GENETIC VARIATION IN THE RODENT MALARIA PARASITE PLASMODIUM CHABAUDI.

ANDREW M. SHARKEY

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Department of Genetics
Abstract.

Genetic variation in the rodent malaria *Plasmodium chabaudi* was studied by comparison of six cloned parasite lines originating in the wild. Protein variation was examined by two-dimensional electrophoresis (2DGE) of metabolically labelled parasite proteins. A set of major reproducible proteins, characteristic of each clone was identified. Variants of 18 of these proteins were found, which differ in isoelectric point or molecular weight between the lines. Each cloned line possessed only a single form of each variant protein. The alternative forms of these proteins probably represent variant alleles.

The function of most of these proteins is unknown, although the variant protein representing the parasite protein Adenosine deaminase (ADA) was identified.

Monoclonal antibodies (mcabs) were used to study variation in parasite antigens by Western blotting and immunoprecipitation. Three novel antigens were identified with alleles which varied in m.wt. between the strains. Two of these variant antigens, recognised by mcabs 12.18 and S-10 were found to correspond to proteins 78 and 16 respectively on the 2D map.

In order to study the molecular basis of the variation in these antigens, a genomic DNA library of the *P. chabaudi* strain CB was constructed in the bacteriophage expression vector lambda gt11. This was screened with a polyclonal antiserum to the 230kDa Polymorphic Schizont Antigen (PSA) of the parasite. Two different DNA clones were isolated which
express epitopes carried on this protein. These share DNA sequence homology with each other, and with a third, as yet uncloned DNA sequence. These three DNA fragments were found to lie on three separate chromosomes, and may represent three separate genes which share some homology. Sequencing studies will be necessary to determine which of these encodes the 230kDa PSA.

Variation in the organisation of the genome between the lines was studied using the technique of Pulsed Field Gradient Electrophoresis (PFGE). This allowed the genome to be resolved into at least 10 large DNA fragments, ranging in size from approximately 650 to 5000kb. These fragments are believed to represent intact chromosomes. All the fragments contained sequences homologous to a *P.berghei* telomere probe. Ribosomal RNA genes and *P.chabaudi* cDNA sequences were mapped to specific fragments. The putative chromosomes vary in size between the six cloned isolates. In a cross between lines AS and CB, which differ in the sizes of chromosomes 4 and 5, the alternative forms of these chromosomes segregated independently.
ACKNOWLEDGEMENTS.

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Finally, a special thanks to Teresa Gorst without whose encouragement this work would not have been completed.

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DECLARATION.

I declare that this thesis is my own composition and that the research described is my own work.

ANDREW M. SHARKEY.

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List of Abbreviations.

ADA  Adenosine deaminase
CAR  Central African Republic
CSP  Circumsporozoite protein
DMSO Dimethyl sulfoxide
IFA  Indirect fluorescence assay
IPTG Isopropyl-B-D-thiogalactopyranoside
kb   Kilobasepairs
kDa  Kilodaltons
mcab Monoclonal antibody
MFM  Methionine free medium
MRPs Major reproducible proteins
m.wt. Molecular weight
PABA Para-aminobenzoic acid
PAGE Polyacrylamide gel electrophoresis
PBS  Phosphate buffered saline
PFGE Pulsed field gradient electrophoresis
pI   Isoelectric point
PSA  Polymorphic schizont antigen
RESA Ring infected erythrocyte surface antigen
SDS  Sodium dodecyl sulphate
X-gal 5-bromo-4-chloro-3-indoyl-B-D-galactopyranoside
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1.1. Introduction.

Intensive efforts have been made to eradicate malaria over the last thirty years. The initial eradication programme was based on the widespread spraying of residual insecticides such as DDT, to kill the mosquito vector of the disease, and the use of antimalarial drugs such as chloroquine to treat clinical cases of malaria. However, more recently they have proved increasingly ineffective, and a huge resurgence of the disease has occurred (WHO, 1987).

The reasons for this resurgence are complex. Resistance to several insecticides has appeared in species of Anopheles, the vector of malaria (WHO, 1983). The malaria parasite itself has also developed resistance to antimalarial drugs. Chloroquine resistance is now widespread, and multidrug resistance has also been reported, (Thaithong et al. 1983)

In the light of these problems the strategy of malaria eradication has been reexamined (WHO, 1979). Many research groups are now attempting to characterise parasite antigens, against which protective immune responses are directed, in the hope that these could be included in a vaccine, (reviewed by Miller et al. 1986). Central to this work is an understanding of the extent to which such candidate antigens exhibit variation within the parasite population. There is an obvious risk that once a vaccine is used widely, alternative forms of the parasite antigens upon which it is based might be selected and come to replace the previous forms in the parasite population. It is therefore important to understand the genetic mechanisms operating in the malaria parasite so that the response of the
Figure 1. Life cycle of a typical mammalian malaria parasite. Reproduced from Cox, 1982.
parasite population to the selection pressure imposed by a vaccine can be predicted.

The purpose of this study has been to develop the rodent malaria species *Plasmodium chabaudi* as a model system in order to investigate the genetic mechanisms by which new antigen forms might arise. This is necessary since genetic experiments with the simian and human malarias are technically more difficult, and subject to the practical and ethical problems inherent in the use of monkeys and primates.

1.2. The Malaria parasite life cycle.

Many species of malaria parasite have been described, infecting a wide variety of hosts, including rodents, primates, birds and man (Garnham 1966). The work in this study has been carried out using the rodent malaria *Plasmodium chabaudi*, first described by Landau (1965), and redefined by Carter and Walliker (1975). The life cycle of *P. chabaudi* is shown in Figure 1, which illustrates the important features common to most malaria species.

The cycle can be considered to start when an infected *Anopheles* mosquito takes a blood meal. Infective sporozoites (stage 1 in the diagram) are injected from the salivary glands of the mosquito into the bloodstream of the vertebrate host. They are carried to the liver where they invade hepatocytes. Here a period of asexual development occurs producing exoerythrocytic schizonts, each containing several thousand merozoites. After some 50 hours the merozoites are released into the blood.

Merozoites invade erythrocytes and undergo further asexual
division to form intraerythrocytic schizonts, containing 6 to 8 merozoites. These are released by rupture of the schizont-infected red cell, and invade other red cells, where they begin further cycles of asexual division. Development of the erythrocytic stage of P.chabaudi in mice is synchronous, with schizont rupture occurring every 24 hours. Alternatively a merozoite may develop into a gametocyte on invasion of red cells. Micro- and macrogametocytes circulate in the blood, and do not develop further unless ingested by a mosquito during a blood meal. On entering a mosquito midgut the macrogametocyte transforms into the female gamete, and the microgametocyte undergoes exflagellation releasing male gametes. These undergo fertilisation and a motile ookinete develops from the zygote, which penetrates the wall of the midgut, where it forms an oocyst. Oocysts take 8 to 12 days to mature, and eventually rupture releasing several thousand sporozoites into the haemocoel. The sporozoites migrate to the salivary glands reaching a peak of infectivity some two weeks after the original blood meal. On the next occasion that the mosquito feeds the sporozoites are injected into the vertebrate host, completing the life cycle.

1.3. Immunity to malaria.

Clinical manifestations of malaria are associated with high levels of the asexual blood forms of the parasite. The acquisition of immunity to malaria requires the activation of immune mechanisms sufficient to limit or inhibit these stages. Both humoral and cellular factors are involved, although the exact nature of the underlying mechanisms remains to be
determined (Cohen, 1979).

The involvement of antibodies was demonstrated by Cohen et al. in 1961, when gamma-globulin from immune adults was given to twelve Gambian infants with severe clinical malaria (P. falciparum). The parasitaemias and clinical effects were dramatically reduced. Similar protection by passive transfer of hyperimmune serum, and by monoclonal and polyclonal antibodies to defined antigens has now been demonstrated in a number of species (Diggs et al. 1969; Holder and Freeman, 1981).

Although the production of protective antibodies is clearly important for host defence, thymus-dependent cell mediated immune mechanisms also play a significant role in the immune response to the disease. Studies involving P. chabaudi adami have demonstrated that B-cell deficient mice resolve acute primary infections with the same kinetics as normal mice, and are immune to subsequent challenge infection (Grun and Weidanz, 1981). In this experimental model, T-lymphocytes from either non-immune or immune donor mice, suppressed infections in histocompatible nude mice (Cavacini et al. 1986). The T cells appear to function either as helper cells, promoting the synthesis of protective antibodies, or as inducer/effecter cells in cell-mediated immune responses which remain to be elucidated (Jayawardena, 1982).

The design of a vaccine against malaria requires an appreciation of both the antibody dependent and independent immune mechanisms, against each stage of the life cycle. Any vaccine would ideally have to incorporate parasite antigens capable of inducing a protective immune response involving both
the humoral and cellular arms of the immune system.

1.4. Vaccination studies with crude parasite preparations.

Vaccination studies on malaria have been carried out for many years, using a variety of whole parasite preparations, and with living parasites (reviewed by Siddiqui, 1980). Protective immunity against sporozoites has been achieved in monkeys, rodents and humans, by inoculation of irradiated parasites and by the bites of infected mosquitoes. The immune response is both stage and species specific, and does not require adjuvant-induced immunopotentiation (reviewed by Cochrane et al., 1980). The protection is at least in part mediated by antibodies. Newborn rodents suckled by sporozoite immunised foster mothers were totally resistant to sporozoite challenge (Orjih et al., 1981).

Irradiated parasite preparations of erythrocytic stages have also been used for vaccination. Wellde et al., (1979) immunised three Aotus monkeys with irradiated P. falciparum blood forms without adjuvant, and two of the monkeys survived after challenge with fresh parasites. Other parasite preparations including dead or lyophilised extracts of P. knowlesi, administered with Freunds Complete Adjuvant (FCA), gave similar protection (Schenkel et al., 1973).

1.5. Need for defined antigens.

Although it was shown that crude parasite preparations could protect hosts to some degree against homologous challenge, adjuvants were used which were often not suitable for human application. Furthermore such preparations could include unwanted material such as red blood cell membranes which might
induce autoimmune reactions in the recipient. Vaccines ideally require well defined, pure malaria antigens. The advent of monoclonal antibody (mcab) technology has enabled many antigens to be identified and purified. In several cases malaria proteins, purified using mcabs have been used for vaccination studies, with varying degrees of success (reviewed below). However difficulties in obtaining parasite material have hampered such studies.

Recombinant DNA technology has solved this problem by providing the means of production of defined parasite proteins in large amounts. Mcabs and immune sera have been used to identify a number of antigens that may be targets of protective immune responses. This coupled with the production of recombinant DNA clones expressing these antigens from expression libraries, has allowed a variety of vaccination studies to proceed in order to determine whether any of these antigens are capable of inducing a protective anti-parasite response in vivo. Most of this work has centred around the antigens of the sporozoite and asexual blood stages of the parasite.

1.6.1. *Antigen studies in sporozoites.*

The antigen responsible for protection in the sporozoite vaccination studies described above has been identified in several malaria species using monoclonal antibodies. It consists of a single type of polypeptide called the Circumsporozoite Protein (CSP), which covers the entire surface of the parasite. The complete CSP genes of several species of malaria have been isolated and sequenced (Arnot *et al.* 1985) The coding regions of these genes range from 1.0 to 1.3 kb in size,
Figure 2. Schematic diagram of some repetitive antigens of *P. falciparum*.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Number of repeats</th>
<th>Amino acids per repeat</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circumsporozoite Protein (PSA)</td>
<td></td>
<td></td>
<td>IMTM22</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>4</td>
<td>ref.(1)</td>
</tr>
<tr>
<td>S-Antigen</td>
<td>100</td>
<td>11</td>
<td>FC27</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>8</td>
<td>NF7</td>
</tr>
<tr>
<td></td>
<td>ref.(2)</td>
<td></td>
<td></td>
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<tr>
<td>Ring-infected erythrocyte surface antigen (RESA)</td>
<td>6</td>
<td>11</td>
<td>)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8</td>
<td>) NF7</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>4</td>
<td>) ref.(3)</td>
</tr>
<tr>
<td>Polymorphic Schizont Antigen (PSA)</td>
<td></td>
<td></td>
<td>MAD20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>K1</td>
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<td></td>
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<td>ref.(4)</td>
</tr>
</tbody>
</table>

conserved sequences signal repeats anchor sequences of PSA which vary between alleles of different strains

Refs.
(1) Dame et. al. 1984
(2) Cowman et. al. 1985
(3) Cowman et. al. 1984
(4) Tanabe et. al. 1987
and have a similar structure in all species studied. They contain a putative N-terminal signal sequence, a carboxy-terminal hydrophobic sequence, and a central repetitive domain (figure 2). This domain consists of tandemly repeated amino-acids comprising approximately half the gene. The CSP's have unusual immunological properties; they have an immunodominant epitope present many times in each molecule. These epitopes are encoded in the repetitive part of the gene. In P.falcparum it is defined by only three consecutive repeats (Zavala et.al.1985). Antibodies to the repeat epitope neutralise sporozoite infectivity, and the epitope is common to P.falcparum isolates throughout the world (Weber and Hockmeyer,1985). It has therefore been used as a basis for vaccine development. Antibodies to other regions of the CSP, which are highly conserved between species, have little or no effect on sporozoite infectivity (Vergera et.al.1985).

1.6.2.Vaccine trials with CSP repeats.

Two candidate vaccines based on the NANP repeats of the P.falcparum CSP have been developed. The first is a conjugate of the 12 amino-acid synthetic peptide (NANP) and Tetanus toxoid. The other consists of a fusion peptide made in E.coli containing NANP repeated 32 times. The results with the two constructs were similar. After challenge by the bites of infected mosquitoes, one vaccinee was completely protected, while the other two showed delayed parasitaemia (Herrington et.al.1987; Ballou et.al.1987).

The results suggest that T-cell stimulation by the vaccines was not significant. This highlights two potential
problems inherent in the use of vaccines based upon simple repeat epitopes. Firstly such vaccines may lack T-cell epitopes and thus be inefficient in eliciting boosting responses in human beings under natural exposure to sporozoites. Secondly, the immune response to any one particular T-cell epitope is known to be genetically restricted (Berzofsky, 1986). Experiments using the (NANP) recombinant vaccine in mice, have shown that this molecule contains a single murine T-cell epitope in the repeat (Good et al. 1986). By use of strains of mice congenic for the H-2 complex, these authors showed that only those strains bearing the 1-Ab allele at the Ir gene locus responded to the T-cell epitope in the repeats (two out of nine strains used).

If an analogous situation occurs in humans, many people might not recognise T-cell epitopes in the repeat, and natural boosting of the immune response would be very restricted. Recognition of malaria-specific T-epitopes would also be crucial for a high level of cellular immunity. Studies in murine malaria suggest an important role for cellular immune mechanisms in sporozoite clearance (Chen et al. 1977).

These studies have broader implications for peptide vaccine development in general. Peptide vaccines must contain invariant B-epitopes, covalently linked to invariant T-epitopes, at least one of which must be recognised by the T-cells of all who receive the vaccine for boosting to occur.

1.7.1. Antigen and vaccine studies in asexual erythrocytic parasites.

The crude whole parasite vaccination studies described in section 1.4. showed that experimentally induced immunity against
blood forms of *P.falciparum* and other species has significant species- and strain-specific components. This indicates inter and intra-species diversity in the antigens involved in the induction of protective immunity (reviewed by Cohen, 1979). As antigen genes have been cloned and sequenced, some idea of the DNA and protein sequence variation underlying these results has begun to emerge. Work on three blood stage antigens, which provide varying degrees of protection is briefly summarised below.

1.7.2. S-Antigens.

S-antigens exhibit great serological and size diversity in different strains, however they are believed to represent alleles of the same genetic locus (Wilson *et al.* 1980; Anders *et al.* 1983). The nucleotide sequences of several S-antigen genes of *P.falciparum* have now been determined (Cowman *et al.* 1985). They consist of a single exon, containing a central block of tandem repeats flanked, as in the CSP, by non-repeat sequences which show considerable homology between strains (figure 2). The repeats are largely conserved within any one gene, but vary in number and sequence between strains, and it is this that accounts for the previously described variation in molecular weight and antigenicity.

Saul *et al.* (1985) have shown that a mcab to an S-antigen could inhibit merozoite invasion *in vitro*. However a vaccination study in *Macaca mulatta* monkeys found that little protection was induced using a *P.knowlesi* S-antigen preparation (Collins *et al.* 1977). The great serological diversity of S-antigens due to variations in the repeat region, suggests that
this antigen is unlikely to be a candidate for inclusion in a vaccine. Work on the possibility that the non-repeat flanking sequences might be useful in a vaccine, is hampered by lack of information on the function of these proteins.

1.7.3. Ring infected erythrocyte surface antigen (RESA).

Perlman et al. (1984) identified a parasite antigen associated with the membrane of erythrocytes containing ring stage parasites, of m.wt.155 KDa. Antibodies to this antigen inhibit merozoite invasion in vitro (Wahlin et al. 1984). Proteins which may represent homologues of RESA have been identified in P. chabaudi and P. yoelii of 105 and 160 KDa respectively (Gabriel et al. 1986, Murakami and Tanabe, 1983), although any firm relationship between these antigens remains to be established. The complete sequence of the RESA gene of P. falciparum has now been published (Favaloro et al. 1986). The gene contains two exons separated by a short intervening sequence (figure 2). The second exon contains two large blocks of tandemly repeated sequence which encode cross-reacting antigenic epitopes.

Vaccination trials in Aotus monkeys with a variety of peptides from the different repeat regions gave some evidence for partial protection (Collins et al. 1986). Interestingly most naturally induced antibodies to RESA are directed against epitopes encoded by the 4 amino-acid repeats. In this trial, antibodies of this specificity were not associated with protection. Therefore high levels of anti-RESA antibodies per se do not necessarily correlate with immunity. Further experiments are underway to determine the potential of the non-
repetitive regions of RESA as vaccine candidates.

1.7.4. Polymorphic Schizont Antigen (PSA).

One of the most extensively studied asexual stage antigens with promise as a vaccine candidate is the PSA of *P. falciparum*, also known as p190 or gp195 (McBride *et al.* 1985; Hall *et al.* 1983; Holder *et al.* 1983). Homologues of this molecule have been identified in several other malaria species, and can induce protective immunity in vivo (Newbold *et al.* 1984). This protection is most effective against homologous parasite challenge, suggesting variability in the epitopes against which the protective immune response is directed (Hall *et al.* 1983; Siddiqui *et al.* 1987). Different alleles of this protein vary in size among isolates, from 190 to 220KDa (Fenton *et al.* 1985). Comparison of the DNA sequence of the gene from several isolates has revealed the genetic basis of the antigen's polymorphism (Tanabe *et al.* 1987). Alleles from different strains appear to be based upon one of two prototypes, with minor sequence variation between alleles of the same sub-type, (figure 2). In addition a relatively small region of the gene consists of tripeptide repeats. This region appears to behave differently, and does not conform to the general dimorphic rule. As well as variation in the repeat sequence in different isolates, the number of repeats varies in a manner analogous to the *S*-antigens.

1.8.1. Antigen diversity and immunity.

The pattern of malaria infection in endemic areas is one of repeated clinical attacks of decreasing severity (Garnham 1966). This is consistent with the existence of a variety of
antigenically distinct parasite strains to which a human host needs to be exposed, before clinical immunity is established. The initial vaccination studies using whole parasite extracts described in section 1.4. supported this interpretation, showing that the resulting immunity had a significant strain-specific component. Comparisons of the nucleotide sequences of antigenically important proteins in several species of *Plasmodium* have clarified the molecular basis for the diversity between alleles of these antigens. Traditionally diversity between alleles of a particular gene is believed to arise due to the accumulation of point mutations, and small deletions and insertions. However in *Plasmodium* antigens such as the S-antigens, the CSP and the PSA, do not seem to conform to this model.

The most remarkable feature which has emerged from the sequencing studies of *plasmodial* antigens is that they often contain extensive arrays of tandemly repeating short amino-acid sequences (described above). The non-repeat regions of the genes encoding the CSP and S-antigens are highly conserved between strains of the same species, and to some extent between species. Diversity in these sequences does appear to arise by accumulation of small mutations in a typical fashion. However the repeat units may vary considerably in sequence and number between isolates, and it is this variation which is responsible for the serological and m.wt. variation of these alleles. The repeats can differ drastically from isolate to isolate, but are usually highly conserved along the molecule in any one isolate. This has led to the suggestion that unequal crossing over or
gene conversion may be involved in the evolution of such repeats (Kemp et al. 1986). This has been postulated in the case of the repetitive satellite DNA present in many eukaryotes (Smith 1976).

1.8.2. Diversity in the PSA.

The pattern of antigenic diversity in alleles of the PSA of *P. falciparum* appears to be very different to that seen in the S-antigen. Many antigenically variant alleles of this protein are known, however the evidence described in section 1.7.5. suggests it exists essentially in two radically different versions, with various alleles being based on one or other of these two prototype alleles. There is also strong evidence that intragenic recombination between alleles can occur to generate further diversity (Tanabe et al. 1987). This recombination seems to occur primarily in the 5' region of the gene, and this may reflect constraints in order to produce a functional gene product. However there seems little doubt that intragenic recombination, shuffling 5' and 3' parts of the gene contributes to antigenic diversity. This suggests that in addition to the identification of peptides from PSA which induce protective immunity, an important aspect of vaccine design is understanding the mechanisms operating to generate variation in the parasite population of peptides included in the vaccine. It is the purpose of genetic studies to understand these mechanisms.

1.9.1. Genetics of malaria parasites.

Extensive work on the basic genetics of malaria parasites by strain hybridisation and progeny analysis has been carried out using the rodent malarias *P. chabaudi* and *P. yoelii*, because
Figure 3 Conducting a cross between two cloned parasite lines.

Parent Line AS pre
\[ \downarrow \]
2 mice
\[ \downarrow \]
Rat
\[ \downarrow \]
Mosquitoes
\[ \downarrow \]
Mouse
\[ \downarrow \]
Parent Line AS post

Parent Line CB pre
\[ \downarrow \]
2 mice
\[ \downarrow \]
Rat
\[ \downarrow \]
Mosquitoes
\[ \downarrow \]
Mouse
\[ \downarrow \]
Progeny
\[ \downarrow \]
Cloning
\[ \downarrow \]
1 2 3 4

Table 1 Origin of parasites.

<table>
<thead>
<tr>
<th>Line</th>
<th>Country of Origin</th>
<th>Species and sub-species</th>
<th>Isolate</th>
<th>Date of Capture</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS</td>
<td>C.A.R.</td>
<td>P. c. chabaudi</td>
<td>1AS</td>
<td>1969</td>
</tr>
<tr>
<td>CB</td>
<td>C.A.R.</td>
<td>P. c. chabaudi</td>
<td>2CB</td>
<td>1970</td>
</tr>
<tr>
<td>ER</td>
<td>C.A.R.</td>
<td>P. c. chabaudi</td>
<td>56L</td>
<td>1965</td>
</tr>
<tr>
<td>EF</td>
<td>Cameroun</td>
<td>P. chabaudi</td>
<td>Biboto37</td>
<td>1973</td>
</tr>
<tr>
<td>DS</td>
<td>Congo</td>
<td>P. c. adami</td>
<td>556KA</td>
<td>1970</td>
</tr>
</tbody>
</table>

(C.A.R. is Central African Republic).
of the relative convenience of these models (reviewed by Walliker, 1983). More recently techniques such as the in vitro culture of gametocytes (Ifediba and Vanderberg, 1981), have enabled a cross to be carried out between two genetically distinct cloned lines of \textit{P. falciparum} (Walliker \textit{et al.} 1987).

The method used for making a cross (figure 3) is to select two cloned parent lines which differ in genetically determined characters such as drug resistance, isoenzymes or antigens. Mosquitoes are allowed to ingest blood containing viable gametocytes from both cloned lines. Cross and self-fertilisation occurs between gametes in the mosquito midgut, eventually leading to the formation of infective sporozoites in the salivary glands. The mosquitoes are then fed on an uninfected host to transmit the infection. The resulting parasites comprise a mixture of the progeny of self and cross-fertilisation. Individual progeny are cloned by limiting dilution from the infected blood, and grown up for analysis of the characters which they have inherited.

1.9.2. Isoenzyme markers.

Isoenzymes have proved particularly useful stable genetic markers in the genetic analysis of Plasmodia. \textit{P. chabaudi} is the best studied since a large number of enzyme variants have been found in this species. Rosario (1976) showed that when two lines were crossed, differing in two different enzyme variants, four types of progeny were produced, two were parental types and two were recombinant. He concluded that the blood forms of the parasite were haploid. Similar results have been found for \textit{P. yoelii} and \textit{P. falciparum} (Knowles \textit{et al.} 1981; Walliker
These genetic studies have revealed that the parasite appears to undergo a normal eukaryotic type of life cycle with haploid blood forms. Fertilisation and meiosis occur in the mosquito. Variant forms of enzymes, antigens and other characters occur due to allelic variation. These alleles undergo segregation and recombination following cross fertilisation between parasite lines, in the manner expected for nuclear genes. However many questions remained unanswered by these studies; for instance the basic organisation of the parasite genome and the number of chromosomes was unknown. Even in the *P. falciparum* cross in which many characters were analysed, no linkage groups have been identified (Fenton et al. in preparation). This is probably due to the small number of progeny that it is possible to examine.

1.9.3. Organisation of the parasite genome.

Although genetic analysis has not revealed linkage groups, the independent assortment of genetically determined characters provides strong evidence for the existence of a genome composed of several chromosomes. Cytological studies have been hampered because condensed chromosomes have never been observed. Recently the technique of Pulsed Field Gel Electrophoresis (PFGE), introduced by Schwartz and Cantor (1984), has provided a powerful new approach for analysing the parasite genome. This technique has allowed the separation of up to 14 distinct DNA fragments of large size in *P. falciparum*, which are considered to be chromosomes (Wellems et al. 1987). Cloned genes can be mapped to the fractionated chromosomes by blotting the DNA to
nitrocellulose followed by hybridisation, thus quickly generating linkage groups (reviewed by Kemp et al. 1986).

The *P. falciparum* chromosomes revealed by PFGE are polymorphic in size in different isolates. Size changes in particular chromosomes have been seen following cross fertilisation between cloned parasite lines in mosquitoes (Walliker et al. 1987). In some cases it has been shown that deletions can be responsible for size changes, and that these can involve either repetitive DNA, or coding sequences (Van der Ploeg et al. 1985; Pologe and Ravetch, 1986). In the latter case, the deletion of a histidine rich protein was associated with the generation of parasites exhibiting the knobless phenotype. Therefore in addition to revealing the fine structure of the parasite genome, these techniques promise a more detailed understanding of the genetic mechanisms which contribute to phenotypic variation of clinical significance.

1.9.5. Genetics and variation.

The genetic studies thus far have revealed several mechanisms which contribute to the rise and spread of variation in *Plasmodia*.

1. Allelic variants of antigens, isoenzymes and other gene products are known. Genetic recombination during meiosis within the mosquito has been shown to occur at a high frequency in rodent malarias, and *P. falciparum*. When this occurs between gametes of parasite lines with different allelic variants, it results in progeny with new combinations of genes.

2. New variants may arise by mutation to increase the variation in alleles of a given gene. This mechanism is
believed to account for the development of drug resistance in malaria (Beale, 1980). Such mutation may involve simple point mutations. However the sequencing studies of antigens such as the CSP and the PSA, have indicated that other more complex mechanisms, perhaps involving intragenic recombination or gene conversion may also be involved in generating allelic variation, which may be advantageous to the parasite.

3. Analysis of the genome structure using PFGE has revealed considerable variation at this level of organisation. Chromosomes exhibit size polymorphism, and are subject to deletions and rearrangements. These may also contribute to phenotypic variation, as has been demonstrated in the case of the Histidine rich protein (Pologe and Ravetch, 1986).

1.10. Aims of the present study.

Much of the work described above has been centred on P. falciparum, because of its obvious clinical importance, and this species is the best characterised at the molecular and genetic levels. A large number of antigens have been isolated and the corresponding genes cloned and sequenced, and the identification of potential vaccine candidates is proceeding rapidly. However many problems remain to be solved before a vaccine could be ready for full-scale testing in humans. A major experimental problem is that a large number of different antigens need to be assessed for vaccine potential. It will be difficult to carry out adequate monkey trials given the small numbers of suitable animals available.

Analysis of the first genetic cross of P. falciparum, has revealed important data about the behaviour of the genome during
meiosis, and the mechanisms which may be responsible for generating the antigen diversity which represents a major obstacle in the control of malaria. Further work is necessary if a full understanding of these mechanisms is to be reached. Rodent malarias are prime candidates for future genetic work in Plasmodia, however these species are relatively poorly characterised at the molecular level. Furthermore the organisation and behaviour of the genome in these species has not been analysed by techniques such as PFGE.

*Plasmodium chabaudi* is considered the rodent malaria most similar to *P.falciparum* in character. Unlike *P.berghei* or *P.yoelii* the infections are synchronous, (as in *P.falciparum*), parasites primarily invade mature erythrocytes, and gametocytes are produced towards the end of infection. Genetic studies are most advanced in this species (McLean,1986), and a wide variety of mcabs are available against antigens of *P.chabaudi*, to aid molecular analysis. It is the broad aim of this study to develop the characterisation of this parasite at the molecular level, and to produce the molecular tools for further genetic studies of the type carried out in *P.falciparum*.

Specific aims of this study were:

1. To compare the proteins of a number of cloned *P.chabaudi* lines by 2-Dimensional (2D) electrophoresis. By this means it was hoped to establish whether proteins varying in charge and/or m.wt. occurred, as has been found in the variant proteins of *P.falciparum*. In addition it was important to show whether the extent of genetic variation detected by this method was comparable to *P.falciparum*, which might indicate that similar
genetic mechanisms for the generation of variation were at work.

2. To use the mcabs available in this laboratory against P.chabaudi, to identify specific antigens possessing alleles which differed in m.wt. In P.falciparum such antigens have frequently been found to be encoded by genes with repeat or other variable regions. Such mcabs would thus provide the molecular tools necessary for the isolation of genes likely to contain variable regions.

3. To produce a parasite genomic library in the expression vector bacteriophage lambda gt11, which could be screened by monoclonal or polyclonal antisera against variant antigens, in order to identify the corresponding genes. In particular to clone the gene encoding the 230 KDa analogue of the PSA of P.falciparum. It was hoped to sequence this gene, in order to establish the molecular basis of the size variation of this protein, to see if it was similar to that found for the PSA.

4. To use the technique of PFGE in order to establish the organisation of the genome of P.chabaudi, and to investigate its behaviour during meiosis.
2. MATERIALS AND METHODS.

2.1 Laboratory Hosts.

Anopheles stephensi were maintained in a 12 hour dark/12 hour light cycle at 24 C and 80% relative humidity. They were supplied with 10% glucose solution supplemented with 0.05% PABA. Seven to ten day old mosquitoes were used for P. chabaudi transmission experiments.

Mus musculus were bred in the Department of Genetics, University of Edinburgh. The inbred line C57Bl was used for parasite preparations in labelling experiments. The outbred line MF1 was used for all other work. All the mice were kept in a reversed light room, with illumination between 5.00 pm. and 8.00 am. Under these conditions, parasite schizogony occurred at around midday.

2.2. Parasite Lines.

2.2.1. Origins.

The parasites used in this work were from a collection of lines of P. chabaudi, as described by Carter and Walliker (1975) and Carter (1978). Two additional lines, EF and ER, are described by Lainson (1983,a). All the parasite lines had been cloned by limiting dilution before this study (Walliker, 1976). Details of the lines are shown in Table 1. (p. 14)

2.2.2. Blood Stages.

The blood stages of the parasites were maintained in rodents by intraperitoneal inoculation of parasitised blood, diluted in citrate saline, (0.9% NaCl, 1.5% Na Citrate, pH 7.2). To estimate the parasitaemia in mice, thin films of infected tail blood were air dried, fixed in methanol and stained with
10% Giemsa's stain in distilled water, for 40 minutes. Parasitaemias were expressed as a percentage of the number of parasites in 1000 red cells.

2.2.3. Cryopreservation of parasites

Blood from an infected mouse was mixed with a heparinised balanced salts solution, and glycerol (Lumsden et al., 1966). The blood was sealed in glass capillary tubes, and stored in liquid nitrogen. Infections were re-established by rapidly thawing the stabilate, mixing the contents with citrate saline and intraperitoneal injection into mice.

2.2.4. Mosquito transmission of parasites.

Successful mosquito transmission requires the mosquito to ingest blood containing infective gametocytes. These can be detected microscopically by observing infected blood under phase contrast and polarised light. Gametocytes appear in the blood on day 5 to 6 after inoculation. The infected mouse was restrained and placed in a cage containing 7 to 10 day old A. stephensi. Feeding was encouraged by depriving the mosquitoes of glucose for 24 hours prior to the blood meal. After a further 24 hours the glucose was returned. This delay selects for the survival of mosquitoes which had taken a blood meal. 15 days after the initial infection, the mosquitoes were allowed to feed on uninfected mice. Infections in mice became patent within 7 days.

2.3. Conducting a cross.

In order to conduct a cross between two cloned P. chabaudi lines, mosquitoes must be infected with blood containing infective gametocytes of both parent lines. The method used was
based upon that of Walliker et al. 1975, and is illustrated in figure 3. Blood forms of the two parent lines was mixed to give equal numbers of gametocytes from each line. The mixture was inoculated intravenously into an uninfected mouse, and mosquitoes allowed to feed. Simultaneously as controls, each parent line was passaged separately through mosquitoes, to ensure the gametocytes of both lines were infective. Infections were re-established in mice, and those from the gametocyte mixture contain both parental and recombinant parasites. These were cloned by limiting dilution, and constitute the progeny of the cross. The presence of progeny clones possessing phenotypic characters of both parents shows that cross-fertilisation between the parent lines has occurred.

2.4. Studies on blood form antigens.

2.4.1. Antigen preparation for IFA.

Blood was collected from mice containing trophozoites and schizonts at a parasitaemia of 10%, into citrate saline. The blood was passed over a column of Whatmans powdered CF11 cellulose, to remove white blood cells (Homewood and Neame, 1976). The red blood cells were pelleted by centrifugation at 2000g, and washed twice in RPMI. The final cell pellet was diluted in RPMI to a haematocrit of 5%. 20μl samples were placed in each well of PTFE coated slides (Hendley), and air dried. Slides were stored desiccated at -20 C.

2.4.2. Monoclonal Antibodies.

The monoclonal antibodies (mcabs) used in this study were kindly supplied in the form of tumour ascites from two sources: a) Dr. A. McLean supplied 7 mcabs. These had been raised
against the CB clone of *P.chabaudi* in this laboratory. They are identified by a number, prefixed by 12., eg. 12.15. All these antibodies had previously been partially characterised by IFA and protein blotting, and the molecular weight (m.wt) of the various proteins with which they reacted, determined in the AS anbd CB lines of *P.chabaudi*, (McLean, 1986). See Table 3.

b) Dr. S. McLean (Zoology Department, Glasgow University), kindly supplied 2 other mcabs which had been raised against the AS clone of *P.chabaudi*, and had not previously been characterised. These were S-10 and S-20.

**2.4.3. Indirect Fluorescence Assay.**

IFA was used to characterise *P.chabaudi* antigens, to give information about the subcellular localisation of the reacting antigens. Multispot preparations of parasite antigens were removed from the deep freeze, fixed in acetone for 5 minutes and air dried. The IFA was carried out as described by McBride *et al*.1985, using 20μl of dilutions of each mcab.

For IFA tests on living parasites, the blood was initially prepared in the same manner as for the multispot slides. After removal of white blood cells, parasitised erythrocytes were washed twice in PBS, and resuspended at 10% hematocrit in RPMI. Monoclonal Antibody was added at 1/100 dilution, and the cells incubated for 30 minutes. After centrifugation and washing in RPMI three times, the cells were incubated with the fluorescein conjugate, washed again and mounted. The preparations were examined using a Leitz Dialux 20 microscope, with an N2-1 filter.

**2.4.4. Immunoprecipitation of parasite antigens.**
Protein A Sepharose (Sigma) was swollen in NET (50mM Tris-HCl pH 8.0, 0.15M NaCl, 5mM EDTA and 0.5% Triton X-100). Extracts of parasite proteins labelled with \( {^{35}}S \) (section 2.6.2.) containing 5x10^6 cpm, were preabsorbed with 50\( \mu \)l of Protein A Sepharose suspension for 30 minutes at 4°C to remove non-specific binding. The Sepharose beads were removed by centrifugation at 11000g for 5 minutes. 12\( \mu \)l of ascites was added to the supernatant and mixed by rotation overnight at 4°C. Antigen-antibody complexes were bound to 80\( \mu \)l of Sepharose beads for an hour at room temperature, the beads were spun down as before, and washed as follows: twice in 1ml NET, once in NET with 0.5M NaCl, then twice more in NET. After each wash the beads were spun down and the supernatant discarded. Finally the beads were resuspended in 50\( \mu \)l of 2x sample buffer containing 2% SDS, 0.1M Tris-HCl pH 7.5, 10% Mercaptoethanol, 4mM PMSF, 2mM EDTA, 10% glycerol, and boiled for 3 minutes. The absorbed antigens were then analysed by SDS-PAGE and autoradiography.

2.4.5. Western Blotting.

Parasite extracts for Western blotting were prepared by removing white blood cells, and subjecting parasitised blood to saponin lysis in the usual way (section 2.6.2.). The freed parasites were solubilised as described in section 2.6.3. and run on a 5 or 10% polyacrylamide gel. Proteins were transferred electrophoretically to nitrocellulose filters at a current of 1Amp for 90 minutes with cooling (Towbin et.al.1978). The nitrocellulose was rinsed in PBS, and non-specific binding sites blocked by incubating the filter for one hour in Blotto (5% non-fat milk powder in PBS, 0.02% Sodium Azide, 0.05% Tween 20)
at room temperature. The filter was then incubated overnight with a 1/200 dilution of ascites in Blotto. This first antibody was removed by washing four times in PBS with 0.1% Tween, 20 minutes per wash. Any antibody specifically bound to parasite antigens was visualised by incubating the filters in 1/300 dilution of Horseradish Peroxidase conjugated anti-mouse IgG, (Scottish Antibody Production Unit, Carluke), in Blotto for three hours. The filters were washed as before, followed by a final wash in PBS. The bound antibody was visualised by developing the blots in 0.1M Imidazole pH 8.0, containing 10mg per ml O-dianisidine and 0.01% hydrogen peroxide for 10 minutes. The reaction was stopped by rinsing in distilled water.

2.5. Polyacrylamide Gel Electrophoresis (PAGE).

2.5.1. One-Dimensional (1D) SDS-PAGE.

This was carried out using a discontinuous buffer system as described by Laemmli (1970), with slight modifications, using a Hoeffer vertical slab gel apparatus. The stock Acrylamide solution contained Acrylamide and Bis-acrylamide in the ratio 29.2 : 0.8. The resolving gel was made up to 0.37M Tris-HCl pH 8.7, 0.1% SDS, 0.032% Ammonium persulphate, 0.0002% TEMED, and either 5.0, 7.5, or 10% Acrylamide. The stacking gel contained 0.11M Tris-HCl pH 6.8, 4.3% Acrylamide, 0.1% SDS, 0.036% Ammonium persulphate and 0.0005% TEMED. Samples were loaded into the sample wells, gently overlaid with electrode buffer (0.025M Tris-HCl, 0.194M Glycine, 0.1% SDS), and electrophoresis of 7.5mm thick gels was carried out at 7mA for 15 hours. The gels were then removed for staining and processing, or for Western Blotting.
2.5.2. Processing of gels.

Gels were stained and fixed by immersion in 0.3% Coomassie Brilliant Blue R, 50% methanol and 7.5% acetic acid for three hours. They were destained by shaking in several changes of 40% methanol, 7.5% acetic acid, and reswollen in 5% methanol, 7.5% acetic acid.

Fluorography was carried out following staining with Coomassie, by impregnating the gels with the scintillant PPO. The gels were shaken in three changes of DMSO for 30 minutes each, then in 22.2% PPO in DMSO for three hours. They were then rinsed for 1 hour, and dried down onto Whatmann 3MM paper, with an LKB gel drier. Fluorographs were made by exposure to Kodak X-OMAT AR film in cassettes at -70°C.

2.5.3. Two Dimensional (2D) Gel Electrophoresis.

The first dimension was performed as described by O'Farrell (1975), at 25°C in a Hoeffer tube gel unit, with gels 12cm long and 3mm in diameter. The composition of the gels was: 9.1M Urea, 4% Acrylamide, 2% v/v NP40, 1.6% v/v 5-7 Ampholines, 0.4% v/v 3-10 Ampholines (from LKB). Polymerisation was initiated by the addition of ammonium persulphate and TEMED to concentrations of 0.01% and 0.0007% v/v respectively. Once set, the gels were overlaid with 20µl of Lysis buffer (section 2.6.4.), and set up with 0.01M H2PO4 as the anode, and 0.02M NaOH as the cathode. The gels were pre-run at 200V for 15 minutes, 300V for 30 minutes and 400V for 30 minutes. The samples were then loaded, overlaid with fresh lysis buffer, and iso-electric focussing was performed for 15 hours at 400V. The gels were removed and gently shaken for 90 minutes in 10ml of eq-
-uilibration buffer (10% v/v glycerol, 5% v/v β-mercaptoethanol, 2.3% w/v SDS, 0.125M Tris-HCl pH 6.8. Gels were then loaded onto the second dimension or stored frozen at -70°C.

The second dimension gels were prepared as described for 1D SDS PAGE, except that no sample wells were formed. Tube gels from the first dimension were loaded onto this, and set in 1% agarose (BDH Isoelectric focussing grade) in equilibration buffer. Electrophoresis and subsequent processing were as described for 1D gels.

2.5.4. Calibration of 1 and 2D gels.

Slab gels were calibrated using molecular weight markers from Sigma. Isoelectric focussing gels were calibrated by direct pH measurement. Immediately after electrophoresis the tube gels were sliced into 5mm segments. Each was shaken for one hour in 1ml of degassed 25mM KCl. and the pH measured directly using a pH electrode. These results were plotted to show the pH profile created during focussing.

2.6. Metabolic labelling of parasite proteins.

2.6.1. Labelling parasites in erythrocytes.

A modification of the method of Newbold et al. (1982) was used to radiolabel parasite proteins biosynthetically with $^{35}$S methionine. Blood from a mouse with a 20-30% parasitaemia was collected into citrate saline. White blood cells were removed as described in section 2.4.1.. The infected red blood cells were washed once in Eagles MEM without methionine (MFM). The pellet was resuspended in MFM to give a 20% hematocrit. 100μCi of $^{35}$S methionine was added to the culture, which was incubated at 37°C for two hours in a candle jar.
Unincorporated methionine was removed by two washes in RPMI. Parasite proteins for 2D electrophoresis or immunoprecipitation were extracted as described in sections 2.6.3. and 2.6.4. Samples for 1D gels were lysed by the addition to the cell pellet of an equal volume of 2x sample buffer and treated as described in section 2.6.3.

2.6.2. Labelling parasites freed from erythrocytes.

Parasites freed from their host cells were labelled by the method of Tait (1981). White blood cells were removed and the blood washed twice in the usual way (section 2.4.1.). The packed erythrocytes were then lysed by the addition of an equal volume of 0.15% Saponin detergent in RPMI, and incubated at 37°C for 5 minutes. 10 ml of RPMI was added and the mixture centrifuged at 2500g for five minutes. The supernatant and red cell ghosts were discarded and the cells were washed twice more, with a final wash in MFM. The freed parasites were resuspended in 200µl of MFM containing 30 µCi 35S methionine, and the culture incubated for two hours in a candle jar at 37°C. Extracts for electrophoresis and immunoprecipitations were made as described below.

2.6.3. Preparation of samples for 1D gel electrophoresis.

Labelled samples of parasitised erythrocytes or freed parasites were solubilised by the addition of an equal volume of 2x sample buffer (84mM Tris-HCl pH 6.8, 2% w/v SDS, 10% v/v mercaptoethanol, 18% v/v glycerol and 0.002% w/v bromophenol blue. The extracts were incubated at 37°C for two hours, centrifuged at 11000g for three minutes to remove insoluble material and stored at -20°C.
2.6.4. Extracts for 2D gels.

Labelled samples of 200μl were solubilised by the addition of 275mg Urea, and 100μl of Lysis buffer containing 10% NP40, 10% (v/v) Ampholines, 25% β-mercaptoethanol. The sample was mixed until clear and then subjected to three cycles of freeze-thawing to lyse the cells. The sample was centrifuged at 11000g for 3 minutes to remove debris and stored at -70°C.

2.6.4. Extracts for immuneprecipitation.

NP40 extraction buffer (NEET) contained 50mM Tris-HCl pH 8.0, 0.15M NaCl, 5mM EDTA, 5mM EGTA, 2mM PMSF and 2% NP40. Parasitised erythrocytes or freed parasites were washed twice in RPMI after metabolic labelling with 35S methionine, and NEET extraction buffer added to the cell pellet up to a final volume of 1ml. The sample was mixed on a rotator at 4°C for 2 hours, insoluble material was removed by centrifugation and the extracts stored at -70°C.

2.6.5. Scintillation counting of radiolabelled extracts.

Samples of labelled parasite proteins were spotted onto glass fibre discs (Whatmann GF/A) and air dried. Radiolabel not incorporated into protein was removed by washing in Trichloroacetic acid solutions according to the method of Bollom (1966). The filters were again air dried, and counted in scintillation fluid, using a Nuclear Chicago scintillation counter.

2.7 Pulsed field Gradient Electrophoresis (PFGE).

PFGE is a recently developed technique, which allows the separation on agarose gels of large pieces of DNA, including small intact chromosomes. DNA is extracted from parasites in a
manner designed to avoid shearing large DNA. This is then subjected to electrophoresis in a horizontal agarose gel, to which an alternating electric field is applied, and intact chromosomes are separated in a size-dependent manner.

2.7.1. Preparation of parasites for PFGE.

Parasites were prepared for electrophoresis as described by Kemp et al. (1985). Infected blood was taken from mice at schizogony, at a parasitaemia of 50%, white blood cells were removed, and saponin lysed in the usual way (section 2.6.2.) The parasites were resuspended in PBS pH 7.4, and embedded in 1% low gelling temperature agarose (BRL), in rectangular moulds of 100μl in volume. Parasite density was approximately 5x10^8 parasites per ml. The agarose blocks were incubated in 0.5M EDTA, 0.01M Tris-HCl pH 8.0, 1% sarcosyl, 2mg/ml Proteinase K at 42°C for 48 hours. Blocks could be stored in this solution for up to 6 months.

Two types of PFGE apparatus were used, one similar to that described by Carle and Olsen (1984), the second was the LKB Pulsaphor apparatus. Most gels were run with the Olsen apparatus. The running buffer was 0.5x Tris/Borate/EDTA (TBE), with the gel consisting of between 1 and 1.5% agarose (Seakem GTG). Pulse time and voltage were varied depending on the resolution required, typical values being 280 volts for 22 hours at 13 °C with a pulse time of 90 seconds. After running, gels were stained in buffer containing 50 μg/ml Ethidium Bromide for 1 hour, destained and photographed. Blocks containing DNA of Saccharomyces cerevisiae strain D273 were used to estimate the size of the Plasmodium DNA bands.
2.8.1. **Parasite DNA extraction.**

After removal of white blood cells and saponin lysis in the usual way (section 2.6.1.), parasites were lysed at 40°C using 2mls of detergent mix containing 100mM Na Acetate, 10mM EDTA, 1% SDS, 0.5% aminosalicylate. Two extractions were carried out using phenol/chloroform (1:1) at 65°C, followed by two chloroform extractions and an ether extraction. DNA was precipitated in ethanol, washed in 70% ethanol, dried and resuspended in TE (10mM Tris-HCl, 1mM EDTA, pH 7.4). DNA concentration was determined using a Perkin Elmer 1000 fluorescence spectrophotometer.

2.8.2. **Digestion with restriction enzymes.**

Restriction enzymes and buffers were obtained from Boehringer. Digests were carried out overnight according to the manufacturer's instructions. For EcoRl "star" activity, the digestion buffer contained 20mM Tris-HCl pH 8.5, 2mM MnCl , 10mM DTT. (Mayer,1978).

2.8.3. **Agarose gel electrophoresis.**

Submarine agarose gel electrophoresis was carried out using the H4 apparatus from BRL. Running buffer was TBE containing 1.0 µg per ml ethidium bromide, and the gel was cast with 0.8% agarose (sigma type 2). The gel was calibrated using the 1 kilobase (kb) ladder marker DNA from BRL, visualised using a short wave uv transilluminator, and photographed with polaroid 667 film.

2.8.4. **Southern blotting and hybridisation.**

Transfer of DNA to nylon membrane (Amersham Hybond-N) was performed as described by Southern (1975), except that PFG gels
were treated with 0.25M HCl for 20 minutes prior to denaturation. Hybridisation of labelled DNA probes was carried out as described by Maniatis (1982), at 65°C for homologous probes, and 59°C for non-homologous ones.

2.8.5. DNA probes used.

In addition to DNA probes produced during the course of this work, two further probes were used. A *P. falciparum* ribosomal gene probe (rib 1), was that described by Langsley *et al.* (1983). A *P. berghei* telomere probe (*Ponzi et al.* 1985), was kindly made available by Dr. C. Frontali.

2.9.1. Construction of a genomic library in the bacteriophage lambda gt11.

5μg of purified parasite DNA from the strain CB was digested for 5 hours with 100 units of the enzyme EcoR1. The buffer contained 20mM Tris-HCL, 2mM MnCl, 10mM DTT, to encourage the appearance of "star" activity, Mayer (1978). The enzyme was then heat inactivated at 68°C for 10 minutes. The digest was diluted to give a DNA concentration of 10 ng per μl, and ligated with 1μg of lambda gt11 arms, (from Promega Biotech, pre-digested with EcoR1 and dephosphorylated). The ligation mix was Ligaid from P and S Biochemicals. The ligated phage were packaged using the Packagene kit from Promega Biotech, and plated as described in section 2.9.4., to ascertain the number of recombinant phage.

2.9.2. Growth of Y1090 and Y1089 plating cells.

Plating cells were grown up and used as described in Maniatis (1982), with 0.2% maltose. The bacteriophage lambda gt11 and the bacterial strains required for cloning with this
vector were obtained from Promega Biotech. The genotypes of these strains are fully described by Young and Davies (1983). Recombinant bacteriophage were plated in agarose containing X-gal and IPTG (Huynh et al. 1985). Plaques produced by non-recombinant phage were blue, while recombinant phage containing inserts appeared white. The library was amplified by plating out on Y1090. Bacteriophage were washed off with SM, and stored at 4°C with a drop of chloroform.

2.9.3. Screening the lambda gt11 library with antibody probes.

Phage were plated at a density of $3 \times 10^5$ plaque forming units (pfu) per 22cm plate, and incubated at 42°C for three to four hours. Each plate was overlaid with dry nitrocellulose, previously soaked in 10mM IPTG. The plates were incubated for a further 2 hours at 37°C, and the nitrocellulose lifts washed briefly in TST (20mM Tris-HCl pH 8.0, 0.15MNaCl, 0.05% Tween 20). The filters were blocked with 5% ovalbumin (Sigma) in TS (TST with no Tween) for one hour at room temperature. Antibodies used for the screen were preabsorbed against E.coli lysates (section 2.9.5.), diluted 1/300 in TS plus 5% ovalbumin and incubated with the filters overnight. Unbound first antibody was removed by washing four times with TST, and the remaining bound antibody visualised with Horseradish peroxidase conjugated anti-mouse gamma globulin antiserum (from SAPU). This was incubated for three hours with the filters at 1/200 dilution, washed as before, and developed in the same buffer as the immunoblots described in section 2.4.5.

2.9.4. Preabsorption of antibodies for library and lysogen screening.
Antisera were preabsorbed using a lysate of a lysogen of wild type lambda gt11 in E.coli Y1089 (gift of Dr.R.Hall). A single colony was grown up and induced as described in section 2.9.7. After growth at 37°C for 2 hours the cells were pelleted at 2000g for 10 minutes and the supernatant removed. The pellet was resuspended in 1/10 the culture volume of TE pH 8.0, and sonicated using a Soniprep 150 tuned to maximum output for one minute. The antisera were preabsorbed with an equal volume of this lysate for one hour, debris pelleted at 11000g in a microfuge, the supernatant removed and preabsorbed twice more. This preparation was used for library and lysogen screening.

2.9.5. DNA minipreparations of recombinant phage.

DNA was prepared from putative recombinants using the rapid small-scale isolation method of Maniatis (1982), from agarose plates. The phage DNA was digested using the restriction enzyme EcoRI, and run on a gel to visualise the cloned insert DNA.

2.9.6. Generation of lambda gt11 recombinant lysogens in Y1089.

Lysogens containing recombinant phage were generated as described by Huynh et.al.1985, in E.coli Y1089 cells. Clones which grew at 32°C but not at 42°C were assumed to be lysogens. This was tested by inducing the phage out of the lysogens. The lysogens were grown up as described in section 2.9.7., and induced by heating to 42°C for 30 minutes but without the addition of IPTG. The cells were grown for a further hour at 37°C, then lysed with three drops of chloroform. The resulting lysate was clarified by centrifugation and the supernatant tested for phage particles by plating in the normal way.
cultures of lysogens in LB medium and 50μg/ml Ampicillin, were stored by the addition of Glycerol to 15% and rapid freezing at -70 °C.

2.9.7. Preparation of a crude lysate from recombinant lysogens.

The lysogen was grown at 30 °C in 5ml LB medium with Ampicillin to an O.D. of 0.5. IPTG was then added to 10mM and the temperature rapidly increased to 42 °C for 30 minutes. The culture was grown for a further 2 hours at 37 °C, and 5ml samples taken at 30 minute intervals. Sampled cells were pelleted and lysed by boiling in 200ul of SDS sample buffer containing 20μl of a cocktail of the following protease inhibitors at 10mM concentration: PMSF, Benzamidine and TLCK, (all from Sigma). After boiling the samples were chilled on ice, a further 20μl of the cocktail was added and they were stored at -20 °C.

2.9.8. Sub-cloning of phage inserts.

DNA inserts contained in recombinant phage were subcloned into the plasmid vector pUC 18 (BRL), for restriction mapping and subsequent manipulations. The plasmid was digested with EcoR1, and ligated with an aliquot of a miniprep of the recombinant phage, which had previously been cut with EcoR1. JM83 cells were transformed as described in Maniatis (1982) and plated on plates containing IPTG and X-gal (section 2.9.2.). Transformants containing inserts appeared as white colonies. These were picked and plasmid minipreps were made by the alkaline lysis method of Maniatis (1982). Digestion of these with EcoR1 and electrophoresis showed which of the minipreps contained the sub-cloned insert.
3 Analysis of parasite proteins by 2D electrophoresis

3.1. Introduction.

Two dimensional (2D) electrophoresis separates proteins first by charge, and then by molecular weight (m.wt.). The system is capable of the very high resolution necessary for the analysis of complex protein mixtures (O'Farrell, 1975). This approach has been used extensively to compare proteins in wild isolates and cloned lines of *P.falciparum* (Tait, 1981; Fenton *et al.* 1985).

Biosynthetically labelled proteins from a number of parasite lines were examined for variation. Over 100 proteins were characterised, the majority of which were identical in all strains; however 15 showed polymorphism in iso-electric point and/or molecular weight. These variant proteins were usually found as clone-specific alternatives, a given clone possessing only a single form of each protein.

From this finding it was assumed that these alternatives were produced by different alleles of a given gene. The allelic basis of the variation in five 2D proteins has been confirmed by a genetic analysis of a cross carried out between two cloned lines of *P.falciparum*, which possess different variant forms of these proteins (Fenton and Walliker, 1988). Each one of a pair of variants was shown to segregate independently during meiosis following mosquito transmission, as expected for proteins determined by alleles of the same genetic locus.

The aims of the work described in this chapter were:

1. To study variation in proteins of the rodent malaria *P.chabaudi* by 2D electrophoresis.
2. To identify and characterise a set of reproducible parasite proteins to provide a framework for subsequent genetic analysis of protein variation.

3. To investigate whether any proteins of *P. chabaudi* have allelic variants exhibiting the considerable m.wt. variations characteristic of proteins containing tandem repeats, as occurs in *P. falciparum*.

3.2. **Biosynthetic labelling of parasite proteins.**

Biosynthetic incorporation of radiolabelled amino acids is a convenient way to label parasite proteins to a high specific activity. Provided white blood cells, reticulocytes and platelets are removed, the absence of protein synthesis in mature red blood cells means that only parasite proteins are labelled (Sweiger, 1962). The method of Tait (1981) was used to radiolabel parasite proteins for 2D electrophoresis. Blood forms of *P. chabaudi* were obtained from mice (section 2.4.1.). The parasite infection is roughly synchronous in mice, with schizogony occurring over a three hour period around midday, with the mice kept in a reversed light regime. Synthesis of many proteins is highly stage-specific and is most active during schizogony (Newbold et al., 1982). Therefore labelling of parasite proteins was always carried out during this period. Typically 6x10⁶ c.p.m. were incorporated into TCA-precipitable material, by 10⁶ parasites in a two hour labelling.

3.3. **Selective incorporation into parasite proteins.**

A Whatman CF11 column was used to remove white blood cells and other host contaminants prior to labelling. In order to check the effectiveness of the column, and the
Figure 4A. Autoradiograph of radiolabelled parasite extract of line CB, subjected to 2D electrophoresis. The origin of the first dimension is marked by "O". The m.wt. (in KDa) and isoelectric point scales are indicated.

Figure 4B. Diagram of the set of major reproducible proteins derived from several gel runs with different labelled samples of parasite line CB. The proteins have been numbered and the molecular weight and isoelectric point of each protein are listed in Appendix 1.
extent of contamination of the parasite proteins with radiolabelled host proteins, equal volumes of infected and uninfected blood were prepared and radiolabelled. Incorporation of radiolabel by the uninfected blood was less than 1% of that in the parasite material. The proteins synthesised by the two samples were compared by 2D electrophoresis (section 2.5.3.). Only three faintly labelled proteins appeared in the extract from uninfected blood (data not shown). These proteins are probably not of parasite origin and were discounted in subsequent analysis.

3.4. Characterisation of a set of major reproducible proteins.

Clone CB of *P. chabaudi* was chosen as the standard clone for the 2D studies. The autoradiograph produced by 2D electrophoresis of labelled parasite proteins of this clone is shown in figure 4A. The pattern of spots was highly reproducible from gel to gel using the same labelled sample. Some variations in the pattern were seen when different samples of the same cloned line were compared. This is probably due to the production of stage-specific antigens to different extents in the separate labellings, as variation in the degree of synchrony of the parasite infection can occur in different mice. However if parasites are always labelled at the same stage of schizogony, reproducible patterns are obtained.

134 of the most intensely labelled proteins, which appeared invariably on the gels of clone CB were identified as shown in figure 4B. The isoelectric focussing gels were calibrated by direct pH measurements, and the SDS gels
by using standard markers (section 2.5.4.). This allowed the 134 proteins to be characterised by their m.wt. and isoelectric point (pI). Each protein in the set was numbered roughly in order of decreasing m.wt. and increasing pI. This allowed specific proteins to be located easily on the gels. Characteristics of these major proteins are listed in Appendix 1.

3.5.1. Protein variation in cloned lines of *P. chabaudi*.

In order to identify parasite proteins which varied in m.wt. or pI., six cloned lines of *P. chabaudi* were compared by 2D electrophoresis. The cloned lines under study originated from wild isolates, and had previously been cloned by limiting dilution (Walliker, 1975). They had been checked to ensure they contained parasites of only one genotype by comparison of isoenzyme and antigen markers (Lainson, 1983; McLean, 1986).

The six lines were analysed by making pairwise comparisons with the set of reproducible proteins of CB. Each clone was labelled with $^{35}$S-methionine, and subjected to 2D electrophoresis at the same time as a sample of the CB line. In order to remove ambiguities due to minor variation between the two gels, a third gel was run in parallel with those of each clone, in which proteins containing the same number of cpm. from each clone were mixed together. Autoradiographs of the two clones were compared, and putative variant proteins identified. These were then compared on the gel of the mixed proteins from each line. Proteins which exhibited variants in each clone were identified according to the same criteria used to identify variant alleles in
Figure 5. Comparison of parasite lines CB and AS. Autoradiographs of 2D separations of labelled proteins from lines CB (5A), AS (5B) and a mixture of equal cpm. of labelled proteins from both lines (5C). Fig. 5D shows a simplified diagram of the autoradiograph in 5C, indicating members of the set of major reproducible proteins which vary in m.wt. and/or isoelectric point between the two lines. Variant proteins are shown as open circles. Some invariant proteins are shown as filled circles to allow orientation. The variant proteins are numbered according to the diagram in figure 4B.
P. falciparum (Walker, 1985):

1. Variant forms of each protein should be reproducible in presence and position in a given clone.

2. New variants should be of similar relative intensity and in close proximity to the corresponding protein of CB.

3. Variant proteins should occur as an alternative form to the corresponding protein in CB, so that in 2D gels of labelled proteins from two mixed lines, the two variant forms should appear as a doublet.

A typical comparison is shown in figure 5, between CB and AS clones. As with P. falciparum the majority of proteins were found to be identical in the two lines by this method. However 8 proteins identified as variant were found (figure 5D), corresponding to proteins 2, 3, 4, 6, 16, 86, 97 and 109 of the reproducible set. Four other cloned lines were compared, both with the CB reference line, and pairwise with one another, in order to identify variant proteins characteristic of each line. The results are shown as a composite map in figure 6A, illustrating all forms of the variant proteins detected in these 6 lines. All of these variant proteins comply with the criteria listed above, and are therefore likely to represent allelic variants of the genes determining them.

3.5.2. Types of variation.

The number and relative positions of some of the different variants of each protein are illustrated in figure 6B, and their m.wt. and pI is detailed in Appendix 1. Three types of variation were seen:

1. Proteins 3, 43, 57, 96, 97, 107, and 109 possessed variants
Figure 6A. Composite map of all variant proteins in P.chabaudi detected by 2D gel electrophoresis, in the six lines which were compared. Variant proteins are shown as open circles, and are numbered according to the system shown in Fig.4B. Variants of the same protein are distinguished by letters, and their characteristics are listed in Appendix 1.

Figure 6B. Diagram illustrating variants of three proteins: 86, 109, and 46. Relevant sections of autoradiographs of 2D gels illustrate two or more variants, and their relative positions are shown diagrammatically.
### Fig. 6A

![Image of Fig. 6A](image)

### Fig. 6B

<table>
<thead>
<tr>
<th>Protein</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>86</td>
<td><img src="image" alt="Image of 86 alleles" /></td>
</tr>
<tr>
<td>109</td>
<td><img src="image" alt="Image of 109 alleles" /></td>
</tr>
<tr>
<td>46</td>
<td><img src="image" alt="Image of 46 alleles" /></td>
</tr>
</tbody>
</table>

*Note: The images of the alleles are not described in detail in the text snippet provided.*
which differed in isoelectric point only.

2. Proteins 7, 12, 19 and 46 had variants which differed in m.wt. only, with no apparent alteration in pI.

3. Proteins 2, 4, 6, 16, 48, 80 and 86 had variants forms which differed in pI or m.wt. only, but which also had variants which differed in both characteristics simultaneously.

The degree of diversity exhibited by particular proteins is clearly variable. Proteins such as no. 43 possess only two variants, differing only in isoelectric point; whereas others such as protein 2, exhibit a different variant in all six isolates, showing considerable differences in pI and m.wt. Variants C and F of this protein differ in m.wt. by as much as 45 KDa. The possible molecular basis for these changes is considered below.

3.6. Identification of Adenosine Deaminase (ADA).

2D electrophoresis has allowed the characterisation of sets of major reproducible proteins in P. chabaudi, P. knowlesi and P. falciparum (Fenton et al., 1985; Howard et al., 1983). However even in P. falciparum, the best characterised species in this respect, only five of these proteins have been identified. Four of these are antigens, all of unknown function: the S-antigen, the PSA, and two other antigens defined by mcabs (Fenton, 1988). The only protein in the 2D map whose function is known is the enzyme Adenosine Deaminase (Fenton and Walliker, in preparation). This was shown by excising a band containing ADA from a starch gel of P. falciparum proteins, and subjecting the material to 2D electrophoresis. The enzyme has a m.wt. of 36 kD. and two
Figure 7. Autoradiographs of 2D gels showing the identity of the parasite enzyme Adenosine deaminase (ADA). Radiolabelled parasite extracts of line CB were subjected to starch gel electrophoresis, and the band identified as the enzyme ADA excised. The proteins were extracted from the excised band and subjected to 2D electrophoresis. Panel A shows the total proteins from the sample; panel B shows the proteins eluted from the ADA band. The single radiolabelled protein spot in B corresponds to protein 36 of the composite map.
Figure 8. Correlation between electrophoretic variants of the enzyme ADA and 2D protein 86, in cloned lines of P. chabaudi.

Figure 8A shows the variants of protein 86 from the composite map, as detected by 2D gel electrophoresis.

Figure 8B shows the electrophoretic forms of the enzyme ADA in the same six cloned lines, as detected by starch gel electrophoresis (from Lainson, 1983).
alternative forms with pI's of 5.8 and 5.9 in \textit{P. falciparum}.

A similar approach was employed in order to identify the enzyme ADA on the 2D map of \textit{P. chabaudi}. Previously at least four alloenzymes were detected by starch gel electrophoresis in the six cloned lines used in this study (Lainson, 1983). A starch gel of radiolabelled \textit{P. chabaudi} proteins was stained and the ADA band excised. The protein in the band was eluted, run on a 2D gel and autoradiographed. A single labelled protein spot was visualised in the same position as protein 86 on the 2D map, suggesting that this protein is the parasite enzyme ADA. This interpretation is supported by a number of observations:

1. There are five 2D variants of protein 86, with four different isoelectric points. These might correspond to the four alloenzymes detected by starch gel electrophoresis (Lainson, 1983).

2. These variants range in pI from 6.3 to 6.5, and in m.wt. from 38 to 41 kD., which are comparable to the values for ADA from \textit{P. falciparum} cited above.

3. By starch gel electrophoresis the order of ADA allozymes from most acidic to most basic is: AJ and ER, CB, EF and DS, and finally AS (figure 8). This is identical to the order of variants of protein 86, where the variant common to AJ and ER is the most acidic and that of AS the most basic. It is therefore likely that protein 86 of the 2D map represents the parasite enzyme ADA.

3.7. Discussion.

3.7.1. Identification of a set of reproducible proteins by 2D electrophoresis has allowed the identification of a
set of major reproducible proteins in *P. chabaudi*, in a similar way to previous studies in *P. falciparum* and *P. knowlesi*. Biosynthetic labelling of parasite proteins is possible because of the absence of synthesis in mature red blood cells (Sweiger, 1962). Comparison of the 2D protein patterns produced by infected and uninfected blood, after a CF11 column had been used to remove white blood cells, showed that almost all the radiolabelled proteins were parasite in origin. A limitation of the use of $^{35}$S-methionine for biosynthetic labelling is that proteins lacking methionine, or with little methionine in their primary structure, would not be detected.

For this reason, the proteins identified as major reproducible proteins (MRPs) in figure 4, only represent a proportion of the total proteins of *P. chabaudi*. In addition, the isoelectric focussing of the first dimension of the 2D gels, covered a limited range from pI 4.5 to 7.1. Proteins focussing outside this range would not have been detected. Finally, the synthesis of many proteins of the asexual forms is highly stage specific (Newbold et al. 1982). Since synchronous infections were labelled at schizogony, proteins synthesised specifically at other stages in the asexual cycle would not be detected.

### 3.7.2. Identification of electrophoretic variants.

18 of the 134 MRPs showed variation in one or more of the six lines studied. This figure is comparable to that determined in the 2D studies of *P. falciparum* (Fenton, 1985). Some proteins had only one or two variants, while others differed in every line examined. In all cases the variants
were found as alternatives, one variant occurring in one clone, and a second variant in another. This is identical to the situation found in *P.falciparum*, where genetic studies have recently proved that these variants represent alleles of their respective genes (Fenton and Walliker 1988). It therefore seems likely that where proteins of *P.chabaudi* have variant forms, these represent alternative alleles of the gene encoding that protein. Formal confirmation of this would require a full-scale genetic analysis to show segregation of the alternative forms of each protein, as described for *P.falciparum*. This was not carried out during the present study. However for the purpose of this thesis, it is assumed that the variant forms of one particular protein spot do indeed represent alleles of the same gene. Some limited evidence in support of this will be presented below and in chapter 4.

The protein variation detected by 2D electrophoresis implies considerable genetic diversity among the six cloned lines studied. A comparison of the variant protein types found in each line is shown in figure 6. It is clear that each line possesses a unique protein profile, presumably reflecting a unique genotype, and that as in *P.falciparum*, the 2D protein map can serve as a sensitive method for strain typing. Overall comparison of the protein profiles shows that the lines isolated from the C.A.R., resemble each other more closely than those from other countries. In addition the line EF from Cameroon is more closely related to those of the C.A.R., than line DS from Congo. This is in agreement with the findings of Lainson (1983), and Carter and Walliker,
(1978), in which the line DS was recognised as the subspecies *P. chabaudi adami*.

3.7.3. **Types of electrophoretic variation**

Three types of electrophoretic variant were detected in this study; variants in pI, or m.wt. only, and then some proteins which differ in both characters simultaneously. Proteins varying in pI, are believed to differ in charge; the resulting alteration in pI being inversely proportional to the size of the molecule (Anderson, 1983). Variants of this type probably arise by substitution of one or a few amino acids in the polypeptide sequence. Variants which differ in m.wt. could do so for a number of reasons, such as alterations in glycosylation or posttranslational modifications. However occasionally point mutations resulting in a single amino acid substitution can cause significant apparent m.wt. changes (Vanderkerckhove *et al.* 1980). Alternatively, although the variants are probably alleles, and hence related in sequence, large scale differences in the primary amino sequence could also be responsible for the m.wt. variants seen in *P. chabaudi*, as has been found for several antigens of *P. falciparum*, (see chapter 1). In some of these antigens the variation is due to differences in large tandem repeat regions of the genes encoding the antigens. Although the molecular basis of the variation in the *P. chabaudi* proteins is unknown, it is possible that some of the genes also possess tandem repeats which vary between alleles.

3.7.4. **Identification of Adenosine Deaminase**.

The functions of the proteins identified in the 2D map
of *P.chabaudi* are unknown, however the identity of the spot representing the enzyme ADA has been determined. This enzyme has been extensively studied in *P.falciparum*, and identified on the 2D map as a protein with two variants, of m.wt. 36 KDa, pI 5.8, or 5.9. It is believed to be involved in the metabolism of adenosine to hypoxanthine. The parasite lacks a *de novo* purine synthesis pathway, and hypoxanthine is the major purine base salvaged by plasmodia (Sherman, 1979).

In *P.chabaudi* starch gel electrophoresis has detected four alloenzymes of ADA (Lainson, 1983). Examination of the *P.chabaudi* 2D map suggested protein 86 might represent the parasite ADA. Indirect evidence supporting this view comes from the correspondance in isoelectric points of the isoenzyme variants detected by starch gel electrophoresis, and the 2D variants (figure 8). Radiolabelled CB parasite ADA was excised from a starch gel, and subjected to 2D electrophoresis. A single radiolabelled spot was obtained, corresponding in position to protein 86 of the CB line. It can be concluded that ADA and protein 86 are probably one and the same gene product. However genetic studies to support this have not been carried out. To confirm this genetically it would be necessary to analyse the progeny of a cross between two lines of *P.chabaudi*, which differed in their ADA alloenzymes by starch gel electrophoresis, and by variant forms of protein 86. If protein 86 does represent ADA, the two markers would be expected to always cosegregate, as was found in *P.falciparum* (Fenton and Walliker, in preparation).

3.7.5. **Summary.**

The use of 2D gel electrophoresis to compare six
lines of *P. chabaudi*, has allowed the identification of a set of major reproducible proteins, characteristic of each clone. Variants of 18 of these proteins have been found, which differ in isoelectric point or m.wt. between the lines. The alternative forms of these proteins probably represent variant alleles. A number show considerable differences in m.wt. between alleles and may be encoded by genes with tandem repeats as has been found in *P. falciparum*. The function of most of these proteins is unknown, although the variant protein representing the enzyme ADA has been identified. The approach used to identify ADA, shows how information from the 2D map can be correlated with that from sources such as enzyme electrophoresis, monoclonal antibody studies, and genetic data to yield useful information.
4. Identification of antigens with monoclonal antibodies

4.1 Introduction

The 2D electrophoresis study described in chapter 3, has revealed a number of proteins which appear to vary in pI and m.wt. between strains of *P. chabaudi*, and which are probably alleles. By analogy with the variant alleles found in *P. falciparum*, some of these *P. chabaudi* variant proteins may possess repeat or other types of variable region, producing the alterations in pI and m.wt. revealed by 2D electrophoresis.

Mcabs have now been produced in several laboratories against *P. chabaudi* antigens. The aim of the work described in this chapter was to define the target antigens of the mcabs available, where this was unknown. These mcabs were then used, together with mcabs of known specificity to investigate whether any of these antigens vary in m.wt between strains. An objective of this approach was to use mcabs reacting with variant proteins to clone the encoding genes from an expression library to determine the structural basis of these size variations.

4.2. Mcabs used and methods of characterisation.

The mcabs used in this study were produced in this laboratory by Dr. A. McLean, and by Dr. S. McLean in the University of Glasgow. In some cases the antigen which these mcabs recognised had been partially characterised. Full details are shown in table 2.

For previously uncharacterised mcabs, IFA was used to investigate the subcellular location and stage specificity of the target antigen. The antigen's m.wt was ascertained by Western blotting and immunoprecipitation of biosynthetically
<table>
<thead>
<tr>
<th>Mcab</th>
<th>Fluorescence Pattern</th>
<th>Previous characterisation of antigen m.wt. (kD)</th>
<th>Results of this study (m.wt. in kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.15</td>
<td>late trophs and schizonts, 'rasberry'</td>
<td>250 kD, variant 245 CB 250 AS 255 EF</td>
<td>Confirmed previous result</td>
</tr>
<tr>
<td>12.1</td>
<td>membrane of infected erythrocyte, all stages</td>
<td>55 kD no variants</td>
<td>Variant 85 AS, AJ 83 CB, ER 82 DS 80 EF</td>
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<tr>
<td>12.18</td>
<td>dull overall with bright inclusions all stages</td>
<td>42 kD</td>
<td>Corresponds to 2D protein 78. Possible variant DS 40 kD?</td>
</tr>
<tr>
<td>12.A</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.B</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.C</td>
<td>As 12.15</td>
<td>--</td>
<td>All probably recognise same antigen as 12.15</td>
</tr>
<tr>
<td>12.D</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-10</td>
<td>dull overall with</td>
<td>--</td>
<td>Variant.</td>
</tr>
<tr>
<td>S-20</td>
<td>bright inclusions, trophs and schizonts</td>
<td>--</td>
<td>105 CB 100 DS 97 AS, AJ, ER 97 EF</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Corresponds to 2D protein 16.</td>
</tr>
</tbody>
</table>

S-10 and S-20 were produced by Dr. S. McLean in Glasgow. All other mcabs were produced by Dr. A. McLean in Edinburgh.

(1) A. McLean Ph.D Thesis, 1986
Figure 9.

IFA patterns produced by mcabs used in this study.

Mcab 12.15
This mcab produced an irregular staining pattern, occasionally being seen localised on the surface of merozoites within schizonts.

Mcab 12.1
This mcab reacted with the membrane of red blood cells infected with all stages of the erythrocytic cycle.

Mcab 12.18
This mcab produced a diffuse fluorescence over the whole infected cell, with a few bright spots. All blood stages.

Mcdabs S-10 and S-20.
These mcabs produce an identical pattern, which is similar to that of mcab 12.18, however the antigen appears to be restricted to trophozoites and schizonts.
labelled parasite extracts. Once the antigen had been identified its m.wt in six different isolates of \textit{P.chabaudi} was examined. The mcabs of previously known specificity were also investigated in this way. Finally attempts were made to determine whether any of the antigens identified by these mcabs corresponded to any of the proteins in the 2D map of \textit{P.chabaudi} produced in chapter 3.

4.3. \textit{Mcab 12.15.}

This mcab had previously been shown to react with an antigen of 250 KDa (McLean,1986). It was used here as a positive control to optimise conditions for Western blotting and immune precipitations. The antigen is synthesised during schizont maturation, and appears localised to the surface of merozoites producing a highly characteristic IFA pattern (figure 9). It has been shown to be equivalent to the PSA of \textit{P.falciparum} (Holder et.al.1983). The alleles of this antigen in clones AS and CB have previously been shown to differ in m.wt. (Newbold et.al.1984).

The results of immunoprecipitations and Western blots against the six lines are shown in figure 10. They confirm the polymorphism previously described, and demonstrate the integrity of the parasite preparations used. Similar extracts were used for subsequent experiments with the other mcabs. In figure 10B three distinct size alleles are visible. In strains EF, ER, and AS, a protein of 230 KDa is precipitated, in CB and AJ it is 225 KDa, and in DS it is 235 KDa. The appearance of a doublet in some samples is surprising, since all samples were labelled and solubilised in the same way. However a similar observation was made by Epstein et.al.(1981), when isolating the analogue of this
Figure 10A. Parasite extracts run on 5% SDS-PAGE and blotted with mcab 12.15. Molecular weight markers are shown in track M in kDa. Parasite extracts probed with normal mouse serum gave negligible reactions under these conditions (data not shown).

Figure 10B. Autoradiograph of six parasite lines metabolically labelled with $^{35}$S methionine, immunoprecipitated with mcab 12.15 and run on a 5% SDS-PAGE. All samples were prepared in the same way using saponin lysed parasites.

Figure 11A. The same parasite extracts as used in figure 10A were run on 10% SDS-PAGE and blotted with mcab 12.1. M.wt. markers are shown in track M in kDa.

Figure 11B. Autoradiograph of three parasite lines metabolically labelled with $^{35}$S methionine, immunoprecipitated with mcab 12.1 and run on 10% SDS PAGE. Saponin freed parasites were used for labelling.
protein in *P. knowlesi*. They suggested that the saponin treatment might activate a protease, however in the present experiment all samples contained protease inhibitors. Alternatively, these extra bands might be processing products since this protein is subject to extensive processing and modification (Boyle et al. 1982). Slight differences in the stage of radiolabelling of the parasite samples could explain the differing degrees of processing.

4.4. Mcab 12.1

The IFA pattern of this mcab indicated that the antigen was localised in the membrane of infected red blood cells (figure 9). The antigen was present in all erythrocytic stages. A surface IFA on infected cells was carried out (section 2.4.3.). No fluorescence was seen, indicating that the antigen is not exposed on the surface of live cells. Presumably the process of fixation in acetone used for normal IFA's results in exposure of the reactive epitopes.

Previously this mcab had been characterised as reacting with a protein of 55 KDa by immunoblotting (McLean, 1986). However on repeating this with the six cloned lines, it was found to react specifically with a doublet of m.wt. 86 KDa (figure 11A). In order to resolve this discrepancy immunoprecipitations were carried out with biosynthetically labelled parasites, using mcab 12.1. The results are shown in figure 11B. This mcab clearly reacts specifically with a labelled protein of m.wt. approximately 85 KDa. This protein appears to vary in m.wt. between strains. Comparison of the results shown in figures 11A and 11B suggests that alleles of this antigen exhibit three or
Figure 12. Identical parasite extracts from line CB run on 10% SDS PAGE and blotted with 5 different mcabs including 12.15. M.wt. markers are shown in track M in kDa.

Figure 13A. Parasite extracts run on 10% SDS PAGE and blotted with mcab 12.18. M.wt.markers are shown in track M in kDa.

Figure 13B. Autoradiograph of parasite extracts of lines DS and CB metabolically labelled with 35S methionine, immunoprecipitated with mcab 12.18, and run on 10% SDS PAGE. Two different extracts were made from each line, one from radiolabelled saponin freed parasites (sap), and the other from parasites radiolabelled within whole blood cells (wc), as in section 2.6.
possibly four different m.wts: 85 KDa (AS and AJ), 83 KDa (CB and ER), 82 KDa (DS-tentative) and 80 KDa (EF).


These mcabs had not previously been characterised. In IFA the pattern of reactivity was identical to that shown by mcab 12.15 (figure 9). On blotting against the line CB, all appeared to react with a single protein of m.wt. 225 KDa. This is probably identical to the antigen recognised by mcab 12.15, which was run as a control in figure 12.

4.6. **Mcb 12.18**

Mcb 12.18 has previously been shown to react with an antigen of m.wt. 42 KDa (McLean,1986). It appears to be present throughout the asexual erythrocytic stages. The IFA pattern appears as a dull fluorescence over the whole erythrocyte with brightly staining inclusions in the red blood cell cytoplasm (figure 9). Figure 13A shows the results obtained when the six cloned lines were probed by Western blotting. The antigen appears to be of identical m.wt. in all lines, except DS, where it was slightly smaller. The poor reaction of the ER sample is not significant, since in other blots the mcab reacted well with this clone.

The IFA pattern indicated that this antigen was localised largely in the erythrocyte cytoplasm. This idea was tested by making two extracts of parasite proteins from equal numbers of infected red blood cells that had been metabolically labelled. In one case the whole cells were solubilised, in the other the cells were saponin lysed and then solubilised. The two samples were immunoprecipitated with 12.18. Results are shown in figure 13B. This shows that the 42 KDa antigen is largely localised in the red blood
**Figure 14A.** Parasite extracts run on 10% SDS PAGE and blotted with mcab S-10. M.wt. markers are shown in track M in kDa. Parasite extracts were made from saponin freed parasites (sap), and also from whole parasitised red blood cells (wc). Identical extracts probed with normal mouse serum gave negligible reaction (data not shown).

**Figure 14B.** Autoradiograph of extracts of parasite lines AJ and CB metabolically labelled with 35S methionine, and immunoprecipitated with mcabs S-10 and S-20. Extracts were made from parasites radiolabelled within whole red blood cells (wc), or from radiolabelled saponin freed parasites (sap).

**Figure 15A.** Serial dilutions of known amounts of mouse (m) and parasite (p) DNA were spotted onto a nitrocellulose filter, and hybridised with nick translated mouse DNA. The extent of contamination of the parasite DNA with mouse DNA can be determined by comparison with the mouse standards.

**Figure 15B.** Agarose gel stained with ethidium bromide. Parasite DNA was digested with the restriction enzyme EcoR1. Aliquots were removed at timed intervals and electrophoresed to monitor progress of the digest. DNA size markers are shown in track M.

**Figure 15C.** Agarose gel stained with ethidium bromide. Minipreps of bacteriophage DNA were made from clones which gave a positive reaction when screened with anti-PSA antiserum. Two sizes of insert are present, 2.5 and 4.0 kb. (arrowed). DNA size markers are shown in track M.
cell cytoplasm, since in the saponin lysed extracts, where most of the erythrocyte membrane and cytoplasm had been removed, the antigen is greatly reduced in amount.

4.7. S-10 and S-20.

These mcabs were produced by Dr. S. McLean, and had not previously been characterised. They are discussed together, since it was found they almost certainly recognise the same antigen. They produce identical patterns by IFA, similar to that of mcab 12.18 (figure 9), and the antigen appears to be present in trophozoites and schizonts. The mcabs reacted with all the *P. chabaudi* isolates tested, and with *P. falciparum* strain T9/96 (figure 9).

It was difficult to identify the antigen with which these mcabs reacted, using immunoblotting. The reactions obtained using the usual saponin lysed parasites were faint and variable. Clearer results were seen with extracts from whole parasitised erythrocytes, although frequently many additional bands were seen (figure 14A). In the whole cell extracts a strong doublet of 102 and 95 KDa was recognised, together with other bands which could be processing or degradation products. In saponin lysed parasites only the upper band was seen. This appears to vary in m.wt. between strains; being 98 KDa (AS), 97 KDa (AJ), and 102 KDa (DS). No binding was seen with uninfected cell extracts.

The two mcabs gave similar results when used for immunoprecipitations, producing a broad band of approximate m.wt. 105 KDa. Much more of the antigen was precipitated from whole cell extracts than from saponin lysed cells (figure 14B). The results for all the mcabs are summarised in Table 2 (page 48).
Figure 16. Autoradiographs of parasite extracts subjected to 2D PAGE. Saponin freed parasites of line CB were metabolically labelled with $^{35}$S methionine. Figs. 16A and 16B show the total parasite proteins run in the normal way, and the composite map of 2D proteins. The same parasite extract was then immunoprecipitated with mAbs 5-10 and 12.18 (as in figures 13 and 14), but this time subjected to 2D electrophoresis, (figs 16 C and D respectively). Immunoprecipitates of the same extract with normal mouse serum produced no significant radiolabelled spots.
4.8. Identification of antigens on the 2D map.

Antigens immunoprecipitated by these mcabs were compared with whole parasite protein extracts, by 2D electrophoresis.

Four mcabs were used for this experiment, 12.15, 12.1, 12.18, and S-10, which recognise antigens of 230, 86, 42, and 105 KDa respectively. Radiolabelled parasites of clone CB were lysed and immunoprecipitated with each mcab. The precipitated antigens were subjected to 2D electrophoresis, and compared to a total protein extract run at the same time. Mcabs 12.18 and S-10 were found to precipitate proteins which could be matched to proteins from whole parasites (fig.15). This allowed the proteins in the composite 2D map, corresponding to these antigens to be positively identified. Mcab 12.18 precipitated three major spots of 42, 36, and 30 KDa. The 42 KDa spot was the most intense and probably corresponds to the 42 KDa antigen identified in the earlier blotting experiments. Mcab S-10 precipitated a strongly labelled complex of proteins, the largest of which matched the m.wt. of the 105 KDa antigen described earlier. The 42 and 105 KDa proteins identified in this way matched proteins 78 and 16 of the composite map (figure 16). Mcabs 12.15 and 12.1 gave no clear result (data not shown).

4.9.1. Discussion.

The 9 mcabs used in this part of the work were found to react with 4 different antigens. When these were screened for m.wt variation, each antigen exhibited variation in at least one clone. This was surprising in view of the fact that only 13% of the total parasite proteins in the 2D map showed such variation. It is possible that these variant
proteins are highly immunogenic and that the immune response is biased towards such proteins. Hence when mcabs are made from an immunised mouse, the population of cells produced contains a large proportion of cells secreting antibody to these immunogenic variant proteins. This is illustrated by the fact that 5 out of 9 mcabs characterised during this work reacted with the same 230 KDa antigen. Several groups of workers who have raised mcabs to the blood stages of *P. chabaudi*, have found that the majority of mcabs produced react with this protein, which appears to be highly immunogenic (Boyle *et al.* 1982; A. McLean 1986).

4.9.2. 230 KDa antigen

The mcab 12.15 had previously been shown to react with a 230 KDa antigen, believed to be the analogue of the PSA, and which varied in m.wt. between the lines AS and CB. These results were confirmed in the present study. The analysis of variation included 3 strains not previously examined, and revealed three size classes of 225, 230, and 235 KDa. These m.wts. were calculated relative to human spectrin, and it is clear from figure 10 that the figure of 250 KDa given in other studies is too high. Only three size classes are detectable by electrophoresis, however all of these alleles can be differentiated by their reaction with a panel of mcabs (McLean, 1986). Clearly antigenic differences do not always result in detectable m.wt. differences.

4.9.3. 86 KDa antigen

Mcab 12.1 had previously been characterised as reacting with an antigen of 55 KDa. However comparison of the six test clones showed that it reacted with an antigen of 86 KDa. The reason for this discrepancy is unclear. In one
sample (EF), there is a faint reaction with a band of 55 KDa. It is possible that this is a degradation product of the larger antigen, and it was this that was previously detected. Immunoprecipitations confirmed the size of the target antigen, which varied between strains, with four different size classes detectable among the six strains. In the absence of a panel of mcabs against this antigen it is not possible to say in clones such as AS and AJ, where the protein appears to be the same size (85 KDa), whether this is because each clone possesses the same allele, or because they have antigenically different alleles of similar size.

4.9.4. 42 KDa antigen

Mcab 12.18 was found to react with an antigen of 42 KDa, confirming the earlier characterisation. It appears the same size in all parasites except DS, where it is slightly smaller. Immunoblotting of different extracts of parasitised red blood cells showed the majority of this antigen appeared to be localised extraparasitically, within the cytoplasm of infected red cells. This was in agreement with the IFA pattern, which showed the antigen localised within small, brightly staining inclusions, possibly vesicles.

4.9.5. 105 KDa antigen

Mcabs S-10 and S-20 were found to give identical reactions in blotting and IFA, and probably recognise the same antigen, which does vary in m.wt. between strains. In both blotting and immunoprecipitations a single band was not obtained. The smaller bands could represent degradation or processing products of the antigen, or non-specific cross reactions with other antigens. Another possibility is that this protein is glycosylated, which could account for the
characteristically broad band seen in the precipitations. This possibility could be examined by biosynthetically labelling the parasites with $^{3}H$-glucosamine. These mcabs cross-reacted by IFA with *P. falciparum*, and may recognise an analogous antigen in the two species.

4.9.6. 2D electrophoresis.

Immunoprecipitates of each antigens were compared with total parasite proteins, to determine whether any of these antigens corresponded to any of the variant proteins previously identified. The reasons for the lack of results with mcabs 12.15 and 12.1 are unclear, although it is possible that the isoelectric points of these antigens fall outside the relatively narrow range covered by the 2D composite map (pI 4.5-7.1). However the antigens recognised by 12.18 and S-10 were found to correspond to proteins 78 and 16 respectively on the 2D map. With both mcabs a number of subsidiary spots of lower m.wt. were seen, possibly degradation products, or proteins coprecipitated with the target antigen.

It is interesting to note that protein 16 had been identified as a protein with variants of 100 KDa (DS), 97 KDa (AS) and 97 KDa (AJ) on 2D gels. These values compare well with the immunoblotting variants of 102, 98, and 97 KDa for the same strains, supporting the view that protein 16 and the 105 KDa antigen are one and the same.
5. Isolation of the gene encoding the 230 KDa antigen of P. chabaudi.

5.1. Introduction.

An antigen which appears important in the induction of immunity, and is therefore a vaccine candidate is the PSA of P. falciparum described in section 1.7.5. Similar molecules have been identified in several other malaria species, which are believed to represent homologues of the PSA (Newbold, 1984). These molecules share the following characteristics:

1. They are high m.wt. polypeptides, synthesised in mature trophozoites and schizonts.

2. This precursor is processed into several discrete fragments, some of which are associated with the surface of merozoites (Hall et al., 1984).

3. The molecules are antigenically complex, with strain specific and species specific epitopes, as well as epitopes that are common to all species so far examined (Holder, Freeman and Newbold, 1983; McBride et al. 1985).

4. Vaccination studies in several species using the whole PSA or processing fragments of them, have shown that these molecules can induce protection, although this is most effective against homologous parasite challenge (Siddiqui et al. 1987; Holder and Freeman, 1981).

Structural studies are most advanced in the PSA of P. falciparum, where sequence comparison of different alleles has led to some understanding of the structural basis underlying the antigenic diversity of these molecules (Tanabe et al. 1987), (see section 1.7.5.). These studies provide
indirect evidence for several mechanisms, that might be responsible for the generation of the antigenic diversity which appears so important to the parasite. In particular, there is evidence that limited intragenic recombination might occur between conserved regions of different alleles, resulting in antigenically novel molecules.

Since this antigen may form an important component of any vaccine, an understanding of the genetic processes which generate diversity in this molecule is vital. Questions of particular interest are:

1. Do the postulated mechanisms, such as intragenic recombination operate in the manner proposed?
2. If so, at what rate are antigenically novel alleles generated?
3. Is the antigenic diversity generated in this way sufficient to impair the efficacy of a vaccine with this molecule as a component?
4. It is likely that if the PSA is indeed included in a vaccine, small polypeptide pieces of the protein will be used rather than the entire molecule. Antigenic alterations to some regions may have little or no effect on the protection achieved, whereas changes in other regions may allow parasites to evade immunity acquired by vaccination. It is therefore important to define which regions of the molecule are most suitable vaccine candidates.

Any of these questions will be difficult to pursue with \textit{P.falciparum}. Rodent malaria parasites are much more convenient models, particularly for genetic studies. However, to date only the PSA of \textit{P.yoelli} has been cloned (J.Burns,
personal communication). This chapter describes an attempt to clone the gene encoding the PSA of *P. chabaudi*.

5.2. **PSA of *P. chabaudi***

The PSA of *P. chabaudi* has been identified as a glycoprotein of m.wt 230 KDa (Boyle *et al.* 1983). It shares all the characteristics of the PSA of *P. falciparum* as listed above. The molecule exhibits considerable antigenic diversity between strains. This is reflected in m.wt. variation between alleles, with three different size classes described in chapter 4. Genetic studies have shown that these different forms of this protein are indeed alleles, segregating independently during meiosis (McLean, 1986). The PSA of *P. chabaudi* has been purified from AS parasites and used to immunise mice (Brown *et al.* 1985). Challenge with CB parasites, which have an antigenically different form of the PSA showed no protection compared with controls. However mice challenged with AS parasites showed a delayed parasitaemia with a reduced peak, indicating some protection was induced by this antigen.

5.3. **The cloning and expression vector lambda gt11.**

The cloning vector used in this work was the bacteriophage lambda gt11 (Young and Davies, 1983). The site used for insertion of foreign DNA is a unique EcoR1 cleavage site, located 53 base pairs upstream of the B-galactosidase translation termination codon, (figure 17). Recombinant phage generate an inactive B-galactosidase fusion protein, and can be distinguished from non-recombinants because they generate colourless plaques when plated onto a lac Z-bacterial host on plates containing X-Gal. The vector can
Figure 17. Restriction map of bacteriophage χgt11. Genomic fragments of up to 7 kb are cloned into the unique Eco RI site in the promoter distal region of the lac Z gene. Coding sequences in the correct reading frame and orientation will be expressed to yield fusion peptides containing most of the β-galactosidase polypeptide chain at the N-terminus.

Figure 18. Scheme for construction of a recombinant library of P. chabaudi genomic DNA. (adapted from Young and Davies 1983). Fragments of P. chabaudi DNA digested with Eco RI were inserted into the unique Eco RI cloning site to produce a library of recombinant phage. The χgt11 phage plaques were then screened for expression of specific antigens using antisera to the PSA of P. chabaudi.

1. Transfer antigen to IPTG-saturated nitrocellulose
2. Probe filter with antibody to antigen
3. Probe position of first antibody using enzyme-linked second antibody
4. Develop with substrate
accommodate up to 7.2 kb of insert (Huynh et al. 1985).

DNA sequences inserted into the lac cloning site may be expressed as fusion proteins joined to β-galactosidase, under the control of the lac Z promoter. Therefore recombinant DNA libraries in this vector may be screened with antibodies as well as with nucleic acid probes, in order to isolate specific recombinant clones. Recombinants are screened in the form of phage plaques on a lawn of lon-protease deficient E. coli cells. Fusion proteins released by the lysis of cells within the plaques are immobilised on a nitrocellulose filter placed over the lawn, and are probed with antibody specific to the antigen of interest. Antibody can bind to any recombinant phage expressing all or part of the antigen of interest in the form of a fusion protein. The filter is probed in a second step with an enzyme-linked antibody, which reveals the position of the phage of interest. This can then be picked from the position on the lawn corresponding to the signal on the filter. Once recombinant phage expressing genes of interest have been isolated, the ability of lambda gt11 to form lysogens can be exploited to produce relatively large amounts of these proteins for further study.

5.4. Construction of a genomic expression library.

A genomic library of P. chabaudi (clone CB), was constructed in lambda gt11, by partial digestion of parasite DNA with the restriction enzyme EcoRI. This fragmented the genome into pieces with sticky ends, which were then ligated into the EcoRI site of lambda gt11 (figure 17).

In order to construct the library, it was desirable to
isolate parasite DNA, free of contaminating mouse host DNA. Parasites were purified from infected blood using a CF11 column and Saponin lysis as described in section 2.8.1. To assess the extent of contamination of parasite DNA by that of the host, serial dilutions of host and parasite DNA were probed with $^{32}$P labelled total mouse DNA in dot blots. Comparison of the signals from the two in figure 15A, shows that contamination of the parasite DNA with mouse sequences is less than 2%. (Page 52)

10μg of parasite DNA was subjected to partial digestion under conditions chosen to enhance EcoR1 "star" activity (Mayer, 1978), and aliquots were removed at timed intervals. Half of each aliquot was analysed by agarose gel electrophoresis to monitor the progress of the digest, while the other half of each sample was pooled. Figure 15B shows that as the digest progressed the size of the genomic DNA reduced. 10 and 50ng amounts of the pooled fractions were ligated with 0.5μg of EcoR1 digested lambda gt11, and packaged in vitro. 10ng of inserts produced the largest library of some 55,000 clones, of which 94% were recombinant. The library was then amplified as described in section 2.9.3. and used for screening.

5.5. Screening the library with anti-PSA mcabs.

In order to isolate the gene encoding the 230 KDa PSA, the library was screened with a cocktail of Mcabs directed against the PSA of P.chabaudi. These reacted with at least 8 different epitopes on the molecule (McLean, 1986), and could therefore be considered to be a polyclonal antiserum, specific to the PSA. When 2x10^5 recombinant phage from the
amplified library were screened with this cocktail, 22 putative positive plaques were obtained, and labelled pc.1 to pc.22. These were purified by three further rounds of screening, producing 16 clones which reproducibly gave a positive signal with the anti-PSA cocktail (figure 19). All of these had the same sized DNA insert (2.5 kb.), except pc.10 where the insert was 4.1 kb. This clone gave a distinctly weaker reaction with the antiserum than those of 2.5 kb. Pc.10 and two of the other clones (6 and 22) were analysed further.

5.6. **Generation of recombinant lysogens in Y1089.**

In order to characterise the fusion proteins produced by these three recombinant clones, lysogens of each were generated in **E.coli** Y1089 (section 2.9.6). In a lysogen, a single phage is integrated into the bacterial genome as a prophage. The prophage produces a temperature sensitive repressor (c1857), which is inactivated at 42°C. On temperature shift from 30 to 42°C, lysogens are induced to accumulate large quantities of phage transcription products in the absence of lysis. It is this property which allows lysogens containing recombinant phage to be exploited to maximise the yield of protein synthesised from transcripts of the phage DNA.

5.7. **Characterisation of the fusion proteins.**

In order to confirm that the clones pc.6, 10 and 22 were producing part of the 230 KDa PSA encoded by the parasite DNA insert, it was necessary to show that a fusion protein was produced which reacted both with the anti-230 KDa cocktail of monoclonal antibodies, and with anti-B-
Figure 19. Screening of recombinant phage clones using anti-230 PSA antiserum. Positive colonies from the primary screen were picked, plated at low density and probed again with the same antiserum. These colonies produced only background signal when the first antibody was omitted from the probing (data not shown).

Figure 20. Western blot of total protein extract from a lysogen of wild type lambda gt11 in Y1089, probed with anti-B-galactosidase antiserum. Samples of the cells were grown with and without the inducer IPTG (IND and N/IND cells), and aliquots removed at timed intervals (minutes). In both cases B-galactosidase accumulates, though to a greater extent in the induced cells due to induction of the lac Z promoter by IPTG. M.wt. markers are shown in track M in kDa. No B-galactosidase was detected when the anti-B-galactosidase antiserum was omitted from the probing (data not shown).

Figure 21. Western blot of total protein extracts from a lysogen of the recombinant clone pc.6. Samples of the cells were grown with and without the inducer IPTG (IND and N/IND cells), and aliquots removed at timed intervals (minutes). Identical blots were probed with the anti 230-PSA cocktail (panel A), or anti-B-galactosidase antiserum (panel B). In both cases a fusion protein of 170 kDa that is inducible with IPTG accumulates (arrowed). Control blots probed with no first antibody showed no reaction (data not shown). M.wt markers are shown in track M in kDa.
galactosidase antiserum. Furthermore the fusion protein should have a m.wt. greater than that of B-galactosidase alone (116 KDa), and should be inducible with IPTG, showing it to be under the control of the lac Z promoter.

Antigen preparations were made from lysogens of each clone as described in section 2.9.7. The cells were harvested and lysed at timed intervals after induction. The lysates were subjected to SDS-PAGE, blotted onto nitrocellulose and probed with the anti-230 PSA cocktail, or a rabbit antiserum raised against B-galactosidase (gift of Dr. R. Hall). Figure 20 shows the results obtained when a lysogen of wild-type lambda gt11 is treated in this way, and probed with the anti-B-galactosidase antiserum. Large amounts of B-galactosidase accumulate, seen as a strong band of m.wt. 116 KDa, due to the induction of synthesis by IPTG. In the same cells subjected to the temperature shift to 42°C, without induction, little of the protein was accumulated. The considerable background is presumably due to components in the antiserum directed against E. coli antigens.

Figure 21 shows the results from a similar experiment using pc.6. The results obtained with pc.22 were the same (data not shown). Identical samples of lysates were probed with the two antisera. In the induced samples from pc.6, there is a strongly reacting doublet of approximate m.wt. 170 KDa, which accumulates during the course of the induction. This doublet appears only weakly in cells uninduced with IPTG, indicating that its synthesis is controlled by the lac Z promoter. The doublet reacted with both anti-B-galactosidase and anti-230 KDa antisera. Similar samples
Figure 22. Western blot of total protein extracts from a lysogen of recombinant clone pc.10. Samples of the cells were grown with and without the inducer IPTG (IND and N/IND cells), and aliquots removed at timed intervals (minutes). Identical blots were probed with anti 230 PSA cocktail (panel A), or anti-β-galactosidase antiserum (panel B). In both cases a fusion protein of 125 kDa, inducible with IPTG, accumulates.

Figure 23. Autoradiograph of Southern blot probed with the 2.5 kb insert from clone pc.6. DNA from minipreps of recombinant clones pc.10, 6, and 22, and parasite genomic DNA were digested with EcoR1, electrophoresed and blotted. The pc.6 insert hybridises to the inserts of clones pc.10 and 22 (arrowed), and to the equivalent bands in parasite genomic DNA, as well as to an additional 3.5 kb fragment. No hybridisation was seen to mouse DNA (track M)
Fig 22

A

B

Fig 23
probed only with second antibody, showed no reaction at all. This protein doublet therefore satisfies the criteria for a bona fide fusion protein.

When lysates made from lysogens of pc.10 were probed, a protein of m.wt. 125 KDa accumulated over the course of induction, which reacted with the anti-B-galactosidase antiserum, as expected for a fusion protein (figure 22). However this protein also accumulates in uninduced cells, though in lower amounts than seen after IPTG induction. Identical samples probed with the anti-230 KDa antiserum reacted more weakly; however there is clearly a series of bands present in the induced cells, which are not detectable in the uninduced samples. The largest band of this series, (indicated by an arrow in figure 22A) is identical in m.wt. with the protein recognised by the anti-B-galactosidase antiserum, and probably represents the fusion protein. The lower m.wt. bands may occur due to degradation of this protein.

5.8. Genomic organisation of the clone pc.6.

In order to facilitate further studies, the insert from pc.6 was subcloned into the plasmid vector Puc18. The purified insert was then used to probe EcoRI digested parasite DNA. The results are shown in figure 23. The insert from pc.6 clearly hybridises to that contained in pc.10, indicating homology between these two inserts. As expected from this, the probe hybridises to fragments in the parasite DNA, corresponding in size to the pc.6 and pc.10 inserts (arrowed). However hybridisation is also seen to a 3.5 kb fragment in the parasite DNA indicating a third DNA
sequence homologous to the pc.6 probe. No hybridisation is seen to mouse DNA. The faint band at about 10 kb is probably due to partial digestion.

5.9. Discussion

5.9.1. Genomic expression library.

A genomic library of *P. chabaudi* clone CB was constructed in lambda gt11, by partial digestion of purified parasite DNA with EcoR1 "star" activity. This approach was chosen because of its technical simplicity, however it suffers from a number of drawbacks. In order for any parasite gene to be correctly expressed as protein by the vector, it must be cloned in the correct reading frame and orientation relative to the EcoR1 cloning site of the vector. For any random DNA insert produced by EcoR1 digestion, there is only a 1 in 6 chance of this. In addition, the EcoR1 recognition sites in and around any particular gene might generate DNA fragments larger than 7.2kb, in which case they would not be cloned at all.

In order to help to overcome these problems, the conditions of digestion of the parasite DNA were altered (section 2.9.1.) to produce a secondary activity of EcoR1, known as "star" activity. The results obtained are variable, but the major secondary recognition site is 5' -AATT- 3' (Mayer, 1978). These ends are ligatable into the EcoR1 cloning site of lambda gt11. This procedure was designed to produce a more random series of fragments than with a normal, complete EcoR1 digest, in order to increase the probability of producing a representative genomic library. The library produced consisted of 55,000 clones of which 94% were
recombinant.

It is possible to estimate the theoretical size required of a genomic library in order to be reasonably certain that all clonable sequences are represented, (Maniatis, 1981). The genome size, and the average size of the inserts in the library must be known. The genome size of P. falciparum has been estimated as between $2 \times 10^7$ and $3.8 \times 10^8$ bp (Goman et al., 1982; Hough-Evans and Howard, 1982). There are no accurate estimates for P. chabaudi, however Dore et al. 1980, have estimated the rodent malaria P. berghei has a genome of $2 \times 10^7$ bp.

The size of 8 randomly picked clone inserts was determined, ranging from 0.5 to 7 kb, giving an average of 4 kb. (data not shown). When these values of insert and genome size are used in the calculation, this method gives an estimate of $4 \times 10^4$ recombinants required, in order to produce a representative library. Therefore the library produced in this work should be large enough to contain all the sequences clonable by this strategy.

5.9.2. Isolation of clones encoding epitopes of the 230 PSA.

The expression library was screened with a mixture of mcabs against different epitopes of the 230 KDa PSA, and 16 clones isolated which reacted with this antiserum. These contained two sizes of insert of 4.1 and 2.5 kb. Lysogens of clones pc.6 and 22, containing the 2.5 kb insert, were found to produce a fusion protein of 170 KDa, suggesting some 50 KDa of parasite polypeptide was fused to the 116 KDa of B-galactosidase. Lysogens of pc.10 containing the 4.1 kb insert produced a smaller fusion protein of 125 KDa. Both of
these proteins appeared to be bona fide fusion proteins: they were larger than 116 KDa, reacted by blotting with both anti-230 PSA and anti-B-galactosidase antisera, and appeared to be under the control of the lac Z promoter, being inducible by IPTG. However the pc.10 fusion protein had some unusual features. It was surprisingly small considering that the insert is some 4.1 kb in length. Presumably the fusion protein contains only a small section of parasite polypeptide. In addition, although the protein is inducible by IPTG as expected for a fusion protein, it was transcribed at a higher rate than expected in uninduced cells. It is possible that the insert is somehow modifying the transcription of the lac Z gene to which it is fused. 5.9.3. Identity of the cloned inserts pc.6 and pc.10.

Having shown that the two cloned inserts encoded epitopes in common with the 230 KDa PSA, the question remained of whether these two inserts resulted from partial digestion of the same parasite gene, or encoded two different parasite antigens, with cross reacting epitopes shared with the 230 KDa PSA. The insert in pc.6 hybridised to that of pc.10, indicating that they shared homology. However pc.6 detected three DNA fragments in parasite DNA digested with EcoR1, two of which corresponded to the two cloned inserts. This proves that the two inserts are not overlapping sections of the same gene, but represent different pieces of parasite DNA, which cross-hybridise to one another, and to a third fragment.

How are these fragments arranged in the genome, and do any or all of them encode the 230 KDa PSA? The three
most likely possibilities are:

1. The 2.5, 3.5, and 4.1 kb fragments represent different regions of the same gene, with common regions of homology, the whole of which possibly encodes the 230 KDa PSA.

2. The three fragments may represent three copies of the 230 KDa PSA present in the genome, all of which might be functional, or some of which might be inactive copies of the functional gene.

3. The three fragments may encode different genes, with extensive regions of homology between them, which is responsible for the cross-hybridisation.

Possibility 1. can be excluded on the basis of data presented later in section 6.6.1. where sequences homologous to the insert pc.6 were found to be located on three separate chromosomes. Therefore it seems clear that the three DNA fragments detected by pc.6 represent three separate genes, which share homology. Two of these, cloned as pc.6 and pc.10 have been shown to encode epitopes shared with the 230 KDa PSA.
6. **Studies on *P. chabaudi* chromosomes.**

6.1. The genetic studies discussed in section 1.9. carried out with both the rodent malarias and *P. falciparum*, have shown that the genes determining variant forms of enzymes, antigens and other characters segregate and undergo reassortment in a typically Mendelian fashion (Walliker, 1983). This provides strong indirect evidence for the existence of a genome composed of several chromosomes.

Cytological studies have been hampered by the fact that condensed chromosomes have never been seen during the life cycle. Serial sections through parasite nuclei undergoing mitosis, analysed by electron microscopy have given estimates of 10 to 14 chromosomes per haploid genome (Prensier and Slomianni, 1987; Sinden, 1978). However these techniques offer little scope for further karyotype analysis.

The recently developed technique of Pulsed field gradient electrophoresis (PFGE), has permitted the separation of chromosome sized molecules in *P. falciparum*. Up to 14 distinct chromosomes have been identified, which range in size from 800 to 3500 kb. Homologous chromosomes can be polymorphic in size in different isolates (Wellems et al., 1987; Langsley et al., 1987). In this chapter work on *P. chabaudi* using the technique of PFGE is described.

6.2. **Pulsed Field Gradient Electrophoresis (PFGE).**

Conventional gel electrophoresis techniques for DNA analysis are limited to molecules of less than 30 kb. in size. However Schwartz and Cantor, (1984) have successfully separated molecules of up to several hundred kb. by the alternating application of two orthogonal electric fields.
Figure 24A. Ethidium bromide stained gel of 6 cloned isolates of *P. chabaudi*, denoted ER, AS, CB, AJ, 16 and EF. Gel run with pulse time of 80 seconds, to resolve seven smallest DNA bands, numbered as shown. Note that the smallest band contains two chromosomes, numbered 1 and 2. Large chromosomes migrate unresolved as compression zone (CZ).

Figure 24B. Southern blot of gel A probed with ribosomal RNA probe rib-1. Note hybridization to chromosomes 5, 6, 8 and to compression zone (containing chromosome 10).

Figure 25B. Ethidium bromide stained gel of *P. chabaudi* clone AS, run at pulse time of 200 seconds for 48 hrs. Increasing amounts of parasite material loaded on tracks 1-7. Note 10 separate chromosomes, numbered as shown. Tracks labelled Y show yeast chromosomes run as size markers, with approximate sizes in kb. as described by Carle and Olsen (1984).

Figure 25A. Southern blot of tracks 1-3 of gel shown in (B) hybridised to *P. berghei* subtelomere probe at low stringency. Note hybridisation to all ten ethidium bromide-stained fragments (most clear in track 1).

Figure 25C. Southern blot of tracks 6, 7 and Y of gel shown in (B) hybridised to *P. falciparum* ribosomal RNA gene rib-1 at low stringency. Note hybridisation specifically to chromosomes 5, 6 (individual chromosomes not distinct), 8 and 10 of *P. chabaudi*, and to yeast chromosomes.
PFGE has been successfully used to separate chromosomes from a variety of organisms, up to several million base pairs in the case of *Saccharomyces pombe*. In general the resolution achieved depends upon the length of the pulse applied to each electrode: the larger the chromosomes, the longer the pulse time required to resolve them.

6.3. **Preparation of DNA for PFGE.**

Large pieces of DNA in solution suffer breakage due to shearing. Several techniques have been devised to prepare large DNA molecules intact for PFGE. The method employed in this study involved suspension of intact live cells in molten agarose, which is then allowed to set in a block mould. Proteins are then removed by incubation with detergents and a protease (Kemp et al., 1985).

6.4. **Karyotype of *P. chabaudi.**

The six cloned isolates of *P. chabaudi* described in section 2.2.1., were examined by PFGE with a variety of pulse times and running conditions. This was necessary to resolve all the chromosomes of different sizes. Using a pulse time of 80 seconds six bands could be clearly resolved (fig. 24). Unresolved bands migrated as a compression zone near the top of the gel. As the pulse time was increased, more DNA bands were resolved. Using a pulse time of 200 seconds 10 bands were resolved, although under these conditions the smaller bands were less well separated (figure 25).

The degree of staining of the DNA bands with ethidium bromide indicated equal stochiometry of all the bands except the fastest moving band 1, and band 10. Under appropriate conditions other workers have resolved band 1 as a doublet.
(Dr.D. Arnot, personal communication). Bands 1 and 10 may therefore contain as yet unresolved components. Evidence is presented below to support the view that the DNA bands resolved by this technique represent intact chromosomes, so the 10 bands may represent at least 11 distinct chromosomes.

The sizes of the putative chromosomes were estimated by comparison with yeast chromosomes, subjected to electrophoresis at the same time (figure 25B). The sizes of the yeast chromosomes have been estimated (Carle and Olsen, 1985). Assuming that the Plasmodium chromosomes obey the same size/mobility relationship as those of yeast, the three smallest chromosomes of *P. chabaudi* were estimated to be 650, 800, and 900 kb.

6.5. **Chromosomal location of DNA sequences.**

Southern blots of PFGE gels can be probed with cloned DNA sequences, to determine their chromosomal location. cDNA and ribosomal gene probes for this work were kindly provided by Dr.G. Langsley of the Institut Pasteur, Paris. A telomere probe of *P. berghei* was kindly provided by Dr.C. Frontali, Rome.

6.5.1. **Telomere probe.**

This probe had previously been shown to cross-hybridise with *P. chabaudi* telomeres (Dore et al. 1986). Figure 25 shows that this sequence hybridised to each DNA band visible with ethidium bromide following PFGE, showing that each band possessed sequences homologous to the *P. berghei* telomeres. In addition the probe hybridised more intensively to bands 1 and 10, supporting the view that these contained more than one chromosome.
Table 1. Chromosomal location of a panel of *P. chabaudi* cDNA probes. Data produced in collaboration with Dr. G. Langsley of the Pasteur Institute, (Sharkey et al. 1988).

<table>
<thead>
<tr>
<th>P. chabaudi cDNA</th>
<th>Chromosome No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pc. 443</td>
<td>1/2</td>
</tr>
<tr>
<td>238</td>
<td>1/2</td>
</tr>
<tr>
<td>276</td>
<td>1/2</td>
</tr>
<tr>
<td>241</td>
<td>1/2</td>
</tr>
<tr>
<td>167</td>
<td>5</td>
</tr>
<tr>
<td>121</td>
<td>8</td>
</tr>
<tr>
<td>365</td>
<td>9</td>
</tr>
<tr>
<td>451</td>
<td>9</td>
</tr>
<tr>
<td>148</td>
<td>10</td>
</tr>
</tbody>
</table>

Additional sequences mapped during this project.

<table>
<thead>
<tr>
<th>rDNA</th>
<th>5, 6, 8, 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>pc.6</td>
<td>1/2, 5, 8</td>
</tr>
</tbody>
</table>
6.5.2. Location of ribosomal genes (rDNA).

The rDNA probe of *P. falciparum* was used to investigate the arrangement of these genes in *P. chabaudi*. There are four copies of rDNA in *P. berghei* (Dame and McCutchen, 1983). Figure 24B shows that this probe hybridised to chromosomes 5 and 6, and also to the unresolved chromosomes in the compression zone. A similarly probed gel run at a longer pulse time to resolve these chromosomes, shows that in fact it also hybridises to chromosomes 8 and 10, (figure 25C).

The degree of hybridisation to each chromosome indicates that there are the same number of copies of rDNA on each. Southern blots of restricted genomic DNA, reveal four EcoR1 fragments, indicating a copy number of four, as in *P. berghei*.

6.5.3. cDNA and other probes.

Figure 26 shows a pulsed field gel blotted and probed with the cDNA probe pc.443. This single copy gene probe clearly hybridises to band 1, and is therefore located on chromosomes 1 or 2. Other probes have been assigned unequivocally to particular chromosomes. This data is summarised in table 3. Note that the probe pc.6 isolated during this study, and possibly encoding the 230 KDa PSA hybridised to three chromosomes.

6.6. Chromosome polymorphism in cloned *P. chabaudi* lines.

The finding that certain cloned gene sequences are located on specific chromosomes allows them to be used as chromosome markers. This provides a means of identifying homologous chromosomes in different isolates. For instance in figure 24, the rDNA probe is shown hybridising to chromosomes 5 and 6 in each strain, which clearly shows that
Figure 26A. Ethidium bromide-stained gel of chromosomes of cloned isolates of *P. chabaudi* separated at a 75 second pulse time.

Figure 26B. Southern blot of gel A hybridised with chromosome band 1-specific probe (cDNA 443). Note hybridization to band 1 (contains chromosomes 1 and 2), and to slot; the latter is thought to be due to chromosomal material trapped in the slot.

Figure 27. Ethidium bromide-stained gel of clones AS and CB and of six clones derived from the progeny of the cross. Note differences in size of chromosomes 4 and 5 in AS and CB. Progeny clone 8 has chromosome 4 of type CB and chromosome 5 of type AS.
these chromosomes vary in size between the strains.

6.7. Analysis of a cross between P.chabaudi clones.

In order to investigate the behaviour of the chromosomes during meiosis, the progeny of a cross between lines CB and AS were analysed. The progeny and parent lines of this cross were kindly made available by Dr. A. McLean. The detailed procedure for making the cross is illustrated in figure 3. Six cloned progeny lines were chosen for analysis by PFGE, because analysis of parental enzyme markers indicated that they resulted from cross fertilisation between gametes from the two parent lines, rather than self-fertilisation (McLean, 1986).

The two parent lines were chosen because in addition to differences in characters such as antigens and isoenzymes which could be used to identify recombinant progeny, they also differed in their karyotypes by PFGE. In AS, chromosome 4 was larger than in CB, while chromosome 5 was smaller (fig. 24). The polymorphisms between the parental chromosomes 4 and 5, allow the segregation of these chromosomes to be followed in recombinant progeny.

Each parent clone was independently transmitted through mosquitoes and analysed by PFGE. The uncloned progeny of each self-fertilisation appeared identical to each parent's initial karyotype. No new DNA bands were visible (data not shown), indicating that they are stable in size by PFGE.

The karyotypes of the six progeny clones resulting from crossing between the two parent lines are shown in figure 27. Three clones (1, 6, and 10), appeared to have
inherited chromosomes 4 and 5 from the AS parent, while clones 3 and 9 inherited them from the CB parent. Clone 8, however inherited chromosome 4 from CB and chromosome 5 from AS. These results indicate that the DNA bands revealed by PFGE, are stably inherited during meiosis, and show that they undergo normal mendelian assortment. Since the blood forms of malaria parasites are known to be haploid (Walliker, 1983), only one chromosome of each homologous pair will appear in any progeny clone.


The long term stability of the first 8 chromosomes of clone CB was investigated both during meiosis and mitosis, in order to see whether novel sized chromosomes were generated. Two CB lines were set up; one was continuously blood passaged for 8 months, the other was repeatedly transmitted through mosquitoes for the same period. the karyotypes of both lines remained the same throughout the experiment (data not shown).

6.9.1. Discussion.

This study has shown that the genome of *P. chabaudi* can be resolved into at least 10 large DNA bands by the technique of PFGE. These are believed to represent intact chromosomes. Their sizes by comparison with yeast marker chromosomes, appeared to range from 650 kb up to 3 Mb. The basis of differences in mobility of the chromosomes in PFGE is not certain. It is presumed that they differ considerably in size, and this is certainly true for yeast (Carle and Olsen, 1985), however it is possible that other factors such as tertiary structure of the DNA may affect their mobility in the gel.
The interpretation that the bands are intact chromosomes is supported by the fact that they all contain sequences homologous to the *P. berghei* telomere probe. This has been shown to recognise *P. chabaudi* telomeres (Dore et al. 1986). In addition, during a cross between the cloned lines AS and CB, which differ in the sizes of chromosomes 4 and 5, the alternative forms of these chromosomes segregated independently.

The precise number of chromosomes in the genome of *P. chabaudi* is not certain, because some DNA bands, such as 1 and 10, may represent more than one comigrating chromosome. These bands hybridise more strongly to the telomere probe than the others, suggesting a chromosome number of at least 12. In a similar study Langsley et al. (1987) suggest up to 14 chromosomes may occur in *P. chabaudi*.

6.9.2. **Chromosome-specific probes.**

The location of a number of different gene probes was determined by probing Southern blots of separated chromosomes. This work was carried out in collaboration with Dr. G. Langsley. Probes which recognise five different chromosomes were identified. No probes specific for chromosomes 3, 4, 6 or 7, have yet been found, however this method should allow further expansion of the linkage map to these chromosomes, as more probes are mapped. (Table 3)

6.9.3. **Location and copy number of the rDNA genes.**

These genes were localised using a *P. falciparum* rDNA probe, on chromosomes 5, 6, 8, and 9, with apparently the same copy number on each. The copy number is probably four since only four bands are seen on probing Southern blots of EcoR1.
digested parasite DNA with this sequence (data not shown). This is similar to \textit{P.berghei}, which has four copies of rDNA which appear to be unlinked (Dame and McCutchen, 1983). \textit{P.falciparum} has seven or eight copies located on chromosomes 1, 5, 6, 11, and 13 (Wellems \textit{et al.} 1987).

The copy number of ribosomal genes in \textit{Plasmodia} is unusually low, and in \textit{P.berghei}, \textit{P.falciparum}, and now in \textit{P.chabaudi} the genes appear to be located on different chromosomes. This is untypical of eukaryotes where commonly there are many ribosomal genes, tandemly repeated (Long and Dawid, 1980). In \textit{P.berghei}, one of the four rDNA's is not active in asexual parasites, and and may be under different transcriptional control (Dame and McCutchen, 1984). The arrangement and copy number of these genes in \textit{P.chabaudi} appears similar, and it is possible that transcriptional differences like those in \textit{P.berghei}, may operate.

6.9.4. \textbf{Chromosome Polymorphism.}

The cDNA and rDNA sequences were used as chromosome-specific markers, to identify homologous chromosomes in different isolates. This has proved particularly valuable in \textit{P.falciparum}, where homologous chromosomes vary in size to such an extent that their relative order of separation with PFGE may differ between isolates (Kemp \textit{et al.} 1987). The probes showed that as in \textit{P.falciparum}, differences in size did occur between homologous chromosomes.

The possibility that novel sized chromosomes might arise during meiosis was investigated, by analysis of recombinant progeny of a cross between lines AS and CB. No new chromosomes were seen. The two parent lines also
underwent self-fertilisation and appeared unaltered. However in this case the progeny were uncloned. It is possible that if a small proportion of the progeny of self or cross-fertilisation exhibited new sized chromosomes, these would not be detected. Although no novel sized chromosomes were detected in the cloned progeny of the cross, the polymorphism between parental chromosomes 4 and 5 allowed their segregation to be followed in the recombinant progeny. These chromosomes clearly showed independent segregation.

These results are in marked contrast to the findings in *P. falciparum*, where over 50% of progeny clones, known to be recombinant by enzyme markers, exhibited novel sized chromosomes (Walliker et.al.1987). Sinnis and Wellems (Genomics, in press), showed that recombination between chromosome 4 of the two parents (1300 and 1520 kb in size respectively), resulted in the generation of a 1400 kb chromosome 4 in one progeny clone. Such large size differences can be clearly resolved by PFGE. Chromosomes 4 and 5 in the *P.chabaudi* cross differed in size by only 20 to 30 kb. Progeny with an intermediate sized chromosome would be very difficult to detect, given the resolution of PFGE with chromosomes of this size.

The chromosome polymorphism found among *P.chabaudi* isolates from the wild indicates that chromosome size variations are common. If they are generated by similar mechanisms to those operating in *P.falciparum*, the results of the cross suggest that the degree of variation in size, and possibly the rate at which novel chromosomes are generated, is lower in *P.chabaudi*. 
7.1. Discussion

The work described in the previous chapters has examined the extent of genetic variation between several cloned lines of *P. chabaudi*. The overall aim of this characterisation has been to develop this species as a suitable model system to investigate some of the questions raised by recent research on vaccine development in the human malarials. This work has been described in chapter 1. One of the key findings to emerge is that variation exists between cloned lines and within the general parasite population, and that this variation represents a major obstacle both in the development of malarial immunity, and an effective vaccine.

The cloning and comparison of variable parasite antigens such as the PSA, and CSP have indicated that different mechanisms may be responsible for generating the variation in different antigens. Antigens such as the S-antigen possess tandem arrays of short repeats within the molecule, that are highly immunogenic. Variation in the number and sequence of these repeats between alleles is responsible for their serological diversity, and is reflected in isoelectric point and m.wt. differences. In other antigens such as the PSA, variation is due to blocks of sequence which differ between alleles, and these are interspersed between common regions.

These findings have provoked a host of speculation regarding the possible mechanisms by which such variation is brought about. More importantly, does this variation delay the acquisition of effective immunity, and if so is the
variation in some antigens more important than others in this respect? It seems unlikely that an effective vaccine will be developed in the absence of answers to these questions.

It is the intention of this discussion to examine critically how the analysis of variation on *P.chabaudi* relates to previous work in *P.falciparum*. This is in order to establish whether similar genetic mechanisms may operate in the two species, and hence whether *P.chabaudi* might prove a suitable model system for investigating genetic mechanisms relevant to vaccine development in *P.falciparum*.

7.2. **Protein variation detected by 2D electrophoresis.**

The examination of protein variation between six cloned lines of *P.chabaudi*, showed that the majority of proteins were invariant, by this method. However a number of the proteins did vary in m.wt and isoelectric point between the clones. This was similar to the situation found in *P.falciparum* when cloned lines were compared in this way. In *P.falciparum* the variants were found as alternatives, each strain possessing only a single variant of a particular protein. These have been shown to represent alternative alleles of the same genetic locus, since they were found to segregate independently during meiosis as alleles. (Fenton, in preparation). Peptide digest studies of each form of the variant proteins have shown that they exhibit similar peptide profiles (Fenton et al. 1987) There is therefore, in *P.falciparum* very strong evidence that the variants of a particular protein spot revealed by 2D electrophoresis, represent allelic variants of the same genetic locus.

In *P.chabaudi*, the initial finding of variant proteins
in 2D gels suggested that in this species too, a number of proteins might have allelic variants differing in electrophoretic characters. This had already been shown for the 230 KDa antigen, where the allele of strain AS was known to be some 5 KDa larger than in CB (Newbold et al. 1984).

7.3. Direct demonstration that 2D variants are alleles.

In order to identify particular proteins with alleles varying in m.wt., the six lines were screened by Western blotting with a panel of mcabs, and three different antigens identified, in addition to the 230 KDa protein. All of these showed m.wt. variation between alleles from different strains. When these antigens were immunoprecipitated and compared with the 2D composite map, two of them (42 KDa and 105 KDa), were found to correspond to proteins 78 and 16 respectively. Moreover protein 16 had been identified as a variant protein in the initial 2D study. It was concluded that the antigen recognised by mcab S-10, was represented on the 2D composite map of *P. chabaudi* by protein 16.

In a similar way, protein 86 of the composite map was identified as the parasite enzyme ADA. Variant alleles of this enzyme detected by starch gel electrophoresis, corresponded to variants of protein 86, as detected by 2D electrophoresis.

These results support the view that the variant proteins identified by 2D PAGE of *P. chabaudi*, do indeed represent variant alleles. In order to confirm this for each variant protein, genetic studies to show segregation of the variants during meiosis would be necessary, as described for *P. falciparum*. Such a study has been carried out in
P. chabaudi for the 230 KDa PSA antigen (McLean, 1986). Using mcabs specific for each allele, she showed that as expected for alleles, they segregated independently at meiosis. The variant alleles identified in this study would be expected to behave in the same way.

7.4. **Extent of genetic diversity between the cloned lines.**

The 2D composite map of the six lines (figure 6A), has shown that each has a unique combination of variant proteins, presumably reflecting a unique genotype. Previous studies to determine the extent of polymorphism in P. chabaudi isolates used electrophoretic variants of enzymes. Of five enzymes tested, all but one showed extensive variation in the population (Lainson, 1983a). It was possible to clearly distinguish the subspecies P. chabaudi (Cameroon), P. c. chabaudi (CAR), and P. c. adami (Congo). Though geographically distinct, it was established that these subspecies could interbreed. The 2D polymorphisms appear to be distributed in a similar way. The P. c. adami line DS, classified by Lainson as a subspecies, was found in this study to have several unique 2D variants. Despite the limitations of examining only a few cloned lines, it seems reasonable to conclude that though there is considerable polymorphism in the population, some proteins probably have variants specific to particular regions. This situation appears very similar to the distribution of variation in the population of P. falciparum. 2D PAGE has shown that there are detectable regional differences within the population, though these may be imposed on a background pattern of intraspecific diversity (Tait, 1981; Walker, 1985).
Overall the 2D PAGE results obtained with *P. falciparum* (Fenton et al. 1985) and in this study with *P. chabaudi* are very similar. Some proteins have alleles differing in isoelectric point or m.wt. alone, in others both vary simultaneously. The type of variation is comparable: the majority of alleles differ only slightly in these characters. However in some cases, as in the S-antigens of *P. falciparum* variation is more extreme, and every strain examined so far has a different allele, varying in m.wt. from 120 to 250 KDa. Protein 2 in *P. chabaudi* varies in a similar way. Polymorphism was detected in 13% of proteins in *P. chabaudi*, compared with 15% in *P. falciparum* (Walker, 1985).

7.5.1. Variant antigens.

A panel of mcabs were used to screen the six *P. chabaudi* strains by western blotting, to identify the target antigen of the mcabs, and to determine whether these antigens varied in m.wt. between the strains. 9 mcabs reacted successfully, although five of them probably recognised the same 230 KDa antigen. Three other parasite antigens were identified of 85 KDa (mcab 12.1), 42 KDa (mcab 12.18), and 102 KDa (mcabs S-10 and S-20). Surprisingly all these antigens possessed alleles which varied in m.wt. One possibility is that these variant proteins are highly immunogenic in comparison with the majority of parasite proteins, and the immune response is biased towards such proteins. This is illustrated by the fact that 5 out of 9 mcabs characterised reacted with the 230 KDa protein.

7.5.2. Previously identified antigens.

The question arises of whether any of the novel
antigens identified in this study correspond to previously characterised antigens, and also whether these represent analogues of antigens identified in *P. falciparum*. Two groups have recently produced mcabs to antigens in the membrane of erythrocytes infected with *P. chabaudi*, of m.wt. 105 and 96 KDa (Gabriel *et al.* 1986; Wanidworanun 1987). Subsequently mcabs to the 105 KDa antigen of one strain of *P. chabaudi*, have been shown to precipitate the 96 KDa antigen from the strain used by the other workers. This suggests that the 96 KDa antigen (pc.96), is in fact the same as the 105 KDa antigen (pc.105). The antigen therefore appears to differ in m.wt. between the two strains of parasite used in each laboratory. This antigen may represent the analogue of the 155 KDa RESA antigen of *P. falciparum*. Wanidworanun *et al.* 1987, report that mcabs to the pc.105 produce a RESA type fluorescence with *P. falciparum*, and recognise a 155KDa antigen in Western blots.

In considering these results, it is possible that the 102 KDa antigen identified in this study by mcabs S-10 and S-20 may be the same as the 96 (or 105) KDa antigen described above:

1. Both antigens produce a similar pattern by IFA, and have similar stage specificity.
2. These antigens are soluble and heat stable.
3. In both cases mcabs to the antigens react with a number of smaller polypeptides, and appear to undergo extensive processing.
4. Both the antigens appear to vary in m.wt. between strains. In this study, the 102 KDa antigen was found to
vary from 97 to 105 KDa, a range which could encompass the 96 and 105 KDa variants identified by Wanidworanun et al. 1987.

This evidence suggests indirectly that the 102 KDa antigen identified in this study, may be the same as the pc.96 or 105 KDa antigen. This could be confirmed directly by exchange of mcab reagents between our laboratories.

7.5.3. **Identity of other variant proteins.**

Variant protein 86 of the 2D map, was identified as the enzyme ADA. This enzyme had previously been shown to be highly polymorphic, with 4 alloenzyme types detectable by starch gel electrophoresis (Lainson, 1983b). In fact 5 alloenzymes were detected by 2D electrophoresis in the six lines studied. Of particular interest was the fact that some of these alleles varied in m.wt. Previously described polymorphism in the ADA of humans and *P. falciparum* have only involved alterations in isoelectric point, (Fenton and Walliker, 1988; Akeson et al. 1987). Recently a number of alleles of the human ADA gene have been cloned and sequenced. In all these cases, the underlying cause of the isoelectric point changes was found to be point mutations (Akeson et al. 1987). In the case of *P. chabaudi* the structural basis of the changes in relative mobility is unknown, nor is it known whether these changes affect the enzyme's activity. The apparent m.wt changes may result from significant changes in the ADA coding sequences. However point mutations can also result in changes in the relative mobilities of proteins on SDS gels, (Vandekerckhove et al. 1980). It would be interesting to clone the gene encoding this enzyme from the
This could be done either by raising antiserum against the purified *P. chabaudi* ADA, or possibly by probing with the human ADA gene probe. Comparison of the gene sequence from different strains, coupled with measurement of the specific activity of the alloenzymes could resolve the question of why these alleles show apparent variation by up to 10% in m.wt. and whether they retain full function.

7.6. **Cloning of polypeptides carrying epitopes of the 230 PSA.**

Although this study has identified several proteins with alleles varying in m.wt., the molecular basis of this size polymorphism is unknown. The genes encoding alleles of variant antigens have been cloned and compared in *P. falciparum* and the simian malarias, and the molecular basis elucidated, however little comparable molecular cloning has been carried out in the rodent malarias. As a first step towards investigating the molecular basis of the size variation identified in the alleles of several proteins in this study, a genomic expression library was constructed in the vector lambda gt11. Two clones specifying fusion proteins, which reacted with mcabs against the 230 KDa PSA, which has variant alleles, were successfully isolated from this library. These parasite DNA fragments of 2.5 and 4.1 kb crosshybridise with one another, and with a third 3.5 kb fragment in the parasite genome. It seems unlikely that these represent different portions of the 230 KDa gene, since they are probably located on three separate chromosomes (Table 3). Other possibilities are that the inserts pc.6 and
pc.10 represent duplicate copies of the same gene, possibly the 230 KDa PSA, or that they encode different antigens, which share homology. In order to resolve this it will be necessary to compare the DNA sequences of the two inserts. This work is underway in collaboration with Dr. W. Deleersnijder, Brussels.

Finally it will be necessary to confirm whether either of the two recombinants actually encodes the PSA of *P. chabaudi*. This could be done by methods such as antibody select (Hall et al. 1983), or by immunisation of mice with the fusion protein, followed by immunoprecipitation of the parent protein from parasite extracts (Coppel et al. 1983).

7.7.1. Genome organisation of *P. chabaudi*.

This study has shown that the technique of PFGE is able to resolve the genome of *P. chabaudi* into at least 10 separate chromosomes. Other workers suggest that the genome may consist of up to 14 chromosomes in total, varying in size from 650 to 3500 kb. In collaboration with Dr. G. Langsley (Institut Pasteur), a number of gene probes were mapped to particular chromosomes to produce a primitive linkage map, (Sharkey et al. 1987). In particular *P. chabaudi* was shown to possess four gene sequences homologous to the rDNA genes of *P. falciparum*, located on chromosomes 5, 6, 8 and 10. The karyotypes of the same six lines of *P. chabaudi* used in the 2D study, showed that homologous chromosomes can exhibit considerable size polymorphism between isolates.

The genomes of *P. falciparum* and *P. chabaudi* appear similarly organised. Most obviously *P. falciparum* also has 14 chromosomes, though the average size of these is larger than
in \textit{P.chabaudi}. Presumably this reflects the larger genome size of \textit{P.falciparum} (Hough-Evans and Howard, 1982). The chromosomes of \textit{P.falciparum} also exhibit size polymorphism. The extent of this seems greater than in \textit{P.chabaudi}, however this may reflect the fact that a greater number of strains, from a wider range of locations have been analysed. The \textit{P.chabaudi} studies need to be extended to more isolates from other areas, to confirm this point.

7.7.2. \textbf{Generation of novel sized chromosomes in \textit{P.chabaudi}}.

The results with \textit{P.chabaudi} in this thesis appear to differ from \textit{P.falciparum} in several respects. Where there are size differences between homologous chromosomes in different isolates of \textit{P.chabaudi}, these are not as great as in \textit{P.falciparum}. Secondly, homologous chromosomes segregate independently during meiosis, and are inherited stably in a simple Mendelian fashion. In contrast, many of the progeny of meiosis in \textit{P.falciparum} exhibit novel chromosomes, unlike either parent.

Detailed analysis of the \textit{P.falciparum} cross progeny, has suggested at least three mechanisms which may give rise to novel sized chromosomes. Sinnis and Wellems (Genomics, in press), have shown that during meiosis homologous recombination between chromosomes which already differ in size, can generate a recombinant chromosome intermediate in size from either parent chromosome. Corcoran et al. 1988 have shown that the generation of novel sized chromosomes may also involve unequal crossing over between blocks of tandemly repeated rep20 sequences located near the telomeres. In addition they have shown that deletions of both coding and
non-coding sequences may occur during mitosis. They suggest that this might involve recombination between the subtelomeric rep20 and homologous sequences located internally within the chromosome.

Both of the studies cited above concluded that most of the size differences between homologous chromosomes were due to variation in sequences near the ends of the chromosomes, such as rep20. The large proportion of progeny with novel sized chromosomes in the *P. falciparum* cross, may be due to variations in the amounts of subtelomeric sequences such as rep20, due to one of the mechanisms described above.

These ideas may help to explain the apparent differences between *P. chabaudi* and *P. falciparum*. If differences in sequences located near the telomeres is largely responsible for size variation in *P. falciparum*, it may be that *P. chabaudi* lacks such sequences, or they are fewer in number. This would reduce the opportunities for unequal recombination in these regions, leading to a lower rate of generation of novel chromosomes during meiosis. This could be investigated by long range restriction mapping of homologous chromosomes of different size in *P. chabaudi*. In particular, this could show whether size variation occurs mainly due to sequences in the subtelomeric region, and what sequences are involved.

Pologe and Ravetch(1986), showed that genome rearrangements could be associated with phenotypic changes in *P. falciparum* (in this case the loss of knobs). However in both *P. falciparum* and *P. chabaudi*, it is not clear whether genome diversity, such as chromosome polymorphisms are
related in any way to the phenotypic diversity, which is so relevant to vaccine development.

7.8. **Conclusions.**

1. The work described in this thesis has examined the extent of genetic variation between several cloned lines of *P. chabaudi*. Variation in the organisation of the genome between the strains was examined using the technique of PFGE. 12 DNA fragments were visualised for the first time in this species, though the true chromosome number may be 14. The chromosomes were found to segregate independently during meiosis, and were relatively stable in size.

Although *P. falciparum* also appears to have 14 chromosomes, comparable gene sequences such as the ribosomal genes (rDNA) are not organised on the same chromosomes in the two species. Expansion of the linkage map of both species can be expected to proceed rapidly, and as additional genes common to both are mapped it should be possible to say whether regions of synteny occur. This may provide some insight into the mechanisms by which the genomes of the two species have evolved.

2. The two species appear to differ in the degree of chromosome stability during meiosis. In *P. chabaudi*, novel sized chromosomes were not detected during crosses, unlike in *P. falciparum*, though it is possible that the two systems differed in the degree of selection against parasites carrying novel chromosomes. Long range restriction mapping studies in *P. falciparum* have suggested that recombination events in repetitive DNA sequences located near telomeres may be responsible for these size changes. *P. chabaudi* may simply
possess less repetitive sequence in these regions. This could be easily tested by restriction mapping homologous chromosomes of different sizes, to determine which regions are responsible for the variation.

3. Protein variation between the *P. chabaudi* lines was examined using 2D PAGE. This showed that a number of proteins vary in relative mobility and isoelectric point between the strains. Some of these variant proteins are believed to represent allelic variants of the same genetic locus. This has been confirmed for two of these proteins, one of which was identified as the enzyme ADA.

4. The protein variation detected by 2D PAGE is qualitatively and quantitatively very similar to the results of more extensive studies in *P. falciparum*. Most importantly, the overall extent and nature of the polymorphism exhibited by particular proteins, suggests that similar genetic mechanisms may operate to generate and maintain variation in the two species.

5. In order to investigate the molecular basis of the size variation between alleles, a genomic expression library of the strain CB was constructed in the vector lambda gt11. Two different clones encoding epitopes common to the 230 KDa PSA were obtained, by screening this library with mcabs to this protein. The two genes share homology with one another and a third, as yet uncloned sequence in the parasite genome. These may represent duplicate copies of the gene encoding the 230 KDa PSA. Alternatively they may encode different antigens, with common epitopes, only one of which may be the PSA. Should one of these encode the PSA, it could be used as
a probe to isolate the corresponding alleles from genomic libraries of other strains. Comparison of the DNA sequences would reveal the molecular basis of the apparent size variation between them. It would be particularly interesting to compare the pattern of variation of the 230 KDa PSA of *P. chabaudi*, with that of its known analogue, the 190 KDa PSA of *P. falciparum*. This would provide a useful test of whether further work on *P. chabaudi* antigens could have predictive value for the *P. falciparum* analogues.

7.9. **Future work.**

Variation in antigens and the mechanisms responsible for it are currently of central importance in malaria research, since the vaccination studies discussed earlier, indicate that diversity in antigens represents a major obstacle to the development of an effective vaccine.

Two major problems therefore face workers attempting to develop a vaccine. Firstly the identification of conserved antigenic determinants that are non-repetitive, and are capable of inducing host protective responses. Secondly genetic studies are necessary to determine the extent to which any candidate antigen might vary under the selective pressure inherent in its use as a vaccine. This might occur due to novel mutations or selection of variants already in the population.

In the first case, a major experimental problem derives from the number of antigens that need to be assessed for vaccine potential. It will be impossible to carry out adequate monkey trials for the many candidate antigens for a *P. falciparum* vaccine, due to the lack of suitable monkeys,
and the ethical problems inherent in their use. These considerations also limit the prospects of future genetic work. There is therefore a compelling need to develop an alternative parasite/host model for the initial assessment of potential antigens, and further genetic studies.

With the establishment of a genomic expression library, and the identification of variant antigens, some of which may be analogues to known \textit{P. falciparum} vaccine candidates, the molecular tools now exist to exploit the natural potential of the \textit{P. chabaudi}/rodent system to tackle the problems of vaccine development outlined above. These, coupled with the analysis of the genome by PFGE, will also allow fuller investigation of the basic genetic mechanisms of the malaria parasite. It does seem likely that mechanisms such as unequal recombination are involved in genomic rearrangements. This process may also be involved in the evolution of some antigen genes, such as the CSP and PSA discussed earlier. Therefore the two phenomena: chromosome size variation, and rapid evolution of repeats in antigens, may be manifestations of the same process of non-homologous recombination; in one case between different regions of the chromosome, in the other, within the gene itself.
References


Cowman, A.F., Sam, R.B., Coppel, R.L., Brown, G.V., Anders, R.F. and


Lainson, F.A. 1983(b) Genetic studies of the population structure of rodent malaria parasites. Ph.D. Thesis


### Table characterising the set of major reproducible 2-D proteins of Plasmodium chabaudi line CB.

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Chromosome size variation in the malaria parasite of rodents, *Plasmodium chabaudi*

Andrew Sharkey¹, Gordon Langsley², Jintana Patarapotikul³, Odile Mercereau-Puijalon², Ann P. McLean¹ and David Walliker¹

¹Department of Genetics, University of Edinburgh, Edinburgh, U.K.; ²Unit of Experimental Parasitology, Institut Pasteur, Paris, France; and ³Department of Parasitology, CHU Pitié-Salpêtrière, Paris, France

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Pulsed field gradient gel electrophoresis has been used to identify at least 10 large DNA fragments in the genome of the rodent malaria species *Plasmodium chabaudi*. The fragments range in size from approximately 650 to 5000 kb. All the fragments contain sequences homologous to a *P. berghei* telomere probe, suggesting that they represent intact chromosomes. Ribosomal RNA genes and *P. chabaudi* cDNA sequences have been mapped to specific fragments. The fragments vary in size in different cloned isolates of the parasite. In a cross between two cloned parasites differing in the sizes of chromosomes 4 and 5, independent segregation of each chromosome occurred during meiosis.

Key words: Chromosome polymorphism; Malaria; *Plasmodium chabaudi*; Pulsed field gradient gel electrophoresis

Introduction

Malaria parasites exhibit diverse forms of characters such as drug-sensitivity and antigens, and this variation is providing an obstacle to control of the disease by chemotherapy or vaccination. Studies on the rodent malaria species *Plasmodium yoelii* and *P. chabaudi*, and more recently on the human parasite *P. falciparum*, have shown that the blood forms possess a haploid genome, and that cross-fertilization between gametes followed by meiosis occurs in mosquitoes [1–3]. The genes determining variant forms of enzymes and other characters have been shown to segregate and undergo reassortment in a typically Mendelian fashion.

The independent assortment of genetically determined characters provides strong evidence for the existence of a genome composed of several chromosomes. However, condensed chromosomes have not been convincingly demonstrated at any stage of the life-cycle, and the limited genetic studies carried out so far have provided no evidence of genetic linkage. Recently, the technique of pulsed field gradient gel electrophoresis (PFG) has allowed the separation of up to 14 distinct DNA fragments of large size in *P. falciparum* which are considered to be chromosomes [4–8]. The chromosomes are found to be polymorphic in size in different isolates. It has been shown, further, that in some instances deletions might be responsible for the changes in size, and that these could involve either repetitive DNA [5] or coding sequences [9,10]. In *P. falciparum*, chromosome size changes have been shown to occur frequently following cross-fertilization between cloned parasite lines in mosquitoes [2].

In this study, we use PFG to examine chromosomal organization of the rodent malaria species *P. chabaudi*. In preliminary work with this...
technique [6], it has been shown that the genome of this species appears to contain up to 14 chromosomes. We show that, as in *P. falciparum*, considerable chromosome polymorphism occurs in different parasite isolates. The chromosomal locations of ribosomal RNA genes and of certain *P. chabaudi* cDNA sequences are established. These are used as chromosome-specific markers to show that homologous chromosomes may vary in size in different cloned isolates. All the fragments also possess telomeres.

**Materials and Methods**

**Parasites.** The *P. chabaudi* clones were from a collection maintained in the World Health Organization Registry of Standard Strains of Malaria Parasites in the Edinburgh University Genetics Department. Routine maintenance of the parasite in mice and mosquitoes (*Anopheles stephensi*), and details of the crossing procedure and cloning of blood forms by limiting dilution are described elsewhere [11].

**Parasite characterization.** Parasites were characterized for pyrimethamine-response by treating infected mice with the drug at 20 mg kg⁻¹ daily for four days [11]. Parasites which survived this treatment were classified as resistant, and those which were eliminated as sensitive. Electrophoretic forms of the enzymes lactate dehydrogenase (LDH, EC 1.1.1.27) and 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.43) were detected by the methods of Walliker et al. [11] and of adenosine deaminase (ADA, EC 3.5.4.4) by the method of Sanderson et al. [12]. Antigen typing of the 250 kDa antigen of *P. chabaudi* schizonts using monoclonal antibody 3.7 was by indirect immunofluorescence, using the method of Boyle et al. [13].

**Pulsed field gradient gel electrophoresis.** Parasites were prepared for electrophoresis as described by Kemp et al. [4] and Van der Ploeg et al. [5]. Parasitised blood was taken from mice, and leucocytes removed by passage over a CF11 cellulose powder column [14]. The parasites were freed from erythrocytes by saponin lysis [15], washed twice in phosphate buffered saline, pH 7.4, and embedded in 1% low gelling temperature agarose at an approximate density of 5 × 10⁸ parasites ml⁻¹. The agarose blocks were incubated in 0.5 EDTA/0.01 M Tris/1% sarcosyl/2 mg ml⁻¹ proteinase K at 42°C for 48 h. Blocks were then stored in this solution at 4°C until used for PFG.

Two types of PFG apparatus were used, one similar to that described by Carle and Olsen [16], and the second the LKB Pulsaphor apparatus. The Carle and Olsen apparatus was used with 0.5 × Tris/borate/EDTA (TBE) buffer and a 1.5% agarose (Sigma type 2) gel, and was run at 280 V for 22 h at 13°C, with a pulse time of 80 s. The Pulsaphor apparatus was used with 0.5 × TBE, a 1% agarose gel, at 300 V for 48 h at 8°C, with a pulse time of 200 s. After running, gels were stained in 0.5 × TBE containing ethidium bromide (50 μg ml⁻¹) for 1 h, then destained in 0.5 × TBE for 1 h and photographed. DNA of *Saccharomyces cerevisiae* strain D273 was used to estimate the size of the *Plasmodium* DNA bands.

**Southern blotting.** Transfer of DNA to nitrocelullose was performed as described by Southern [17], except that gels were treated with 0.25 M HCl for 25 min prior to denaturation. After hybridization at 59°C, the blots were washed at low stringency in 2 × SSC (1 × SSC = 150 mM NaCl/15 mM sodium citrate), 0.1% sodium dodecyl sulfate (SDS), again at 59°C. The *P. falciparum* ribosomal gene probe (rib.1) was that described by Langsley et al. [18], and the *P. berghei* telomere probe was that of Ponzi et al. [19]. The cDNA sequences are those described by Da Silveira et al. [20] and Langsley et al. [6].

**Results**

**Karyotype of *P. chabaudi*.** Six cloned isolates of *P. chabaudi* were examined by PFG using two different pulse times (Figs. 1 and 2). A total of ten DNA bands, considered to contain intact
chromosomes, could be clearly resolved in each clone. A pulse time of 80 s was the most satisfactory for resolving the six smallest bands (Fig. 1A). Under these conditions larger bands co-migrated as a ‘compression zone’ in the gel. When a pulse time of 200 s was used, four further bands were separated from the compression zone, although with this pulse time the smaller bands were less well resolved (Fig. 2B).

The fastest moving band frequently showed more intense fluorescence than others in ethidium bromide-stained gels (e.g. Fig. 1, tracks ER and AS). This is most probably due to the presence in this band of two chromosomes of similar size [6]. We therefore consider that the ten DNA bands represent at least 11 distinct chromosomes. In addition to the ten bands resolved so far, significant quantities of DNA remained in the gel slots which might include further larger chromosomes.

It was not possible to estimate precisely the sizes of each chromosome, although from a comparison with yeast marker chromosomes subjected to PFG at the same time, the three smallest *P. chabaudi* chromosomes appeared to range in size from approximately 650 to 900 kb (Fig. 2B). This assumes that the *P. chabaudi* chromosomes obey the same size-mobility relationships as the yeast chromosomes.

**Chromosomal location of DNA sequences.** Southern blots made from PFG gels of *P. chabaudi* were probed with a selection of DNA sequences in order to determine their chromosomal locations. The following probes were used:

(i) A *P. berghei* telomere sequence [19]. This sequence hybridised to each DNA band visible by ethidium bromide staining (Fig. 2A), showing that each band most probably represented an individual chromosome. This probe appeared to hybridise more intensively to the smallest band than to the other bands, supporting the view that this
Fig. 2. (B) Ethidium bromide stained gel of *P. chabaudi* clone AS, run at pulse time of 200 s for 48 h. Increasing amounts of parasite material loaded on tracks 1 – 7. Note 10 separate chromosomes, numbered as shown. Tracks labelled Y show yeast chromosomes run as size markers, with approximate sizes in kb as described by Carle and Olsen [16]. (A) Southern blot of tracks 1 – 3 of gel shown in (B) hybridised to *P. berghei* telomeric probe at low stringency. Note hybridisation to all ten ethidium bromide-stained fragments (most clear in track 1). (C) Southern blot of tracks 6, 7 and Y of gel shown in (B) hybridised to *P. falciparum* ribosomal RNA gene rib-1 at low stringency. Note hybridisation specifically to chromosomes 5, 6 (individual chromosomes not distinct), 8 and 10 of *P. chabaudi*, and to yeast chromosomes.

(ii) *P. falciparum* ribosomal RNA sequence [6]. This sequence (rib.1) hybridised to chromosomes 5, 6, 8 and 10 (Figs. 1B and 2C) with apparently equal intensity. As the ribosomal RNA gene copy number in malaria parasites has been estimated to be between 4 and 8 [18,21,22], it is probable that only one copy of this sequence is present on each respective chromosome.

(iii) *P. chabaudi* cDNA sequences. 9 clones derived from a cDNA library of *P. chabaudi* [20] were mapped to chromosomes in PFG blots. Results are given in Table I. Four of these clones hybridised to chromosome band 1; however, since this band is thought to contain two chromosomes (see (i) above) it is unknown whether they are specific for chromosome 1 or 2. All the other probes hybridised to single chromosome bands and were thus suitable as chromosome specific markers.

**TABLE I**

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<tr>
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Chromosome polymorphism in cloned *P. chabaudi* isolates. The finding that certain cDNA and ribosomal RNA sequences are located on specific chromosomes enables them to be used as chro-
mososome markers, making it possible to identify homologous chromosomes in different isolates. Fig. 1B illustrates the chromosomal locations of the ribosomal RNA genes in six independently obtained cloned *P. chabaudi* isolates. Chromosomes 5 and 6 show especially marked size variation in the different clones. Thus in clones CB and ER, chromosome 5 appears larger than in the other clones; in clone 16, chromosome 6 is smaller than in the others. Fig. 3 shows hybridization of a cDNA probe (cDNA 443) to band 1 (containing chromosomes 1 and 2) in 7 isolates. The hybridisation of the ribosomal RNA genes, of cDNA 443, and of the other cDNA probes shown in Table I (results not shown), shows that despite their size polymorphisms, the relative order of the chromosomes is the same in the different cloned isolates.

**Analysis of cross between *P. chabaudi* clones.** In order to investigate the behaviour of the chromosomes during meiosis, a cross was performed between the two cloned isolates AS and CB (Fig. 4). Mosquitoes (*Anopheles stephensi*) were fed on a mixed infection of gametocytes of each clone, and the resulting sporozoites used to infect mice. The blood forms which developed in these animals were cloned by limiting dilution, and each clone examined for isoenzyme, antigen and drug-resistance markers which distinguished the parent clones AS and CB (Table II). Six clones which exhibited recombination between these markers were examined by PFG, using a pulse time of 80 s to investigate variation in chromosomes 1-6. The parent clones were also independently transmitted through mosquitoes, and examined for their karyotypes at the same time.

Differences in karyotypes of the parent clones could be detected in chromosomes 4 and 5 (Fig. 114).
TABLE II

| Iso-enzyme type, pyrimethamine-response, antigen type and PFG karyotype of parents and selected progeny clones of cross between *P. chabaudi* clones AS and CB |
|---|---|---|---|---|---|---|
| Parents | LDH | PGD | ADA | Pyr. | Mab 3.7 | PFG karyotype |
| AS | 3 | 2 | 6 | S | + | AS |
| CB | 4 | 3 | 8 | R | - | CB |
| Progeny | | | | | | |
| 1 | 3 | 2 | 8 | S | - | AS |
| 3 | 3 | 2 | 8 | S | - | CB |
| 6 | 3 | 3 | 6 | S | + | AS |
| 8 | 3 | 3 | 8 | R | - | Recombinant |
| 9 | 4 | 3 | 8 | S | - | CB |
| 10 | 3 | 3 | 6 | R | + | AS |

ADA, adenosine deaminase; LDH, lactate dehydrogenase; PGD, 6-phosphate gluconate dehydrogenase. Numbers represent electrophoretic variants of each enzyme. Pyr., pyrimethamine response: R = resistant, S = sensitive. Mab 3.7, monoclonal antibody recognising 250 kDa antigen of *P. chabaudi* schizont antigen.

5). In AS, chromosome 4 was larger than in CB, while chromosome 5 was smaller. The sizes of each chromosome were unchanged following mosquito transmission of each parent clone (data not shown). Three of the progeny clones (nos. 1, 6 and 10) possessed karyotypes which appeared identical to AS, and two (nos. 3 and 9) were the same as CB. In the sixth clone (no. 8), chromosome 4 corresponded in size to that of the CB parent, while chromosome 5 corresponded to that of AS.

The DNA bands revealed by PFG, therefore, appear to undergo a normal Mendelian independent assortment. Note that, since the blood forms of malaria parasites are haploid [1], only one chromosome of each pair will appear in any progeny clone.

**Discussion**

This study has shown that the genome of *P. chabaudi* can be resolved into at least 10 large DNA bands by the technique of pulsed field gradient gel electrophoresis. It can be concluded that these bands are most probably intact chromosomes because (a) a *P. berghei* telomeric sequence hybridised to all bands in Southern blots of PFG gels, and (b) the alternative forms of chromosomes 4 and 5 segregated independently at meiosis during mosquito transmission of the mixed AS and CB clones. The precise number of chromosomes in the genome of *P. chabaudi* is not clear, because each DNA band may represent more than one chromosome, as shown here for the smallest band. In an earlier preliminary study using different conditions of electrophoresis, Langsley et al. [6] considered that *P. chabaudi* may possess up to 14 chromosomes, the number now known to be characteristic of *P. falciparum* [8,23].

Previous genetic studies have demonstrated that *Plasmodium* possesses haploid blood forms, and
cytological work has demonstrated that meiosis occurs during early development of the zygote in mosquitoes. Recombination between genetically determined characters such as enzymes, drug-resistance and antigens has been shown to occur at a high frequency. In the present study, two parent clones, AS and CB, which differed by enzyme, pyrimethamine-sensitivity and antigen markers as well as the sizes of chromosomes 4 and 5, were crossed by feeding mosquitoes on a mixture of gametocytes of each clone. Both self- and cross-fertilisation of gametes of each clone were likely to have occurred in the mosquito midgut, resulting in the production of parental and hybrid-type zygotes. Meiosis of parental-type zygotes yielded progeny clones exhibiting the characteristics of each respective parent. Meiosis of hybrid zygotes resulted in independent assort-ment of parental markers, with the production of recombinant clones identifiable by non-parental combinations of the markers. Progeny clones of parental and recombinant classes were obtained in the cross between AS and CB. The six clones which were selected for PFG study were recombinant for phenotypic characters (Table II). One clone (no. 8) possessed a CB-type chromosome 4 and an AS-type chromosome 5. These two chromosomes, therefore, had segregated independently, in the manner expected for eukaryotic cells. Segregation of chromosomes other than 4 and 5 could not be detected as they were similar in size in both parent lines. In the other five clones, the chromosomes appeared to have segregated as parental types. Thus, while segregation of phenotypic markers had clearly occurred in these clones (Table II), this was not detectable by the PFG technique.

In *P. falciparum*, cross-fertilisation between two clones differing in karyotypes resulted in the production of forms in which certain chromosomes were of different size from those of either parent [2]. The genetic mechanisms involved in generating such novel-sized chromosomes are not understood, but could include unequal crossing-over events, deletions, etc., following pairing of unequal sized chromosomes at meiosis. Although chromosomes of new size appear not to have been produced during the *P. chabaudi* cross, at least among the six progeny clones examined so far, it seems likely that such events do occur, in view of the variation in chromosomes seen among clones of natural isolates of this species (Fig. 1A). These observations are similar to those with *P. falciparum*, in which variations in chromosome size are seen in both uncloned parasites isolated from patients in endemic regions and in cloned lines maintained in the laboratory [4,5,7,9]. This subject is being examined further in crosses between *P. chabaudi* clones differing more extensively in their karyotypes than AS and CB.

This work confirms that the genome of malaria parasites is organised into chromosomes which undergo segregation in the manner expected for eukaryotic organisms. Previous genetic studies have shown that recombination occurs readily following cross-fertilization between strains in mosquitoes. It is not known whether this recombination is due to re-assortment of unlinked genes on separate chromosomes, or to crossing-over between linked genes. In future, it can be expected that the use of PFG combined with conventional genetic studies will provide information on the nature of recombination mechanisms in these organisms.

Acknowledgements

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