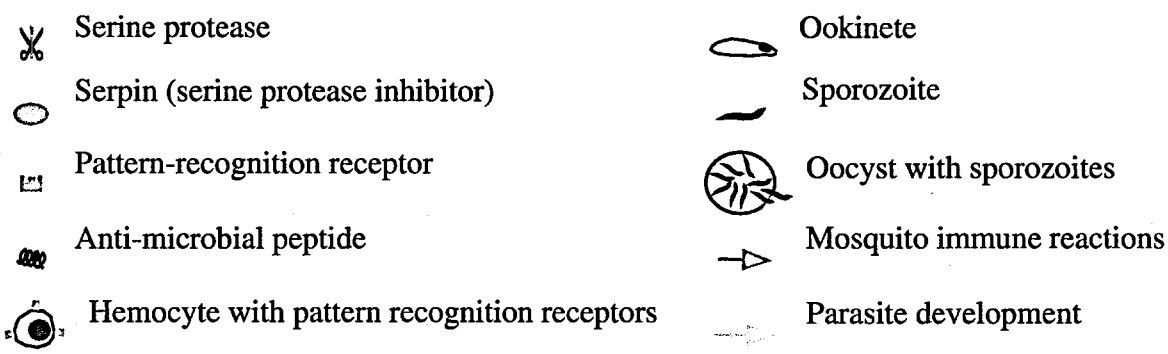
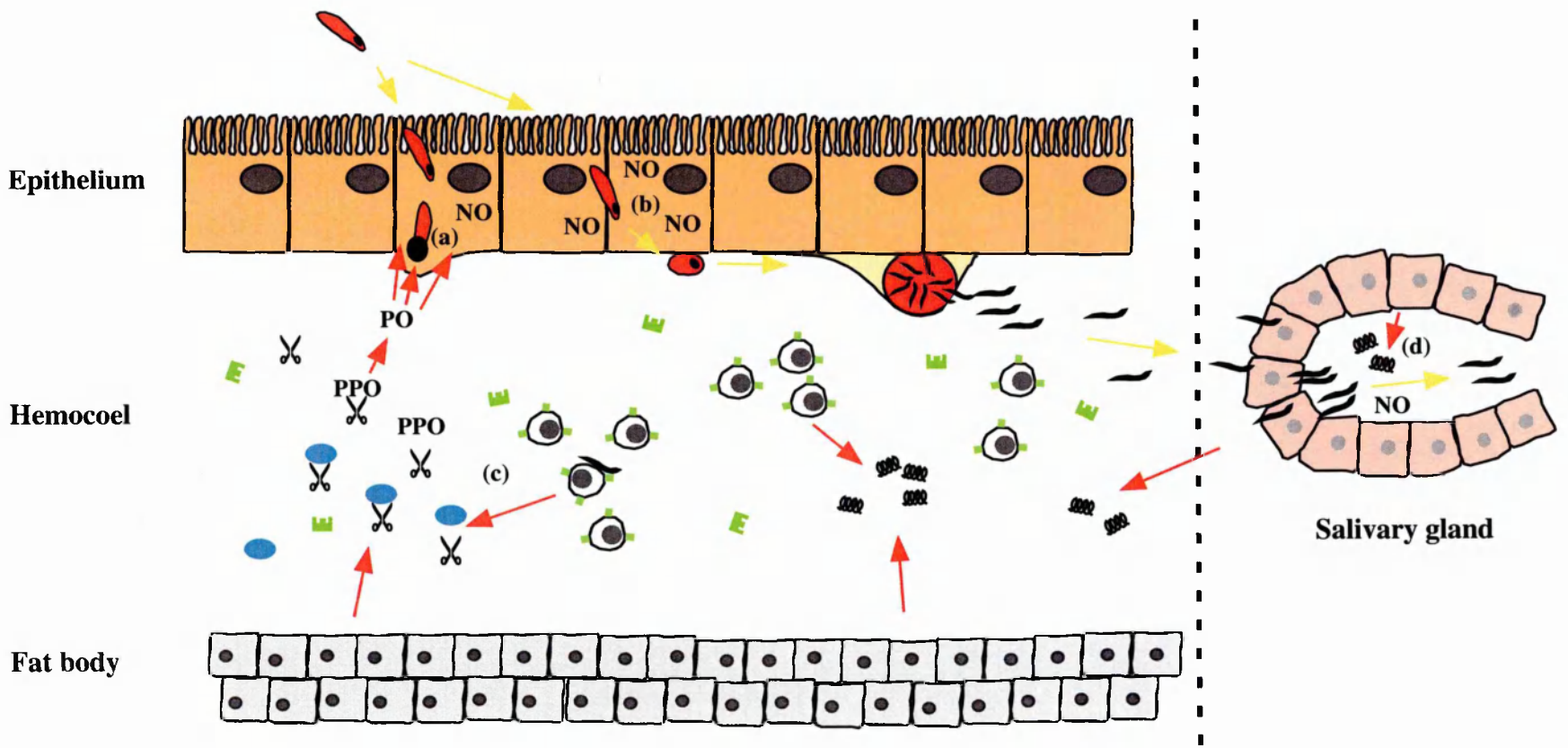


Figure modified from Dimopoulos *et al.*, (2001)


Epithelium
Hemocoel
Fat body
NO
(a)
NO
NO
(b)
NO
PO
PPO
PPO
(c)
(d)
NO
Salivary gland

Chapter Two

Materials and methods

2.1 Media, solutions and buffers

All chemicals and reagents were of analytical grade and purchased from BDH Chemicals Ltd., Poole, UK, or Sigma, Poole, UK, unless otherwise stated. Compositions of media, solutions and buffers are given in the Appendix.

2.2 Bioinformatics

Alignments and editing of nucleotide and protein sequences were performed using the MegAlign®, EditSeq® and MapDraw® programmes (all DNASTAR Inc). BLAST® (Basic Local Alignment Search Tool) searches were carried out using the NCBI Genbank Database (http://www.ncbi.nlm.nih.gov/) and the PlasmoDB server (http://plasmodb.org). Searches for protein sequence motifs were carried out using the Simple Modular Architecture Research Tool (SMART) programme (http://smart.embl-heidelberg.de/). Post-translational modifications and localisation signals were predicted by running the *Pc*SLAP sequence through the following programmes: SignalP (http://www.cbs.dtu.dk/services/SignalP/) for hydrophobic signal sequences, TargetP (http://www.cbs.dtu.dk/services/TargetP/) and PATS (Prediction of Apicoplast Targeted Sequences) programmes; http://gecco.org.chemie.uni-frankfurt.de/pats/pats-index.php) for localisation sequences in general and apicoplast transit peptides, respectively, and big-PI Predictor (http://mendel.imp.univie.ac.at/sat/gpi/gpi_server.html) for GPI modification sites.

2.3 Parasites and experimental animals

2.3.1 Parasites

Plasmodium chabaudi chabaudi (AS strain) was originally obtained from Dr K. N. Brown, National Institute for Medical Research (NIMR, London).

Plasmodium berghei ANKA, originally isolated in Katanga, Congo, in 1965, was supplied by Professor D. Walliker (WHO Registry of Standard Strains of Malaria Parasites, Edinburgh University, Scotland) to Dr. W Jarra, (NIMR, London) as a cloned, mosquito transmitted, re-cloned and enzyme typed line (Beale *et al.*, Rodent Malaria). A frozen stabilate from this line was kindly given to us by Dr Jarra.

Cryopreserved stabilates (Section 2.3.2) of these two parasite lines, no more than six syringe passages from the original materials, were used for animal infections. Parasites were syringe passaged every 5-10 days, for a maximum of four times before the line was discarded.

2.3.2 Cryo preservation of parasites

Mice were culled by terminal anaesthesia, the blood collected into 50 µL heparin per mouse (5000 U/mL; Leo Laboratories Ltd., Aylesbury, UK) and centrifuged at 1000 x g, 10 minutes at 4°C. The packed cell volume was estimated and two volumes of parasite freezing medium (Appendix) was added as follows: 1/5 of the volume to be added was mixed in gently and the parasites left for five minutes. The remaining 4/5 of freezing medium were then added drop wise with gentle mixing. Stocks were initially frozen at –80°C and then transferred to liquid nitrogen.

2.3.3 Experimental animals

6-10 week old female BALB/c mice were obtained from the NIMR Special Pathogen Free breeding unit and conventionally housed in sterilised cages, with sterile food and bedding.

6–10 week old female µMT mice, deficient in B cells due to a targeted mutation in the immunoglobulin (Ig) µ-chain, were also used (Kitamura *et al.*, 1991). These were bred and maintained at the breeding facilities of NIMR, and housed as described above.

Before any procedures were carried out, animals were left to acclimatise for seven days in the facilities subsequently used for the experiments.

2.3.3 Infection of experimental animals

Cryopreserved parasite stabilates were thawed on ice, 0.9% saline added to give a total volume of approximately 300 µL, and 100-150 µL injected intraperitoneally (ip) into two mice. Animals infected with *Plasmodium chabaudi chabaudi* (AS) were kept in an artificial, 12 hour reversed light cycle, so that parasite schizogony took place in the early afternoon (13.00 -16.00) rather than during the night. Parasitemia was monitored by light microscopy on methanol-fixed, Giemsa-stained thin blood films (10% Giemsa's stain in phosphate buffer (Appendix), pH 7.4; Garnham, 1966), and determined as follows:

$$\% \text{ Parasitemia} = \frac{\text{Total number of infected erythrocytes in } n \text{ fields}}{(\text{Total number of erythrocytes in a representative field}) \times n} \times 100$$

At parasitemias ≥1% 10 fields were counted, whereas below this percentage a total of 50 fields were scanned.

To initiate new infections, infected blood containing 10^5-10^7 pRBC was diluted in 0.9% saline to a volume of 100 µL and syringe passaged into a naïve mouse.

2.3.4 Collection of blood & plasma preparation

Blood was collected into 0.1 mL heparin (5000 U/mL; Leo Laboratories Ltd., Aylesbury, UK) and centrifuged at 13000 x g, 4°C for 10 minutes. The plasma fraction was removed and used immediately or stored at -20°C.

2.4 DNA and RNA preparation and manipulation

2.4.1 Preparation of parasite genomic DNA

Plasmodium chabaudi chabaudi (AS)- or *Plasmodium berghei* ANKA-infected BALB/c mice were bled into heparin at 4°C, at approximately 20% parasitemia. The blood was washed twice in ice-cold phosphate buffered saline (PBS; Appendix) centrifuging for 5 minutes at 400 x g, 4°C. The pelleted cells were resuspended in sterile ice-cold PBS (Life Technologies, UK; 30 mL PBS per mL pellet) and passed through a Plasmodipure filter (Euro-Diagnostica, The Netherlands) according to the manufacturer's instructions, to remove mouse leukocytes. The cells were washed as above and extraction buffer

(Appendix) and Proteinase K (10mg/mL) were added to the pellet in the following volume proportions: pellet:extraction buffer:10% SDS:Proteinase K -1:1:0.05:0.01. The mixture was incubated overnight at 50°C in a rotating Hybaid hybridisation oven. The following morning additional Proteinase K was added (same volume as before) and the incubation continued at 50°C until the evening. An equal volume of phenol:chloroform (1:1, pH 10.5; Life Technologies) was added and the solution incubated overnight at room temperature in a rotating hybridisation oven.

The second morning the mixture was centrifuged for 10 minutes at 2000 x g, 4°C, and a second phenol:chloroform extraction performed as above. 2.5 volumes of ice-cold 100% ethanol was subsequently added, resulting in the DNA falling out as a brown precipitate which was transferred to a clean Eppendorff tube. One mL of 70% ethanol was added, the tube inverted several times followed by centrifugation for five minutes at 13000 x g, room temperature. The DNA was then left to air-dry for ten minutes, re-dissolved in Tris-ethylenediaminetetraacetic acid (EDTA; TE) buffer (Appendix) and stored at 4°C.

2.4.2 Amplification of DNA by Polymerase Chain Reaction (PCR)

All primers used in standard PCR were synthesised by Oswel (Southampton, UK) and are listed in Tables 2.1 and 2.2. Reactions were carried out in a total volume of 50 µL and were performed using a Hybaid PCR Express machine.

2.4.2.1 PCR using Pfx DNA Polymerase

For amplification of sequences where accuracy was of great importance, the high-fidelity Pfx system (Life technologies) was used. Reactions included the following: 1 mM $MgSO_4$, 50 µM of each dNTP (Pharmacia), 1 x Pfx amplification buffer, 250 nM of each primer, 100 ng template DNA and 1.2 units Pfx DNA Polymerase.

2.4.2.2 PCR using AmpliTaq DNA Polymerase

For standard PCR AmpliTaq (Roche) was used. Reactions included the following: 50 µM of each dNTP (Pharmacia), 1 x AmpliTaq amplification buffer, 250 nM of each primer, 100 ng template DNA and 1.2 units AmpliTaq Polymerase.

Reactions were run as follows:

Step 1	94°C	5 minutes	Initial denaturation
Step 2	94°C	30 seconds	Denaturation
Step 3	50°C	30 seconds	Annealing
Step 4	72°C	1 minute	Extension, to Step 2 for 30 cycles
Step 5	72°C	10 minutes	Final extension

2.4.3 Cloning of PCR products

PCR products that were to be sequenced were cloned straight into the pCR®4Blunt-TOPO® vector (Zero Blunt® TOPO® PCR Cloning Kit for sequencing, Invitrogen, Gottingen, FRG) according to the manufacturer's instructions.

PCR products amplified from primers containing 5' restriction sites were purified using PCR Clean Columns (Qiagen, Hilden, The Netherlands), digested with the appropriate restriction enzyme (Restriction enzyme digestion of DNA), purified again, and ligated with the vector at a 1:3 vector:insert ratio. Ligation products were transformed into TOP10 One Shot chemically competent cells (Invitrogen) by heat shock and transformed bacteria selected for on agar plates (100 μg ampicillin /mL). Colonies were picked and overnight cultures set up in LB broth containing ampicillin (100 μg/mL). Plasmids were isolated as described in Section 2.4.4 and 2.4.5.

2.4.4 Small-scale preparation of plasmid DNA (minipreps)

Isolation of plasmid DNA was performed using the Qiagen Miniprep Kit (Qiagen), according to the manufacturer's instructions.

2.4.5 Large-scale preparation of plasmid DNA (maxipreps)

Isolation of plasmid DNA was performed using the Qiagen Maxiprep Kit (Qiagen), according to the manufacturer's instructions.

2.4.6 Restriction enzyme digestion of DNA

Digestion of genomic and plasmid DNA was performed as directed by the enzyme manufacturer.

2.4.7 Sequencing of cloned PCR products

Positive clones were identified by restriction digestion of isolated plasmids (Sections 2.4.4 and 2.4.6), and sequenced by MWG Biotech (Milton Keynes, UK).

2.4.8 Bacterial cultures and stocks

Bacterial cultures were grown in standard LB Broth (Appendix), with shaking, at 37°C. The medium was supplemented with ampicillin at 100 μg/mL. Bacterial stocks were made from 85% bacterial culture and 15% glycerol and stored at -70°C.

2.4.9 The Vectorette II System

All work with the Vectorette System was carried out using the Vectorette II Starter Pack (Sigma Genosys, Cambridge, UK), according to the manufacturer's instructions.

2.4.9.1 Construction of *Plasmodium chabaudi chabaudi* (AS) gDNA Vectorette libraries

Plasmodium chabaudi chabaudi (AS) gDNA was isolated as described previously (Section 2.4.1) and aliquots of 1μg digested with RsaI (Roche, UK), Sau3AI (Promega, UK), SspI (Roche) or XhoII (Promega). Vectorette units were subsequently ligated onto the ends of the gDNA fragments to generate four *Plasmodium chabaudi chabaudi* (AS) gDNA libraries.

2.4.9.2 Vectorette PCR

Vectorette PCR was carried out as described (Section 2.4.2), but using the supplied Vectorette PCR primer and one of two primers based on the known *Pc*SLAP gene sequence, either one for 5' extension, or one for 3' extension of the gene (Table 2.3). Reaction volumes included the following: 1 μL Vectorette library, 10 μL 10 x PCR amplification buffer, 1mM $MgSO_4$, 50 μM of each dNTP (Pharmacia), 250 nM

Vectorette primer, 250nM *Pc*SLAP primer, 1.2 units of Pfx DNA Polymerase and sterile dH_2O to 100 µL. Reactions were performed using the following programme:

Step 1	94°C	5 minutes	Initial denaturation
Step 2	94°C	1 minute	Denaturation
Step 3	50°C	1 minute	Annealing
Step 4	72°C	3 minutes	Extension, to Step 2 for 40 cycles
Step 5	72°C	10 minutes	Final extension

PCR products were cloned into the pCR®4Blunt-TOPO® vector and sequenced as described (Figure 2.1; Sections 2.4.3 and 2.4.7).

2.4.10 Preparation of parasite total RNA

Infected BALB/c mice were bled into heparin at 4°C and the erythrocytes pelleted by centrifugation at 1000 x g, 4°C, for five minutes. Ten pellet volumes of Trizol® (pre-warmed to 37°C, Invitrogen), was added to the cells and the tube shaken vigorously until all clumps were dissolved. At this stage the preparation was either frozen at -80°C, or left for five minutes (RT). Two pellet volumes of chloroform were added to the Trizol®-mix followed by shaking for 15 seconds. The mixture was left to separate at RT for three minutes, then centrifuged for 30 minutes at 3000 x g, 4°C. The supernatant was transferred to clean tubes, mixed with isopropanol in the following proportions: 0.6 pellet volumes supernatant + 0.5 pellet volumes isopropanol, and left on ice for 60 minutes. Samples were centrifuged for 25 minutes at 13000 x g, 4°C, in a microfuge, each pellet washed with 1 mL 70% ethanol (13000 x g, 10 minutes, 4°C) and the tubes inverted and air-dried for five minutes. Pellets were resuspended in 10-50 µL of formamide (volume depending on pellet size), heated to 60°C for 10 minutes then placed on ice. Samples were transferred to new tubes excluding any drops on the side of the old tubes. RNA concentrations were estimated by measuring optical density at 260 nm. Samples were stored at -80°C.

2.4.11 Preparation of total RNA from mouse (BALB/c) blood

Isolation of RNA from mouse blood was performed as parasite RNA isolation, but using blood from uninfected, female BALB/c mice.

2.5 DNA and RNA electrophoresis

2.5.1 Gel electrophoresis of DNA

Electrophoresis of DNA was performed as described in Current Protocols of Molecular Biology using Multi Purpose Agarose (Roche) at concentrations between 0.8% and 3%, in TBE buffer (Appendix).

2.5.2 Gel electrophoresis of RNA

Total RNA from *P. c. chabaudi* (AS), *P. berghei* ANKA or BALB/c peripheral blood mononuclear cells was resolved in 1% agarose gels containing five mM guanidine thiocyanate, in TBE running buffer. Each sample contained 10-20 mg of RNA in 10 mL formamide and a very small amount of ethidium bromide (a pipette tip was dipped in a 10 mg/mL solution of ethidium bromide and then into the sample) Samples were incubated at 60°C for 10 minutes, chilled on ice and loaded. Loading dye alone was added to the final well, with unused wells in between to avoid contamination. Gels were run for an initial 10-15 minutes at 110 volts, after which the voltage was decreased to 70 volts. Electrophoresis was continued until the bromphenol blue dye was approximately 10 cm from the wells.

2.5.3 Extraction of DNA from agarose gels

DNA was extracted from agarose gels using the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's instructions.

2.6 Southern and Northern blotting

2.6.1 Southern blotting

0.5 μg of DNA per sample was digested and electrophoresed as described (Sections 2.4.6 and 2.5.1). The gel was incubated in depurination buffer for 15 mins (Appendix), washed in transfer buffer for 30 minutes (Appendix) and the DNA blotted onto a positively

charged nylon membrane (Hybond N+, Amersham) by capillary action overnight (all incubations on a rotating platform). The membrane was rinsed in 2xSSC (Appendix) and cross-linked at 120 mJoules (Auto-crosslink setting) using a Stratalinker® UV Crosslinker.

2.6.2 Northern blotting

Electrophoresed RNA was transferred by capillary action over night onto a positively charged nylon membrane (Hybond N+, Amersham) after washing with rotation in RNA transfer buffer for 30 minutes (Sambrook 1977). The membrane was rinsed in 2xSSC and cross-linked under UV-light as above.

2.6.3 Radio-labelling of DNA probes

Radio-labelleling was performed using ≥25 ng of probe and [^{32}P]-dATP (Amersham), using the Prime-It® II Random Primer Labelling Kit (Stratagene, La Jolla, USA) according to the manufacturer's instructions.

2.6.4 Hybridisation with radio-labelled probes

Membranes were pre-hybridised for at least 1 hour in PerfectHyb™ hybridisation solution prior to probe addition. After probe addition DNA membranes were hybridised at 60°C, for 6 hours or longer. RNA membranes were hybridised with probe at 42°C, over night. Membranes were then washed in 0.1% SDS and reducing concentrations of SSPE (Appendix; from 2x to 0.5x) for 15 minutes per wash, rinsed in 2xSSC (no SDS) and exposed to Kodak Biomax MR Single emulsion film at -70°C.

2.7 Gametocyte purification

BALB/c mice were inoculated with 10^7 parasitised erythrocytes. On 4 successive days, starting 24 hours post inoculation, pyrimethamine was administered by intraperitoneal injection (10 mg kg^{-1} bodyweight). At the end of drug treatment blood was collected for gametocyte isolation. All steps were performed at 37°C, except centrifugations. Animals were bled into heparin, the blood diluted in RPMI 1640/10% FCS (Invitrogen; Globepharm, UK) and run through a Plasmodipure filter to remove leukocytes. This was

followed by centrifugation seven minutes at 200 x g, the pellet resuspended in RPMI/10% FCS and overlaid onto a 48% Nycodenz/(RPMI/10% FCS) gradient (Robins Scientific, UK; 35 mL culture over 10 mL Nycodenz). The gradient was centrifuged for 15 minutes at 350 x g with slow acceleration and no brake. The interface was isolated and washed with RPMI for seven minutes at 300 x g. The purified gametocytes were used for RNA isolation or for making thin blood smears for indirect immunofluorescence assays (Sections 2.4.10 and 2.13).

2.8 Analysis of protein

2.8.1 Expression of recombinant *Pc*SLAP protein

A bacterial stock containing a 270 bp cDNA fragment of *pcslap* was provided by Maria Mota and Anthony Holder (Division of Parasitology, NIMR). The fragment had been cloned into the *Pst*I and *Hin*dIII restriction sites of the pTrcHisC expression vector (Invitrogen), creating the pTrcHisC-$pcslap_{270}$ construct, and subsequently transformed into *E. coli* DH10B cells. The vector contained an N-terminal hexa-histidine tag (His_6) for purification on nickel-matrices.

Five mL of an overnight culture with the above transformed bacteria was inoculated into 500 mL of LB broth and incubated with shaking (250 rpm) at 37°C. Expression was induced when the OD_{600nm} of the bacterial culture reached 0.6, by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1mM, and incubation continued as described above. A preliminary assay showed that maximal expression was achieved at five hours post-induction. These conditions were used to prepare a large scale, 40 liter culture (Margaret Goggin, NIMR) for subsequent purification.

2.8.2 Purification of recombinant *Pc*SLAP protein

Frozen bacterial extracts were thawed and solubilised overnight in lysis buffer (Appendix). Samples were then centrifuged at 15000 x g at 4°C for 1 hour, ultracentrifuged at 30000 x g, 4°C for an additional hour and applied to a nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography column (Qiagen) pre-equilibrated with lysis buffer. The column was washed with lysis buffer and the bound protein eluted with 200 mM imidazole in lysis buffer. A second step of purification was performed

using an FPLC gel filtration column with Superdex 200 prep grade matrix (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was washed and the protein eluted with lysis buffer. The fractions containing the protein were then pooled and slowly dialysed against PBS to remove the urea, by dilution of the urea by a factor of two each time the dialysis solution was changed. The protein was then concentrated under pressure in an Amicon concentrator (Sartorius, Epsom, UK) with a 10kD cut-off membrane (Sartorius), before use in immunisations of mice and rabbits.

2.8.3 Protein separation by SDS-PAGE

Proteins were separated over gels consisting of a 4% acrylamide stacking gel and a 10% acrylamide resolving gel (both Appendix). 10 µL sample was mixed with 10 µL SDS sample buffer (Appendix), boiled for five minutes, transferred to ice and loaded. Electrophoresis was carried out at 25mA in SDS running buffer (Appendix). Gels were stained in Coomassie blue stain (Appendix) for approximately one hour and incubated in de-stain (Appendix) until the protein bands were clearly visible.

2.9 Immunisations

2.9.1 Protein immunisations for antibody production - mice

Groups of five female BALB/c mice received intraperitoneal injections of 100 µL RIBI adjuvant -antigen emulsion containing 25 µg of *P c*SLAP$_{90}$ antigen per injection (adjuvant-antigen ratio according to the manufacturer's instructions). Four booster injections were subsequently administered with the same amount of antigen, at two to three week intervals and plasma collected one week after every boosts as described in Section 2.3.4. Antibody responses in each animal were monitored by enzyme-linked immunosorbent assay (ELISA).

2.9.2 Protein immunisations for antibody production - rabbits

Rabbit immunisations were carried out at Abbott Murex Biotech Ltd. (Dartford, UK). Briefly, two rabbits received subcutaneous injections of 200 µL Freund's complete adjuvant-antigen emulsion, containing 0.5 mg of *Pc*SLAP$_{90}$ antigen per injection. Booster injections were administered at two-week intervals, at a total of four times, with the same

amount of antigen in Freund's incomplete adjuvant. Sera were collected one week after the boosts and antibody responses monitored by ELISA. Before the start of the immunisations, samples of pre-immune serum had been collected from each rabbit to be as negative controls.

2.9.3 Preparation of murine hyperimmune plasma

Mice were repeatedly infected with 10^6 *P. c. chabaudi* (AS) infected erythrocytes up to a total of seven times. Blood was collected and plasma prepared as described as described in Section 2.9.1.

2.10 ELISA

2.10.1 Preparation of *P. c. chabaudi* (AS) parasite lysate for ELISA and Western analysis

Trophozoite- and schizont stage parasite-infected blood was collected into heparin, on ice, from *P. chabaudi*-innoculated BALB/c mice. Mouse leukocytes were removed by passing the blood through Plasmodipure filters according to the manufcturer's instructions, the remaining erythrocytes washed three times in 50 mL ice-cold PBS at 800 x g, 8 minutes at 4°C, and resuspended in 5 mL PBS. Drops of 10% saponin (in PBS) were slowly added until the solution started turning brown (erythrocyte lysis), at which point 30 mL PBS was immediately added and the cells centrifuged as before. The wash was repeated twice, the supernatant carefully discarded and the parasite pellet resuspended in 1 mL PBS with 100 µg of Aprotinin protease inhibitor added. For Western analysis the preparation was used immediately whereas for ELISA, aliquots were frozen and stored at –20°C (Sections 2.11 and 2.10.4, respectively). When needed, an aliquot was thawed, carefully resuspended in parasite lysis buffer (Appendix) until parasite lysis occurred (clear light brown colour), and optical density measured at 280 nm

to determine protein concentration. The preparation was used at OD_{280}=1.5 for both ELISA and Western analysis.

2.10.3 *Pc*SLAP-specific ELISA assays to determine antibody levels to the *Pc*SLAP fusion protein

2.10.3.1 α-*Pc*SLAP antibody levels in immunised mice and rabbits

50 µL of coating solution containing 2.5 µg/mL purified *Pc*SLAP fusion protein in carbonate buffer (Appendix), was added to 96-well Polysorp plates (Nalge Nunc International) and incubated over night. Non-specific binding sites were blocked with 200 µL blocking buffer (Appendix) at 37°C for 1 hour. Plasma from immunised animals was added, starting at a dilution of 1:100 and then two-fold serially diluted 12 times in blocking buffer. Hyper-immune mouse plasma (Section 2.9.3) was used as a standard/positive control and normal mouse plasma (Section 2.3.4) or pre-immune rabbit serum (Section 2.9.2) as a negative control. The samples were incubated at 37°C for 1 hour. To detect bound antibodies, alkaline phosphatase (AP)-conjugated- goat α-mouse IgG (1:1000 dilution in PBS; Bio-Rad) or goat α-rabbit IgG (1:15,000 dilution in PBS; Bio-Rad) was added and the plate incubated 1 hour at 37°C. p-Nitrophenylphosphate-sodium (PNPP) salt in diethanolamine buffer (Appendix) was used as a substrate to produce a colour reaction. Fully developed plates were read at OD_{405nm} when the sample from the lowest serial dilution of standard was 1 or greater, using Revelation software and an MRX TC II microplate reader (Dynex Technologies, Billinghurst, UK)

2.10.3.2 α-*Pc*SLAP antibody levels in hyperimmune plasma

Coating of plates and blocking were performed as in Section 2.10.3.1. Test samples containing BALB/c and C57Bl/6 hyperimmune plasma (Section 2.9.3) were serially diluted as above but starting at a 1:10 dilution. Immune plasma from *Pc*SLAP immunisations was used as a standard/positive control and normal mouse plasma from BALB/c and C57Bl/6 as negative controls. Incubations, detection, development and reading of the plate were performed as described in Section 2.10.3.1.

2.10.4 Malaria specific ELISA assay to determine whether α-*Pc*SLAP antibodies recognise antigens in a parasite preparation

50 µL of parasite lysate preparation (Section 2.10.1) in PBS (final OD_{280}=0.075) was used to coat 96-well Polysorp plates (Nalge Nunc International). Blocking was performed as in Section 2.10.3.1. Test samples containing α-*Pc*SLAP plasma, starting at a dilution of 1:10 and then two-fold serially diluted 12 times in blocking buffer were added along with hyper-immune plasma as a standard/positive control and normal mouse plasma as a negative control, and incubated at 37°C for 1 hour. Incubations, detection, development and reading of the plate were performed as in Section 2.10.3.1.

2.11 Western analysis of a *P. chabaudi* lysate using a semi-dry blotting system

10 µL samples (prepared as described in Section 2.10.1) were mixed with 2 x reducing or non-reducing loading buffer (Appendix) and boiled for five minutes. The samples were resolved by SDS-PAGE on a 10% gel along with a protein marker, by running at 120 V until the dye front reached the bottom of the gel. The separated proteins were subsequently transferred onto a nitrocellulose membrane using a horizontal transfer unit at 0.8 mA/cm^2 for approximately 45 minutes. A transfer unit is defined as a stack of (starting from the bottom) filter paper (Whatman) soaked in cathode buffer, gel, membrane, filter paper soaked in anode buffer 1, and filter paper soaked in anode buffer 2 (anode and cathode buffers in Appendix). Following transfer, the membrane was washed in PBS on a shaker for 10 minutes at RT, and incubated with blocking buffer (Appendix) overnight at room temperature to block free, non-specific binding sites. The following morning it was washed in wash buffer (Appendix) twice for five minutes, at RT, and probed with primary antibody (in Antibody dilution buffer, Appendix) for one hour at 37°. Plasma or antibodies varied between experiments and are referred to in Chapter Four. The membrane was again washed twice for five minutes in wash buffer (Appendix), before incubated with the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG, at 1/1000 dilution (in Antibody dilution buffer), for one hour at room temperature. After washing again as above, labeled proteins were visualised using the Supersignal® West Pico Chemiluminescence system (Pierce, Rockford, IL, USA).

2.12 Indirect immunofluorescence assay (IFA)

Blood was collected from malaria-infected female μMT mice at 15-25% parasitemia, centrifuged at 13000 rpm, five minutes at 4°C, and the pellet mixed with three pellet volumes FCS. Thin blood smears were prepared on glass slides, left to dry at RT, and fixed in acetone (BDH), acetone-methanol (1:1) or left unfixed. If fixed, slides were left to dry for a further 30 minutes at RT, wrapped in aluminium foil and frozen at –20°C.

On the day of the assay, slides were removed from the freezer, immediately fixed in acetone or acetone-methanol (1:1) and left to dry at room temperature before incubated with primary antibodies, plasma or sera (Table 2.4) for 1 hour at 37°C in a humid chamber. The slides were washed 2 x 5 minutes in PBS + 1% (w/v) bovine serum albumin and incubated as before with fluorochrome-conjugated secondary antibodies (Table 2.5). At the end of the assay, slides were submerged briefly in a solution of 4,6-diamidino-2-phenylindole (DAPI, 1μg/mL) to stain nuclear material. Slides were mounted in Citifluor and viewed under a Zeiss microscope or an Olympus DeltaVision fluorescence microscope, both with a 100 x magnification objective with oil.

For co-localisation studies, antibodies were added in the following order: anti-*Pc*SLAP plasma – anti-mouse secondary antibody - 85RF.45.3 rat monoclonal antibody - anti-rat secondary antibody.

2.13 Transfection of *Plasmodium berghei* ANKA parasites

All transfection and selection procedures in Section 2.13 follow the protocol outlined by Waters *et al.* (1997).

2.13.1 Construction of *pbslap* transfection vectors

The transfection vectors pSLAPInt and pSLAPRep were constructed by cloning sequences from the *P. berghei* ANKA *slap* gene into the pBSK-*dhfr-ts* vector (a gift from R. Tewari, Imperial College, London; Fig 5.3). The pBSK-*dhfr-ts* vector was made from the pBluescript II SK (+) phagemid vector backbone (Stratagene), with the DHFR-TS transcription unit (5.2 kilobases) inserted between the HindIII and the EcoRI restriction sites in the pBluescript II SK multiple cloning site (MCS; Figure 2.2). The DHFR-TS transcription unit comprises the pyrimethamine resistant form of the *Toxoplasma gondii*

dihydrofolate reductase-thymidilate synthase (*dhfr-ts*) gene, flanked by the *Plasmodium berghei* 5'- and 3' *dhfr-ts* UTR sequences.

The *pbslap* gene fragments were amplified from *Plasmodium berghei* ANKA genomic DNA (isolated as described in Section 2.4.1) by PCR, using the high fidelity Platinum Pfx system (Life Technologies) and the primers listed in Table 2.6. All primers were designed using MacVector software. The following was included in each 50 µL PCR reaction: 1 mM $MgSO_4$, 50 µM of each dNTP (Pharmacia), 1 x amplification buffer, 250 nM of each primer, 100 ng *Plasmodium berghei* ANKA gDNA and 1.2 units Pfx DNA Polymerase. PCRs were performed on a Hybaid PCR Express machine using the following program:

Step 1	94°C	5 minutes	Initial denaturation	
Step 2	94°C	1 minute	Denaturing	
Step 3	50°C	1 minute	Annealing	
Step 4	72°C	3 minutes	Extension	, to Step 2 for 30 cycles
Step 5	72°C	10 minutes	Final extension	

Products were analysed by gel electrophoresis, followed by cloning into the pBSK-*dhfr-ts* transfection vector (described in Sections 2.13.2 and 2.13.3).

2.13.2 Construction of the pSLAPIns insertion construct

The 911 bp *pbslap* gene fragment was amplified by PCR with the SLAPInsForw and SLAPInsRev primers and blunt-ligated into the pCR®4Blunt-TOPO® vector, using the Zero Blunt® TOPO® PCR Cloning Kit for sequencing (Invitrogen), according to the manufacturer's instructions. The vector was propagated and purified (Sections 2.4.8 and 2.4.4), the cloned fragment excised from the vector using the EcoRI restriction sites flanking the cloning site, gel purified (Section 2.5.3) and re-cloned into pBSK-*dhfr-ts* at the EcoRI site. Cloning and selection procedures were performed as described (Section 2.4.3).

2.13.3 Construction of the pSLAPRep replacement construct

Fragment 2 was digested with EcoRI and XbaI restriction enzymes and directionally cloned into the pBSK-*dhfr-ts* vector between the EcoRI and XbaI restriction sites. The vector was propagated and purified (Sections 2.4.8 and 2.4.4), and Fragment 1, digested with ApaI and HindIII, was cloned in between the ApaI and HindIII restriction sites in the vector. Cloning and selection procedures were performed as described (Section 2.4.3).

2.13.4 Preparation of *Plasmodium berghei* ANKA donor culture

Female BALB/c mice were infected by intraperitoneal injection with 10^6 or 10^7 parasitised BALB/c-derived erythrocytes. At 15-20% parasitemia the animals were culled, the blood collected into heparin, transferred into RPMI 1640 and centrifuged for 8 minutes at 450 x g, RT. The rbc pellet was equally divided between two 500 mL Erlenmeyer flasks each containing 60 mL of CO_2-saturated RPMI 1640 with 20% heat inactivated FCS. The culture was saturated with gas mixture of 10% O_2, 5% CO_2, 85% N_2, sealed and incubated on a shaker at 38°C for approximately 20 hours (overnight).

2.13.5 Schizont isolation for electroporation

When the majority of parasites were at the late schizont stage (as examined by Giemsa-stained thin blood smears), the culture was overlaid on a 55% Nycodenz/PBS gradient (Robins Scientific; 35 mL culture over 10 mL Nycodenz) and centrifuged without a brake at 300 x g, 25 mins, RT. The interphase was collected and washed at 800 x g for 10 minutes, RT. This yielded approximately 10^8 schizonts, which were subsequently mixed with cytomix (Appendix) to a total volume of 100 µL.

2.13.6 Preparation of transfection vectors for electroporation

50 µg of each transfection vector was digested with the appropriate restriction enzyme(s) and buffer in a total volume of 50 µL, at 37°C over night. Digested vectors were subsequently purified, either over PCR Purification columns (Qiagen), or by phenol-chloroform extraction followed by ethanol precipitation. Each vector was made up with cytomix to a final volume of 300 µL.

2.13.7 Electroporation

The parasite- and vector preparations were mixed, transferred to a 0.4 cm electroporation cuvette and subjected to a single pulse of 1.1 kV and 25 μF (time constant between 0.6 and 1 ms), using a Bio-Rad electroporator. Cuvettes were immediately transferred to ice and 0.2 mL of the mixture injected intravenously into the tail vein or intraperitoneally into each mouse.

2.13.8 Pyrimethamine injections

A stock solution of 2.5 mg mL^{-1} pyrimethamine (Appendix) was diluted with saline to prepare injection volumes of 0.1 mL, to give a final dose of 10 mg kg^{-1} bodyweight, injected intraperitoneally.

2.13.9 PCR screening of transfected parasites

PCR analysis for screening of transfected parasites was performed as described (Section 2.4.2.2), using the primer combinations described in Section 5.3.4.1. All primer sequences are listed in Table 2.2.

2.13.10 Southern analysis screening of transfected parasites

Southern analysis for screening of transfected parasites was performed as described (Section 2.6), with the exception of using two μg of DNA per lane rather than 0.5μg. The blotted membrane was probed with a radio-labeled 1202 basepair fragment corresponding to the sequence between nucleotides 439 and 1640 of the *pbslap* gene. The probe was amplified by PCR, using primers SLAPRepF1 and SLAPRepR2, gel purified and radio-labeled, as described (Sections 2.5.3 and 2.6.3).

Table 2.1 Primers used in conventional PCR to identify the 5'- and 3' ends of the *pcslap* gene

Primer	Sequence
5' end of *pcslap* gene:	
PSLAPext.F1 (forward)	GACGTGATTGTATTAGTTCATG
PSLAPext.F2 (forward)	CTAGCTGTTCAGAAGATTGTGC
PSLAP 5':1 (reverse)	TTAACCACATACTAGTGCAAG
PSLAP 5':2 (reverse)	TGTGTGCAATAAATTATTAACAGGG
3' end of *pcslap* gene:	
PSLAP 3':1 (forward)	TTTCAGTATATTTTTTAGCTAC
PSLAP 3':2 (forward)	CTAATTTGTATTGGTTGAATAG
PSLAPext.R1 (reverse)	CACGTATATGACAATCTATGTC
PSLAPext.R2 (reverse)	ATAGCTGCAGCACATATAGAGG

Table 2.2 Primers used for PCR screening of transfected parasites

Primer	Sequen ce
SLAPRepF1 / 1* (forward)	CCCCCGGGCCCGAAGAAAAACGAGAAGCAAC
SLAPRepR1 / 5* (reverse)	CCGGAAGCTTCCCCATTTATTATTTACACGG
SLAPRepF2 / 6* (forward)	GGCCGAATTCGCTGTTTGTAAAAAAGGTCC
SLAPRepR2 / 4* (reverse)	CCGGTCTAGACCATTAGATGTAGGTGAACC
DHFR2 / 3* (forward)	AATAATAACTTATAAATATGAG
DHFR3 / 2* (reverse)	GTGGAAATAAAATAACATATAA

* For clarity, instead of using their names, these primers were given numbers in Section 5.3.4.2.

Table 2.3 Primers used in Vectorette PCR

Primer	Sequence
Forward primers (for 3' extension):	
Forw1	CCACATGAAACATCTGAAGATATTTACTGTTCTC
Forw2	AGCAGCTATACATGCAGGTGTTCTATCGAG
Forw3	CCAAGTAGTAAAGGGAAAAATACATGG
Forw4:1	ACAAGCAACTGATTACTTTATAGGGG
Forw4:2	TGTTCAGATAATTTACTCAGTGAGCG
Reverse primers (for 5' extension):	
Rev1	GCTTCATCTCCTTTACATCTAAAATTCGTTG
Rev2	GAATGGTTCCGTTTTCTATCGAATCGTTAC
Rev3:1	AACTATGACAGTTTTGTGGGCATCGAAC
Rev3:2	CGGAACTATGACAGTTTTGTGGGCATCGAAC

Table 2.4 Primary antibodies, plasmas and sera used in IFA

Antibody	Working dilution	Source
α-*Pc*SLAP mouse immune plasma (BALB/c)	1:40	Cecilia Waller, Langhorne laboratory, NIMR
α-*Pc*SLAP rabbit immune plasma	1:40	Abbott Murex Biotech Ltd.
α-*Pf*SLAP mouse immune sera (BALB/c)	1:40	Isabelle Delrieu, Holder laboratory, NIMR
85RF.45.3 (α-*Pf*s48/45), rat monoclonal IgG	10μg/mL	Dr. R. Sauerwein, University Hospital Nijmegen, The Netherlands
NIMP23-FITC, mouse monoclonal IgG	1:50	Ching Li/Latifu Sanni, Langhorne laboratory, NIMR
Normal mouse plasma (BALB/c)	1:40	Langhorne laboratory, NIMR
Pre-immune rabbit sera	1:40	Abbott Murex Biotech Ltd.
Rat IgG, purified	10μg/mL	Sigma
Mouse IgG, purified	30μg/mL	Sigma
Hyperimmune α -*P. c. chabaudi* (AS) plasma	1:100	Langhorne laboratory, NIMR

Table 2.5 Secondary antibodies used in IFA

Antibody	Working dilution	Source
Goat α-mouse IgG-FITC (green)	1:160	Sigma
Goat α-mouse IgG-FITC (green)	1:1500	BD Pharmingen
Goat α-mouse IgG-Alexa Fluor® 594 (red)	1:400	Molecular Probes
Goat α-rat IgG-FITC (green)	1:200	Sigma
Goat α-rat IgG-Alexa Fluor® 488 (green)	1:200	Molecular Probes
Goat α-rat IgG-Alexa Fluor® 594 (red)	1:500	Molecular Probes

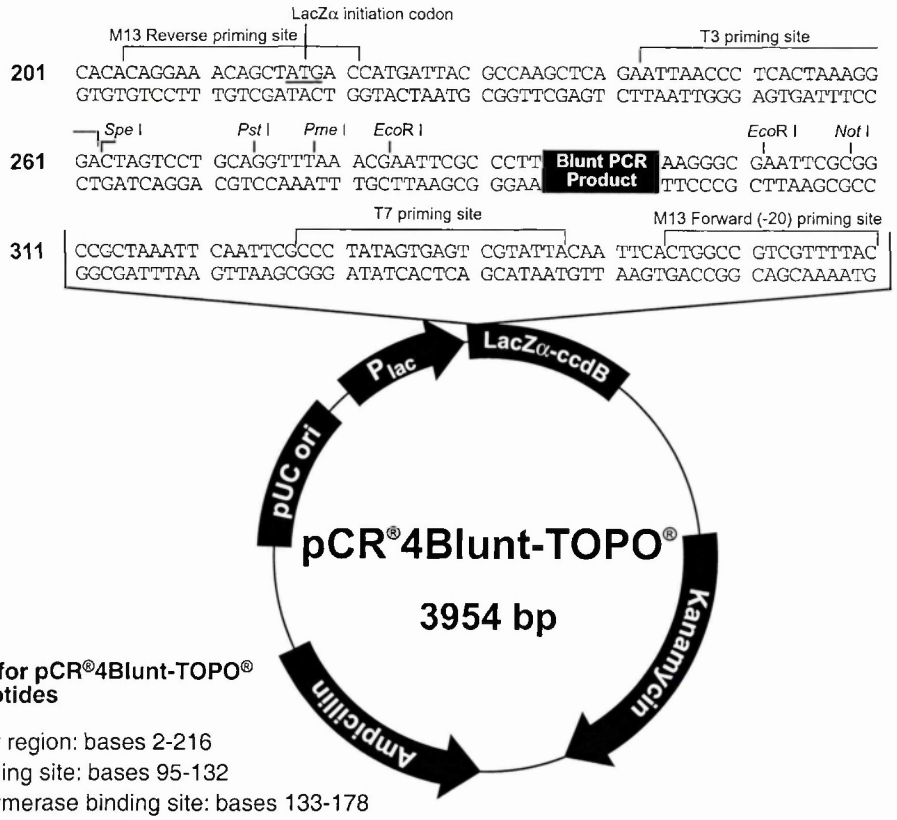
Table 2.6 Primers used to amplify sequences used in transfection vector constructs

Primer	Sequence (restriction sites underlined)	Added 5' restriction site	Amplified fragment
For pSLAPIns (insertion vector):			
SLAPInsForw (forward)	AAGACATAGATTGCCATATCCGTG	-	Insertion vector Fragment (InsF)
SLAPInsRev (reverse)	TGATAACCTAATTCTAAACATGC	-	
For pSLAPRep (replacement vector):			
SLAPRepF1 (forward)	CCCCCGGGCCCGAAGAAAAACGAGAAGCAAC	ApaI	Fragment 1 (F1)
SLAPRepR1 (reverse)	CCGGAAGCTTCCCCATTTATTATTTACACGG	HindIII	
SLAPRepF2 (forward)	GGCCGAATTCGCTGTTTGTAAAAAAGGTCC	EcoRI	Fragment 2 (F2)
SLAPRepR2 (reverse)	CCGGTCTAGACCATTAGATGTAGGTGAACC	XbaI	

Figure 2.1

pCR4Blunt-TOPO (Invitrogen)

Vector map for pCR4Blunt-TOPO, used for cloning of PCR products for subsequent sequencing.



Comments for pCR®4Blunt-TOPO®
3954 nucleotides

lac promoter region: bases 2-216
  CAP binding site: bases 95-132
  RNA polymerase binding site: bases 133-178
  Lac repressor binding site: bases 179-199
Start of transcription: base 179
M13 Reverse priming site: bases 205-221
LacZα-*ccdB* gene fusion: bases 217-810
  LacZα portion of fusion: bases 217-497
  *ccd*B portion of fusion: bases 508-810
T3 priming site: bases 243-262
TOPO® Cloning site: bases 294-295
T7 priming site: bases 328-347
M13 Forward (-20) priming site: bases 355-370
Kanamycin promoter: bases 1021-1070
Kanamycin resistance gene: bases 1159-1953
Ampicillin (*bla*) resistance gene: bases 2309-3061 (c)
Ampicillin (*bla*) promoter: bases 3062-3158 (c)
pUC origin: bases 3159-3832

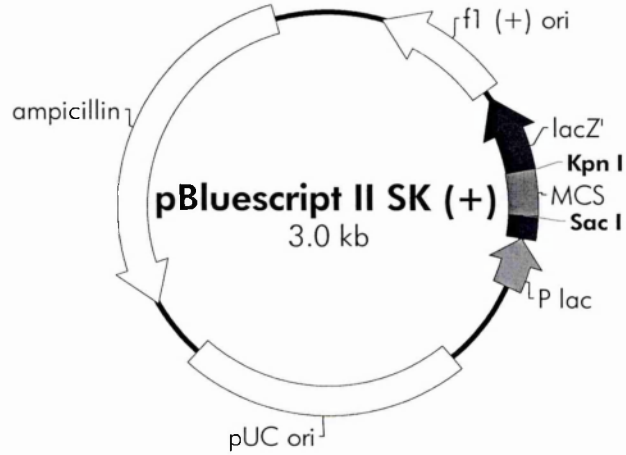
(c) = complementary strand

Figure 2.2

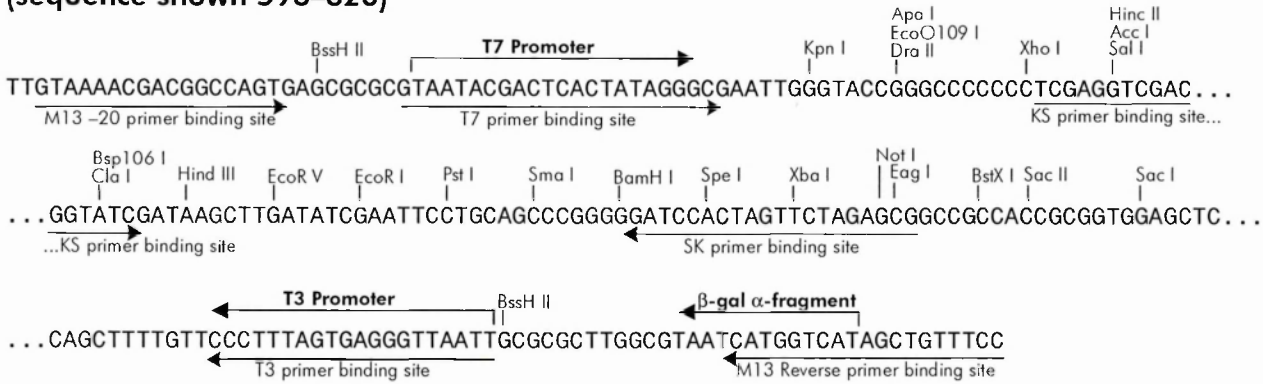
pBluescript II SK (Stratagene)

Vector map for pBluescript II SK, used for construction of the pSLAPIns and pSLAPRep transfection vectors for disruption of the *pbslap* gene.

f1 (+) origin 135–441
β-galactosidase α-fragment 460–816
multiple cloning site 653–760
***lac* promoter** 817–938
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1976–2833



pBluescript II SK (+/–) Multiple Cloning Site Region (sequence shown 598–826)



Chapter Three

*Pc*SLAP – Identification and analysis of a novel *Plasmodium chabaudi chabaudi* (AS) gene and protein

3.1 Introduction

The Apicomplexa is an ancient phylum made up entirely of parasitic species. Phylogenetic reconstruction based on comparisons of small subunit ribosomal RNA sequences and conserved protein sequences predict that Apicomplexa has diverged extensively from the other eykaryotic kingdoms of animals, plants and fungi (Gajadhar *et al.*, 1991; Escalante and Ayala, 1994; Baldauf *et al*, 2000). It is therefore likely that a number of genes will be unique to this phylum. Indeed, the *Plasmodium falciparum* sequencing project found that of 5,268 predicted proteins, over 60% (3,208) did not have homologues in any other organisms. These species-specific molecules are of particular interest as their study will allow a better and more complete understanding of the unique biology of the parasite, as well as the opportunity to discover new drug targets and vaccine candidates. Proteins that are restricted to a pathogen are especially well suited as therapeutic targets as they pose less of a risk of unwanted side effects if used for medical intervention.

This chapter describes the identification of a novel gene in *Plasmodium chabaudi chabaudi* (AS). In previous studies a small fragment of a gene (270 nucleotides) had been identified (Maria M Mota, PhD thesis, 1998). Examination of preliminary sequences on the TIGR website revealed un-annotated homologues to this sequence in both *Plasmodium falciparum* and *Plasmodium yoelii*. These were single exon genes of approximately 3.8 (*P. falciparum)* and 3.9 (*P. yoelii*) kilobases. Alignments of the open reading frames (ORFs) of these genes showed a 75% identity at the nucleotide level and 63% at the protein level (data not shown), suggesting a well conserved, novel Plasmodial protein. Thorough searches through sequence databases from other organisms, including the NCBI non-redundant database, failed to detect any orthologues, indicating that this could be a gene unique to Apicomplexa. Work by us on the *P. falciparum* homologue revealed a multi-domain protein with several types of adhesive motifs, including

revealed a multi-domain protein with several types of adhesive motifs, including Scavenger Receptor Cysteine-Rich (SRCR) domains and *Limulus* factor C, Coch-5b2 and Lgl1 (LCCL) modules, and was subsequently named *Plasmodium* SRCR LCCL Adhesive-like Protein (*P*SLAP; Delrieu *et al.*, 2000).

At the start of this study very little sequence data was available from the *P. chabaudi* genome and thus BLAST searches with the 270 nucleotide sequence did not produce any hits. In order to identify the full sequence of the *P. chabaudi slap* (*pcslap*) gene two experimental approaches were used: Vectorette PCR and conventional PCR. As more data was being made available in the *P. chabaudi* sequence database towards the end of the study, the 5′ end of the gene that still remained to be identified was obtained from a sequence database search.

This chapter describes the cloning of the full sequence of the *P. chabaudi* gene, as well as structural and functional analysis of secondary structural motifs within the protein.

3.2 Results

3.2.1 Cloning of the *pcslap* gene

3.2.1.1 Identification of *pcslap* gene sequence using Vectorette PCR

In order to extend the original *pcslap* sequence of 270 base pairs the Vectorette II System (Sigma Genosys) was used. This is a method that allows amplification of unidentified nucleotide sequences upstream and downstream of known DNA.

First a "Vectorette library" is created by digesting DNA with a restriction enzyme and adding on short DNA adaptor sequences, Vectorettes (Fig 3.1), to the fragment ends. The Vectorette library is then used as template DNA in a PCR. Vectorette PCR is a "one-sided" reaction performed with one primer based on the known gene sequence and the other primer binding to the Vectorette adapter sequence (Fig 3.2). Amplification from DNA fragments lacking the known sequence is avoided by the fact that the Vectorette primer sequence is complementary only to the strand that is created by extension from the known primer. This is because the sequence of the Vectorette primer is identical to one of the strands in the mismatched portion of the Vectorette unit, and so will only anneal once a complementary strand of this portion has been produced.

In order to avoid a situation where further amplification would be difficult and time consuming due to too many restriction sites of the chosen enzyme(s) being present within the *pcslap* gene sequence, it was decided to construct four Vectorette libraries. *Plasmodium chabaudi chabaudi* (AS) genomic DNA was digested with the restriction enzymes RsaI, Sau3A1, SspI and XhoII. The first three enzymes cut frequently in Plasmodium DNA: RsaI and Sau3A1 have short, 4-base recognition sequences (GT$^{\downarrow}$AC and $^{\downarrow}$GATC, respectively) whereas SspI recognises a six-base sequence, but one made up of only adenine and thymine nucleobases (AAT$^{\downarrow}$ATT), both of which are abundant in *Plasmodium* DNA. XhoII, on the other hand, cuts infrequently as it has a six-base recognition sequence that includes at least one guanine and one cytosine nucleobase (R$^{\downarrow}$GATCY, (R=either purine and Y=either pyrimidine)). The Vectorette Kit contains adapters with five different ends: four sticky end adapters and one blunt end adapter. For the present project, the RsaI- and SspI-digested genomic DNA samples were ligated to the blunt Vectorette adapters, and the Sau3AI- and XhoII-digested genomic DNA with the BamH1 adapter.

Primers based on the 270 bp original *pcslap* sequence were subsequently designed, one for amplification in the 5′ direction, Rev1, to obtain sequence upstream of the known *pcslap* sequence, and one for 3′ amplification, Forw1, for downstream sequence identification (Figures 3.3, 3.4 and Tables 3.1 and 2.3). All four Vectorette libraries were screened with both of the above primers, and the results analysed by gel electrophoresis. This first screen generated products from upstream as well as and downstream amplification of $pcslap_{270}$, both approximately 1100 nucleotides long, and from the SspI and Sau3AI libraries, respectively (Figure 3.5A). The products, designated 5′:Seq1 for the product extending *pcslap* in the 5′ direction, and 3′:Seq1 for the extension in the 3′ direction, were cloned into the pCR®4Blunt-TOPO® sequencing vector (Invitrogen) and sequenced (Figure 3.5B). Both sequences were confirmed as being derived from the *pcslap* gene as they were identical to $pcslap_{270}$ at the gene specific primer ends. The new sequences also displayed a high degree of identity with the *slap* gene in *P. falciparum* (data not shown).

The addition of the 5′:Seq1 and 3′:Seq1 products, 1045 and 1065 nucleotides long, respectively, onto $pcslap_{270}$ resulted in a 2126 nucleotide long sequence (Figure 3.6). This translated into an open reading frame without a stop codon at the 3′ end (data not shown). This, in conjunction with the size of the *P. falciparum slap* gene (3816 nucleotides), indicated that the full *P. chabaudi* homologue had not yet been identified.

3.2.1.1.2 The Second Vectorette Screen

The 5′:Seq1 and 3′:Seq1 sequences were thus used to design a second set of primers, Rev2 and Forw2, to "walk" further up and down the gene (primers 1 and 3, respectively, in Figure 3.5B, and Tables 3.1 and 2.3). Again, all Vectorette libraries were screened in the PCR. This second screen generated an approximately 700 nucleotide product using the Forw2 primer and the RsaI library (Figure 3.7A), but no upstream amplification with the Rev2 primer. The 700 base pair product, designated 3′:Seq2, was cloned and sequenced as before (Section 3.2.1.1.1), and produced a sequence of 621 base pairs (Figure 3.7B). Again, as there was a significant overlap (282 nucleotides) of complete identity between 3′:Seq2 and the previously obtained *pcslap* sequence, the product was considered to be a true continuation of the gene. In order to try and generate more 5′

Table 3.1 Experimental strategies used to clone *pcslap*.

PCR	Forward primer(s)*	Reverse primer(s)*	Product
Vectorette PCR, 1st screen			
5′ amplification	Vectorette primer	Rev1 (*pcslap* specific)	5′:Seq1
3′ amplification	Forw1 (*pcslap* specific)	Vectorette primer	3′:Seq1
Vectorette PCR, 2nd screen			
5′ amplification	Vectorette primer	Rev2, Rev2:1 (*pcslap* specific)	-
3′ amplification	Forw2 (*pcslap* specific)	Vectorette primer	3′:Seq2
Conventional PCR			
5′ amplification	pslap.5′:1, pslap.5′:2	Rev3:1, Rev3:2	-
3′ amplification	Forw3:1, Forw3:2	pslap.3′:1, pslap.3′:2	3′:Seq3

*Primer sequences are listed in Chapter 2, Tables 2.1 and 2.3.

- No product obtained

sequence, a second primer, Rev2:1 (primer 2 in Figure 3.5B), was designed and run in a Vectorette PCR. However, again no product was obtained.

The 3′:Seq2 product added 367 nucleotides to the existing *slap* sequence (Figure 3.8). However, when translated, the amino acid sequence was still lacking a 3′ stop codon, again suggesting that the end of the gene had not yet been cloned (data not shown).

3.2.1.2 Conventional PCR for cloning of the 5′ and 3′ ends of *pcslap*

At the start of this project no rodent *slap* sequences had yet been identified and so they were not available in public data bases. However, during the Vectorette sequencing, both the *P. yoelii slap* and the *P. berghei slap* (*pyslap* and *pbslap*, respectively) sequences were entered into the PlasmoDB database. This made it possible to use a different strategy that could recover the remaining sequence at the 5′ and the 3′ ends of *P. chabaudi slap* in a single attempt. This approach relied on conventional PCR with primers based on the up- and downstream un-translated regions (UTRs) of *pyslap* and *pbslap*. This would most likely be a faster method, as with the Vectorette approach it could potentially be necessary to do several more screenings to clone the full gene, if

there were several RsaI, Sau3AI, SspI and XhoII restriction sites present in the missing sequence.

The *P. yoelii* and *P. berghei slap* 5′ and 3′ UTRs were aligned and shown to share 93% identity over 183 nucleotides for the 5′ UTR and 89% identity over 209 nucleotides for the 3′ UTR (Figure 3.9 A and B, respectively). This high degree of identity, in addition to an 86% identity between the 2.5 kilobases of *P. chabaudi slap* sequence identified so far and the corresponding sequence in these other two rodent malarias (data not shown), indicated that the *slap* gene itself and the sequence surrounding it are well conserved and that the *P. chabaudi* UTRs would most likely be similar enough to bind primers based on these regions. Thus primers were designed from regions in the alignment with no or one differing nucleotide. Primers pslap.5′:1 and pslap.5′:2 were based on the *P. yoelii/P. berghei* 5′ UTR, and pslap.3′:1 and pslap.3′:2 on the *P. yoelii/P. berghei* 3′ UTR (Figure 3.9A and B, respectively, Tables 3.1 and 2.1). These were run together with primers designed from the previously obtained *pcslap* sequence: Rev3:1 and Rev3:2 were used for 5′ amplification (new primers were made as primers Rev2:1 and Rev2:2 had not worked in the Vectorette PCR), and Forw3:1 and Forw3:2 for 3′ amplification (Figures 3.5B, 3.7B, 3.8, and Table 3.1). *P. chabaudi* genomic DNA, as well as the four Vectorette libraries, were used as templates. An approximately 750 base pair long product was produced in the 3′ amplification with primers Forw3:2 and pslap.3′:2 from *P. chabaudi* genomic DNA (Figure 3.10A), and was subsequently cloned and sequenced (Figure 3.10B). Because the product, designated 3′:Seq3, had sequence overlapping with the identified *pcslap* sequence it was considered part of the gene and added to the existing sequence (Figure 3.11). It translated into an ORF terminated by a stop codon towards the C-terminal, marking the end of the gene.

3.2.3 Identification of the 5′ end of *pcslap* by BLAST

Because no products had been generated in the 5′ sequencing attempt, the beginning of *pcslap* was still missing. However, at this time more sequence had been made available in the Sanger Center *P. chabaudi* database. A BLAST search using the known *pcslap* sequence as the query for searching *P. chabaudi* sequence data produced a hit with a 2053 base pair long sequence (P score 3.7 e^{-174}, Figure 3.12) in the genomic contigs

database. The contig was aligned with the *pcslap* sequence (data not shown), adding 1205 base pairs of new sequence to the 5′ end of the gene (Figure 3.13). A translation showed that the ORF was initiated by a start codon 460 bases into the contig sequence (underlined in Figure 3.12).

The complete coding *pcslap* sequence proved to be 3897 nucleotides long and translating into a 1298 residue protein (Figure 3.14).

3.2.2 Determination of *pcslap* copy number in *P. c. chabaudi* (AS)

To investigate the copy number of *pcslap* in the *P. chabaudi* genome, Southern blot analysis was performed. Parasites were harvested from *P. chabaudi* infected mice, genomic DNA isolated and aliquots digested with RsaI, Sau3AI, SspI and XhoII restriction enzymes. A restriction map for *pcslap*, along with the relative position of the probe within the sequence, is shown in Figure 3.15. The fragments were resolved on an agarose gel (Figure 3.16A) and probed with radio-labelled $pcslap_{270}$ (the 270 bp original sequence). Hybridisation was carried out at 65°C and resulted in single bands of the expected sizes in all four digests (Table 3.2 and Figure 3.16B).

However, hybridisation was subsequently also performed at 55°C with the $pcslap_{270}$ probe. This resulted in a second, smaller band appearing in each digest, of the following approximate sizes: RsaI - 400 nucleotides, Sau3AI – 350 nucleotides, SspI – 650 nucleotides and XhoII - 5000 nucleotides (Figure 3.16C). This suggested that there is another gene in the *P. chabaudi* genome with sequence similarity to the probe sequence.

Table 3.2. Expected band sizes from restriction digests of *P. chabaudi* genomic DNA probed with $pcslap_{270}$.

Restriction enzyme	No of sites in probe sequence	Sizes of fragments (nucleotides)
RsaI	0	2661
Sau3AI	0	1417
SspI	0	1883
XhoII	0	>4014

In order to try and identify this potentially related gene, the *pcslap*$_{270}$ sequence was BLASTed through the *P. chabaudi* genomic contigs database and the *P. chabaudi* shotgun reads database and the highest scoring sequences in each of these analysed. As there is still redundancy in the data within these databases, the sequences in each set were aligned and any identical sequences removed, so that six unique sequences from each database remained. The highest scoring sequence in these searches was a part of the *P. chabaudi slap* gene itself - a 615 nucleotide long sequence in the shotgun reads database with a P value of 4.8 x e^{-56} and 100% identity. The rest of the hits had P scores between 0.00011 and 0.00038, and identities ranging from 59% to 65% (data not shown). In order to investigate whether any of these sequences could represent the second band seen in the Southern analysis, the RsaI, Sau3AI, SspI and XhoII restriction sites were mapped out for each sequence (data not shown) and the sizes of fragments compared with the size of the second band. However, none of the sequences obtained in the BLAST searches had a restriction map that would produce the fragments seen in the Southern analysis.

Southern analysis was subsequently repeated using *pcslap*$_{2130}$, a 2.1 kilobases long probe comprising *pcslap*$_{270}$ and the sequences identified in the first Vectorette screen (5′:Seq1 and 3′:Seq1). *pcslap*$_{2130}$ carried restriction sites of the enzymes used to digest the DNA and would therefore bind more than one fragment, resulting in more than one band in each digest (Figure 3.15 and Table 3.3). The smallest fragments produced in the RsaI and Sau3AI digests (64 and 18 nucleotides, respectively), were however unlikely to generate bands as they would have been lost into the running buffer during electrophoresis of the DNA. Likewise, the 606 base pair long fragment generated in the Sau3AI digest only contained 6 nucleotides of the *pcslap*$_{2130}$ probe sequence, and would therefore not be expected to bind the probe. As shown in Figure 3.18, the expected bands are all present on the autoradiograph. In addition, the extra bands seen previously with the *pcslap*$_{270}$ probe are also visible, although very faintly. This was expected as *pcslap*$_{270}$ is contained within the *pcslap*$_{2130}$ sequence (Figure 3.15).

These results indicate that *pcslap* is a single copy gene in the *P. chabaudi* genome.

Table 3.3. Expected band sizes from restriction digests of *P. chabaudi* genomic DNA probed with *pcslap*$_{2130}$.

Restriction enzyme	No of sites in probe sequence	Sizes of fragments (nucleotides)
RsaI	2	393, (64), 2661
Sau3AI	3	822, (18), 1417, (606)
SspI	1	1883, 952
XhoII	0	>4014

Fragments in brackets are unlikely to be visible on the blot (see further Section 3.2.2 and Figure 3.15).

3.2.3 pslap copy number in *Plasmodium yoelii* and *Plasmodium berghei*

In order to investigate the number of genes in other rodent malarias, a second Southern analysis was subsequently performed, using genomic DNA from *Plasmodium yoelii yoelii* (17X strain) and *Plasmodium berghei* ANKA as well as from *P. chabaudi*. To control for hybridisation to contaminating, co-purified mouse DNA from leukocytes and reticulocytes, BALB/c genomic DNA from an un-infected mouse was included as a negative control to ensure that the generated bands were from hybridisations to parasite, and not murine DNA. This sample was prepared alongside the parasite preparations. Because of the high level of conservation of the *slap* gene it was possible to use *pcslap*$_{270}$ as a probe for all genomic DNAs. Alignments with the homologous sequences in these rodent species showed 91% identity with the *P. berghei* ANKA sequence and 90% identity with the *P. yoelii* sequence (Figure 3.18).

The genomic DNAs were digested with either Sau3A1 or SspI restriction enzymes, the fragments separated by gel electrophoresis and probed with *pcslap*$_{270}$ at 55°C (Figure 3.19). Expected hybridisation patterns are shown in Table 3.4. No binding to the negative control, BALB/c genomic DNA, was detected, demonstrating that the generated bands were not from contaminating mouse DNA. As before (Section 3.2.2) two bands appeared in the digested *P. chabaudi* genomic DNA. Interestingly, the *P. berghei* digests also

Table 3.4. Expected band sizes from restriction digests of *P. berghei* and *P. yoelii* genomic DNA probed with $pcslap_{270}$.

Parasite	Restriction enzyme	No of sites in probe sequence	Sizes of band (nucleotides)
P. chabaudi	Sau3AI	0	1417
	Ssp	0	1883
P. berghei	Sau3AI	0	683
	SspI	0	1475
P. yoelii	Sau3AI	0	1364
	SspI	0	1475

showed a second weaker band. Only one band was detected in the *P. yoelii* digested DNA. A BLAST search was thus carried out to search the *P. berghei* assembled contigs and genome shotgun reads databases to try and identify the second gene, in the same way the *P. chabaudi* database had been screened previously (Section 3.2.2). However, again no sequence was detected that would have given the restriction pattern that was observed.

3.2.4 Summary of results - the *pslap* gene in *Plasmodium chabaudi chabaudi* (AS)

Starting with a 270 basepair fragment, most of the *pcslap* gene was identified using Vectorette PCR, but the 3′ end obtained through conventional PCR and the 5′ end from a sequence in the Sanger *P. chabaudi* database. The identification and analysis of *pcslap* showed that the full coding sequence of the gene is 3897 basepairs, with an AT-content of 73%, a typical high value for Plasmodial sequences. *pcslap* consists of a single exon and southern analysis showed that it exists as a single copy gene in the *P. chabaudi chabaudi* (AS) genome. The gene was also detected as a single copy in *P. yoelii* and *P. berghei* using southern analysis.

3.2.5 Analysis of the *Pc*SLAP protein

The 3897 nucleotide long *pcslap* gene translates into an open reading frame of 1298 amino acids, with a calculated molecular weight of 146,785 Daltons. In order to identify

sequences related to *P*SLAP, protein data bank searches were performed using PSI-BLAST. Significant hits with E values less than $1e^{-5}$ included a number of proteins containing Scavenger Receptor Cysteine-Rich (SRCR) domains. To further analyse the sequence, motif and pattern prediction programs were used. Analysis of the *Pc*SLAP protein using the Simple Modular Architecture Research Tool (SMART) revealed two centrally located Scavenger Receptor Cysteine-Rich (SRCR) domains, flanked by two *Limulus* factor C, Coch-5b2 and Lgl1 (LCCL) modules (Figure 3.20). A third LCCL motif is present at the C-terminal of the protein and a Lipoxygenase Homology 2/Polycystin-1, Lipoxygenase and α-Toxin (LH2/PLAT) domain towards the N-terminal. The protein also carries an N-terminal signal sequence.

3.2.5.1 Scavenger Receptor Cysteine–Rich (SRCR) domains

SRCR domains belong to an ancient, highly conserved super family of proteins that was first recognised during the analysis of the type 1 macrophage scavenger receptor (Aruffo *et al*, 1997; Resnick *et al*, 1994; Freeman *et al*, 1990). The domain is around 110 amino acids in length and contains six or eight conserved cysteine residues, predicted to participate in intra-domain disulphide bonds to produce a structural domain. SRCR domains are most commonly located in the ecto-domains of membrane bound proteins, but are also found in secreted molecules. Many of the proteins in this family are involved in the development of the immune system or in regulation of immune responses (Aruffo *et al*, 1997).

The SRCR family can be further divided into two sub families, Group A and B, based on the number of cysteines present in the domains, and the spacing between these residues. Both groups have six conserved cysteines (denoted by stars in Figure 3.21) in common, predicted to form three disulphide bridges, but Group B also has an additional pair, forming a fourth disulphide bond. In addition, in Group A each SRCR domain is coded for by an individual exon (Aruffo *et al.*, 1997).

Two SRCR domains were identified in the *Pc*SLAP protein, and subsequently designated SRCR-1 and SRCR-2 (expectation values $5.16 \times e^{-6}$ and $1.91 \times e^{-6}$, respectively; Figure 3.20). SRCR-1 is 113 residues long and SRCR-2 115 residues, with both domains containing eight cysteine residues. Since the full *pcslap* gene is coded for by an single exon, the above classification groups the *Pc*SLAP SRCR domains into the Group B sub

family. Group B modules build up the extra-cellular domains of several lymphocyte cell surface proteins, most importantly CD5 and CD6 (Aruffo *et al.*, 1997).

PSI-BLAST and multiple sequence alignment show that *Pc*SLAP SRCR-1 and -2 share up to 35% identity and 51% similarity with other SRCR domains (Fig 3.21). Excluding *P*SLAP in other *Plasmodium spp.*, *Pc*SLAP SRCR-1 is most similar to mouse (*Mus musculus*) Sp-alpha (mSpα), a secreted protein containing three SRCR domains (Gebe *et al.*, 2000; Fig 3.22). mSpα has been suggested to play a role in development and/or maintenance of the lymphoid compartment. The gene is transcribed in most lymphoid tissues and knock-out mice have a reduction in numbers of double positive thymocytes, suggesting that mSpα may be an inhibitor of apoptosis of maturing thymocytes (Miyazaki *et al.*, 1999). *Pc*SLAP SRCR-2 most closely resembles Scavenger Receptor Cysteine-Rich Protein Variant 1 (SpSRCR1), a protein found in the purple sea urchin *Strongylocentrotus purpuratus.* SpSRCR1 belongs to a large family of SRCR genes specifically expressed in coelomocytes, the immune effector cells in these organisms (Pancer *et al.*, 1997; Pancer, 2000). The primary function of these cells is to protect against invasive marine pathogens.

3.2.5.2 *Limulus* factor C, Coch-5b2 and Lgl1 (LCCL) domains

The LCCL module was named after *Limulus* factor C, the cochlear protein Coch-5b2 and Late gestation lung protein-1 (Lgl1), the best characterised proteins it was first discovered in. The module is typically 100 residues long and forms a structural domain due to the presence of disulphide bridges. Alignments of known LCCL sequences have shown eight potential cysteine locations within the module, four of which are highly conserved and found in the majority of LCCL proteins (Fig 3.23). The C-terminal end is the most conserved part of the domain and believed to be the core of the module. A highly conserved histidine within a conserved motif in this region, YxxxSxxCxAAVHxGVI (underlined in Figure 3.23), is present in nearly all LCCL domains identified so far (Trexler *et al.*, 2000).

The three LCCL domains identified in *Pc*SLAP, LCCL-1, -2 and –3 (expectation values 1.84 x e^{-4}, 8.49 x e^{-5} and 2.47 x e-17) are 101, 91 and 86 amino acids long respectively, and contain four cysteines each. The conserved region is clearly present in LCCL-2 and LCCL-3, but not in LCCL-1, which most importantly lacks the histidine (Fig 3.23).

LCCL domains have been identified previously in both *Plasmodium* and other Apicomplexa. CCP2, a protein present in several *Plasmodium spp.*, including *P. falciparum* and the rodent species *P. yoelii* and *P. berghei* (GenBank accession number AF491294) contains one LCCL and one Ricin B domain (Figure 3.24). Putative orthologues to CCP2 were also found in other Apicomplexa, including a complete gene in *Cryptosporidium parvum* (Cpa135, GenBank accession number AJ006593.2), and a partial sequence in *Toxoplasma gondii* (cDNA, GenBank accession number BM131310; Tosini *et al.*, 1999; Tang *et al* (Toxoplasma EST project), 2001). Furthermore, a mass spectrometric proteomic analysis of different developmental stages of *P. falciparum* parasites revealed the presence of four LCCL-containing proteins, including *P*SLAP, CCP2 and two previously unidentified molecules, PFA0445w and PF14_0532 (Figure 3.24; Lasonder *et al.*, 2002).

3.2.5.3 The Lipoxygenase homology 2/Polycystin-1, lipoxygenase and α-toxin (LH2/PLAT) domain

LH-2/PLAT domains are found in a variety of membrane and lipid associated proteins. The domain is a β-sandwich composed of two sheets with four strands each. It is about 90 residues in length, with most of the conservation found in the β-strands (Bateman and Sandford 1999). LH2 domains are present in several membrane or lipid-associated proteins, and thus appear to be involved in either protein-lipid, or protein-protein interactions. In *Pc*SLAP a region with some similarity to this domain type was discovered, although with a rather high expectation value ($2.24 \times e^{-2}$). The LH-2/PLAT domain is the most N-terminal motif identified in the *Pc*SLAP sequence (Fig 3.20).

3.2.5.4 Further sequence analysis - Signal peptides and localisation signals

Newly synthesized proteins have intrinsic signal sequences that function as "address tags" and are necessary for efficient and effective translocation to their respective destinations. Eukaryotic proteins that are destined for organelles, membranes or for secretion out of the cell carry an N-terminal hydrophobic signal sequence. This sequence directs them to the rough endoplasmic reticulum and allows translation immediately into

the lumen of this compartment (Lodish *et al.*, 1995). Additional sequence motifs subsequently determine the final destination of the proteins.

The N-terminal of *Pc*SLAP consists of a sequence that potentially is a signal peptide for translocation into the endoplasmic reticulum. The methionine start codon is immediately followed by a positively charged arginine, after which there is a twenty residue stretch of mainly (eleven out of twenty) hydrophobic amino acids (Figure 3.20). The *Pc*SLAP protein sequence was therefore run through SignalP (Nielsen *et al.*, 1997). The program predicted a signal peptide from residues 1-22, followed by a von Heijne-type cleavage motif between amino acids 22 and 23: AYG-KE (Figure 3.20).

To further investigate the destination/localisation of *Pc*SLAP the TargetP V1.0 and PATS (Prediction of Apicoplast Targeted Sequences) prediction programs were used. Both programs use neural networks to predict subcellular locations of proteins based on N-terminal sequence information. TargetP discriminates between eukaryotic proteins destined for the mitochondrion, the chloroplast and the secretory pathway. The program screens the 120 most N-terminal residues and detected a secretory peptide in *Pc*SLAP but no mitochondrial pre-sequence (chloroplast prediction was turned off) and thus classified it as a secreted protein.

PATS was specifically designed to analyse *Plasmodium* sequences for apicoplast targeting signals. The program analyses the first 80 amino acids of the query and gives the sequence a score between 0 and 1, where values close to 1 signify proteins likely to be apicoplast targeted and values near 0 denote unlikely apicoplast candidates (Zuegge *et al.*, 2001). *Pc*SLAP was scored 0.026 and thus categorised as a non-apicoplast protein.

Furthermore, no trans-membrane sequence was identified in the *Pc*SLAP protein sequence. These results strongly indicate that *Pc*SLAP is a secreted protein.

3.2.6 *P*SLAP is highly conserved among *Plasmodium* species

As previously mentioned, homologues of the *pcslap* gene are present in several other *Plasmodium* genomes, including *P. falciparum, P. knowlesi, P. yoelii* and *P. berghei.* An alignment between the three rodent parasites using the coding part of the gene showed an 82% identity at the nucleotide level (Figure 3.25) and 79% identity at the protein level (Figure 3.26). The same alignment with *Pc*SLAP and the *P. falciparum* homologue

displayed 73% identity between the genes (Figure 3.27), and 63% identity between the compared proteins (Figure 3.28).

In contrast to the high degree of conservation of *P*SLAP among *Plasmodium* species, until recently no significant homology was found with genes from other apicomplexan parasites, other metazoans, protists or bacteria. However, a recent BLAST search identified an orthologue in another member of Apicomplexa, *Toxoplasma gondii*. This protein, *Toxoplasma gondii* Scavenger receptor 1 (TgSR1; accession no AAN74967), is 1247 residues long and is made up of an identical set of domains, apart from having an extra C-terminal LCCL module (Figures 3.22 and 3.23). An alignment of the protein sequences of *Tg*SR1 and *P. falciparum* SLAP showed that the molecules share 30% identity (data not shown). Furthermore, when looking for sequence similarities within Plasmodia, the SRCR and PLAT motifs were not present in any other available sequences, including the fully sequenced *P. falciparum* genome, whereas the LCCL motif was found in other genes. However, as more genomic sequence data is retrieved from other organisms, homologues to *P*SLAP may be found in other Apicomplexan parasites.

3.3 Discussion

We have identified and analysed a novel gene and protein in *Plasmodium chabaudi chabaudi* (AS). Using the Vectorette system and standard method PCR, a 270 base pair fragment of a gene was extended in the 3′ and 5′ directions, and a 3897 nucleotide sequence, translating into a 1298 residue ORF, was obtained.

We have identified and analysed a novel gene and protein in *Plasmodium chabaudi chabaudi* (AS). Using the Vectorette system and standard method PCR, a 270 base pair fragment of a gene was extended in the 3′ and 5′ directions, producing 2692 base pairs of open reading frame of the gene. The remaining sequence at the 5′ end was obtained from a BLAST search of the Sanger Center *P. chabaudi* database. The full gene sequence of 3897 nucleotide sequence had thus been identified, translating into a 1298 residue ORF. As expected from a *Plasmodium* gene the sequence has a typical AT-bias, with 73% (adenosine+thymine) nucleotides (the fully sequenced *P. falciparum* genome has an 80.6% (A+T) content (Gardner *et al*, 2002). Southern analysis using high stringency conditions showed that *pcslap* is present as a single copy gene in the *P. chabaudi* genome. This agrees with the data from work on *P. falciparum* (3D7 strain) *slap*, which has been shown to be a single copy gene on chromosome 14, as reported previously by us, as well as confirmed by the presence of only one *slap* gene within the sequence data from the completed *P. falciparum* genome sequencing project (Delrieu *et al.*, 2002; Lasonder *et al.*, 2002). However, lowering of the stringency revealed hybridisation to a second fragment in the digests, indicating the existence of a sequence with a high degree of similarity to *pcslap*. Interestingly, analysis of *pslap* copy numbers in other rodent parasites also showed hybridisation to a second fragment in *P. berghei*, but not in *P. yoelii*. However, searches through *P. chabaudi* and *P. berghei* sequence databases failed to identify this potential related gene, as they did not generate any sequences with a high degree of identity. However, the genomes of these two rodent species have been sequenced to a coverage of 2x and 3x, respectively, and it is possible that this second hybridising sequence has simply not been cloned and sequenced yet, and therefore not entered into the public sequence databases. The other possibility is that the second sequence only has a weak similarity to $pcslap_{270}$ but still enough to support hybridisation. Clearly, it would be interesting to identify this second gene, as so far no other genes have been observed in *Plasmodium* with any primary structure similarity to *pslap*, or with a

similar domain composition (at least one copy of each domain found in the query sequence). As more sequence data is made available it may be possible to identify the sequence *in silico*. Alternatively, the gene could be isolated by screening a genomic library using $pcslap_{270}$ as a probe.

Analysis of the *P*SLAP secondary structure revealed a mosaic protein composed of several types of domains, including two SRCR domains, three LCCL modules and one LH2/PLAT domain. A 22 residue signal peptide is present at the N-terminal end of the protein but no further translocation signals were identified, suggesting that the protein is secreted rather than retained within the parasite.

SRCR domain proteins make up an ancient and highly conserved super family, present in species from several phyla (Aruffo *et al.*, 1997). The SRCR domains themselves are well conserved across species boundaries and were first recognised during the analysis of type 1 Macrophage scavenger receptor, a membrane-bound cell surface molecule on mammalian macrophages (Kodama *et al.*, 1990). They have since been found in a wide range of proteins such as CD5 and CD6 on mammalian T cells (and some B cells), *WC1* on γδ T cells in pigs, sheep and cattle, Mac-2 Binding Protein in human and murine carcinoma cells, 18-B in chicken serum, Complement factor 1 in mammalian and amphibian plasma, Sp22D in *Anopheles gambie* mosquito hemocytes, and the Speract receptor on sperm cells and SpSRCR1 and SpSRCR5 on coelomocytes (invertebrate immune cells) in sea urchins (Jones *et al.*, 1986; Aruffo *et al.*, 1991; Wijngaard *et al.*, 1992; Koths *et al.*, 1993; Chicheportiche and Vassalli, 1994; Iwasaki *et al.*, 2001). Although in some proteins, SRCR domains are present as one of several secondary structures, such as *P*SLAP, they are in many instances the only identifiable motif, as is the case with among others CD5 and CD6. This proves that SRCR domains play important functional roles and do not only serve as structural elements in molecules.

So far, all identified Group B SRCR domain proteins have functions relating to immunity, such as the mammalian antigens CD5, CD6 and CD163. However, the role of the SRCR domain itself is still unclear but it appears to be involved in receptor-ligand interactions. To date only a few Group B SRCR interactions have been characterised in detail at a molecular level. The best documented interaction is that between CD6 on T

cells and Activated Leukocyte Cell Adhesion Molecule (ALCAM) on lymphocytes (Bowen *et al.*, 2000). CD6 contains three SRCR domains, of which the most membrane proximal binds one of the V-like immunoglobulin domains of ALCAM. This interaction is believed to modulate T cell receptor signalling. Recently it was shown that the bacteria binding and agglutinating properties of salivary agglutinin can be attributed to its SRCR domains. A sixteen residue synthetic peptide, based on a consensus sequence of the salivary agglutinin SRCR domains, showed binding to a wide range of gram positive and –negative bacteria, including *Esherichia coli, Staphylococcus aureus, Streptococcus mutans, Heliobacter pylori* and *Prevotella intermedia*, as well as mediated the agglutination of *S. mutans* (Bikker *et al.*, 2002).

Interestingly, SRCR domains are also present in proteins in *Anopheles gambie*, the invertebrate vector of *Plasmodium* parasites. Sp22D (Serine protease from chromosomal division 22D) is a multi-domain serine protease that contains two SRCR domains (Figure 3.22). It is constitutively expressed in mosquito hemocytes, fat body and midgut epithelial cells – all tissues involved in immunity (Section 1.8.4). Infection experiments have shown that Sp22D mRNA is upregulated upon bacterial challenge, indicating a role in host defense against invading pathogens (Danielli *et al.*, 2000). It has been suggested that Sp22D may have an immune recognition function similar to that of *Limulus* factor C in the horseshoe crab (Gorman *et al*, 2000). A putative homologue to Sp22D named GRAAL, coded for by the gene *tequila*, was recently identified in *Drosophila melanogaster* (accession number AJ251803; Adams *et al.*, 2000). GRAAL shares all domains of Sp22D, except for having one rather than two N-terminal chitin binding domains. An alignment between the protein sequences of the two molecules showed a 29% identity (data not shown). The function of GRAAL is also unknown.

A BLAST search using *Pc*SLAP SRCR-1 as the query sequence also identified a novel gene from the *Anopheles gambie* sequencing project. A large open reading frame coding for a 3069 residue protein labeled agCP4856 (accession number EAA15063.1) included two SRCR domains and 11 parallel β-helix repeats (Fig 3.22). This sequence has not been curated by experts and no publication on the gene or protein is yet available.

Unlike the SRCR domain, which is frequently the only identifiable motif in a protein (Sp-α, CD5, CD6), the LCCL domain has so far always been found alongside other modular

domains such as epidermal growth factor (EGF)-like domains, serine protease motifs and von Willebrand type A domains. It has therefore not been clear whether it has a purely structural function or if it is involved in interactions with other molecules. However, studies of the human deafness disorder DFNA9 showed that the mutations that cause it are all located in the LCCL part of the Coch-5b2 protein (reviewed in Trexler *et al.*, 2000). More importantly, one of these mutations, Trp91Arg, did not affect the overall structure of the protein, indicating that the progressive loss of hearing that is a result of the disease is not due to a structural change in the protein, but most likely caused by a failing ligand interaction between the LCCL domain and a yet unknown binding partner (Liepinsh *et al*, 2001).

In the horse shoe crab, the LCCL-containing *Limulus* clotting factor C protein is part of an antibody-independent host defense system against invading pathogens. It has been shown that the N-terminal part of the protein is involved in binding of LPS but it has not yet been proven that the LCCL domain mediates this interaction. Similarly, the mammalian late gestation lung protein-1 (lgl1) is expressed at the same time during development as a number of anti-microbial proteins, raising the possibility that it has a similar function to these molecules.

A follow-up study to the *P. falciparum* sequencing project, using high-accuracy mass spectrometry, analysed the expression of developmentally regulated proteins in the parasite. Among proteins expressed during the sexual stages, four molecules containing LCCL domains, including *Pf*SLAP, were identified (Lasonder *et al.*, 2002; *Pf*SLAP, PF14_0732, PF14_0732 and PFA07445w in Figure 3.24). Interestingly, all of these also carried lectin-like domains: Ricin B domains, found on CCP2, have been shown to bind simple sugars such as galactose and lactose, discoidin domains on PF14_0732 bind cell surface carbohydrates, a galactose-binding motif was identified on PFA07445w, and SRCR domains, found in *P*SLAP, have been suggested to bind carbohydrate surface molecules on bacteria (Bikker *et al.*, 2002). Thus, binding of carbohydrates appear to be an important characteristic of sexual-stage parasites, perhaps playing a role in fertilization, evasion of host or vector immune responses, or in development within the mosquito. Indeed, it has been shown that lectins with specificities for N-acetyl-D-glucoseamine (GlcNAc) and N-acetyl-D-galactoseamine (GalNAc) residues bind to

midgut sections from *Aedes aegypti* and *Anopheles stephensi*, respectively (Rudin and Hecker, 1989). The authors suggested that carbohydrates play a role in parasite recognition of, and migration within, the vector, stating that "If the parasite's recognition of the vector tissue and subsequent penetration of the midgut epithelium depends on surface-exposed sugars, stage-specific lectin-like proteins should be on the parasite surface."

Up until recently, extensive searches throughout available databases failed to identify any homologues of SLAP, or any molecules with similar domain composition (ie at least one copy of each domain in the query sequence). However, a recent search using BLAST identified an orthologue to *Plasmodium* SLAP in another apicomplexan parasite, *Toxoplasma gondii*. This protein, *Toxoplasma gondii* Scavenger receptor 1 (TgSR1; accession no AAN74967), is 1247 residues long, shares 30% identity with *P. falciparum* SLAP and is made up of an identical set of domains, but with an additional C-terminal LCCL module (Figure 3.22). As in *Plasmodium*, this is the first SRCR-containing protein that has been identified in *T. gondii*, and nothing is yet known about its function. Extensive searches through a number of sequence databases, including the NCBI non-redundant database, failed to detect any other genes homologous to *pslap*.

Clearly, *P*SLAP is an interesting novel protein, not previously seen in any other species. Its high conservation within *Plasmodium spp.* indicates that *P*SLAP has an important biological function in the parasite. The multi-domain composition with several adhesive motifs suggests that *P*SLAP is involved in some type of interaction with other molecules. The finding that the SRCR-containing mosquito protein Sp22D responds to invading pathogens raises the possibility that *Pc*SLAP might be used in molecular mimicry. It is known that *Plasmodium* infections cause up-regulation of mosquito genes involved in insect immune responses (Dimopoulos *et al.*, 1997; 1998). A molecule with the ability to act in a similar way to Sp22D could potentially divert the host immune response away from pathogen destruction and thereby increase the parasite's chances of survival.

The LCCL domain also has potential for ligand-binding. Multiple alignment showed that LCCL-1 has diverged more from the domain consensus sequence than LCCL-2 and -3.

This could indicate an adaptation to a species-specific ligand interaction, whereas the other two modules may serve as structural elements within the *P*SLAP protein.

Figure 3.1

Structure of the Vectorette adapter

The Vectorette adapter is a short DNA sequence with a sticky end that allows it to be ligated onto DNA digested with the appropriate restriction enzyme. The adapter is double stranded with a central mis-matched region where the DNA strands are non-complementary. The Vectorette primer, (hatched line underneath the Vectorette) has a sequence identical to one of these strands (hatched bottom strand), and therefore cannot anneal in the PCR until a complementary region has been amplified, initiated from the gene-specific, known primer.

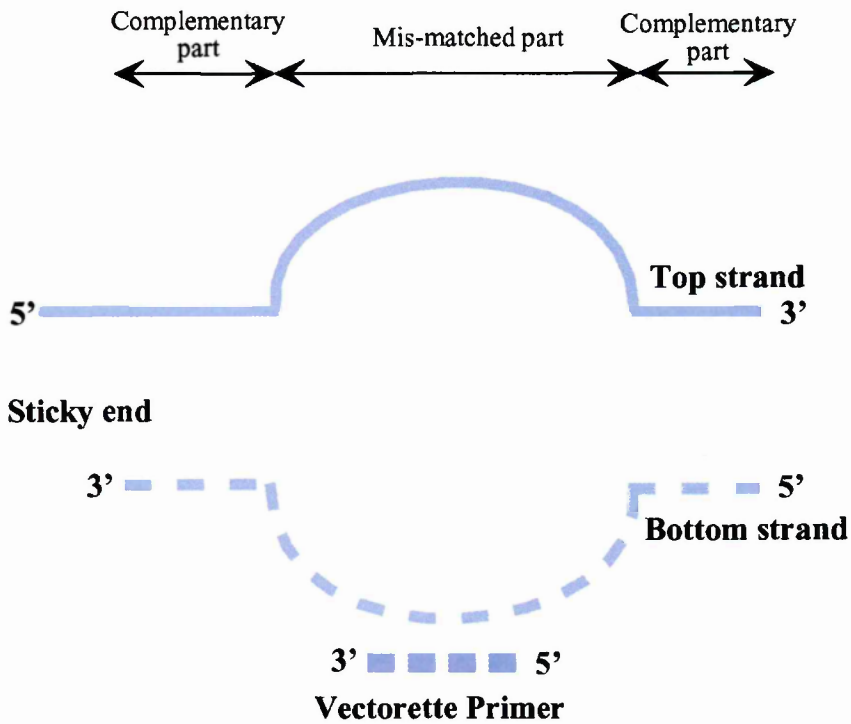

Complementary part
Mis-matched part
Complementary part
Top strand
5'
3'
Sticky end
3'
5'
Bottom strand
3'
5'
Vectorette Primer

Figure 3.2

The Vectorette II System

The Vectorette II System allows identification and amplification of unknown DNA sequences upstream and downstream of a known sequence. The method includes three steps:

I. Target DNA is digested with a restriction enzyme (R), generating fragments containing the known sequence (red) and fragments without this sequence.

II. Vectorette adapters (blue), with a central mismatched region, are ligated onto the ends of the DNA fragments.

III. In the Vectorette PCR, the first round of amplification is from the gene-specific, known primer (KP) only. Since fragments without the known sequence cannot bind this primer, no amplification is initiated. In the second round, amplification takes place from both primers. The Vectorette primer (VP) has the same sequence as the mismatched portion of the bottom strand of the Vectorette, and can therefore anneal to the new strand produced in the first round of amplification.

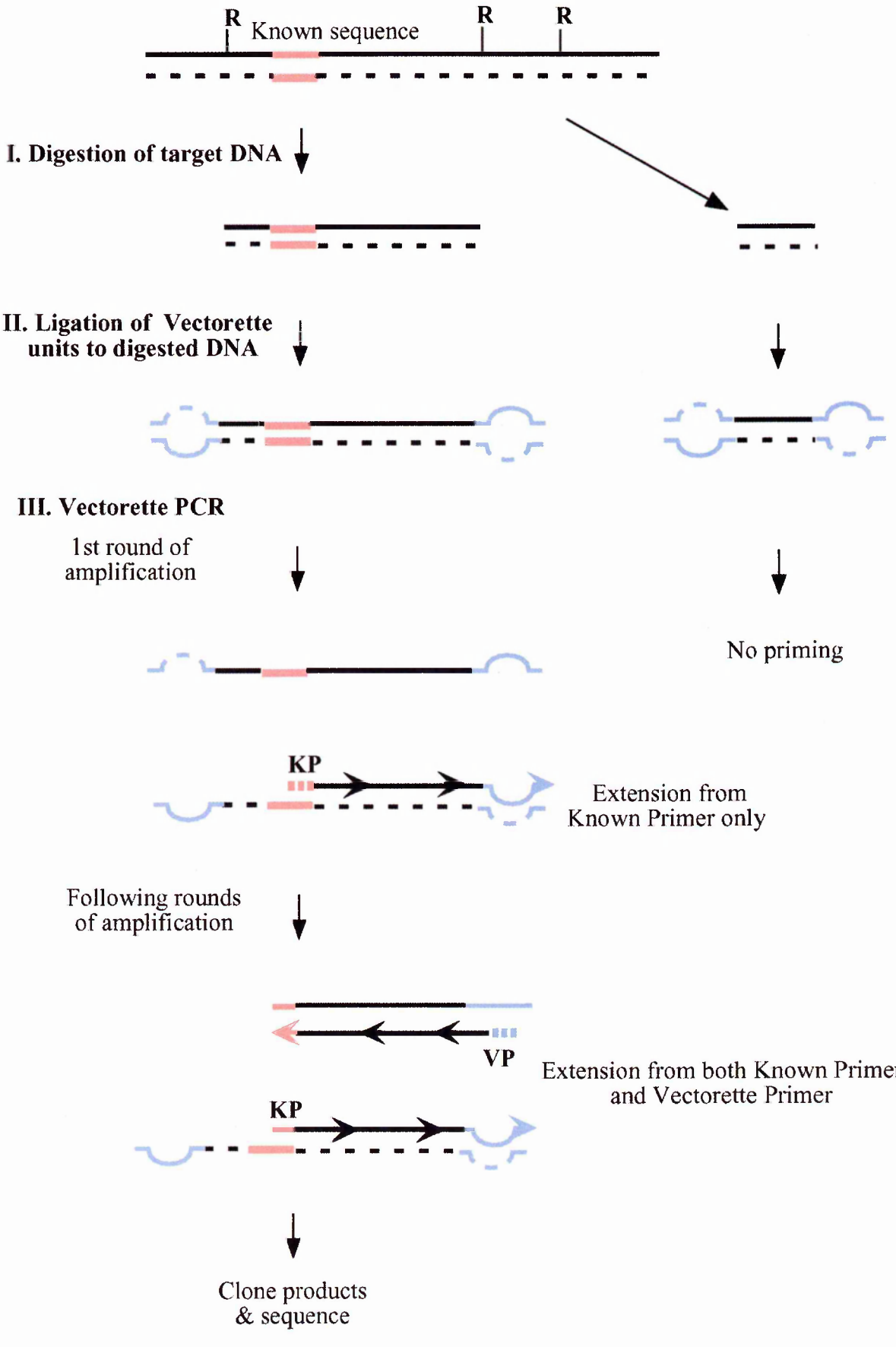

R
Known sequence
R
R
I. Digestion of target DNA
II. Ligation of Vectorette units to digested DNA
III. Vectorette PCR
1st round of amplification
No priming
KP
Extension from Known Primer only
Following rounds of amplification
VP
Extension from both Known Primer and Vectorette Primer
KP
Clone products & sequence

Figure 3.3

Primers used in the first Vectorette screen

Primers for the first Vectorette screen were based on the original $pcslap_{270}$ sequence. One reverse primer, Rev1 (red), for amplification of sequence upstream of $pcslap_{270}$, and one forward primer, Forw1 (blue), for downstream amplification, were designed. These were run in separate PCR reactions together with the Vectorette primer (not shown).

***pcslap*$_{270}$**

AATGGATTTTCTAAACAACTATGTAGTGATATAGCAGGTGAAAATTTATGTGGTCATGAT

ACAGAAAGAATTAATGCAACGAATTTTAGATGTAAAGGAGATGAAGCTAATTTAAAAAAT 120

TGCCCACATGAAACATCTGAAGATATTTACTGTTCTCACGAAGAAGATATAATTATTGGT

TGTGCAAATGCAGAAGAAGATGGAGATGCTTCGACCAGTTCAACTAGCATTTCTAAATAT 240

GGATTAAACAAACATATAATGTCAATGGA

Figure 3.4

Schematic representation of the position of *pcslap*$_{270}$ in relation to the expected size of the full *pcslap* gene

Based on the sizes of the *P. yoelii* and *P. falciparum slap* genes (3816 and 3900 basepairs, respectively) the full length of *P. chabaudi slap* was estimated to approximately 4000 basepairs. The schematic shows the predicted position of *pcslap*$_{270}$ (red) within the gene, based on sequence alignments between *pcslap*$_{270}$ and the other two genes (data not shown). Primers Rev1 and Forw1, used for upstream and downstream amplification, respectively, are shown as half-head arrows.

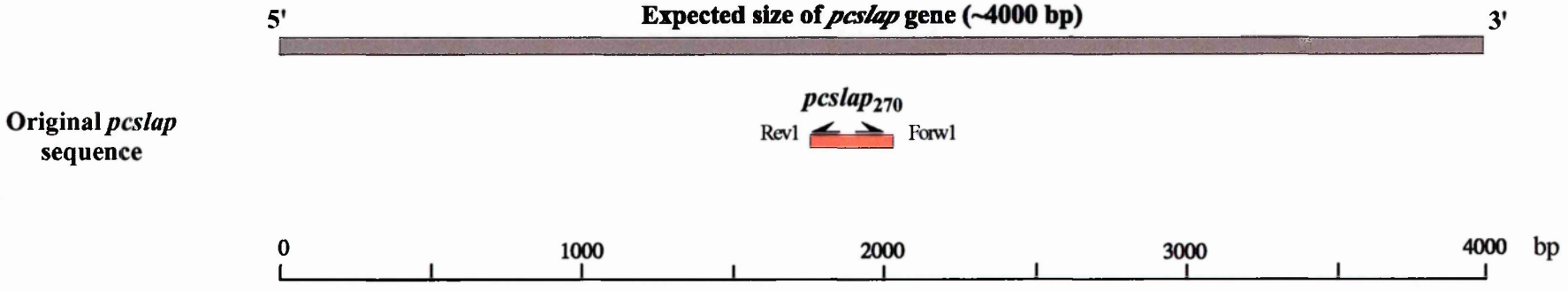

5'
Expected size of pcslap gene (~4000 bp)
3'
Original pcslap sequence
pcslap270
Rev1
Forw1
0
1000
2000
3000
4000
bp

Figure 3.5

PCR products obtained in the first Vectorette screen

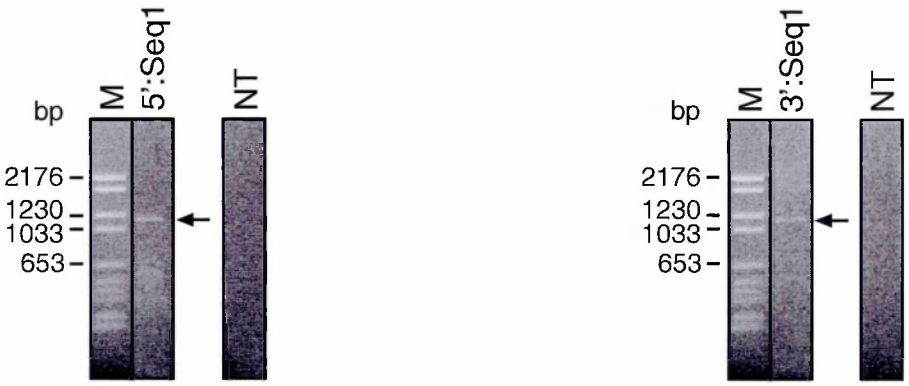
(A) Gel electrophoresis of PCR products from the first Vectorette screen. Ten µL of each product was loaded onto a 1% agarose gel. Shown on the left is the result from the 5′ amplification, showing a band of approximately 1100 nucleotides (5′:Seq1, arrow) using the Rev1 primer and the SspI library. On the right is the result from the 3′ amplification with primer Forw1 and the SauAI library. An approximately 1100 basepair product was generated and designated 3′:Seq1 (arrow). M = Marker VI, Roche. NT = negative control containing sterile water instead of template DNA.

(B) Products 5′:Seq1 and 3′:Seq1 were sequenced and their authenticity as extensions of *pcslap* verified. Primers for further gene identification were subsequently based on these sequences.

1 = Primer Rev2
2 = Primer Rev2:1
3 = Primer Forw2
} Used in Second Vectorette screen

4 = Primer Rev3:1
5 = Primer Rev3:2
} Used in conventional PCR for identification of 5′ and 3′ ends of *pcslap*

A



B

5′:Seq1

```
                                                                                4
ATGGAAATGCAAAAGACATAGATTGTCATATACGTGCTAATGATTTAATAGATACAACTAAATCATTAAATAATAGTTTT
                                                                    1       2
GTTTTACAAAATAAGGTACATATATTTAAAGTTCGATGCCCACAAAACTGTCATAGTTCCGATTTTTCTATCATAGAAGG 160
                                                                5
TACTTCTATACATCCTGCATCTACCTCTATATGTGCTGCAGCTATTTATGATGGTTCATTAACAGAAAGTGGAGGCGAAA
TTATTGTTACTATAACAAAAGGATTAAATTATTATTATGCAATGGATGGCACATATAATAATTTAAAAGCAATTGAATTT 320
TCTACCAAAGGTGATGAAAGTGATGAAAATAATTTCTCTTTTTATACATATCATTTGACATCTATTGATGATATAAAAAG
TAATGTTAGAATCGTTGATTCTTTTGGAAAATTATCTTCTTTAGGAAGATTAGAAATTCGTGTAAATAATAAATGGGGAG 480
CTGTTTGTAAAAAAGGTCCAAACTTTGAATTTTCAGAAGAGGCAGCAAAAAGAGCTTGTAAAGATTTAGGTTTTCCAAAT
GGTATATATATTAGAGATACTTGCTCAAATATCAATGAACAAAATTATTGTGCTGGGTATAAATATCCATTTAATGCTTC 640
TGGTATTTTATGTTCTGGAAATGAACAGAATTTATTATCCTGTAATACTGACGATCCTTCATATTGTATAGATCATCATG
ATGATGTTATAATACAATGTGTTAATCAATTAGGTAACGATTCGATAGAAAACGGAACCATTCGACTTTTAGACAGCACT 800
GGTGCACCTACATCTAATGGAATAGGAAGATTACAAATATATTACAATGGGGTATTCGGTTCTGTTTGTTCAGAAGGATG
GACAAAAGAAACAGAAAAAATTGCATGTTTAGAATTAGGTTATCATAATGTAAAAGCA
```

3′:Seq1

```
AAAAAAACAATTTCATCCCAAAATCGAATTAAGTTGCTTTGACAAAATAAGTTCTAAAGCTGAATTGAGTAAAGGAAATG
TTGGAGATATATTTTTAGTCAGCTGCCCAGAAAAATGTGATGAAGATAGTGGTGTAATAAAAGGAACTTTTGTATACACA 160
TTCGATTCTTATATATGTAAAGCAGCTATACATGCAGGTGTTCTATCGAGTAATGTTGCTGATGATGTAGTTCTTATCAT
TACCCATAGTAGAAATAAATTTATAGGAACCAAAAGAAATAATGTAGAATCAAAAGAATTTAATGGTGAATCGAAAAGTT 320
TTAGCTTAAGTATACCAACAAATTATATCATAATGGAAGAAAGACAAAATAATTCAAAATATGAAGATGAAATTTTAAAA
GATGATAATGATTTTTATTATGAACATATATTTAATAAACAAAATAATAATGAAAAAGGAAAAAATAAAACGTTTTTTGA 480
ACATAATATGCATCTAGAACCCACTTTTCAATGGATAGCTCCATCCTCTTTTACTGGATTCAATGGAAATGAAAATGAAT
                                                                                3
ATATAAATGCTAGTAATCTTCCTAATGAAAAATATATTAGAACTCTTTCTAATTTTACTTTTATTACACACTTCATTCCA 640
AGTAGTAAAGGGAAAAATACATGGAGAACAATATTATCTCATAGTTTATGTGAAGGTATATCTATATCTATTGATGAAGA
AAATGAATTAGTAATAGAACAAAATTGTAACCCTCATTTAATAAAAACTAAATTTATCCCAAAATTTGAACACCCTTATC 800
ATCTGGTTCTAATTTATAACAAGCCAAATAAATCAATTTCTTTATATATTAATCAGAAAAAAATTAACCTTGAAAATATG
AAAATCGATTTTACCCTAAATGGAGATTTAACTATTGGTAGATC
```

Figure 3.6

Schematic representation of sequences obtained in the first Vectorette screen relative to *pcslap*$_{270}$ and the full length gene

Product 5′:Seq1 extended the known sequence of *pcslap* 938 nucleotides in the 5′ direction, and 3′:Seq1 identified 924 nucleotides in the 3′ direction (both products shown in blue). Primers Rev2, Rev2:1 and Forw2 designed for further sequence identification up- and downstream of the known sequence are shown as half-head arrows by the 5′:Seq1 and 3′:Seq1 sequences.

The original gene sequence, *pcslap*$_{270}$, is shown in red and sequences obtained by Vectorette PCR are shown in blue.

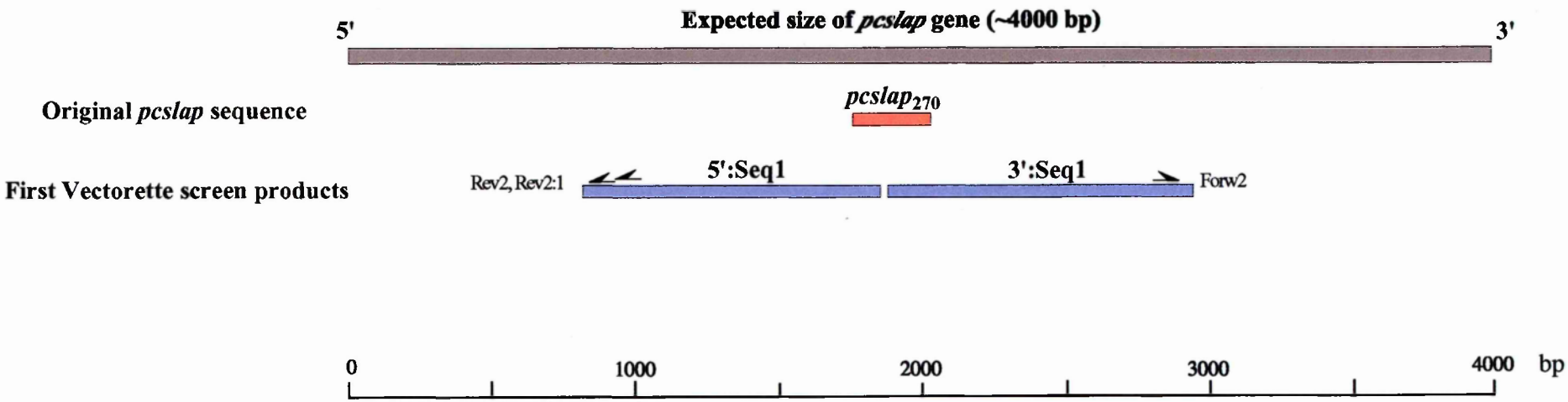

5'
Expected size of pcslap gene (~4000 bp)
3'
Original pcslap sequence
pcslap270
First Vectorette screen products
Rev2, Rev2:1
5':Seq1
3':Seq1
Forw2
0
1000
2000
3000
4000
bp

Figure 3.7

PCR product obtained in the second Vectorette screen

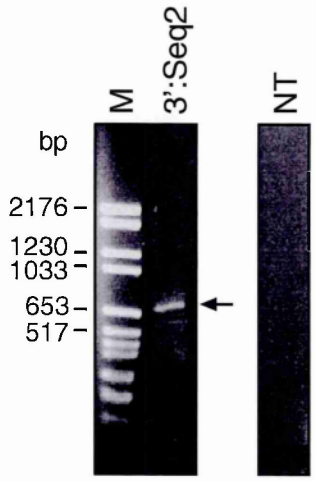
(A) Gel electrophoresis of PCR reactions from the second Vectorette screen. Ten μL of each product was loaded onto a 1% agarose gel. A 700 basepair product, designated 3′:Seq2, was generated in the 3′ amplification attempt using the RsaI library (arrow). M = Marker VI, Roche. NT = negative control containing sterile water instead of template DNA.

(B) Product 3′:Seq2 was sequenced and subsequently used to design primers for further sequence identification:

1 = Forw3:1

2 = Forw3:2

A



B

3′:Seq2

```
CCAAGTAGTAAAGGGAAAAATACATGGAGAACAATATTATCTCATAGTTTATGTGAAGGT
ATATCTATATCTATTGATGAAGAAAATGAATTAGTAATAGAACAAAATTGTAACCCTCAT 120
TTAATAAAAACTAAATTTATCCCAAAATTTGAACACCCTTATCATCTGGTTCTAATTTAT
AACAAGCCAAATAAATCAATTTCTTTATATATTAATCAGAAAAAAATTAACCTTGAAAAT 240
ATGAAAATCGATTTTACCCTAAATGGAGATTTAACTATTGGTAGATCAAACAAACAAGCA
ACTGATTACTTTATAGGGGATATTAATTTTGTTAAAATTTATAAATATATATTAACAGAA 360
CAAGAAATTAAAGAGTCCTTTGATTCAGTTCTTTCAAATAATTATTTAAATGATGGGATG
TCTGGAAATGGAAACATAAATAGTAGAAAAATACAAAATAAAAAAACAAAAAATCAACGA 480
AAAACTATAGATGGACGTGATTGTATTAGTTCATGTAAATCCAAAACGAATGTAAATAAA
AATATACAAATTAATACGGAAGAATTTTACCTGAACTGTTCAGATAATTTACTCAGTGAG 600
CGATTTAGTGGAAAAATTGGAACTCAGTTTTTGGCTAGCTGTTCAGAAGATTGTGCTAGC
TCAAAATATATAGTAAAAGGGTCTAACAATTACTACACACCAGAT
```

1 →

2 →

Figure 3.8

Schematic representation of sequences obtained in the second Vectorette screen relative to previously obtained sequence and the full length *pcslap* gene

Product 3′:Seq2 (short blue bar) added 367 nucleotides to the known sequence of *pcslap* in the 3′ direction. Primers Rev3:1, Rev3:2, Forw3:1 and Forw3:2, subsequently used for conventional PCR for further sequence identification, are shown as half-head arrows.

The original gene sequence, $pcslap_{270}$, is shown in red and sequences obtained by Vectorette PCR are shown in blue.

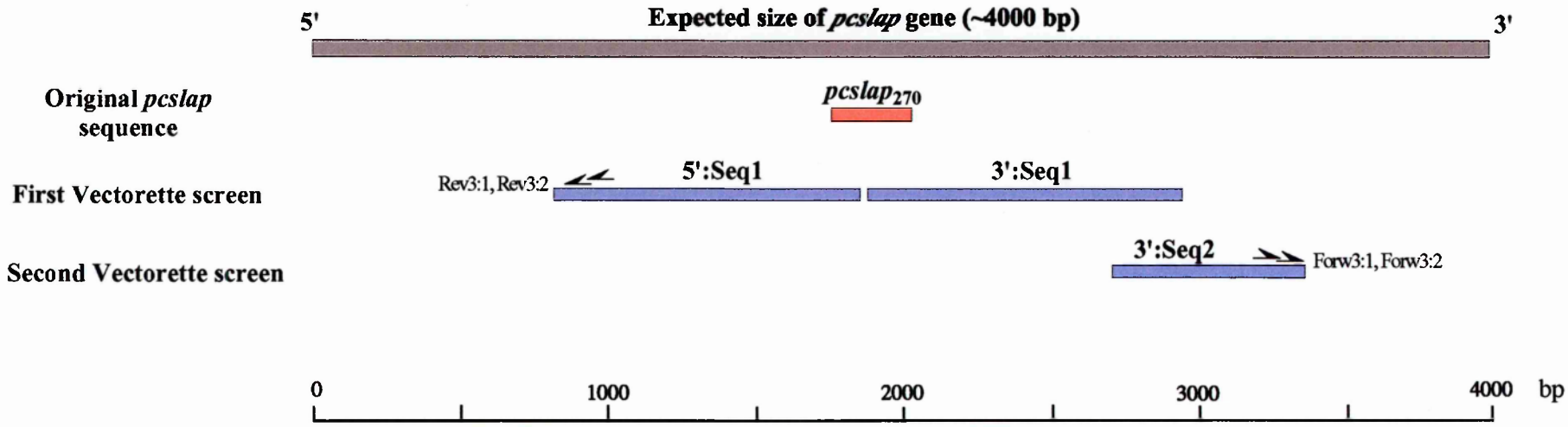

5'
Expected size of pcslap gene (~4000 bp)
3'
Original pcslap sequence
pcslap270
First Vectorette screen
Rev3:1, Rev3:2
5':Seq1
3':Seq1
Second Vectorette screen
3':Seq2
Forw3:1, Forw3:2
0
1000
2000
3000
4000
bp

Figure 3.9

Alignment of 5′- and 3′ un-translated regions (UTRs) from the *P. yoelii* and *P. berghei slap* genes

The 5′- and 3′ UTRs from *P. yoelii* and *P. berghei slap* were aligned in order to find regions of high conservation that could be used for designing degenerate primers to be used in the identification of the 5′- and 3′ ends of *P. chabaudi slap*. Primers are shown as black half-head arrows in the figure.

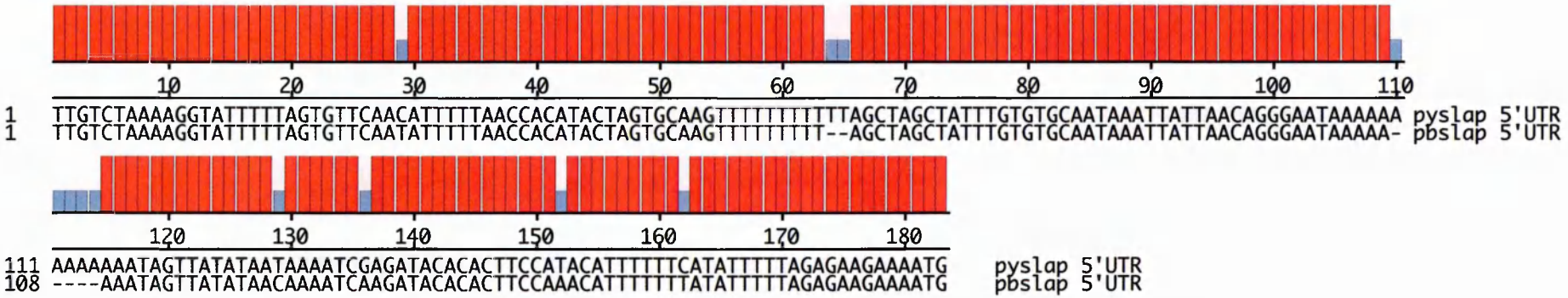
(A) Alignment of the 5′ UTRs from *P. yoelii* and *P. berghei slap*. Primer pslap.5′:1 was based on nucleotides 34-55 and pslap.5′:2 on nucleotides 77-101. Both of these sequences shared complete identity between *P. yoelii* and *P. berghei.*

(B) Alignment of the 3′ UTRs from *P. yoelii* and *P. berghei slap*. Primer pslap.3′:1 was based on nucleotides 61-82 in the alignment and pslap.3′:2 on nucleotides 136-157. These two sequences each had one nucleotide differing between the *P. yoelii* and *P. berghei* sequences.

Red colour denotes identity between nucleotides, blue colour denotes difference between nucleotides.

The alignment was generated with the MegAlign sequence alignment program using the ClustalW algorithm.

A


10 20 30 40 50 60 70 80 90 100 110
1 TTGTCTAAAAGGTATTTTTAGTGTTCAACATTTTTAACCACATACTAGTGCAAGTTTTTTTTTTTTAGCTAGCTATTTGTGTGCAATAAATTATTAACAGGGAATAAAAAA pyslap 5'UTR
1 TTGTCTAAAAGGTATTTTTAGTGTTCAATATTTTTAACCACATACTAGTGCAAGTTTTTTTTT--AGCTAGCTATTTGTGTGCAATAAATTATTAACAGGGAATAAAAA- pbslap 5'UTR
120 130 140 150 160 170 180
111 AAAAAAATAGTTATATAATAAAATCGAGATACACACTTCCATACATTTTTTCATATTTTTAGAGAAGAAAATG pyslap 5'UTR
108 ----AAATAGTTATATAACAAAATCAAGATACACACTTCCAAACATTTTTTTATATTTTTAGAGAAGAAAATG pbslap 5'UTR

B

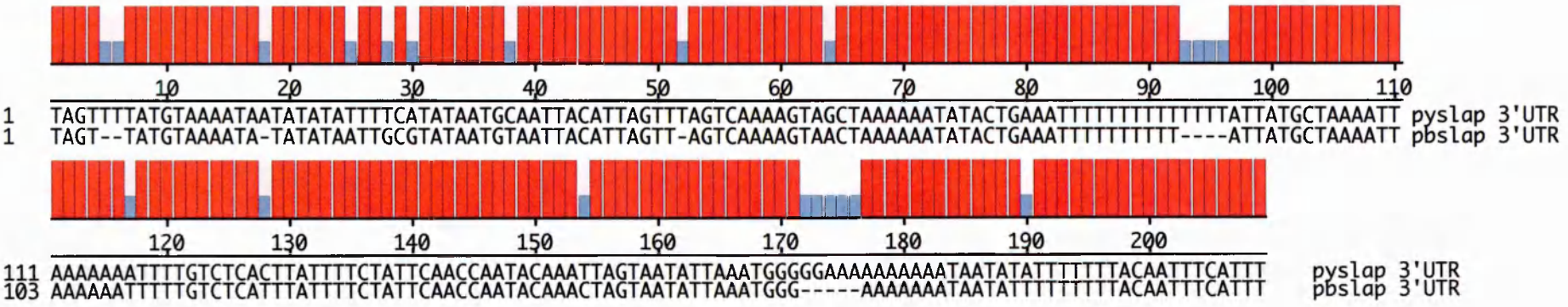

10 20 30 40 50 60 70 80 90 100 110
1 TAGTTTTATGTAAAATAATATATATTTTCATATAATGCAATTACATTAGTTTAGTCAAAAGTAGCTAAAAAATATACTGAAATTTTTTTTTTTTTTTATTATGCTAAAATT pyslap 3'UTR
1 TAGT--TATGTAAAATA-TATATAATTGCGTATAATGTAATTACATTAGTT-AGTCAAAAGTAACTAAAAAATATACTGAAATTTTTTTTTT----ATTATGCTAAAATT pbslap 3'UTR
120 130 140 150 160 170 180 190 200
111 AAAAAAATTTTGTCTCACTTATTTTCTATTCAACCAATACAAATTAGTAATATTAAATGGGGGAAAAAAAAAAATAATATATTTTTTTACAATTTCATTT pyslap 3'UTR
103 AAAAAATTTTTGTCTCATTTATTTTCTATTCAACCAATACAAACTAGTAATATTAAATGGG-----AAAAAAATAATATTTTTTTTTACAATTTCATTT pbslap 3'UTR

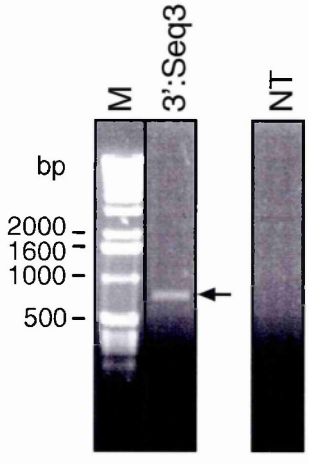
Figure 3.10

PCR product obtained from conventional PCR

(A) Gel electrophoresis of products from a conventional PCR using *pcslap*-specific primers and degenerate primers based on the 5′- and 3′ *pslap* UTRs of *P. yoelii* and *P. berghei*. A product of approximately 750 nucleotides was generated and designated 3′:Seq3.
M = Marker VI, Roche. NT = negative control containing sterile water instead of template DNA.

(B) Sequence of product 3′:Seq3. Translation of 3′:Seq3 revealed a C-terminal stop codon at position 597 (underlined) marking the end of the ORF.

A



B

3′:Seq3

CTAGCTGTTCAGAAGATTGTGCTAGCTCAAAATATATAGTAAAAGGGTCTAACAATTACT
ACACACCAGATACGTCAATATGTAAAGCAGTTATGCATTCAGGAATAATGCGTAAAAATA 120
ATGAGCATGATAAGGATGATAACGAAAATAATAAAAATTCGTTTATTATAAAAATTGTTG
AAGGCTTAACAGAATATAAATCATCTAGAGGTCATTTTGGAATCATTTCAAAAGCAGAAA 240
AACAATCACAATTAAGATCCTTTTCTCTTTTTTCAAAAAATGAAGATGATATTTTTACAT
GTTTTACAGATGGATCATTTTTATTTGAATTACCTATTGGAAGTACAAAAAATATTATTT 360
GTCCCGAGAATTGCCATAAAATCGATAAACAAATATACGGAACTAATACATATAGTCCTT
TATCTTCTGTTTGTAAAGCAGCTATACATGCAGGTGTTATTTCAATAAAGGGAGGTCAAA 480
TACAAGTTGTTGTTGGGAAAGGTCAACAAGAATTTAAACCATCCACACAAAATAATGTTC
AATCATATATAGCAGAAAAACAAGATCGTTCATTCACCTTTTTCAAGCGCTTATATTAGT 600
TATGCAAAATATGTATACTTACATAATGTGTAATTACATTAGTCTAACCAAAAGTAAGTA
AAAAATAGAATGATATTTTTTTTTATTAAGTTCAAATTAAAACAATTTTGTCTTTCTTTC 720
TATTCAACCAATACAAATTAG

Figure 3.11

Schematic representation of product obtained from conventional PCR relative to previously obtained sequence and the full length *pcslap* gene

The 741 nucleotides long product 3′:Seq3 (green) translated into an ORF terminated by a C-terminal stop codon at position 597 in the sequence. The position of the end of the gene was predicted from alignments with the *P. yoelii slap* gene.

The original gene sequence, $pcslap_{270}$, is shown in red, sequences obtained by Vectorette PCR are shown in blue and sequence generated by conventional PCR is shown in green.

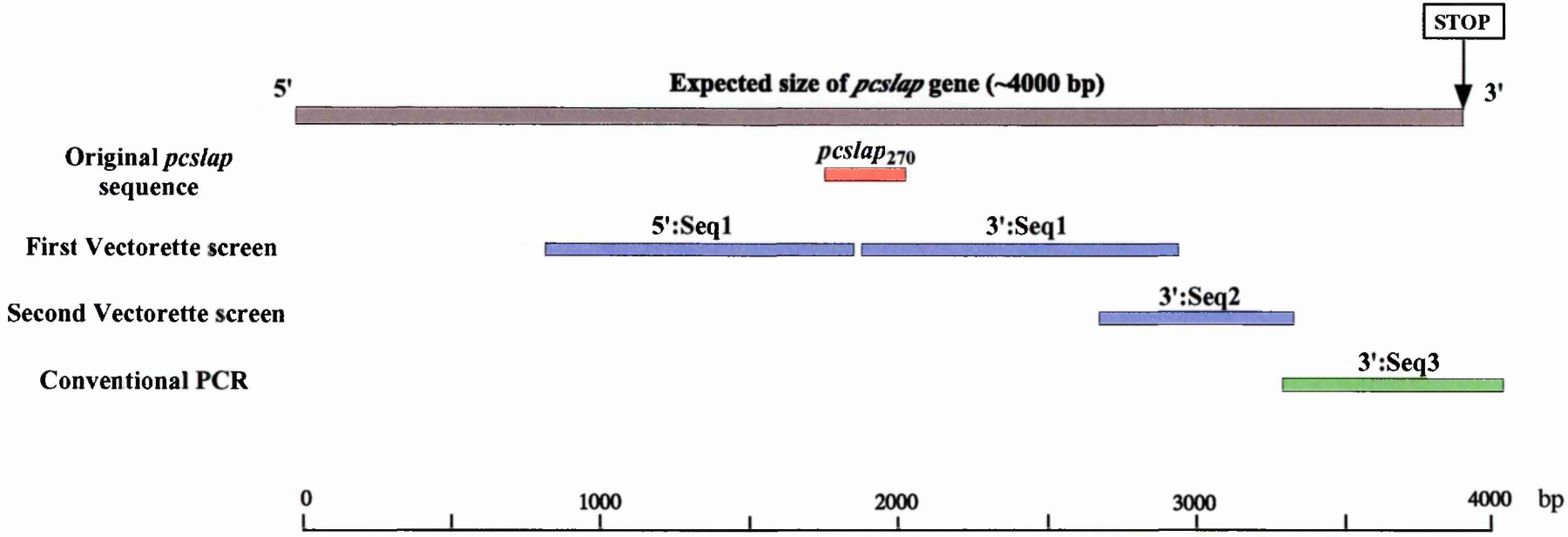

STOP
5'
Expected size of pcslap gene (~4000 bp)
3'
Original pcslap sequence
pcslap270
First Vectorette screen
5':Seq1
3':Seq1
Second Vectorette screen
3':Seq2
Conventional PCR
3':Seq3
0
1000
2000
3000
4000
bp

Figure 3.12

Contig from the *P. chabaudi* genomic contigs sequence database

Reverse complement sequence of a *P. chabaudi* 2053 nucleotide long contig, identified in a BLAST search through the genomic contigs database. The 3′ end of the contig aligned with the identified *pcslap* sequence. The ATG start codon initiating the *pcslap* ORF at position 460 in the sequence is underlined.

>1202868.c000703295.Contig1 (reverse complement)

TTCATATATCATTTAAAATGGGATGTGCATAAATGTACAGAAACATATGAAATTTTGTTA
TTGAACATTTAGTAATAAGAAAAGCATTTGCTTAATTTGGTGAATTTGTTGAACAGATTC 120
GTGATAAATAACAACAATAGTTACTTGGGTTATTAAATTCTCGCACACACACATACTGTG
TATATATATATTCATAAGTTTATTTTCATATTATAGTTTTTTTATACATTGTAAGTTTTT 240
TTTTTCAGACTAGCATAGTGCGCCTTTTTAACCTATAAATTTTTGTCTAAAAGGTATTTT
TAGTGTCCAATATTTTTAAGCATATTACTTGTGCAGGTTTTTTTTTTTAGCTAGCTATTT 360
GTGTGCAAAAAATTATTAACAGGACATAAAAAAAAAATAGTTATATAATAAAGTTGAGATA
CACCCTTCCATATATTTTTTCATATTTTTAGTGAAGAAAATGAGAATCTATAACTGGGTT 480
AGTTGTGGATTTATATTTTTGCAATTTTTTCTGAGCGCATATGGAAAGGAATGGTGCAAA
GCAAAATTTGAATATGGAATGTCTGATTATGCAGAGTGCCAAAAAGAAGGAGATAATCTA 600
ACGAAATATATGATTGAAATAATTCCTGCTAAAGCAAATAATATAGATTTATATAGTGAT
GTTTCAATAGTTTTATCGAATTCTCAAGGTATTAATACTAAAGAAATAATTATTGGTAGT 720
GAAAAAGAAGGACTATTTAGAAAAATATATTCGGTTAGAAAAGATATAGATAATCCAGAA
TATATTCATGTAAAATTAAATTCGAAAATAAATAATAATAGAAATTGGAAATGTAAAAAA 840
ATAAAAGTTTGGAAAGAATATAAGTATTGGACTTTTGAttgTATAGGTATATTAAATGAA
GAAAAACAAGAAGCAACATATTTCTTGTCTGGTAATAAATTATATACTGCTTATGTTCAA 960
ACAGGAAAAGATATAGAAGCAGGTACTACTGGAATTATAGATATAATATTACTAGGTAAT
AACAACAAAAGAAGTAATACAAAAATGCTACATGAAGGATTTACATCTGGCGGATTAAAA 1080
AAAATAAAATTTCAAGCATCAGATGTAGGAAATTTAGAAAATATAATATTAATAAACAAT
TCTTATAATGATCCATGGTATTGTGATTTTGTTAAAATAAAAGGTGATGATAACAAAGTT 1200
TATGTTTTCAATGTAAAAAGTTGGATTGGTTATCCATATAATAATAAAATAAAAATAAAT
ATAAATACAAATAATATTGATGGAAATGCAAAAGACATAGATTGTCATATACGTGCTAAT 1320
GATTTAATAGATACAACTAAATCATTAAATAATAGTTTTGTTTTACAAAATAAGGTACAT
ATATTTAAAGTTCGATGCCCACAAAACTGTCATAGTTCCGATTTTTCTATCATAGAAGGT 1440
ACTTCTATACATCCTGCATCTACCTCTATATGTGCTGCAGCTATTTATGATGGTTCATTA
ACAGAAAGTGGAGGCGAAATTATTGTTACTATAACAAAAGGATTAAATTATTATTATGCA 1560
ATGGATGGCACATATAATAATTTAAAAGCAATTGAATTTTCTACCAAAGGTGATGAAAGT
GATGAAAATAATTTCTCTTTTTATACATATCATTTGACATCTATTGATGATATAAAAAGT 1680
AATGTTAGAATCGTTGATTCTTTTGGAAAATTATCTTCTTTAGGAAGATTAGAAATTCGT
GTAAATAATAAATGGGGAGCTGTTTGTAAAAAAGGTCCAAACTTTGAATTTTCAGAAGAG 1800
GCAGCAAAAAGAGCTTGTAAAGATTTAGGTTTTCCAAATGGTATATATATTAAAGATAAT
TGCTCAAATATCAATGAACAAAATTATTGTGCTGGGTATAAATATCCATTTAATGCTtcT 1920
GGTATTTTATGTTCTGGAAATGAACAGAATTTATTATCCTGTAATACTGACGATCCTTCA
TATTGTATAGATCATCATGATGATGTTATAATACAATGTGTTAATCAATTAGGTAACGAT 2040
TCGATAGAAAACG

Figure 3.13

Schematic representation of position of the *P. chabaudi* contig relative to the identified *pcslap* gene sequence

The *P. chabaudi* contig was translated and an ORF with a start codon 460 nucleotides into the sequence was identified. This sequence (orange) was added to the already obtained sequence of *pcslap* and completed the gene. The ORF of the gene was shown to be a total of 3897 base pairs.

The original gene sequence, $pcslap_{270}$, is shown in red, sequences obtained by Vectorette PCR are shown in blue, the sequence generated by conventional PCR is green and the sequence from the contig is shown in orange.

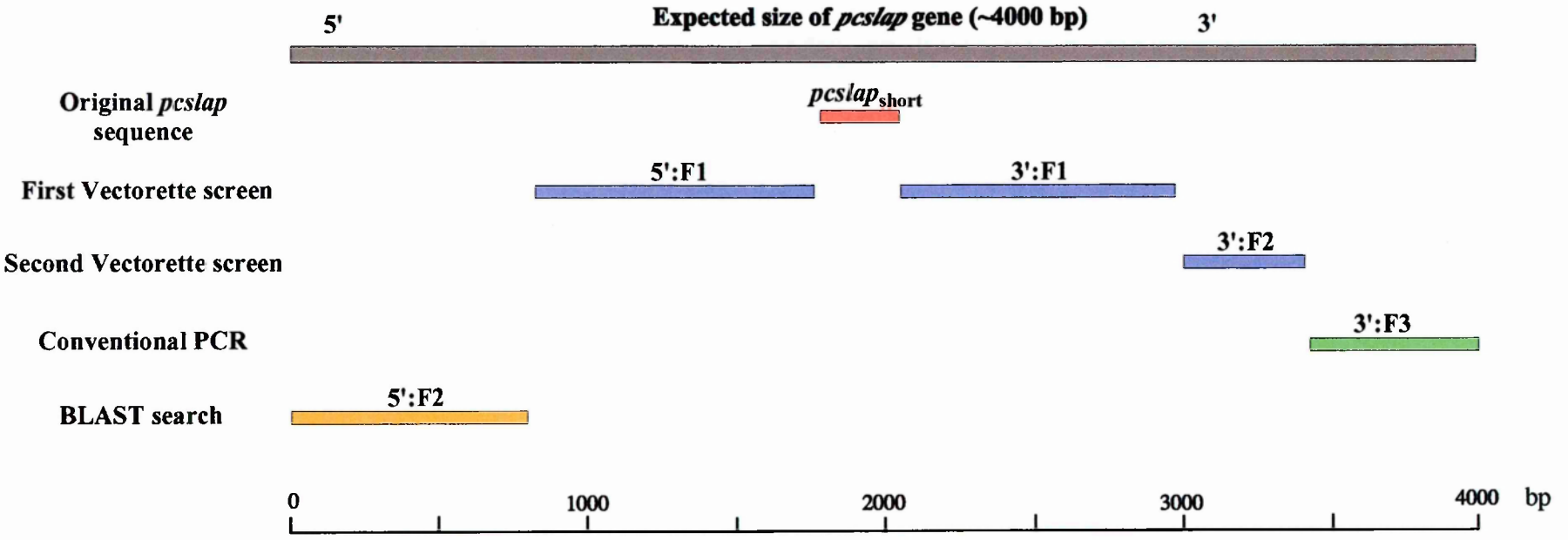
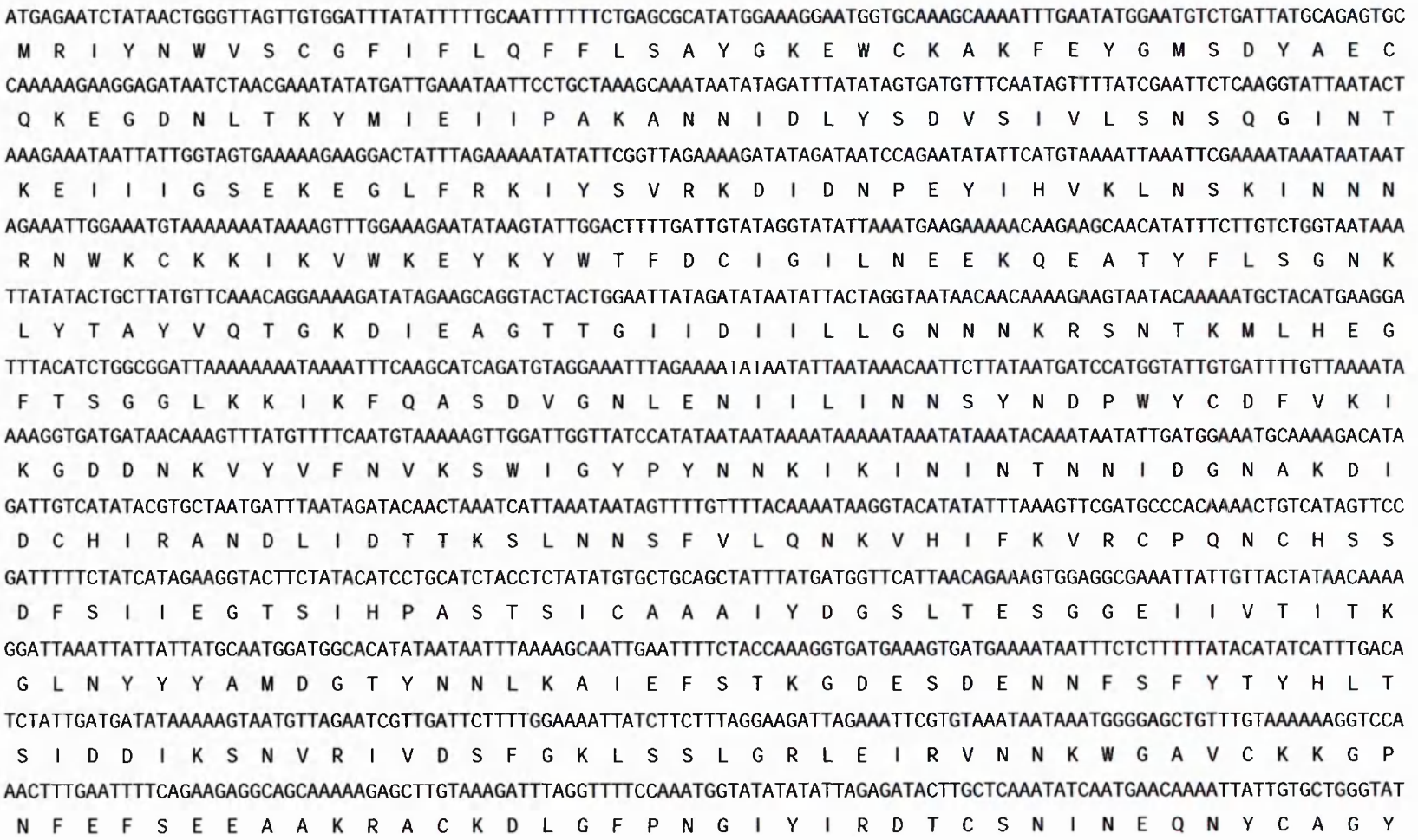

5'
Expected size of pcslap gene (~4000 bp)
3'
Original pcslap sequence
pcslap short
First Vectorette screen
5':F1
3':F1
Second Vectorette screen
3':F2
Conventional PCR
3':F3
BLAST search
5':F2
0
1000
2000
3000
4000
bp

Figure 3.14

Sequence of the full-length coding sequence of *pcslap*

The identified sequence of *P. chabaudi slap* was shown to be 3897 nucleotides long, translating into a continuous ORF of 1298 amino acids without introns.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M R I Y N W V S C G F I F L Q F F L S A Y G K E W C K A K F E Y G M S D Y A E C
CAAAAAGAAGGAGATAATCTAACGAAATATATGATTGAAATAATTCCTGCTAAAGCAAATAATATAGATTTATATAGTGATGTTTCAATAGTTTTATCGAATTCTCAAGGTATTAATACT
Q K E G D N L T K Y M I E I I P A K A N N I D L Y S D V S I V L S N S Q G I N T
AAAGAAATAATTATTGGTAGTGAAAAAGAAGGACTATTTAGAAAAATATATTCGGTTAGAAAAGATATAGATAATCCAGAATATATTCATGTAAAATTAAATTCGAAAATAAATAATAAT
K E I I I G S E K E G L F R K I Y S V R K D I D N P E Y I H V K L N S K I N N N
AGAAATTGGAAATGTAAAAAAATAAAAGTTTGGAAAGAATATAAGTATTGGACTTTTGATTGTATAGGTATATTAAATGAAGAAAAACAAGAAGCAACATATTTCTTGTCTGGTAATAAA
R N W K C K K I K V W K E Y K Y W T F D C I G I L N E E K Q E A T Y F L S G N K
TTATATACTGCTTATGTTCAAACAGGAAAAGATATAGAAGCAGGTACTACTGGAATTATAGATATAATATTACTAGGTAATAACAACAAAAGAAGTAATACAAAAATGCTACATGAAGGA
L Y T A Y V Q T G K D I E A G T T G I I D I I L L G N N N K R S N T K M L H E G
TTTACATCTGGCGGATTAAAAAAAATAAAATTTCAAGCATCAGATGTAGGAAATTTAGAAAATATAATATTAATAAACAATTCTTATAATGATCCATGGTATTGTGATTTTGTTAAAATA
F T S G G L K K I K F Q A S D V G N L E N I I L I N N S Y N D P W Y C D F V K I
AAAGGTGATGATAACAAAGTTTATGTTTTCAATGTAAAAAGTTGGATTGGTTATCCATATAATAATAAAATAAAAATAAATATAAATACAAATAATATTGATGGAAATGCAAAAGACATA
K G D D N K V Y V F N V K S W I G Y P Y N N K I K I N I N T N N I D G N A K D I
GATTGTCATATACGTGCTAATGATTTAATAGATACAACTAAATCATTAAATAATAGTTTTGTTTTACAAAATAAGGTACATATATTTAAAGTTCGATGCCCACAAAACTGTCATAGTTCC
D C H I R A N D L I D T T K S L N N S F V L Q N K V H I F K V R C P Q N C H S S
GATTTTTCTATCATAGAAGGTACTTCTATACATCCTGCATCTACCTCTATATGTGCTGCAGCTATTTATGATGGTTCATTAACAGAAAGTGGAGGCGAAATTATTGTTACTATAACAAAA
D F S I I E G T S I H P A S T S I C A A A I Y D G S L T E S G G E I I V T I T K
GGATTAAATTATTATTATGCAATGGATGGCACATATAATAATTTAAAAGCAATTGAATTTTCTACCAAAGGTGATGAAAGTGATGAAAATAATTTCTCTTTTTATACATATCATTTGACA
G L N Y Y Y A M D G T Y N N L K A I E F S T K G D E S D E N N F S F Y T Y H L T
TCTATTGATGATATAAAAAGTAATGTTAGAATCGTTGATTCTTTTGGAAAATTATCTTCTTTAGGAAGATTAGAAATTCGTGTAAATAATAAATGGGGAGCTGTTTGTAAAAAAGGTCCA
S I D D I K S N V R I V D S F G K L S S L G R L E I R V N N K W G A V C K K G P
AACTTTGAATTTTCAGAAGAGGCAGCAAAAAGAGCTTGTAAAGATTTAGGTTTTCCAAATGGTATATATATTAGAGATACTTGCTCAAATATCAATGAACAAAATTATTGTGCTGGGTAT
N F E F S E E A A K R A C K D L G F P N G I Y I R D T C S N I N E Q N Y C A G Y

AAATATCCATTTAATGCTTCTGGTATTTTATGTTCTGGAAATGAACAGAATTTATTATCCTGTAATACTGACGATCCTTCATATTGTATAGATCATCATGATGATGTTATAATACAATGT

K Y P F N A S G I L C S G N E Q N L L S C N T D D P S Y C I D H H D D V I I Q C

GTTAATCAATTAGGTAACGATTCGATAGAAAACGGAACCATTCGACTTTTAGACAGCACTGGTGCACCTACATCTAATGGAATAGGAAGATTACAAATATATTACAATGGGGTATTCGGT

V N Q L G N D S I E N G T I R L L D S T G A P T S N G I G R L Q I Y Y N G V F G

TCTGTTTGTTCAGAAGGATGGACAAAAGAAACAGAAAAAATTGCATGTTTAGAATTAGGTTATCATAATGTAAAAGCAAATGGATTTTCTAAACAACTATGTAGTGATATAGCAGGTGAA

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

AATTTATGTGGTCATGATACAGAAAGAATTAATGCAACGAATTTTAGATGTAAAGGAGATGAAGCTAATTTAAAAAATTGCCCACATGAAACATCTGAAGATATTTACTGTTCTCACGAA

N L C G H D T E R I N A T N F R C K G D E A N L K N C P H E T S E D I Y C S H E

GAAGATATAATTATTGGTTGTGCAAATGCAGAAGAAGATGGAGATGCTTCGACCAGTTCAACTAGCATTTCTAAATATGGATTAAACAAACATATAATGTCAATGGAAAAAAAACAATTT

E D I I I G C A N A E E D G D A S T S S T S I S K Y G L N K H I M S M E K K Q F

CATCCCAAAATCGAATTAAGTTGCTTTGACAAAATAAGTTCTAAAGCTGAATTGAGTAAAGGAAATGTTGGAGATATATTTTTAGTCAGCTGCCCAGAAAAATGTGATGAAGATAGTGGT

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

GTAATAAAAGGAACTTTTGTATACACATTCGATTCTTATATATGTAAAGCAGCTATACATGCAGGTGTTCTATCGAGTAATGTTGCTGATGATGTAGTTCTTATCATTACCCATAGTAGA

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

AATAAATTTATAGGAACCAAAAGAAATAATGTAGAATCAAAAGAATTTAATGGTGAATCGAAAAGTTTTAGCTTAAGTATACCAACAAATTATATCATAATGGAAGAAAGACAAAATAAT

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

TCAAAATATGAAGATGAAATTTTAAAAGATGATAATGATTTTTATTATGAACATATATTTAATAAACAAAATAATAATGAAAAAGGAAAAAATAAAACGTTTTTTGAACATAATATGCAT

S K Y E D E I L K D D N D F Y Y E H I F N K Q N N N E K G K N K T F F E H N M H

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L E P T F Q W I A P S S F T G F N G N E N E Y I N A S N L P N E K Y I R T L S N

TTTACTTTTATTACACACTTCATTCCAAGTAGTAAAGGGAAAAATACATGGAGAACAATATTATCTCATAGTTTATGTGAAGGTATATCTATATCTATTGATGAAGAAAATGAATTAGTA

F T F I T H F I P S S K G K N T W R T I L S H S L C E G I S I S I D E E N E L V

ATAGAACAAAATTGTAACCCTCATTTAATAAAAACTAAATTTATCCCAAAATTTGAACACCCTTATCATCTGGTTCTAATTTATAACAAGCCAAATAAATCAATTTCTTTATATATTAAT

I E Q N C N P H L I K T K F I P K F E H P Y H L V L I Y N K P N K S I S L Y I N

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Q K K I N L E N M K I D F T L N G D L T I G R S N K Q A T D Y F I G D I N F V K

ATTTATAAATATATATTAACAGAACAAGAAATTAAAGAGTCCTTTGATTCAGTTCTTTCAAATAATTATTTAAATGATGGGATGTCTGGAAATGGAAACATAAATAGTAGAAAAATACAA

I Y K Y I L T E Q E I K E S F D S V L S N N Y L N D G M S G N G N I N S R K I Q

AATAAAAAAACAAAAAATCAACGAAAAACTATAGATGGACGTGATTGTATTAGTTCATGTAAATCCAAAACGAATGTAAATAAAAATATACAAATTAATACGGAAGAATTTTACCTGAAC

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

TGTTCAGATAATTTACTCAGTGAGCGATTTAGTGGAAAAATTGGAACTCAGTTTTTGGCTAGCTGTTCAGAAGATTGTGCTAGCTCAAAATATATAGTAAAAGGGTCTAACAATTACTAC

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

ACACCAGATACGTCAATATGTAAAGCAGTTATGCATTCAGGAATAATGCGTAAAAATAATGAGCATGATAAGGATGATAACGAAAATAATAAAAATTCGTTTATTATAAAAATTGTTGAA

T P D T S I C K A V M H S G I M R K N N E H D K D D N E N N K N S F I I K I V E

GGCTTAACAGAATATAAATCATCTAGAGGTCATTTTGGAATCATTTCAAAAGCAGAAAAACAATCACAATTAAGATCCTTTTCTCTTTTTTCAAAAAATGAAGATGATATTTTTACATGT

G L T E Y K S S R G H F G I I S K A E K Q S Q L R S F S L F S K N E D D I F T C

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F T D G S F L F E L P I G S T K N I I C P E N C H K I D K Q I Y G T N T Y S P L

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S S V C K A A I H A G V I S I K G G Q I Q V V V G K G Q Q E F K P S T Q N N V Q

TCATATATAGCAGAAAAACAAGATCGTTCATTCACCTTTTTCAAGCGCTTATATTAG

S Y I A E K Q D R S F T F F K R L Y .

Figure 3.15

Restriction map of *pcslap*

Sites of cleavage by each of the four restriction enzymes RsaI, Sau3AI, SspI and XhoII are shown as black lines across grey bars representing the *pcslap* gene. The numbers above the restriction sites denote the exact position of cleavage in the nucleotide sequence of *pcslap*. The positions of $pcslap_{270}$ and $pcslap_{2130}$, used as probes in Southern analysis, within *pcslap* are shown in red and orange, respectively.

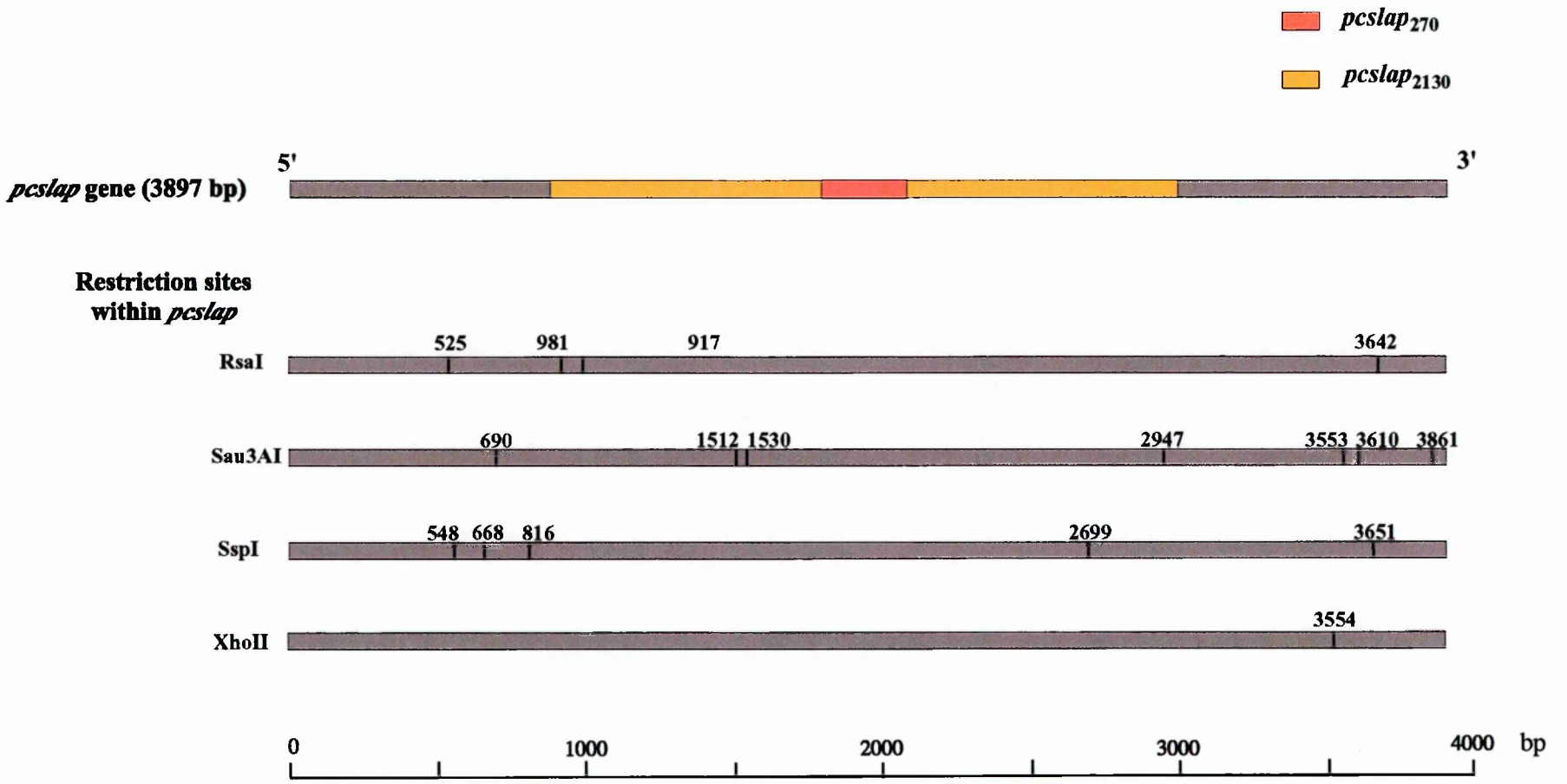

pcslap270
pcslap2130
5'
3'
pcslap gene (3897 bp)
Restriction sites within pcslap
RsaI
525
981
917
3642
Sau3AI
690
1512
1530
2947
3553
3610
3861
SspI
548
668
816
2699
3651
XhoII
3554
0
1000
2000
3000
4000
bp

Figure 3.16

Southern analysis of *P. chabaudi* genomic DNA using $pcslap_{270}$ as a probe

P. chabaudi genomic DNA was isolated and aliquots digested with RsaI (lanes denoted 1), Sau3AI (lanes denoted 2), SspI (lanes denoted 3) and XhoII (lanes denoted 4). Expected bands are listed in Table 3.2.

(A) The digested DNA was resolved on a 1% agarose gel along with λ/HindIII marker (M) and transferred to a nylon membrane.

(B) Hybridisation was performed over night with [α-32P]-dATP-labelled $pcslap_{270}$, at 65°C. A single band can be seen in each restriction digest.

(C) Hybridisation was performed over night with [α-32P]-dATP-labelled $pcslap_{270}$, at 55°C. Two bands appear in each restriction digest.

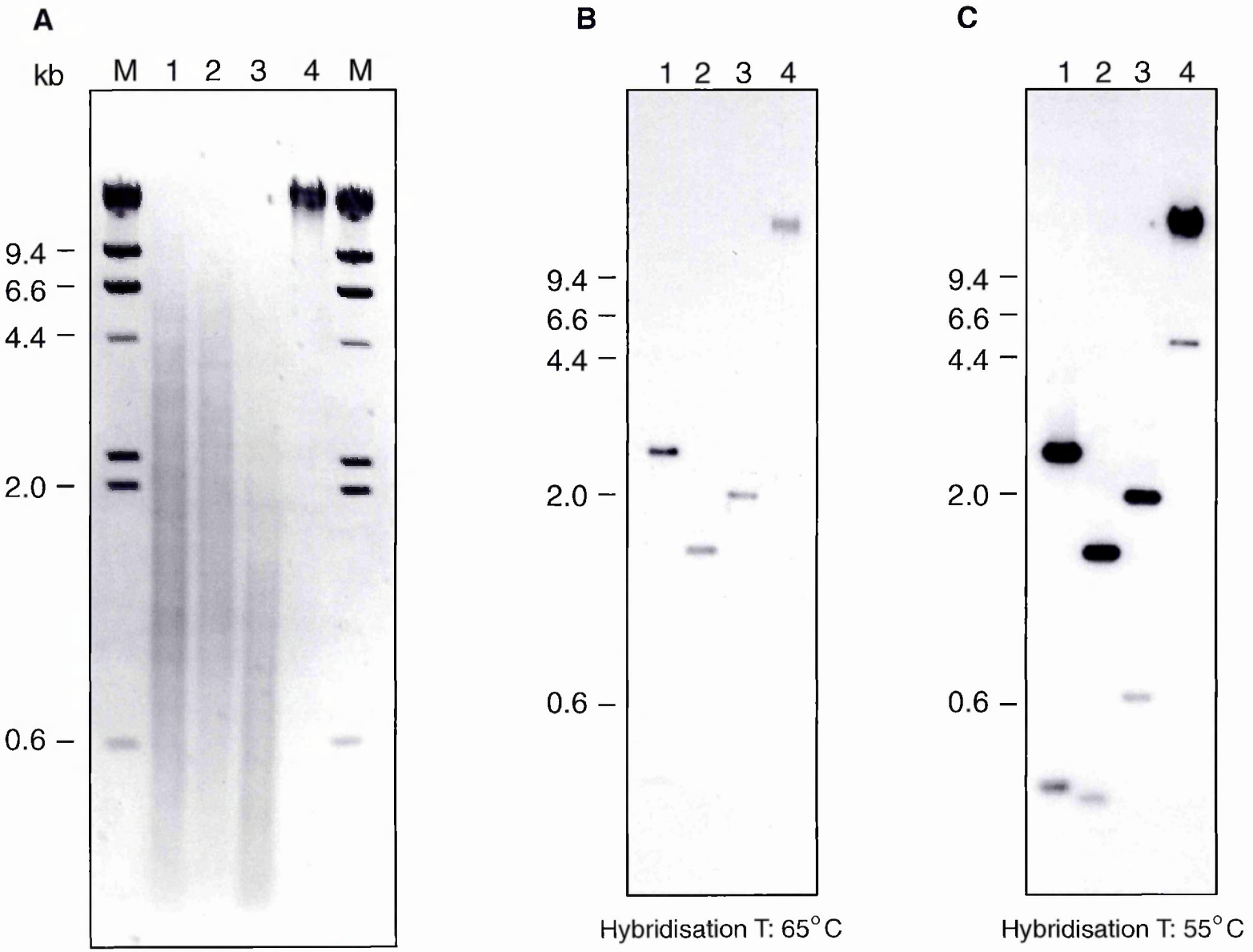

A
kb
M 1 2 3 4 M
9.4
6.6
4.4
2.0
0.6
Ethidium bromide - stained gel
B
1 2 3 4
9.4
6.6
4.4
2.0
0.6
Hybridisation T: 65°C
C
1 2 3 4
9.4
6.6
4.4
2.0
0.6
Hybridisation T: 55°C

Figure 3.17

Southern analysis of *P. chabaudi* genomic DNA using $pcslap_{2130}$ as a probe

P. chabaudi gDNA, digested with RsaI (lane 1), Sau3AI (lane 2), SspI (lane 3) and XhoII (lane 4) was blotted as in Figure 3.19, and probed over night with [α-32P]-dATP-labelled $pcslap_{2130}$ at 55°C. As the restriction enzymes used to digest the DNA cut within the probe sequence, several bands are expected in each digest (Table 3.3).

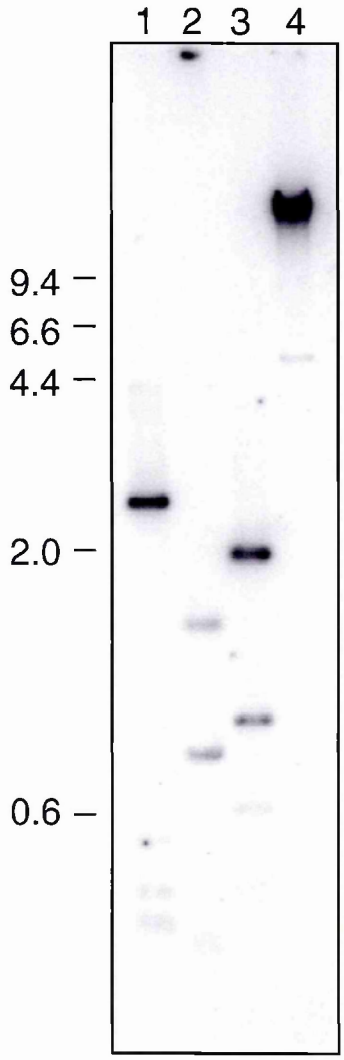

1 2 3 4
9.4
6.6
4.4
2.0
0.6

Figure 3.18

Alignment of *P. chabaudi pcslap*$_{270}$ and the homologous sequences in *P. berghei* and *P. yoelii*

Alignments of *pcslap*$_{270}$ from *P. chabaudi* with the homologous sequences in *P. berghei* and *P. yoelii*. The comparison was carried out in order to ensure that there was sufficient identity between the sequences for Southern analysis hybridisation of *pcslap*$_{270}$ to *P. berghei* and *P. yoelii* genomic DNA.

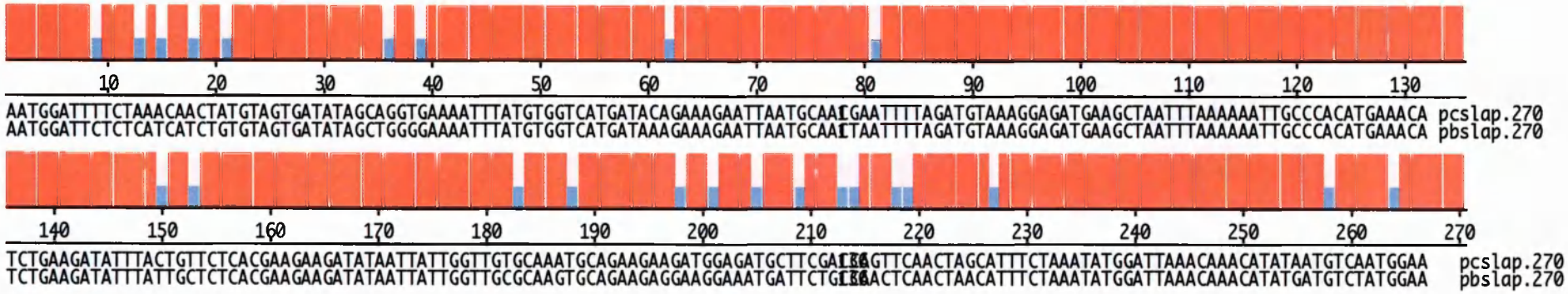
(A) Alignment of *pcslap*$_{270}$ nucleotide sequence in *P. chabaudi* with the homologous sequence in *P. berghei* ANKA. The overall identity was 91%.

(B) Alignment of *pcslap*$_{270}$ nucleotide sequence in *P. chabaudi* with the homologous sequence in *P. yoelii*. The overall identity was 90%.

Red colour denotes identity between the residues in all sequences, green colour denotes residue dissimilarity in one sequence and blue colour denotes dissimilarity between residues in all three sequences.

The alignment was generated with the MegAlign sequence alignment program using the ClustalW algorithm.

A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AATGGATTCTCTCATCATCTGTGTAGTGATATAGCTGGGGAAAATTTATGTGGTCATGATAAAGAAAGAATTAATGCAACTAATTTTAGATGTAAAGGAGATGAAGCTAATTTAAAAAATTGCCCACATGAAACA pbslap.270
140 150 160 170 180 190 200 210 220 230 240 250 260 270
TCTGAAGATATTTACTGTTCTCACGAAGAAGATATAATTATTGGTTGTGCAAATGCAGAAGAAGATGGAGATGCTTCGAGTTCAACTAGCATTTCTAAATATGGATTAAACAAACATATAATGTCAATGGAA pcslap.270
TCTGAAGATATTTATTGCTCTCACGAAGAAGATATAATTATTGGTTGCGCAAGTGCAGAAGAGGAAGGAAATGATTCTGGAACTCAACTAACATTTCTAAATATGGATTAAACAAACATATGATGTCTATGGAA pbslap.270

B

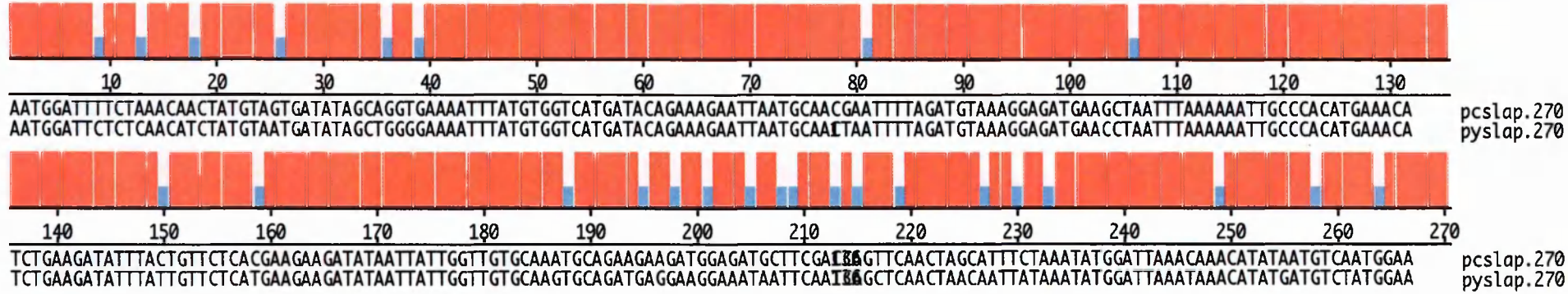

10 20 30 40 50 60 70 80 90 100 110 120 130
1 AATGGATTTTCTAAACAACTATGTAGTGATATAGCAGGTGAAAATTTATGTGGTCATGATACAGAAAGAATTAATGCAACGAATTTTAGATGTAAAGGAGATGAAGCTAATTTAAAAAATTGCCCACATGAAACA pcslap.270
AATGGATTCTCTCAACATCTATGTAATGATATAGCTGGGGAAAATTTATGTGGTCATGATACAGAAAGAATTAATGCAACTAATTTTAGATGTAAAGGAGATGAACCTAATTTAAAAAATTGCCCACATGAAACA pyslap.270
140 150 160 170 180 190 200 210 220 230 240 250 260 270
TCTGAAGATATTTACTGTTCTCACGAAGAAGATATAATTATTGGTTGTGCAAATGCAGAAGAAGATGGAGATGCTTCGAGTTCAACTAGCATTTCTAAATATGGATTAAACAAACATATAATGTCAATGGAA pcslap.270
TCTGAAGATATTTATTGTTCTCATGAAGAAGATATAATTATTGGTTGTGCAAGTGCAGATGAGGAAGGAAATAATTCAATGGCTCAACTAACAATTATAAATATGGATTAAATAAACATATGATGTCTATGGAA pyslap.270

Figure 3.19

Southern analysis of *P. yoelii* and *P. berghei slap* using $pcslap_{270}$ as a probe

Genomic DNA from *P. chabaudi, P. yoelii* and *P. berghei* was digested with Sau3AI and SspI, resolved on a 1% agarose gel and probed with [α-32P]-dATP-labelled $pcslap_{270}$ at 55°C. Expected bands are listed in Table 3.4

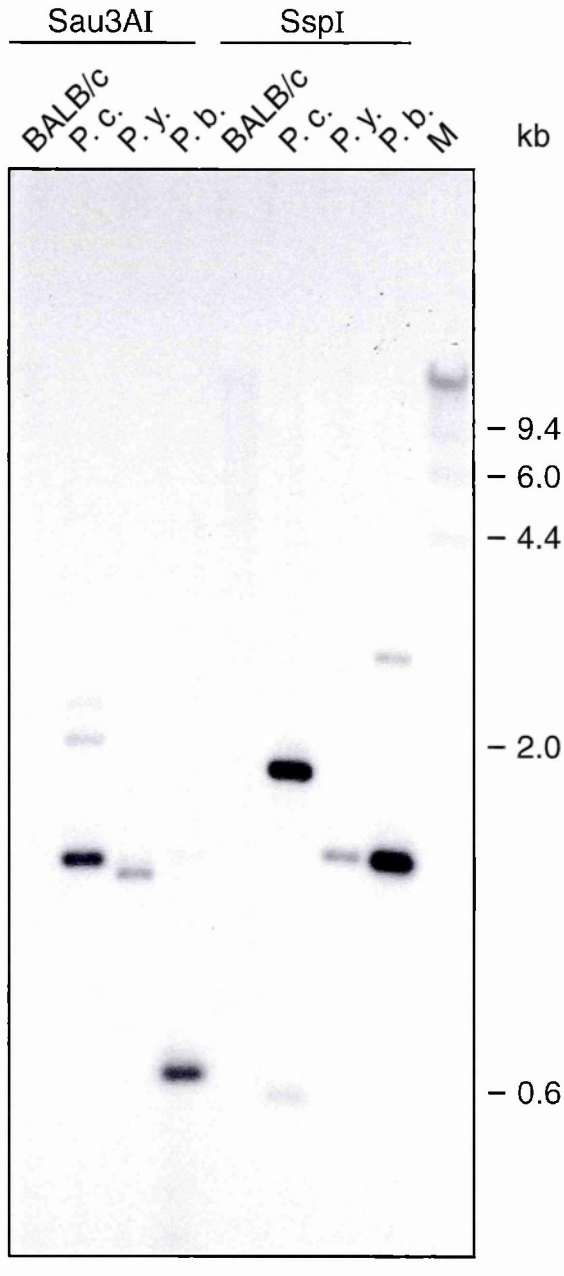

Sau3AI
SspI
BALB/c
P. c.
P. y.
P. b.
BALB/c
P. c.
P. y.
P. b.
M
kb
9.4
6.0
4.4
2.0
0.6

Figure 3.20

***Pc*SLAP secondary structure**

Analysis of the *Pc*SLAP protein showed three types of sequence motifs: two Scavenger Receptor Cysteine-Rich (SRCR) domains (green), three *Limulus* factor C, Coch-5b2 and Lgl1 (LCCL) modules (blue) and one Lipoxygenase Homology 2/Polycystin-1, Lipoxygenase and α-Toxin (LH2/PLAT) domain (red). A signal sequence (dark red) is located at the N-terminal of the protein.

MRIYNWVSCGFIFLQFFLSAYGKEWCKAKFEYGMSDYAECQKEGDNLTKYMIEIIPAKANNIDLYSDVSIVLSNSQGINT
KEIIIGSEKEGLFRKIYSVRKDIDNPEYIHVKLNSKINNNRNWKCKKIKVWKEYKYWTFDCIGILNEEKQEATYFLSGNK 160
LYTAYVQTGKDIEAGTTGIIDIILLGNNNKRSNTKMLHEGFTSGGLKKIKFQASDVGNLENIILINNSYNDPWYCDFVKI
KGDDNKVYVFNVKSWIGYPYNNKIKININTNNIDGNAKDIDCHIRANDLIDTTKSLNNSFVLQNKVHIFKVRCPQNCHSS 320
DFSIIEGTSIHPASTSICAAAIYDGSLTESGGEIIVTITKGLNYYYAMDGTYNNLKAIEFSTKGDESDENNFSFYTYHLT
SIDDIKSNVRIVDSFGKLSSLGRLEIRVNNKWGAVCKKGPNFEFSEEAAKRACKDLGFPNGIYIRDTCSNINEQNYCAGY 480
KYPFNASGILCSGNEQNLLSCNTDDPSYCIDHHDDVIIQCVNQLGNDSIENGTIRLLDSTGAPTSNGIGRLQIYYNGVFG
SVCSEGWTKETEKIACLELGYHNVKANGFSKQLCSDIAGENLCGHDTERINATNFRCKGDEANLKNCPHETSEDIYCSHE 640
EDIIIGCANAEEDGDASTSSTSISKYGLNKHIMSMEKKQFHPKIELSCFDKISSKAELSKGNVGDIFLVSCPEKCDEDSG
VIKGTFVYTFDSYICKAAIHAGVLSSNVADDVVLIITHSRNKFIGTKRNNVESKEFNGESKSFSLSIPTNYIIMEERQNN 800
SKYEDEILKDDNDFYYEHIFNKQNNNEKGKNKTFFEHNMHLEPTFQWIAPSSFTGFNGNENEYINASNLPNEKYIRTLSN
FTFITHFIPSSKGKNTWRTILSHSLCEGISISIDEENELVIEQNCNPHLIKTKFIPKFEHPYHLVLIYNKPNKSISLYIN 960
QKKINLENMKIDFTLNGDLTIGRSNKQATDYFIGDINFVKIYKYILTEQEIKESFDSVLSNNYLNDGMSGNGNINSRKIQ
NKKTKNQRKTIDGRDCISSCKSKTNVNKNIQINTEEFYLNCSDNLLSERFSGKIGTQFLASCSEDCASSKYIVKGSNNYY 1120
TPDTSICKAVMHSGIMRKNNEHDKDDNENNKNSFIIKIVEGLTEYKSSRGHFGIISKAEKQSQLRSFSLFSKNEDDIFTC
FTDGSFLFELPIGSTKNIICPENCHKIDKQIYGTNTYSPLSSVCKAAIHAGVISIKGGQIQVVVGKGQQEFKPSTQNNVQ 1280
SYIAEKQDRSFTFFKRLY.

1298 residues

Figure 3.21

Multiple alignment of Scavenger Receptor Cystein-Rich (SRCR) domain sequences

*Pc*SLAP SRCR-1 and SRCR-2 were aligned with SRCR domains from other proteins from the SRCR superfamily. Similar residues are aligned and shown in the same colour. Gaps in the sequences signify positions that represent deletions within one sequence relative to another. The stars mark the six conserved cysteine residues shared by all members of the family.

The alignment was generated using the SMART database and Hmmalign with the ClustalW algorithm.

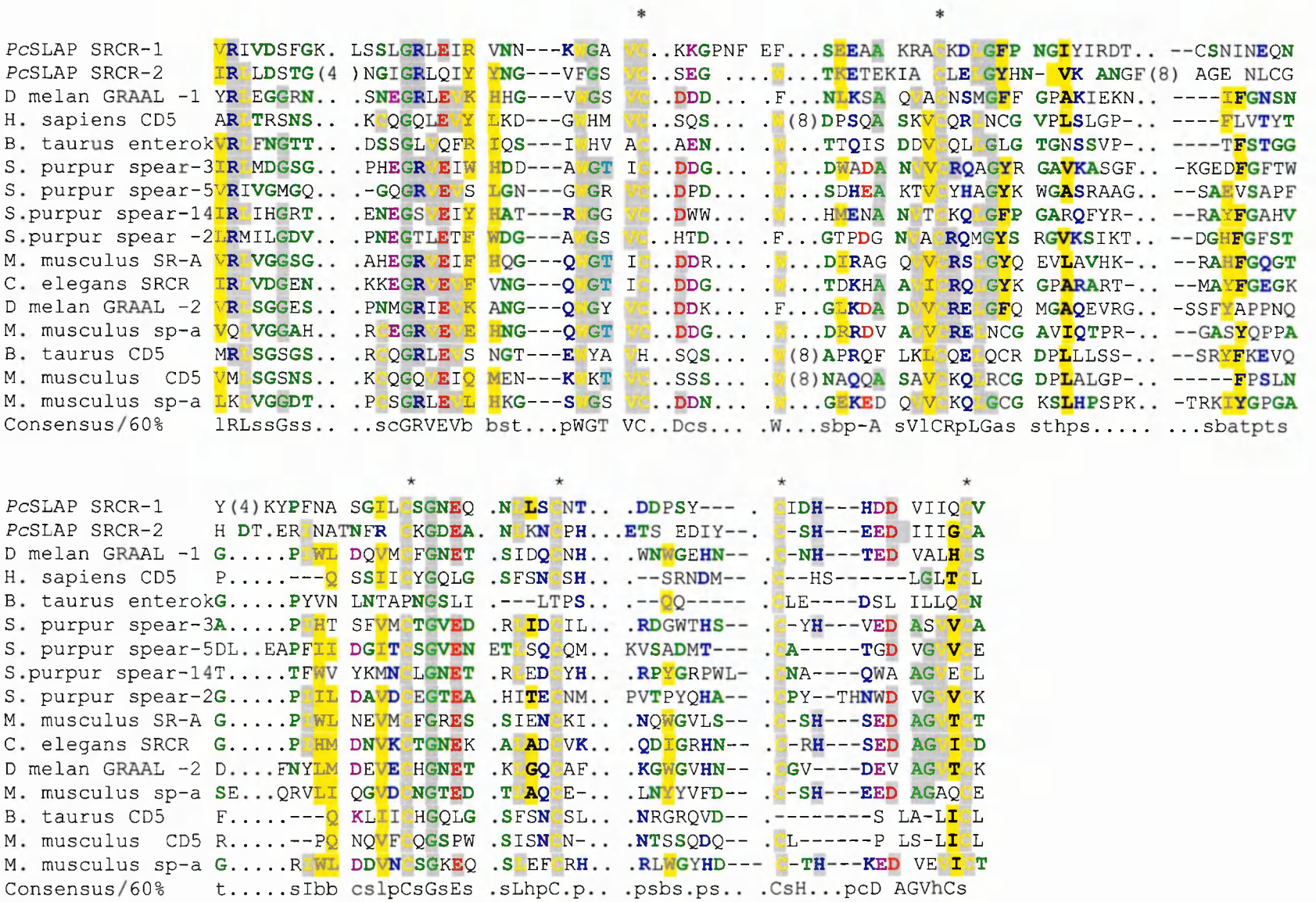
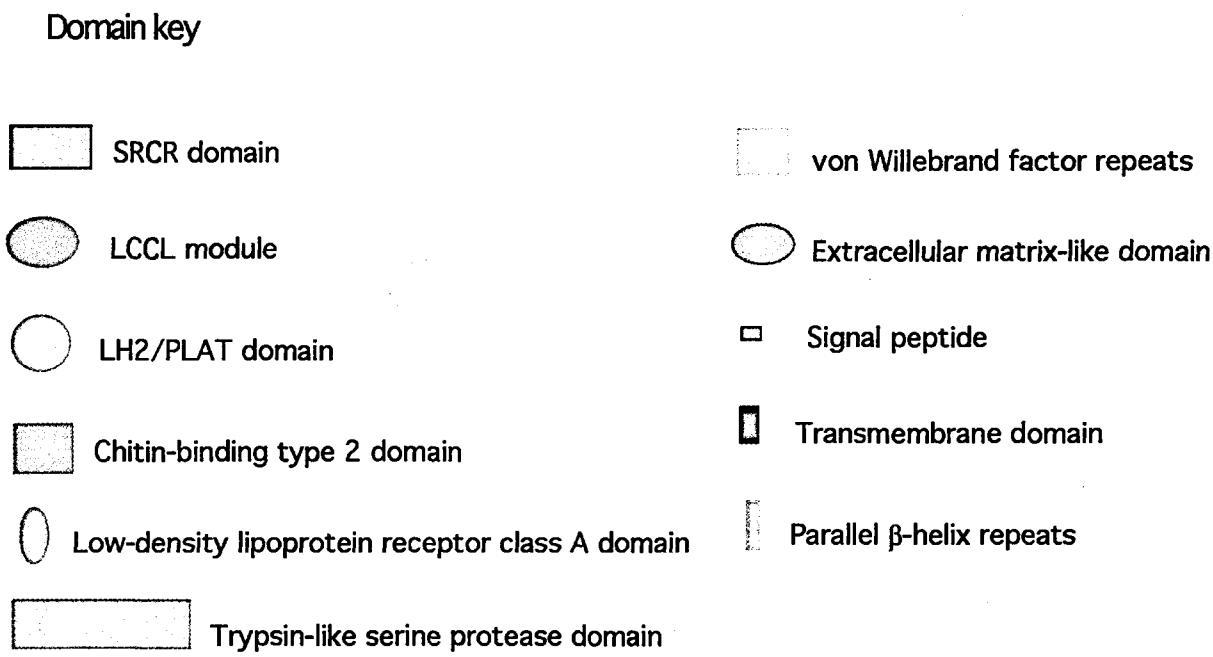


Fig 3.22

Proteins with Scavenger Receptor Cysteine-Rich domains



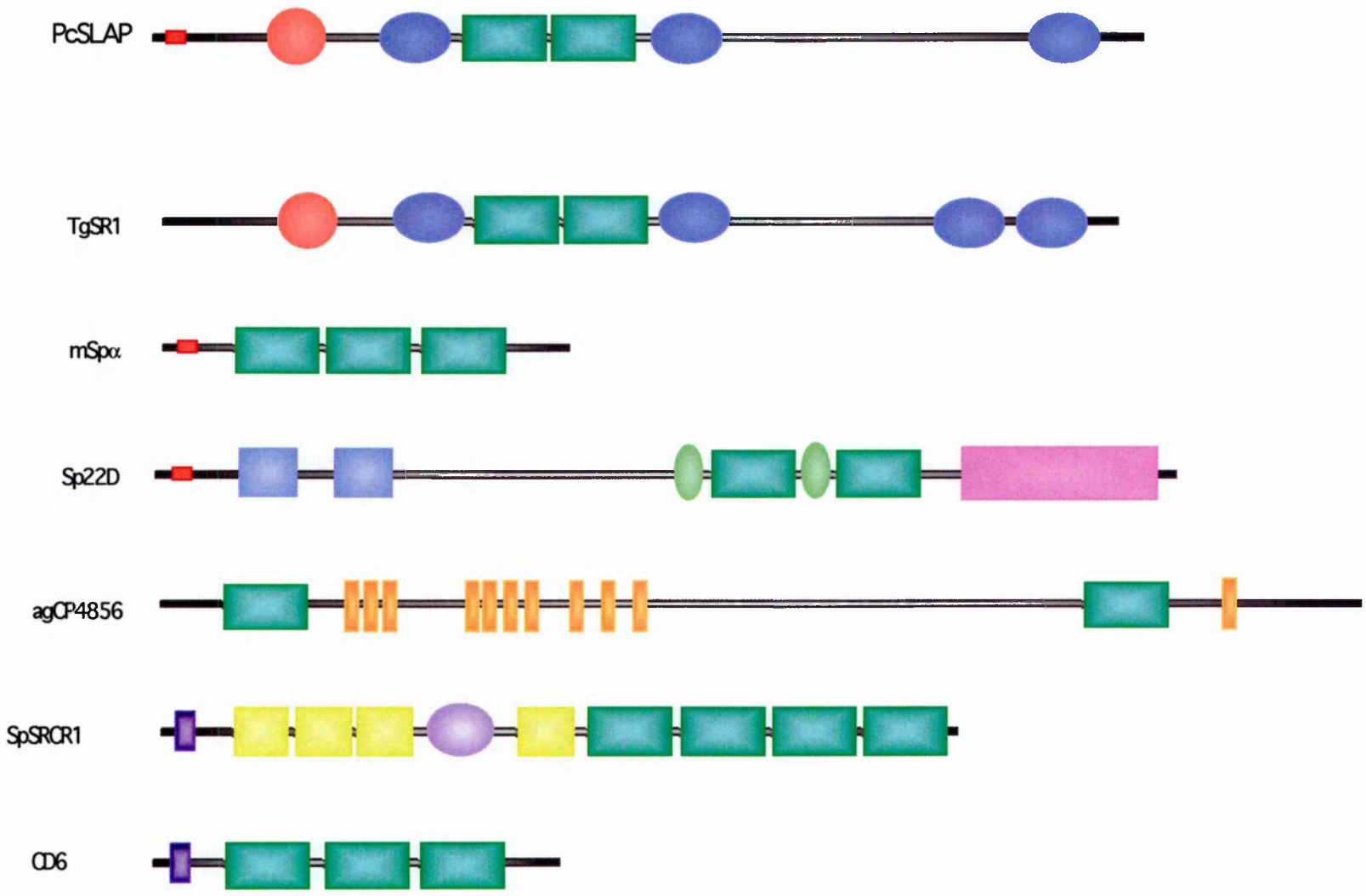
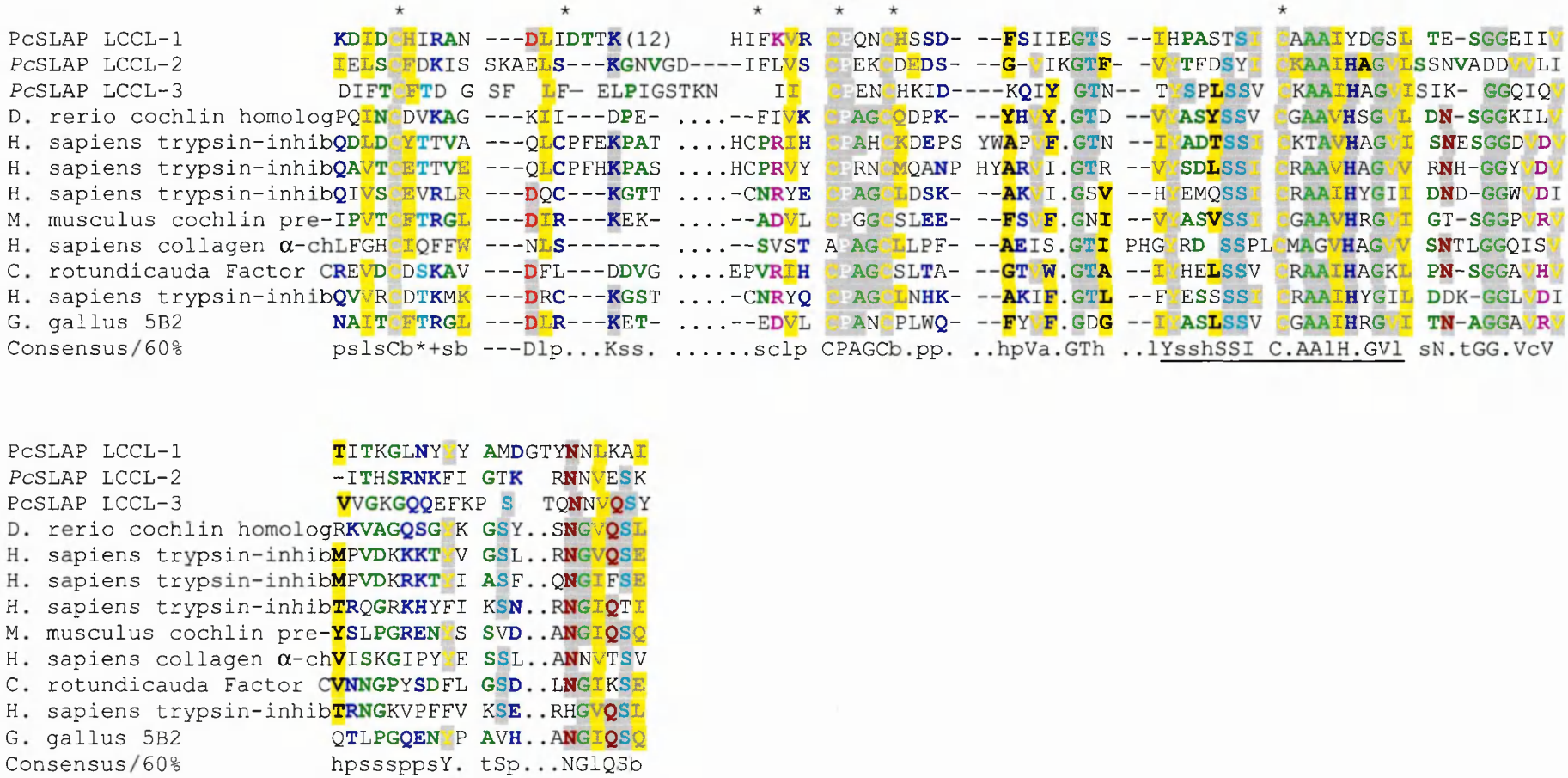

PcSLAP
TgSR1
mSpα
Sp22D
agCP4856
SpSRCR1
CD6

Figure 3.23

Multiple alignment of LCCL domain sequences

*Pc*SLAP LCCL-1, LCCL-2 and LCCL-3 were aligned with other LCCL-containing proteins from the SMART sequence database. LCCL domains contain up to eight cysteine residues (potential positions marked by stars), four of which are highly conserved. A seventeen residue conserved motif is located near the C-terminal end (underlined in the consensus sequence) and is believed to be the core of the module.

The alignment was generated using the SMART database and Hmmalign with the ClustalW algorithm.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PcSLAP LCCL-2
PcSLAP LCCL-3
D. rerio cochlin homolog
H. sapiens trypsin-inhib
H. sapiens trypsin-inhib
H. sapiens trypsin-inhib
H. sapiens trypsin-inhib
M. musculus cochlin pre-
H. sapiens collagen α-ch
C. rotundicauda Factor C
H. sapiens trypsin-inhib
G. gallus 5B2
Consensus/60%
KDIDCHIRAN ---DLIDTTK(12) HIFKVR CPQNCHSSD- --FSIIEGTS --IHPASTSI CAAAIYDGSL TE-SGGEIIV
IELSCFDKIS SKAELS---KGNVGD----IFLVS CPEKCDEDS- --G-VIKGTF- -VYTFDSYI CKAAIHAGVLSSNVADDVVLI
DIFTCFTD G SF LF- ELPIGSTKN II CPENCHKID----KQIY GTN-- TVSPLSSV CKAAIHAGVISIK- GGQIQV
PQINCDVKAG ---KII---DPE- ....--FIVK CPAGCQDPK- --YHVY.GTD --VYASYSSV CGAAVHSGVL DN-SGGKILV
QDLDCYTTVA ---QLCPFEKPAT ....HCPRIH CPAHCKDEPS YWAPVF.GTN --IYADTSSI CKTAVHAGVI SNESGGDVDV
QAVTCETTVE ---QLCPFHKPAS ....HCPRVY CPRNCMQANP HYARVI.GTR --VYSDLSSI CRAAVHAGVV RNH-GGYVDV
QIVSCEVRLR ---DQC---KGTT ....-CNRYE CPAGCLDSK- --AKVI.GSV --HYEMQSSI CRAAIHYGII DND-GGWVDI
IPVTCFTRGL ---DIR---KEK- ....--ADVL CPGGCSLEE- --FSVF.GNI --VYASVSSI CGAAVHRGVI GT-SGGPVRV
LFGHCIQFFW ---NLS------- ....--SVST APAGCLLPF- --AEIS.GTI PHGVRD SSPLCMAGVHAGVV SNTLGGQISV
CREVDCDSKAV ---DFL---DDVG ....EPVRIH CPAGCSLTA- --GTVW.GTA --IVHELSSV CRAAIHAGKL PN-SGGAVHV
QVVRCDTKMK ---DRC---KGST ....-CNRYQ CPAGCLNHK- --AKIF.GTL --FYESSSSI CRAAIHYGIL DDK-GGLVDI
NAITCFTRGL ---DLR---KET- ....--EDVL CPANCPLWQ- --FYVF.GDG --IYASLSSV CGAAIHRGVI TN-AGGAVRV
pslsCb*+sb ---Dlp...Kss. ......sclp CPAGCb.pp. ..hpVa.GTh ..lYsshSSI C.AAlH.GVl sN.tGG.VcV
TITKGLNYYY AMDGTYNNLKAI
-ITHSRNKFI GTK RNNVESK
VVGKGQQEFKP S TQNNVQSY
RKVAGQSGYK GSY..SNGVQSL
MPVDKKKTYV GSL..RNGVQSE
MPVDKRKTYI ASF..QNGIFSE
TRQGRKHYFI KSN..RNGIQTI
YSLPGRENYS SVD..ANGIQSQ
VISKGIPYYE SSL..ANNVTSV
CVNNGPYSDFL GSD..LNGIKSE
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QTLPGQENYP AVH..ANGIQSQ
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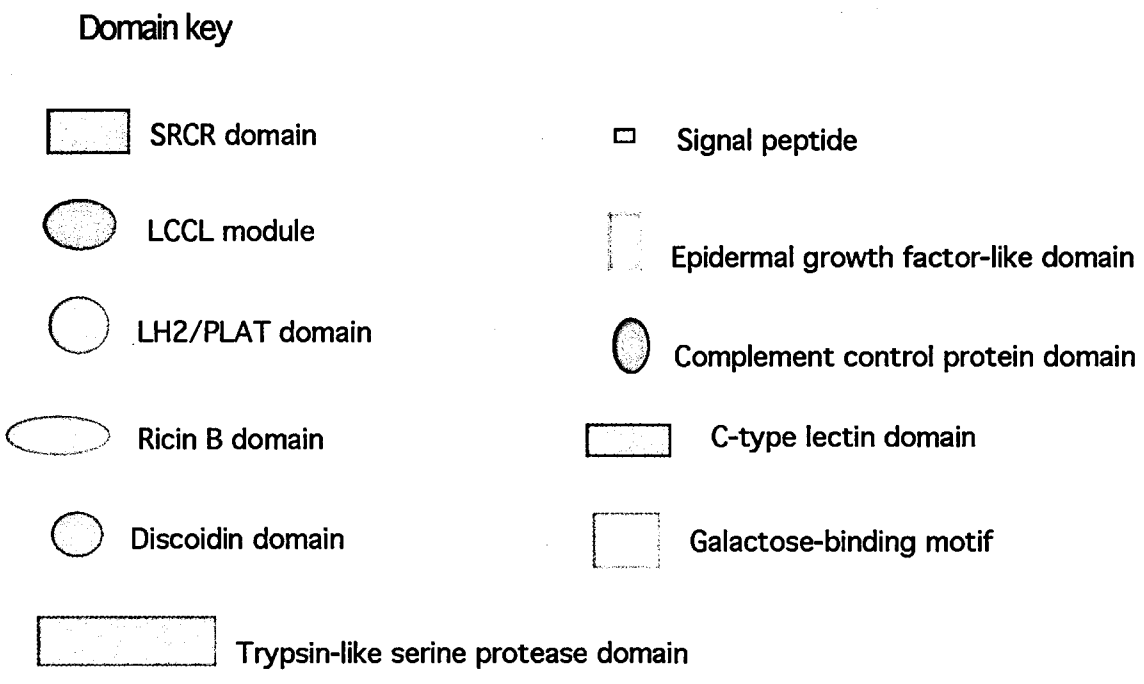
Fig 3.24

Proteins with *Limulus* factor C, Coch-5b2 and Lgl1 (LCCL) domains



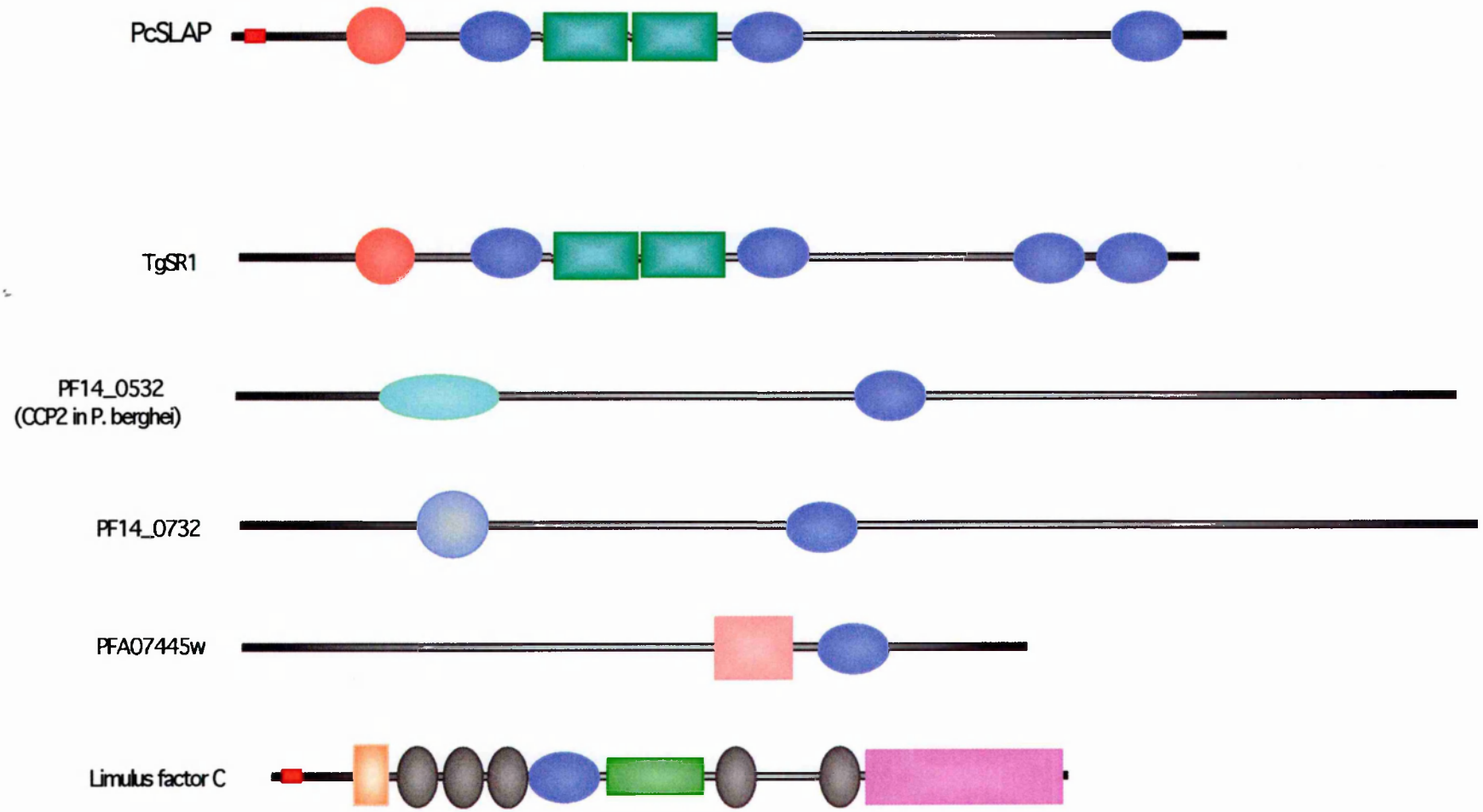

PcSLAP
TgSR1
PF14_0532
(CCP2 in P. berghei)
PF14_0732
PFA07445w
Limulus factor C

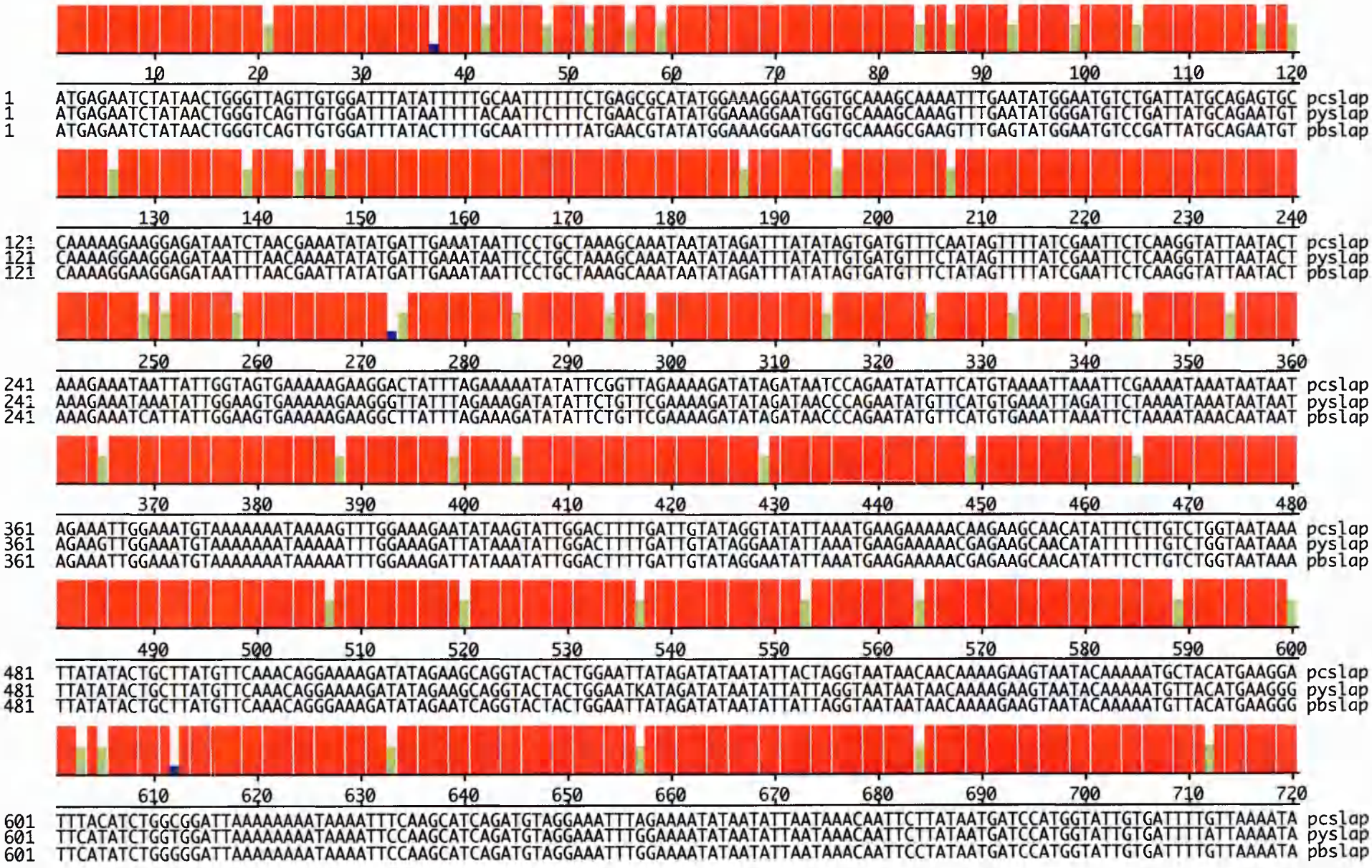
Figure 3.25

Multiple sequence alignment of the nucleotide sequence of the *slap* gene in *P. chabaudi, P. yoelii* and *P. berghei*

Alignment of the open reading frames of *P. chabaudi* (3897 nucleotides), *P. yoelii* (3900 nucleotides), and *P. berghei* (3915 nucleotides).

Red colour denotes identity between nucleotides of all sequences, green colour denotes nucleotide dissimilarity in one sequence and blue colour denotes nucleotide dissimilarities between all three sequences.

The alignment was generated with the MegAlign sequence alignment program using the ClustalW algorithm.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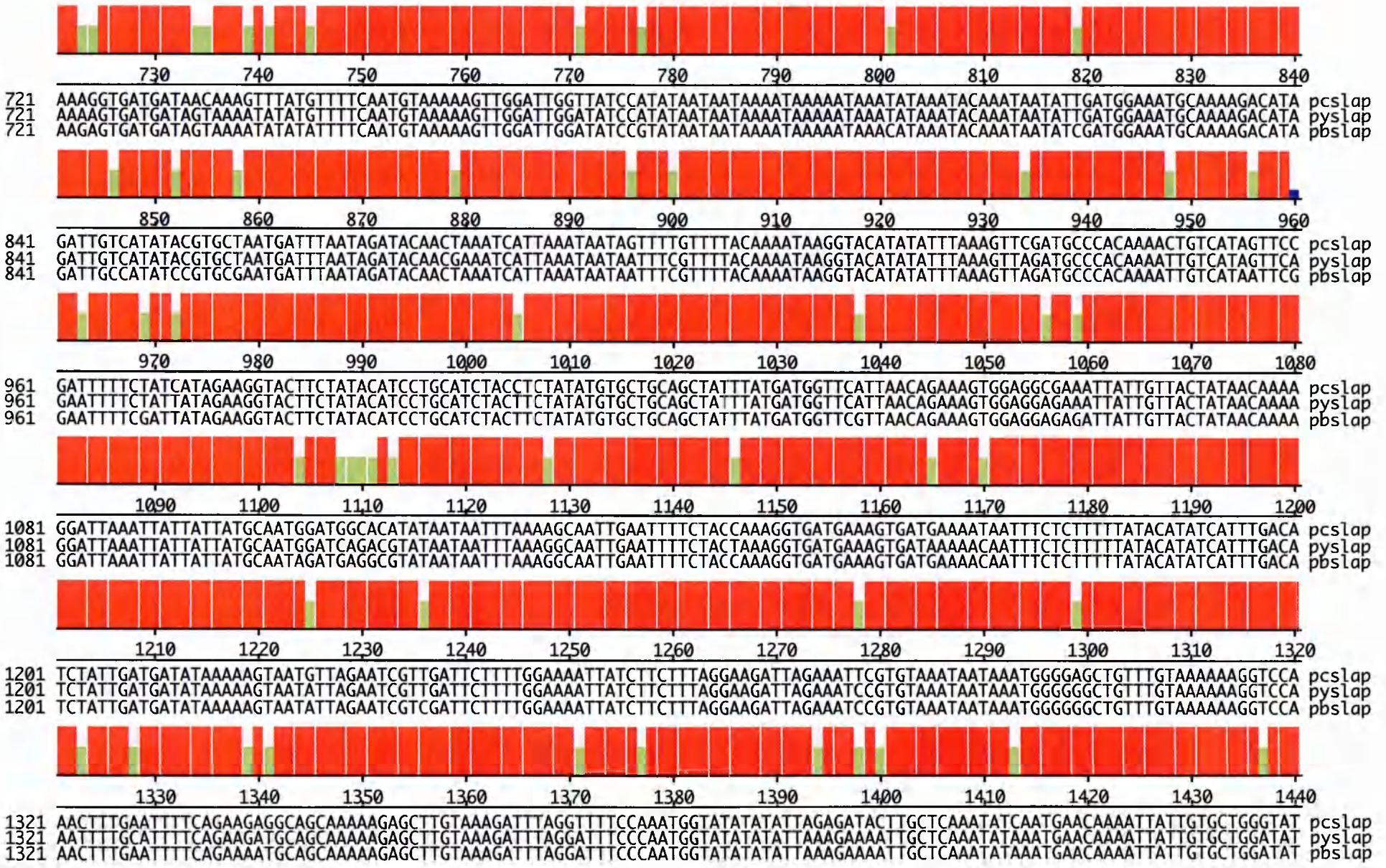
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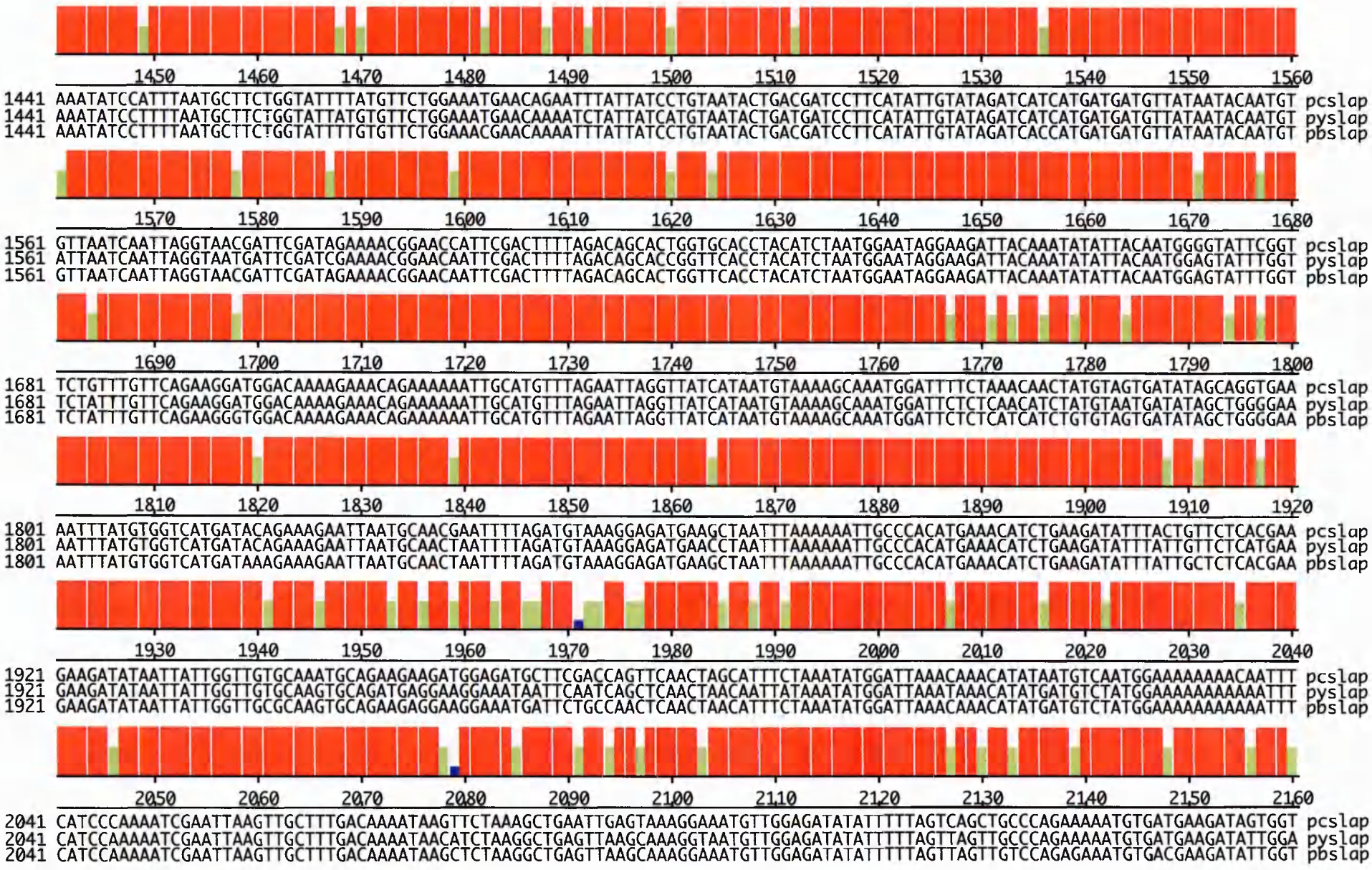
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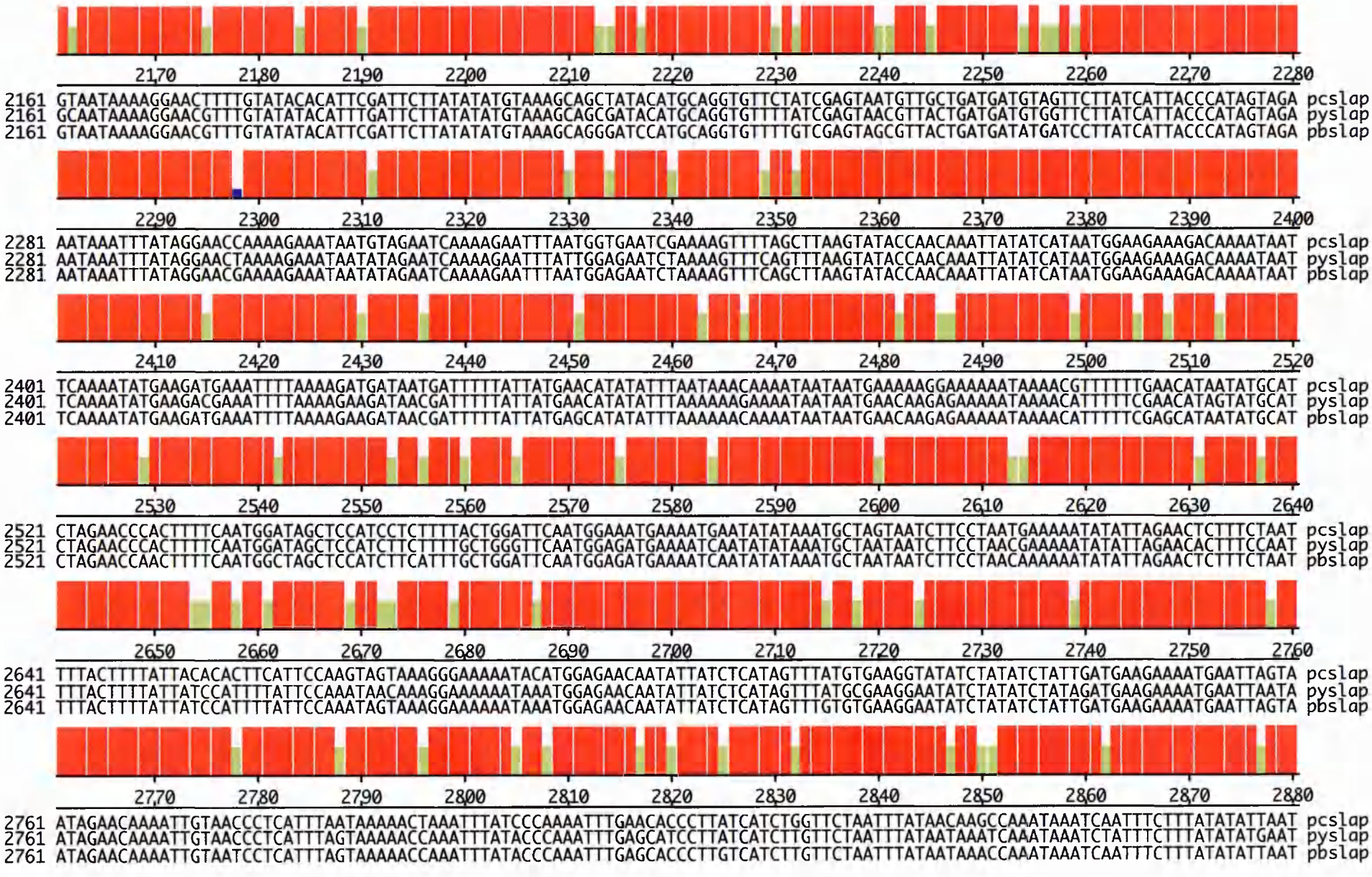
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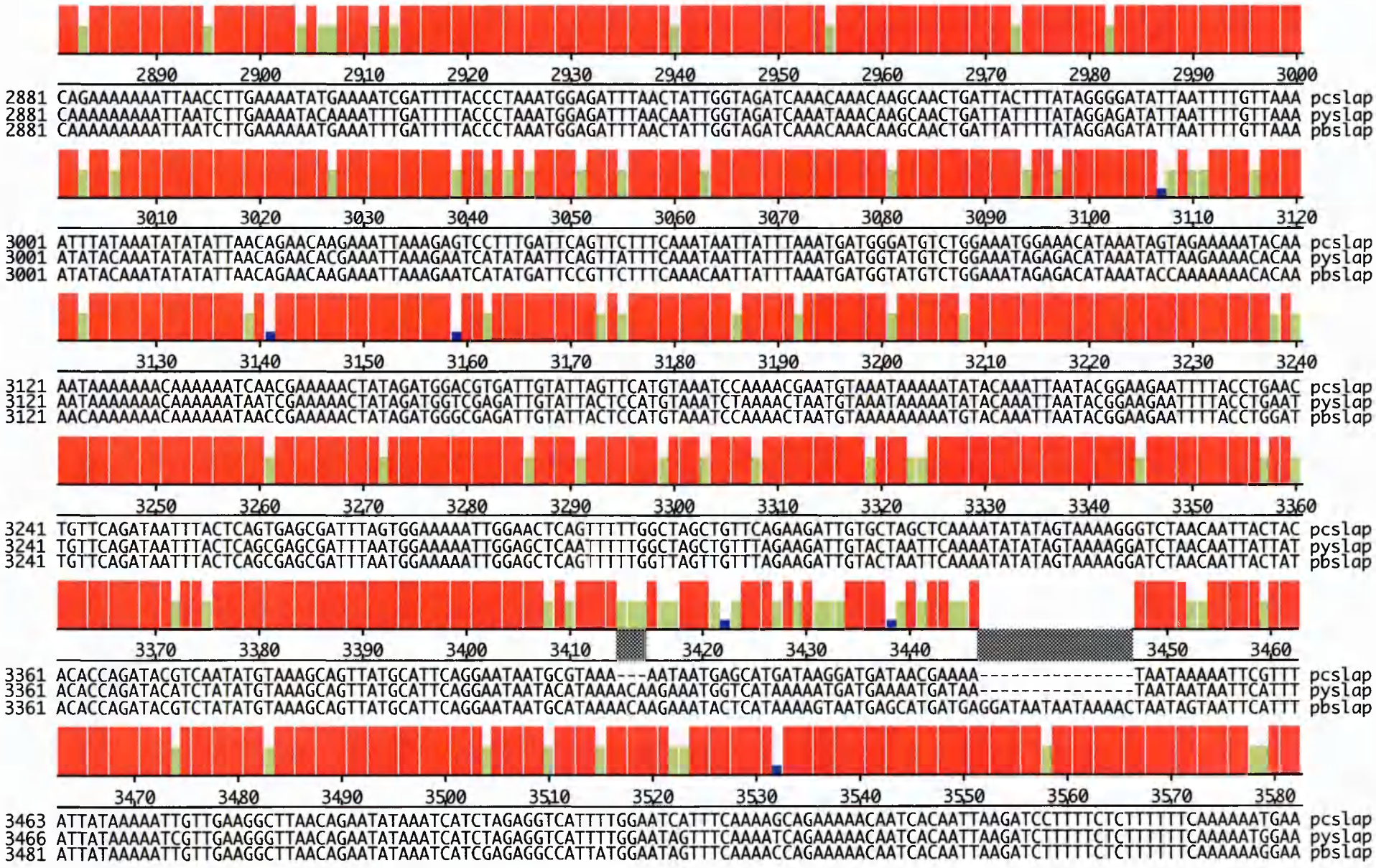
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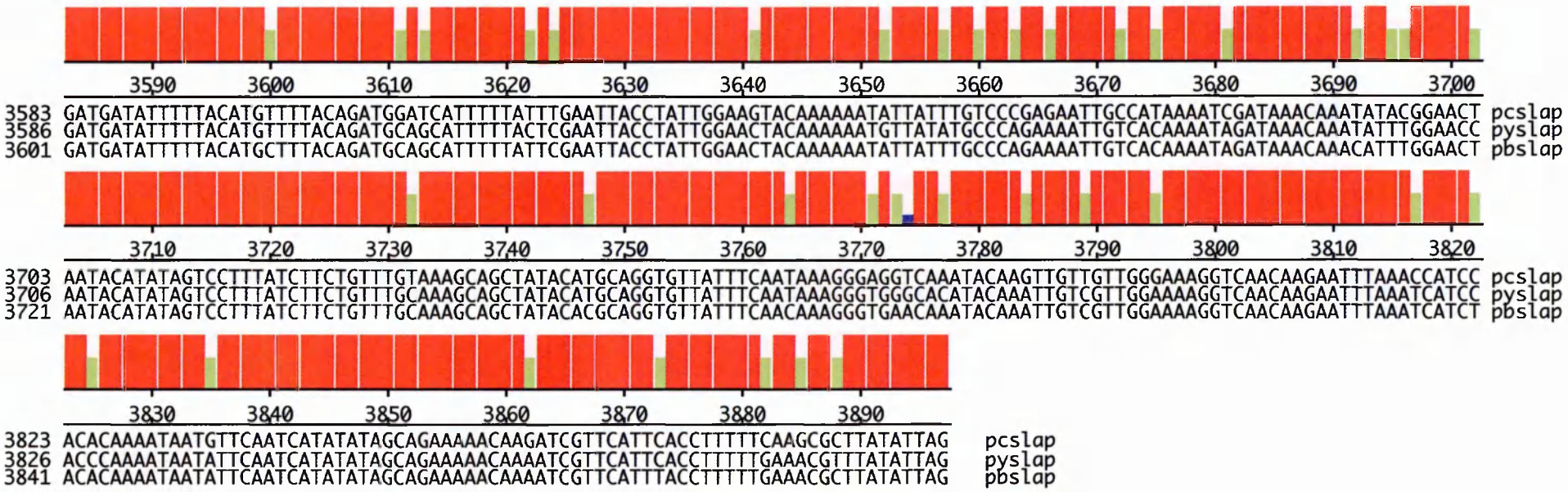
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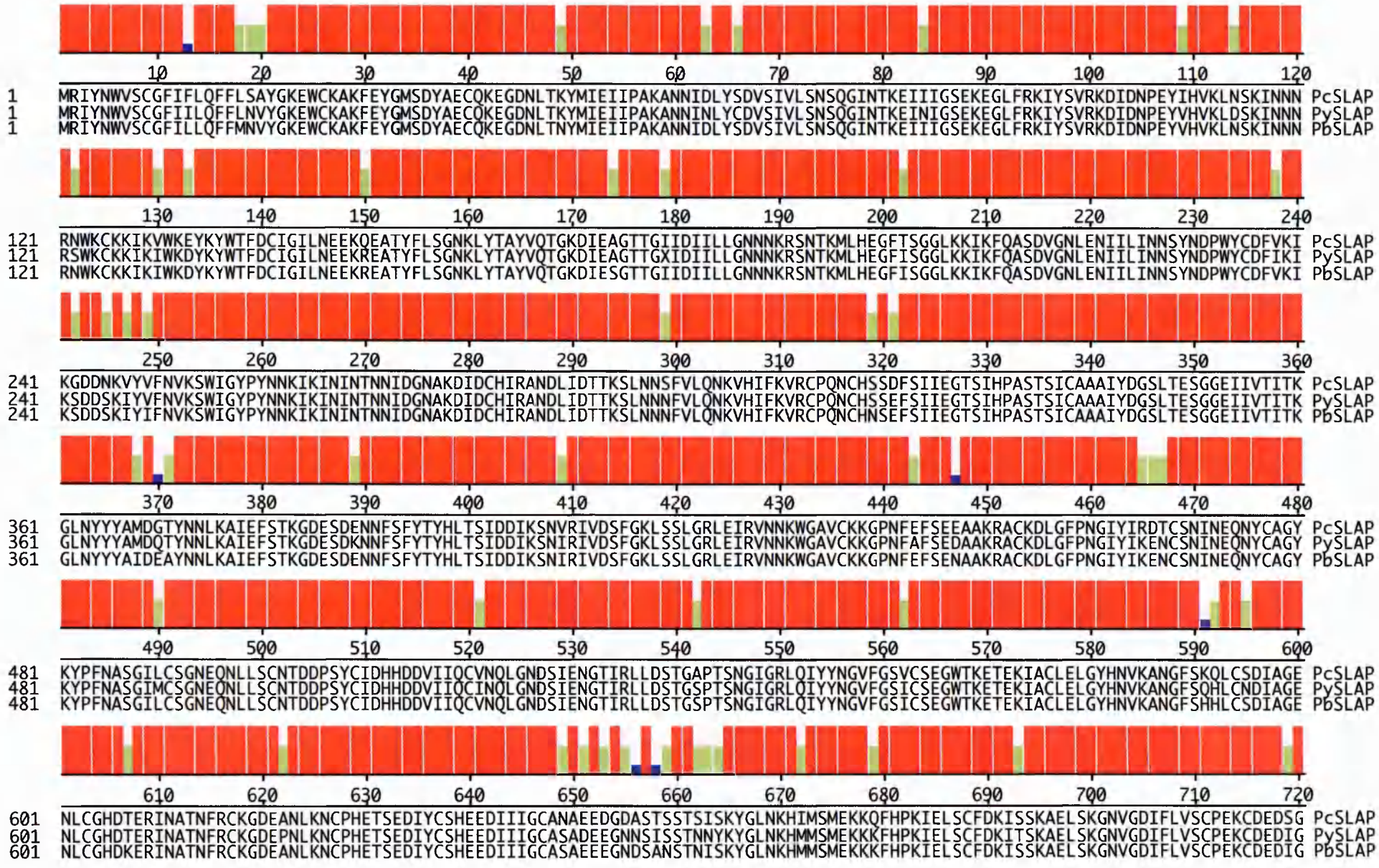
Figure 3.26

Multiple sequence alignment of the *P*SLAP protein sequences in *P. chabaudi, P. yoelii* and *P. berghei*

Alignment of *P*SLAP coding sequence from *P. chabaudi* (1298 residues), *P. yoelii* (1300 residues), and *P. berghei* (1305 residues).

Red colour denotes identity between residues in all sequences, green colour denotes residue dissimilarity in one sequence and blue colour denotes dissimilarities between residues of all three sequences.

The alignment was generated with the MegAlign sequence alignment program using the ClustalW algorithm.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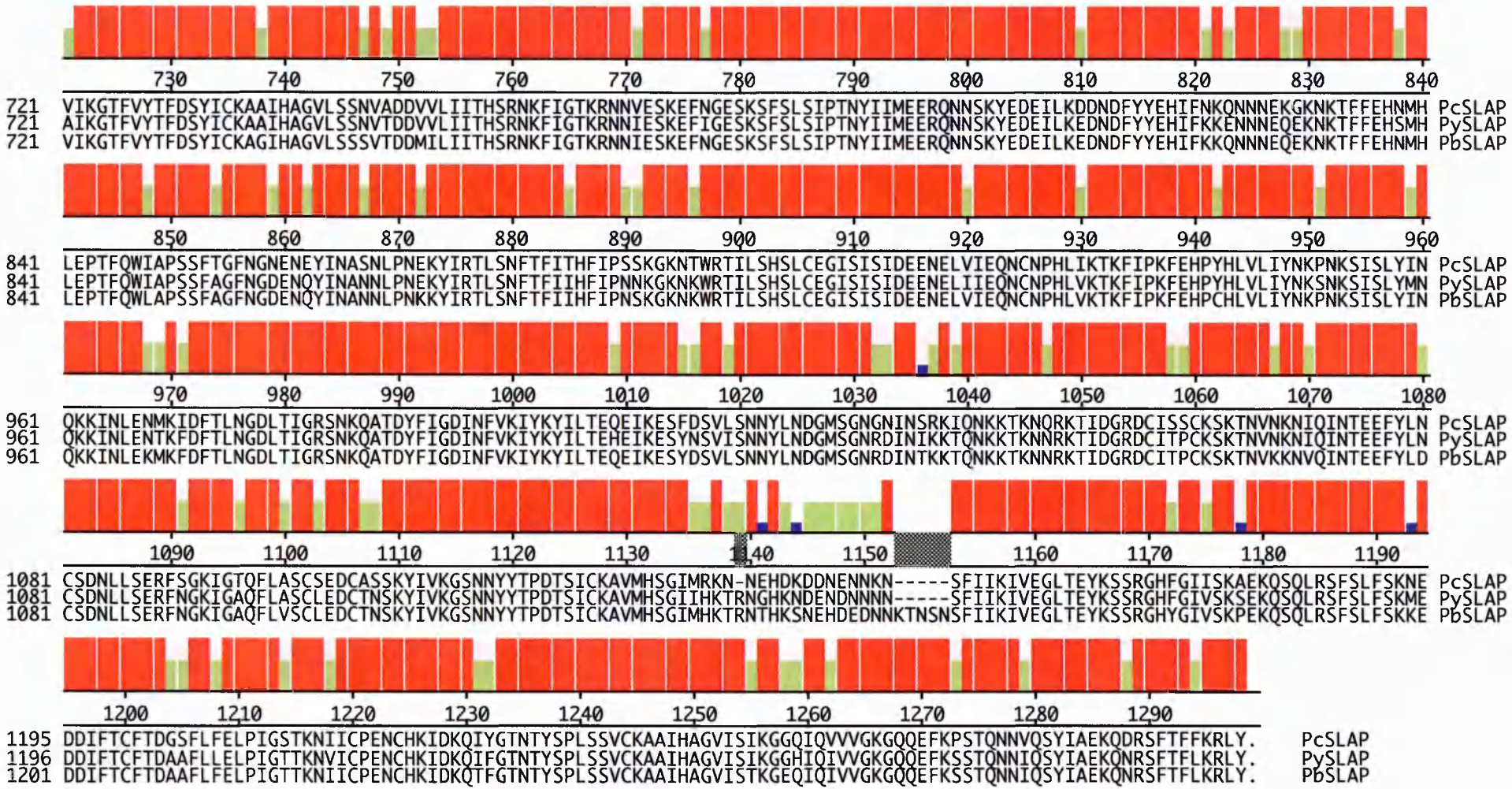
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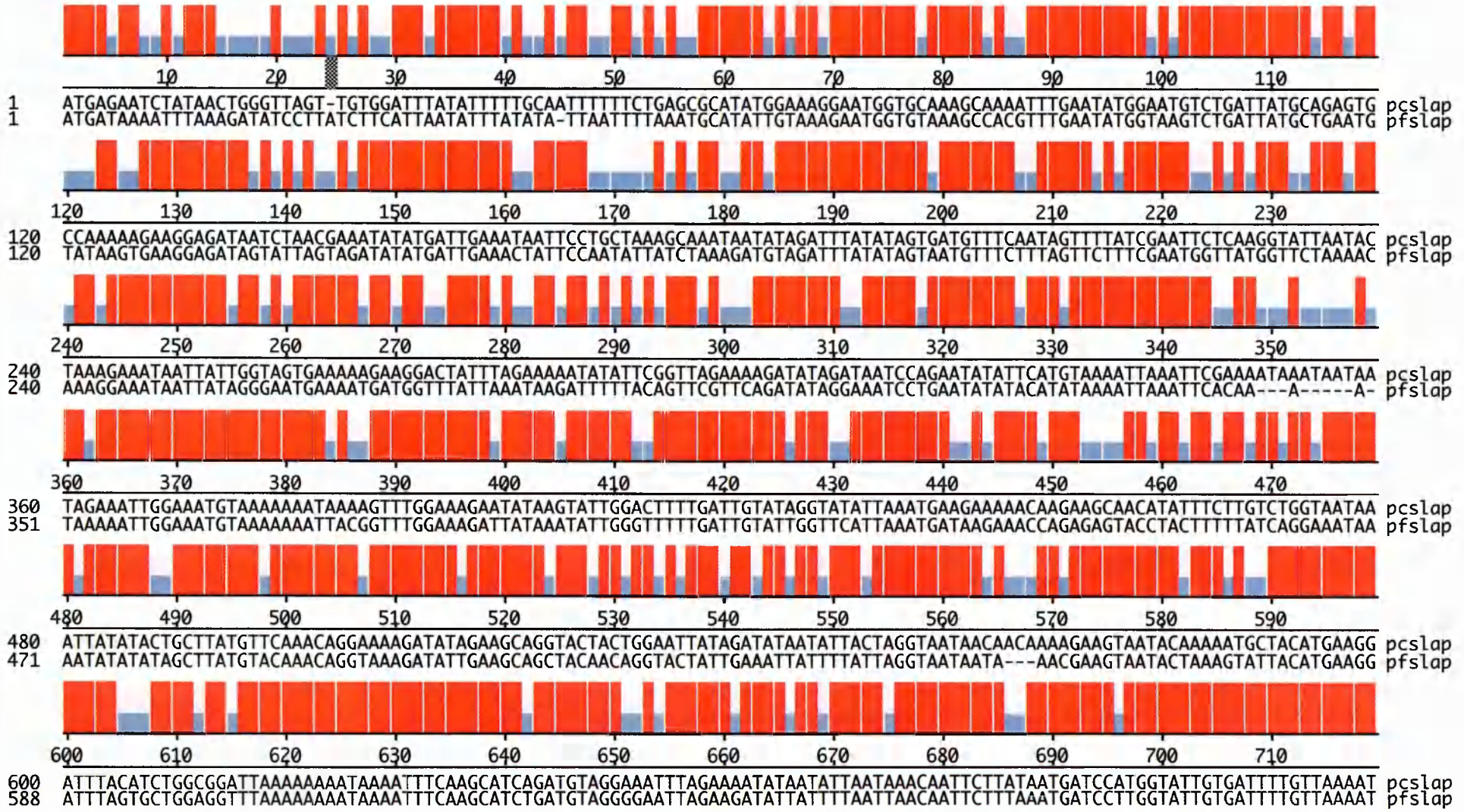
Figure 3.27

Alignment of the nucleotide sequences of *Plasmodium slap* in *P. chabaudi* and *P. falciparum*

Alignment of the open reading frames of *P. chabaudi* (3897 nucleotides) and *P. falciparumi* (3816 nucleotides).

Red colour denotes identity between nucleotides in all sequences, green colour denotes nucleotide dissimilarity in one sequence and blue colour denotes nucleotide dissimilarities between all three sequences.

The alignment was generated with the MegAlign sequence alignment program using the ClustalW algorithm.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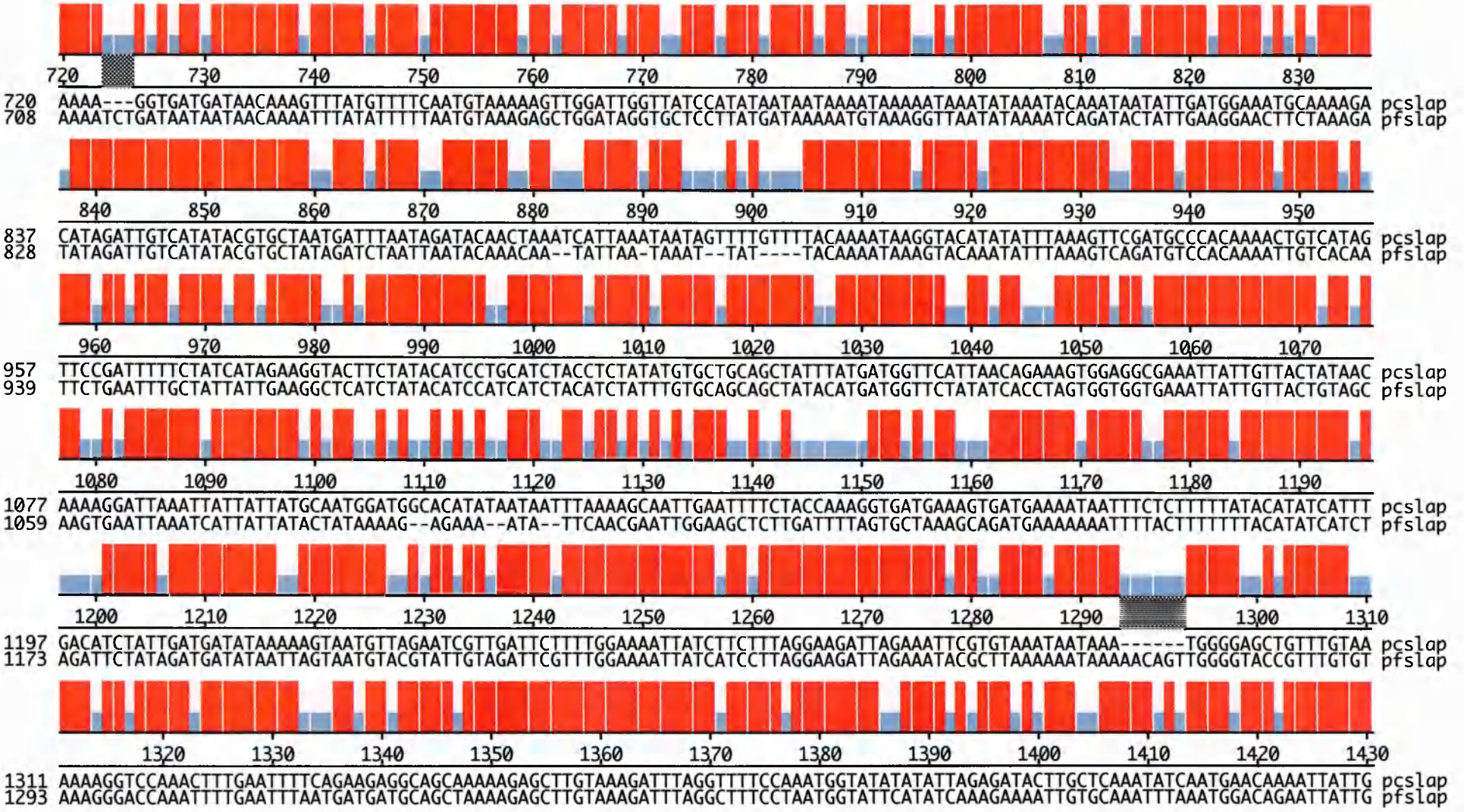
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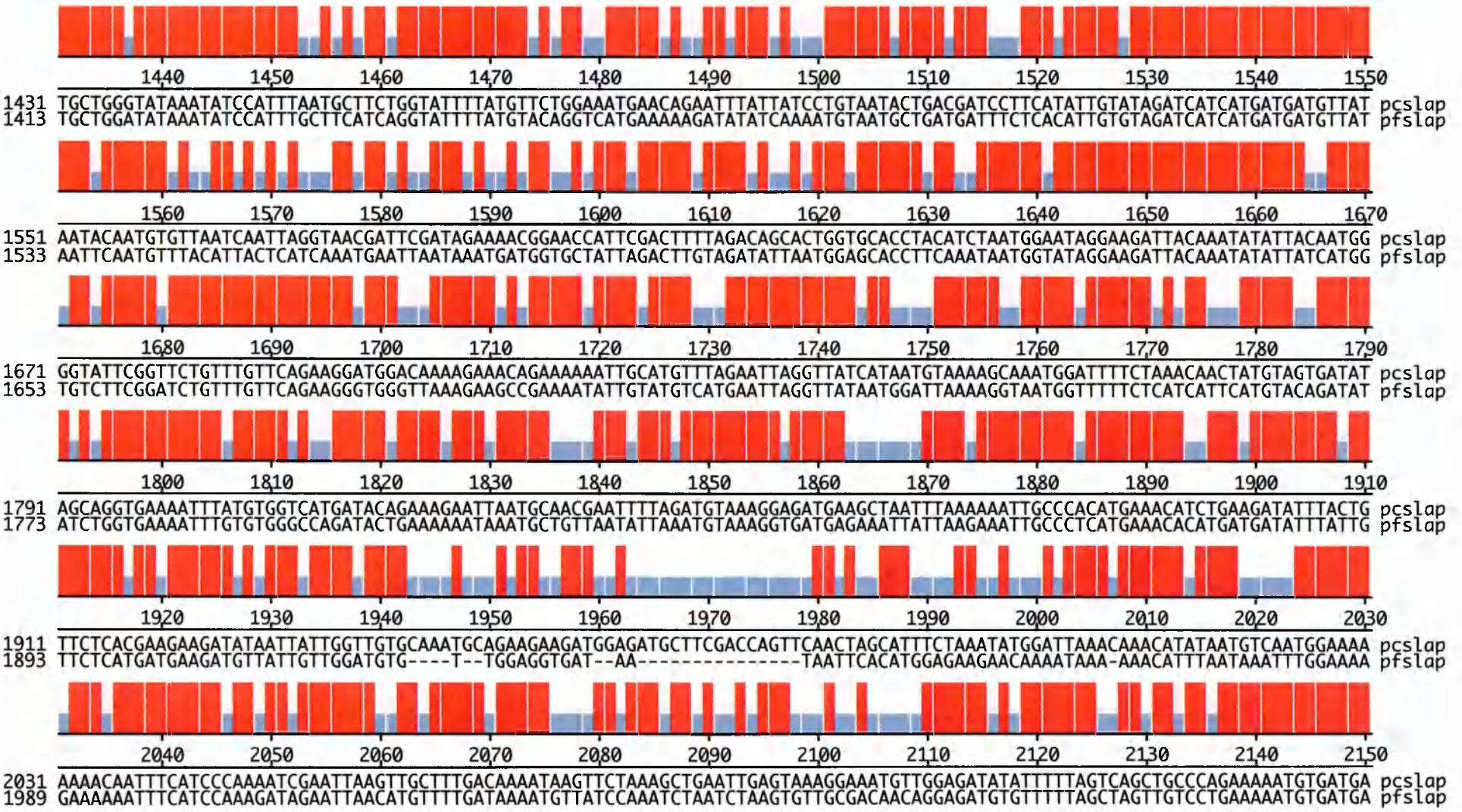
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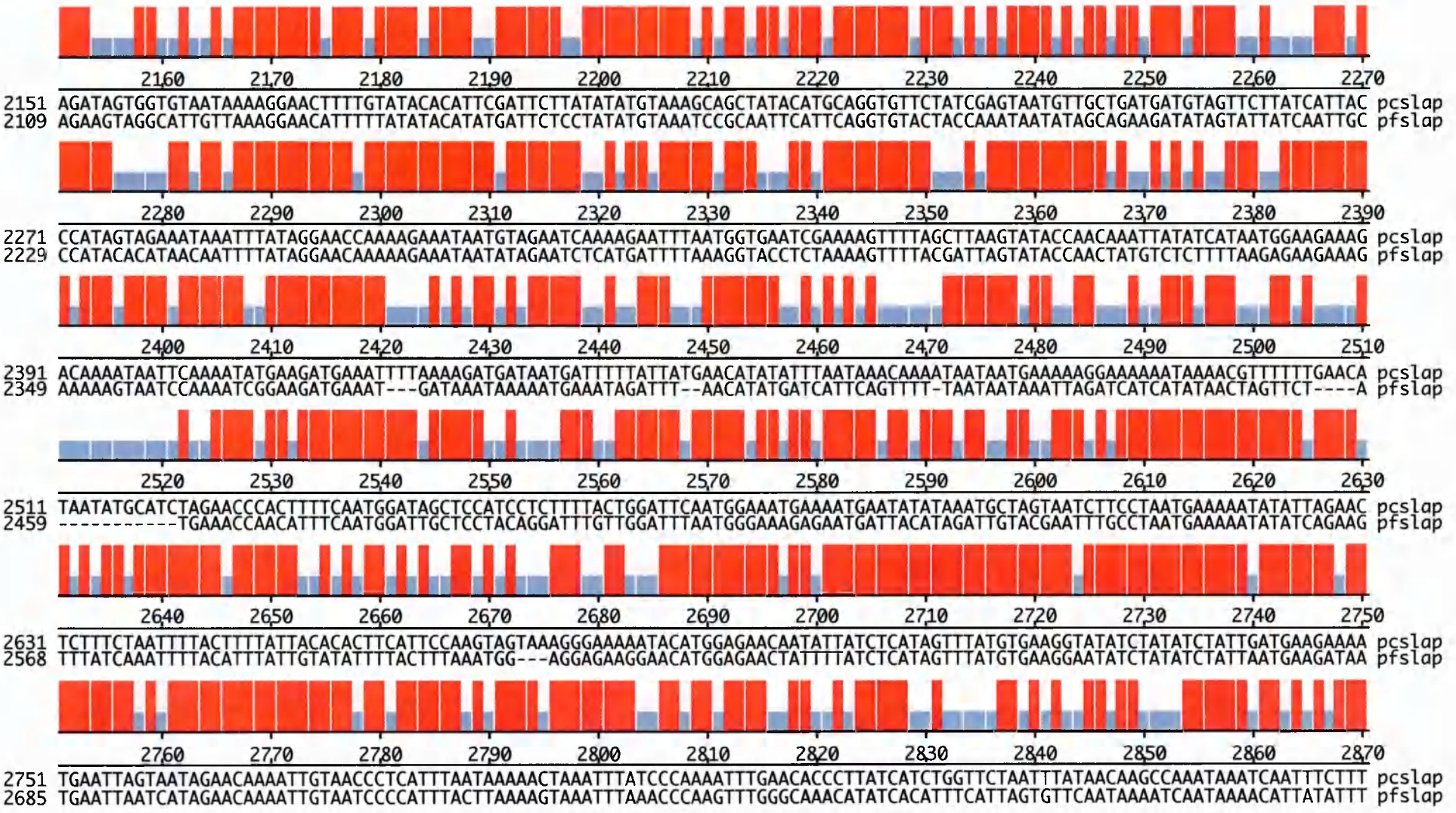
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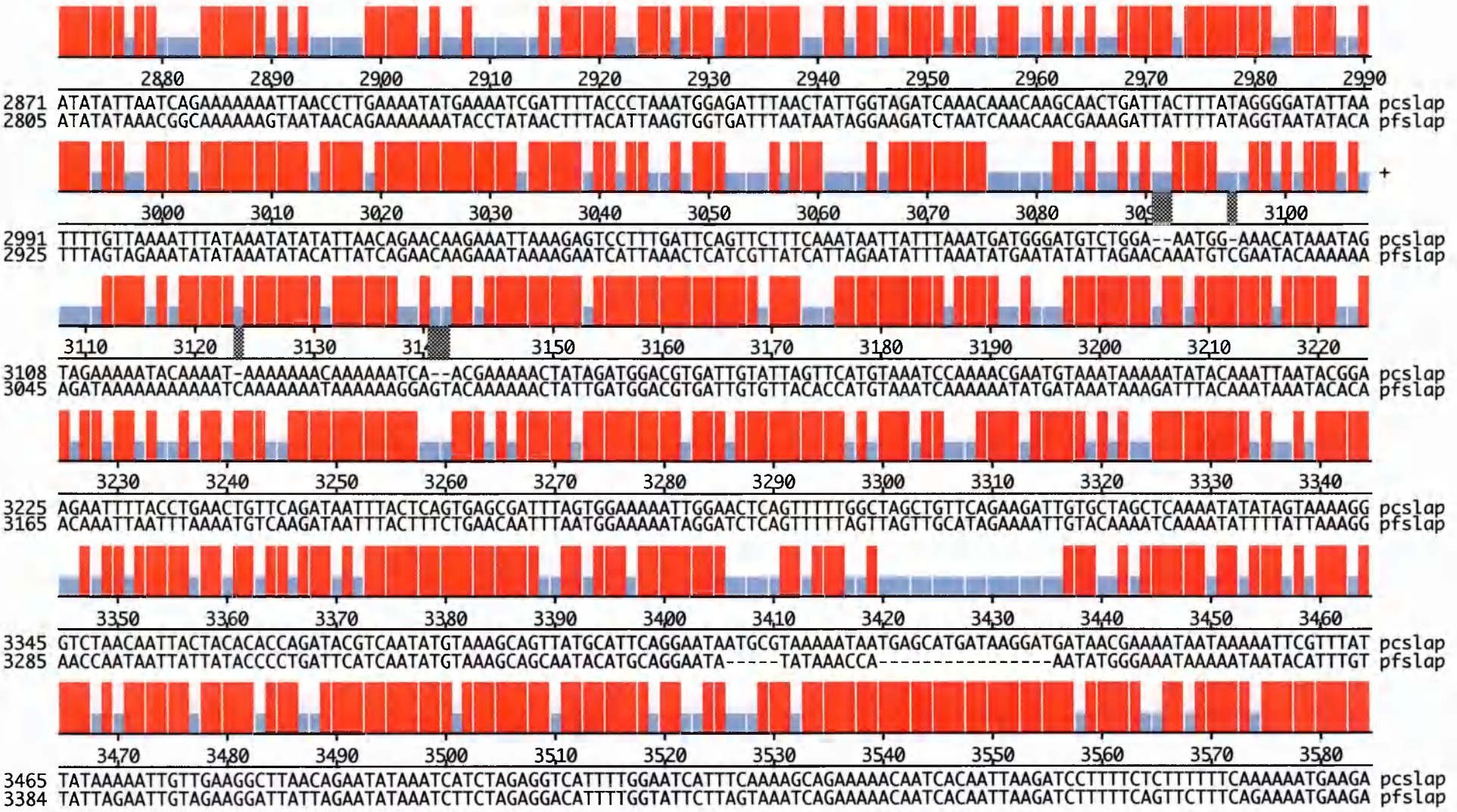
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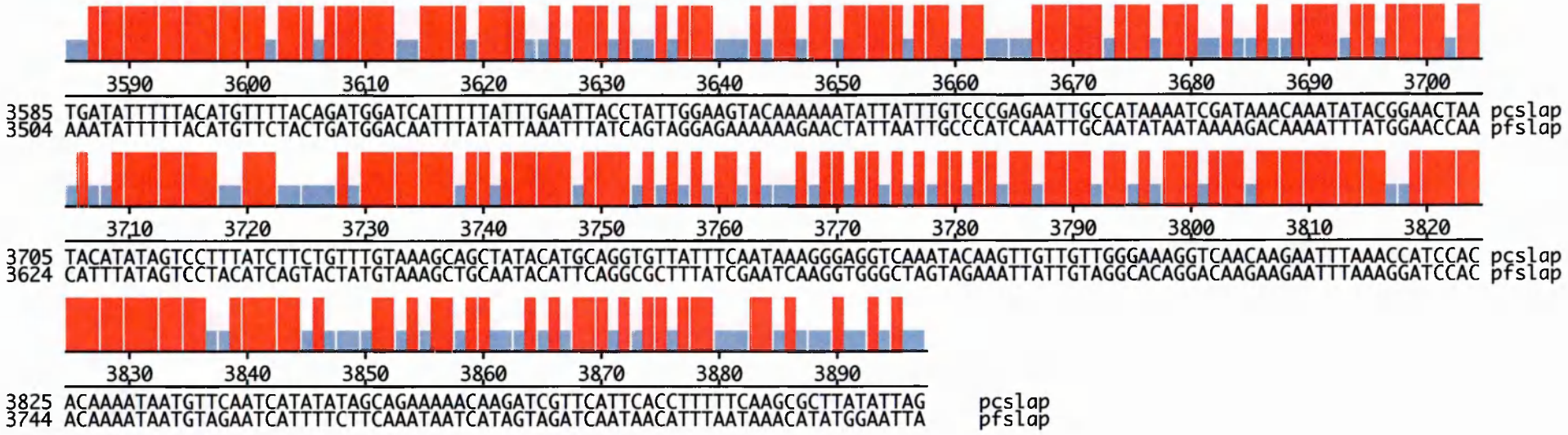
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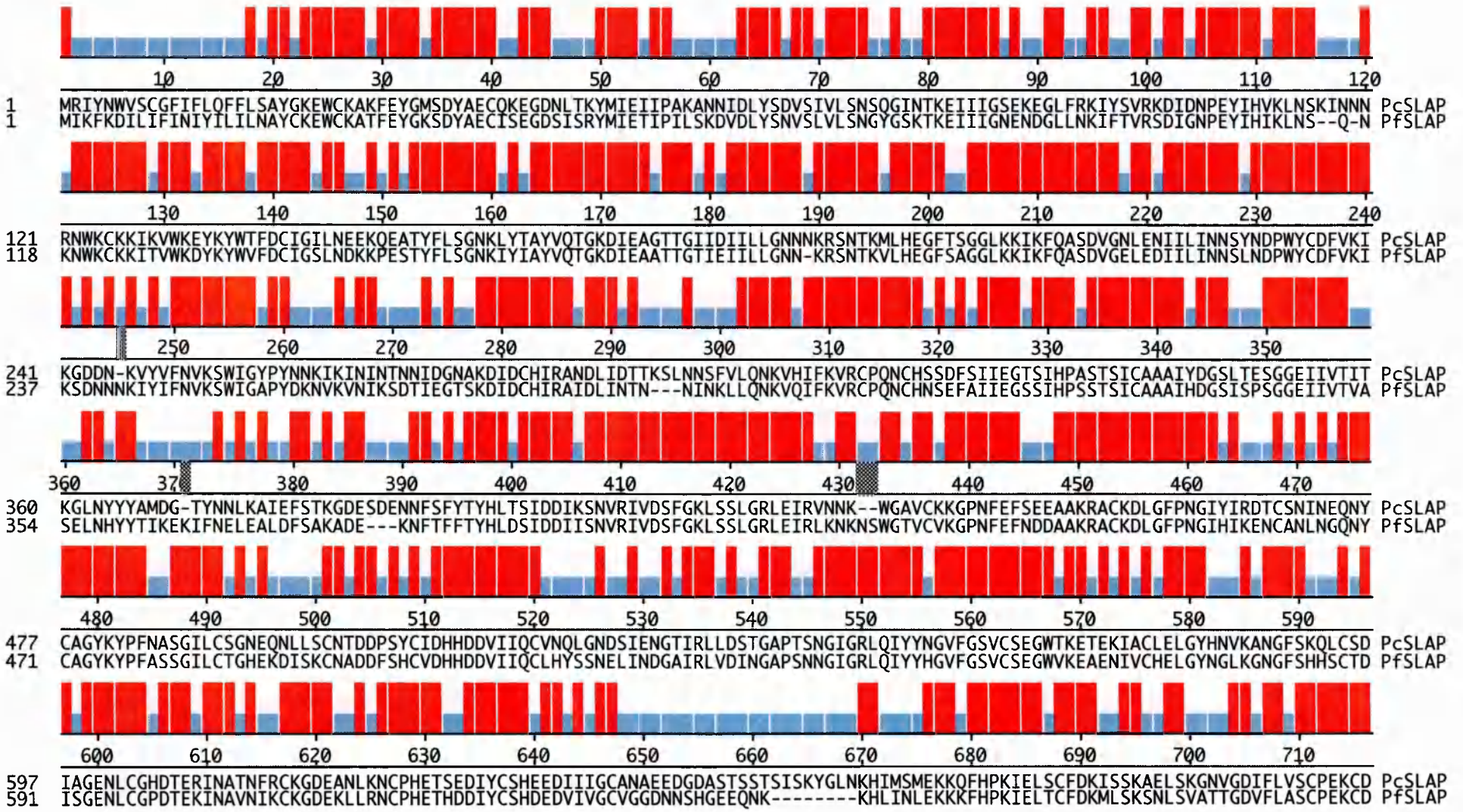
Figure 3.28

Alignment of the *P*SLAP protein sequences of *P. chabaudi* and *P. falciparum*

Alignment of the *P*SLAP coding sequence from *P. chabaudi* (1298 residues) and *P. falciparum* (1269 residues).

Red colour denotes identity between the residues in all sequences, green colour denotes residue dissimilarity in one sequence and blue colour denotes dissimilarity between residues in all three sequences.

The alignment was generated with the MegAlign sequence alignment program using the ClustalW algorithm.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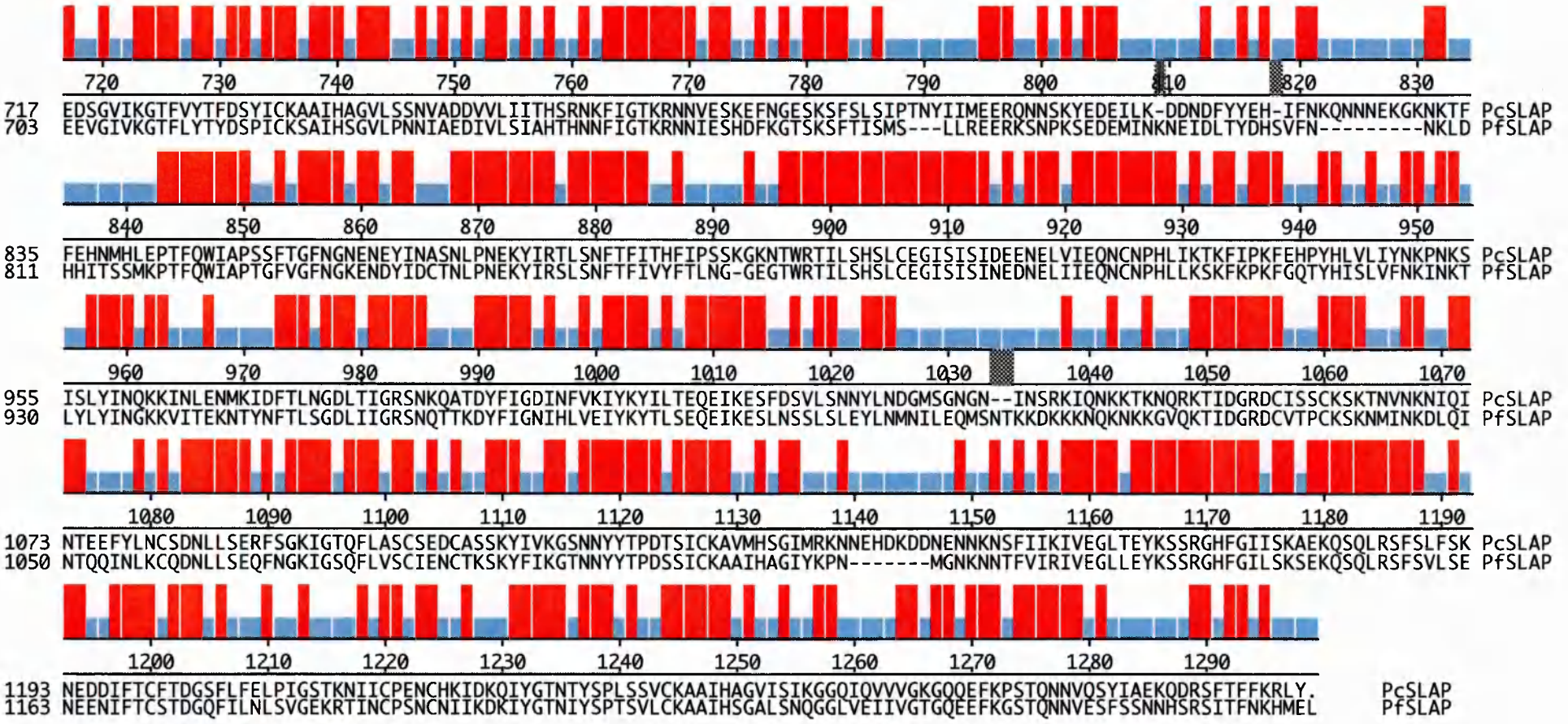
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Chapter Four

*P*SLAP transcription, expression and localisation

4.1 Introduction

Chapter Three identified *P*SLAP as a novel molecule with a complex domain composition not previously reported in biology. The analysis showed that *P*SLAP carries two SRCR domains, two LCCL modules and an LH2/PLAT domain, all motifs implicated in receptor-ligand interactions. Recent studies suggest that SRCR domains bind surface carbohydrates, whereas the LH2 motif is associated with membrane or lipid interactions (Bikker *et al.*, 2002). Although no precise function or ligand has yet been proposed for the LCCL module, recent studies suggest that it is involved in binding to other molecules (Liepinsh *et al*, 2001).

As a parasite *Plasmodium* is highly dependent on molecular interactions with its hosts in order to survive and develop. Critical events such as evasion of host immune responses, host cell invasion and migration are all reliant on receptor-ligand interactions between parasite and host. The importance is clearly reflected in the parasite's genome, where a very small part consists of genes encoding enzymes and transporter proteins, and as much as 1.3% of identified genes code for molecules involved in either adhesion or immune evasion (Section 1.9; Gardner *et al.*, 2002).

A number of different adhesive domains have been identified in *Plasmodium*. Some of these motifs are present in several molecules, making up families of proteins with similar characteristics, such as the cysteine-rich Duffy Binding-Like (DBL) domain in the Erythrocyte Binding Protein (EBP) family. The DBL motif determines ligand-specific recognition and binding of vertebrate erythrocytes, and the family includes the *P. falciparum* protein Erythrocyte-Binding Antigen-175 (EBA-175) and the *P. vivax* Duffy Binding Protein (DBP), both involved in erythrocyte invasion, but also the variant surface antigen *Pf*EMP-1, responsible for cytoadhesion to the vertebrate endothelium (Section 1.7.1). Because of a relatively high sequence variation, the DBL domains of different

EBP proteins interact with a diverse set of erythrocyte receptors, including ICAM-1, complement receptor-1 and heparan sulphate (*Pf*EMP-1), Glycophorin A (EBA-175) and Duffy blood group antigen (DBP; Smith *et al.*, 2000; Rowe *et al.*, 1997; Chen *et al.*, 1998; Orlandi *et al.*, 1992; Miller *et al.*, 1976).

The expression profiles of *Plasmodial* proteins that carry adhesive domains are specific both in time and space, and reflect the different functions of the molecules. An understanding of the timing of transcription of the genes encoding these proteins, as well as their location, is crucial for identifying a function. For example, the *var* genes that encode *Pf*EMP-1 are transcribed during the erythrocytic blood stages in asexual parasites. This is significant since, as mentioned above, *Pf*EMP-1 functions as a sequestration receptor that allows the infected erythrocyte to adhere to various ligands on the endothelium in the vertebrate host. Cytoadhesion prevents destruction of parasitised erythrocytes in the spleen, which filters the cells in the blood by recognizing subtle changes in the rigidity of the red blood cell membrane. Adhering to the host blood vessels allows the parasite population to survive and expand, thereby establishing an infection.

At present, the molecular parasite-host interactions that take place during the vertebrate stages are known in more detail than the mosquito phases of parasite development. This may be, at least in part, due to the greater difficulty in obtaining pure and large enough quantities of parasite material from mosquito stage parasites (Ghosh *et al.*, 2003). However, the complex series of developmental transitions inside the mosquito involves numerous interactions of parasite proteins with host cell molecules. One such event is the involvement of the ookinete protein CSP- and TRAP-related protein (CTRP) in mosquito midgut invasion. Sequence analysis of *P. falciparum* CTRP had shown a predicted structure of several domains with adhesive properties, including six von Willebrand factor type-A related domains and seven human thrombospondin type-1 domains. Transcription studies of the *P. berghei* homologue using Northern analysis demonstrated that the *ctrp* gene was active in ookinetes but not in erythrocytic stage parasites. Indirect immunofluorescence confirmed the presence of the protein in mosquito-stage parasites, showing expression throughout ookinete development, mainly located to the micronemes (Yuda *et al.*, 1999). The micronemes, together with the rhoptries and dense granules,

make up the apical complex, a collection of secretory organelles whose contents are thought to be involved in host cell recognition and attachment (Dubremetz *et al.*, 1993). Consequently, a theory that CTRP played a role in midgut invasion was formed, and later verified by *ctrp* gene disruption in *P. berghei.* It was shown in dissections of parasite-infected mosquitoes that CTRP-deficient ookinetes were unable to enter the midgut epithelium, as well as to migrate through the epithelium to the hemocoelomic side of the midgut (Dessens *et al.*, 1999).

In a similar way, the function of the parasite protein MAEBL was suggested by its location. Immunofluorescence analysis showed expression of MAEBL in the micronemes of mosquito-stage sporozoites. However, closer examination revealed that the protein was present in midgut sporozoites collected from oocysts, but not in salivary gland sporozoites. This indicated that MAEBL played a role during the stages between late oocyst development and sporozoite translocation to the salivary glands, but not later for maturation within the glands or subsequent development inside the vertebrate host. This was confirmed when disruption of the *P. berghei maebl* gene resulted in an accumulation of sporozoites in the mosquito hemolymph, but none were found inside, or associated with, the salivary glands, suggesting that MAEBL functions as a sporozoite binding ligand for the initial attachment to the surface of mosquito salivary glands prior to invasion (Kariu *et al.*, 2002).

Chapter Three discussed the nucleotide sequence and protein structure of *P*SLAP. The present chapter continues the examination of the *P*SLAP gene and protein in blood-stage parasites. Analysis of *pslap* transcription, as well as the generation of anti-*Pc*SLAP antibodies, and their use in determining the stage-specificity of expression of the *P*SLAP protein and its sub-cellular localization is described.

4.2 Results

4.2.1 Expression and purification of *Pc*SLAP for generation of antibodies

In order to study *Pc*SLAP, antibodies were raised against part of the molecule in mice and rabbits. A 270 base pair nucleotide sequence of the *pcslap* gene, cloned into the pTrcHisC expression vector (Invitrogen), was translated and expressed in *Escherichia coli* DH10B cells as a hexa-histidine-tagged fusion protein. The expressed sequence, designated $Pc\text{SLAP}_{90}$, was 90 residues long and included the C-terminal half of SRCR-2 and 28 residues of the sequence between SRCR-2 and LCCL-2 (Figure 4.1).

Expression was induced by addition of IPTG (one mM final concentration) and a small-scale test-run was performed with culture samples collected at one hour intervals for seven hours (Section 2.8.1). The samples were solubilised and centrifuged, and pellets and supernatants analysed separately by SDS-PAGE (Figure 4.2 A). The expressed protein was identified in the pellet fractions, demonstrating that it is not soluble. Maximum expression was found to take place at five hours post-induction, thus this time point was used for subsequent expression and purification of $Pc\text{SLAP}_{90}$ on a larger scale.

Purification was performed by capture on a nickel-nitrilotriacetic (Ni-NTA) matrix, and the protein was eluted with 200 mM imidazole. A second purification step was carried out over an FPLC gel filtration column (Superdex 200 prep grade matrix, Amersham Pharmacia Biotech; Section 2.8.2). Eluted fractions that corresponded to high OD_{280}-values were analysed by SDS-PAGE in order to identify the peak corresponding to $Pc\text{SLAP}_{90}$ (data not shown). The correct fractions were pooled and concentrated under pressure in an Amicon concentrator. The concentrated sample was run on a polyacrylamide gel, followed by Coomassie-staining to ensure that no major contaminants were present in the preparation (Figure 4.2 B). Lastly, the protein was re-folded in order for it to be used in mouse and rabbit immunisations, by removal of the high level of urea (eight molar) through slow dialysis against PBS. Recovery in the final preparation was determined spectrophotometrically by absorption at 280 nm and by comparison with a quantitative protein marker in SDS-PAGE, and found to be approximately 11 mg (data not shown).

4.2.2 Immunisation with *Pc*SLAP$_{90}$ and characterisation of anti-*Pc*SLAP plasma by ELISA

Four female BALB/c mice were immunised and boosted with the purified *Pc*SLAP$_{90}$ protein and subsequently bled for plasma, which was tested for anti-*Pc*SLAP reactivity using a *Pc*SLAP-specific ELISA (Sections 2.9.1 and 2.10.3.1). The assay showed high levels of *Pc*SLAP$_{90}$-specific antibodies, with plasmas from all mice having titers over 100,000 (Figure 4.3). To control for non-specific binding to *Pc*SLAP$_{90}$ by naturally occurring antibodies present in normal plasma, the assay also included plasma from un-treated mice as a background control (NP control in Figure 4.3). These results were negative, confirming that normal BALB/c plasma did not react with the expressed portion of *Pc*SLAP.

Polyclonal anti-sera to *Pc*SLAP$_{90}$ were also raised in rabbits (Section 2.9.2). Reactivity of the sera with *Pc*SLAP$_{90}$ was tested for as with the mouse plasmas above, and again showed titers of above 100,000 (419 and 431 in Figure 4.4). Pre-immune sera from the rabbits were used as negative controls and displayed no binding to the *Pc*SLAP$_{90}$ protein, demonstrating that the reactivities seen with the immune sera were due to the presence of induced anti-*Pc*SLAP$_{90}$ antibodies (Figure 4.4).

All plates in both assays also included duplicate wells of diluent in place of plasma or serum, so called "blanks". An average of the two blank values was subtracted from the OD_{405}-readings obtained from immune and normal plasma/serum on the same plate and then used for plotting the graph, and therefore no separate blank readings are shown in the figures.
The readings from the blank wells were in all cases below OD_{405}=0.1.

4.2.3 Anti-*Pc*SLAP plasma recognises *P. chabaudi* blood stage parasites

To test whether the generated anti-*Pc*SLAP antibodies recognised the native *Pc*SLAP protein, and whether this is expressed during the blood stages of the parasite life cycle, an ELISA was performed using a lysate made from predominantly late trophozoite- and schizont stage *P. chabaudi* parasites (Section 2.10.4).

Negative controls using plasma from un-treated animals showed that there was no non-specific binding of components in normal mouse plasma to the parasite preparation (NP control in Figure 4.5). To ensure that the assay itself worked, plasma from mice repeatedly infected with *P. chabaudi*, referred to as "hyperimmune plasma" (HIP; Section 2.9.3), was included. A strong reactivity with the parasite lysate confirmed that this was the case (HIP control in Figure 4.5).

Although low, all anti-*Pc*SLAP plasmas showed reactivity with the parasite preparation. End point titers ranged between 500 (Mouse 2) and 2000 (Mice 3 and 4; Figure 4.5). Repeats of the experiment produced similar results, confirming that the polyclonal anti-*Pc*SLAP antibodies recognised the *Pc*SLAP protein, but that the levels of the protein present in the blood-stage lysate were low.

4.2.4 Western analysis of *Pc*SLAP

The results from the ELISA suggested that *Pc*SLAP was present in the *P. chabaudi* blood-stage parasite lysate, however, the reactivity was low and this prompted examination of the expression of *Pc*SLAP by Western analysis (Section 4.2.4). Western analysis also gives an experimental estimation of protein size. The theoretical molecular weight of *Pc*SLAP had been determined to 147 kilodaltons (EditSeq sequence analysis program).

The first parasite preparation tested was a lysate of *P. chabaudi* (mainly trophozoites and schizonts) similar to that previously recognized by the *Pc*SLAP antibodies in ELISA (Section 4.2.4). The lysate was resolved by SDS-PAGE (10% gel) under both reducing and non-reducing conditions, blotted onto nitrocellulose and tested for immunoreactivity with the four individual $PcSLAP_{90}$-specific plasmas. Hyperimmune plasma was used as a positive control in the assay and normal mouse plasma from un-infected mice was included as a negative control. Hyperimmune plasma reacted strongly with proteins of a wide range of sizes, demonstrating that the assay worked. However, no reactivity was observed with the anti-*Pc*SLAP antibodies (data not shown). Despite repeats of the experiment no bands were produced.

It was subsequently reasoned that the *P*SLAP protein may have been lost in the process of lysate preparation. As it was shown in Chapter Three that *Pc*SLAP has a signal

sequence, the protein could be secreted into the erythrocyte and would thus be lost when the cells were lysed and centrifuged. The protein could also be sensitive to freezing and may have degraded since the lysate was made. It was therefore decided to use whole infected erythrocytes, prepared without discarding any material. Fresh, parasitised blood was obtained from a *P. chabaudi*-infected mouse (Table 4.1), mouse leukocytes removed by Plasmodipure filtration and the contents of the lysed cells resolved under reducing and non-reducing conditions by SDS-PAGE. The assay included the same controls as above, as well as samples of non-infected blood, prepared identically to the parasitised blood and incubated with the same antibodies. Three out of four of the anti-*Pc*SLAP plasmas reacted with the lysate and produced some bands, however these were also seen with the normal mouse plasma. Two bands at approximately 45 kDa and 80 kDa appeared to be specific to the plasma from Mouse 3, and could represent breakdown products of *Pc*SLAP. However, this result was not reproducible despite numerous attempts, and therefore no conclusions can be drawn from this record (data not shown). The Western analysis was repeated several times with amendments to the protocol, including longer incubations and more concentrated plasma samples, but without success (data not shown).

4.2.5 Anti-*Pc*SLAP plasmas react with fixed *P. chabaudi* parasites

In order to further analyse the expression of the *P*SLAP protein in blood-stage parasites, and to investigate its subcellular location, indirect immunofluorescence assays (IFA) were performed. Thin blood films were made from *P. chabaudi*-infected mice bled at parasitemias of 15-25 %, fixed with acetone and stored at –20°C (Section 2.12). Giemsa-staining of the slides and analysis by light microscopy was carried out in order to determine the relative proportion of different developmental stages in the preparation (Table 4.2). To avoid binding of the secondary antibody used in the assay, goat anti-mouse IgG-FITC, to antibody present in the collected blood, *P. chabaudi*-infected B cell- and antibody-deficient μMT mice were used as a source of infected erythrocytes (Kitamura *et al*, 1991).

The four plasmas from the *Pc*SLAP$_{90}$-immunized mice were tested individually on the fixed blood smears. The slides were also stained with 4,6-diamidino-2-phenylindole

(DAPI) to distinguish infected erythrocytes from un-infected ones (mature erythrocytes lack nuclear material). A previously characterized monoclonal antibody against *P. chabaudi* MSP-1, NIMP23, was used as a positive control and produced a distinct circular pattern of staining, demonstrating that the assay worked (McKean *et al.*, 1993; Figure 4.6 C). A negative control using plasma from un-treated BALB/c mice, included to ensure that there was no binding of antibodies present in normal plasma, did not show any fluorescence (Figure 4.6 E). A control for non-specific binding of the fluorochrome-conjugated secondary antibody (anti-mouse IgG-FITC) to the cells was also negative (Figure 4.6 F).

When testing the anti-*Pc*SLAP plasmas, specific staining was observed with antibodies from Mouse 1 and Mouse 3, producing a similar pattern of staining. The fluorescence had a punctate appearance with a more intense staining at the center of the fluorescent area and somewhat blurred edges, suggesting cytosolic, rather than surface labeling (Figure 4.6 A). The close proximity to the parasite nucleus suggested that the plasmas were recognizing a component of the parasite and not the erythrocyte. A manual increase in the fluorescence intensity visualised the outline of the host cell membranes, and revealed that the staining ranged from occupying a small area immediately surrounding the nucleus, to extending throughout the entire cell. It also showed that the boundaries of fluorescence were well-defined, suggesting that the labeled molecule is expressed and contained within the parasite cytosol, and not exported into the erythrocyte cytoplasm (Figure 4.6 B). Figure 4.7 show further examples of the observed *Pc*SLAP staining patterns. These experiments were repeated at least five times with the same result.

As compared with the total number of infected cells, identified by DAPI-stained nuclei, very few were also labeled with the anti-*Pc*SLAP plasmas (Figure 4.8). Several repeats of the assay, as well as the fact that the positive control worked, confirmed that the scarceness of labeled cells was not due to an experimental artifact. Approximately 0.3% of the total number of parasitised erythrocytes were positive for *Pc*SLAP. This small proportion of labeled cells suggested that the antibodies recognized a specific subset of blood stage parasites, and suggested that the expression of *P*SLAP is carefully controlled in space and/or time. Interestingly, the percentage matched the small numbers of

gametocytes normally present in *P. chabaudi* blood stage infections, and prompted a careful examination of the numbers of parasites at different developmental stages on the slides used in this experiment. Table 4.2 shows the differential counts, with a percentage of gametocytes at approximately 0.4%, a number that compared well with the proportion of *Pc*SLAP-positive cells (0.3%). Alternatively, the protein may be expressed only during a very short window of time during another stage of development.

The two $PcSLAP_{90}$ anti-sera raised in rabbits were also tested in indirect immunofluorescence assays. Pre-immune sera from each respective rabbit were included as negative controls. However, a high level of background fluorescence was observed in these controls. In addition, the *Pc*SLAP-specific sera stained infected as well as uninfected erythrocytes (data not shown). The secondary antibody (goat anti-rabbit IgG-TRITC) had been used previously and so was known not to cross-react with mouse preparations. However, non-specific reactivity is not an uncommon problem with polyclonal rabbit sera and is probably due to naturally occurring anti-mouse antibodies in rabbits. To overcome these difficulties and remove the pre-existing anti-mouse antibodies, the rabbit sera (immune and pre-immune) were adsorbed onto fresh uninfected mouse erythrocytes. Staining with lower concentrations of sera and secondary anti-rabbit antibody was also tried. However, the sera kept reacting with uninfected erythrocytes on the fixed blood preparations. It was therefore decided to use the mouse-derived plasmas instead of the rabbit sera in subsequent assays.

4.2.6 Northern analysis of *pslap* transcription

The ELISAs and immunofluorescence assays had shown that *Pc*SLAP is present during the erythrocytic stages of the *P. chabaudi* life cycle, with an indication towards specific expression in gametocytes (Sections 4.2.3 and 4.2.5). In order to investigate the time and stage of transcription of the *pslap* gene, Northern analysis was performed using all developmental forms present in a blood-stage infection.

During the blood-stages the majority of parasites are asexual. However, the only intra-erythrocytic form that can survive transmission to a mosquito is the gametocyte, and therefore a small number of asexual parasites transform into sexual forms (Section 1.3).

The number of gametocytes generated in an infection varies between parasite species. *P. chabaudi* produces relatively few gametocytes, which makes isolation difficult (Figure 4.9 top graph). However, other species, such as *P. berghei*, generate a higher proportion of sexual parasites, and it was therefore decided to include a sample of *P. berghei* purified gametocytes, as well as a sample of mixed asexual *P. berghei* parasites in the assay (Figure 4.9, bottom graph). This was possible due to the high degree of conservation of the *slap* gene within rodent *Plasmodia*, as verified by an alignment of the probe sequence used for the hybridization from the two species, which showed 93% identity (Figure 4.10). The probe had been specifically chosen to corresponded to a region of *pcslap* that did not code for any secondary motifs, as some of these (the LCCL modules) are known to be present in other proteins in *Plasmodium*, and could potentially bind the probe and produce a false positive result (Figure 4.12). The 252 base pair long srequence, named $pcslap_{252}$, was part of the 3':F1 Vectorette PCR fragment that was produced when the full sequence of the *pcslap* gene was being identified (Chapter Three, Section 3.2.1.1).

To control for hybridization to contaminating, co-purified mouse RNA from leukocytes and reticulocytes, a sample of blood from an un-infected mouse was prepared alongside the parasite preparations (Section 2.4.11). In order to avoid contamination with asexual parasites within the *P. berghei* gametocyte preparation, the infected mice were treated with pyrimethamine prior to parasite collection (Section 2.13.8). Pyrimethamine is a folate antagonist that interferes with DNA synthesis (Section 5.1). In a blood stage malaria infection, treatment with pyrimethamine kills replicating asexual parasites, whereas non-replicating gametocytes survive (Ferone *et al*, 1969).

Parasitised blood was collected from a highly synchronous infection of *P. chabaudi* at specific time points in order to obtain as high as possible a proportion of ring-, trophozoite- and schizont stage parasites, respectively. The *P. berghei* ANKA infection is inherently asynchronous and all developmental stages are present at all times of the day, thus collection at any hour will result in a mixed-stage parasite population. *P. chabaudi*-infected mice were kept in a reverse light cycle, resulting in schizogony taking place in

the early afternoon, whereas animals infected with *P. berghei* ANKA were kept under normal light conditions (Section 2.3.3).

For each preparation, blood was collected from two or three mice, pooled and total RNA extracted (Section 2.4.10). A thin blood film was prepared from each pooled blood sample, Giemsa-stained and examined by light microscopy, in order to determine the proportion of parasites at different developmental stages (Table 4.3). The purity of the preparations ranged between 94 and 99%. No attempt was made to remove mouse RNA, such as using Plasmodipure filters to collect mouse leukocytes, as it was reasoned to be safer to extract a larger amount of RNA per sample to minimise losses of parasite RNA during the preparation process. The extracted RNA was separated on a 1% agarose gel, with ribosomal RNA bands of both parasite and mouse origin being clearly visible (Figure 4.11 A; Section 2.5.2). Although care had been taken to use similar quantities of RNA from all samples, the electrophoresis showed obvious differences in loaded amounts, something that had to be taken into consideration when interpreting the results of the hybridisation analysis. The immobilized RNA was transferred to a nylon membrane and subsequently hybridised with the 252 basepair probe sequence (Section 2.6.2). To make the probe, the plasmid carrying the 3':F1 sequence was propagated, restriction digested and the $pcslap_{252}$ fragment gel purified and radioactively labeled (Sections 2.4, 2.5 and 2.6).

A signal at approximately six kilobases was detected in preparations of *P. chabaudi* ring- and trophozoite-stage parasites, as well as in *P. berghei* asexual and gametocyte RNA, but not in the BALB/c preparation (Figure 4.11 B). Taking into account the differing amounts of RNA loaded into each lane, the strongest relative signal was generated by probe hybridization to the *P. berghei* gametocyte sample, followed by the *P. berghei* mixed asexual preparation, and a weaker signal in the *P. chabaudi* ring- and trophozoite samples. This suggested that peak transcription of *pcslap* takes place in gametocytes, but that the gene may also be active in early- and mid-stage asexual parasites. Alternatively, because the asexual *P. chabaudi* preparations contained contaminating gametocytes, it may be that the signal originated from *pslap* transcripts within these parasites (Table 4.3).

The bands on the blot were approximately six kilobases in size, although the coding regions of *pcslap* and *pbslap* are about four kilobases. This is not an abberant result as it is not uncommon to have both 5' and 3' untranslated regions (UTRs), as well as a poly(A) tail of up to 200 base pairs for mRNA to be translated (Lewin *et al.*, 1997).
The radiograph also shows a weaker signal in all samples at approximately four kilobases. This is the size of the largest rRNA band on the agarose gel shown in Figure 4.11 A, indicating that the high amounts of RNA collected at this level caused non-specific hybridisation of the probe due to quantity rather than sequence complementarity.

The Northern analysis was repeated with a second set of RNA samples (prepared as before) and produced similar results to those described above (data not shown).

4.2.7 Immunofluorescence staining of *P. chabaudi* gametocytes

The results from the ELISA and the immunofluorescence assays had shown that *Pc*SLAP is expressed in *P. chabaudi* blood stage parasites, while the Northern analysis indicated a strong gene expression in gametocytes (Sections 4.2.3, 4.2.5 and 4.2.6). It was therefore necessary to investigate whether the parasites that had been reacting with the *Pc*SLAP antibodies in the previous immunofluorescence assays were gametocytes or asexual forms.

In *P. falciparum*, gametocytes are easily identifiable due to the crescent-like shape of the host erythrocyte (apart from very young, stage I gametocytes which are round). In rodent *Plasmodia*, on the other hand, gametocytes and their host cells retain the circular shape of the asexual parasite, making them more difficult to distinguish from other developmental stages (Wemsdorfer and McGregor, 1988). To overcome this problem in assays such as IFA, dual staining can be performed, using antibodies against the molecule of interest in combination with antibodies against a known sexual-stage antigen. A previously characterized rat monoclonal antibody, 85RF.45.3, raised against the P48/45 gametocyte-specific antigen in *P. falciparum* was used in the present studies (Roeffen *et al.*, 2001; Section 1.7.2). A homologue to *Pf*s48/45 had recently been reported in *P. yoelii*, suggesting that this protein could be conserved within *Plasmodium*, and also present in other rodent parasite species (Carlton *et al.*, 2002). Indeed, a BLAST search through the

PlasmoDB rodent Plasmodia sequence database identified partial sequences of both *P. chabaudi* and *P. berghei Pf*s48/45 homologues (data not shown).

The 85RF.45.3 antibody was used in the immunofluorescence assays following the same protocol as before and *P. chabaudi* fixed blood films from the same batch as for the staining with the anti-*Pc*SLAP plasmas (Section 4.2.5). However, although intense fluorescence was observed with the positive control, the NIMP23 monoclonal antibody, no staining could be detected with 85RF.45.3 (data not shown). The assay was repeated with amendments to the protocol, including different fixatives, different lengths of fixation and variations in primary and secondary antibody concentrations, but without success (data not shown).

4.2.8 Immunofluorescence staining of *P. berghei* gametocytes

Because the immunofluorescent staining of *P. chabaudi* gametocytes was unsuccessful it was decided to investigate whether 85RF.45.3, as well as the plasmas against *P. chabaudi* SLAP, could be used in the same assay but with parasites of another rodent species. *P. berghei* ANKA had been used previously for isolation of gametocyte RNA for Northern analysis and was therefore the obvious second choice after *P. chabaudi* (Section 4.2.1).

Thin blood films of mixed-stage blood stage parasites were made from *P. berghei* ANKA infected blood and differential counts of the maturation stages present in the preparation obtained from Giemsa-stained blood films examined by light microscopy (Table 4.2). The slides were stained with the 85RF.45.3 antibody and appropriate controls, followed by goat anti-rat IgG-Alexa®488 (green). Examination of the stained slides showed fluorescence with a somewhat punctate appearance throughout positively stained cells. Quantitative analysis revealed that approximately 2% of the total number of parasitised cells stained with the 85RF.45.3 antibody (Figure 4.13 A). However, light microscopy of Giemsa-stained parasites from the same preparation had shown gametocyte numbers of almost 6%, indicating that not all sexual stage parasites were labeled in the fluorescence assay (Table 4.2).

Staining with NIMP23, used as a positive control, confirmed that the assay worked (data not shown). No fluorescence was observed with the negative controls, which included purified rat IgG (for 85RF.45.3) and diluent in place of plasma (for non-specific binding of the secondary antibody; Figure 4.13 B and C, respectively).

4.2.9 Anti-*Pc*SLAP plasmas react with fixed *P. berghei* blood-stage parasites

Before co-localisation of P48/45 and SLAP could be carried out, the *P. berghei* blood films also had to be tested for reactivity with the plasma raised against the *P*SLAP protein. Although the plasma had been raised against *P. chabaudi* SLAP, it was likely that it would also bind the *P. berghei* homologue, due to the high level of conservation between the two species. An alignment of *P. chabaudi* *Pc*SLAP$_{90}$, the part of the protein used in the immunisations, and the homologous sequence in *P. berghei*, had an overall identity of 88%, with a 41 residue central stretch of complete identity (Figure 4.14). With this high degree of similarity it seemed highly probable that the immunisations would have generated antibodies to identical sequences, which would be able to react with SLAP from both species.

The reactivity of the anti-*Pc*SLAP plasmas with *P. berghei* ANKA blood-stage parasites was assessed using the same blood films as for the staining with 85RF.45.3 (Section 4.2.7 and Table 4.2). The fluorescence showed the pattern previously seen in staining of *P. chabaudi* with anti-*Pc*SLAP plasma: a punctate fluorescence surrounding the parasite nucleus, the extent of which ranged from a small area around the nucleus to the entire cell (Section 4.2.5; Figure 4.15 A). Between three and four percent of the parasitised cells stained positive with the plasmas. As with the labeling of *P. chabaudi* with the *Pc*SLAP-specific plasma, this number is close to the proportion of gametocytes present in the preparation, another indication that *P*SLAP may be a sexual-stage protein (Section 4.2.5).

No staining was observed using normal plasma from un-treated mice, or with the conjugated secondary antibody (anti-mouse IgG-Alexa® 594) on its own (Figure 4.15 C and D, respectively).

4.2.10 *P. berghei* PSLAP co-localises with the gametocyte-specific antigen P48/45

To test whether *P*SLAP was expressed in gametocytes, blood films were made from *P. berghei* ANKA-infected mice treated with pyrimethamine (Section 2.13.8). Pyrimethamine eliminates asexual parasites but does not affect the viability of sexual stages (Section 4.2.1). The animals were treated with a dose of 10 mg pyrimethamine per kilo bodyweight on four consecutive days and the parasite counts followed by microscopic examination of Giemsa-stained blood smears from the mice. On day four of treatment only sexual parasites were detected on the smears. *P. berghei* gametocytes have distinctive morphological characteristics that are visualized by the stain. Macro-gametocytes (female) have compact nuclei and a blue staining cytoplasm due to a basic pH caused by an abundance of ribosomes (Figure 4.16 A). Micro-gametocytes (male) are pink due to acidic cytoplasms and have large nuclei (Figure 4.16 B). Both sexes contain scattered pigment granules and entirely fill up the host erythrocyte, making them clearly distinguishable from asexual parasites.

After finished treatment, blood was collected and thin blood films made. Although the films had a very low percentage of infected cells (approximately 0.5%) due to the small proportion of gametocyte-infected cells in a blood-stage infection, the purity of the preparation was very high (Figure 4.16 A and B, Table 4.2).

Co-localisation studies were subsequently performed using the anti-*Pc*SLAP plasmas, anti-*Pfs*48/45 monoclonal antibody 85RF.45.3 and the *P. berghei* gametocyte thin blood films. Analysis of the slides by single colours showed the previously observed fluorescence patterns for the *P*SLAP-specific plasmas (Alexa®594 – red) and 85RF.45.3 (Alexa®488 green). Moreover, double colour analysis showed the same cells staining for both *P*SLAP and P48/45 (Figure 4.17). However, not all parasitised cells were labeled with the antibodies. Approximately 93% of DAPI-staining cells were positive for *P*SLAP and 35% also labeled with the anti-P48/45 antibody.

The fluorescence produced by the two antibodies in dual-stained cells in some cases displayed very similar patterns, with a punctate staining throughout the infected cells. However, merging of the images revealed small regions stained only with one of the two antibodies, as well as a variation in the fluorescence intensity over different areas of the

cells, verifying that the similarity in pattern was not due to cross-reacting secondary antibodies. These results strongly indicate that *P. berghei* ANKA *P*SLAP is a sexual stage protein present in gametocytes.

4.2.11 *P. falciparum* SLAP is expressed in gametocytes

The results obtained by Northern analysis and IFA suggested that rodent (*P. berghei and P. chabaudi*) *P*SLAP is expressed in both asexual and sexual stage parasites. Due to the high degree of conservation between the rodent *slap* and the homolog in *P. falciparum* (Section 3.4), they would be expected to share similar patterns of expression. However, a parallel project in the division where the present work was carried out, investigating the *P. falciparum* homolog of *Pc*SLAP, had showed transcription of the *slap* gene in *P. falciparum* gametocytes but not in asexual parasites. To investigate the expression profile of the *Pf*SLAP protein, immunofluorescence assays were performed, following the same protocol as for the rodent parasites (Section 2.12).

In vitro systems for culturing of *P. falciparum* are well established and specific techniques allow enrichment for gametocytes within parasite populations. By restricting the addition of fresh erythrocytes and new medium to a culture, the parasites start preparing for transmission (to a new host) by developing into sexual forms. Parasites were thus grown under such conditions, purified and thin blood films were made. The smears were stained with polyclonal sera against three different regions of the *Pf*SLAP protein: the LCCL-1, LCCL-2 and SRCR-1 motifs (Figure 4.18). As before, the 85RF.45.3 antibody was used for dual labeling, to identify sexual stage parasites, and DAPI to distinguish infected cells.

Analysis of the staining showed that *P*SLAP was expressed on the same cells as the gametocyte-specific protein *P*48/45. No fluorescence was detected in cells that did not stain with the 85RF.45.3 antibody. The appearance and pattern of the *Pf*SLAP staining was similar to that of *P*SLAP in *P. chabaudi* and *P. berghei*, with a punctate fluorescence distributed throughout or part of the parasite cytosol (Figure 4.19 A; Sections 4.2.5 and 4.2.7). However, a punctate pattern following the outline of the erythrocyte membrane was also observed (Figure 4.19 B). This was not present in the staining of parasitised rodent cells. *Pf*SLAP appeared to be expressed in a stage-specific fashion during

gametocytogenesis: it could not be detected in early gametocytes (stages I-II) but became abundant in more mature stages (stages III-V).

4.3 Discussion

The previous chapter (Chapter Three) demonstrated that *Pc*SLAP has a complex and unique domain composition, not reported anywhere outside of Apicomplexa. However, its high degree of conservation across different species of *Plasmodium* suggests it would possess an important function. Determining the location and time of expression of *P*SLAP is essential in trying to understand its role in the parasite.

In order to characterise *Pc*SLAP, antibodies against a 90-residue portion of the protein were raised in mice and rabbits for use in expression- and localization studies. It is notable that, to reduce the risk of non-specific reactivity, this sequence of *Pc*SLAP did not include any domains, or parts of domains, that have been identified in other Plasmodial proteins. The plasmas obtained from the immunised animals were first used in ELISAs that showed binding to a lysate made from *P. chabaudi* blood-stage parasites. However, the reactivity was low. There are several plausible explanations for this: (i) low levels of *Pc*SLAP expression, (ii) loss of *Pc*SLAP during the preparation of the lysate due to its location, (iii) loss of *Pc*SLAP antibody epitopes during lysate preparation, or (iv) degradation of *Pc*SLAP during lysate preparation. The first explanation (i) could be true if *Pc*SLAP is only expressed within a very short window of time, if it is a protein only required and synthesised by the parasite in very small quantities, or if maximal *Pc*SLAP expression takes place at a different stage in the life cycle, and what is seen are low levels of non-induced expression. Explanations (ii), (iii) and (iv) are technical: the method to prepare the parasite lysate uses saponin to lyse the erythrocyte membranes, after which parasite constituents are immediately centrifuged down and collected. However, if saponin is left in with the infected erythrocytes too long, it starts breaking up the parasite parasitophorous vacuole membrane, and any soluble proteins located in the parasitophorous vacuole, or anchored to its membrane, are lost (ii). It is also possible that some of the proteins in the preparation will adopt non-native configurations when extracted from the erythrocyte, thereby losing some epitopes recognized by the anti-*Pc*SLAP plasmas (iii), or if very sensitive to degradation, that *Pc*SLAP may even have been broken down by the time the lysate was ready to use (iv). Although showing a relatively low level of reactivity, this assay demonstrated that *P*SLAP is expressed during the intra-erythrocytic stages of the *Plasmodium* life cycle.

In order to further analyse the presence of *Pc*SLAP in blood-stage parasites, Western analysis was performed. All four *Pc*SLAP-specific plasmas were tested for reactivity with i) a lysate of parasites isolated from *P. chabaudi*-infected mouse blood, and ii) a lysate of whole erythrocytes from *P. chabaudi*-infected mouse blood. However, no specific bands were reproducibly obtained with either of the preparations despite several repeats of the assay. The obvious explanation for the negative result would be that the *Pc*SLAP protein was absent in the tested preparations. This appears unlikely though, since all plasmas had previously shown reactivity with a similar parasite extract in ELISAs, and the two assays work on the same principle: the binding of specific antibodies to a mixture of immobilized proteins containing the target molecule. The most likely explanation for the absence of reactivity would be that only a very low amount of *Pc*SLAP protein was present in the lysate, in combination with too low a concentration of *Pc*SLAP-specific plasma in the test samples. The plasmas were used at dilutions of 1:1000 and 1:500 due to the high sensitivity of the detection reaction. However, it may be that a dilution of 1:50 or 1:100 would have been required for *Pc*SLAP-detection.
It is also possible that the interaction with solid phases altered the conformation of the protein, producing steric restrictions to antibody binding or loss of epitopes. This problem could be circumvented by using a different technique, such as immunoprecipitation. Immunoprecipitation provides the same information as Western analysis, ie size of an antigen and whether it is present in a protein mixture or not, but allows antibody-antigen interactions to take place in solution rather than on a solid support.

Immunofluorescence microscopy was subsequently used to study *Pc*SLAP and its location within the parasite. Both the mouse plasmas and the rabbit sera were tested for reactivity with *P. chabaudi* blood-stage parasites. The mouse-derived antibodies reacted exclusively with parasite-infected erythrocytes. However, the rabbit immune sera stained both infected and un-infected cells. Despite adsorption of the sera onto un-infected mouse erythrocytes, as well as a lowering of the concentration of primary and secondary antibodies, the non-specific reactivity of the sera could not be eliminated. It is possible that using even lower concentrations of sera and fluorochrome-conjugated antibody could have reduced the high background. Careful titrations of both primary and secondary antibodies down to concentrations where no fluorescence is seen would have verified

this. Alternatively, the sera could have been affinity purified. This method involves conjugation of the target protein onto a column matrix, over which the serum is passed, allowing any target-specific antibodies to bind and subsequently be eluted and collected, while non-specific antibodies are washed off and discarded. Alternatively, antibodies could have been raised against other regions of *Pc*SLAP. This would have increased the chances of obtaining plasma that recognized the protein in Western analysis, as well as strengthened the findings from the immunofluorescence assays regarding the expression and location of *Pc*SLAP.

Using indirect immunofluorescence, the anti-*Pc*SLAP plasmas produced a pattern with a punctate appearance that suggested the protein is stored in subcellular vesicles. This is consistent with the presence of a 22-residue long signal sequence at the amino terminal of the molecule, which would direct it into the endoplasmic reticulum, from where it may be translocated into vesicles (Section 3.3.4). The fluorescence was not distributed throughout the infected erythrocyte but appeared to be contained within clear boundaries, indicating that *Pc*SLAP is retained inside the parasite rather than exported into the red cell cytoplasm. Whether this is the final location of *P*SLAP or whether it is subsequently transported out of the parasite remains to be investigated.

Interestingly, only a small proportion of blood-stage infected cells stained positive for *P*SLAP in indirect immunofluorescence assays. Northern analysis, performed to analyse the transcription profile of *pslap*, suggested that this was due to *P*SLAP being expressed in gametocytes. Examination of *P. chabaudi* and *P. berghei* blood-stage parasites indicated that the highest level of transcription was seen in a preparation of *P. berghei* gametocytes. Although a weaker signal was also detected in samples of *P. chabaudi* rings and trophozoites, these preparations contained approximately 0.33% and 0.5% contaminating gametocytes respectively, consequently the signal could originate from these parasites. However, it cannot be excluded that *pslap* transcripts are also present in asexual stage parasites, and a recent report by Claudianos *et al.* (2002) showed transcription of *P. berghei slap* by reverse transcriptase (RT)-PCR, in gametocytes as well as in asexual stages from a clone unable to produce sexual parasites.

Because transcription of a gene does not invariably equate with presence of the protein, analysis of the *P*SLAP protein was continued. Co-localisation studies were performed using 85RF.45.3, a monoclonal antibody against the sexual-specific antigen *Pf*s48/45 (Roeffen *et al.*, 2001). *Pf*s48/45 is expressed in stage II to V *P. falciparum* gametocytes, as well as in gametes and zygotes (Vermeulen et al., 1985). The assays were performed using blood films made from *P. berghei* infected mice treated with pyrimethamine in order to eliminate asexual parasites and obtain a preparation highly enriched for sexual parasites. The results showed that *P*SLAP was co-expressed in the same cells as the sexual-stage antigen P48/45, demonstrating that *P*SLAP is a sexual-stage protein. Interestingly, however, some cells stained positive with the *Pc*SLAP-specific plasmas but not with 85RF.45.3. In *P. falciparum*, P48/45 is first seen in stage II gametocytes. The protein is present inside the gametocyte but is later expressed on the surface of the emerging gamete (Vermeulen *et al.*, 1985). It may be that some of the gametocytes on the blood films were approaching the gamete stage and that the epitope recognized by 85RF.45.3 is inaccessible once the protein is being, or has been, translocated, and therefore these parasites could not be labeled in the assay. It is also possible that because 85RF.45.3 was raised against the *P. falciparum* P48/45 protein, the distortion caused by fixing the cells could have made the *P. berghei* P48/45 protein on some cells unrecognizable to the antibody. Alternatively, the *P. berghei* P48/45 homologue may have a more restricted expression that starts later in gametocyte delopment than the *P. falciparum* homologue, and therefore gametocytes at the earliest stages are only labeled with the anti-*P*SLAP antibodies.

Surprisingly, the transcription profiles of *slap* in rodent Plasmodia and the homologue in the human parasite *P. falciparum* differ. A parallel project in the division where the present work was carried out, showed that transcription of the *P. falciparum* homologue of *pcslap* takes place in gametocytes but not in asexual parasites (Delrieu *et al.*, 2002). Nonetheless, subsequent immunofluorescence assays showed that expression and sub-cellular location of *Pf*SLAP was very similar between the species: *Pf*SLAP was detected exclusively in gametocytes, with fluorescence of a punctate appearance, reminiscent of vesicular distribution. However, an additional pattern of staining was observed in *P. falciparum*: a circle of punctate staining running along the erythrocyte membrane. This

suggested that *P*SLAP stored in vesicles in the parasite cytosol may be translocated to the inside of the erythrocyte membrane, perhaps upon a developmental signal. The time of expression and location of *P*SLAP in the parasite are suggestive of a role in the mosquito, and it is possible that, upon transmission to the mosquito and initiation of gametogenesis, as the erythrocyte membrane is lost the vesicles are released and the protein inside secreted. Indeed, a recent study investigating *P. berghei* SLAP reported expression of the protein throughout the parasite life cycle, including the stages within the mosquito (Truman *et al.*, manuscript in preparation).

Figure 4.1

Sequence of *Pc*SLAP used in immunisations for generation of polyclonal antisera

A 90 residue sequence of *Pc*SLAP was expressed and purified in an *E. coli* system, and subsequently used to immunize four BALB/c mice for production of polyclonal antibodies. The sequence, designated $PcSLAP_{90}$ comprised the C-terminal half of SRCR-2 and the following 28 residues. It is noteworthy that the sequence did not include any part of the LCCL-2 module, as LCCL motifs have been identified in other proteins in *Plasmodium*, and antibodies raised against them could potentially react with proteins other than *Pc*SLAP, producing abberant results in subsequent assays.

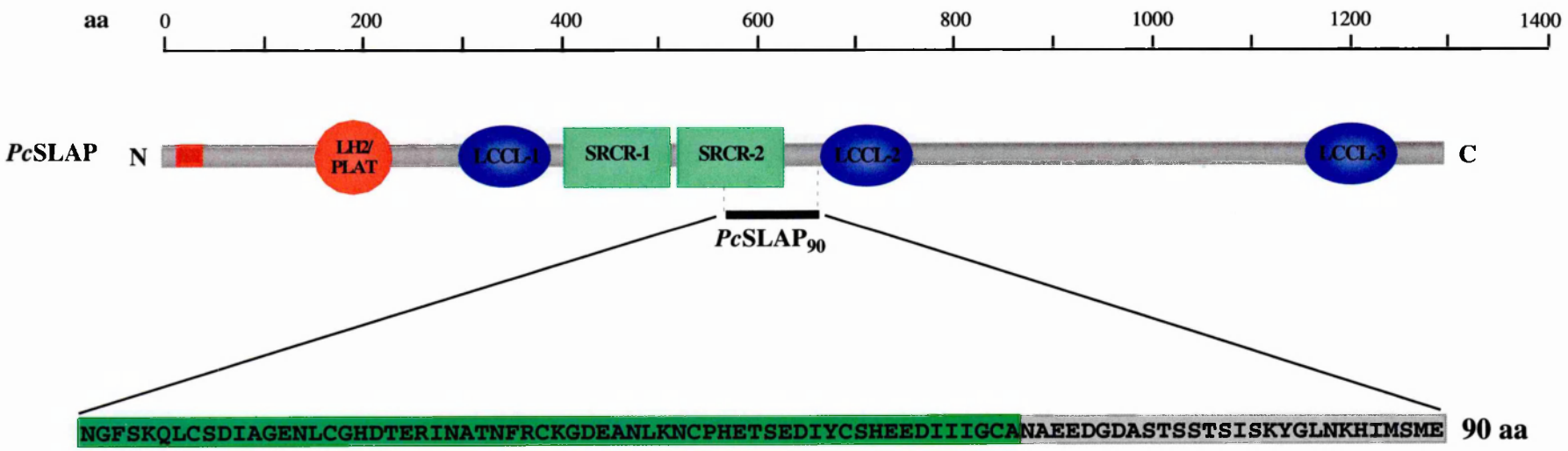

aa
0
200
400
600
800
1000
1200
1400
PcSLAP
N
LH2/
PLAT
LCCL-1
SRCR-1
SRCR-2
LCCL-2
LCCL-3
C
PcSLAP90
NGFSKQLCSDIAGENLCGHDTERINATNFRCKGDEANLKNCPHETSEDIYCSHEEDIIIGCANAEEDGDASTSSTSISKYGLNKHIMSME
90 aa

Figure 4.2

Expression and purification of *Pc*SLAP$_{90}$.

(A) A culture of *E. coli* transformed with the pTrcHisC-*pcslap*$_{270}$ construct was sampled hourly, for seven hours, after induction of expression with IPTG. Samples were centrifuged and pellets (P) and supernatants (S) analysed separately on a 12.5% polyacrylamide gel under reducing conditions. Maximum expression was observed at five hours post-induction (arrow).

(B) The purified *Pc*SLAP$_{90}$ protein was analysed on a 12.5% polyacrylamide gel (reducing conditions) to ensure that no contaminants were present in the preparation.

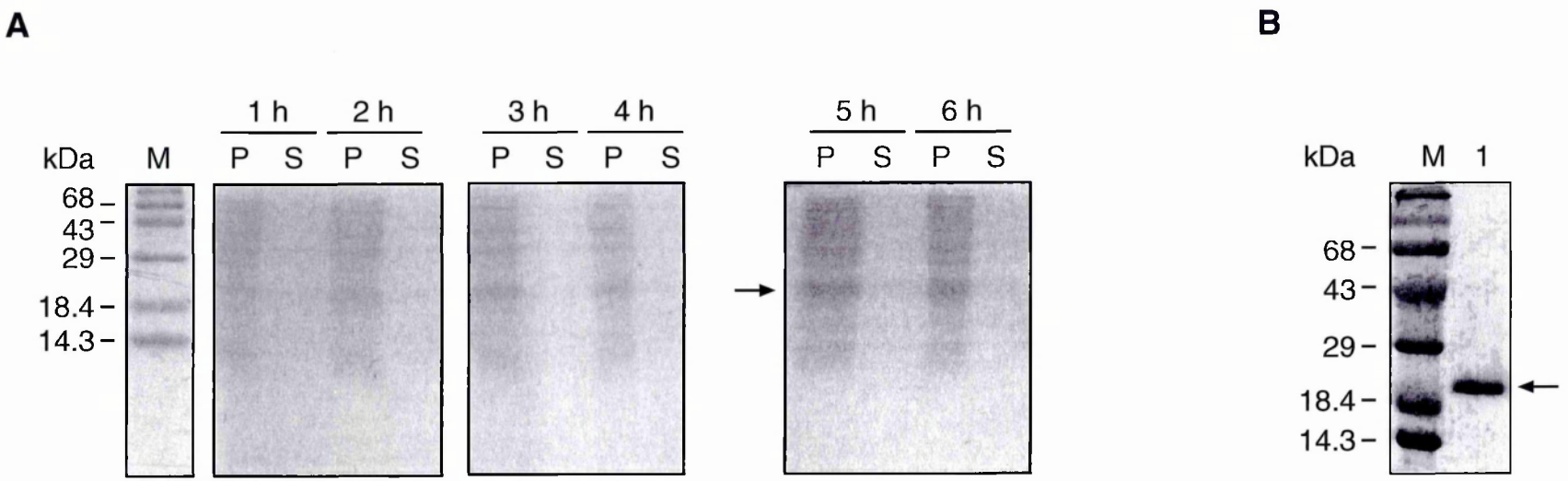

A
1 h
2 h
3 h
4 h
5 h
6 h
kDa
M
P S P S P S P S P S P S
68
43
29
18.4
14.3
B
kDa
M
1
68
43
29
18.4
14.3

Figure 4.3

ELISA to measure levels of anti-*Pc*SLAP antibodies in plasma from immunised mice

Four BALB/c mice (M1- M4) were immunised with $PcSLAP_{90}$ and plasma collected to test for presence of anti-*Pc*SLAP antibodies. The plasma was titrated in duplicate using 2-fold serial dilutions starting at 1:200. Normal mouse plasma (NP) was included as a negative control and diluted identically to the plasmas from the immunised mice. Plasma from mice 1 and 2 were assayed on a separate plate from plasma from mice 3 and 4, hence two normal plasma controls were required (NP control for M1 & M2, and NP control for M3 & M4).

The assay also included duplicate wells of diluent in place of plasma. An average value of the readings from these was used to subtract from the OD_{405}-readings obtained from the immune- and normal plasma samples on the same plate, and is therefore not shown in the figure.

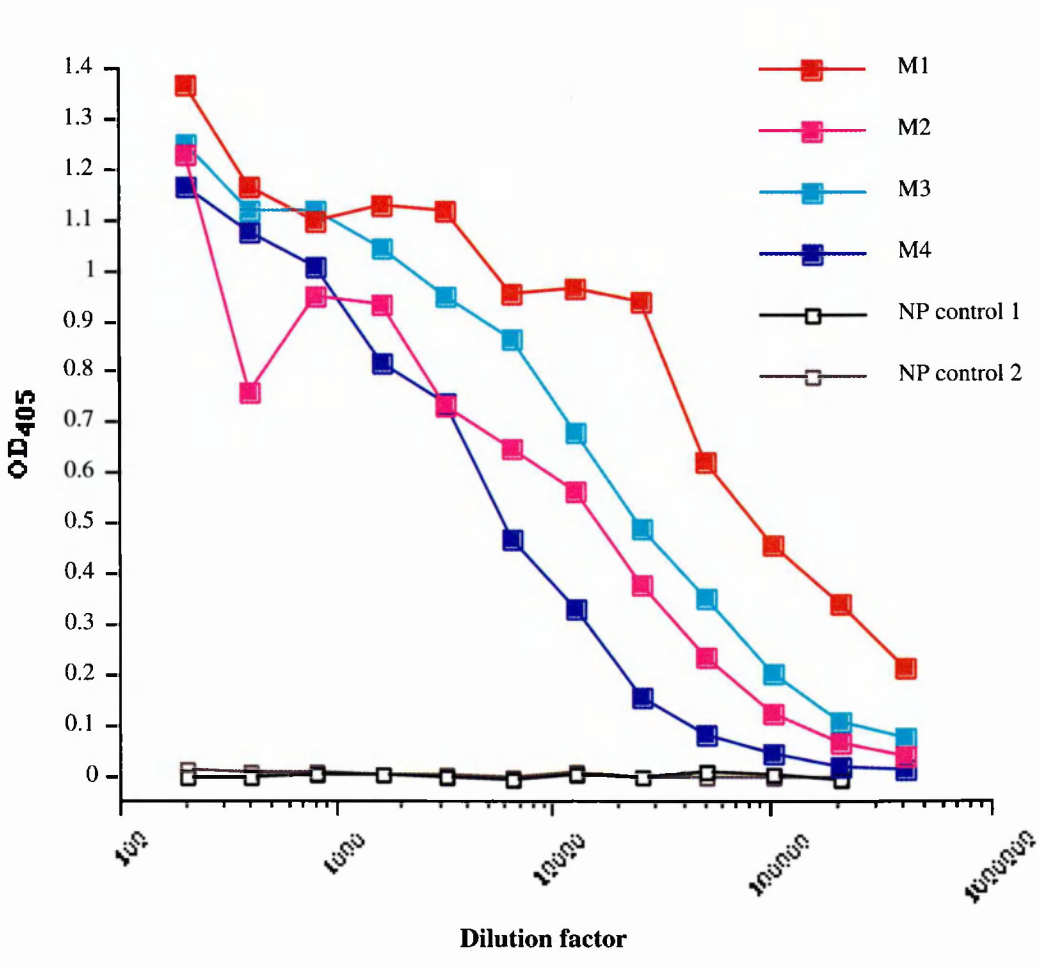

1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD405
M1
M2
M3
M4
NP control 1
NP control 2
Dilution factor

Figure 4.4

ELISA to measure levels of anti-*Pc*SLAP antibodies in sera from immunised rabbits

Two rabbits (419 and 431) were immunised with $PcSLAP_{90}$ and serum collected to test for presence of anti-*Pc*SLAP antibodies. The sera were titrated using 2-fold serial dilutions starting at 1:200. Pre-immune serum from each rabbit, collected before the start of the immunizations, was included as a negative control and diluted identically to the immune sera (419 pre-immune control and 431 pre-immune control).

The assay also included duplicate wells of diluent in place of serum ("blanks"). An average value of the readings from these was used to subtract from the OD_{405}-readings obtained from the immune- and pre-immune sera samples on the same plate, and is therefore not shown in the figure.

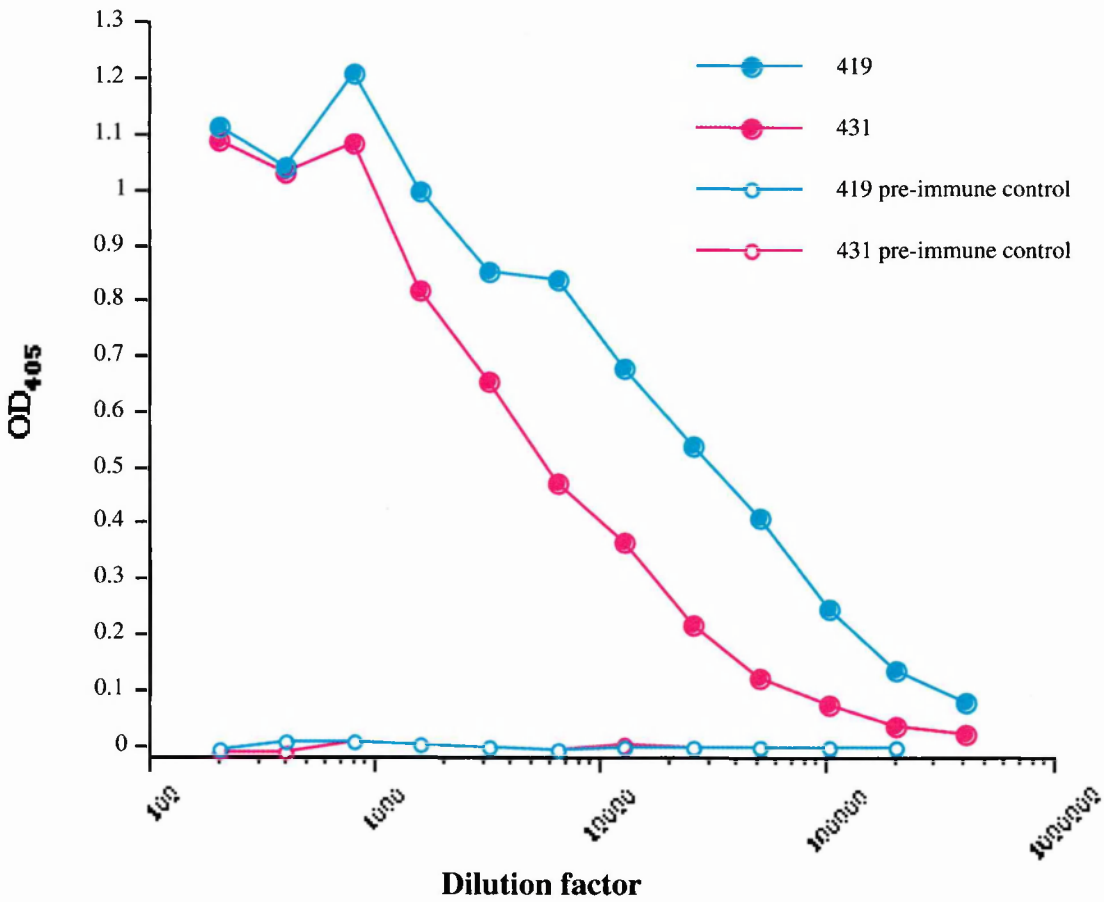

1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD405
419
431
419 pre-immune control
431 pre-immune control
Dilution factor

Figure 4.5

Malaria-specific ELISA to investigate *Pc*SLAP blood-stage expression

The plasmas from the *Pc*SLAP$_{90}$-immunised mice were tested for reactivity with a lysate made from blood-stage *P. c. chabaudi* parasites. The plasmas were titrated in duplicate using two-fold serial dilutions, starting at 1:4. Normal mouse plasma (NP) was included as a negative control and diluted identically to the plasmas from the immunised mice. Plasmas from mice 1 and 2 were assayed on a separate plate from plasmas from mice 3 and 4, hence two normal plasma controls (NP control for M1 & M2, and NP control for M3 & M4), as well as two hyperimmune plasma controls (HIP control for M1 & M2, and HIP control for M3 & M4) were required.

Both plates also included duplicate wells of diluent in place of plasma. An average value of the readings from these wells was used to subtract from the OD_{405}-readings obtained from immune-, hyperimmune- and normal plasma on the same plate, and is therefore not shown in the figure.

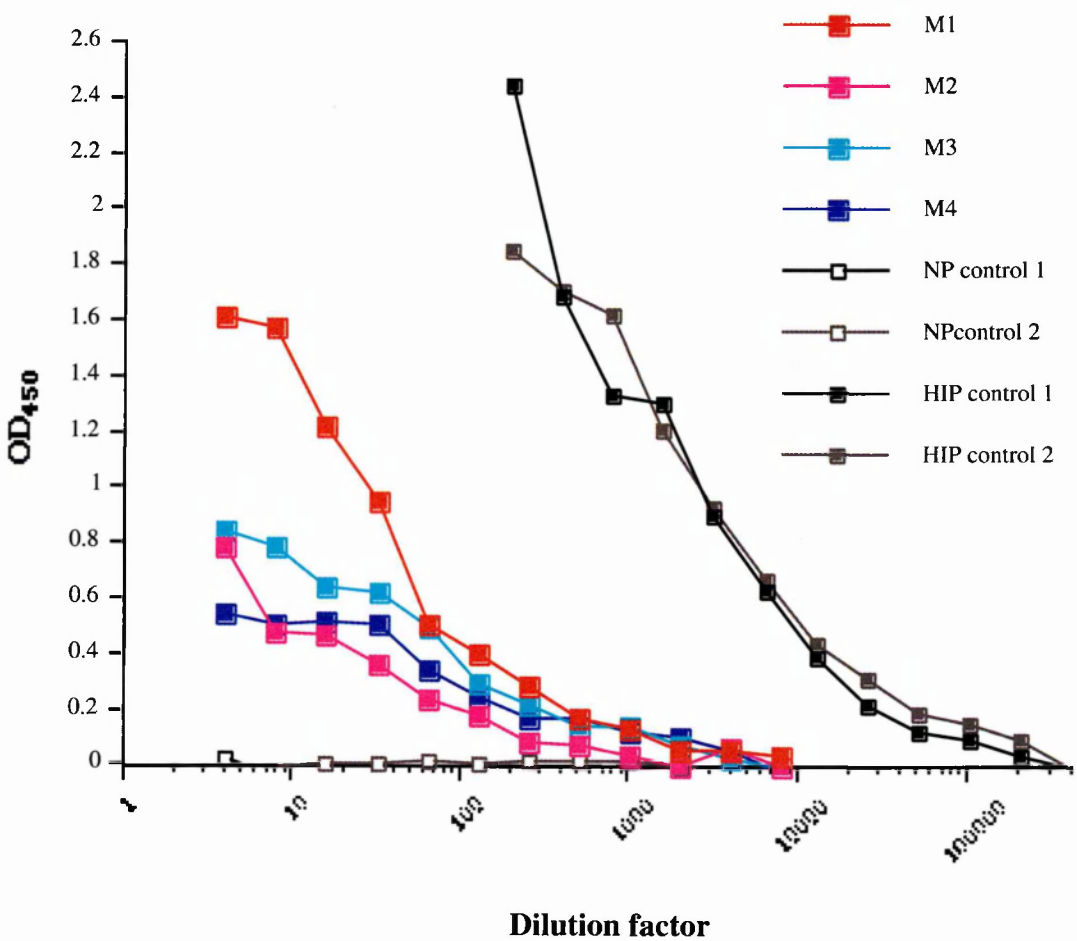

M1
M2
M3
M4
NP control 1
NPcontrol 2
HIP control 1
HIP control 2
OD450
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Dilution factor

Figure 4.6

IFA with fixed *P. chabaudi* blood stage parasites and anti-*Pc*SLAP plasma

Parasitised erythrocytes were stained with DAPI (blue) and the following primary antibodies:

(A) Anti-*Pc*SLAP plasma

(B) Same field as **(A)**, manually increased fluorescence intensity

(C) NIMP23 (anti-MSP-1, mouse IgG), positive control for the assay

(D) Purified mouse IgG, negative control for **(C)**.

(E) Normal mouse plasma, negative control for **(A)**.

(F) Diluent, negative control for the secondary antibody

Goat anti-mouse IgG-FITC (green) was used as secondary antibody on all samples apart from **(C)**, which was directly FITC-conjugated (green). The cells were visualised on an Olympus DeltaVision fluorescence microscope under 1000 x magnification with oil.

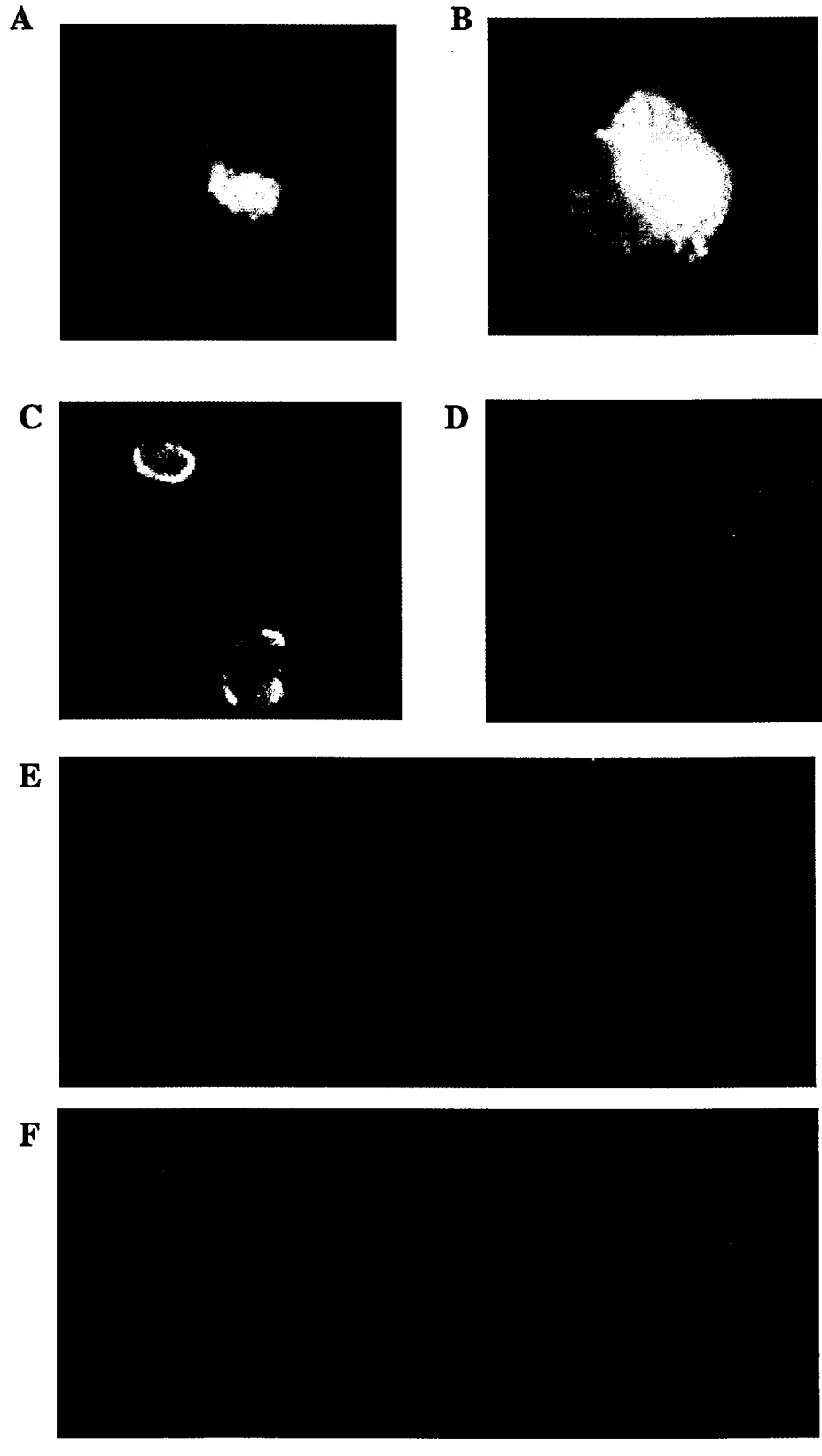

A
B
C
D
E
F

Figure 4.7

***Pc*SLAP staining patterns in IFA with fixed *P. chabaudi* blood stage parasites and anti-*Pc*SLAP plasma**

Staining of parasitised erythrocytes with DAPI (blue) and anti-*Pc*SLAP plasma.

(A) Examples of the different patterns of staining observed with the anti-PcSLAP plasma.

(B) A manual increase in fluorescence intensity visualised the outline of the infected erythrocytes and the distribution of fluorescence within the host cells.

Cells were stained with goat anti-mouse IgG-FITC (green) as secondary antibody and viewed on an Olympus DeltaVision fluorescence microscope under 1000 x magnification with oil.

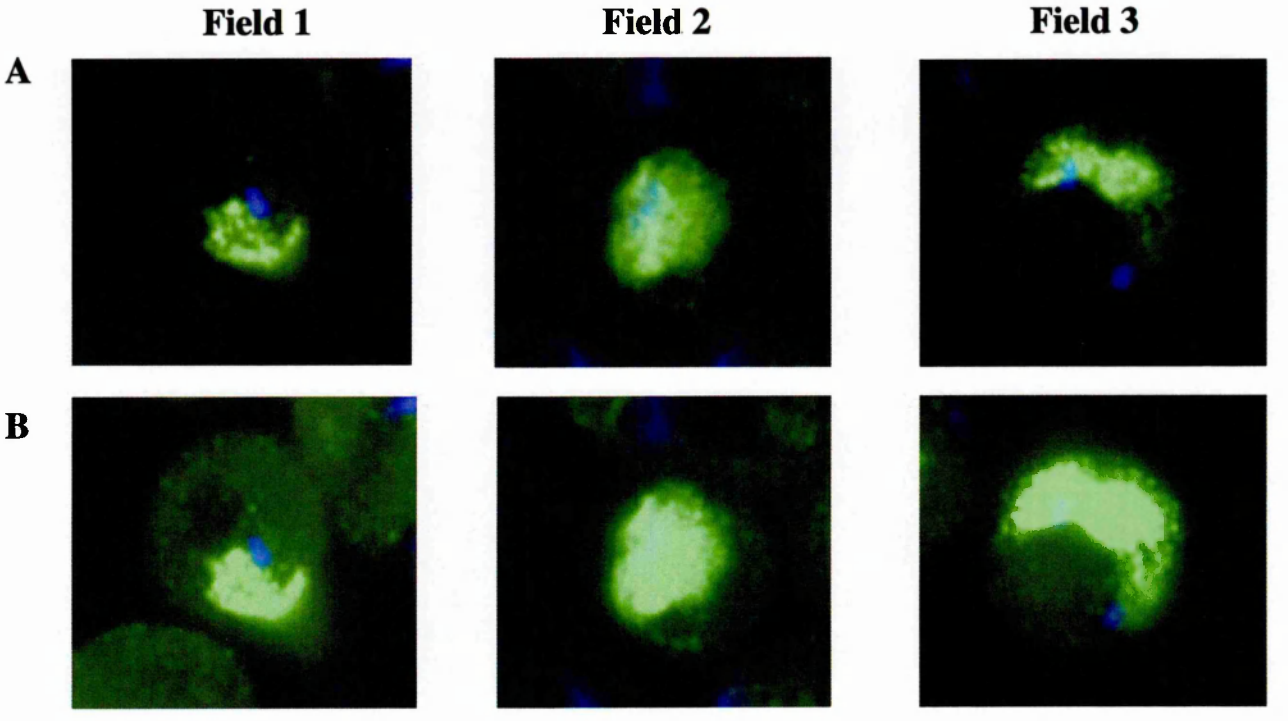

Field 1
Field 2
Field 3
A
B

Figure 4.8

Few parasitised cells stained positive for *Pc*SLAP in IFA

Staining of parasitised erythrocytes with DAPI (blue) and anti-*Pc*SLAP plasma followed by goat anti-mouse IgG-FITC (green). Of the total number of infected cells, as identified by DAPI-staining, very few were also labeled with the anti-*Pc*SLAP plasmas. Approximately 0.3% of the total number of parasitised cells stained positive for *Pc*SLAP. The image shows a representative field with ten infected erythrocytes, one of which stained for *Pc*SLAP.

The cells were viewed on an Olympus DeltaVision fluorescence microscope under 1000 x magnification with oil.

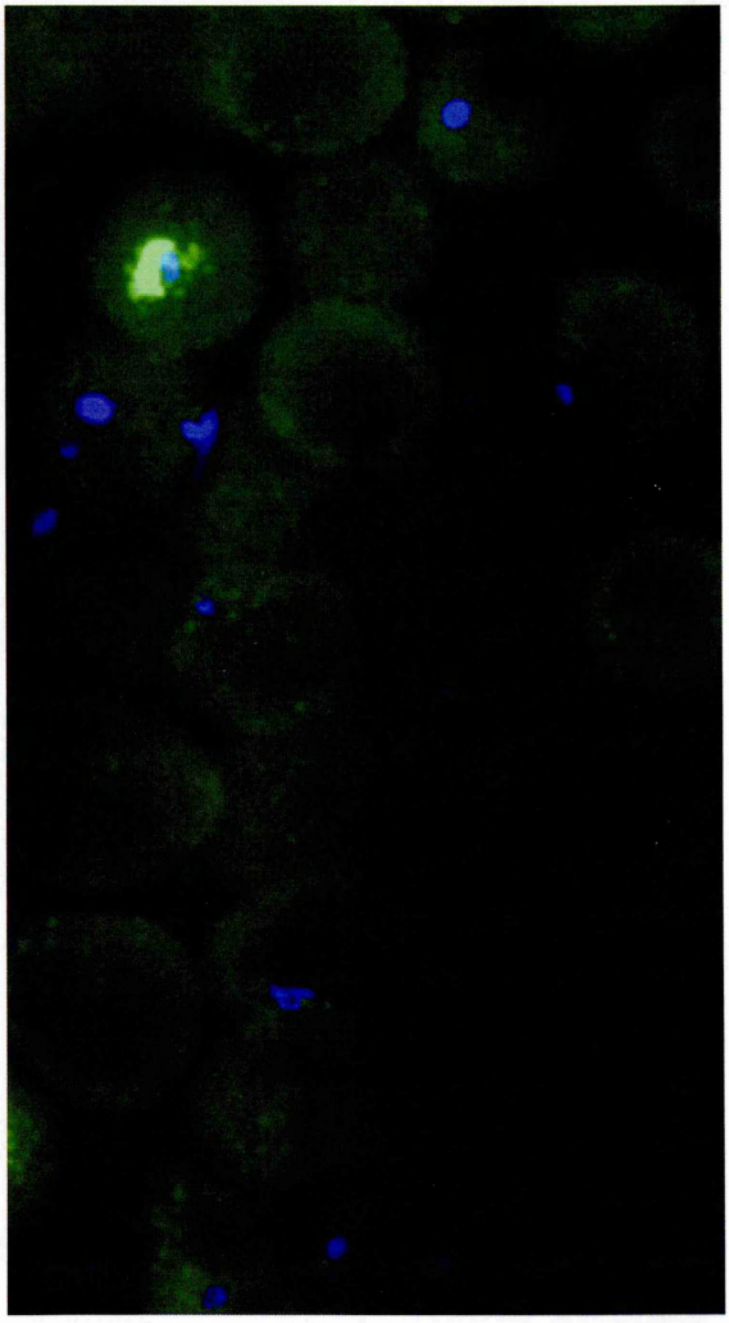


Figure 4.9

***Plasmodium chabaudi chabaudi* (AS) and *Plasmodium berghei* ANKA infections – levels of asexual and gametocyte infected erythrocytes**

Female BALB/c mice were inoculated with 10^6 parasitised erythrocytes from either *P. c. chabaudi* (AS) (top graph) or *P. berghei* ANKA (bottom graph), and the infections followed by microscope analysis of Giemsa-stained thin blood smears for 14 days. The percentages of asexual and sexual-stage parasites in each infection are shown in blue and red, respectively.

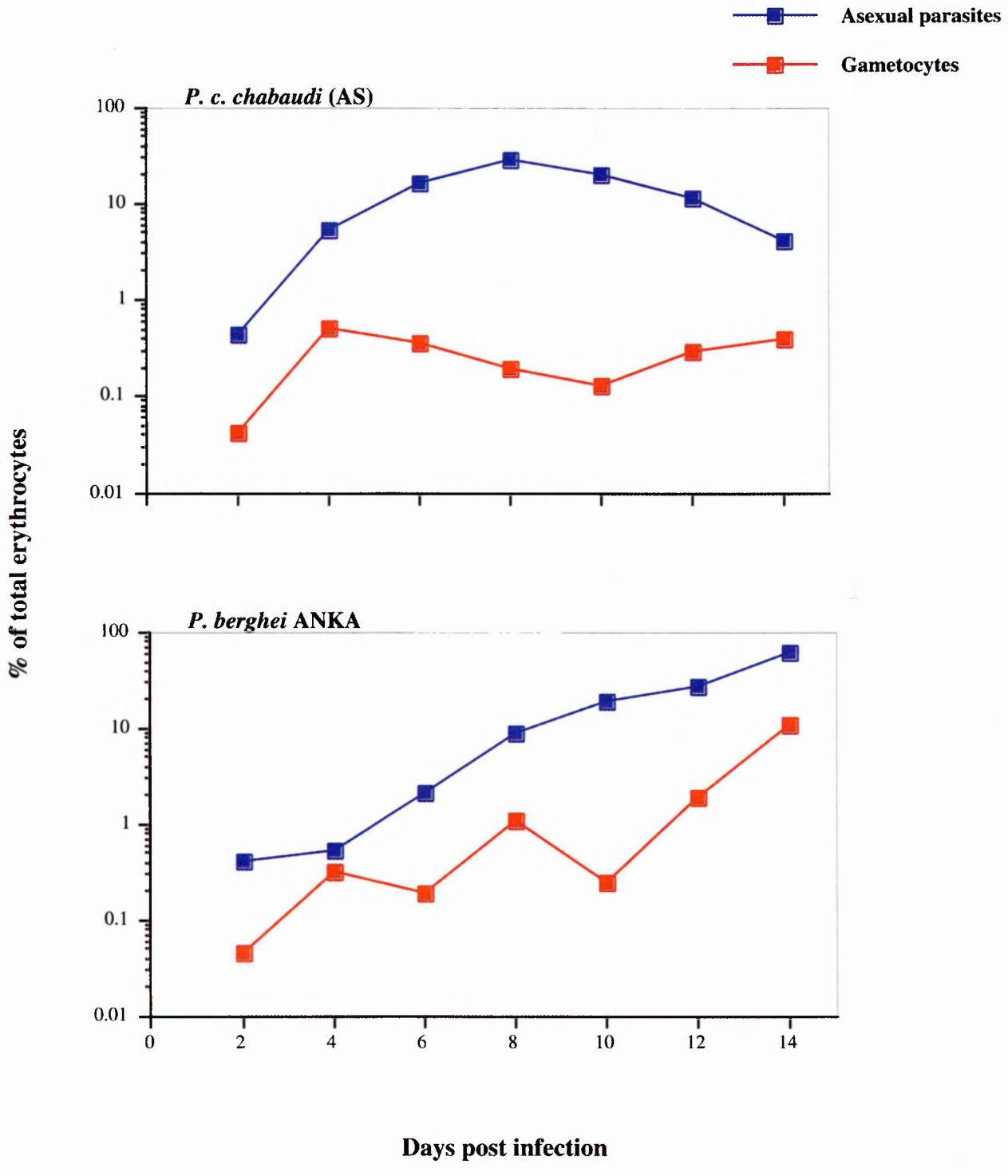

Asexual parasites
Gametocytes
P. c. chabaudi (AS)
P. berghei ANKA
% of total erythrocytes
100
10
1
0.1
0.01
0
2
4
6
8
10
12
14
Days post infection

Figure 4.10

Alignment of *P. chabaudi pcslap*$_{252}$ and the homologous sequence in *P. berghei*

Alignment of *pcslap*$_{252}$ from *P. chabaudi* with the homologous sequence in *P. berghei*, *pbslap*$_{252}$. The comparison was carried out in order to ensure that there was sufficient identity between the two sequences for Northern analysis hybridisation of *pcslap*$_{252}$ to *P. berghei* RNA. The result showed an overall identity of 93.3%, with 17 mismatches out of a total of 252 bases.

Red colour denotes identity between sequences, blue colour denotes difference between sequences.

The alignment was generated with the MegAlign sequence alignment program using the ClustalW algorithm.

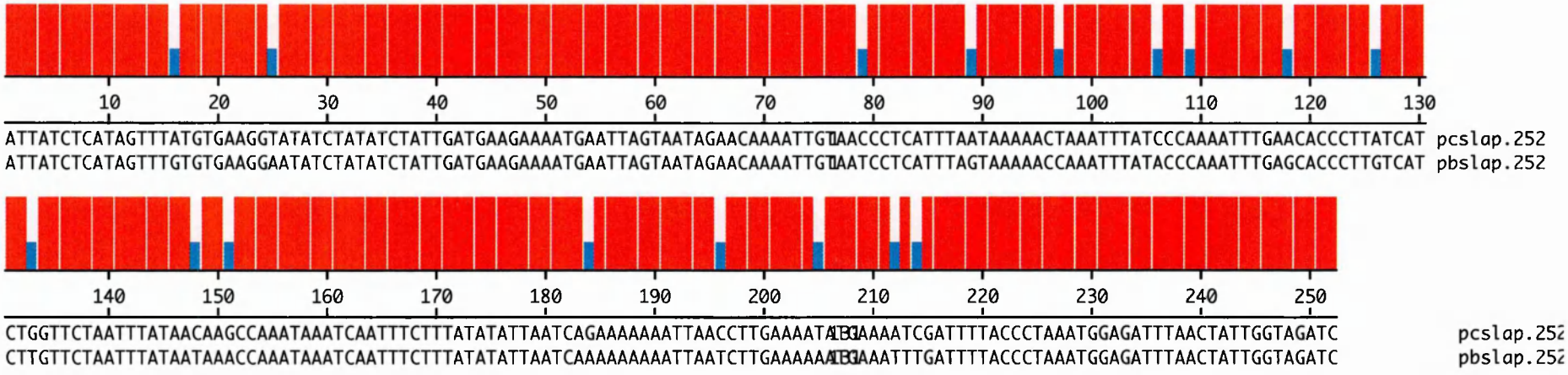

10 20 30 40 50 60 70 80 90 100 110 120 130
ATTATCTCATAGTTTATGTGAAGGTATATCTATATCTATTGATGAAGAAAATGAATTAGTAATAGAACAAAATTGTAACCCTCATTTAATAAAAACTAAATTTATCCCAAAATTTGAACACCCTTATCAT pcslap.252
ATTATCTCATAGTTTGTGTGAAGGAATATCTATATCTATTGATGAAGAAAATGAATTAGTAATAGAACAAAATTGTAATCCTCATTTAGTAAAAACCAAATTTATACCCAAATTTGAGCACCCTTGTCAT pbslap.252
140 150 160 170 180 190 200 210 220 230 240 250
CTGGTTCTAATTTATAACAAGCCAAATAAATCAATTTCTTTATATATTAATCAGAAAAAAATTAACCTTGAAAATATGAAAATCGATTTTACCCTAAATGGAGATTTAACTATTGGTAGATC pcslap.252
CTTGTTCTAATTTATAATAAACCAAATAAATCAATTTCTTTATATATTAATCAAAAAAAAATTAATCTTGAAAAAATGAAATTTGATTTTACCCTAAATGGAGATTTAACTATTGGTAGATC pbslap.252

Figure 4.11

Northern analysis of *pslap* transcription

Total RNA was isolated from whole mouse blood (BALB/c), *P. berghei* ANKA mixed asexual parasites (PbA asex) and gametocytes (PbA gam), and *P. chabaudi* ring-stage parasites (Pc rings), trophozoites (Pc trophs) and schizonts (Pc schiz). An RNA marker was included in the analysis and run in the left-most lane (M).

(A) The RNA was separated on a 1% agarose gel containing guanidine thiocyanate. Both parasite and mouse ribosomal RNA bands are clearly visible (red and black arrows, respectively).

(B) The RNA was transferred to a nylon membrane and probed with $pcslap_{252}$, resulting in a signal at approximately six kilobases.

The radiograph shows the results of one of two experiments with similar results.

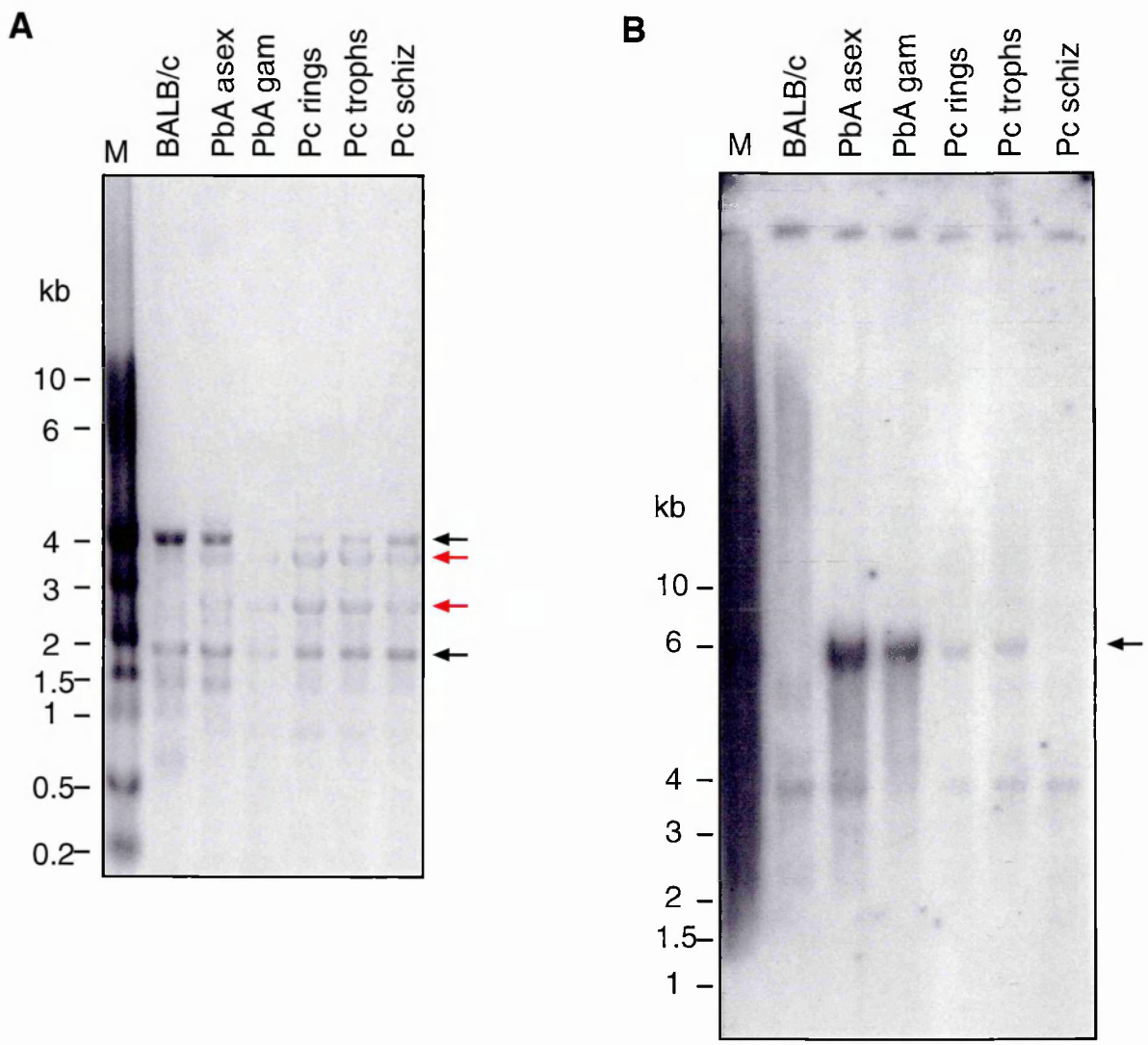

A
M
BALB/c
PbA asex
PbA gam
Pc rings
Pc trophs
Pc schiz
kb
10
6
4
3
2
1.5
1
0.5
0.2
B
M
BALB/c
PbA asex
PbA gam
Pc rings
Pc trophs
Pc schiz
kb
10
6
4
3
2
1.5
1

Figure 4.12

Relative location of *pcslap*$_{252}$ within the *pcslap* gene

The probe used in Northern analysis, *pcslap*$_{252}$, was located between LCCL-2 and LCCL-3. It was chosen so that it would not include regions of the gene coding for any of the identified domains in the *Pc*SLAP protein, so as to avoid hybridization to other proteins than *Pc*SLAP that may carry the same types of domains.

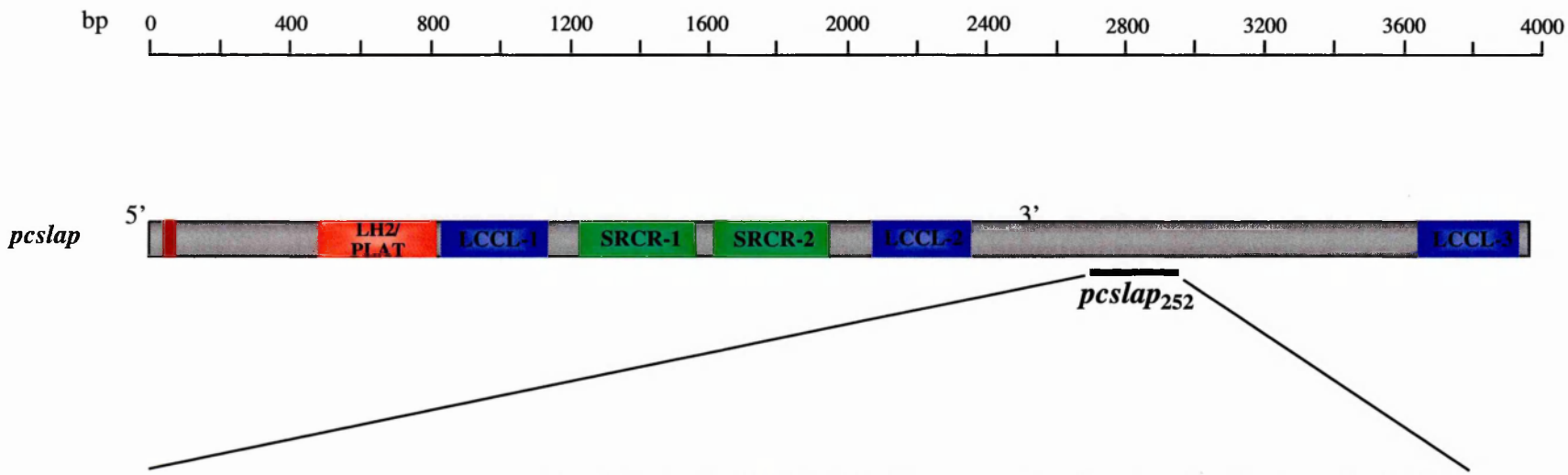

bp
0
400
800
1200
1600
2000
2400
2800
3200
3600
4000
pcslap
5'
3'
LH2/
PLAT
LCCL-1
SRCR-1
SRCR-2
LCCL-2
LCCL-3
pcslap252
ATTATCTCATAGTTTATGTGAAGGTATATCTATATCTATTGATGAAGAAAATGAATTAGTAATAGAACAAAATTGTAACCCTCA
TTTAATAAAAACTAAATTTATCCCAAAATTTGAACACCCTTATCATCTGGTTCTAATTTATAACAAGCCAAATAAATCAATTTC
TTTATATATTAATCAGAAAAAAATTAACCTTGAAAATATGAAAATCGATTTTACCCTAAATGGAGATTTAACTATTGGTAGATC 252 bp

Figure 4.13

Staining of fixed *P. berghei* ANKA blood stage parasites with anti-*Pfs*48/45 monoclonal antibody

Staining of a mixed-stage infection of *P. berghei* ANKA parasites with 85RF.45.3, a monoclonal antibody against the sexual-stage antigen *Pfs*48/45 from *P. falciparum*. Parasitised erythrocytes were stained with DAPI (blue) and the following primary antibodies:

(A) 85RF.45.3 (anti-*Pfs*48/45, rat IgG). Fields 1 and 2 show two examples of the observed pattern of fluorescence

(B) Purified rat IgG, negative control for **(A)**.

(C) Diluent, negative control for the secondary antibody

Goat anti-rat IgG-Alexa®488 (green) was used as secondary antibody. The preparations were visualised on an Olympus DeltaVision fluorescence microscope under 1000 x magnification with oil.

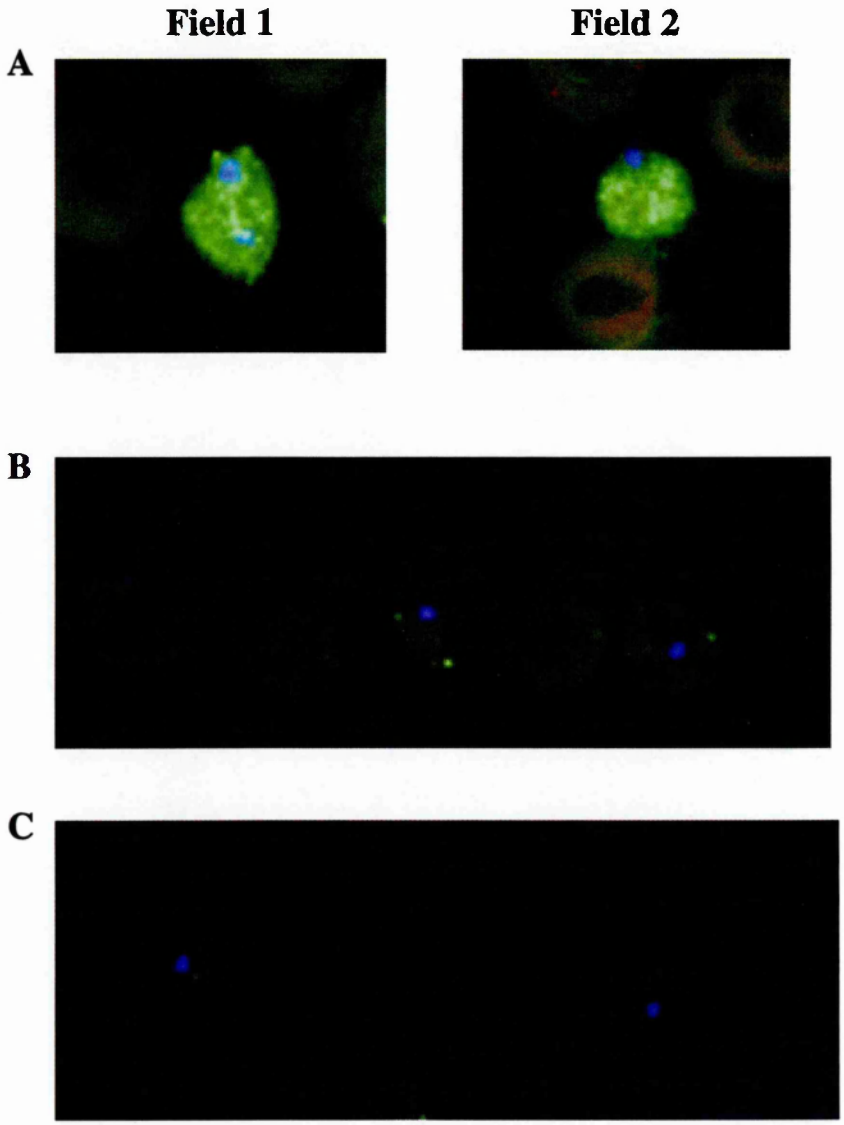

Field 1
Field 2
A
B
C

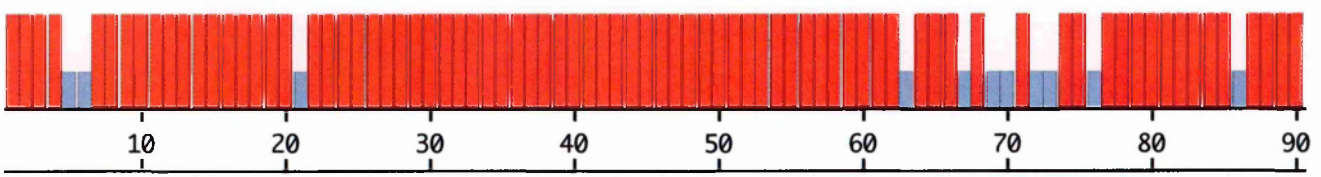
Figure 4.14

Alignment of *P. chabaudi Pc*SLAP$_{90}$ and the homologous sequence in *P. berghei*

An alignment of *Pc*SLAP$_{90}$ and *Pb*SLAP$_{90}$ was perfomed in order to assess the likelihood that a polyclonal plasma raised against the *P. chabaudi* sequence would react with the homologous sequence in *P. berghei*. The comparison showed an overall identity of 88%, with a 41 residue central stretch of complete identity (residues 22-62).

Red colour denotes identity between residues, blue colour denotes difference between residues.

The alignment was generated with the MegAlign sequence alignment program using the ClustalW algorithm.



1 NGFSKQLCSDIAGENLCGHDTERINATNFRCKGDEANLKNCPHETSEDIYCSHEEDIIIGCANAEEDGDASTSSTSISKYGLNKHIMSME PcSLAP.90

1 NGFSHHLCSDIAGENLCGHDKERINATNFRCKGDEANLKNCPHETSEDIYCSHEEDIIIGCASAEEEGNDSANSTNISKYGLNKHMMSME PbSLAP.90

Figure 4.15

IFA with fixed *P. berghei* ANKA blood stage parasites and anti-*Pc*SLAP plasma

P. berghei parasitised erythrocytes were tested for reactivity with the anti-*Pc*SLAP plasmas raised to the *P. chabaudi* $PcSLAP_{90}$ sequence. The cells were stained with DAPI (blue) and the following primary antibodies:

(A) Fields 1-3 show examples of the different patterns of fluorescence observed with the anti-*Pc*SLAP plasma
(B) Same fields as in **(A)**, manually increased fluorescence intensity
(C) Normal mouse plasma, negative control for **(A)**.
(D) Diluent, negative control for the secondary antibody

Goat anti-mouse IgG-Alexa®594 (red) was used as secondary antibody. The cells were viewed on an Olympus DeltaVision fluorescence microscope under 1000 x magnification with oil.

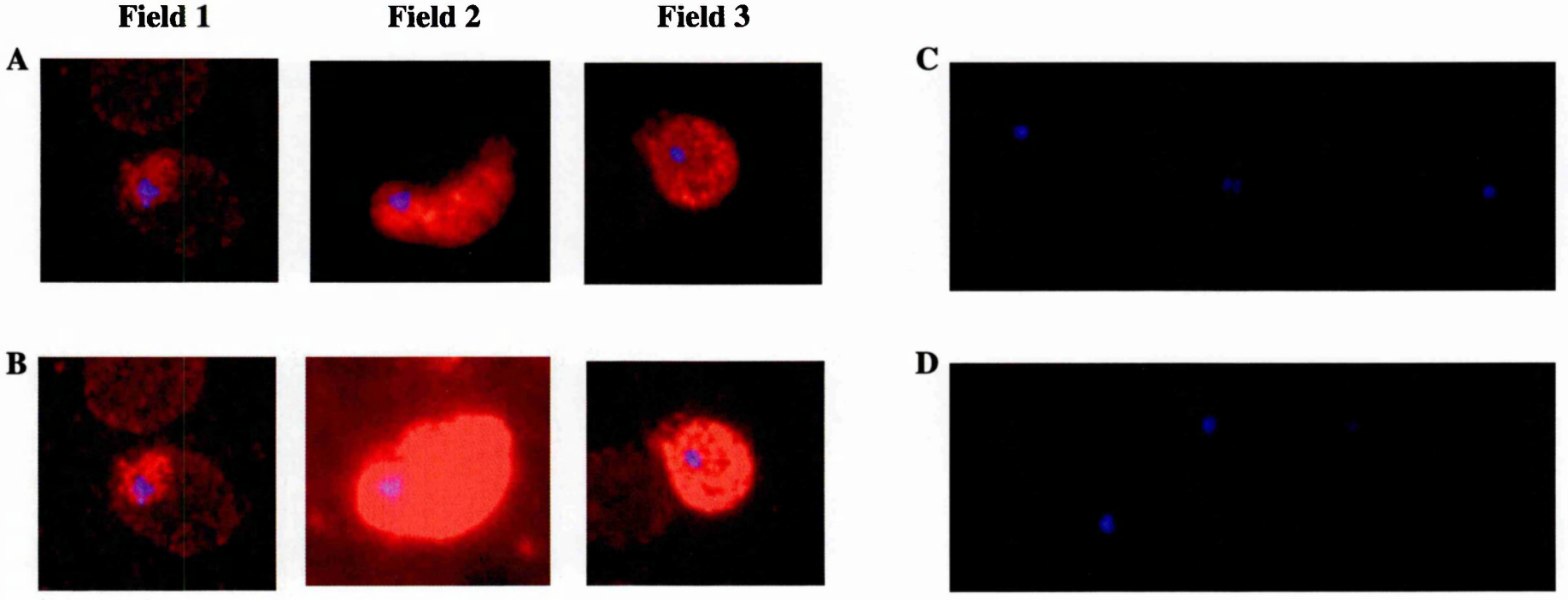

Field 1
Field 2
Field 3
A
B
C
D

Figure 4.16

***P. berghei* ANKA gametocyte preparations used in IFA**

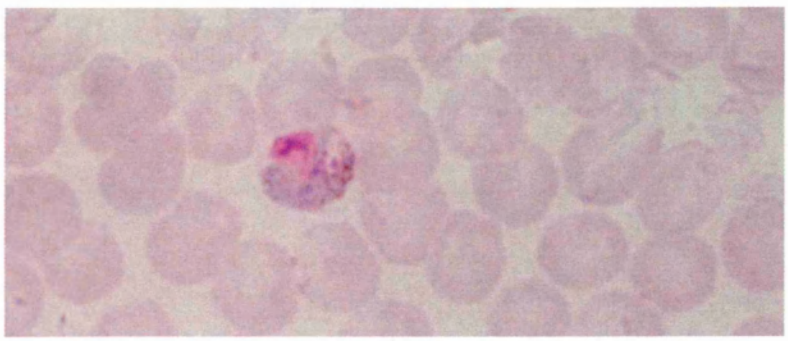
P. berghei ANKA-infected mice were treated with 10 milligram pyrimethamine per kilo bodyweight on four consecutive days, for elimination of asexual parasites in order to obtain an infection with only gametocytes. Blood was subsequently collected and thin blood films made to be used in indirect immunofluorescence assays. Smears were also stained with Giemsa to assess the quality of the preparations. Two independent preparations were made and representative images are shown in **(A)** and **(B)**, respectively.

(A) Giemsa-staining of Preparation 1. A female gametocyte can be seen at the center of the image.

(B) Giemsa-staining of Preparation 2. A male gametocyte is positioned at the center of the image.

The images were acquired using a Nikon Coolpix 5000 digital camera and the bright field setting on an Olympus DeltaVision fluorescence microscope, at 1000 x magnification with oil.

A



B

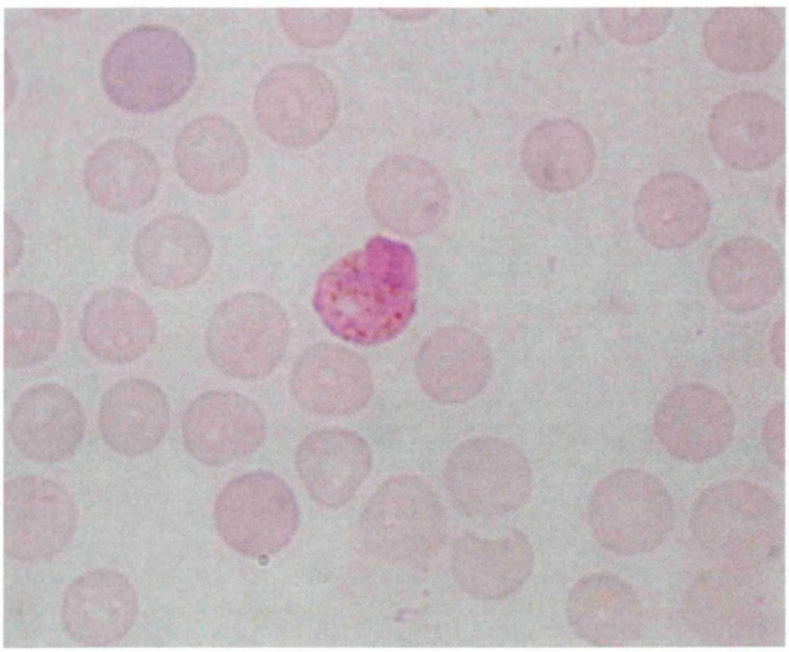


Figure 4.17

Co-localisation IFA using anti-*Pc*SLAP plasma and the 85RF.45.3 anti-*Pf*s48/45 antibody

P. berghei gametocytes were double stained with the anti-*Pc*SLAP plasma (red, left-hand side panels) and the 85RF.45.3 monoclonal antibody against *P. falciparum* sexual-stage protein *Pf*s48/45 (green, center panels). A merging of the staining produced with the two antibodies demonstrated that the labelled proteins are expressed in the same cells but with distinct patterns (right-hand side panels). Fields 1 and 2 show two examples of the observed fluorescence.

Goat anti-mouse IgG-Alexa®594 (red) and goat anti-rat IgG-Alexa®488 (green) were used as secondary antibodies. The cells were viewed on an Olympus DeltaVision fluorescence microscope under 1000 x magnification with oil.

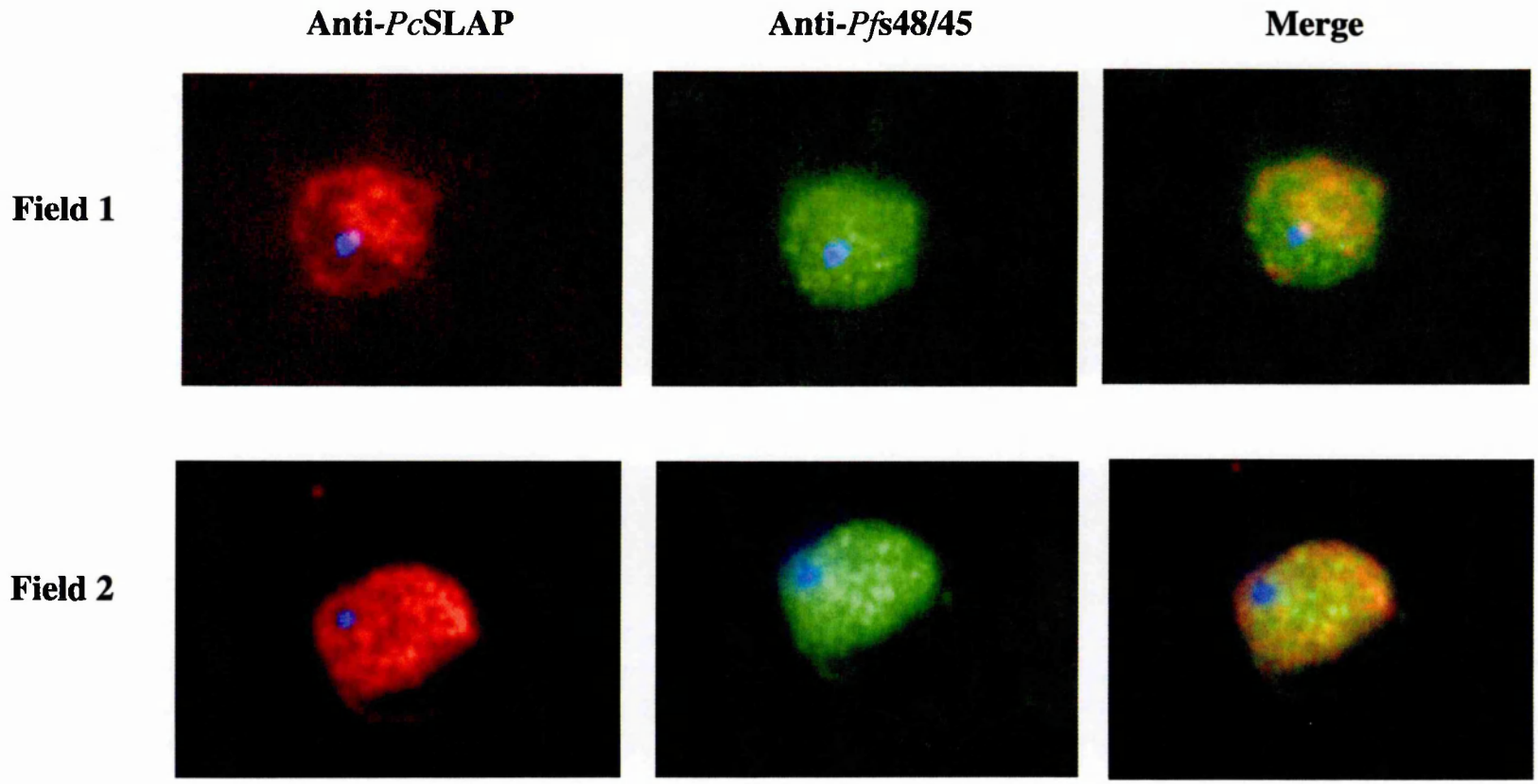

Anti-*Pc*SLAP
Anti-*Pfs*48/45
Merge
Field 1
Field 2

Figure 4.18

Sequences of *Plasmodium falciparum* SLAP used in immunizations for generation of polyclonal antisera

Three sequences, marked by black bars below the full protein sequence, corresponding to the structural motifs LCCL-1, LCCL-2 and SRCR-1 of *P. falciparum* SLAP, were expressed in an *E. coli* expression system. Following purification, the proteins were used to immunize five BALB/c mice for production of polyclonal antibodies.

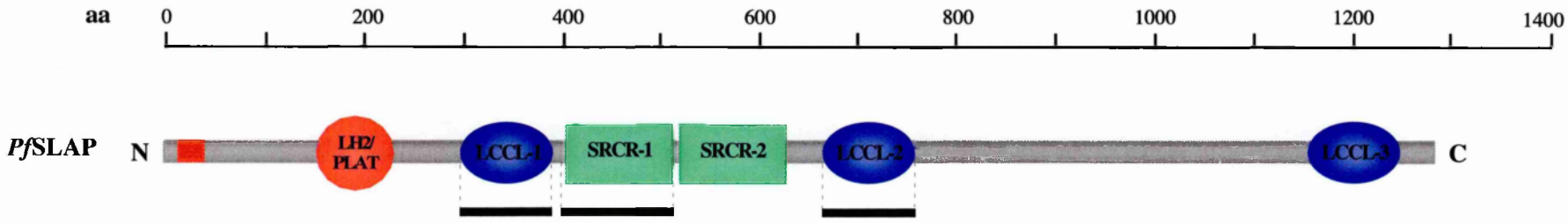

aa
0
200
400
600
800
1000
1200
1400
PfSLAP
N
LH2/
PLAT
LCCL-1
SRCR-1
SRCR-2
LCCL-2
LCCL-3
C

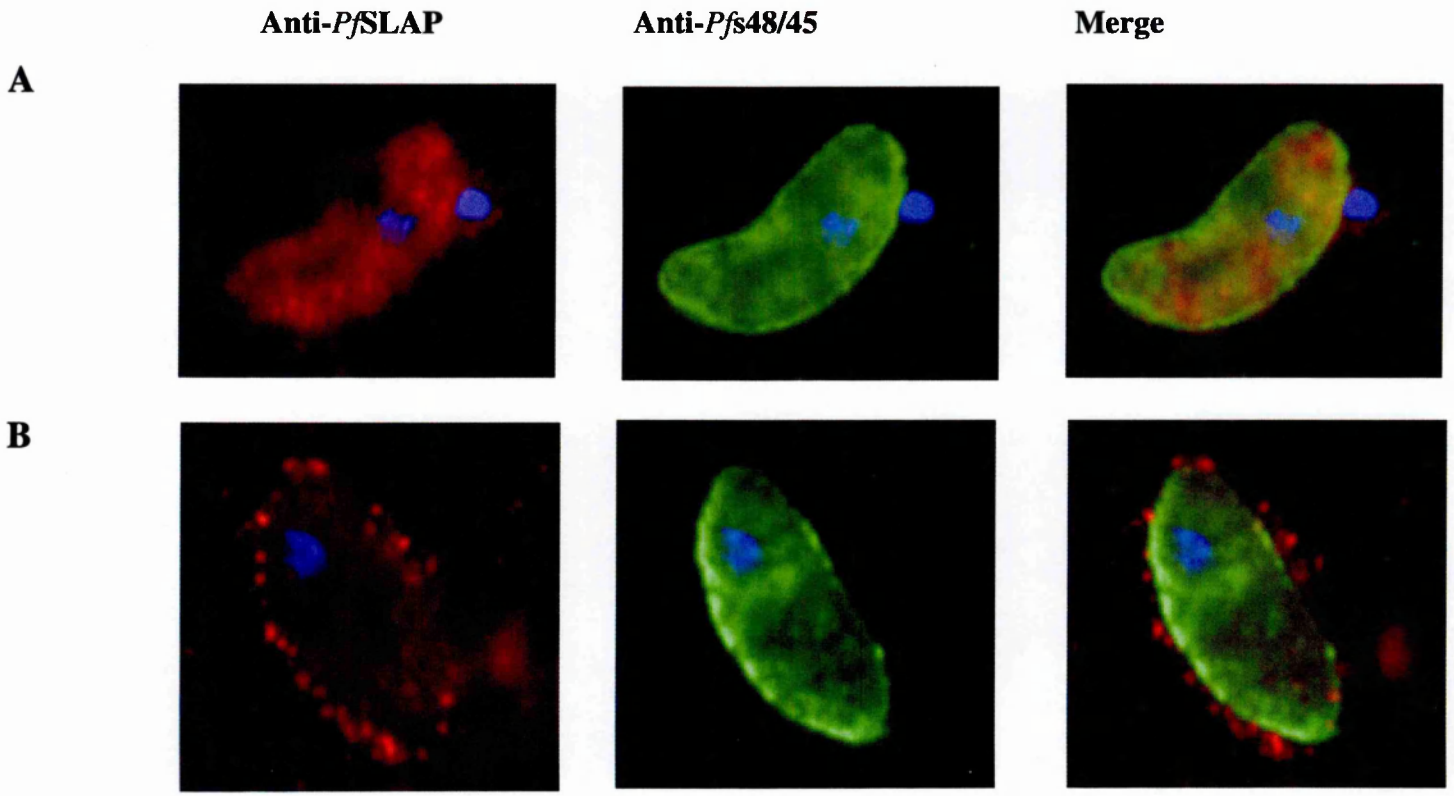
Figure 4.19

Co-localisation IFA using *Pf*SLAP antisera and the 85RF.45.3 anti-*Pf*s48/45 antibody

P. falciparum gametocytes were double stained with the *Pf*SLAP antisera (red, left-hand side panels) and the 85RF.45.3 monoclonal antibody against *P. falciparum* sexual-stage protein *Pf*s48/45 (green, center panels). A merging of the patterns produced with the two antibodies demonstrated that the labelled proteins are expressed in the same cells (right-hand side panels).

(A) *Pf*SLAP anti-serum against LCCL-1
(B) *Pf*SLAP anti-serum against SRCR-1

Goat anti-mouse IgG-Alexa®594 (red) and goat anti-rat IgG-Alexa®488 (green) were used as secondary antibodies. The cells were viewed on an Olympus DeltaVision fluorescence microscope under 1000 x magnification with oil.


Anti-*Pf*SLAP
Anti-*Pf*s48/45
Merge
A
B

Chapter Five

Disruption of the *pslap* gene in *Plasmodium berghei* ANKA

5.1 Introduction

A useful approach to study the function and importance of a particular gene is to subject it to genetic manipulation. The techniques needed to do this have been available for many years for organisms such as the bacterium *Escherichia coli* and the yeast species *Saccharomyses cerevisiae*. However, protocols for genetic modification of *Plasmodium spp.* have been particularly difficult to develop for a number of reasons. One is the inaccessibility of the parasite nuclear material, due to the presence of four membranes separating the external environment from the intra-erythrocytic parasite nucleus. This is believed to be a major factor contributing to the inefficiency of *Plasmodium* transfections, as compared with other systems. The difficulty of *in vitro* culturing of many of the parasite's developmental stages, and cloning problems due to the extreme AT-richness of Plasmodial DNA, are another two.

However, efforts made to overcome these problems eventually resulted in the first successful transfection of a malaria parasite in 1993, using the avian species *Plasmodium gallinaceum* (Goonewardene *et al.*, 1993, transient transfection and expression of firefly luciferase in gametes and zygotes). This was soon followed by another two laboratories simultaneously, but independently of each other, succeeding in transfecting *Plasmodium falciparum* (Wu *et al.*, 1995, transient transfection and expression of bacterial chloramphenicol acetyltransferase in ring-stage parasites and schizonts) and *Plasmodium berghei* (van Dijk *et al.*, 1995, stable transfection of *P. berghei dhfr-ts* gene in merozoites). Since then, stable genetic transformation has been achieved in the simian parasites *Plasmodium knowlesi* and *Plasmodium cynomolgi* (van der Wel *et al*, 1997; Kocken *et al*, 1999, both transfection of *T. gondii dhfr-ts* gene in schizonts), and most recently, also in the rodent species *Plasmodium yoelii* (Mota *et al*, 2001, transfection and expression of green fluorescent protein, and disruption of *thrombospondin-related anonymous protein* gene).

Plasmodium transfection experiments have since provided insights into a number of areas including promoter structures, reporter gene expression, the requirements for stage-specific localisation of malaria proteins, organelle targeting and imunomodulation by parasite expression of host cytokines (Crabb *et al.*, 1997; de Koning-Ward *et al.*, 1999; de Koning-Ward *et al.*, 1998; Margos *et al.*, 1998; Kocken *et al.*, 1998; Waller *et al.*, 1998; Ozwara *et al.*, 2003).

Genetic manipulation is the alteration (mutation, substitution or disruption) of a specific sequence in the genome of an organism. It mostly relies on homologous recombination between two identical sequences, one present in the incoming DNA and the other in the genome. For selection of successfully transformed parasites, a transfection construct also contains a selectable marker gene. Most constructs used for *Plasmodium* gene targeting carry a pyrimethamine resistant form of the *Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) gene (Waters *et al.*, 1997). The DHFR enzyme catalyses reduction of dihydrofolate to tetrahydrofolate, the latter of which is required for single carbon transfer reactions in the synthesis of purines. Pyrimethamine is a folate antagonist that binds to the DHFR-TS protein and inhibits its reductase activity, thereby causing disruption of DNA synthesis (Ferone *et al*, 1977). However, two point mutations in the *T. gondii dhfr* gene result in the amino acid changes $Ser^{36}\prod Arg$ and $Thr^{83}\prod Asn$, and an enzyme that is highly resistant to pyrimethamine (Donald & Roos, 1993). The *T. gondii dhfr-ts* gene is preferred over *Plasmodium dhfr-ts* genes because it reduces the chances of unwanted recombination with the endogenous copy of the *dhfr-ts* gene. The *T. gondii dhfr-ts* gene also appears to confer a 10- to 100-fold higher resistance to pyrimethamine than the *P. berghei* homolog, and when co-administered into *P. knowlesi* parasites, the *T. gondii dhfr-ts* constructs were preferentially selected for *in vivo* (van der Wel, *et al.*, 1997). To ensure efficient and reliable expression from this heterologous gene once it is inside the *P. berghei* parasite, it is placed under the control of the *P. berghei dhfr-ts* 5′- and 3′ un-translated regions (UTRs), containing promoter sequences and transcriptional stop signals, respectively (Waters *et al.*, 1997).

Two types of transfection constructs have been used for *Plasmodium* transfections,

insertion constructs and replacement constructs (Figure 5.1). The difference between the two is in the length of the inserted sequence and the route of entry into the genome. The insertion vector contains just one target sequence that integrates through a single cross-over event, inserting the entire plasmid into the target locus (Ménard and Janse, 1997). Although reports from several eukaryotic systems suggest that this vector type integrates at a higher frequency than replacement constructs, the integration is reversible and therefore not very stable (Hasty *et al.*, 1991). This is due to the fact that no gene sequence is lost in the integration, resulting in two identical copies of the target sequence being left in the transfected parasite (Figure 5.1, left schematic). These can pair up and recombine again and thereby exclude the construct sequence in the same way it was once inserted. The replacement construct, on the other hand, carries two sequences from the gene of interest and so requires a double cross-over for integration. No plasmid backbone is integrated with this type of vector (Figure 5.1, right schematic). Once replacement has taken place, the construct sequence is permanently inserted since the wild-type sequence has been removed and lost (Ménard and Janse, 1997).

The parasite maturation stage used for transfection is the bloodstage merozoite. The asexual blood-stages are not only of most clinical relevance but are also the phase in the life cycle where the parasite population undergoes the most rapid expansion. The cycle of invasion-development-**multiplication**-release-invasion allows for selection and growth of very few individuals up to detectable levels within a short period of time. The merozoite is a suitable target cell for several reasons: it is no longer dependent on its host cell for growth and therefore damage to the erythrocyte membrane caused by electroporation is not a problem, it appears to withstand the stress from electroporation well without losing viability, and it is easy to collect from cultures in relatively large quantities due to the fact that rodent malaria parasites do not re-invade uninfected erythrocytes when cultured *in vitro*, but instead halt at the mature schizont stage (Janse and Waters, 2002).

The aim of the work described in this chapter was to disrupt the *pslap* gene. *P*SLAP is, because of its multi-domain composition and high degree of inter-species conservation, without a doubt an interesting novel molecule. As has been shown previously by us, *Plasmodium falciparum* SLAP is expressed in blood stage gametocytes and has been proposed as a potential candidate for a transmission blocking vaccine (Delrieu *et al.*,

2002). Disrupting the gene would show whether SLAP is essential for parasite survival .If this is not the case, a rodent model would allow studies on the interactions between parasite and host, such as parasite growth and development in both the vertebrate host and the insect vector.

As there is no protocol available for transfections of *Plasmodium chabaudi*, it was decided to attempt disruption of the gene in *Plasmodium berghei* ANKA. Transfection and selection procedures described in the present chapter follow the protocol outlined by Waters *et al.* (1997; Section 2.13).

5.3 Results

5.3.1 Susceptibility of *Plasmodium berghei* ANKA to pyrimethamine

In order to assess the susceptibility to pyrimethamine of the strain of *Plasmodium berghei* ANKA that was to be used for the transfection experiments, a drug sensitivity test was performed. The dose of ten milligram pyrimethamine per kilogram bodyweight of host animal was used, as this is standard in transfection experiments using *P. berghei* ANKA (refs). Three groups of four BALB/c mice were infected with 10^6 *P. berghei* infected erythrocytes each. On day 1 of infection (24 hours post infection), one of the groups of mice was treated with pyrimethamine (ten milligram per kilogram bodyweight), one group was injected with saline and one group left untreated (Section 2.13.8). These treatments were repeated on days 2, 3 and 4, and the parasitemia followed daily in all mice by Giemsa-stained thin blood smears. Non-pyrimethamine treated animals were monitored for 12 days after which they were culled due to sickness, whereas drug-treated mice were monitored for an additional two days.

The experiment showed that the parasites were highly susceptible to pyrimethamine. A few parasitised reticulocytes were detected in the pyrimethamine treated animals on days two and three (up to 0.2% parasitemia), however, all asexual stage parasites had been eradicated by day four (Figure 5.2, top graph). In contrast, all animals that received saline or no injections had parasitemias of 2% or higher at the same time (Figure 5.2, center and bottom graphs, respectively). Drug-treated animals were parasite free up until day ten, while saline injected and non-treated mice had parasitemias of between 13 and 35% on the same day, with a continued rise in parasitemia up to levels of 50% by day 12.

The result of the assay demonstrated susceptibility of *P. berghei* ANKA to pyrimethamine at a dose of ten milligram per kilogram bodyweight. However, the emergence of parasites in treated animals by day ten and after showed that the drug did not eradicate the parasites completely.

5.3.2 Transfection vectors to disrupt *pbslap*

Two transfection vectors were constructed for the subsequent disruption of *pbslap*, one insertion vector and one replacement vector, designated pSLAPIns and pSLAPRep, respectively. Both vectors were made from the pBSK-*dhfr-ts* plasmid, a pBluescript SK II (+) backbone (Stratagene) with the DHFR-TS transcription unit cloned in between the

HindIII and EcoRI restriction sites in the multiple cloning cassette (Figure 5.3; Section 2.13.1).

For the insertion vector, pSLAPIns, a 911 basepair fragment corresponding to the sequence between bases 835 and 1745 of *pbslap* was used. The pSLAPRep replacement construct carried fragments corresponding to the sequences from position 439 to position 1298 (Fragment 1), and from position 1300 to 1640 (Fragment 2) in the *pbslap* gene (Figures 5. 4 and 5.5). All three fragments were amplified using the high fidelity Pfx system, as, although not known for *Plasmodium*, it has been shown in mammalian systems that even a single nucleotide mismatch between target gene and target construct can decrease the efficiency of recombination dramatically (Deng *et al*, 1992, teRiele *et al.*, 1992).

5.3.2.1 Isolation of *Plasmodium berghei* ANKA genomic DNA

The *pbslap* gene fragments for the transfection vectors were to be amplified by PCR from genomic DNA (gDNA) from *P. berghei* ANKA, the same strain that was going to be used for the transfections. Therefore, female BALB/c mice were inoculated with *Plasmodium berghei* infected erythrocytes, blood collected at a parasitemia of approximately 30% and parasite gDNA extracted (Section 2.4.1). To test the DNA, it was used as a template in a PCR with the forward and reverse primers designed to amplify the insertion vector fragment (SLAPInsForw and SLAPInsRev, respectively; Table 2.7, Section 2.13.1). The result was analysed by gel electrophoresis and showed a band at approximately 911 bases (Figure 5.6). This product was blunt-cloned into the pCR4Blunt-TOPO sequencing vector (Invitrogen), sequenced and found to have the correct sequence of the *P. berghei slap* gene (data not shown).

5.3.2.2 pSLAPIns insertion construct

Since the sequence of the cloned Insertion vector fragment (InsF) had already been amplified and verified, it was used for the subsequent construction of the pSLAPIns vector. The pCR4Blunt-TOPO vector cloning site had two flanking EcoRI sites, which were used to excise the fragment and re-clone it into the EcoRI site in the pBSK-*dhfr-ts* vector (Figure 2.1, Figure 5.7, Section 2.13.2). The pSLAPIns vector was propagated,

purified and the sequence confirmed, initially by restriction digestion and later by sequencing (Figures 5.8 and 5.9).

5.3.2.3 pSLAPRep replacement construct

Two sets of primers were designed in order to amplify Fragments 1 and 2 for the replacement construct. SLAPRepF1 and SLAPRepR1 were used to generate Fragment 1, and SLAPRepF2 and SLAPRepR2 to obtain Fragment 2 (Table 2.7). Restriction sites were added onto the 5′ ends of the primers to allow directional cloning into the pBSK-*dhfr-ts* vector: ApaI and HindIII sites were added to the forward and reverse primers, respectively, for Fragment 1; and EcoRI and XbaI sites to the forward and reverse primers, respectively, for Fragment 2 (Table 2.7).

The two fragments were amplified by PCR and the products analysed by gel electrophoresis (data not shown). The sequences were cloned into the pBSK-*dhfr-ts* vector sequentially, starting with Fragment 2, followed by Fragment 1 (Section 2.13.3; Figure 5.10). Restriction digests and vector sequencing confirmed that the correct fragments had been cloned (Figures 5.11, 5.12 and 5.13).

5.3.3 Parasite transfection and selection procedures

The transfection and selection procedures used in the present work follow the protocol by Waters *et al.* (1997). However, with valuable advice from Dr. R. Tewari (Imperial College, London) some amendments were made, due to the use of mice throughout the experiment rather than rats (Section 2.13).

Briefly, it was agreed that three to four mice be used in place of one rat, as is suggested in the standard protocol. Although this is not an equivalence in terms of blood volume, *P. berghei* infections reach higher parasitemias in mice than in rats, and thus the same number of parasites were obtained this way. Therefore, four female BALB/c mice were inoculated with 10^6 *P. berghei*-infected erythrocytes five days prior to transfection (Day 0). The day before electroporation, at 15-20% parasitemia, the animals were culled and the blood collected. A "donor" culture was set up and incubated overnight to allow all parasites to develop into schizonts. To prepare the transfection vectors for electroporation, 50 μg of construct was digested overnight: pSLAPRep with ApaI and

XbaI, to release the transfection part of the vector (the DHFR-TS unit and the flanking *pbslap* sequences) from the plasmid backbone, and pSLAPIns with AvaII, to produce a single cut for linearisation. The following day, the maturation of the culture was carefully followed by Giemsa-stained thin blood films. When the majority of parasites had matured into schizonts, the culture was harvested, the parasitised cells purified on a Nycodenz density gradient and mixed with either of the digested transfection vectors. The mixture was electroporated by delivery of a single electric pulse, divided into two equal aliquots and injected intravenously or intraperitoneally into two female BALB/c mice (Figure 5.14).

In order to allow the parasites to complete one cycle of asexual division in the absence of drug pressure, pyrimethamine treatment was initiated on day one (24 hours) post-injection, and continued on days two, three and four (48, 72 and 96 hours), at ten milligram per kilo bodyweight. At the beginning of the selection, asexual parasites were frequently observed, however, as the drug started to take effect, only gametocytes were detected. The growth of the parasites were monitored daily by Giemsa-stained thin blood smears. When parasites emerged they were passaged to new mice for a second round of selection (Figure 5.15). If parasites appeared after the second selection it was considered a high probablility that they were truly transfected, and they were subsequently cloned by limiting dilution.

5.3.4 Screening assays for transfected parasites

In order to screen for successful transfections, DNA was isolated from growing parasites and analysed by PCR and Southern analysis (Sections 2.13.9 and 2.13.10). The two methods complement each other in that the PCR would confirm the presence of the *T. gondii dhfr-ts* gene, but would not differentiate between genomicly integrated and episomally maintained DNA. The Southern analysis, on the other hand, is based on restriction digestion that produces fragments of differing sizes depending on whether the hybridising DNA is episomal, genomic wild type or genomic transfected.

5.3.4.1 Screening by PCR

For the PCR analysis, isolated parasite DNA was used as a template for amplifications with four sets of primers (Figure 5.16). Two of the sets (primer 1 + primer 2, and primer

3 + primer 4) contained one primer based on the *dhfr-ts* sequence (primers 2 and 3), and would therefore only amplify regions present in transfected parasites, resulting in products of 1026 basepairs and 464 basepairs ("Transfection products"). The other two sets (primer 1 + primer 5, and primer 4 + primer 6) amplified regions present in both wild type and transfected parasites, producing 860 basepair- and 341 basepair long products ("Control products"). Some of the above primers were used previously under different names, but have been numbered from 1 to 6 in this section and on Figure 5.17 for clarity. All primer sequences are listed in Table 2.3.

To verify the validity of the assay the following DNA template controls were included: pSLAPRep transfection vector as a positive control for amplification of both transfection products and control products, and wild-type *P. berghei* ANKA DNA as a negative control for amplification of transfection products, but positive control for the control products. Reactions without template DNA were also run to control for non-specific contamination.

5.3.4.2 Southern analysis

For the Southern analysis, two µg of parasite DNA was digested with HindIII and EcoRI restriction enzymes, separated on an agarose gel and blotted onto a nylon membrane. The DNA was probed with a 1202 bp sequence comprising the two *pbslap* fragments used to construct the pSLAPRep vector (Fragment 1 and Fragment 2), and the single nucleotide separating these two fragments in the *pbslap* gene (Figures 5.17 and 5.5). The probe was amplified by PCR from *P. berghei* ANKA gDNA, using the forward primer for Fragment 1, SLAPRepF1, and the reverse primer for Fragment 2, SLAPRepR2, isolated and radio-labelled (Section 2.13.10 and Table 2.3).

Hybridisation with F1-F2 produced different band patterns in transfected and wild-type parasites. In *P. berghei* wild type DNA, the F1-F2 probe hybridises to the identical *pslap* gene sequence, producing one band in a Southern analysis. However, in a transfected parasite, where the *dhfr-ts* sequence has been inserted into the *slap* gene, Fragments 1 and 2 would have been separated by the restriction digest, and therefore the probe would hybridise to two fragments, generating two bands in the analysis (Figure 5.17).

The sizes of the mentioned bands depend on the location of EcoRI and HindIII sites within *pbslap* and in the sequence surrounding the gene. An EcoRI site is present at position 219 in the gene (Figure 5.17). However, no other EcoRI or HindIII sites exist within the *pbslap* sequence. Because the *P. berghei* genome is not yet fully sequenced, the position of next EcoRI/HindIII downstream of the gene is not known. In an attempt to identify this restriction site, a BLAST search was performed. Sequence up to 1785 bases downstream of the *pbslap* stop codon was obtained and a restriction map of this region produced (data not shown). Still, no EcoRI or HindIII sites were found, demonstrating that the next EcoRI/HindIII site is further downstream than 1785 bases from the 3′ end of the *pbslap* open reading frame. Therefore, the absolute sizes of the fragments produced in the digest cannot be exactly calculated, but their minimum lengths can be determined. Despite the lack of information on all restriction sites, the result of this analysis is clearly diagnostic of whether disruption has taken place or not due to the difference in the number of bands generated (one for wild-type, two for disruption). Expected sizes of hybridising fragments for transfected and wild type parasites are shown in Figure 5.17. Wild-type *P. berghei* ANKA DNA was included as a positive control for the assay, and as a size comparison for DNA from non-transfected parasites, and the pSLAPRep transfection vector as a size comparison for episomal DNA.

5.3.5 Analysis of results of *pbslap* transfection experiments

Initially both transfection constructs were used for transfections. However, it was later decided to continue only with the replacement construct, as it generates a more stable integration (Section 5.1). A total of five attempts were made to try and disrupt the *Plasmodium berghei slap* gene. Three of these resulted in parasite growth after the first round of pyrimethamine selection and are described below. All of these were obtained using the pSLAPRep transfection vector.

5.3.5.1 Transfection experiment 1

Parasites were cultured, electroporated and injected into two female BALB/c mice, as described (Section 5.3.3). Asexual parasites were observed in peripheral blood in both recipient mice on day one or two (Mouse 1 and Mouse 2, respectively; Figure 5.18 A).

However, by day three the parasitemia had been reduced to non-detectable levels by the drug pressure. On day seven parasites re-appeared in Mouse 2, and so were passaged into three new mice (Figure 5.18 A). No parasites were observed after the second round of treatment, indicating that the transferred parasites were not transfected, and therefore not pyrimethamine resistant (Figure 5.18 B).

However, in the first round of selection, parasites in Mouse 2 had appeared relatively soon after drug treatment (three days after the last injection; Figure 5.18 A). This suggested that the population was resistant and DNA was therefore isolated and screened. PCR reactions were set up with the parasite DNA as a template to amplify the products with the primers specified in Section 5.3.4.1. However, analysis by gel electrophoresis showed that control products but not transfection products had been amplified (Figure 5.19 A). Moreover, only one band of the same size as the wild type control sample was seen in Southern analysis (Figure 5.19 B). These results showed that disruption of the *pbslap* gene had not taken place.

5.3.5.2 Transfection experiment 2

Following injection of electroporated parasites into three female BALB/c mice, one animal subsequently developed an infection 12 days post infection (Mouse 0, Figure 5.20 A). Although this was a very late appearance, it was decided to passage this population for a second round of pyrimethamine selection. Two mice were infected with the transferred parasites and subjected to four days of drug treatment (Figure 5.20 B). Mouse 0 subsequently became parasitemic on day nine, and Mouse 1 on day ten, post infection. However, screening by PCR failed to amplify either of the transfection products whereas amplification of both control products verified that the assay worked (Figure 5.21 A). Similarly, a single band was observed in the Southern analysis (Figure 21 B) showing that again the transfection had not been successful. DNA was isolated and screened at an early stage from these animals, thus the parasites were not cloned despite having passed through two rounds of selection.

5.3.5.3 Transfection experiment 3

Parasites were cultured, electroporated and injected into one mice as described (Section 5.3.3). Newly invaded parasites could be seen on day one (24 hours post transfection) but

were undetectable on day three, after two days' pyrimethamine treatment (Figure 5.22 A). Six days post transfection, asexual parasites emerged and were subsequently transferred to four female BALB/c mice for a second round of drug selection (Figure 5.22 B). One of these mice (Mouse 3) developed an infection that was detectable eight days post transfer. The remaining three mice stayed parasite free for a total of three weeks, at which point they were culled. In order to generate a homogeneous population, the parasites growing in Mouse 3 were cloned by limiting dilution. Infected blood was extensively diluted and groups of 10 female BALB/c mice were inoculated with 0.6, 1 or 3 parasitised erythrocytes each. Three animals, Mouse 0 (M0), Mouse 1 (M1) and Mouse 2 (M2), all inoculated with 3 infected cells, subsequently developed infections. The parasite populations from each of these mice were recovered, as well as from the uncloned parental population (UC = Mouse 3 in Figure 5.22 B), and parasite genomic DNA was extracted for screening.

Despite having been subjected to two rounds of pyrimethamine selection, PCR analysis showed no amplification of either Transfection product 1 (expected size 1026 bp) or Transfection product 2 (expected size 464 bp) from the DNA extracted from any of the cloned parasites (Figure 5.23 A). Amplification of both of these products from the pSLAPRep transfection vector (TV) confirmed that both sets of primers worked. Both control products, 860 bp and 341 bp, could be amplified from the same parasite DNAs, showing that the absence of Transfection product amplification was not due to a problem with the extracted DNA (too much or too little template DNA, inhibiting contaminants etc), but because the parasites had not been successfully transfected.

This result was confirmed in Southern analysis (Figure 5.23 B) of the uncloned parasite population (UC) by the presence of only one band of approximately 7 kilobases, the same size as the band in the *P. berghei* wild type control sample, indicating that integration of the *dhfr-ts* gene had not taken place. A band at approximately 4 kilobases in the digested pSLAPRep transfection vector (TV) sample confirmed that the restriction digest had worked.

5.4 Discussion

Two transfection vectors were constructed for disruption of the *pslap* gene in *Plasmodium berghei* ANKA parasites. The insertion vector pSLAPIns contained a single *pbslap* sequence of 911 basepairs, and was designed for integration via a single cross-over event. The pSLAPRep construct carried two sequences, 860 and 341 basepairs, from *pslap*, and would require double homologous recombination to integrate into the parasite genome. Furthermore, susceptibility of the *Plasmodium berghei* ANKA parasite strain to the anti-malarial pyrimethamine was assessed and verified. An inoculum of 10^6 parasitised erythrocytes was followed by drug administration at ten milligrams per kilo bodyweight on four consecutive days, which eliminated detectable levels of asexual parasites from peripheral blood for a total of ten days. However, as analysed by PCR and Southern blot, a successful disruption of *P. berghei slap* using the described transfection constructs was not achieved.

Why was disruption of pbslap not successful?

There are several factors that may have contributed to the failure of generating a *pbslap* knock out parasite. Some general conditions were changed from the standard transfection procedure, which may have decreased the overall efficiency and success rate of the experiments:

A critical stage in the protocol is the invasion of fresh erythrocytes of the recipient animal by the newly electroporated parasites. To increase the efficiency of this event, recipient animals are treated with phenylhydrazine, a chemical that increases the production of reticulocytes, the preferred cell type of *P. berghei* for invasion and growth. This drug was however not administered in the present study due to animal project license regulations. For the same reason, BALB/c mice were used throughout the experiments rather than Wistar rats for donor cultures and as recipients of electroporated parasites. Furthermore, injection of electroporated parasites is usually performed through the intravenous route. This part of the protocol was followed to begin with, however, due to unexpected deaths of several injected mice, future injections had to be administered intraperitoneally. No published reports were found where this has resulted in successful parasite transformation, although it has been shown to work, albeit with lower transfection efficiency than normally observed with intravenous injections (Rita Tewari, personal

communication). This is probably due to the fact that the peritoneal cavity is a more hostile environment for pathogens due to the presence of an abundant population of peritoneal macrophages.

Although few, there are a small number of reports describing successful transfections using mice in place of rats, such as Mota *et al* (2001). Although *P. berghei* successfully infects both mice and rats, the parasitemia in the latter does not exceed a few percent, whereas in mice more than half of the population of erythrocytes can be parasitised. The standard protocol uses one rat (5-10 millliliter blood) with a parasitemia of 1-3% per transfection experiment. However, in order to obtain similar numbers of parasites in the present work, three mice (approximately 1.5 millliliter blood) at 15-20% parasitemia were used. Although this equals the number of parasites used per experiment, it results in a different cell density in the culture, as well as changes the ratio of infected versus uninfected erythrocytes. Cells tend to grow best in cultures that mimic their normal environment so it is possible that the low density affected the overall health and viability of the cultured parasites in a negative manner.

Most published transfection work to date on *P. berghei* follows the guidelines outlined by Waters *et al* (1997). Interestingly, alongside their standard method, these authors also suggested a simplified transfection procedure. This version uses Swiss mice throughout the experiment. The *in vitro* culturing step is omitted, and instead a parasite preparation consisting of mainly trophozoites is used, which after electroporation is intraperitoneally injected into mice. This protocol is similar to the one used in the present project. However, its usefulness is doubtful as the authors admitted that they had had no success at integrating any constructs into the *P. berghei* genome, and no work has since been published using this technique (Waters *et al* 1997).

It is also possible that the shorter one of the target sequences used in the pSLAPRep replacement vector, the 341 basepair Fragment 1, was insufficiently long for efficient recombination to take place. Although it has been shown in the parasite *Leishmania enriettii* that as little as 200 basepairs is enough in for homologous integration to take place, transfection efficiency was low (Tobin and Wirth, 1992). Successful transformations in *Plasmodium* have used fragments of approximately 500 basepairs,

with an upper limit of 1000 basepairs, as longer sequences tend to cause problems with stability (Ménard and Janse, 1997).

Why did non-transfected parasite populations appear after pyrimethamine selection?
The assay on pyrimethamine sensitivity, carried out at the beginning of this study, verified that the asexual *P. berghei* ANKA parasites that later were used in the transfection experiments were susceptible to pyrimethamine at a dose of ten milligrams per kilo bodyweight (Section 5.3.1). However, it also demonstrated that elimination of the infection was not complete, as parasites started appearing ten days post inoculation. The administered dose is part of the standard protocol for *P. berghei* transfections and has been carefully chosen for drug selection in order to eliminate a potentially large population of non-transformed, susceptible parasites, without eradicating a comparatively very small proportion of resistant individuals (van Dijk *et al.*, 1994). Therefore this amount of drug may not, as was shown in the present study, completely wipe out non-resistant parasites. It is true that non-transfected parasites in some cases appeared at earlier time points in the described transfection experiments than in the pyrimethamine sensitivity experiment, such as after the first injection of electroporated parasites in Transfection experiment 1 (six days). However, the inoculum of parasites in the drug sensitivity study was known to be 10^6 whereas the number of live parasites injected into a recipient animal after electroporation is difficult to estimate. It is likely that with a greater number of parasites injected, more are able to avoid elimination by the drug and can therefore expand to detectable levels sooner. Thus, a direct comparison between the pyrimethamine sensitivity study and the *pbslap* transfection experiments cannot be made.

Lastly, there is also a possibility that non-targeted parasites became truly resistant to pyrimethamine. Drug-insensitive clones can arise both from spontaneous mutations in the endogenous *dhfr-ts* gene, and from replacement of the endogenous *dhfr-ts* gene by the incoming mutated copy in the transfection construct. The latter is to some degree avoided by the use of a heterologous selectable marker, rather than the gene from *P. berghei.* However, although the *dhfr-ts* coding sequence originates from *Toxoplasma gondii*, the 5′ and 3′ UTRs flanking the gene are endogenous (Figure 5.3). They can therefore pair up

with the identical sequences in the *P. berghei* genome, resulting in integration of the DHFR-TS transcription unit into the *dhfr-ts* locus, rather than into the target gene.

Does pbslap have a function that is essential for parasite survival during the blood stages?

An obvious explanation for the failure in creating a *pbslap* knock out parasite would be that the SLAP protein is essential for asexual blood stage development or survival. If this was true, transformed parasites would never expand to detectable levels, as an individual lacking the protein could not grow or multiply. However, this is not the case, as during the present study, a paper was published reporting on the effects of *pbslap* disruption on parasite development within the mosquito (Claudianos *et al.*, 2002). The *slap* gene had been knocked out using a replacement construct carrying a 606 basepair long sequence at the 5′ end of the gene and a 481 basepair sequence towards the 3′ end (Figure 5.25). The report stated that the SLAP protein was exclusively expressed in sporozoites, and disruption of *pbslap* showed that it is essential for sporozoite development, as sporozoite formation was abrogated in developing oocysts. (A 35% increase in oocyst size as compared with wild type parasites was also observed.) However, the work presented in Chapter Four of the present project, clearly indicates that the SLAP protein of both *P. falciparum* and *P. berghei* is expressed in blood stage gametocytes. The presence of *Pf*SLAP in sexual stage parasites has recently also been confirmed by a high-accuracy mass spectrometric analysis of protein expression at different maturation stages (Lasonder *et al.*, 2002). Protein expression in *Plasmodium* is tightly regulated and organized on several levels, with transcriptional and translational control, as well as chromosomal clustering of genes involved in common processes (Florens *et al.*, 2002). This regulation is also reflected in the distinct proteomes (protein expression profiles) of the different developmental stages (Lasonder *et al.*, 2002; Florens *et al.*, 2002). It therefore appears unlikely that a protein that is expressed already in the gametocyte does not play a functional role in the parasite until the sporozoite stage. For example, expression of the sexual specific protein *Pf*s48/45 is initiated in stage III gametocytes. A subsequent disruption of the *pfs48/45* gene was shown to affect the next developmental stage, the gamete, with an impairment in male gamete fertility that resulted in a reduction

of the numbers of zygotes that were formed (van Dijk *et al.*, 2001). Similarly, synthesis of the mosquito stage protein MAEBL has been shown to begin in sporozoites as they are developing inside oocysts. Disruption of the *maebl* gene was later shown to produce an accumulation of *maebl*$^{-}$ sporozoites in the mosquito hemocoel but an almost complete absence in the salivary glands, strongly indicating a role for MAEBL in sporozoite salivary gland invasion (Kariu *et al.*, 2002).

Although *P*SLAP clearly plays a role in sporozoite formation, it is fully possible that a protein with an extensive and unique domain composition like *P*SLAP could have more than one function. Complex protein structures resulting in multi-functional molecules are not uncommon and include a number of proteins and protein families, such as the proteases of the Disintegrin And Metalloprotease (ADAM) family (Evans, 2001) and thrombospondins (Adams, 2001).

Indeed, disruption studies of the genes encoding the two Plasmodial proteins P25 and P28 revealed not only that they to a large degree have redundant functions, but also that the proteins play multiple roles during more than one developmental stage: in double knock-out parasites ($p25^{-/-}/p28^{-/-}$) ookinate formation was significantly inhibited, those ookinates that did develop had a much reduced capacity to traverse the midgut epithelium, and the few that did cross showed a compromised ability to continue their maturation into oocysts (Tomas *et al.*, 2001).

Figure 5.1

Insertion and replacement transfection vectors

Genetic modification of a gene can be achieved with two types of vectors that use different modes of integration. The insertion construct (shown on the left) enters the parasite genome via a single cross-over event. This is achieved through a single homologous recombination between one parasite gene sequence (yellow) cloned into the vector, and the identical sequence in the parasite genome (also yellow), resulting in the insertion of a duplicated target sequence, the selectable marker (the *dhfr-ts* gene, shown in red) and the vector backbone (white).

The replacement construct (shown on the right), on the other hand, carries two sequences from the target gene (green and blue), flanking the selectable marker. For integration to take place a double cross-over is required. The result is a *loss* of the original genomic gene sequences, (and any sequence in between these two), *replaced* by the sequences in the vector with the selectable marker gene in the middle.

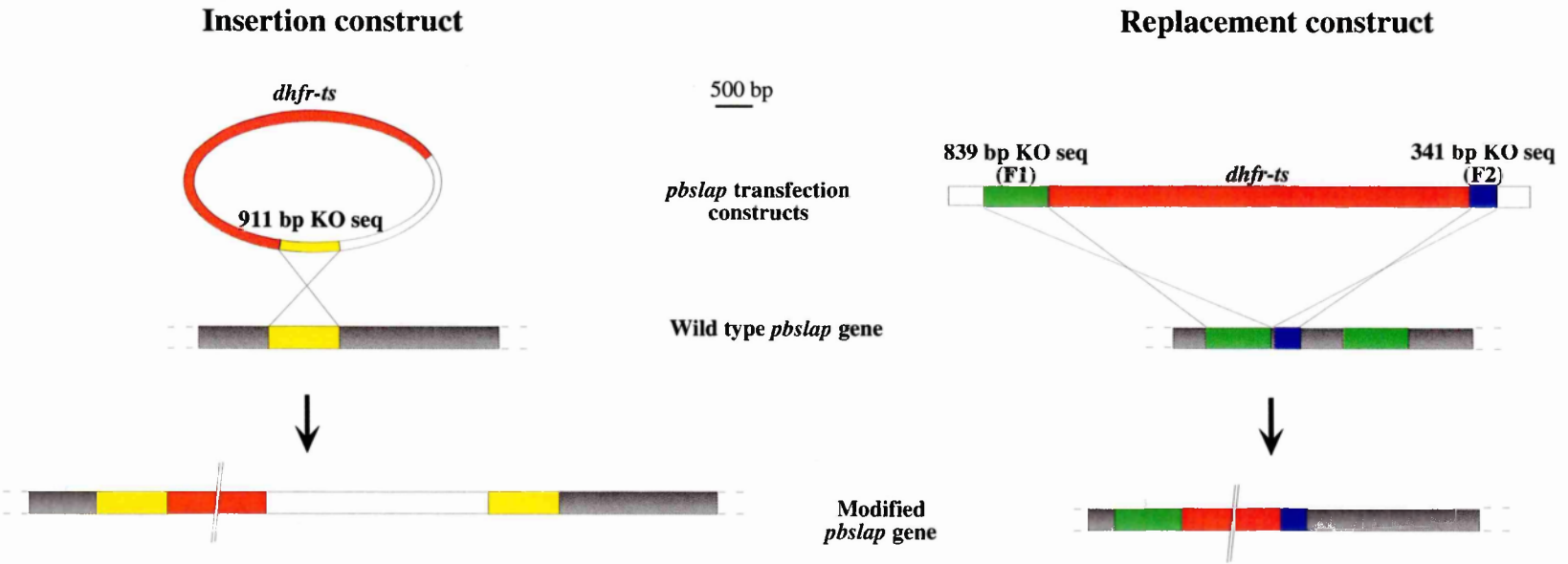

Insertion construct
Replacement construct
dhfr-ts
500 bp
911 bp KO seq
839 bp KO seq
(F1)
dhfr-ts
341 bp KO seq
(F2)
pbslap transfection constructs
Wild type pbslap gene
Modified pbslap gene

Figure 5.2

Pyrimethamine sensitivity of *Plasmodium berghei* ANKA blood stage parasites

Groups of four mice were inoculated intraperitoneally with 10^6 *Plasmodium berghei*-infected erythrocytes. Starting on day 1 (24 hours post innoculation), one group was treated with pyrimethamine (10mg/kg bodyweight; top graph), one group received saline injections (center graph) and the third group was left un-treated (bottom graph). The injections (indicated by arrows) were repeated on days 2, 3 and 4, and the parasitemia monitored daily on Giemsa stained thin blood smears.

Arrows indicate days of injections of pyrimethamine or saline

The cross (†) denotes the death of an animal.

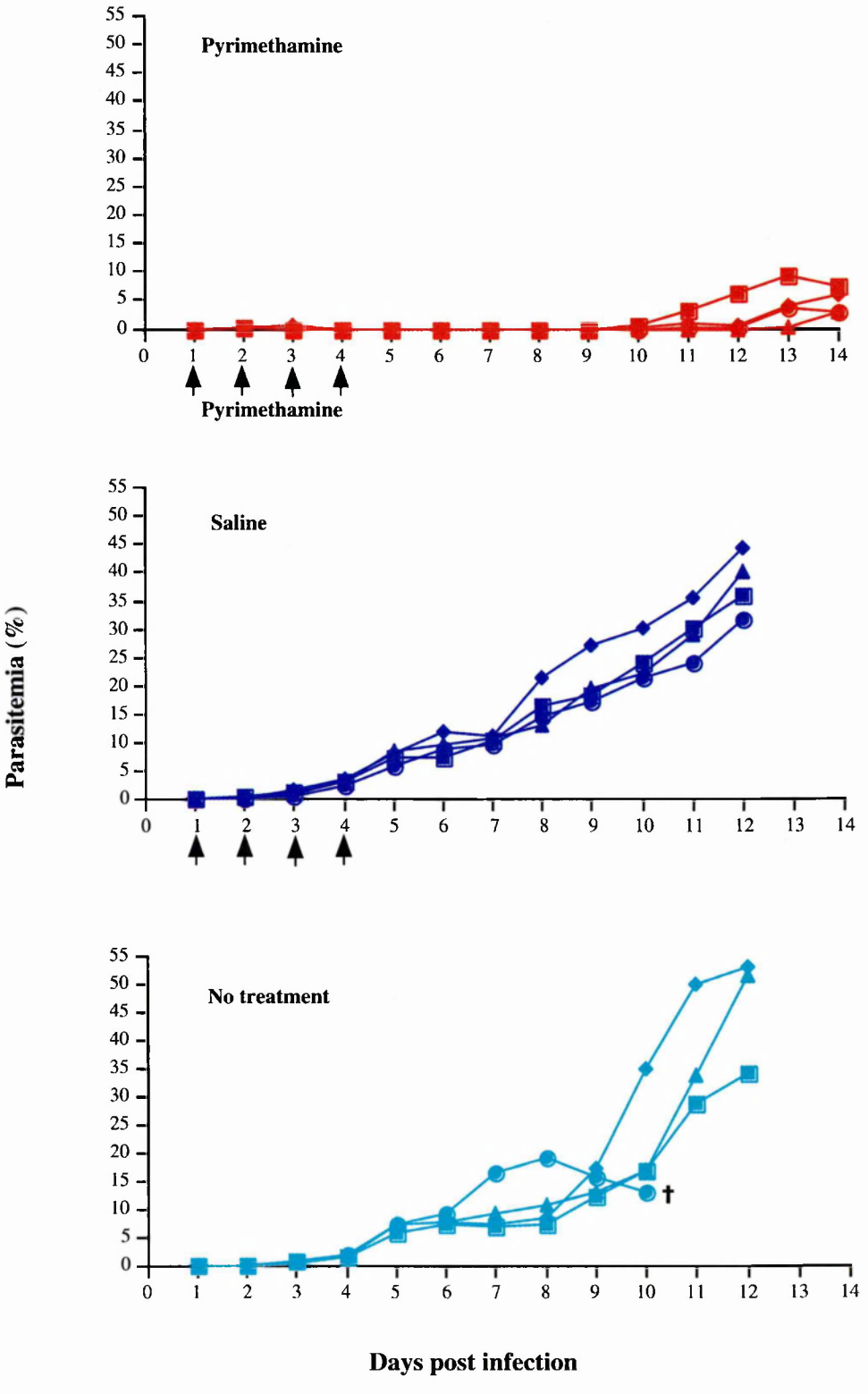
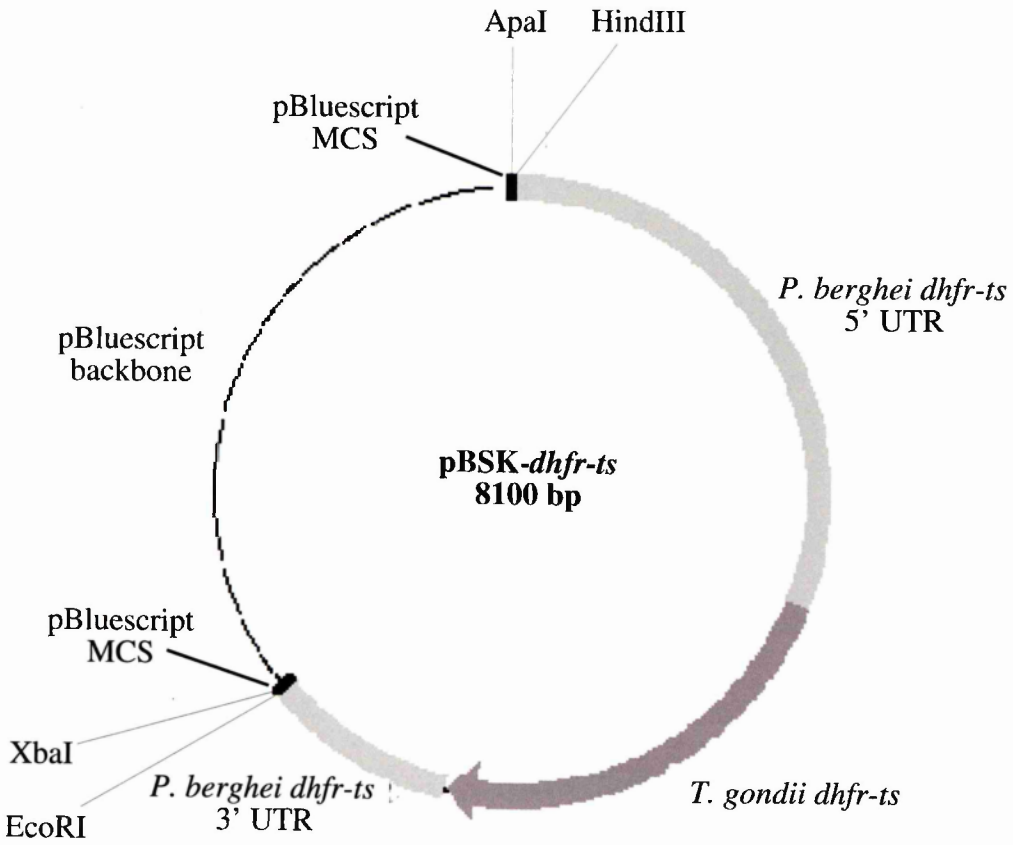

Pyrimethamine
Pyrimethamine
Saline
No treatment
†
Parasitemia (%)
Days post infection

Fig 5.3

The pBSK-*dhfr-ts* plasmid used for constructing *pbslap* transfection vectors

The pBSK-*dhfr-ts* plasmid was created by cloning of the 5.2 kilobases DHFR-TS transcription unit between the HindIII and EcoRI restriction sites in the multiple cloning cassette (MCS, black) of the pBluescript SK II (+) phagemid vector (Stratagene). The DHFR-TS transcription unit consisted of the pyrimethamine resistant form of the *Toxoplasma gondii* dihydrofolate reductase-thymidilate synthase (*dhfr-ts*) gene (dark grey), flanked by the *Plasmodium berghei* 5'- and 3' *dhfr-ts* UTR sequences (light grey). Restriction sites that were subsequently used for construction of the pSLAPIns and pSLAPRep transfection vectors are shown in the schematic.

The pBKS-*dhfr-ts* vector was a gift from Dr. Rita Tewari, Imperial College, London.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pBluescript
MCS
P. berghei dhfr-ts
5' UTR
pBluescript
backbone
pBSK-dhfr-ts
8100 bp
pBluescript
MCS
XbaI
EcoRI
P. berghei dhfr-ts
3' UTR
T. gondii dhfr-ts

Figure 5.4

Location of fragments used in the *pbslap* transfection constructs in relation to nucleotide sequence coding for structural motifs in the SLAP protein

The fragments chosen for the transfection constructs (represented by black bars below the *pbslap* gene) were located towards the 5' end of the gene so that in the event that transcription took place despite the disruption, only a very short polypeptide with very few structural or functional motifs would be translated.

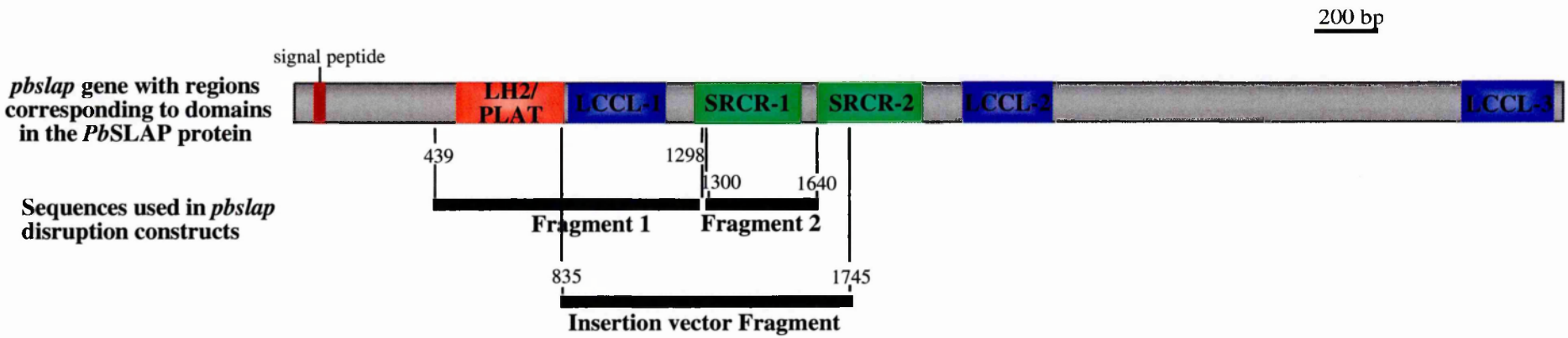
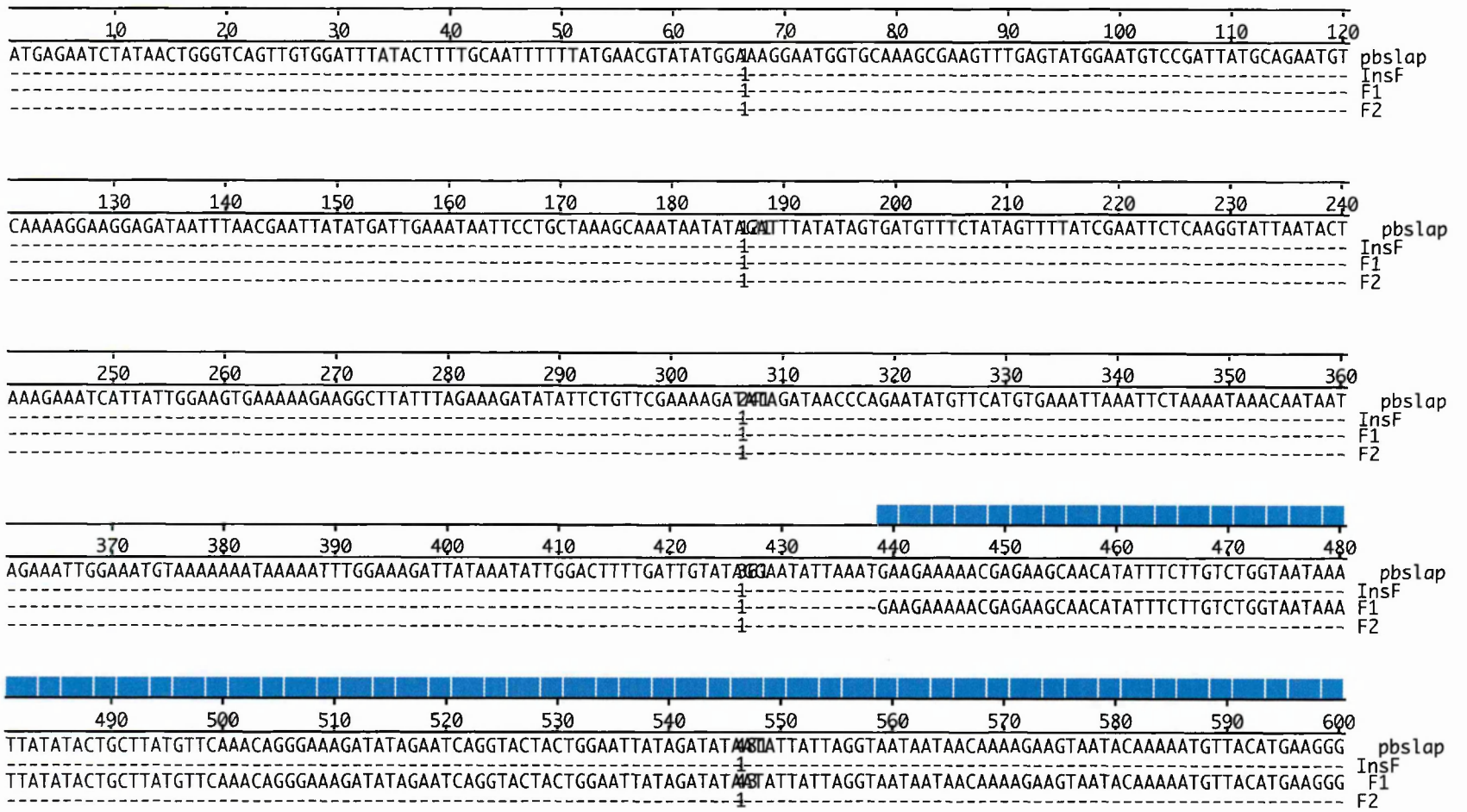

200 bp
signal peptide
pbslap gene with regions corresponding to domains in the *Pb*SLAP protein
LH2/ PLAT
LCCL-1
SRCR-1
SRCR-2
LCCL-2
LCCL-3
439
1298
1300
1640
Sequences used in *pbslap* disruption constructs
Fragment 1
Fragment 2
835
1745
Insertion vector Fragment

Figure 5.5

Alignment of *pbslap* and sequences used in transfection vectors

Multiple sequence alignment showing the exact positions of the Insertion vector fragment from pSLAPIns, and Fragments 1 and 2 from the pSLAPRep vector, in relation to the *pbslap* gene.

The alignment was performed using CLUSTAL W. Sequence identity is represented as a coloured bar above the aligned sequences, where blue represents two identical sequences and green three identical sequences.
Overlapping stretches of sequences are represented by blue colour and non-overlapping sequence is either without colour (beginning of gene) or green (end of gene).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F1
F2
130 140 150 160 170 180 190 200 210 220 230 240
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InsF
F1
F2
250 260 270 280 290 300 310 320 330 340 350 360
AAAGAAATCATTATTGGAAGTGAAAAAGAAGGCTTATTTAGAAAGATATATTCTGTTCGAAAAGATAAAGATAACCCAGAATATGTTCATGTGAAATTAAATTCTAAAATAAACAATAAT pbslap
InsF
F1
F2
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InsF
GAAGAAAAACGAGAAGCAACATATTTCTTGTCTGGTAATAAA F1
F2
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InsF
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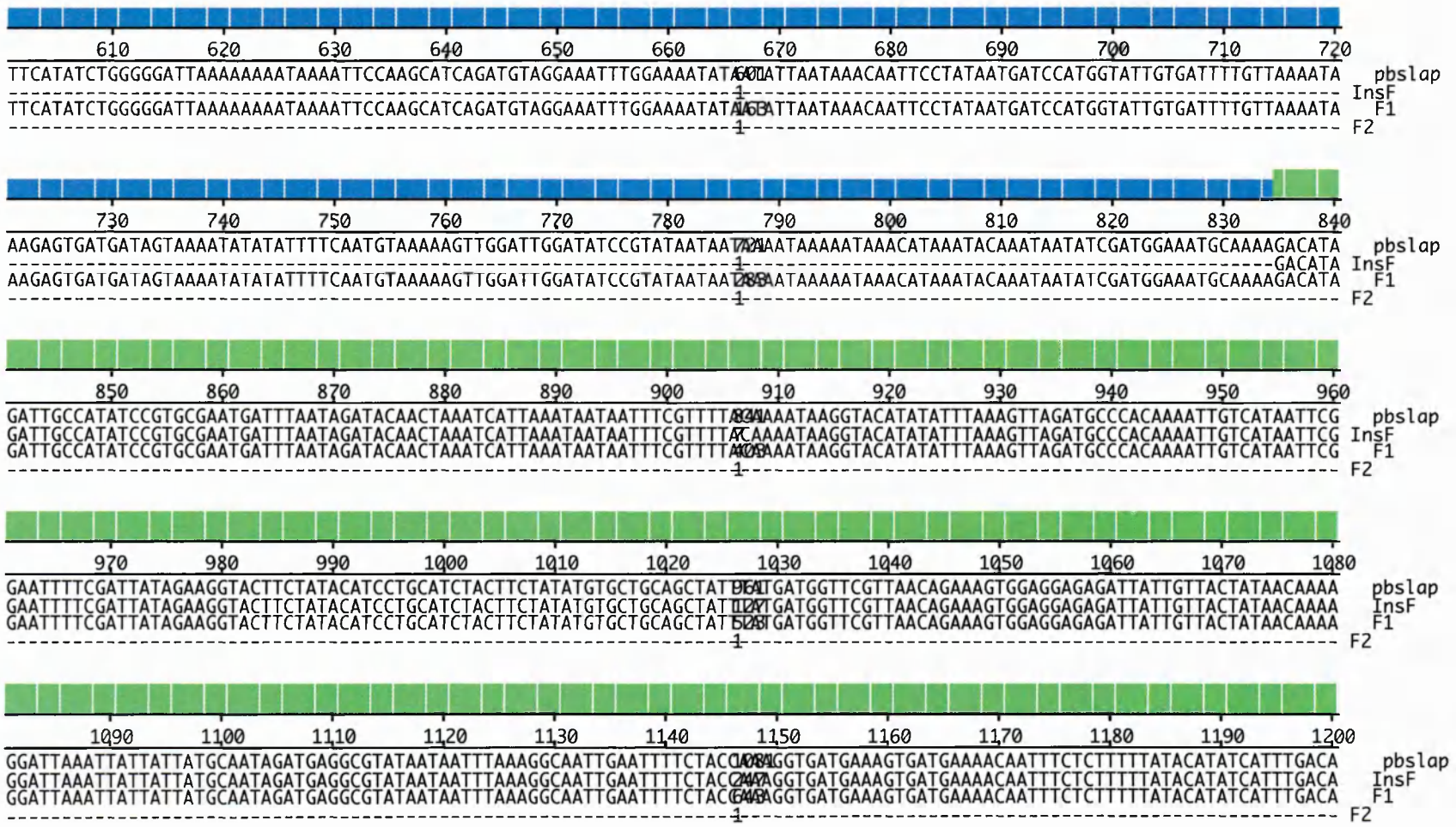
F2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F2
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F2
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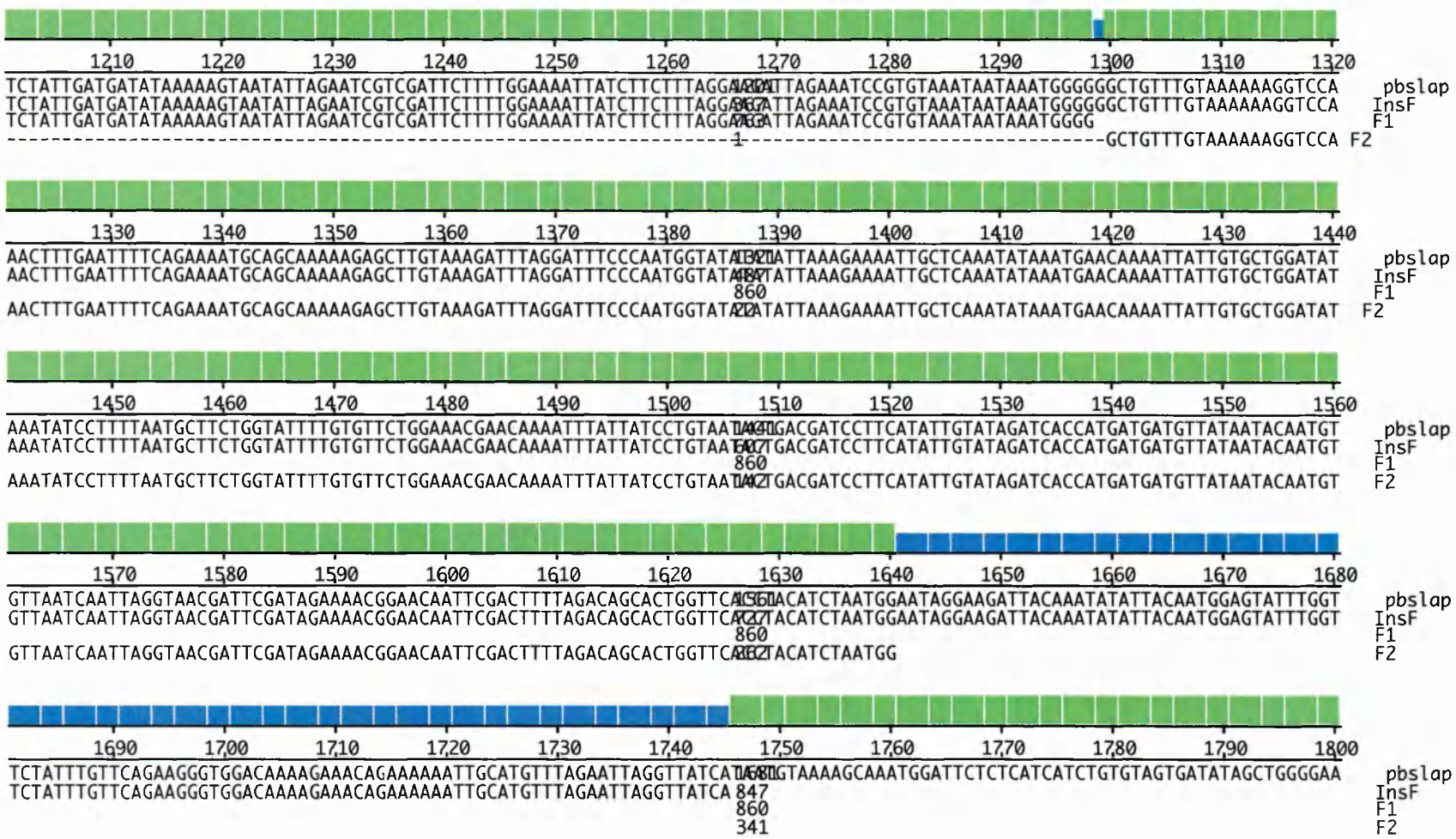
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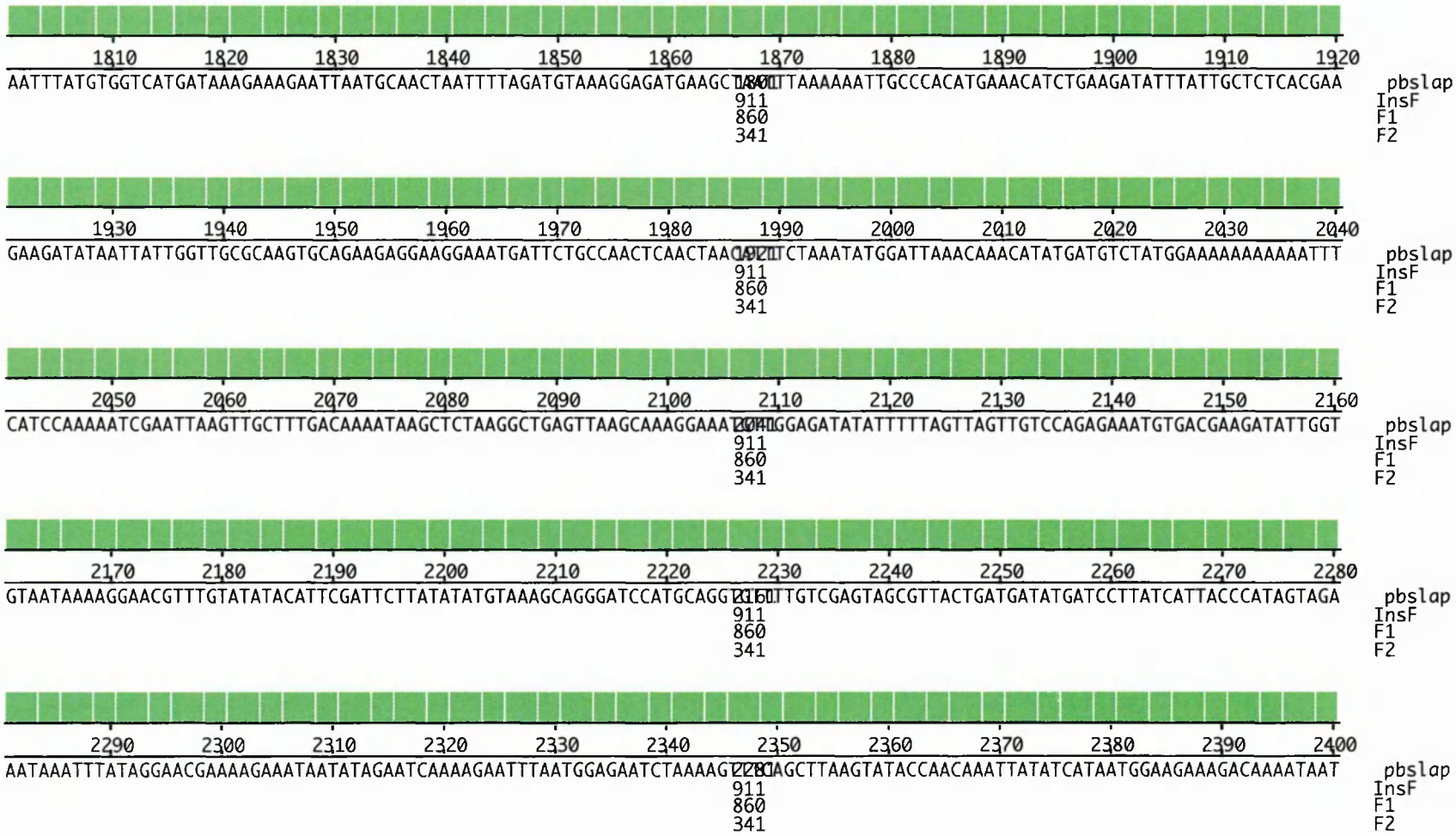
F1
F2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pbslap
InsF
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F2
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341
pbslap
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F2
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InsF
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F2
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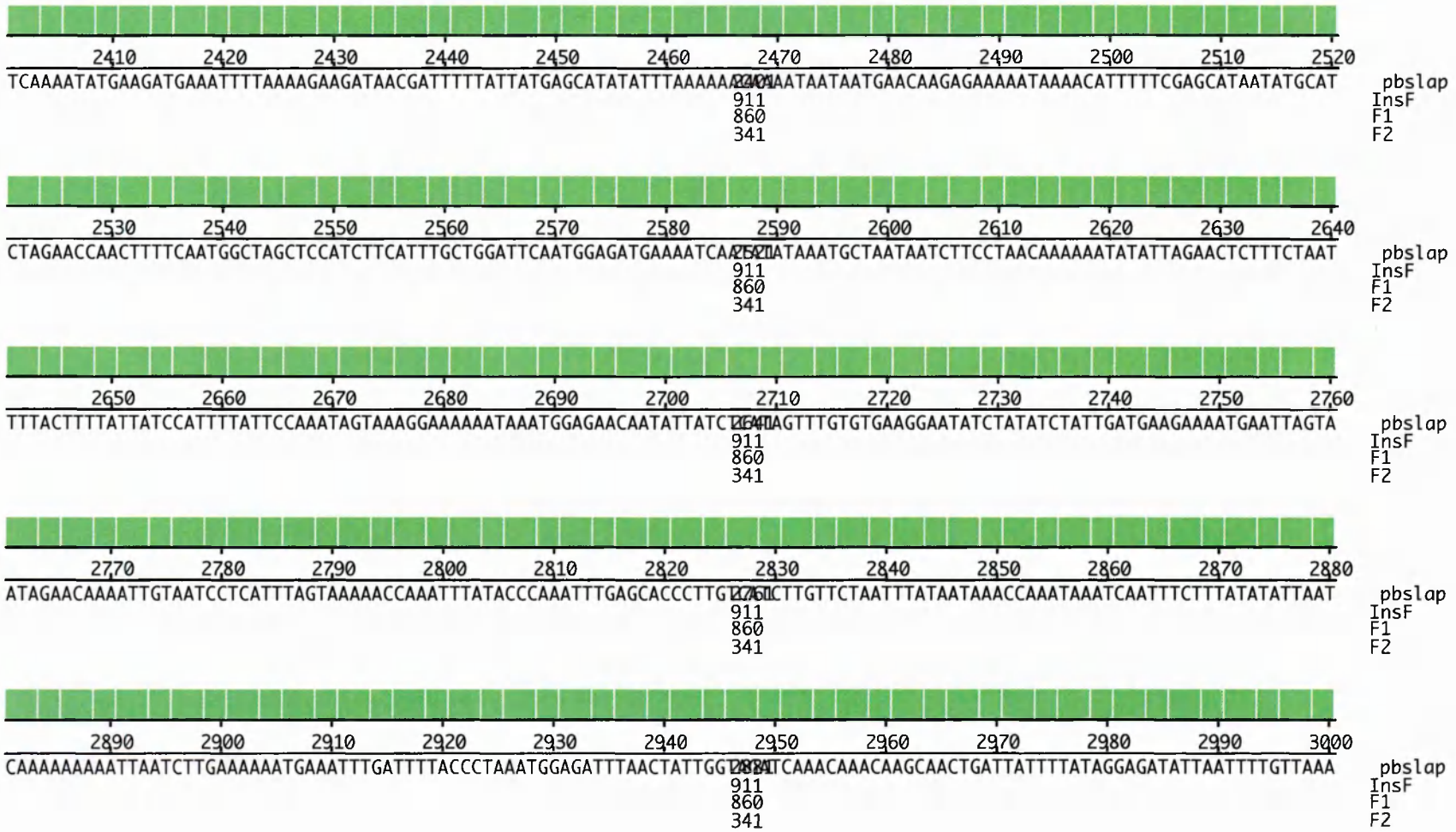
pbslap
InsF
F1
F2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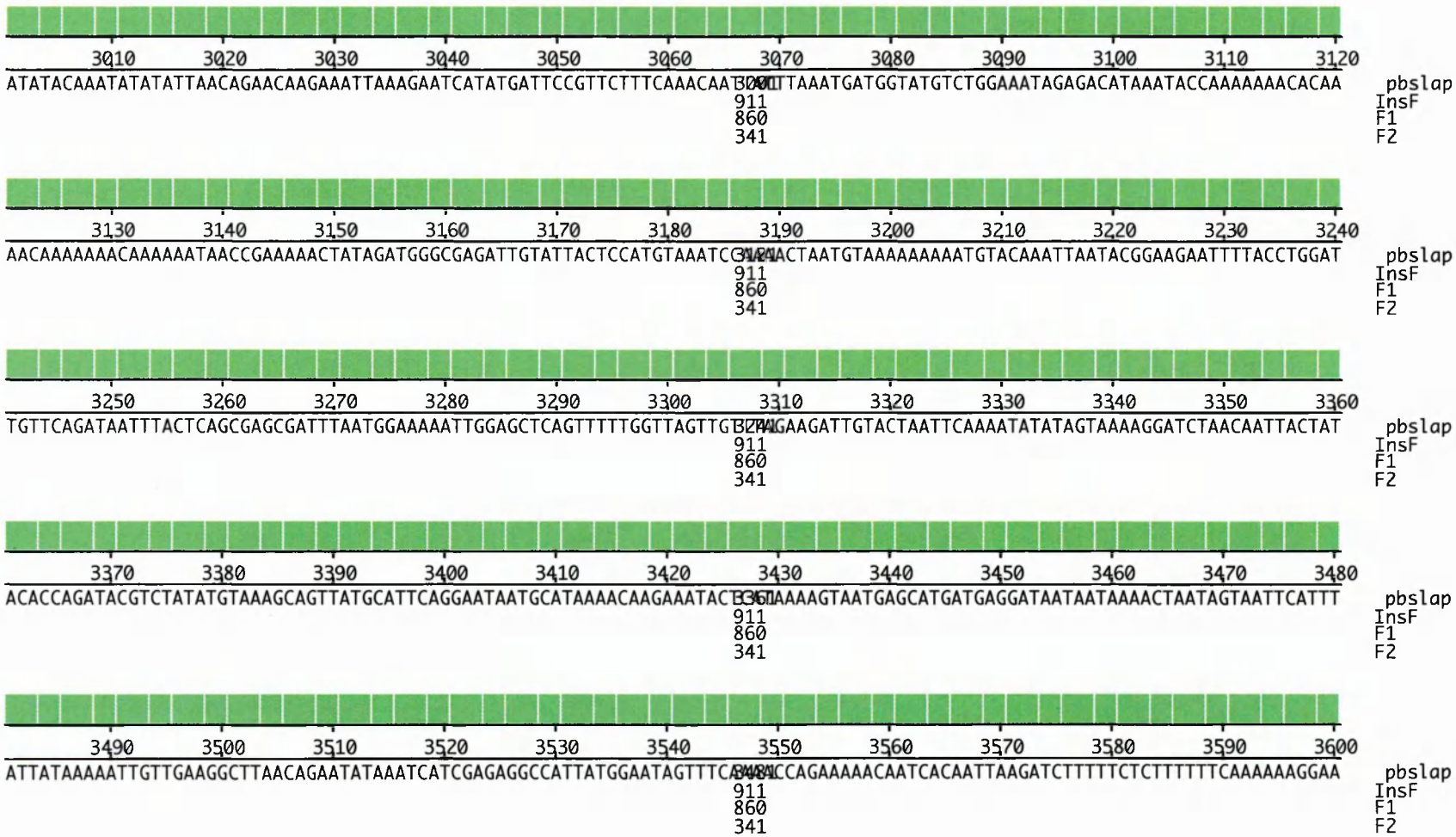
911
860
341
pbslap
InsF
F1
F2
2650 2660 2670 2680 2690 2700 2710 2720 2730 2740 2750 2760
TTTACTTTTATTATCCATTTTATTCCAAATAGTAAAGGAAAAAATAAATGGAGAACAATATTATCTAAAAGTTTGTGTGAAGGAATATCTATATCTATTGATGAAGAAAATGAATTAGTA
911
860
341
pbslap
InsF
F1
F2
2770 2780 2790 2800 2810 2820 2830 2840 2850 2860 2870 2880
ATAGAACAAAATTGTAATCCTCATTTAGTAAAAACCAAATTTATACCCAAATTTGAGCACCCTTGTAAACTTGTTCTAATTTATAATAAACCAAATAAATCAATTTCTTTATATATTAAT
911
860
341
pbslap
InsF
F1
F2
2890 2900 2910 2920 2930 2940 2950 2960 2970 2980 2990 3000
CAAAAAAAAATTAATCTTGAAAAAATGAAATTTGATTTTACCCTAAATGGAGATTTAACTATTGGTAAATCAAACAAACAAGCAACTGATTATTTTATAGGAGATATTAATTTTGTTAAA
911
860
341
pbslap
InsF
F1
F2


3010 3020 3030 3040 3050 3060 3070 3080 3090 3100 3110 3120
ATATACAAATATATATTAACAGAACAAGAAATTAAAGAATCATATGATTCCGTTCTTTCAAACAATTTTAAATGATGGTATGTCTGGAAATAGAGACATAAATACCAAAAAAACACAA
pbslap
InsF
F1
F2
911
860
341
3130 3140 3150 3160 3170 3180 3190 3200 3210 3220 3230 3240
AACAAAAAAAACAAAAAATAACCGAAAAACTATAGATGGGCGAGATTGTATTACTCCATGTAAATCGTTACTAATGTAAAAAAAAAATGTACAAATTAATACGGAAGAATTTTACCTGGAT
pbslap
InsF
F1
F2
911
860
341
3250 3260 3270 3280 3290 3300 3310 3320 3330 3340 3350 3360
TGTTCAGATAATTTACTCAGCGAGCGATTTAATGGAAAAATTGGAGCTCAGTTTTTGGTTAGTTGTTGAAGAAGATTGTACTAATTCAAAATATATAGTAAAAGGATCTAACAATTACTAT
pbslap
InsF
F1
F2
911
860
341
3370 3380 3390 3400 3410 3420 3430 3440 3450 3460 3470 3480
ACACCAGATACGTCTATATGTAAAGCAGTTATGCATTCAGGAATAATGCATAAAACAAGAAATACTTTTAAAAGTAATGAGCATGATGAGGATAATAATAAAACTAATAGTAATTCATTT
pbslap
InsF
F1
F2
911
860
341
3490 3500 3510 3520 3530 3540 3550 3560 3570 3580 3590 3600
ATTATAAAAATTGTTGAAGGCTTAACAGAATATAAATCATCGAGAGGCCATTATGGAATAGTTTCAAAACCAGAAAAACAATCACAATTAAGATCTTTTTCTCTTTTTTCAAAAAAGGAA
pbslap
InsF
F1
F2
911
860
341

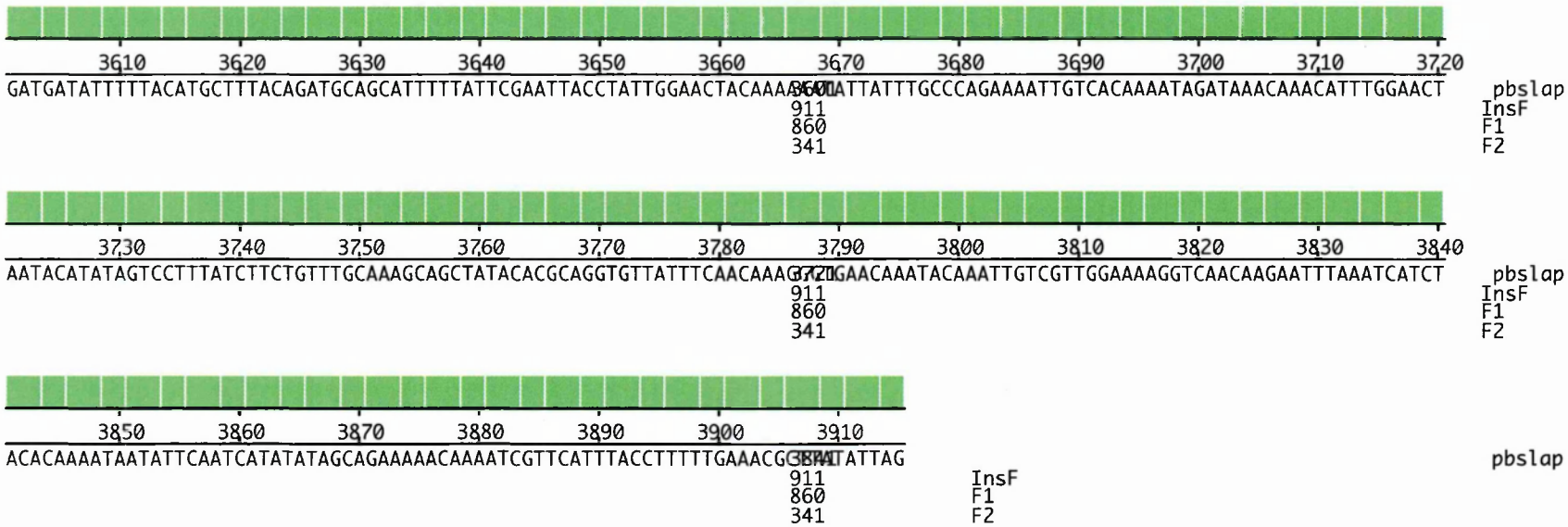

3610
3620
3630
3640
3650
3660
3670
3680
3690
3700
3710
3720
GATGATATTTTTACATGCTTTACAGATGCAGCATTTTTATTCGAATTACCTATTGGAACTACAAAAGAATTATTTGCCCAGAAAATTGTCACAAAATAGATAAACAAACATTTGGAACT
pbslap
911
860
341
InsF
F1
F2
3730
3740
3750
3760
3770
3780
3790
3800
3810
3820
3830
3840
AATACATATAGTCCTTTATCTTCTGTTTGCAAAGCAGCTATACACGCAGGTGTTATTTCAACAAAGAAGAACAAATACAAATTGTCGTTGGAAAAGGTCAACAAGAATTTAAATCATCT
pbslap
911
860
341
InsF
F1
F2
3850
3860
3870
3880
3890
3900
3910
ACACAAAATAATATTCAATCATATATAGCAGAAAAACAAAATCGTTCATTTACCTTTTTGAAACG
pbslap
911
860
341
InsF
F1
F2

Figure 5.6

Isolated *Plasmodium berghei* ANKA genomic DNA used as template in PCR

P. berghei genomic DNA was used as template in PCR for amplification of the 911 base pair long Insertion vector fragment (InsF), using primers SLAPInsF and SLAPInsR (Table 2.7). Ten µL product was analysed by gelelectrophoresis.

M. Marker IX, Roche

InsF Insertion vector fragment amplification

A band of the correct size, 911 basepairs, was amplified (arrow).

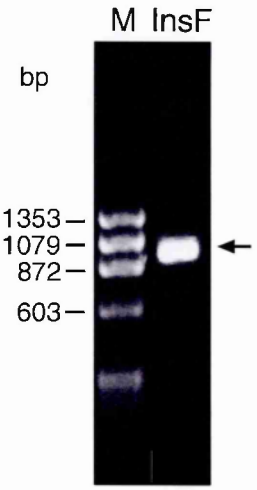

M InsF
bp
1353
1079
872
603

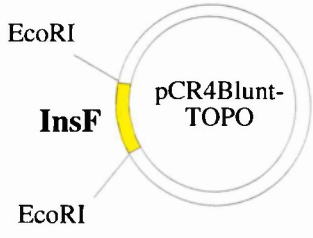
Figure 5.7

Schematic of construction of the *pbslap* insertion vector

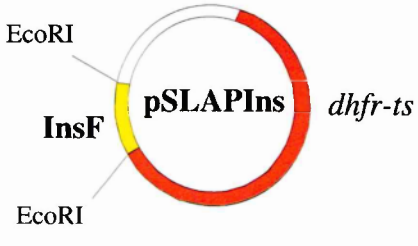
The SLAPInsFor and SLAPInsRev primers were used to amplify the 911 basepair InsF sequence of the *pbslap* gene from *P. berghei* gDNA. The fragment, shown in yellow, was blunt-cloned into the pCR4Blunt-TOPO vector for sequencing. Once the correct sequence was verified it was re-cloned into the EcoRI site of the pBluescript-*dhfr-ts* vector, analysed by restriction digestion and sequenced.

Insertion vector Fragment (InsF) amplified by PCR from *P. berghei* ANKA genomic DNA

Insertion vector Fragment (InsF) cloned into pCR4Blunt-TOPO for sequencing



Insertion vector Fragment excised from pCR4Blunt-TOPO and cloned into pBSK-*dhfr-ts*



Sequence verified by restriction digest and sequencing (Figure 5.8)

Figure 5.8

Analysis of the Insertion vector fragment sequence

(A) EcoRI digest of the pSLAPIns vector after cloning of the 911 nucleotide sequence into the EcoRI site of pBKS-*dhfr-ts*.

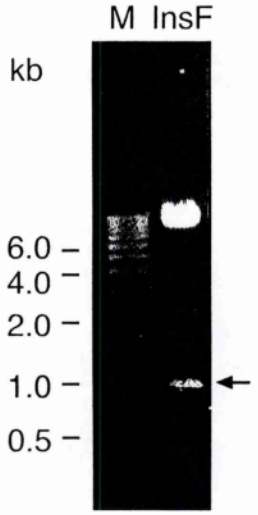
M 1 kb marker, Invitrogen

InsF EcoRI digest of the pSLAPIns vector

A band of the expected size was excised from the vector (arrow) indicating that the correct fragment had been cloned into the pSLAPIns vector.

(B) Sequencing of the pSLAPIns vector verified that the cloned fragment was the InsF sequence of *pbslap*.

A



B

Insertion vector Fragment:

AAGACATAGATTGCCATATCCGTGCGAATGATTTAATAGATACAACTAAATCATTAAATAATAA
TTTCGTTTTACAAAATAAGGTACATATATTTAAAGTTAGATGCCCACAAAATTGTCATAATTCG
GAATTTTCGATTATAGAAGGTACTTCTATACATCCTGCATCTACTTCTATATGTGCTGCAGCTA
TTTATGATGGTTCGTTAACAGAAAGTGGAGGAGAGATTATTGTTACTATAACAAAAGGATTAAA
TTATTATTATGCAATAGATGAGGCGTATAATAATTTAAAGGCAATTGAATTTTCTACCAAAGGT
GATGAAAGTGATGAAAACAATTTCTCTTTTTATACATATCATTTGACATCTATTGATGATATAA
AAAGTAATATTAGAATCGTCGATTCTTTTGGAAAATTATCTTCTTTAGGAAGATTAGAAATCCG
TGTAAATAATAAATGGGGGGCTGTTTGTAAAAAAGGTCCAAACTTTGAATTTTCAGAAAATGCA
GCAAAAAGAGCTTGTAAAGATTTAGGATTTCCCAATGGTATATATATTAAAGAAAATTGCTCAA
ATATAAATGAACAAAATTATTGTGCTGGATATAAATATCCTTTTAATGCTTCTGGTATTTTGTG
TTCTGGAAACGAACAAAATTTATTATCCTGTAATACTGACGATCCTTCATATTGTATAGATCAC
CATGATGATGTTATAATACAATGTGTTAATCAATTAGGTAACGATTCGATAGAAAACGGAACAA
TTCGACTTTTAGACAGCACTGGTTCACCTACATCTAATGGAATAGGAAGATTACAAATATATTA
CAATGGAGTATTTGGTTCTATTTGTTCAGAAGGGTGGACAAAAGAAACAGAAAAAATTGCATGT
TTAGAATTAGGTTATCA

Figure 5.9

pSLAPIns insertion vector

A 911 base pair long sequence (shown in yellow) from the *P. berghei slap* gene was cloned into the EcoRI site of the pBSK-*dhfr-ts* vector, to create the pSLAPIns transfection construct.

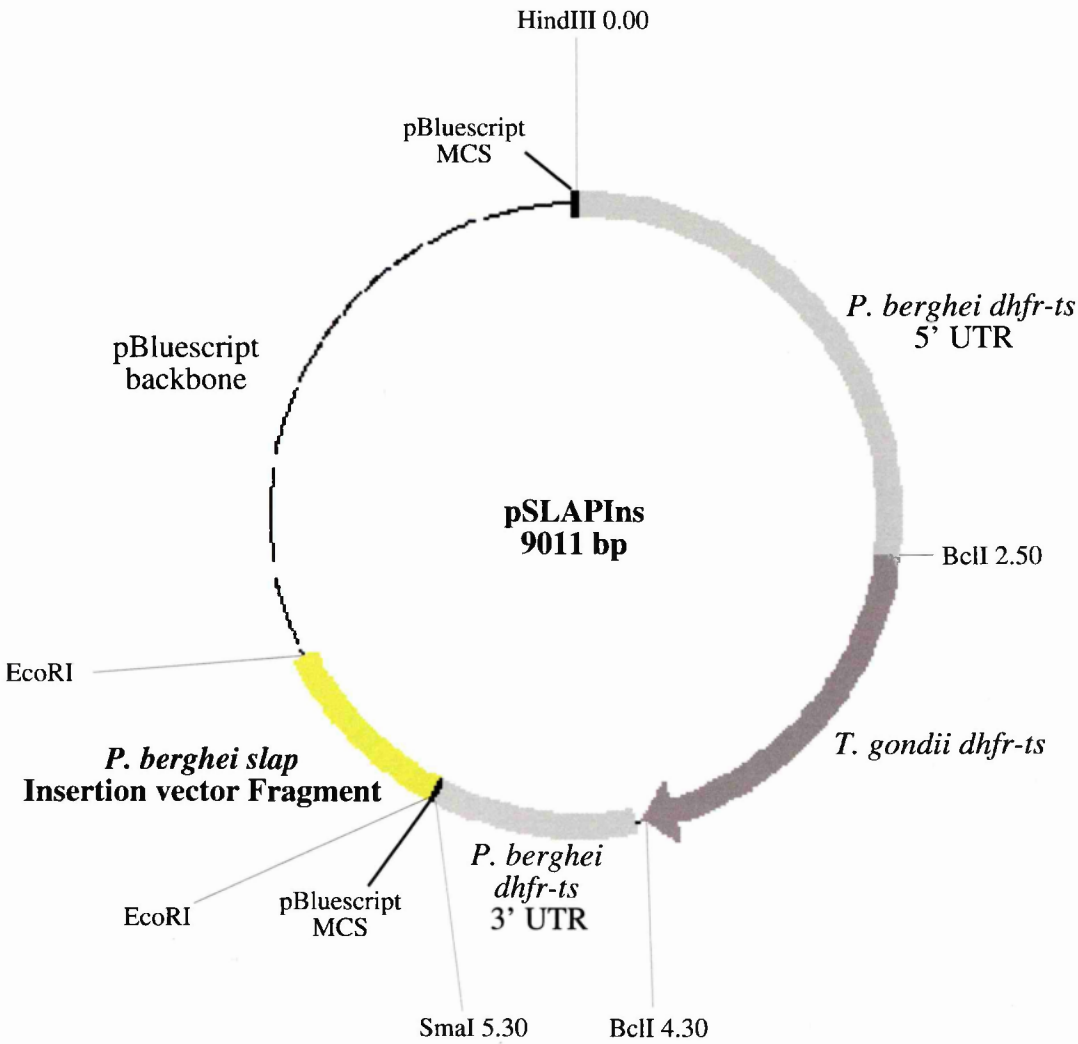
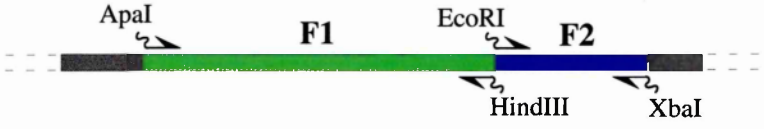

HindIII 0.00
pBluescript
MCS
P. berghei dhfr-ts
5' UTR
pBluescript
backbone
pSLAPIns
9011 bp
BclI 2.50
EcoRI
T. gondii dhfr-ts
P. berghei slap
Insertion vector Fragment
P. berghei
dhfr-ts
3' UTR
EcoRI
pBluescript
MCS
SmaI 5.30
BclI 4.30

Figure 5.10

Schematic of construction of the *pbslap* replacement vector

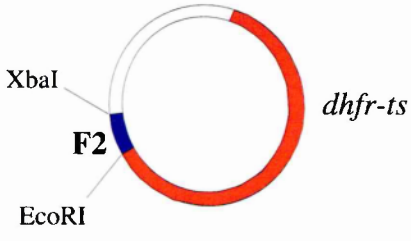
The fragments for the pSLAPRep vector were amplified by PCR using the primers SLAPRepF1 + SLAPRepR1 for Fragment 1 (F1, shown in green), and SLAPRepF2 and SLAPRepR2 for Fragment 2 (F2, shown in blue). To create the pSLAPRep vector, the fragments were cloned sequentially into pBSK-*dhfr-ts*, starting with F2 being inserted between the EcoRI and XbaI sites, followed by F1 between the ApaI and HindIII sites.

Fragment 1 (F1) and Fragment 2 (F2) amplified by PCR



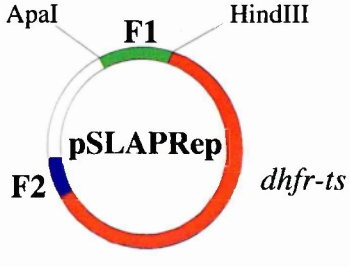
↓

Fragment 2 cloned into pBSK-*dhfr-ts*



↓

Fragment 1 cloned into Fragment 2-containing clone



↓

Sequences verified by restriction digest and sequencing (Figures 5.11 and 5.12)

Figure 5.11

Analysis of the cloned pSLAPRep Fragment 2 sequence

(A) The pSLAPRep construct was digested with EcoRI and XbaI to verify that the 341 base pair long Fragment 2 (F2) had been cloned.

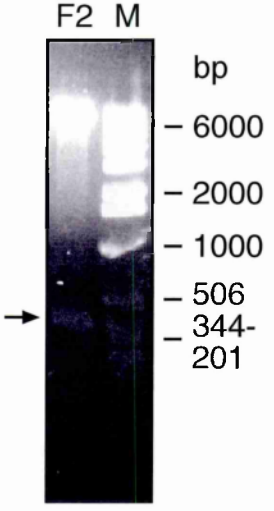
F2 EcoRI and XbaI digest of the pSLAPIns vector

M 1 kb ladder, Invitrogen

A band corresponding to the size of F2 was excised from the vector (arrow) indicating that the correct sequence had been cloned.

(B) Sequencing of the pSLAPRep vector verified that the cloned fragment was the F2 sequence of *pbslap*.

A



B

Replacement vector Fragment 2 (F2):

GCTGTTTGTAAAAAAGGTCCAAACTTTGAATTTTCAGAAAATGCAGCAAAAAGAGCTTGT
AAAGATTTAGGATTTCCCAATGGTATATATATTAAAGAAAATTGCTCAAATATAAATGAA
CAAAATTATTGTGCTGGATATAAATATCCTTTTAATGCTTCTGGTATTTTGTGTTCTGGA
AACGAACAAAATTTATTATCCTGTAATACTGACGATCCTTCATATTGTATAGATCACCAT
GATGATGTTATAATACAATGTGTTAATCAATTAGGTAACGATTCGATAGAAAACGGAACA
ATTCGACTTTTAGACAGCACTGGTTCACCTACAT

Figure 5.12

Analysis of the cloned pSLAPRep Fragment 1 sequence

(A) The pSLAPRep construct was digested with ApaI and HindIII to verify that the 860 base pair Fragment 1 (F1) had been cloned.

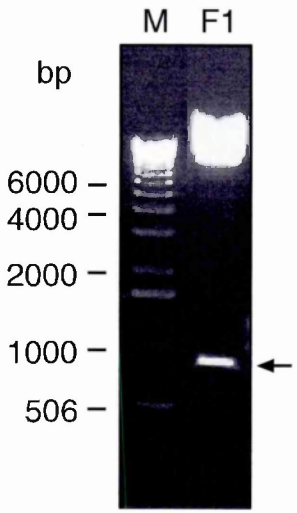
M 1 kb ladder, Invitrogen

F2 ApaI and HindIII digest of the pSLAPIns vector

A band corresponding to the size of F1 was excised from the vector (arrow) indicating that the correct sequence had been cloned.

(B) Sequencing of the pSLAPRep vector verified that the cloned fragment was the F1 sequence of *pbslap*.

A



B

Replacement vector Fragment 1 (F1):

GAAGAAAAACGAGAAGCAACATATTTCTTGTCTGGTAATAAATTATATACTGCTTATGTT
CAAACAGGGAAAGATATAGAATCAGGTACTACTGGAATTATAGATATAATATTATTAGGT
AATAATAACAAAAGAAGTAATACAAAAATGTTACATGAAGGGTTCATATCTGGGGGATTA
AAAAAAATAAAATTCCAAGCATCAGATGTAGGAAATTTGGAAAATATAATATTAATAAAC
AATTCCTATAATGATCCATGGTATTGTGATTTTGTTAAAATAAAGAGTGATGATAGTAAA
ATATATATTTTCAATGTAAAAAGTTGGATTGGATATCCGTATAATAATAAAATAAAAATA
AACATAAATACAAATAATATCGATGGAAATGCAAAAGACATAGATTGCCATATCCGTGCG
AATGATTTAATAGATACAACTAAATCATTAAATAATAATTTCGTTTTACAAAATAAGGTA
CATATATTTAAAGTTAGATGCCCACAAAATTGTCATAATTCGGAATTTTCGATTATAGAA
GGTACTTCTATACATCCTGCATCTACTTCTATATGTGCTGCAGCTATTTATGATGGTTCG
TTAACAGAAAGTGGAGGAGAGATTATTGTTACTATAACAAAAGGATTAAATTATTATTAT
GCAATAGATGAGGCGTATAATAATTTAAAGGCAATTGAATTTTCTACCAAAGGTGATGAA
AGTGATGAAAACAATTTCTCTTTTTATACATATCATTTGACATCTATTGATGATATAAAA
AGTAATATTAGAATCGTCGATTCTTTTGGAAAATTATCTTCTTTAGGAAGATTAGAAATC
CGTGTAAATAATAAATGGGG

Figure 5.13

pSLAPRep replacement vector

Two sequences, Fragment 1 (860 nucleotides, shown in green) and Fragment 2 (341 nucleotides, shown in blue), were cloned from the *P. berghei slap* gene into the pBSK-*dhfr-ts* vector, to construct the pSLAPRep transfection vector.

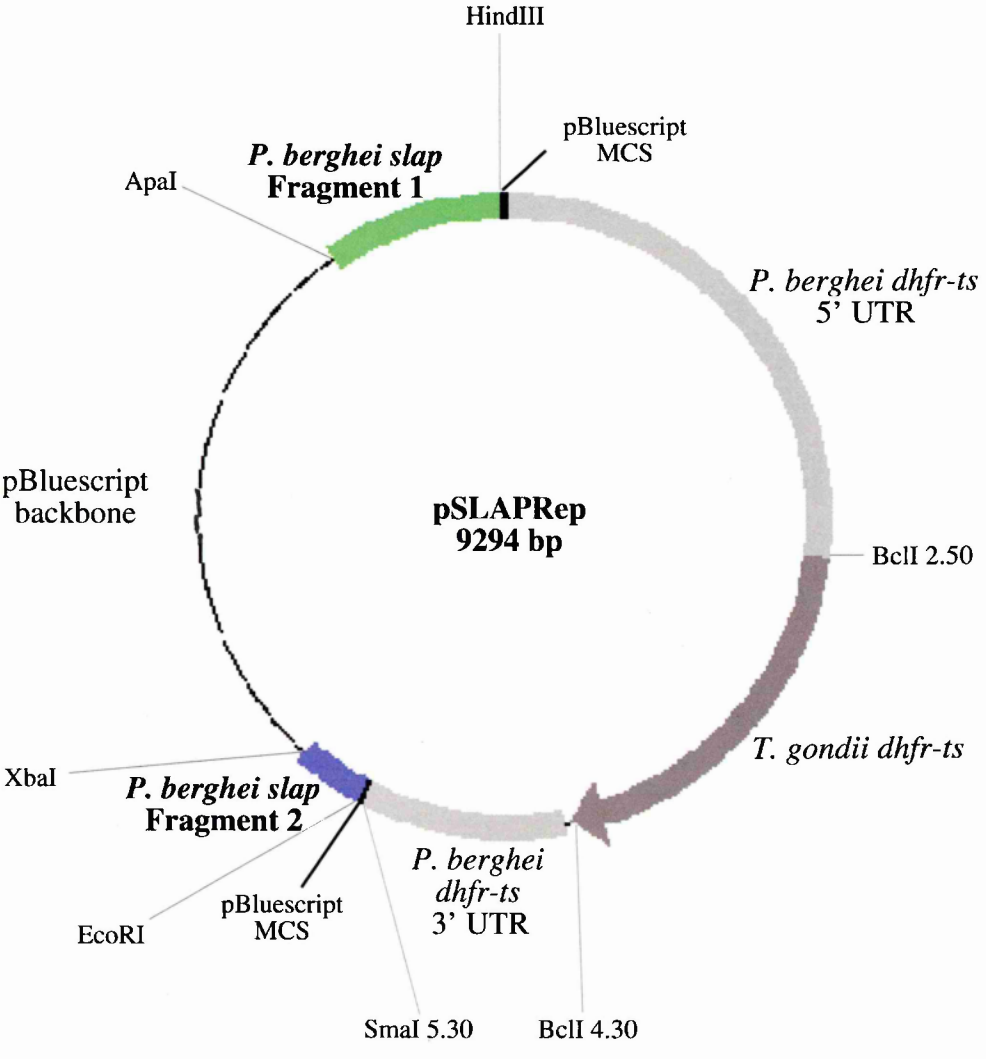

HindIII
pBluescript
MCS
P. berghei slap
Fragment 1
ApaI
P. berghei dhfr-ts
5' UTR
pBluescript
backbone
pSLAPRep
9294 bp
BclI 2.50
T. gondii dhfr-ts
XbaI
P. berghei slap
Fragment 2
P. berghei
dhfr-ts
3' UTR
pBluescript
MCS
EcoRI
SmaI 5.30
BclI 4.30

Figure 5.14

Plasmodium berghei transfections

The transfection constructs pSLAPIns and pSLAPRep were digested over night, purified and mixed with *Plasmodium berghei* ANKA schizonts from a 20 hour *in vitro* culture. The mixture was electroporated and injected into female BALB/c mice. The parasites were allowed one cycle of asexual division before put under selective pressure by administration of pyrimethamine (ten milligram per kilo bodyweight) 24 hours post electroporation.

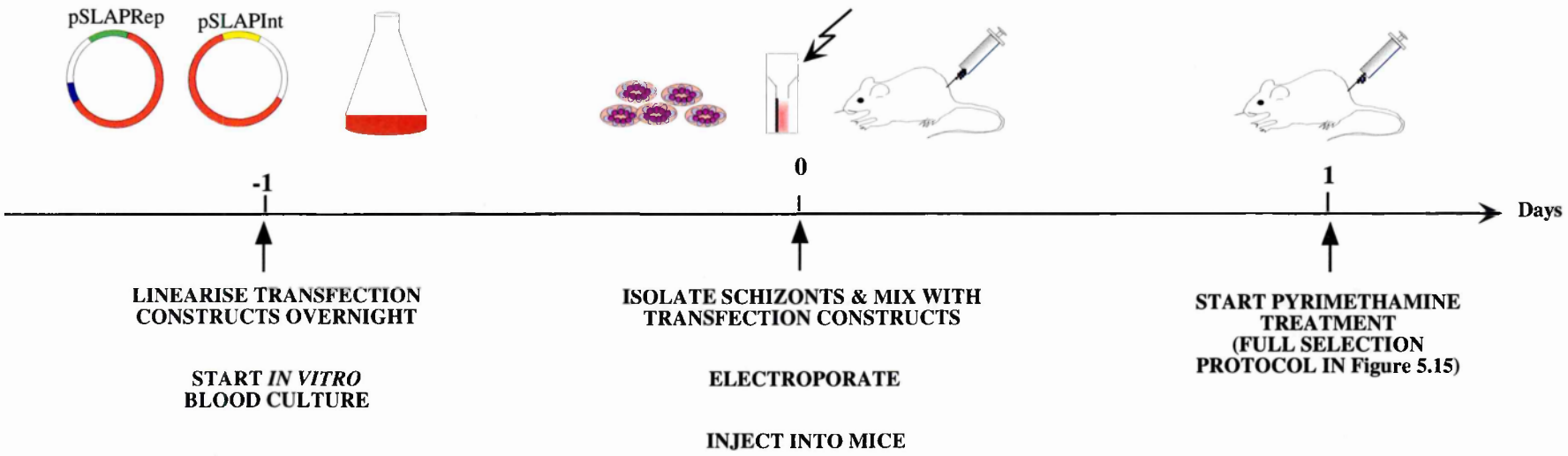

pSLAPRep
pSLAPInt
-1
0
1
Days
LINEARISE TRANSFECTION CONSTRUCTS OVERNIGHT
START *IN VITRO* BLOOD CULTURE
ISOLATE SCHIZONTS & MIX WITH TRANSFECTION CONSTRUCTS
ELECTROPORATE
INJECT INTO MICE
START PYRIMETHAMINE TREATMENT (FULL SELECTION PROTOCOL IN Figure 5.15)

Figure 5.15

Protocol for selection of transfected parasites

To select for transformed parasites infected mice were treated with pyrimethamine (arrows; ten milligrams per kg body weight) on four consecutive days, starting 24 hours post infection. Emerging asexual parasites were transferred to new mice and subjected to a second round of pyrimethamine. Parasites appearing after a second selection were cloned by limiting dilution.

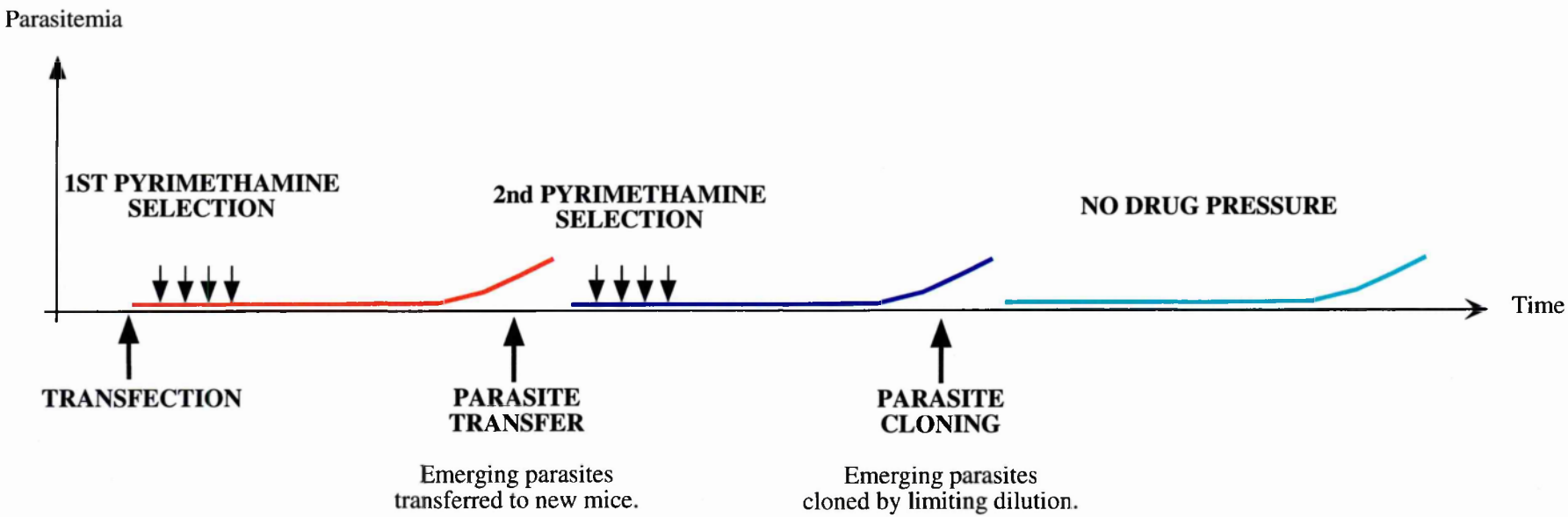
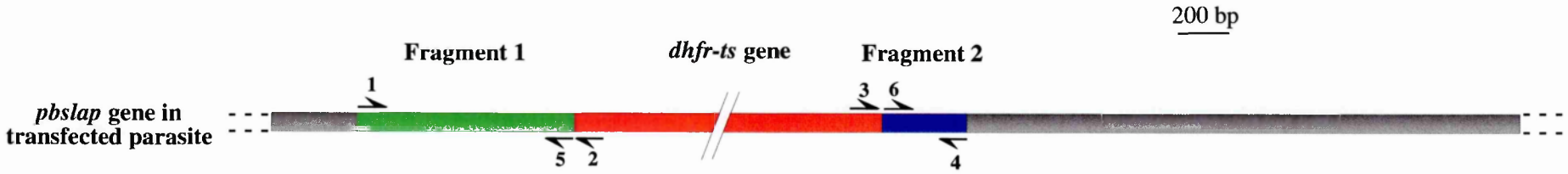

Parasitemia
1ST PYRIMETHAMINE SELECTION
2nd PYRIMETHAMINE SELECTION
NO DRUG PRESSURE
Time
TRANSFECTION
PARASITE TRANSFER
PARASITE CLONING
Emerging parasites transferred to new mice.
Emerging parasites cloned by limiting dilution.

Figure 5.16

Screening for parasites transfected with pSLAPRep by PCR

Plasmodium berghei DNA was isolated from infected mice from the transfection experiments, and used as template in PCR. Four sets of primers were used for the screening (see below). Combinations 1 + 2 and 3 + 4 amplify sequences that are partly from the *dhfr-ts* gene and therefore only present in transfected parasites ("Transfection products"). Combinations 1 + 5 and 4 + 6 amplify sequences that are present in both transfected and wild type parasites and were used as controls ("Control products").

The above primers are listed under the following names in Table 2.3:
Primer 1 - SLAPRepF1, Primer 2 - DHFR3, Primer 3 - DHFR2, Primer 4 - SLAPRepR2, Primer 5 - SLAPRepR1, Primer 6 - SLAPRepF2.



Products:

Primer pairs	**1 + 2**	**→ 1026 bp product**	**Transfection products**
	3 + 4	**→ 464 bp product**	**Only from transfected *pbslap* gene containing *dhfr-ts* sequence**
	1 + 5	**→ 860 bp product**	**Control products**
	4 + 6	**→ 341 bp product**	**From both wild type and transfected *pbslap* gene**

Figure 5.17

Screening for parasites transfected with pSLAPRep by Southern analysis

Plasmodium berghei DNA was isolated from infected mice from the transfection experiments, and digested alongside wild type *P. berghei* gDNA, using EcoRI and HindIII restriction enzymes. The DNA was blotted onto a nylon membrane and probed with a sequence comprising F1 (green) and F2 (blue), shown in the figure as F1-F2 probe. With transfected parasite DNA, the probe would hybridise to two fragments, giving rise to two bands on the blot. With wild type parasite DNA on the other hand, the probe would only hybridise to one fragment, appearing as a single band on the blot. Expected bands in the Southern analysis are shown as black bars underneath the genes.

Colour key

Green	pSLAPRep Fragment 1 (F1)
Blue	pSLAPRep Fragment 2 (F2)
Grey	*pbslap* gene
Red	*dhfr-ts* transcription unit

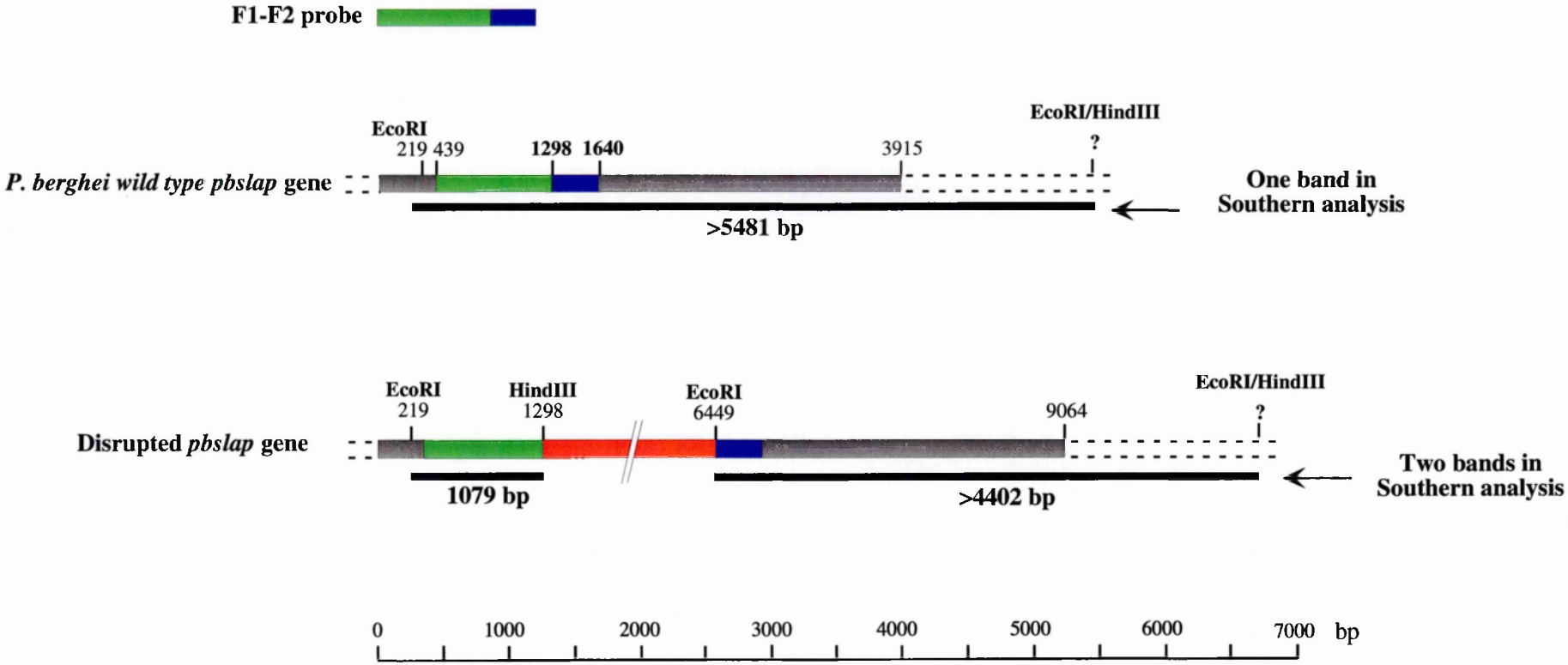
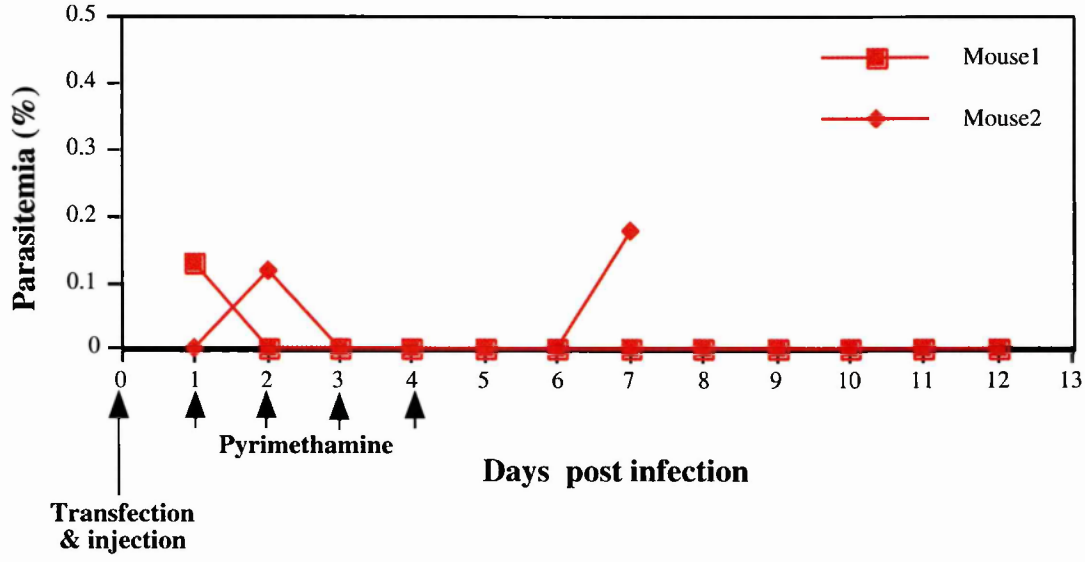

F1-F2 probe
EcoRI
EcoRI/HindIII
219 439
1298 1640
3915
?
P. berghei wild type pbslap gene
One band in Southern analysis
>5481 bp
EcoRI
HindIII
EcoRI
EcoRI/HindIII
219
1298
6449
9064
?
Disrupted pbslap gene
Two bands in Southern analysis
1079 bp
>4402 bp
0
1000
2000
3000
4000
5000
6000
7000
bp

Figure 5.18

Transfection experiment 1 - parasite growth

(A) Parasites were electroporated and injected into two female BALB/c mice on day zero (long vertical arrow). The animals were treated with pyrimethamine on days one, two, three and four (short vertical arrows). Parasites in the peripheral circulation were observed on day six post inoculation and were passaged into three new BALB/c mice. As before, these mice, shown in **(B)**, received pyrimethamine on days one, two, three and four (short vertical arrows). No parasites emerged after the second drug treatment.

A


Parasitemia (%)
0.5
0.4
0.3
0.2
0.1
0
Mouse1
Mouse2
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Pyrimethamine
Days post infection
Transfection
& injection

B

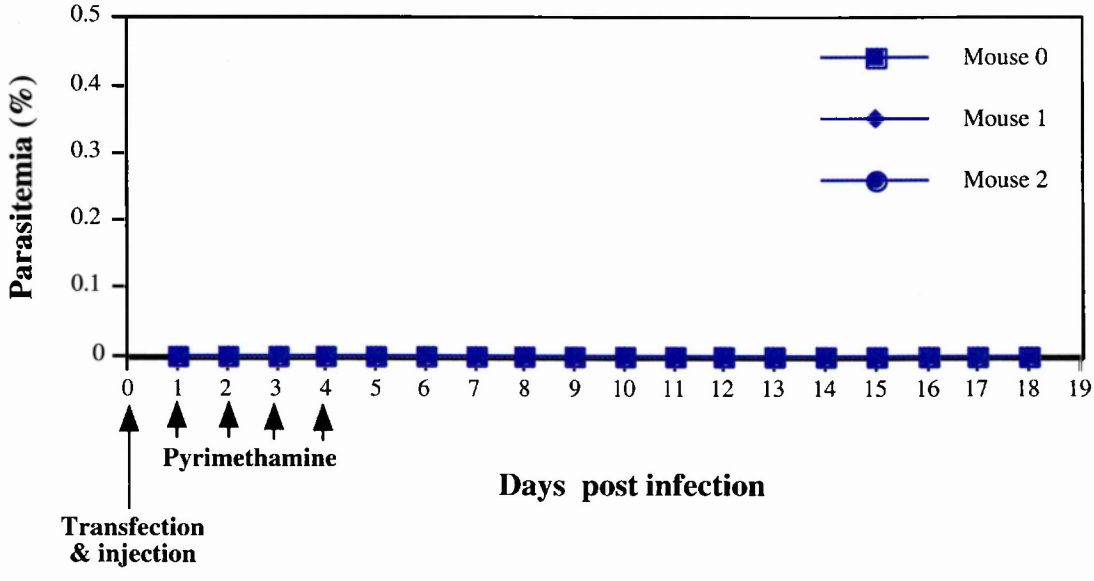

Parasitemia (%)
0.5
0.4
0.3
0.2
0.1
0
Mouse 0
Mouse 1
Mouse 2
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Pyrimethamine
Days post infection
Transfection
& injection

Figure 5.19

Transfection experiment 1 – screening

An un-cloned parasite population was isolated after one round of pyrimethamine treatment from Mouse 2 (uncloned=UC), gDNA extracted and screened:

(A) Results from PCR amplification using gDNA from the uncloned (UC) parasite population from Mouse 2, from the transfection vector (TV) and from wild type *P. berghei* gDNA, analysed by gelelectrophoresis (1% agarose). The two top panels show amplification of a product of the correct size from the transfection vector only (arrows). The two lower panels show amplification of products of the expected sizes from all templates (arrows). A sample without DNA template (no template=NT) was used as a negative control.

(B) Southern analysis of the uncloned (UC) parasite population, using the F1-F2 sequence as a probe (Figure 5.17). The blot shows a single band of approximately 7 kilo bases (arrow, left panel), the same size as the band in the *P. berghei* wild type negative control sample. Transfection vector (TV) was included as a positive control for the restriction digest and produced a band at the expected 4 kilo bases (arrow, right panel).

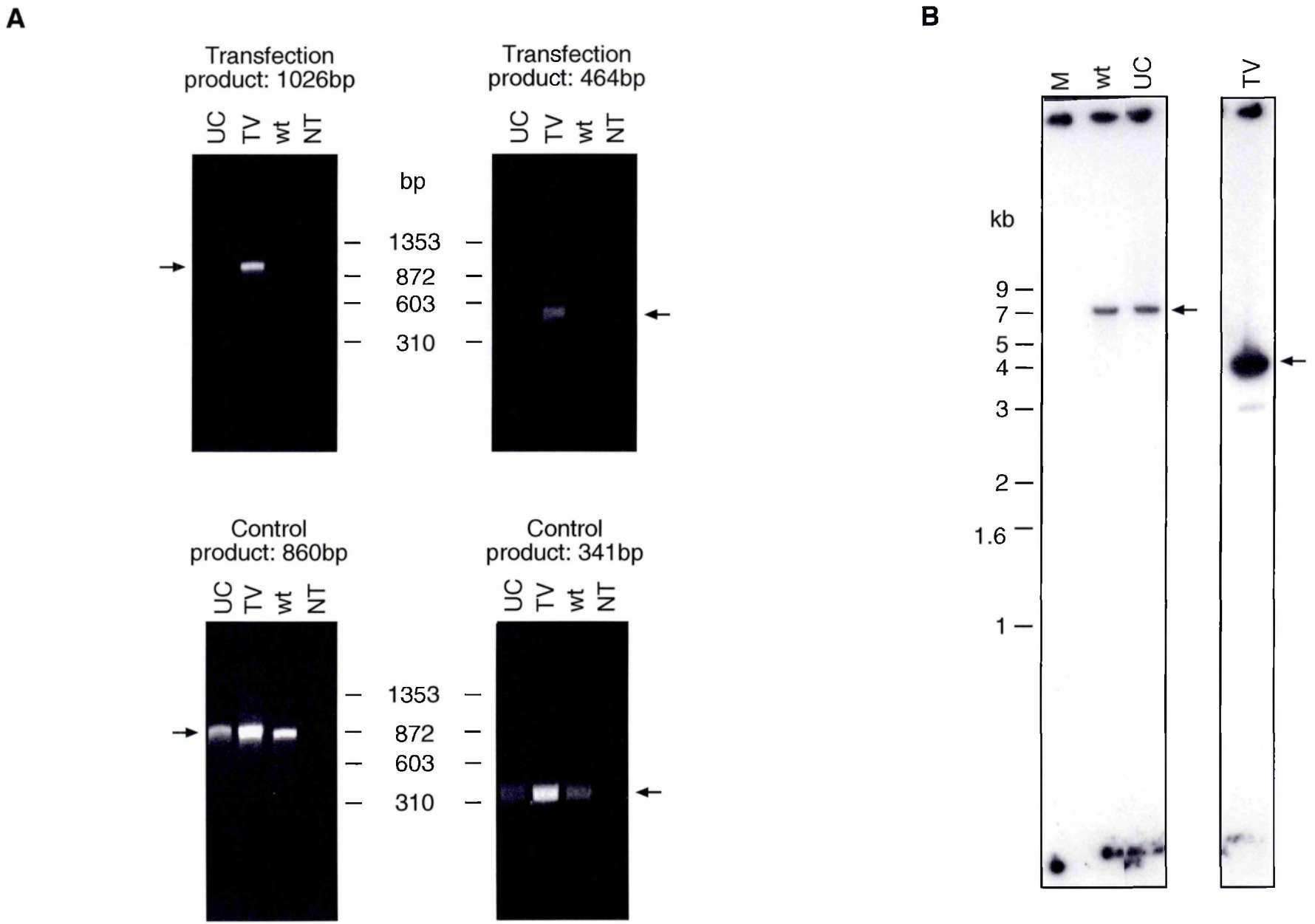
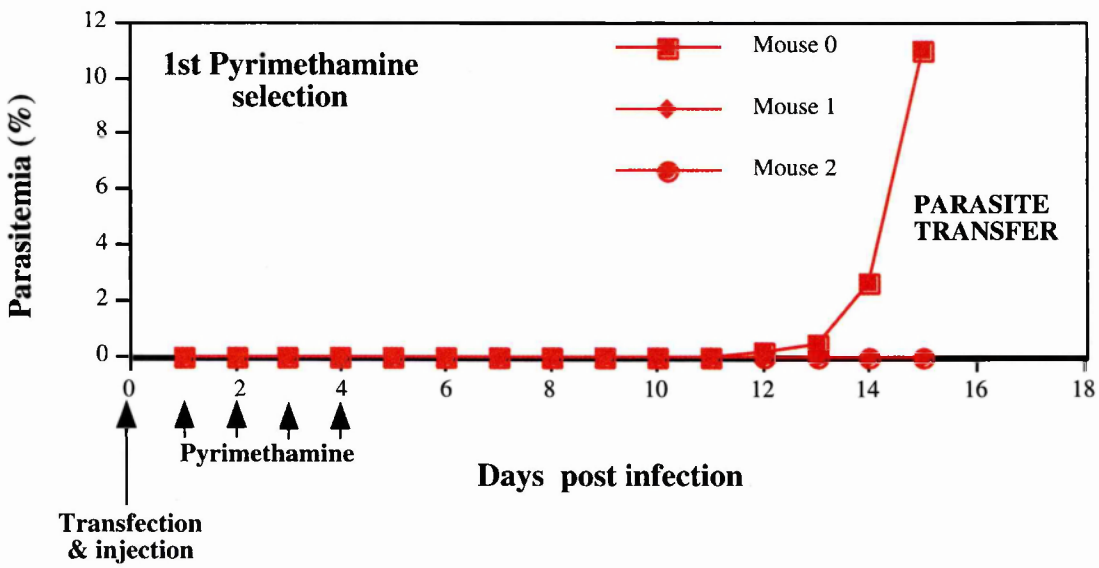

A
Transfection product: 1026bp
UC TV wt NT
bp
1353
872
603
310
Transfection product: 464bp
UC TV wt NT
Control product: 860bp
UC TV wt NT
1353
872
603
310
Control product: 341bp
UC TV wt NT
B
M wt UC
TV
kb
9
7
5
4
3
2
1.6
1

Figure 5.20

Transfection experiment 2 - parasite growth

(A) Parasites were electroporated and injected into two female BALB/c mice on day zero (long vertical arrow). The animals were treated with pyrimethamine on days one, two, three and four (short vertical arrows). Twelve days post inoculation parasites were observed in Mouse 0 and so were passaged to two new mice. These mice, shown in **(B)**, also received pyrimethamine on days one, two, three and four (short vertical arrows). Both animals subsequently developed infections, detectable on day nine and ten post-transfer. Parasites from both mice were screened by PCR and Southern blot ("UC1" and "UC2" in Fig 5.21).

A


1st Pyrimethamine selection
Mouse 0
Mouse 1
Mouse 2
PARASITE TRANSFER
Parasitemia (%)
Pyrimethamine
Transfection & injection
Days post infection

B

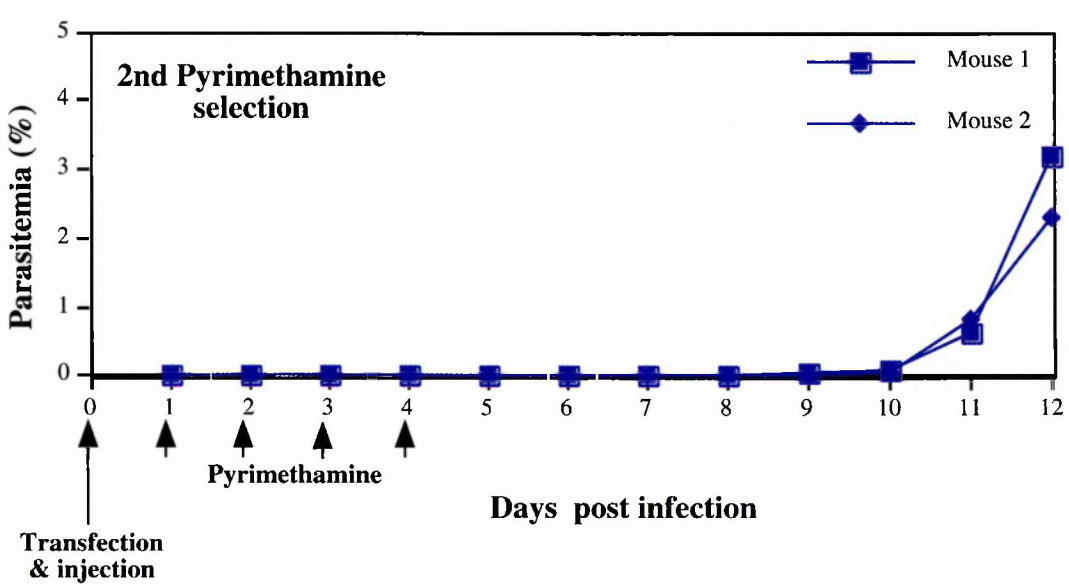

2nd Pyrimethamine selection
Mouse 1
Mouse 2
Parasitemia (%)
Pyrimethamine
Transfection & injection
Days post infection

Figure 5.21

Transfection experiment 2 – screening

Two un-cloned parasite populations were isolated from Mouse 1 (UC1) and Mouse 2 (UC2) after two rounds of pyrimethamine treatment, DNA extracted and screened by PCR and Southern analysis.

(A) Products from PCR amplifications using template DNA from un-cloned parasites from Mouse 1 (UC1) and Mouse 2 (UC2), the pSLAPRep transfection vector (TV), and wild-type *P. berghei* DNA (wt) were analysed by gelelectrophoresis (1% agarose). The two top panels show amplification of products of the expected sizes (1026 basepairs and 464 basepairs) from the transfection vector only (arrows).The lower panels show amplification of the expected products (860 basepairs and 341 basepairs) from all templates (arrows). No amplification has taken place in the reactions without template (NT).

(B) Southern analysis of DNA from Mouse 1 and 2 (UC1 and UC2, respectively). A single band of approximately seven kilobases (arrow, left panel), identical to the band in the *P. berghei* wild type negative control sample, was generated by both samples. The transfection vector (TV) was included as a positive control for the restriction digest and produced a band at the expected 4 kilo bases (arrow, right panel).

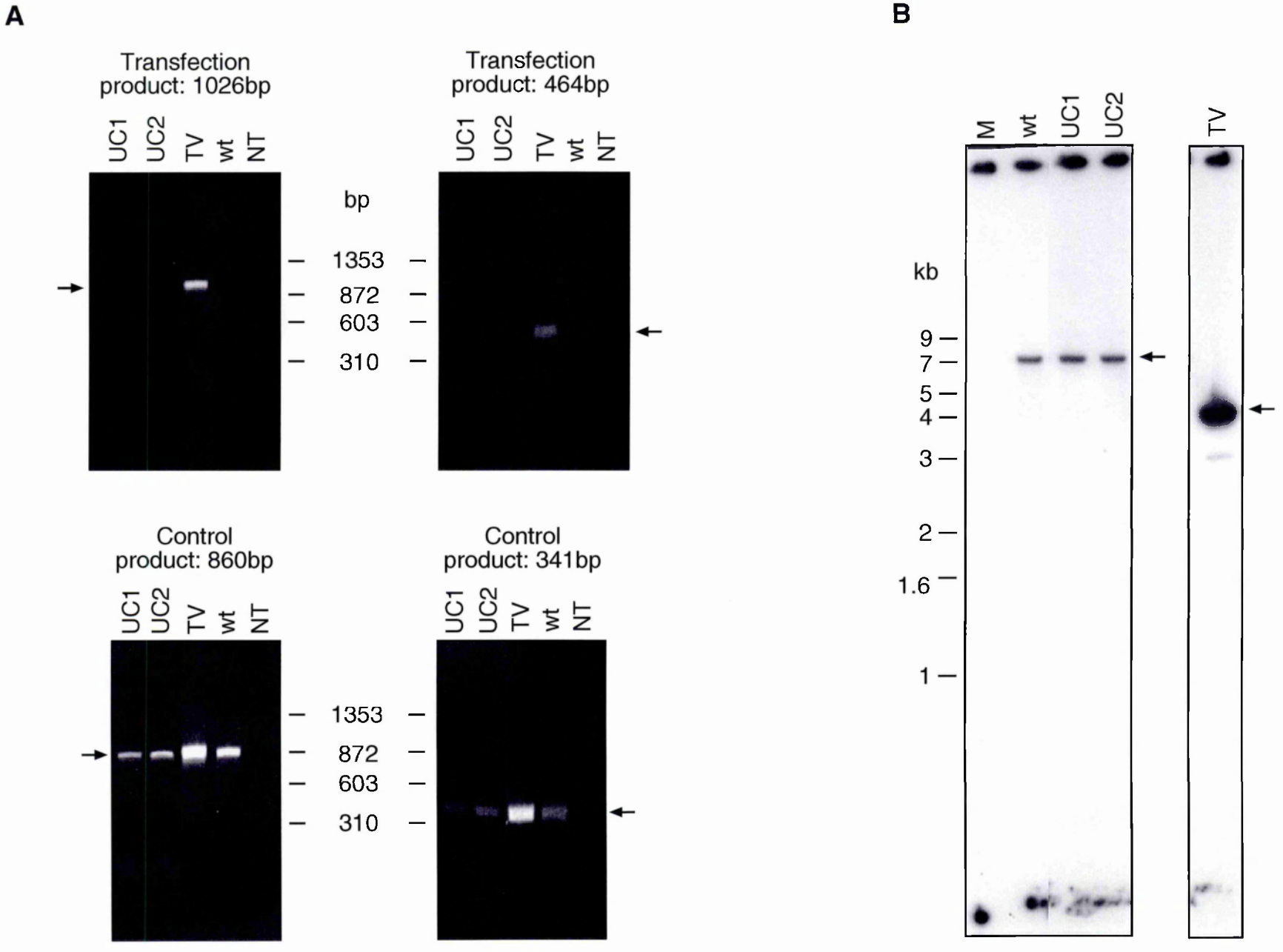
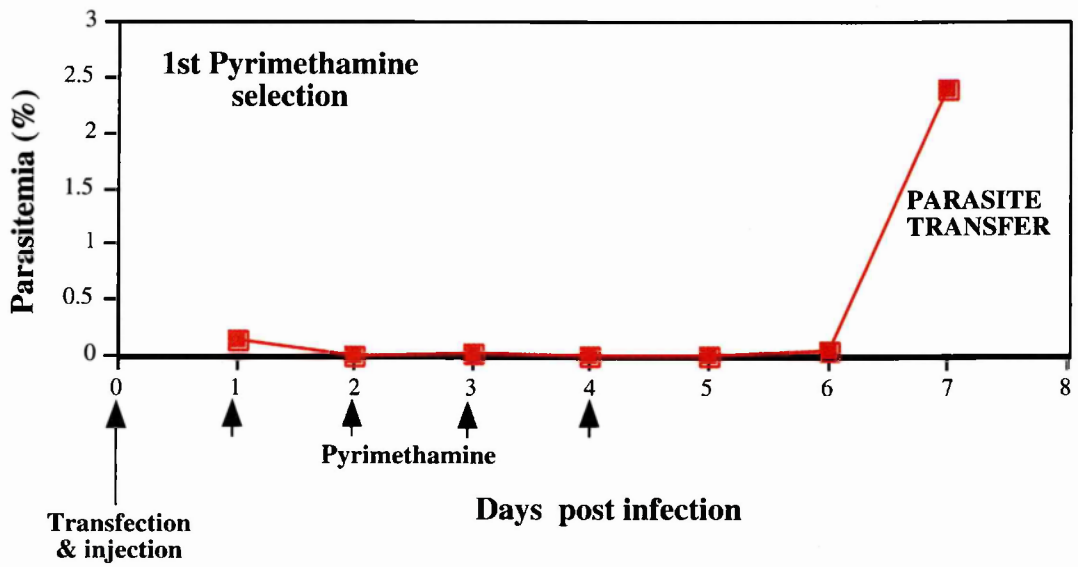

A
Transfection product: 1026bp
UC1
UC2
TV
wt
NT
bp
1353
872
603
310
Transfection product: 464bp
Control product: 860bp
Control product: 341bp
B
M
wt
UC1
UC2
TV
kb
9
7
5
4
3
2
1.6
1

Figure 5.22

Transfection experiment 3 - parasite growth

(A) Parasites were electroporated and injected into one BALB/c mouse on day zero (long vertical arrow). The mouse was treated with pyrimethamine on days one, two, three and four (short vertical arrows). Peripheral parasites were observed on day six post inoculation and were transferred to four new BALB/c mice. These mice, shown in **(B)**, also received pyrimethamine on days one, two, three and four (short vertical arrows). Mouse 3 subsequently developed an infection, detectable on day eight post-transfer. Parasites from Mouse 3 were cloned by limiting dilution and screened by PCR and Southern blot ("UC" in Fig 5.23).

A


1st Pyrimethamine selection
Parasitemia (%)
PARASITE TRANSFER
Pyrimethamine
Transfection & injection
Days post infection

B

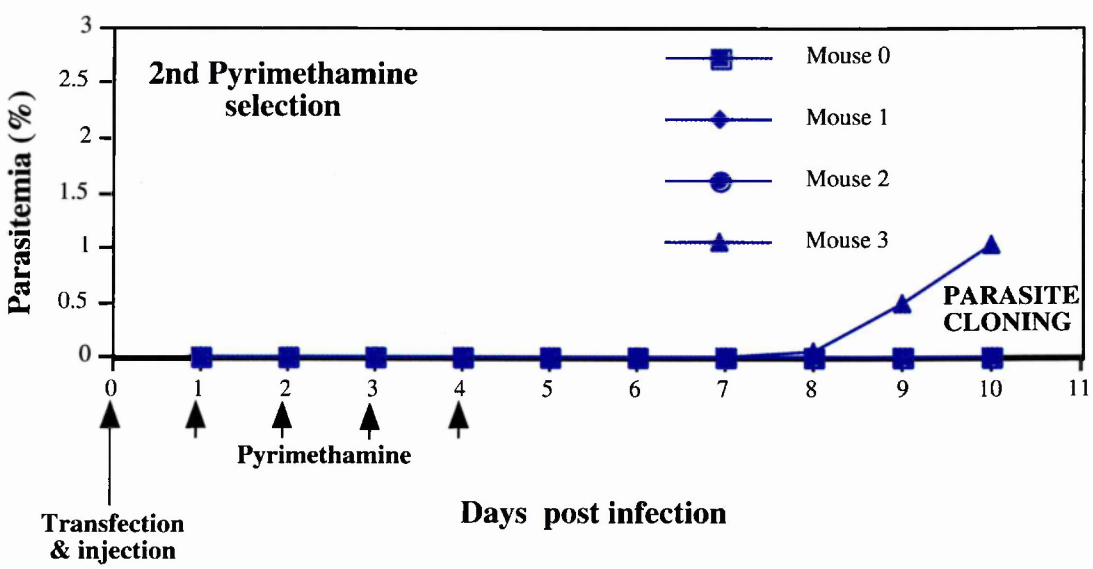

2nd Pyrimethamine selection
Mouse 0
Mouse 1
Mouse 2
Mouse 3
Parasitemia (%)
PARASITE CLONING
Pyrimethamine
Transfection & injection
Days post infection

Figure 5.23

Transfection experiment 3 – screening

Parasites were isolated after two rounds of pyrimethamine treatment from Mouse 3 (uncloned=UC), and from three mice after cloning by limiting dilution (M0, M1 and M2).

(A) Results from PCR amplification using gDNA from the uncloned (UC) parasite population, from cloned parasites from three different mice (M0, M1 and M2), from the transfection vector and from wild type *P. berghei* gDNA. In the two top panels amplification products can be seen only from the transfection vector template. The two lower panels show amplification of products of the correct sizes from all templates. A sample without DNA template (no template=NT) was used as a negative control.

(B) Southern analysis of the uncloned (UC) parasite population showed a single band of approximately 7 kilo bases (arrow, left panel), identical to the band in the *P. berghei* wild type negative control sample. The transfection vector (TV) was included as a positive control for the restriction digest and produced a band at the expected 4 kilo bases (arrow, right panel).

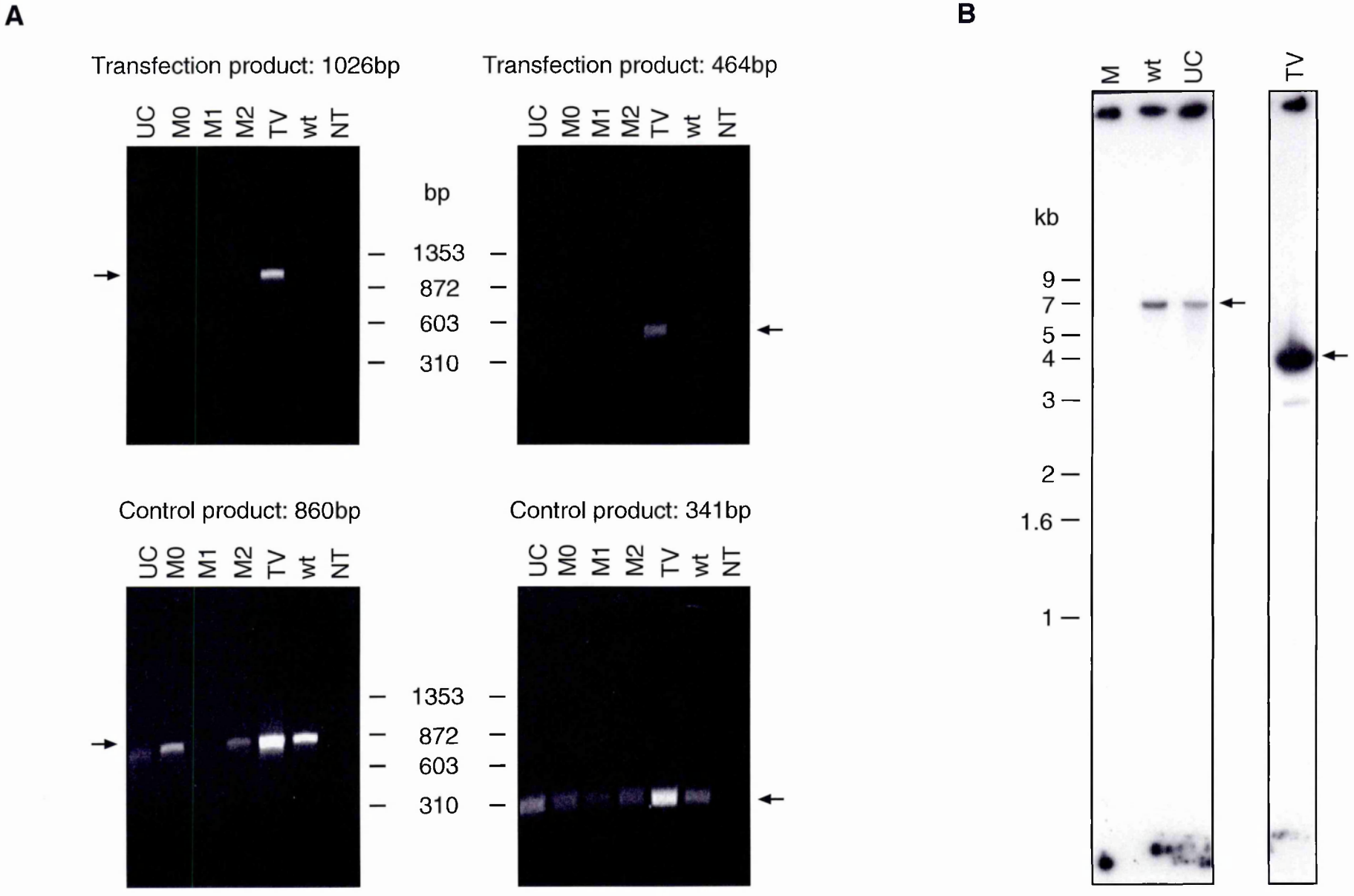
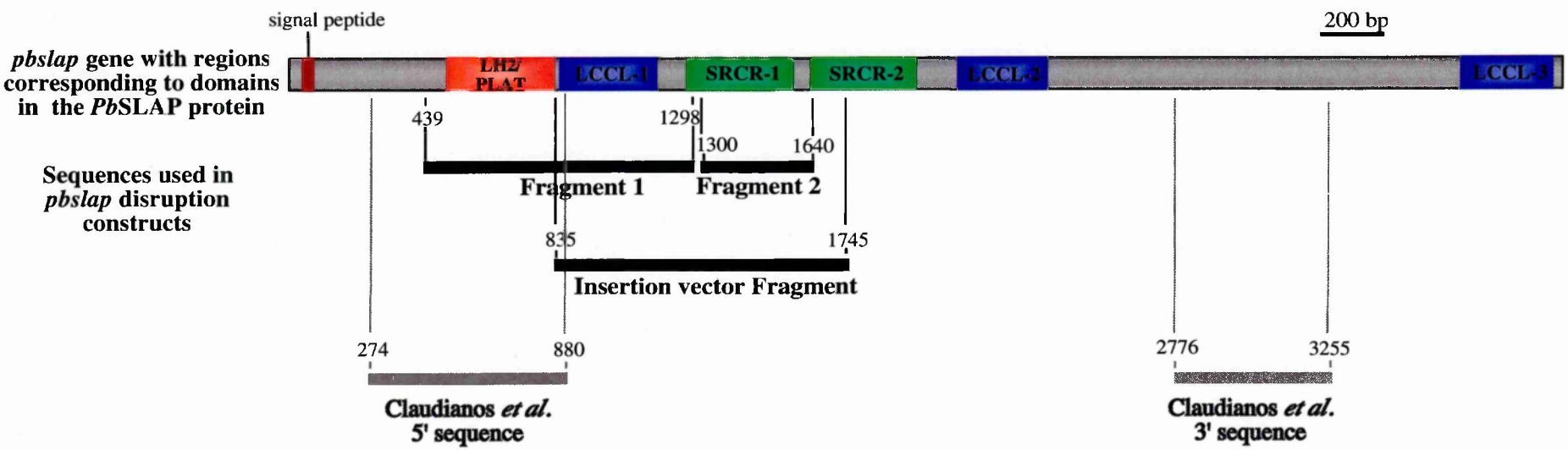

A
Transfection product: 1026bp
Transfection product: 464bp
UC
M0
M1
M2
TV
wt
NT
bp
1353
872
603
310
Control product: 860bp
Control product: 341bp
B
M
wt
UC
TV
kb
9
7
5
4
3
2
1.6
1

Figure 5.24

Locations of gene sequences used in *pbslap* disruption constructs

A paper by Claudianos et al. (2002) reported on a disruption of the *pbslap* gene, using a replacement vector containing sequences from nucleotide 274 to 880 and 2776 and 3255. The schematic shows the location of these sequences in relation to the sequences used in the present study for the pSLAPRep vector.


signal peptide
200 bp
pbslap gene with regions corresponding to domains in the *Pb*SLAP protein
LH2/ PLAT
LCCL-1
SRCR-1
SRCR-2
LCCL-2
LCCL-3
439
1298
1300
1640
Sequences used in *pbslap* disruption constructs
Fragment 1
Fragment 2
835
1745
Insertion vector Fragment
274
880
2776
3255
Claudianos *et al.* 5' sequence
Claudianos *et al.* 3' sequence

Chapter Six

Conclusions and Future work

It is clear that a more detailed knowledge of the unique biology of *Plasmodium* is required to in order to control malaria. Understanding the life cycle of the parasite and how it interacts with its vectors is essential for developing new strategies to combat the disease. The recently completed sequencing projects of the genomes of *Plasmodium falciparum* and *Anopheles gambiae* now provide an invaluable source of information for malaria research. The availability of the genomic data has also allowed the development of new approaches to study transcription and expression on a whole organism level. However, in order to gain a detailed understanding of the role and significance of a specific gene product, there is still a need for analysis of individual proteins.

This project aimed to identify the full sequence of a previously unknown gene in *Plasmodium chabaudi chabaudi* (AS), and to analyse the structure, expression and function of the encoded protein.

Using Vectorette and conventional PCR, *pcslap* was found to be a single exon gene of 3897 basepairs. Southern analysis demonstrated that the gene was present in a single copy in the *P. chabaudi chabaudi* (AS) genome, as well as in *P. yoelii yoelii* (17X strain) and *P. berghei* ANKA. Translation of the nucleotide sequence revealed a 1298 residue protein with a complex and unique domain structure. Two regions show similarity to Scavenger Receptor Cysteine-Rich (SRCR) domains, an ancient superfamily found in a diverse range of organisms, that includes both vertebrates and invertebrates. Although no unifying function or ligand has yet been identified, many SRCR-domain-bearing molecules are involved in immunity and the domains themselves are thought to mediate receptor-ligand interactions. Flanking the two SRCR domains are two *Limulus* factor C, Coch-5b2 and Lgl1 (LCCL) modules, with a third LCCL module located near the C-terminal of the protein. The LCCL module is a recently identified motif with an unknown function, although recent studies suggest it is involved in ligand binding. The third motif

identified in *Pc*SLAP is the N-terminal LH2/PLAT domain, found in many membrane-associated molecules and believed to mediate protein-protein or protein-lipid interactions. The presence of an N-terminal signal sequence and the lack of a transmembrane region or GPI-binding site suggest that *Pc*SLAP is a secreted molecule.

Interestingly, multiple sequence alignments showed that *P*SLAP is highly conserved across *Plasmodium spp.*, with a 79% identity between the *P*SLAP proteins of *P. chabaudi*, *P. yoelii* and *P. berghei*, and 63% identity between the *P. chabaudi* and the *P. falciparum* homologous proteins. This strongly implies that *P*SLAP has the same function within *Plasmodium spp.* and allowed some of the expression- and localization studies in this project to be performed on species related to *P. chabaudi*. However, despite the high degree of conservation within *Plasmodium*, the only orthologue found so far is the recently identified *Toxoplasma gondii* Scavenger Receptor 1 (*Tg*SR1). *Tg*SR1 is made up of a set of domains identical to *P*SLAP, but has an additional LCCL domain at the C-terminal. Alignments show an overall identity of 30% with *P. falciparum* SLAP, but 37-56% identity between the structural motifs. This indicates that *P*SLAP and its homologues may possess a function that is both vital and specific to apicomplexan biology.

To study the stage of expression of *P*SLAP, as well as its location, antibodies were raised against the protein and used in indirect immunofluorescence assays. Using a monoclonal antibody against the sexual-stage antigen *Pf*s48/45 for co-localisation studies, it was demonstrated that *P. berghei* and *P. falciparum* *P*SLAP are expressed in blood-stage gametocytes. The fluorescence of *P*SLAP within the infected erythrocyte displayed a granular expression pattern, suggesting that the protein may be stored in vesicles, perhaps for later secretion. This is consistent with the identification of a signal sequence that would direct the protein through a secretory pathway. However, the distribution of the protein appeared to be restricted to the parasite, indicating that, at the gametocyte stage, *P*SLAP is not transported into the erythrocyte cytoplasm but retained inside the parasite.

Finally, in order to analyse the function of *P*SLAP, an attempt was made to eliminate the protein in *P. berghei* by targeted gene disruption. Two types of transfection vectors were successfully constructed, however, disruption of the gene was unsuccessful.

The findings in this project, together with data from recent reports in the literature, raise interesting questions regarding the role of *P*SLAP and related molecule(s) in

Apicomplexa, and allow some speculation on possible functions for this novel protein, and how these questions could be addressed.

Towards the end of this project, a highly relevant study was published reporting on a successful disruption of the *pslap* gene in *P. berghei* ANKA (Claudianos *et al* 2002). The report confirmed the hypothesis previously put forward by us that *P*SLAP is likely to play a role in the mosquito due to its expression in the transmission stages of the erythrocytic life cycle (Delrieu *et al.*, 2002). The study demonstrated that despite normal development up until the early oocyst stage, *Pb*SLAP-deficient parasites were unable to produce sporozoites. The report stated that *Pb*SLAP-negative parasites developed fully through the erythrocytic cycle and formed normal numbers of gametocytes in mice. There was no reduction in viability in gametocytes and analysis of the mosquito stages of the life cycle showed that ookinetes and early oocysts, as analysed by confocal microscopy, developed in numbers comparable to those in wild-type parasites. However, from day 10 post parasite ingestion and onwards, there was a clear difference between wild-type and disrupted parasites. Although DNA staining showed normal nuclear organization within all oocysts, in SLAP-deficient parasites there was no evidence of cytokinesis (division of cytoplasm) and subsequent sporozoite growth. The absence of sporozoites was confirmed by mosquitoes fed on *Pb*SLAP-negative parasites being unable to transmit the infection to mice. In addition, five days post infection, oocyst growth curves started diverging, and by day 15, *Pb*SLAP-deficient oocysts were on average 35% larger (measurement of mean diameter of 100 oocysts) than wild-type oocysts.

Although this data clearly shows that *P*SLAP plays an essential role in the oocyst to sporozoite transformation, the actual function of the protein is still not understood.

Does *P*SLAP function in development or in immunity?

Disruption of the *slap* gene resulted in a failure of maturing oocysts to support the development of growing sporozoites. Although the result of an absence of *P*SLAP is clear, the cause of the phenotype is not.

Perhaps *P*SLAP is a developmental molecule, required for the initiation of sporozoite morphogenesis within the oocyst? Sporogony is a complex developmental process that is

highly dependent on interactions between the developing oocyst and the mosquito basal lamina. It begins in the young oocyst, which is surrounded by an electron-dense capsule. As the oocyst develops, it enlarges progressively and the nucleus divides repeatedly. The plasma membrane starts retracting from the capsule, giving rise to subcapsular vacuoles. These enlarge and neighboring vacuoles merge into larger clefts that penetrate and divide the oocyst cytoplasm, forming the sporoblast. Sporozoites evaginate along the surface of the membranes of the sporoblast, and a nucleus and cytoplasmic components are transferred into the sporozoites, before they bud off from the sporoblast "like rays from the sun" (Figure 6.1; Menard *et al.*, 1997; Vanderberg *et al.*, 1967).

However, considering the structural features of *P*SLAP, it is also possible that the protein plays a protective role against mosquito immune factors that target the parasite and inhibit sporozoite growth. The oocyst is an extracellular stage of the parasite life cycle that develops in the mosquito hemocoel, and is therefore highly exposed to the insect immune response. Malaria infections induce strong innate immune defense reactions in the mosquito, with temporal and spatial activation of immune-related genes in a pattern that follows the development of the parasite (Dimopoulos *et al.*, 1998; Section 1.8.4). The anti-pathogenic response includes up-regulation of genes encoding pattern-recognition receptors, serine proteases and antimicrobial peptides.

To study the effects of the mosquito response to *P*SLAP-deficient parasites and specifically oocyst- and sporozoite development, an *in vitro* culturing system would be useful. Methods for culturing of the whole parasite life cycle, including the developmental phases in the mosquito, have recently been described and would provide an environment to study parasite development and survival without interference from an activated insect immune system (Al-Olayan *et al.*, 2002). This would allow an analysis of whether the arrested development of sporozoites in *P*SLAP-deficient parasites is due to damaging effects of the mosquito immune response, or whether it is a result of a developmental defect.

What are the ligands interacting with *P*SLAP?

Another question is that of potential ligands for *P*SLAP. What molecule(s) is *P*SLAP interacting with, and which structural motif(s) is/are involved in the interaction(s)? The

complex structure of *P*SLAP and the fact that all identified domains have previously been implicated in ligand-binding, strongly suggests a role in inter-molecular interactions.

Analysis of a molecule by gene disruption is an "all or nothing" approach, resulting in a null mutant that completely lacks the protein in question. However, in order to elucidate specifically what domain(s) of *Pb*SLAP that is/are involved in the processes supporting sporozoite development, generation of parasites with partial gene deletions, "domain knockouts", are likely to be more informative. This approach was successfully employed by Tewari *et al.* (2002) for analysis of the functions of regions I and II of the *Plasmodium* circumsporozoite protein (CSP). Region I is based around a short, conserved amino acid motif and region II is a sequence with homology to the type I repeat in human thrombospondin (TSP). It was found that, while parasites lacking region I showed no impairment in locomotion or host cell invasion, removal of region II abolished sporozoite motility and greatly reduced the ability of parasites to invade mosquito salivary glands and vertebrate hepatocytes.

Once the domain(s) in *P*SLAP responsible for binding has/have been determined, the molecular interactions may be characterised in further detail by transfection of *pslap* genes with mutated versions of the binding region, in order to pinpoint the specific residues contributing to the interaction.

Does PSLAP function at stages other than in oocysts/sporozoites?

It is still unclear whether *P*SLAP functions at several stages in the parasite life cycle. It is surprising that *pslap* gene disruption only produced a phenotype different from that of wild type parasites in oocysts/sporozoites, as studies by us and others on the expression pattern of *P*SLAP have demonstrated protein synthesis in all stages from blood stage gametocytes through to salivary gland sporozoites (Delrieu *et al.*, 2002; Lasonder *et al.*, 2002; Truman *et al.*, manuscript in preparation). Because of its multi-domain structure, it is not unreasonable to think that *P*SLAP may also have multiple functions.

Development through the insect stages of the parasite life cycle require specific interactions between parasite and mosquito molecules, as they involve a series of differentiation and migration events, such as attachment to and traversion of the mosquito midgut epithelium, interactions with the midgut basal lamina for oocyst formation, and sporozoite entry into the mosquito salivary glands (Figure 6.1). However, disruption of

the *pbslap* gene showed no reduction in viability in either ookinetes or (early) oocysts, indicating that *Pb*SLAP is not involved in these stages of parasite maturation (Claudianos *et al.*, 2002). Still, in advanced biological systems there is frequently redundancy and complementation between molecules, and this also appears to be true for *Plasmodium*. Knock out studies on the sexual-stage proteins P25 and P28 in *P. berghei* (*Pb*s25 and *Pb*s28) showed that disruption of either one of the two genes produced essentially no phenotype, whereas disruption of both genes had multiple deleterious effects on several stages of parasite development (Tomas *et al.*, 2001). However, while the P25 and P28 proteins have great structural similarities, and therefore would be expected to perform somewhat similar functions, the domain composition of *P*SLAP is unique. Still, although the full repertoire of motifs in *P*SLAP seems to be unique, individual domain types have been identified in other Plasmodial molecules. While the SRCR motif appears to be an exclusive feature of *P*SLAP, the LCCL module is present in additional proteins. A mass spectrometry study of the *P. falciparum* blood-stage proteome by Lasonder *et al.* (2002) reported on four LCCL-containing proteins, one of which was *Pf*SLAP. All four proteins were expressed in sexual-stage parasites (gametocytes and gametes), and all contained signal sequences, predicting that they are secreted like *P*SLAP. A fifth gene coding for an LCCL-containing protein was recently identified by Truman *et al.* (manuscript in preparation), prompting these authors to propose that the molecules belong to a small protein family characterized by the presence of LCCL or lectin-like motifs. Together with a sixth molecule carrying an "anthrax protective antigen"-domain (but no LCCL module) they make up the LCCL/Lectin Adhesive-like Protein (LAP) family. MudPIT analysis of *P. berghei* showed that four out of the five LCCL-bearing members of the family are expressed in gametocytes and ookinetes, and two also in oocysts. These results are suggestive of a specific role for the LCCL module during the parasite stages in the mosquito midgut. It is therefore possible that if *P*SLAP is indeed a multi-functional protein, a role dependent on the LCCL motif could perhaps also be performed by another LCCL-containing molecule. If this were the case, a disruption of the *slap* gene would not produce a phenotype during these stages of the life cycle.

In the report by Claudianos *et al.* (2002) the effects of *P*SLAP depletion in the developmental stages beyond the late oocyst stage, such as sporozoite release and

migration, mosquito salivary gland infection and hepatocyte invasion in the vertebrate host, were not investigated. However, a recent study used functional complementation to address this issue (Truman *et al.*, manuscript in preparation). Functional complementation makes use of the heterokaryotic state of the ookinete: gametocytes of different genotypes are mixed *in vitro* and fed to mosquitoes, where they through fertilisation produce parasites with a heterozygous genotype. Depending on the role of the protein encoded by the disrupted gene, this may or may not complement the loss of function and rescue diploid individuals. In the case of *pbslap*, *pbslap*$^{-}$/*pbslap*$^{+}$ parasites were able to develop fully through the oocyst stage, producing both *pbslap*$^{-}$ and *pbslap*$^{+}$ sporozoites. Transmission of the these sporozoites to mice, and analysis by diagnostic PCR of parasite gDNA isolated from the mice, showed that the animals had been successfully infected with the *pbslap*$^{-}$ genotype. These results demonstrated that *Pb*SLAP is not essential for sporozoite migration to, or invasion of, mosquito salivary glands, or for infection of vertebrate (murine) hepatocytes.

Is *P*SLAP a gametocyte sequestration receptor?

Studies on the stage-specific expression of *P*SLAP in the present project showed that the protein was present in blood-stage gametocytes, a finding that was later confirmed by a high-accuracy mass spectrometry study of the *P. falciparum* proteome (Delrieu *et al.*, 2002; Lasonder *et al.*, 2002). The last event in blood-stage maturation before transmission of *Plasmodium* to its insect host can take place is gametocytogenesis – the sexual differentiation of asexual parasites into male and female gametocytes. In *P. falciparum*, an interesting characteristic of these sexual forms is their ability to sequester in the microvasculature of the vertebrate host, primarily in the spleen and bone marrow (Thomson, 1914; Garnham, 1931; Smalley *et al.*, 1981). This phenomenon is thought to play a critical role in the development and survival of the sexual stages. Cytoadhesion of asexual-stage parasites to the host endothelium of organs such as the spleen, brain and kidneys is well documented and involves the parasite-encoded molecule *Plasmodium falciparum* Erythrocyte Membrane Protein-1 (*Pf*EMP-1), expressed on the infected erythrocyte surface, and several host receptors including ICAM-1 and CD36 (Section 1.7.1). However, sequestration of sexual stage parasites is less well understood. It appears that very young *P. falciparum* gametocytes (stages I and IIa) retain the adhesive

properties of asexual parasites, such as expression of *Pf*EMP-1, knob (electron dense macromolecular complexes surrounding *Pf*EMP-1) formation and ability to bind CD36. However, from stage IIb onwards these characteristics change or are lost, and so the nature of adhesion is altered (Day *et al.*, 1998; Hayward *et al.*, 1999). A study by Rogers *et al* (2000) found that, out of a panel of monoclonal antibodies with different specificities, that against ALCAM (CD166) was the one which most strongly inhibited binding of gametocytes to two bone marrow cell lines. Interestingly, ALCAM is the ligand for the lymphocyte antigen CD6, a membrane-bound molecule composed of three SRCR domains. The interaction between ALCAM and CD6 has been characterized in some detail and found to involve the most membrane proximal SRCR domain of CD6 and the most membrane distal Ig-like domain of ALCAM (Bowen *et al.*, 2000). This raises the question of whether *P*SLAP could be the so far un-identified receptor on gametocyte-infected erythrocytes that mediates adhesion to human bone marrow. Is it possible that the SRCR domains of *P*SLAP interact with the Ig domains on ALCAM?

Gametocyte sequestration is also a feature of murine Plasmodia, and has been observed in *P. chabaudi, P. yoelii* and *P. vinckei.* Examination of these species have shown that the stage of gametocytes that sequesters also appears to be that with the highest degree of infectivity to mosquitoes (Landau *et al.*, 1979; Gautret *et al.*, 1996). If *P*SLAP is involved in gametocyte adhesion it would be expected to see a difference in infectivity in *P*SLAP-negative parasites, as compared with wild-type parasites. In the report on the disruption of the *slap* gene in *P. berghei* by Claudianos *et al* (2002) the authors stated that *Pb*SLAP KO parasites formed normal numbers of gametocytes in mice, and that they developed into oocysts in numbers comparable to wild-type parasites. Oocyst counts were obtained from assays where mosquitoes had been fed either on gametocyte-infected mice or on ookinetes from membrane feeders. However, a closer examination of the oocyst counts reveal greater numbers from knock out- than from wild type parasites when using gametocytes as feed (Table 6.1). If *P*SLAP plays a role in gametocyte sequestration, it would be expected that a lack of the protein would have reduced the number of sequestered gametocytes in an infected mouse, and thereby increased the number of highly infective gametocytes in the circulation, resulting in a rise in the numbers of oocysts formed in mosquitoes fed on these gametocytes, as was observed in the study by Claudianos *et al.* (2002).

Table 6.1 Effect of *pbslap* disruption on *P. berghei* infectivity to *A. stephensi*, as analysed by numbers of oocyst formed in mosquitoes. Modified from Claudianos *et al.* (2002).

Exp	Feed	WT	*Pb*SLAP KO	% increase/decrease in KO compared to WT
1	Gametocytes	18	39.2	118% increase
2		111	143	29% increase
3	Ookinetes	38.7	43.7	13% increase
4		46.6	41.7	11% decrease

Exp = experiment, WT = wild type, KO = knock out

In order to function as a gametocyte sequestration receptor, *P*SLAP would have to be expressed on the surface of the infected red blood cell. Although there is as yet no evidence for this, it is still possible that the level of expression is too low to be detected by the methods used so far. *P*SLAP does not carry a transmembrane domain or a typical GPI anchor site but could be attached to a membrane protein via an interaction with one of its many adhesive structures, perhaps the LH2 domain, a type of motif often found in membrane or lipid-associated proteins (Bateman and Sandford 1999). Although this would be a weak interaction, parasites sequestered in the bone marrow are protected from the forces of shear flow encountered in the microvasculature of major organs, and therefore may still be able to successfully sequester in this environment through such an interaction. Moreover, it is consistent with the lower avidity observed in *in vitro* adhesion assays reported for gametocyte sequestration, as compared with similar assays using asexual parasites (Rogers *et al.*, 2000).

To address the question of whether *P*SLAP may be involved in gametocyte sequestration the same *in vitro* adhesion assays used by Rogers *et al* (2000) could be employed. Antibodies directed against the two SRCR domains of *P*SLAP could be used with the objective of determining whether binding of gametocyte-infected erythrocytes is inhibited or not, to investigate whether the interaction is mediated by the *P*SLAP SRCR domains.

Is *P*SLAP a modulator of the mosquito immune response?

Parasite survival in the insect host requires not only a carefully tuned developmental program, but also efficient and successful protection from the mosquito immune response. The mosquito is capable of mounting a range of anti-parasitic reactions, including both humoral and cellular responses. These are initiated approximately 24 hours post parasite ingestion, at the time of ookinate midgut invasion, and continue throughout the parasite residence in the insect (Dimopoulos *et al.*, 1998). A central role in invertebrate immunity is played by serine proteases, which regulate anti-pathogenic defense mechanisms such as melanotic encapsulation, hemolymph coagulation and antimicrobial peptide synthesis (Gorman and Paskewitz, 2001). It is therefore interesting to find structural motifs that are shared between a mosquito immune molecule and a Plasmodial protein expressed during the insect-stages of development. Sp22D is a serine protease composed of two SRCR domains, two Chitin-binding type 2 domains (CBDs), two Low-density lipoprotein receptor class A (LDLRA) domains and a Trypsin-like serine protease domain (Gorman *et al.*, 2000; Figure 3.22). Although the function of Sp22D is still unknown, Gorman *et al.* (2000) suggest a role in immunity due to the fact that most of its domains are similar to immune-related proteins, it is up-regulated in response to bacterial challenge but not to wounding, and it is constitutively transcribed in hemocytes, fat body, and midgut epithelium – all tissues with important immune functions. A general feature of invertebrate immunity is the use of defense systems that respond to antigens exposed on the surface of invading pathogens. The composition of Sp22D resembles a pathogen-recognition molecule: SRCR domains were recently shown to bind a wide repertoire of gram positive and –negative bacteria and CBDs bind chitin, a surface component of fungi and nematode eggs (Bikker *et al.*, 2002; Shen and Jacobs-Lorena, 1999). This suggests that Sp22D could be involved in detection of a broad range of micro-organisms, including parasites. To avoid destruction while inside its insect host, it is likely that *Plasmodium* would have developed mechanisms to deflect the mosquito immune response. Structural and functional mimicry is a wide-spread strategy for immune evasion among pathogens and involves the use of a self molecule that functionally and/or structurally resembles a host protein. Thus, *P*SLAP may act as such an immunomodulator, competing for the same ligand as Sp22D, thereby impairing the parasite eradication process and increasing the chances of survival.

Does *P*SLAP have a function similar to Circumsporozoite Protein?

Studies on the expression pattern of *P*SLAP have shown protein synthesis from gametocytes through to sporozoites (Delrieu *et al.*, 2002; Truman *et al.*, manuscript in preparation). Surprisingly, however, *slap* gene disruption only produced a phenotype different from that of wild type parasites in sporozoites. Early oocyst development was normal, but there was no evidence of cytokinesis and subsequent sporozoite formation (Claudianos *et al.*, 2002). Interestingly, disruption of the gene encoding the *P. berghei* circumsporozoite protein (CSP), produced a similar phenotype: oocyst development up until day ten was identical in CSP-negative and wild-type parasites, however, starting on day 11 as sporozoites began appearing in wild-type oocysts, CSP-negative oocysts displayed an empty, vacuolated structure. Due to its expression in the unsegmented oocyst and on the membrane invaginations in the sporoblast cytoplasm, the authors suggested that CSP may have a regulatory function in early oocyst growth, perhaps guiding membrane formation during sporogony by interacting with receptors in the intersporozoite matrix (Menard *et al.*, 1997; Figure 6.1). Considering the many adhesive-like domains of *P*SLAP and similarities between the CSP- and *P*SLAP- deficient phenotypes, it seems possible that the two proteins could have similar functions. This is also supported by the follow-up to the *pbslap*-disruption study that used functional complementation to restore the *Pb*SLAP-negative phenotype. Complementation of the loss of function in *pbslap*$^-$ was successful and diploid *pbslap*$^-$/*pbslap*$^+$ parasites were able to develop fully through the oocyst stage. However, infection of mosquitoes with a mixed population of *pbslap*$^-$ and *pbslap*$^+$ homozygous ookinetes did not rescue *pbslap*$^-$ oocysts (Truman *et al.*, manuscript in preparation). This finding suggests that *Pb*SLAP functions inside the immature oocyst, an idea that is more compatible with a developmental rather than immuno-regulatory function.

A possible approach to investigating the role of *P*SLAP in oocyst differentiation would be to carry out ultrastructural studies using recombinant parasites with reduced production of *P*SLAP, and compare the morphology of these with wild type and *P*SLAP-negative oocysts. This may provide a correlation between the level of development of specific structures within the oocyst with the amounts of protein being produced, and thereby identify a more specific role for *P*SLAP.

Are *P*SLAP and *Toxoplasma gondii* Scavenger Receptor 1 functional orthologues?

It is interesting that an orthologue of *P*SLAP, TgSR1 was recently identified in another apicomplexan parasite, Toxoplasma *gondii*. So far no function has been ascribed to this protein, but the almost identical domain organisation suggests a conserved and essential function for the SLAP/TgSR1 proteins, specifically evolved in *Apicomplexa*.

Most Apicomplexans exhibit an overall general life cycle, characterised by three distinct processes: sporogony, merogony and gametogony. However, differences between *Plasmodium* and *Toxoplasma* include host species, host cell specificity and mode of transmission. *T. gondii* infects a wide range of mammals, including birds, pigs and rodents, and infections are widely prevalent in humans causing the disease toxoplasmosis.

The parasite exhibits a predator-prey type life cycle. The definite hosts (where sexual replication occurs) are members of the cat family (*Felidae*), in which the parasite invades the enterocytes (intestinal cells) of the animal's gut. After several rounds of asexual replication, the parasites differentiate into male and female gametocytes, which fuse and transform into an oocyst. The oocyst is shed into the environment with the cat's feces and divides into four sporoblasts, each of which forms a sporocyst containing two sporozoites. These are highly resistant to damage and may persist in the soil for years under favorable (moist) conditions. After ingestion by a secondary, intermediate host, for example a mouse, the sporozoites develop into rapidly dividing tachyzoites, causing an acute infection. In most hosts, tachyzoites transform into the slowly dividing bradyzoites, thereby establishing a chronic infection that results in the formation of latent tissue cysts. Interestingly, tachyzoites, bradyzoites and sporozoites are all infectious, and therefore, carnivorous ingestion of the cysts by a second intermediate host, such as a rat, leads to infection of a naïve individual, allowing an indefinite non-sexual propagation of the parasite, or if the predator is a cat, to initiation of the sexual development and completion of the life cycle (Figure 6.2; Dubey, 1998).

If the role of SLAP in *Plasmodium* is similar to that of *Tg*SR1 in *Toxoplasma*, it would be expected that the proteins function in a situation that exists in both species. *Plasmodium falciparum* is as yet the only apicomplexan parasite with a fully sequenced genome, and

the extent of homology between different members of *Apicomplexa* is therefore not known. However, studies on individual genes and proteins have shown conservation of specific functions. One such example is the involvement of TRAP and orthologous proteins in other apicomplexans, in host cell invasion and parasite gliding motility. Members of the TRAP family of proteins have been found in *Plasmodium* ookinetes and sporozoites, as well as in the invasive stages of *Toxoplasma* (tachyzoite), *Eimeria* (sporozoite) and *Cryptosporidium* (sporozoite). Gene transfection experiments with inter-species substitutions showed that the cytoplasmic tail of *Plasmodium berghei* TRAP was interchangeable with that of the homologous protein in *T. gondii*, Micronemal Protein 2 (MIC2). *Plasmodium* sporozoite gliding motility and cell penetration, both processes that rely on TRAP, were comparable in mutant and wild-type parasites, suggesting that these mechanisms are conserved within *Apicomplexa* (Sultan *et al.*, 1997; Kappe *et al.*, 1999).

It is interesting to note that the life cycle of *Toxoplasma* does not include parasite maturation inside an insect vector. If *P*SLAP has the same function in *Plasmodium* as *Tg*SR1 in *Toxoplasma*, this fact indicates that the protein could not be involved in defense against an insect-specific anti-parasitic immune response, but is more likely to be required for progression of normal development. Alternatively, *P*SLAP may play an additional role in *Plasmodium* in protection against mosquito immune molecules. As gene disruption techniques are also available for manipulation of *T. gondii*, it would be interesting to investigate what effect a disruption of *Tg*SR1 has on the development and survival of *T. gondii* parasites, as this may also shed some light on the function on *Plasmodium* SLAP.

Figure 6.1

Developmental cycle of *Plasmodium* in *Anopheles* mosquitoes

Shortly after the mosquito ingests an infected blood meal, male and female gametocytes escape from the erythrocyte inside the midgut lumen (1) and mature into gametes (2). Fertilisation (3) occurs rapidly generating a zygote (4), which elongates to become an ookinete (5). The motile ookinete penetrates between or through the midgut epithelial cells and moves to the space between the epithelial cells and the basal lamina, where it transforms into an oocyst (6). The young oocyst undergoes nuclear division while its plasma membrane retracts from the outer capsule (7), giving rise to clefts and invaginations that divide the cytoplasm, forming the sporoblast (8). Sporozoites evaginate along the membranes of the sporoblast (8 and 9) producing a mature oocyst filled with needle-shaped sporozoites (10). The sporozoites are released (11) and migrate to and invade the mosquito salivary glands (12). Sporozoites contained within the acinar cells of the glands are injected into vertebrate hosts during subsequent feedings.

ML, midgut lumen; H, hemocoel; ISM, intersporozoite matrix; SG, salivary glands.

Modified from Menard *et al.*, 1997.

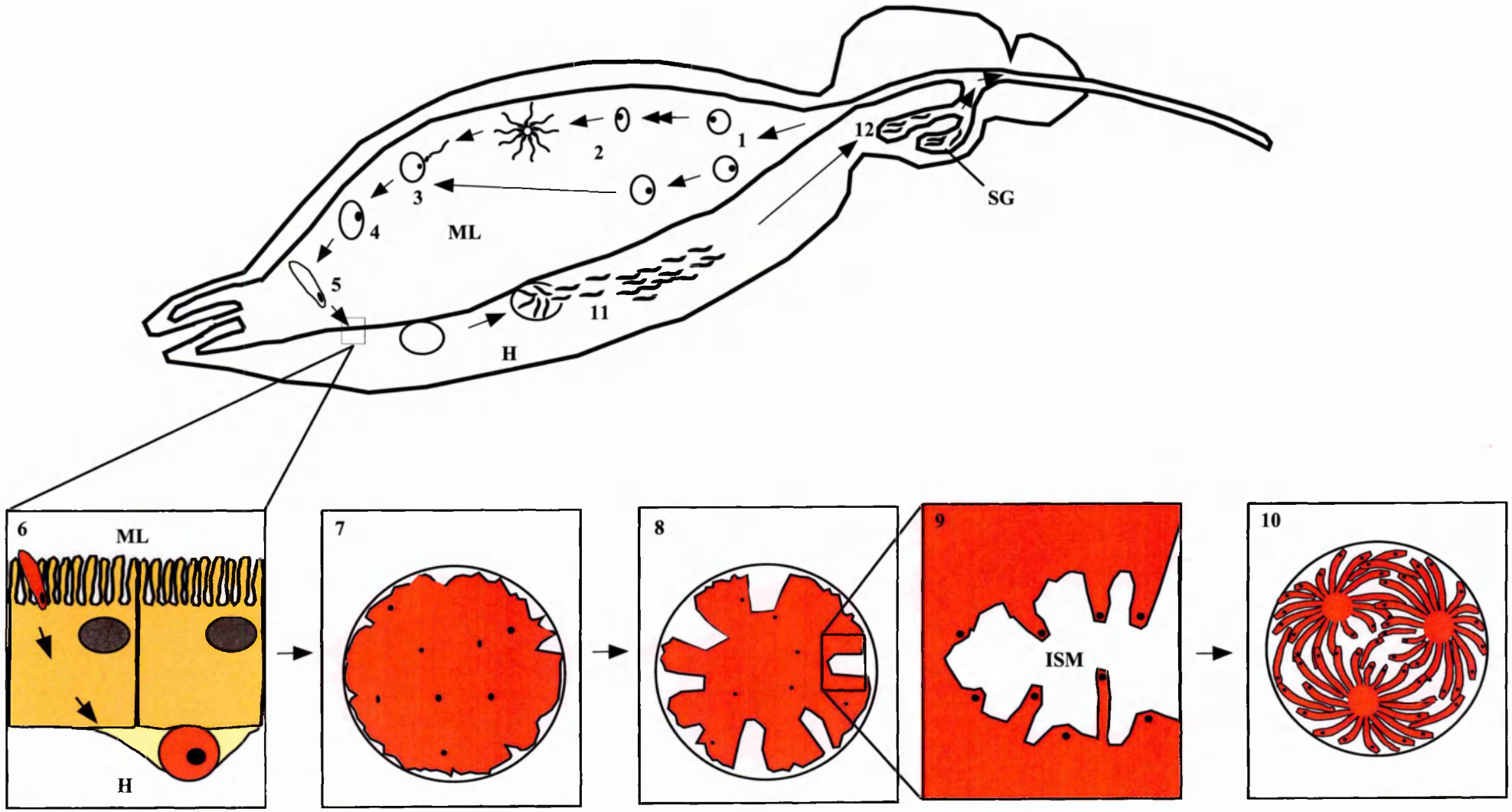
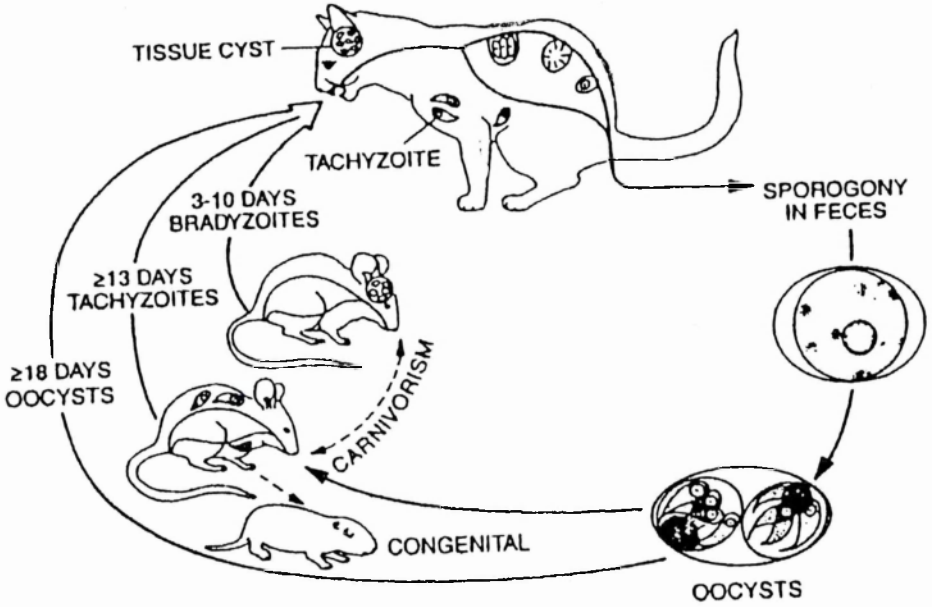

1
2
3
4
5
ML
11
H
12
SG
6
ML
H
7
8
9
ISM
10

Figure 6.2

Life cycle of *Toxoplasma gondii*

Toxoplasma gondii has three infectious stages: tachyzoites, bradyzoites and sporozoites (contained within oocysts). In cats, the definitive host, ingestion of any of these results in infection. Following invasion of enterocytes in the gut, the parasites replicate asexually and differentiate into male and female gametocytes. These fuse and develop into an oocyst, which is excreted with the animal's feces. Sporogony takes place in the environment, within the highly resistant walls of the oocyst. The matured oocyst is subsequently ingested by an intermediate host, such as a mouse, and the sporozoites first develop into tachyzoites and later into bradyzoites, thereby establishing a chronic infection. Because both of these stages are transmissable, carnivorous ingestion by a second intermediate host results in infectious disease and allows indefinite non-sexual propagation of the parasite. The cycle is eventually completed as a cat ingests tachyzoites or bradyzoites through carnivorism, or sporozoites from the environment.

Modified from Dubey, 1998.


TISSUE CYST
TACHYZOITE
SPOROGONY
IN FECES
3-10 DAYS
BRADYZOITES
≥13 DAYS
TACHYZOITES
≥18 DAYS
OOCYSTS
CARNIVORISM
CONGENITAL
OOCYSTS

Appendix

Media, buffers and solutions

All buffers and solutions were made up in dH_2O unless otherwise stated, using chemicals and reagents of analytical grade purchased from BDH Chemicals Ltd., Poole, UK, or Sigma, Poole, UK, unless otherwise stated.

Acrylamide 10% homogenous separating gel (for 5 gels)

8 mL	Lower gel buffer
10.7 mL	30% acrylamide
200 µL	10% ammonium persulphate (APS)
30 µL	N,N,N',N'-tetramethylethylenediamine (TEMED)
13.3 mL	dH_2O

Acrylamide stacking gel (for 5 gels)

2.5 mL	Upper gel buffer
1.58 mL	30% acrylamide
40 µL	10% APS
13 µL	TEMED
5.92 mL	dH_2O

Anode buffer 1 (10x), Western analysis

300	mM Tris-base
20% (v/v)	Methanol (100%)

Anode buffer 2 (10x), Western analysis

3 M	Tris-base
20% (v/v)	Methanol (100%)

Antibody dilution buffer

To be made up in PBS

0.1%	BSA
0.3%	Tween 20

Blocking buffer, ELISA

To be made up in PBS

1%	Bovine serum albumine (BSA)
0.3%	Tween 20
0.05%	NaN_3

Blocking buffer, Western analysis
To be made up in PBS
1% BSA
0.3% Tween 20

Carbonate buffer, 0.1M, ELISA
8.40g $NaHCO_3$
3.56g Na_2CO_3
Make up to 1L and adjust pH to 9.5.

Cathode buffer (10x), Western analysis
250 mM Tris-base
400 mM ε-aminocaproic acid
20% (v/v) Methanol (100%)
Coomassie stain
45% (v/v) Methanol
10% (v/v) Ethanoic acid
0.1% (w/v) Coomassie Brilliant Blue R

Cytomix
120 mM KCl
0.15 mM $CaCl_2$
10 mM K_2HPO_4/KH_2PO_4 pH 7.6
25 mM Hepes pH 7.6
2 mM EGTA pH 7.6
5 mM $MgCl_2$
Make up to a total volume of 100 µL.

Depurination buffer
0.25 M HCl

Destain
5% (v/v) Methanol
10% (v/v) Acetic acid

Diethanolamine buffer, ELISA
4.85% (v/v) diethanolamine pH 9.8
0.2 M $MgCl_2 \bullet 6H_2O$
0.1% NaN_3

Extraction buffer for parasite gDNA
50mM Tris, pH 7.5
50mM Ethylenediaminetetraacetic acid (EDTA)
100mM NaCl

Flow cytometry buffer (PBNE)
To be made up in PBS

1% (w/v)	BSA
5 mM	EDTA
0.01% (w/v)	NaN_3

pH to 7.2

Gel buffer for acrylamide gels, Lower

1.5 M	Tris Base
0.4% w/v	Sodium dodecyl sulphate (SDS, final pH 8.8)

Gel buffer for acrylamide gels, Upper

0.5 M	Tris Base
0.4% w/v	SDS (final pH 6.8)

Iscove's Modified Dulbecco's Medium, complete

500 mL	Iscove's Modified Dulbecco's Medium
10%	FCS (heat inactivated; Globepharm, UK)
10,000 units	Penicillin
10,000 µg	Streptomycin
100 mM	Sodium pyruvate
200 mM	L-glutamine
1 M	HEPES buffer
50 mM	2-mercapto-ethanol

All additives filter sterilized (except FCS) before included in the medium.

LB Agar
To be made up in LB Broth

15g/L	Agar

LB Broth

10g/L	NaCl
10g/L	Bacto-tryptone
5g/L	Yeast extract

Loading buffer (6x), DNA gels

0.25%	Bromphenol blue
40% (w/v)	Sucrose

Lysis buffer for bacteria

50 mM	Na_2HPO_4/NaH_2PO_4
0.5 M	NaCl
10 mM	Imidazole
8 M	Urea

Adjust pH to 7.4

Nycodenz, 100%
27.6g Nycodenz powder (Robins Scientific, UK) in Nycodenz buffer to a total volume of 100 mL

Nycodenz buffer
5 mM Tris-HCl pH 7.5
3 mM KCl
0.3 mM EDTA

Parasite freezing medium
40% v/v Glycerol
0.14M Sodium lactate
5mM Potassium chloride
Adjust pH to 7.4

Parasite lysis buffer
50 mM Tris-HCl
1 mM EDTA
0.5% (v/v) SDS
Adjust pH to 7.5

PBNE See Flow cytometry buffer

Phosphate Buffered Saline (PBS)
0.1 M NaCl
80 mM Na_2HPO_4
20 mM NaH_2PO_4
Adjust pH to 7.5

Pyrimethamine
25 mg powder in 6 mL DMSO
Make up to a total volume of 10 mL with sH_2O

RNA transfer buffer
7.5 mM NaOH

SDS sample buffer (2x) for protein preparations
0.125 M Tris base
20% (w/v) Glycerol
4.5% (w/v) SDS
0.01% (w/v) Bromophenol blue

SDS-PAGE running buffer (1x)
25 mM Tris-base

192 mM Glycine
0.1% SDS
Adjust pH to 8.3

SSC, 2x, pH 7.0
0.3M NaCl
0.33M $Na_3C_6H_6O_7$

SSPE, 1x
120mM NaCl
15mM tri-Na citrate
13mM KH_2PO_4
1mM EDTA

TBE buffer
100 mM Tris-HCl
100 mM sodium borate
5 mM EDTA
Adjust pH to 8.0

Transfer buffer
1.5 M NaCl
0.5 M NaOH

Wash buffer, Western analysis
To be made up in PBS
0.3% Tween 20

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