MALARIA ANTIGENS INVOLVED IN PROTECTIVE IMMUNITY.

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ABSTRACT.

The rodent malaria *Plasmodium yoelii* is used as a laboratory model for human malaria. The invasion of erythrocytes by *Plasmodium* merozoites is thought to be mediated by rhoptries; the mechanism of invasion is unknown. A monoclonal antibody recognising a 235kD *P. yoelii* rhoptry antigen was isolated by Freeman et al. The monoclonal antibody, which was protective on passive transfer appeared to alter the specificity of the parasite causing preferential invasion of reticulocytes followed by clearance of infection (1).

The first section of the thesis describes the isolation of a *P. yoelii* DNA fragment thought to code for a blood stage merozoite rhoptry antigen. Antisera raised against a fusion protein and a synthetic peptide appear to recognise rhoptries by indirect immunofluorescence. Antibodies selected from *P. yoelii* immune serum immunoprecipitate a blood stage protein of 235kD and recognise a similar sized protein on Western blots. Sequence data, Southern blots and PCR amplification suggest that cloned DNA fragments may be present as more than one copy in the *P. yoelii* genome. Cross species hybridisation shows the gene sequence to be present in other rodent *Plasmodia*.

The second section of the thesis covers experiments attempting to identify the components of isoelectrically focussed *P. yoelii* fractions. De Souza and Playfair have shown that certain fractions can protect mice against challenge infection as effectively as a total parasite lysate (2). Antibodies to
focussed fractions were used to immunoprecipitate labelled P. yoelii proteins and in immunofluorescence studies. Antibody depletion experiments were carried out on focussed fractions to try to identify protective components.

References.
ACKNOWLEDGEMENTS.

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<tr>
<td>amp</td>
<td>Ampicillin.</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxy 5’ adenosine triphosphate.</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloroindolyl phosphate.</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair.</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin.</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie.</td>
</tr>
<tr>
<td>CIP</td>
<td>Calf intestinal phosphatase.</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute.</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate.</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide.</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol.</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate conjugate.</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography.</td>
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<tr>
<td>x g</td>
<td>Acceleration due to gravity.</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography.</td>
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<td>HPV</td>
<td>Human Papilloma virus.</td>
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<tr>
<td>IEF</td>
<td>Isoelectric focussing.</td>
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<td>IIF</td>
<td>Indirect immunofluorescence.</td>
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<tr>
<td>i.m.</td>
<td>Intra muscually.</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intra peritoneally.</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl thio-β-D-galactopyranoside.</td>
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<tr>
<td>i.v.</td>
<td>Intra venously.</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase.</td>
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<tr>
<td>kd</td>
<td>Kilodalton.</td>
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<tr>
<td>KLH</td>
<td>Keyhole limpet haemocyanin.</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>mA</td>
<td>Milliamps.</td>
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<tr>
<td>NBT</td>
<td>Nitro blue tetrazolium.</td>
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<tr>
<td>QPP</td>
<td>Quick plasmid preparation.</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis.</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline.</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction.</td>
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<tr>
<td>PMMSA</td>
<td>Precursor to the major merozoite surface antigen</td>
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<tr>
<td>PMSF</td>
<td>Phenyl methyl sulphonyl fluoride.</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA.</td>
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<tr>
<td>tRNA</td>
<td>Transfer RNA.</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute.</td>
</tr>
<tr>
<td>TE</td>
<td>Tris/EDTA.</td>
</tr>
<tr>
<td>TNP</td>
<td>Trinitrophenyl.</td>
</tr>
<tr>
<td>TX-100</td>
<td>TritonX-100.</td>
</tr>
<tr>
<td>SMPS</td>
<td>Simultaneous multiple peptide synthesis.</td>
</tr>
<tr>
<td>STE</td>
<td>Sodium/Tris/EDTA.</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneously.</td>
</tr>
<tr>
<td>SM</td>
<td>Sodium/magnesium.</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium chloride/sodium citrate.</td>
</tr>
<tr>
<td>V</td>
<td>Volts.</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per unit volume.</td>
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<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.</td>
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INTRODUCTION.
Malaria infections are caused by parasitic protozoa of the genus *Plasmodium*. Four species infective to man are *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. *P. falciparum* is the most deadly, killing approximately one million African children per year and is, therefore, the most intensively studied species. *P. vivax* infection is a major cause of morbidity; giving rise to relapses of clinical malaria. *P. malariae* and *P. ovale* are less prevalent. Other species infect primates eg. *P. knowlesi* and *P. cynomolgi*, rodents eg. *P. yoelii*, *P. berghei* and *P. chabaudi* and birds eg. *P. lophurae* (Bruce-Chwatt, 1984). Although these animal infections are used as laboratory models, none truly mimics the course of natural *P. falciparum* infection since laboratory animals are often permissive rather than natural hosts. Historically the primate malarias were thought to be most closely related to those infective to humans, on the grounds that the parasite evolved in parallel with the host. McCutchan et al examined the genome organisation and dG:dC content of DNA from different *Plasmodia* species infective to humans, monkeys, mice and birds. This comparison suggested that rodent and avian malarias may bear more resemblance to human than primate species and that *P. vivax* has similar characteristics to *P. cynomolgi* a primate malaria (McCutchan et al, 1984). Some antigens isolated from rodent and primate malarias show a high degree of homology with *P. falciparum* analogues (Lewis, 1989. Nussenzweig and Nussenzweig, 1985).
1.1 **THE PLASMODIUM LIFE CYCLE.**

The life cycle of the malaria parasite is illustrated in figure 1.1. Malaria infections are initiated by the bite of the female *Anopheles* mosquito when it takes a blood meal. Sporozoites, present in the saliva of the mosquito are injected into the blood stream and within approximately thirty minutes are carried to the liver where they invade hepatocytes. Each sporozoite develops through a trophozoite stage into a multinucleate schizont containing thousands of merozoites, a process defined as exoerythrocytic schizogony. Rupture of liver schizonts releases merozoites into the liver sinusoid where they quickly invade erythrocytes. Following invasion of the red blood cell, merozoites develop into early ring forms characterised by the presence of a vacuole which can be seen microscopically when parasitised erythrocytes are treated with Giemsa's stain. Differentiation of the parasites within the erythrocyte produces trophozoites followed by multinucleate schizonts containing many merozoites. Erythrocyte rupture releases the merozoites into the blood stream and these invade new red cells within minutes. The invading merozoite attaches to the red blood cell and re-orientates to bring its apical region into contact with the plasma membrane; discharge of material from the paired, apical organelles, or rhoptries, is thought to be involved in invasion. Following merozoite attachment, the erythrocyte membrane invaginates, the merozoite enters the cell, the membrane reseals and the parasite becomes surrounded by a vacuole (Mitchell and Bannister, 1988). This erythrocytic cycle of parasite development
Figure 1.1 The life cycle of Plasmodium.
is responsible for the clinical symptoms of the disease. A proportion of merozoites differentiate into male and female gametocytes which are taken up by feeding mosquitoes. The sexual phase of the cycle is initiated when ingested gametocytes undergo development in the mosquito gut to form gametes; fertilisation produces zygotes which subsequently develop into oocinetes. This stage invades the gut wall where sporozoites form within oocysts. Oocyst rupture releases sporozoites which migrate to the salivary glands and are injected into the vertebrate host when the mosquito takes its next blood meal (Bruce-Chwatt, 1984).

1.2 THE MALARIA GENOME.

Asexual forms of the malaria parasite are haploid and divide by mitosis during liver and blood stages (Walliker et al, 1987). Meiosis occurs at the sexual, zygote stage within the mosquito, the only stage of parasite development where classical genetic recombination can occur following fertilisation of gametes (Walliker et al, 1987). Pulsed field gel electrophoresis has shown *P. falciparum* to contain fourteen chromosomes, correlating with the number of kinetochores observed by electron microscopy (Kemp et al, 1987a); the size of individual chromosomes appears to vary between strains (Weber, 1988). A karyotype comparison between *P. falciparum* and the rodent malaria, *P. chabaudi* has shown both species to contain fourteen chromosomes (Langsley et al, 1987). The size of the malaria genome is estimated at 2-4x10^7 bp per haploid genome depending on the species. The genome is generally AT rich with variations in the content dependent on species, for
example, *P. yoelii* contains approximately 76% AT and *P. falciparum* approximately 82% (Weber, 1988). The coding regions of malaria genes have been shown to be less AT rich than flanking, non-coding sequences. Introns, also with a high AT content, have been found in some *Plasmodium* genes but all are relatively short (Weber, 1988). Many of the malaria genes sequenced so far code for antigens and contain tandemly repeated oligonucleotide sequences. The function of the repetitive amino acid sequences is unknown but it has been suggested that these immunogenic structures may direct the host immune response to non-essential antigens or to non-essential regions of a particular antigen. Alternatively, because many of the repeats have some structural homology it has been suggested that the presence of cross reacting epitopes may prevent the generation of a high affinity antibody response to crucial antigens (Weber, 1988).

1.3 IMMUNITY TO MALARIA.

Immunity to *P. falciparum* malaria is acquired and depends on both the degree and duration of exposure to the parasite. Sterilising immunity can be induced in rats by *P. berghei* infection or in mice immunised with *P. yoelii* or *P. chabaudi* but, in man, immunity to *P. falciparum* is rarely sterile (Playfair, 1982a). Acquired immunity is stage specific reflecting the stage specific expression of genes encoding antigens exposed to the immune system. Rats immunised with *P. berghei* sporozoites have been shown
to be immune to sporozoite challenge (Spitalny and Nussenzweig, 1973) but mice immunised with sporozoites are not resistant to blood stage challenge (Nussenzweig et al, 1969). Superinfection experiments using cloned lines of \textit{P.berghei} or \textit{P.chabaudi} suggest that the murine immune response to infection is largely species specific and markedly strain specific (Jarra and Brown, 1985). The intracellular development of the malaria parasite prevents the exposure of many antigens to the immune system. However, immunisation experiments have shown that two stages transitorily free in the bloodstream, merozoites and sporozoites, carry antigens that are targets of the protective immune response to the disease. Other target antigens may be expressed on the surface of the infected host cell, for example proteins associated with knobs on the infected erythrocyte, that may be involved in parasitic sequestration ie. the cytoadherence of parasitised cells to the endothelial cells of deep capillary venules (Kemp et al, 1987b). Experiments using animal models have shown the protective immune response to malaria to be both B cell and T cell dependent (Clark and Allison, 1974. Weinbaum et al, 1976. Jayawardena et al, 1977. Finerty and Krell, 1977. Playfair et al, 1979. Roberts and Weidanz, 1979. Freeman and Parish, 1981). Recent experiments using molecular techniques, and T cell cloning, support past findings implicating the involvement of T cells in the immune response to the disease (Good, 1988c. Schofield, 1987, 1989).
1.31 CELL MEDIATED IMMUNITY.

Evidence that T cells are involved in immunity to malaria has come from several sources using immuno-deficient animals (Clark and Allison, 1974. Weinbaum et al, 1976. Jayawardena et al, 1977. Roberts and Weidanz, 1979) and from studies correlating T cell activation with immunity (Playfair et al, 1977, 1979, 1985. Kabilan et al, 1987. De Souza and Playfair, 1988. Recently, protection against P. chabaudi adami infection in nude mice by adoptive transfer of a cloned T cell line has been described by Brake et al (1988). These experiments show that a single T cell site on one parasite antigen can induce a protective immune response and the clone was shown to release gamma interferon when stimulated with antigen in vitro. Weiss et al (1988) demonstrated that mice immunised with irradiated P. yoelii sporozoites were no longer resistant to live sporozoite challenge when depleted of their CD8+ suppressor / cytotoxic T cell subset. In similar experiments with mice immunised with P. berghei sporozoites, Schofield et al (1987) showed that immune animals depleted of their CD8+ T cell subset developed infections when challenged with sporozoites but remained immune when depleted of their helper T cell subset bearing the CD4+ antigen. Passive transfer of immune immunoglobulin alone or immune T cells alone conferred partial immunity to naive recipients suggesting that a combination of antibody and T cell effector mechanisms was required for protection. The same group showed that administration to immune hosts of neutralising antibody against gamma interferon also reversed sterile immunity to sporozoite
challante, however, a recent study by Hoffman et al (1989) has shown that spleen cells from mice immunised with irradiated sporozoites can eliminate P. berghei parasites from hepatocytes in vitro and that the effect cannot be inhibited by a monoclonal antibody to gamma interferon. Schofield et al suggested that the response of CD8+ cells to parasite antigen, in the context of class 1 MHC, is to produce gamma interferon which exerts an effect on the exoerythrocytic form of the parasite. Romero et al (1989) have recently provided direct evidence that CD8+ T cells, specific for a defined epitope of the circumsporozoite protein (CSP), can confer protection against challenge infection. A 12 amino acid peptide corresponding to amino acids 249-260 of the P. berghei CSP was recognised by H-2Kd restricted cytotoxic T cells and passive transfer of of cytotoxic T cell clones recognising this epitope provided a high degree of protection to mice. The protection observed was species and stage specific.

The primary structure of the P. falciparum CSP (Nussenzweig and Nussenzweig, 1985. Miller et al, 1986) has shown a central amino acid sequence, consisting of Asn-Ala-Asn-Pro (NANP) repeated many times, to be the immunodominant B cell epitope on the molecule. Although high antibody titres to this repeated sequence are associated with protection, immunisation of human volunteers with either (NANP),-tetanus toxoid, or a recombinant protein containing 30 repeat elements has shown the sequence to be a poor inducer of antibody in man (Herrington et al, 1987. Ballou et al, 1987). Experiments suggest that the recognition of T cell sites within the molecule is an important factor in the
cellular immune response to sporozoites. De la Cruz et al (1987) and Good et al (1988a, 1988b) have identified helper T cell epitopes within the \textit{P. falciparum} CSP which map to regions of the molecule shown to be genetically polymorphic in different parasite strains. They suggest that the occurrence of these sites within variant domains may be a strategy for evasion of the immune response by the parasite. Guttinger et al (1988) synthesised peptides corresponding to predicted T cell sites within polymorphic and non-polymorphic non-repetitive regions of the molecule and used them to isolate T cell clones from immune and non-immune donors. The T cell clones were then tested for reactivity against isolated CS protein. The experiments showed that one amino acid change, due to a single nucleotide substitution in the polymorphic region of the CSP gene, could affect T cell recognition of the protein. Furthermore the response appeared to be HLA restricted. All clones were of the CD4+/CD8- phenotype. Sinigaglia et al (1988) have identified an additional invariant, putative T cell epitope within the \textit{P. falciparum} CSP. Peptides corresponding to this non-polymorphic region stimulated CD4+/CD8- T cell clones from a non-immune donor, overcame murine MHC class II restriction of the NANP response and were recognised by many HLA-DR elements. However, there is evidence that this epitope is not immunodominant \textit{in vivo} (Good et al, 1988a). In recent experiments to assess the extent of sequence diversity in the putative T helper epitope region, Lockyer et al (1989b) have used polymerase chain reaction (PCR) amplification to isolate DNA coding for the polymorphic region of
the *P. falciparum* CSP. The DNA was prepared from blood samples collected from infected children living in a small, defined area of the Gambia. Cloning and sequencing of the DNA obtained has shown that most children carry mixed infections and that the sequence corresponding to the T cell epitopes identified by Good *et al* (1988a, 1988b) and Guttinger *et al* (1988) is variant in many of the clones isolated. Moreover the degree of T-cell epitope polymorphism within this small sample population is greater than that found among geographically diverse laboratory isolates. Laboratory isolates are propagated as blood forms *in vitro* and are therefore not exposed to immune pressure in the host.

There is also limited evidence that the suppressor function of the CD8+ subset of T cells may play a part in the immune response to malaria. Experiments carried out in The Gambia by Riley *et al* (1989), suggest that the presence of the CD8+ subset of T cells can inhibit *P. falciparum* induced lymphoproliferation in some individuals. Lymphoproliferation and gamma interferon production were significantly enhanced when CD8+ cells were removed from cultures of peripheral blood mononuclear cells that responded poorly to a soluble malaria antigen.

1.32 LYMPHOKINE MEDIATED IMMUNITY.

The experiments discussed above show that T cell mediated gamma interferon release plays a part in immunity to sporozoites in the mouse. Other experiments confirm its involvement in the immune response to malaria. T cells from mice immunised with irradiated *P. berghei* sporozoites produce interferon *in vitro*
after challenge with homologous CS protein and recombinant interferon injected into mice before challenge with *P. berghei* sporozoites had a significant inhibitory effect on infection (Nussenzweig and Nussenzweig, 1986). Recombinant human gamma interferon and interleukin-1 have been shown to strongly inhibit *P. falciparum* sporozoite development in cultures of human hepatocytes. Timed experiments suggest that gamma interferon exerts its effects after penetration of hepatocytes by sporozoites whilst interleukin-1 acts at the very early phase of infection (Mellouk et al., 1987).

There is evidence that macrophages, activated in response to malaria infection, release reactive oxygen intermediates, capable of killing intracellular parasites (Dockrell and Playfair, 1983). In *P. chabaudi*, peak oxidative burst activity occurs as mice begin to recover from infection (Dockrell et al., 1986). Administration of recombinant gamma interferon to mice reduces *P. chabaudi* infection and is suggested by Clark et al. (1987) to simulate interferon release by antigen activated T cells which induces macrophages to release H$_2$O$_2$. The same group also fed mice with butylated hydroxyanisole, a scavenger of free radicals and noted an enhancement of infection. Recent work by Rockett et al. (1988) suggests that the parasiticidal activity of tumour necrosis serum (TNS) may be due to lipid peroxides, which have a longer half life than reactive oxygen intermediates. TNS was no longer parasiticidal when depleted of lipoproteins and separation of TNS by ultracentrifugation showed the lipoprotein fraction to contain 70% of the total parasiticidal activity.
A "crisis form factor" (CFF), previously observed to retard the development of erythrocytic stage parasites in animal models, has been shown to exist in serum from Sudanese individuals (Jensen et al, 1982. Jensen et al, 1987). Epidemiological studies carried out in Sudan showed a correlation between CFF concentration and malaria immunity. In the presence of CFF, intracellular parasites are slow to develop and become distinctly deformed (Jensen et al, 1987). The effect cannot be reproduced in vitro using recombinant gamma interferon or tumour necrosis factor (TNF) but can be reproduced with tumour necrosis serum (Carlin et al, 1985. Taverne et al, 1987). However, Clark et al (1987) delivered recombinant TNF intra peritoneally to P. chabaudi infected mice by means of an osmotic pump and noticed the appearance of parasite "crisis forms" in mice receiving the highest dose of TNF (2.88μg TNF/day). Jensen et al (1987) have reported the presence of CFF in serum from rabbits immunised with BCG (an attenuated form of Mycobacterium tuberculosis, a vaccine that can be used as an adjuvant), followed by LPS (bacterial lipopolysaccharide or endotoxin). The rabbit serum, which contains TNF, induced "crisis forms" in cultured P. falciparum but no correlation was found with TNF concentration or CFF concentration, furthermore, TNF has not been identified in CFF containing serum from Sudanese individuals. CFF activity has been demonstrated in serum from people suffering from TB and Jensen et al (1987) suggest that CFF is a so far unidentified cytokine, capable of destroying intracellular parasites.
1.33 HUMORAL IMMUNITY.

The importance of antibody in resistance to malaria is evident from the protection conferred to babies by maternal antibody (Bruce-Chwatt, 1963). Children in endemic areas are most susceptible at age 3 months to 5 years after which they begin to develop resistance to the disease. Experimentally, the protective role of antibody was demonstrated by Cohen et al (1961) when multiple injection of gamma globulin from immune adults was shown to reduce parasitaemia in children. Immunity to *Plasmodium* infection is often accompanied by elevated serum antibody levels and immunoglobulin from adults living in endemic areas has been shown to inhibit *P. falciparum* multiplication *in vitro* (Mitchell et al, 1976). Passive immunisation of naive mice with serum from animals immune to *P. yoelii* has been shown to confer protection demonstrating that humoral factors can control the progression of infection (Diggs and Osler, 1969. Freeman and Parish, 1981). Immunisation with purified *P. knowlesi* merozoites protects monkeys against challenge infection (Mitchell et al, 1978) and *in vitro* studies using serum from monkeys infected with *P. knowlesi* showed that immune serum reduced invasion of erythrocytes by causing agglutination of merozoites (Miller et al, 1975). Passive transfer of *P. yoelii* immune serum was observed to inhibit reinvasion of new erythrocytes suggesting that antibody effects are exerted against schizonts and/or merozoites (Freeman and Parish, 1981). Monoclonal antibodies to *P. falciparum* blood stages and polyclonal antibodies affinity purified from human immune serum have been shown to inhibit merozoite invasion *in vitro* (Wahlin et al, 1984).
Schofield et al., 1986). Monoclonal antibodies raised against blood stage *P. yoelii* antigens have also been shown to protect mice against challenge infection (Majarian et al., 1984. Freeman et al., 1980a).

Studies on a class of blood stage *Plasmodium* antigens with potential as vaccine candidates, have provided evidence to suggest that variation in B cell epitopes may also affect the immune response to malaria. The amino acid sequence of regions of the *P. falciparum* precursor to the major merozoite surface antigen (PMMSA) has been shown to vary between strains. The differences are thought to be due, in part, to intragenic recombination at meiosis between two parental alleles (Holder, 1988b). Furthermore, a protective monoclonal antibody recognising the C terminus of the *P. yoelii* PMMSA has been shown to recognise a variant specific epitope of the protein (Burns et al., 1989). The variation observed within these molecules has obvious implications for vaccine development and shows that sequence comparison of B cell epitopes is a valuable method of identifying invariant regions of parasite proteins. The development of molecular immunological techniques has enabled the use of immune sera to identify parasite antigens exposed to the immune system.

1.4 IDENTIFICATION OF PLASMODIA ANTIGENS.

Many *Plasmodia* antigens have been identified by using immune serum to immunoscreen DNA libraries induced to produce recombinant parasite proteins (Kemp et al., 1983. Ozaki et al., 1986. Coppel et al., 1987. Smythe et al., 1988. Galinski and
Antibodies raised against a fusion protein or affinity selected from immune serum enables further characterisation of antigens including localisation by indirect immunofluorescence (IIF) or immuno electron microscopy (IEM). Using recombinant DNA technology, the genes encoding many *Plasmodia* antigens identified by antibodies, have been cloned (Weber, 1988). In a different approach, monolayers of *P. falciparum* infected erythrocytes were used to select antibodies from human immune serum which identified a merozoite antigen thought to be involved in red cell invasion (Perlmann *et al.*, 1984). Monoclonal antibodies raised against different stages of the parasite exposed to the immune system, have been used to identify antigens such as the *P. falciparum* CSP, the precursor to the major merozoite antigen (PMMSA) and a number of proteins thought to be associated with merozoite invasion of erythrocytes (Yoshida *et al.*, 1980. Nussenzweig and Nussenzweig, 1986. Holder and Freeman 1982. Holder *et al.*, 1985a. Sam-Yellowe *et al.*, 1988. Howard *et al.*, 1984. Schofield *et al.*, 1986. Bushell *et al.*, 1988. Clark *et al.*, 1987. Roger *et al.*, 1988). Two monoclonal antibodies were identified that protected mice against virulent *P. yoelii* infection (Freeman *et al.*, 1980a). One of the protective monoclonal antibodies was used to purify a *P. yoelii* rhoptry antigen by immunoaffinity chromatography. The isolated 235kd antigen also protected mice against challenge infection (Holder and Freeman, 1981).

A novel approach to the identification of *P. yoelii* blood stage antigens that may be important in immunity has been
employed by De Souza and Playfair (1988), using an assay based on
the ability of fractionated polypeptides to protect mice against
challenge infection. Formalin fixed preparations of \textit{P.yoelii} and
a Triton X-100 lysate of parasitised erythrocytes had previously
been shown to protect mice (Playfair et al, 1977. Playfair and De
Souza, 1986). The protection seen was described as falling into
two categories; an "early" response where parasites were cleared
from the bloodstream by day 8-12, and a "late" response with
clearance by day 17-28 (Playfair and De Souza, 1986). Two
dimensional gel analysis of metabolically labelled \textit{P.falciparum}
and \textit{P.yoelii} asexual blood forms, showed lysates to contain
and De Souza separated TritonX-100 soluble lysates, prepared from
parasitised erythrocytes, by isoelectric focussing. Recovered
fractions were then tested for their ability to protect mice
against \textit{P.yoelii} challenge. Spleens from protected mice were
assayed for their capacity to stimulate parasite specific, helper
T cell activity, measured by an anti-trinitrophenyl (TNP)
response to TNP coated, parasitised erythrocytes.

The majority of proteins focussed in the pH4.1-4.6 range with the
pH4.2 fraction consistently conferring 100% protection, mainly of
the "early" type. Two other peaks of "late" type protection were
observed; the pH6.5 fraction provided 90% protection and the
pH8.0 fraction 80%. The three protective peaks were separated by
fractions of very low protein concentration, with little or no
protective capacity. Control immunisations using focussed
non-parasitised erythrocytes were not protective. In the helper T
cell assay, priming correlated well with protection of the
"early" type. In spleens from mice exhibiting the "late" form of
protection, correlation with helper T cell priming was less
marked.

1.5 SPOROZOITE ANTIGENS.

There is little information on sporozoite antigens save the
immunodominant circumsporozoite protein (CSP) which is the major
antigen on the outer membrane of the sporozoite (Nussenzweig and
Nussenzweig, 1985). The protein and the gene encoding it have been
well characterised in a number of Plasmodium species (Nussenzweig
Lockyer, 1989a). The polypeptide contains an N-terminal region
that includes a putative signal sequence, a central area of
species specific tandem repeats and a C-terminal domain with a
sequence thought necessary to anchor the protein in the
sporozoite outer membrane. In P. falciparum the central repeat
consists of Asn-Ala-Asn-Pro (NANP); the minimum number of repeats
constituting an epitope is three. The surface of an infective
sporozoite has been estimated to carry more than $10^7$ such
epitopes (Zavala et al, 1985). Studies have shown that antibodies
to synthetic peptides corresponding to these repeats show similar
properties to those raised against the intact sporozoite (Young
chickens against sporozoite challenge was first demonstrated by
immunisation with X-ray attenuated sporozoites of P. gallinaceum
(Mulligan et al, 1941). Similar immunisation experiments using
sporozoites from *P. berghei* in mice, *P. knowlesi* in monkeys and *P. falciparum* and *P. vivax* in human volunteers followed. Protection in humans was short lived and species but not strain specific (Cochrane *et al.*, 1980). Two recent vaccine trials using peptides or recombinant proteins corresponding to the NANP repeats have met with limited success. In both studies, antibody responses were poor with little or no evidence of boosting. The conclusions drawn were that the antigens failed to stimulate the cell mediated arm of the immune response (Herrington *et al.*, 1987. Ballou *et al.*, 1987).

1.6 **BLOOD STAGE ANTIGENS.**

At the time of release from schizont infected erythrocytes, merozoites are exposed to serum antibodies that can prevent reinvasion of red blood cells. Studies by Miller *et al.* (1975) showed that incubation of *P. knowlesi* infected erythrocytes with immune serum caused aggregation of merozoites rendering them incapable of red cell invasion. In the presence of immune serum, changes in the morphology of the merozoite surface coat occurred, resulting in agglutination via the surface coat of adjacent merozoites. Affected merozoites appeared to adhere to red cells but eventually detached from the erythrocyte membrane. Such observations have led to efforts to define merozoite antigens targetted by antibody. EM studies by Ladda *et al.* (1969), Bannister *et al.* (1975) and Aikawa *et al.* (1978) have provided much information on the ultrastructure of the merozoite, the organelles thought to be involved in invasion and the
morphological changes that accompany merozoite entry into erythrocytes. Electron micrograph studies have shown that merozoite invasion of red blood cells involves initial attachment, followed by reorientation to bring the apical end of the merozoite into contact with the erythrocyte membrane (Dvorak et al., 1975). Erythrocytes undergo waves of deformation, the merozoite enters the cell and the membrane reseals. Fine, fibrillar material has been seen at the junction connecting the merozoite and the red cell membrane (Aikawa et al., 1978). The attached merozoite appears to release the contents of its rhoptries onto, or into the erythrocyte membrane leading to invagination of the membrane and formation of a parasitophorous vacuole which surrounds the intracellular parasite Ladda et al., 1969. Bannister et al., 1975. Mitchell and Bannister, 1988).

Figure 1.2 shows a merozoite entering an erythrocyte. There is some evidence that the discharge of rhoptry material is also involved in the exit of merozoites from late stage erythrocytic schizonts (Seed et al., 1976. Stewart et al., 1986). Rhoptries are found in Plasmodia and other closely related members of the Apicomplexa which are obligate, intracellular parasites. In Toxoplasma gondii, a factor with lytic activity is believed to be secreted by the rhoptries (Nicholls et al., 1983). The apical complex generally consists of polar rings, rhoptries, micronemes and sub-pellicular microtubules and is thought to contain both proteins and lipidic substances since whorls of concentric lamellae are seen in rhoptries when sections are fixed with tannic acid (Bannister et al., 1986). Membranous whorls of
Diagram showing the different stages of invasion including (A) initial attachment, (B) reorientation of merozoite with bending of the attached red cell; (C) apical contact; (D) invagination of the invasion pit; (E) closure of the parasitophorous vacuole; (F, G) extrusion of the microsphere contents with further expansion of the parasitophorous vacuole membrane; and (H) final transformation into a ring stage.

Figure 1.2  Merozoite invasion of erythrocytes. Reproduced by kind permission of Dr. L.H. Bannister, Guy's Hospital, (Mitchell and Bannister, 1988).
1.61 MEROZOITE SURFACE ANTIGENS.

The most extensively studied blood stage antigen is the Precursor to the Major Merozoite Surface Antigen (PMMSA) which has been characterised in a number of species. The apparent molecular weight of the molecule varies between species, eg. 195kd in *P.falciparum*, 250kd in *P.chabaudi* AS and 230kd in *P.yoelii* (Holder and Freeman, 1982, 1984a. Holder, 1988b). The proteins share features which form the basis for their classification into a single group. They are synthesised as high molecular weight precursors at the onset of schizogony and undergo proteolytic processing to a number of lower molecular weight fragments. Interspecific serological cross reactivity has been demonstrated within the class and the 230kd *P.yoelii* PMMSA displays extensive sequence homology with the *P.falciparum* protein (Holder, 1988b. Lewis, 1989). The *P.falciparum* PMMSA was first identified by Holder and Freeman (1982) using a monoclonal antibody. The 195kd glycoprotein is synthesised throughout schizogony and is present on the surface of the intracellular parasite where it is processed to fragments of 83kd, 28-30kd, 38kd and 42kd. The 42kd polypeptide undergoes further processing to two fragments of 15-18kd and 19kd (Holder and Freeman 1984b. Holder et al, 1985b, 1987). Indirect immunofluorescence studies using antibodies specific for each of the processed fragments have shown that the 19kd C-terminal fragment is present in ring stage parasites, carried through with the merozoite on invasion of the new erythrocyte. Antibodies specific for the 83kd N-terminal fragment do not react with ring forms but the
polypeptide can be recovered from culture supernatants, suggesting that this portion of the molecule is shed from the merozoite before red cell invasion (Holder, 1988b). DNA sequencing of the PMMSA from different strains of *P. falciparum* has revealed regions of conserved, semi-conserved and variable sequence (Holder, 1988b). Alignment of the deduced amino acid sequences from semi-conserved and variable regions suggested that there are two basic alleles for the PMMSA with strain differences resulting, in part, from intragenic recombination (Holder, 1988b).

Siddiqui et al (1987) reported complete protection of *Aotus* monkeys against homologous challenge infection by vaccination with the *P. falciparum* PMMSA and its processed fragments, however, immunisation experiments by Holder et al (1988a), with recombinant polypeptides encoded by the *P. falciparum* PMMSA gene, provided no evidence of protection in *Aotus* monkeys. A monoclonal antibody specific for the 230kd *P. yoelii* PMMSA recognises fragments of 197kd, 160kd, 151kd, 126kd, 90kd, 56kd and 28kd. The 230kd precursor and its processed fragments protected mice against homologous challenge infection (Holder and Freeman, 1981) and protection correlated with T cell mediated immunity (Playfair et al, 1985).

### 1.62 APICAL COMPLEX ANTIGENS.

Much work has concentrated on proteins which may be involved in merozoite invasion of the erythrocyte both from the standpoint of vaccine research and the biochemistry of the invasion process.
The Ring Infected Surface Antigen (RESA) is an intensively studied blood stage antigen thought to be involved in erythrocyte invasion (Coppel et al, 1984. Brown et al, 1985). It is located in the micronemes of merozoites and is also seen in the membranes of ring infected erythrocytes. The gene encoding RESA has been sequenced. It contains two blocks of tandem repeats. At the 3' end two different repeated sequences form a block whilst at the 5' end the block has one component which is also present in degenerate form (Coppel et al, 1984). The protein has an apparent molecular weight of 155kd, is present as a non-ionic detergent soluble species in merozoites and is insoluble in the same detergent when isolated from newly invaded red blood cells. The same protein was identified in parallel by Perlmann et al (1984) by purifying antibodies from human immune serum on monolayers of P.falciparum infected erythrocytes. Human IgG eluted from monolayers efficiently inhibited merozoite invasion in vitro and the major antibody in the eluate was directed against a 155kd antigen (Wahlin et al, 1984). In experiments carried out by Collins et al (1986), immunisation of monkeys with recombinant peptides corresponding to the 11 amino acid 5' repeat or the 8 amino acid 3' RESA repeat provided partial protection against challenge infection and antibody titres correlated with protection. The alternative, 4 amino acid 3' repeat provided no protection in this study although this region appears to be immunodominant in humans. In experiments by Patarroyo et al (1987), a synthetic peptide representing the 8 amino acid 3' repeat did not protect monkeys against P.falciparum challenge. In
a later experiment, human volunteers were immunised with a synthetic vaccine containing sequences from the *P. falciparum* CSP and PMMSA plus one copy of the 5' RESA repeat. The vaccine failed to protect and no antibodies specific for the RESA sequence, or the CSP sequences, were detected in the serum of immunised volunteers (Patarroyo et al., 1988).

Holder et al. have isolated a 140kd *P. falciparum* rhoptry antigen using a monoclonal antibody which reacted with $^{35}$S-Met labelled blood stage proteins of 140kd and a doublet of 155kd (Holder et al., 1985a). Dissociation experiments showed that the 155kd species co-precipitated with the 140kd molecule, when the two proteins were dissociated, the monoclonal antibody did not react with the 155kd species.

Coppel et al. (1987) used serum from immune humans to isolate a *P. falciparum* cDNA clone, Ag44. The expressed, fused polypeptide was used to select specific antibodies from immune serum which precipitated a parasite protein of 105kd, identified as a rhoptry protein by IIF. Further experiments by Lustigman et al. (1988) showed that the antigen, RhopH3 is synthesised as a 103kd protein in mature trophozoites, is present in schizonts and merozoites as a 105kd protein and is discharged from rhoptries and found on the surface of newly invaded red cells as a 110kd species. Anti-Ag44 antibodies also immunoprecipitate proteins of 135kd and 150kd from lysates of infected cells and culture supernatants; these proteins are thought to be non-covalently associated with RhopH3 forming a high molecular weight, HMW, complex. The 135kd component of this complex appears to be the
same protein as that isolated by Holder et al (1985a) since it can be immunoprecipitated with the monoclonal antibody to the 140kd protein. Similar complexes have also been described by Cooper et al (1988) and Campbell et al (1984). Sam-Yellowe et al (1988) have described a similar *P. falciparum* rhoptry complex containing a major component of 110/100kd and minor components of 155kd, 140kd and 130kd as identified by monoclonal antibody. The proteins appear to have intra molecular disulphide bonds based on comparisons of electrophoretic mobility under reducing or non-reducing conditions. The higher molecular weight proteins are thought to co-precipitate with the 110kd species. Biosynthetic labelling studies suggest the 110kd protein is synthesised at the trophozoite stage and is processed, at the schizont stage, to the 100kd species which is also seen in newly invaded rings. Preliminary investigations suggest the 110kd protein may be a protease.

A lower molecular weight *P. falciparum* rhoptry antigen complex containing proteins of 80kd, 66kd and 42kd has been described by several laboratories (Howard et al, 1984. Schofield et al, 1986. Bushell et al, 1988. Clark et al, 1987). Schofield et al (1986) reported that monoclonal antibodies to this complex inhibited merozoite invasion of erythrocytes in vitro. Using protease inhibitors, the same group deduced that the 66kd component of the complex was a processed product of the 80kd polypeptide and that the 42kd species was co-precipitated.

A 55kd *P. falciparum* rhoptry antigen has been isolated by Smythe et al (1988) using a solubilisation procedure intended to
identify membrane proteins. Immobilised antigens were used to affinity purify antibodies from complex, human immune serum and the antibodies were used to screen a recombinant *P.falciparum* cDNA library in bacteriophage. Two clones were isolated, one coding for a 45kd merozoite surface antigen and another coding for a 55kd rhoptry antigen. Immunoprecipitation of parasite proteins using anti 55kd protein antibodies suggested that the molecule was unrelated to other proteins of a similar molecular weight present in previously identified rhoptry complexes.

Three high molecular weight rhoptry antigens have been documented that do not appear to exist as part of a complex. A 225kd antigen was identified by Roger *et al* (1988) using monoclonal antibodies raised against *P.falciparum* culture supernatants. Pulse chase experiments showed the 225kd protein to be the product of a 240kd precursor processed 0-4 hours after synthesis during schizogony.

Galinski and Barnwell (1989) have evidence for a 250kd *P.vivax* rhoptry antigen identified by IIF using immuno affinity purified antibodies selected by a recombinant bacteriophage clone. The lambda gt11 clone was identified using antiserum from *Saimiri* monkeys hyperimmune to *P.vivax* infection. Limited sequence data suggest the protein is very highly charged.

Two anti *P.yoelii* monoclonal antibodies raised by Freeman *et al* (1980a) were shown to recognise rhoptries by IIF. In passive transfer experiments, both protected mice against *P.yoelii* challenge infection. The effect of the antibodies was seen during the week following transfer when the course of virulent YM
infection displayed characteristics similar to those of a 17X non-lethal infection. Monoclonal antibody 25.77 immunoprecipitated a \textit{P.yoelii} merozoite-specific antigen of 235kd which was localised to rhoptries by immunogold EM (Oka et al,1984). In IIF studies monoclonal antibody 25.77 cross reacted with \textit{P.yoelii} 17X, \textit{P.yoelii} 33X, \textit{P.yoelii killicki}, \textit{P.yoelii nigeriensis}, \textit{P.vinckei petteri}, \textit{P.chabaudi chabaudi} and \textit{P.chabaudi adami} merozoite antigens whilst monoclonal antibody 25.37 reacted with only \textit{P.yoelii} sub-species. Neither antibody cross reacted with \textit{P.falciparum} antigens (Holder and Freeman,1984c). \textit{P.yoelii} strain 17X synthesises the 235kd protein but the monoclonal antibodies did not affect the course of 17X infection (Holder and Freeman,1984c). Using monoclonal antibody 25.77, Holder and Freeman (1981) purified the 235kd \textit{P.yoelii YM} protein from infected erythrocytes and immunised mice with the antigen. Mice challenged with \textit{P.yoelii YM} parasitised erythrocytes developed parasitaemias that were initially supressed relative to controls. After day eight, an influx of reticulocytes into the circulation was coincident with an increase in parasitaemia with all parasites confined to reticulocytes. Parasitaemias were cleared by day sixteen.

1.7 EXPERIMENTAL STRATEGY.

The experiments detailed below describe attempts to isolate and characterise blood stage antigens of \textit{P.yoelii} using serum from mice hyperimmune to \textit{P.yoelii}. \textit{P.yoelii} hyperimmune serum is protective on passive transfer and was observed by Freeman and
Parish (1981) to impair reinvasion of new host erythrocytes suggesting that antibody acts against schizonts and/or merozoites. The selection of monospecific antibodies from complex immune serum by binding to recombinant proteins is a method of dissecting the immune response to malaria infection. Antigens can be identified by IIF, EM, western blotting and immunoprecipitation and purified by immuno affinity chromatography. This strategy allows the cloning and analysis of genes encoding *P. yoelii* blood stage antigens and the possibility of expressing recombinant proteins for immunisation and antiserum production. The first section of this thesis describes the immunochemical screening of a *P. yoelii* genomic library in the bacteriophage expression vector lambda gt11 using *P. yoelii* hyperimmune serum. Following isolation, one of the clones was used to immuno affinity select antibodies from *P. yoelii* hyperimmune serum. The purified antibodies immunoprecipitated a *P. yoelii* blood stage protein of 235kd and recognised rhoptries by indirect immunofluorescence. The gene sequences thought to code for part of a 235kd *P. yoelii* rhoptry protein are detailed and preliminary experiments using antibodies raised against the protein encoded by a fragment of cloned *P. yoelii* DNA are also described.

The second section of the thesis records attempts to identify individual *P. yoelii* blood stage antigens present in protective isoelectrically focussed fractions (De Souza and Playfair, 1988). It is not known whether the protective effects of focussed fractions are due to single proteins or the synergistic
action of more than one antigen. Further separation of
isoelectrically focussed material would enable immunisation
experiments using single antigens and combinations of proteins
recovered from protective, focussed fractions. Antisera to
individual proteins could be used to locate proteins by indirect
immunofluoresence or immunoelectron microscopy and allow
subsequent cloning of antigen coding genes. Isolation of
individual proteins from protective fractions has, so far, proved
unsuccessful, however, antibodies raised against protective pH4
and pH6 fractions suggest that the 230kd PMMSA and other
unrelated proteins are present in these fractions.
CHAPTER TWO.
MATERIALS AND METHODS.

2.01 PREPARATION OF P.YOELII DNA.

2.01.1 Plasmodium yoelii yoelii.

Plasmodium yoelii 17X is an avirulent line of P.yoelii yoelii, discovered in the Republique Centreafricain (Landau and Chabaud, 1965), which produces a mild infection in mice with clearance of parasites from the bloodstream in approximately three weeks. Line 17X initially prefers to invade reticulocytes; briefly switches preference to mature erythrocytes but by day six is again restricted to reticulocytes (Knowles and Walliker, 1980). The virulent line, YM, was derived from the avirulent line 17X, after a sudden increase in virulence of the parasite following storage (Yoeli et al, 1975. Walliker, 1981). The YM line also shows an initial preference for reticulocytes but by day four invades mature red blood cells and infections continue, uncontrolled, until mice die at approximately day seven to ten. Cloned lines of P.yoelii used in this laboratory were kindly provided by Dr. D. Walliker, University of Edinburgh. The YM line was maintained by serial passage in Balb/c mice.

2.01.2 Harvest of Parasitised Erythrocytes.

DNA was prepared using the method described by Martin et al (1971). Giemsa stained blood smears from three P.yoelii infected passage mice were examined and parasitaemia estimated. Blood from all three mice was pooled and diluted to 20ml with Alsever's
solution (70mM NaCl, 0.1mM glucose, 27mM NaHCO₃, 3.0mM citric acid). Five CD1 mice were injected intra peritoneally (i.p.) with 0.2ml of diluted blood parasitised to approximately 3%. Three days later when parasitaemias were approximately 30% pooled blood was diluted to 30ml with Alsever's solution. Two hundred microlitres of diluted blood parasitised to 5% was injected i.p. into 30 CD1 mice. On the following day 0.15ml of 40 mg.ml⁻¹ cylophosphamide (Koch Light Cat.No.1392-60) in isotonic saline was injected sub cutaneously (s.c.) into each mouse (Berenbaum, 1975). Two days later pooled blood from thirty mice was spun at 2000 rpm 4° for 10 min in a Chilspin centrifuge and the cell pellet washed and resuspended in PBS (0.13M NaCl, 0.3mM KCl, 8mM Na₂HPO₄, 1.5mM KH₂PO₄). Total red cell count was estimated using a haemocytometer. A Giemsa stained cytocentrifuge slide was examined, the total number of mouse lymphocytes calculated and the parasite : mouse lymphocyte ratio determined.

2.01.3 Extraction and purification of *P. yoelii* DNA.

Erythrocytes suspended in 50ml PBS were centrifuged at 2000 rpm for 10 minutes at 4° in a Chilspin centrifuge, resuspended in lysis buffer, (0.83% NH₄Cl, 0.17M TrisHcl pH8.0) incubated at 37° for 3 minutes and centrifuged at 4000 rpm for 10 minutes at room temperature in a benchtop centrifuge. The supernatant was discarded and the cell pellet resuspended in 10ml buffer A, (10mM TrisHcl pH8.0, 10mM EDTA, 10mM NaCl, 0.5% SDS). Freshly prepared proteinase K (Boehringer Mannheim Cat.No. 745723) in TE, (10mM
TrisHCl, 10mM EDTA pH 8) was added to 25μg.ml\(^{-1}\) and the suspension incubated at 37° for 17 hours. Nucleic acid was extracted three times in phenol equilibrated with buffer B, (500mM TrisHcl pH 8.0, 10mM EDTA, 10mM NaCl, 0.5% SDS) by swirling gently for 10 minutes. The phases were separated by centrifugation for 5 min at 1000rpm in a benchtop centrifuge at room temperature and the aqueous phase dialysed against buffer C, (50mM TrisHcl, 10mM EDTA 10mM NaCl) for 48 hours. When the absorbance of the dialysis buffer at 270nm reached 0.045 the preparation was treated with RNase A at 50 μg.ml\(^{-1}\) and incubated at 37° for 4 hours. SDS was added to a final concentration of 0.5% followed by proteinase K to 50μg.ml\(^{-1}\) and incubated overnight at 37°. DNA was extracted twice in phenol equilibrated with buffer B and dialysed for 48 hours against buffer D, (10mM TrisHcl pH 8.0, 0.5mM EDTA, 10mM NaCl). DNA was precipitated with a tenth volume 2.5M sodium acetate pH 6.2 and 2 volumes of 100% ice cold EtOH and gently spooled out using a glass rod. After washing in 70% EtOH the DNA was resuspended in TE and the final concentration adjusted to 0.2-0.5 mg.ml\(^{-1}\).

2.01.4 Calculation of mouse lymphocyte DNA contamination of \textit{P. yoelii} DNA.

The murine genome is approximately 200 times larger than the \textit{P. yoelii} genome therefore each lymphocyte contaminating the parasite DNA preparation will contribute 200 times more DNA than a single parasite. Infected mice were treated with
cyclophosphamide to minimise murine lymphocyte numbers but some residual cells were present in the collected blood.

Doubling dilutions of mouse genomic DNA and two dilutions of P. yoelii DNA were spotted onto Biodyne nylon membrane (Pall Ultrafine Filtration Corporation. Cat.No. BNN G82), denatured (1.5M NaCl, 0.5M NaOH), neutralised (3M Na acetate pH5.5) and baked at 80° for 1 hour. The membrane was prehybridised and hybridised in buffer containing 50% formamide (5x Denhardts [0.5% Ficoll MW 400,0000, 5% polyvinyl-pyrrolidone MW 40,0000, 5% BSA], 5xSSC [0.15M NaCl, 15mM sodium citrate], 50mM sodium phosphate pH6.5, 0.1%SDS, 250µg.ml⁻¹ denatured salmon sperm DNA, 50% formamide). One microgram of mouse genomic DNA was nick translated with ³²PdATP (Amersham International plc Cat.No.10204) using DNAsel and PolI (Rigby et al,1977). Nick translated DNA labelled to 5x10⁶ cpm was added to the hybridisation mix and the filter shaken overnight at 37°. The dot blot was washed under stringent conditions (0.1xSSC, 0.1% SDS) at 50° for 30 minutes and exposed to film. To assess lymphocyte DNA contamination, of the P. yoelii preparation autoradiographs were scanned using a densitometer and the radioactive signal from hybridised DNA on nitrocellulose filters estimated by scintillation counting.
2.02  **P.yoelii Genomic DNA Libraries.**

2.02.1  **P.yoelii** genomic library in lambda gtll.

Lambda gtll is an insertion vector which has the capacity to accept fragments of foreign DNA ranging in size from 0-4.8kb.

Insertion of foreign DNA into the unique EcoRl site enables expression of cloned sequences as lac\(^Z\) fusion proteins. Recombinants are recognised as white plaques when grown on plates containing the chromogenic indicator X-gal (5-bromo-4chloro-3-indolyl-\(\beta\)-D-galactopyranoside. Boehringer Mannheim Cat.No. 745-740). Fusion protein expression is induced with IPTG (isopropyl thio-\(\beta\)-D-galactopyranoside. Boehringer Mannheim Cat.No. 724815) from the bacteriophage \(\beta\)-galactosidase promotor. Phage are propagated in bacteria carrying the lacU169 mutation, a deletion complemented by the recombinant phage. Other mutations prevent restriction and modification of foreign DNA and the lon- mutation causes a protease deficiency, maximising the stability of recombinant proteins. Production of the lac repressor protein by the bacteria prevents leaky expression of recombinant proteins prior to IPTG induction (Young and Davis, 1983).

Lambda gtll arms, predigested with EcoRl and treated with calf intestinal phosphatase (CIP) to prevent vector religation, were used from a Stratagene kit. (Stratagene, Cat.No.GTll) The lyophilised arms were resuspended in TE to 1 \(\mu\)g.\(\mu\)l\(^{-1}\).

For insertion into lambda gtll, **P.yoelii** genomic DNA was partially digested with EcoRl under EcoRl\(^*\) conditions to generate random sized fragments. EcoRl recognises the palindromic sequence
GAATTC under normal conditions (10mM Mg$^{++}$, 50mM NaCl, 100mM TrisHCl pH7.5) but under conditions of low ionic strength, (2mM Mg$^{++}$, 10mM TrisHCl pH8.0), or elevated pH, the enzyme recognises only the integral four nucleotides of the sequence. It is not known exactly how the enzyme cuts nor whether an overhang is generated; fragments can be cloned into EcoR1 sites but cannot always be recovered by digestion with EcoR1. Timed digestion of DNA under EcoR1* conditions was monitored by electrophoresis of aliquots on 1% agarose gels and staining with ethidium bromide. DNA examined after 120 minutes digestion appeared to contain a good distribution of random sized fragments.

Ten nanograms of EcoR1* digested P.yoelii was mixed with 0.1µg lambda gt11 arms and 10x ligation buffer (0.66M TrisHCl pH7.6, 50mM MgCl$_2$, 50mM DTT, 10mM ATP) to a final volume of 5µl. Five units of T4 DNA ligase (New England Biolabs. Cat.No.202) was added and the ligation incubated overnight at 15°. One hundred nanograms of lambda gt11 arms were ligated in the same way to 10ng rheo test insert (2.5kb) supplied with the lambda gt11 kit. Reactions were terminated by incubation at 65° for 10 minutes.

Hohn and Murray (1977) and Enquist and Sternberg (1977) developed a method of in vitro packaging phage DNA utilising mutations in genes coding for major capsid genes of bacteriophage lambda. Head and tail assembly occurs separately and extracts from two cultures of phage with mutations in head and tail genes can be combined with recombinant lambda DNA in vitro to produce viable bacteriophage. Commercial in vitro packaging extracts were used to package recombinant DNA (Gigapack plus, Vector Cloning...
Systems, Cat.No.GP6-P). Five microlitres ligation mix was added to freeze thaw and sonicated packaging extracts and incubated at 22° for 2 hours. Packaged phage were diluted with 250μl SM buffer (100mM NaCl, 50mM TrisHCl pH7.5, 10mM MgSO₄.7H₂O, 0.01% gelatin) and 10μl CHCl₃ added. Five microlitres of Rheo test insert DNA, supplied with the kit was processed in exactly the same way.

Ten microlitres of diluted, packaged recombinant DNA and rheo test DNA was plated on agar with top agarose containing 10mM MgCl₂, 60μg.ml⁻¹ IPTG and 60μg.ml⁻¹ Xgal. Y1088 plating cells, supplied with the Stratagene kit, were used from an overnight culture grown in LB (1% tryptone 0.5% yeast extract 0.5% NaCl) plus 0.2% maltose. Plates were incubated overnight at 37° and the library titred.

2.02.2  *P.yoelii* genomic Dral library in pUC9.

pUC 9 is one of a series of bacterial plasmid vectors developed by Vieira and Messing (1982). The 2.77 kb insertion vector was designed for insertion mutagenesis and sequencing using universal primers. The recognition sequence of Dral is TTTAAA; the enzyme is expected to generate fragments with an average size of 0.5kb from *Plasmodium* DNA. The *P.yoelii* library was constructed by Dr. M.J. Lockyer by digesting genomic DNA to completion with Dral in buffer recommended by the enzyme manufacturer. Digested DNA was ligated into phosphatase treated pUC9 (Pharmacia Cat.No.050199) via the Smal site as described below (2.05.1).
2.02.3 **P.yoelii** genomic EcoRl library in pUC9.

EcoRl digestion of *Plasmodium* DNA is expected to produce fragments with an average size of 2.9kb. A *P.yoelii* library in pUC9 was prepared by Dr. A. Lewis by digesting genomic DNA to completion with EcoRl and ligation into the EcoRl site of the plasmid vector pUC9 (Vieira and Messing, 1982).

2.03 SCREEN OF LAMBDA gt11 AND PLASMID DNA LIBRARIES.

2.03.1 Immunochemical screen of lambda gt11 library.

Recombinant phage were screened with serum from mice hyperimmune to *P.yoelii* using the method detailed in section 2.10.4. This antiserum (with a titre of 1:50,000 by IIF) was raised by R.R. Freeman by infecting mice with *P. yoelii*, 17X, and challenging with *P.yoelii* YM (Freeman and Parish, 1981).

2.03.2 Screen of plasmid libraries by hybridisation.

Recombinant bacteriophage DNA containing the J7\(^{144}\) insert was prepared using the infection method described by Maniatis et al (1982, p.90). Insert DNA was isolated by double digestion with the enzymes KpnI and SstI in buffers recommended by the enzyme manufacturer followed by electroelution of the appropriate band from a 1% agarose gel and purification of the DNA on an Elutip column using buffers recommended by the manufacturer (Schleicher and Schuell Cat.No. NA010/2). A second double digestion using the
enzymes Banll and Pvull followed by electroelution and purification produced the J7\textsuperscript{144} fragment.

The Dral digested \textit{P. yoelii} genomic DNA library in pUC9 was plated onto LB agar plates containing ampicillin (50\mu g.ml\textsuperscript{-1}) after transformation of MAX Efficiency DH5α\textsuperscript{TM} competent cells (BRL Cat.No. 8258SA) with 2\mu l (0.04\mu g) of ligation mix using the transformation procedure detailed by the manufacturer. A total of 32,000 colonies on 20 82mm plates were transferred to Biodyne nylon membranes (Pall Ultrafine filtration Corporation Cat.No. BNNG82). After denaturation (1.5M NaCl, 0.5M NaOH), neutralisation (3M Na acetate pH5.5) and baking (80° 1 hour) the filters were shaken at 55° for 30 minutes in a proteinase K solution (0.2xSSC, 0.1% SDS, 50\mu g.ml\textsuperscript{-1} proteinase K) to remove bacterial debris. The filters were rinsed in 2xSSC, air dried and split into 2 batches of 10. Prehybridisation and hybridisation was carried out in 10ml buffer containing 50% formamide as described in the Biodyne manufacturer's handbook. One hundred nanograms of J7\textsuperscript{144} DNA was labelled with \textsuperscript{32}PdATP (Amersham International Cat.No. PB10204) by nick translation. Unincorporated nucleotides were separated from labelled DNA on a Nick Column using buffers recommended by the manufacturer (Pharmacia Cat.No. 17-0855-02). Five million cpm of nick translated J7\textsuperscript{144} probe/10ml hybridisation mix was added to each set of filters which were shaken overnight at 37°. Filters were washed at room temperature (2xSSC, 0.1% SDS) followed by a high stringency wash (0.1xSSC, 0.1% SDS) at 50° and exposure to film overnight.
Probe Oligo IV was a 21mer oligonucleotide corresponding to a short repeated nucleotide sequence present in pPyS6. The oligonucleotide was synthesised by Mr. H. Spence by the phosphate triester method using Biosyntech reagents (Biosyntech GmbH, Hamburg, Germany.) using a Milligen 7500 DNA synthesiser.

The EcoR1 digested P. yoelii genomic DNA library in pUC9 had been screened previously and this probe was stripped from the Biodyne nylon membranes by shaking them in 0.4M NaOH for 30 minutes at 42°. After rinsing in 2xSSC at room temperature the filters were exposed to film overnight to ensure all radioactive signal had been removed. A total of 16,000 colonies were probed with 100ng of oligo IV, 5'end labelled with 100μCi ³²PdATP (Amersham International plc. Cat.No. PB10168) using T₄ polynucleotide kinase (Pharmacia Cat.No. 27-073-01) in kinase buffer (10mM MgCl₂, 100mM TrisHcl pH7.6, 20mM 2-mercapto-ethanol) and incubated at 37° for 1 hour followed by 10 minutes at 65° to terminate the reaction. Filters were shaken in prehybridisation buffer (6xSSC, 1xDenhardts, 0.5% SDS, 100μg.ml⁻¹ salmon sperm DNA, 0.05% sodium pyrophosphate) for 1 hour and transferred to hybridisation buffer (6xSSC, 1xDenhardts, 20μg.ml⁻¹ tRNA, 0.05% sodium pyrophosphate) containing the labelled oligonucleotide and incubated overnight at 37°. The filters were washed (6xSSC, 0.05% sodium pyrophosphate) at 37° for 1 hour, followed by a second wash in the same buffer at 47° for 10 minutes and exposure to film overnight.
2.04 ANALYSIS OF RECOMBINANTS.

2.04.1 Analysis of lambda gt11 recombinants.

Bacteriophage plaques recognised by P. yoelii hyperimmune serum and visualised by $^{125}$I protein A were picked, rescreened three times with P. yoelii hyperimmune serum and plate lysate stocks prepared by plating $1 \times 10^5$ plaques per plate and washing phage into 5ml SM buffer. 50$\mu$l CHCl$_3$ was added to each stock which were titred and stored at 4°.

DNA was prepared from five, purified lambda gt11 recombinants by the infection method described by Maniatis et al (1982, p77). To determine P. yoelii insert sizes the DNA was digested with Kpni and SstI followed by BanII and PvuII, as described in section 2.03.2. The digested DNA was electrophoresed on a 1% agarose gel and stained with ethidium bromide.

The five purified bacteriophage clones were used to immunochemically select specific antibodies from P. yoelii hyperimmune serum. Selected antibodies were used in indirect immunofluorescence (IIF) studies on acetone fixed smears of P. yoelii parasitised erythrocytes and to immunoprecipitate $^{35}$S methionine labelled P. yoelii blood stage proteins. See Immunochemical Methods, section 2.10.

2.04.2 Analysis of pUC9 recombinants.

Thirteen colonies isolated by hybridisation with probe J7 from the Dral digested P. yoelii genomic library were picked and DNA prepared by a quick plasmid preparation (QPP) method
described by del Sal et al (1988). DNA was digested with HindIII and EcoR1, separated on a 1% agarose gel and stained with ethidium bromide. Overnight cultures of 4 recombinants of interest were streaked onto LB agar ampicillin (50μg.ml⁻¹) plates and grown overnight at 37°. Colonies were blotted onto Biodyne membranes and probed with nick translated J7²⁴⁴ as described in section 2.03.2. Isolated, single colonies from each of these plates were picked and used to seed cultures for large scale DNA preparation by the alkaline lysis method (Maniatis et al, 1982 p90). The four recombinant plasmids, pPyS6, pPyS7, pPyS8 and pPyS12 were mapped by digestion with the enzymes EcoR1, BamH1, HindIII and Pst1, plus double digests using EcoR1 and HincII. The DNA was electrophoresed on 1% agarose gels, depurinated (0.25M Hcl) and Southern blotted (Southern, 1975) onto Genescreen Plus nylon membrane (Du Pont NEN Products Cat.No. NEF976) by transfer in 0.4M NaoH (Reed and Mann, 1985). The filter was rinsed (2xSSC) and air dried. Immobilised DNA was incubated in prehybridisation buffer (50% formamide, 1% SDS, 1M NaCl, 10% dextran sulphate, 250μg.ml⁻¹ denatured salmon sperm DNA) for 1 hour, 5x10⁶ cpm of nick translated J7²⁴⁴ probe added /10ml mix and the membrane shaken at 37° overnight. The filter was washed under stringent conditions (2xSSC, 1% SDS, 65° 30 minutes and 0.1xSSC, room temperature 30 minutes) and exposed to film.

pUC9 recombinants hybridising with Oligo IV were picked and DNA prepared by the QPP method above was digested with EcoR1 and electrophoresed on a 1% agarose gel. Digested DNA was Southern blotted onto Genescreen Plus hybridisation membrane as above and
probed with oligoIV as described in section 2.03.2. Two recombinants, pPyE3 and pPyE8 were selected and DNA from both of these clones was prepared by the large scale alkaline lysis plasmid preparation method (Maniatis et al, 1982 p.90). pPyE8 insert DNA was prepared by EcoR1 digestion of DNA, electrophoresis on a 1% agarose gel, electroelution of a band of approximately 6kb and purification on an Elutip column.

2.04.3 Analysis of pGex-3x recombinants.

Colonies recognised by oligonucleotide probe IV were picked and grown in 2ml overnight cultures of LB broth containing 50μg.ml\(^{-1}\) ampicillin. One in ten dilutions of overnight cultures were shaken at 37° for 1 hour, induced with 1mM IPTG and shaken at 37° for a further 3.5 hours. Cells were harvested, resuspended in distilled water and an equal volume of polyacrylamide gel electrophoresis (PAGE) loading buffer (150mM TrisHcl pH6.8, 4% SDS, 20% glycerol, 0.2M DTT, 0.02% bromophenol blue) added. Samples were electrophoresed on 10% SDS polyacrylamide gels (Laemmli and Favre, 1973), stained with 0.05% Coomassie blue and expression of fusion proteins monitored by comparison to a preparation of induced, religated vector electrophoresed on the same gel.
2.05 MANIPULATION OF DNA FRAGMENTS BY SUBCLONING.

2.05.1 Subcloning into pUC9.

To enable sequencing of *P. yoelii* DNA fragments the insert from recombinant phage J7 was subcloned into pUC9. J7 insert DNA, isolated from lambda gt11 by digestion with Kpn1 and Sst1 followed by Ban11 and Pvu11 as described in section 2.03.2, was treated with the Klenow fragment of *E.coli* DNA polymerase 1 (Pharmacia Cat.No. 27-0928-19) to fill in any recesses at the ends of the fragment using the method described by Maniatis *et al* (1982, p113). A 2 fold molar excess of J7 insert DNA was ligated to commercially prepared Smal cut phosphatased pUC9 DNA (Pharmacia Cat.No.27-4918-01) using 1.5 units of *T₄* DNA ligase (New England Biolabs Cat.No. 202). The ligation was incubated overnight at 16°. Ligated DNA was transformed into Library Efficiency™ DH5α competent cells (BRL Cat.No. 8262SA) following the small scale transformation method described by the manufacturer.

2.05.2 Subcloning into the expression vector pGEX-3x.

*P. yoelii* insert DNA was prepared by polymerase chain reaction amplification (PCR) using the thermostable DNA polymerase (Taq polymerase) from the bacterium *Thermus aquaticus* (Chien *et al*,1976. Saiki *et al*,1985. Scharf *et al*,1986). Two 30bp oligonucleotide primers, synthesised by Mr. H. Spence, representing conserved sequences at the 3' and 5' ends of pPyS6, pPyS7 and pPyS8 sequences were used to amplify inserts from pPyS6, pPyS7 and pPyS8 using a Geneamp kit (Perkin Elmer Cat.No.
N801-0043) and a Hybaid Intelligent Heating Block (Hybaid Model HB 2024). Ten times concentrate reaction buffer and nucleotides were supplied with the kit and used according to manufacturers' instructions. DNA was denatured at 94°, primers were annealed at 37° and a complementary strand synthesised at 72°. Twenty five rounds of this cycle are predicted to produce a $10^6$ fold amplification of specific DNA sequences. Amplified DNA was electrophoresed on a 1% preparative agarose gel, electroeluted and purified on an Elutip column.

The pGex expression vectors were constructed by Smith and Johnson (1988) to direct the synthesis of foreign peptides in *E. coli* as fusions with the COOH-terminus of Sj26, a 26kd glutathione S-transferase encoded by the parasitic helminth *Schistosoma japonicum* (Johnson et al, 1989). Soluble fusion proteins can be purified from crude bacterial lysates by affinity chromatography on immobilised glutathione. Three vectors are available and the presence of a protease cleavage site enables the expressed protein to be separated from the Sj26 peptide after digestion and re-chromatography on the affinity column. Figure 2.1 shows a diagram of the pGex vector. The open reading frame of the pPyS6 insert required the use of pGex-3x for fusion protein expression. pGex-3x DNA was prepared by streaking cells from a glycerol stock onto LB agar containing 50μg.ml$^{-1}$ ampicillin and grown overnight at 37°. A single colony was used to seed a 400ml bacterial culture and vector DNA was prepared by the alkaline lysis method (Maniatis et al, 1982, p90). Ten micrograms of pGex-3x DNA was digested with Sma1 and purified
Nucleotide sequence and coding capacities of pGex vectors at the COOH terminus of Sj26

Figure 2.1  The pGex expression vector system. Reproduced by kind permission of Dr. K.S. Johnson, University of Cambridge, (Smith and Johnson, 1988).
on an Elutip column. Linear DNA was treated with 0.04u calf intestinal phosphatase (CIP) (Boehringer Mannheim Cat.No.713023) in 2 successive incubations at 37° in CIP buffer (50mM TrisHcl pH9.0, 1mM MgCl$_2$, 0.1mM ZnCl$_2$, 1mM spermidine) to dephosphorylate protruding 5' termini. The reaction was stopped by addition of 1xSTE (10mM TrisHcl pH8.0, 100mM NaCl, 1mM EDTA), 5μl 10% SDS and heating to 68° for 15 minutes. The DNA was purified on an Elutip column, precipitated and dissolved in TE. PCR amplified pPyS6 insert was digested with Hpal at 37° for 2 hours then 1M NaCl was added to the reaction mix to adjust the salt concentration to 100mM, followed by the addition of 1 unit of Ssp1 per μg DNA. Following purification on an Elutip column, a 2 fold molar excess of Hpal/Ssp1 digested pPyS6 DNA was ligated to Smal cut pGex-3x using T$_4$ DNA ligase in buffer specified by the manufacturer. Ligated DNA was transformed into DH5α MAX efficiency competent cells and plated onto 10 agar plates containing 50μg.ml$^{-1}$ ampicillin. Approximately 10,000 colonies were transferred to Biodyne membranes and treated with Proteinase K as described in section 2.03.2. Filters were probed, as previously detailed in section 2.03.2, with 100ng of $^{32}$P 5' end labelled oligonucleotide IV representing a repeat region found in pPyS6.

2.06 DNA SEQUENCING.

2.06.1 pPyJ7, pPyE3 and pPyE8.

The DNA sequence of *P. yoelii* DNA subcloned into pPyJ7, and initial sequence from pPyE3 and pPyE8 was obtained by the Sanger
chain termination method using a Sequenase kit (United States Biochemical Corporation Cat.No.70700) (Sanger et al,1977. Tabor and Richardson,1987). pUC forward and reverse universal primers (United States Biochemical Corporation. Reverse Cat.No. 70708 Forward Cat.No. 70706) were annealed to single stranded template DNA to initiate chain elongation. The sequence was visualised by the incorporation of of $^{35}\text{S} \text{dATP}$ (Amersham Cat.No.ST304) into the synthesised fragments. The 6% denaturing gels were fixed in 10% acetic acid, dried under vacuum on a slab gel dryer at 80° and exposed to film overnight.

2.06.2 pPyS6, pPyS7, pPyS8 and pPyE8.

\textit{P.yoelii} insert fragments in pPyS6, pPyS7 and pPyS8 were sequenced by the chain termination method using a Sequenase kit as above. A series of oligonucleotides, kindly prepared in house by Mr.H. Spence, were used to progress from the known \textit{J}7\textsuperscript{144} sequence in both directions. Figure 2.2 shows the position of the oligonucleotide sequences used for sequencing. The same oligonucleotides were used for initial sequence determination of pPyE8. Sequences were analysed using Microgenie software, version 5 (Queen and Korn, supplied by Beckman).
Figure 2.2 Oligonucleotides used to sequence pPyS6, pPyS7, pPyS8 and pPyS12 P. yoelii insert DNA.
2.07 GENOMIC SOUTHERN BLOTS.

2.07.1 P. yoelii Southern Blots.

Aliquots of P. yoelii DNA were digested to completion with EcoR1, Dral and Rsal. Digests were split into three, electrophoresed on 1% agarose gels and blotted onto Genescreen Plus membrane by the method described above. After a 1 hour prehybridisation in buffer recommended by the manufacturer, containing 50% formamide, 5x10^6 cpm of nick translated pPyJ7, pPyS6 or pPyE8 insert was added to each of the 3 filters. After an overnight incubation filters were washed under stringent conditions, as described in section 2.04.2 and exposed to film.

2.07.2 Cross Species Southern Blots.

Predigested genomic DNA from P. berghei, P. chabaudi and P. falciparum was kindly donated by Dr. C. Davies, Imperial College, Dr. N. Brown, NIMR and Dr. M. Lockyer, Wellcome Biotech. One microgram of P. yoelii genomic DNA was digested with Dral or Rsal and electrophoresed on 1% agarose gels. DNA was blotted onto Genescreen Plus membrane and hybridised with nick translated pPyS6 or pPyE8 insert as described above. Filters were washed in 2xSSC, 1% SDS at 37°, 42° or 65°.
2.08 NORTHERN BLOT ANALYSIS.

2.08.1 Electrophoresis of *P. yoelii* RNA.

*P. yoelii* total mRNA was prepared by Dr. V. Riveros-Moreno by lysing parasitised erythrocytes with NH$_4$Cl as described for DNA preparation in section 2.01.3 and extracting mRNA in guanidine HCl following the method of Deeley *et al* (1977).

All electrophoresis equipment was treated with a 0.01% solution of diethyl pyrocarbonate (Sigma D5758) to destroy RNase. All solutions were autoclaved and prepared using distilled water previously maintained at 80° for 8 hours. Thirty five micrograms of RNA was denatured with a 40% solution of glyoxal (Sigma G3140) and dimethyl sulphoxide (DMSO) (Sigma D5897) (1M glyoxal, 0.5% DMSO, 10mM Na$_2$HPO$_4$) and incubation at 50° for 1 hour. Sterile loading buffer (50% glycerol, 10mM Na$_2$HPO$_4$, 0.4% bromophenol blue) was added to samples which were loaded immediately onto a 1% agarose gel prepared with sterile 10mM Na$_2$HPO$_4$ pH7.0 (Maniatis *et al*, 1972 p.200). The RNA was electrophoresed in 10mM Na$_2$HPO$_4$ pH7.0 at 100V with constant recirculation of running buffer and was blotted onto Biodyne nylon membrane in 10XSSC.

2.08.2 Hybridisation of RNA.

Im mobilised RNA was prehybridised in 5ml buffer (5xDenhardtts, 5xSSC, 0.2% SDS, 250µg.ml$^{-1}$ denatured salmon sperm DNA). Denatured, nick translated pPyE8 insert DNA labelled to a specific activity of 2.5x10$^6$ cpm was added to the filter and a 500bp fragment of the *P. yoelii* PMMSA gene, known to recognise a
message of 7.4kb, a gift from Dr. A. Lewis, was nick translated and hybridised as a control. Membranes were shaken at 37° overnight, washed at 50° (5mM NaH₂PO₄, 1mM EDTA, 0.2% SDS) for 30 minutes, air dried and exposed to film for one week.

2.09 Antisera used in immunochromical analysis.

2.09.1 Anti pGex-3xS6 fusion protein antiserum.

An overnight culture of the pGex-3xS6 transformant was diluted 1:10 in 400ml of LB broth containing ampicillin and grown for 1 hour at 37°. IPTG was added to 0.1mM and the culture shaken at 37° for 3-5 hours. Cells were harvested, resuspended in 10ml PBS and passed 3x through a French press. TritonX-100 (BDH Ltd.) was added to 1% and the preparation spun at 15,000 rpm in an SS34 rotor for 5 minutes at 4°. The supernatant was mixed with 2ml 50% glutathione sulphur linked agarose (Sigma Cat.No. G4510) in PBS. The fusion protein was eluted by competition with freshly prepared 5mM reduced glutathione (Sigma Cat.No. 4251), in 50mM TrisHCl pH8.0. Glutathione agarose beads were washed in PBS and stored as a 50% suspension at 4°.

Purified pGex-3xS6 fusion protein was electrophoresed on a 10% preparative SDS polyacrylamide gel, lightly stained with Coomassie blue, destained and soaked in water for 30 minutes. The major fusion protein band was cut from the gel and ground finely in a pestle and mortar, marker proteins were run on a similar gel and processed in the same way for control immunisations. Table 2.1 shows the volumes and concentration of antigen used for the
<table>
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Control groups 6, 7 and 8 were immunised with marker proteins.

Table 2.1 Immunisation of mice with pGex-3xS6 fusion protein.
primary intra peritoneal immunisation of groups of four Balb/c female mice. Twenty micrograms of saponin or an equal volume of Freund's incomplete adjuvant was used per dose. For control immunisations gel was suspended in 0.85% saline. A boosting immunisation was given intra peritoneally on day 28 and serum from 2 mice in each group collected on day 42. Antibodies were titred by immunofluorescence as described in section 2.10.1.

Mouse antiserum raised against a pGex-2x Human Papilloma Virus fusion protein, a gift from Miss S. Comerford, was used as a control.

2.09.2 Anti peptide antiserum.

Two peptides, an 18mer and a 21mer, were kindly synthesised by Dr. R. Campbell using the "T-bag" or SMPS (simultaneous multiple peptide synthesis) method (Houghton et al, 1985. Houghton et al, 1986). The 18mer (P2) corresponds to the short repeated sequence found in the P.yoelii Dral DNA fragment in pPyS8. The 21mer (P1) represents a similar sequence found in the Dral fragment in pPyS6. Both peptides were coupled to the carrier keyhole limpet haemocyanin (KLH) using N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (Sigma Cat.No. E6383). Two milligrams of each peptide, dissolved in 40μl water was added to 10mg KLH dissolved in 1ml water. One hundred and twenty milligrams of carbodiimide in 150μl water was added and the solution mixed at room temperature for 1 hour. The coupled peptides were dialysed overnight against PBS.
and the concentration adjusted to 2mg protein/ml (Pfaff et al., 1982).

One hundred micrograms of each conjugate in 50μl PBS was mixed with an equal volume of Freund's complete adjuvant and used to immunise rabbits intra musculy (i.m.). Boosting doses of 20μg of each coupled peptide in Freund's incomplete adjuvant were injected i.m. on days 21 and 49. A fourth boost of peptide P1 was administered subcutaneously on day 69 at five sites using 20μg total protein / site. Antibody titres were monitored by IIF on acetone fixed P. yoelii preparations as described in section 2.10.1. Serum from the rabbit immunised with peptide P2 was collected on day 90 and from the rabbit immunised with peptide P1 on day 126.

2.09.3 P. yoelii monoclonal antibodies.

Monoclonal antibody (McAb) 25.77 which recognises a P. yoelii rhoptry protein of 235kd, was raised by intra peritoneal immunisation of mice with a fusion of myeloma cells and spleen cells from mice recovered from P. yoelii 17X infection and rechallenged 3 times with P. yoelii YM (Freeman et al., 1980b). An aliquot of hybridoma cells was grown up in RPMI 1650 (Flow Labs. Cat.No. 29-101-54) in CO₂ in air and 0.5ml of cells suspended in PBS were injected intra peritoneally into Balb/c mice previously treated with Pristane (Aldrich Chemical Co. Cat.No. T2,280-2). Ascitic fluid was recovered from mice, filtered and stored at -20°.
McAb 25.1 which recognises the 230kd \textit{P.yoelii} PMMSA and McAb 25.23 which recognises a 66kd protein present on all \textit{P.yoelii} blood stages were raised by Freeman \textit{et al} as described above (1980a, 1980b). McAb 25.41 and McAb 46.11 were raised against \textit{P.yoelii} YM blood stages and McAbs 45.2, 45.3, 46.4 and 46.6 were raised against \textit{P.yoelii} 17X proteins. All monoclonal antibodies reacted with \textit{P.yoelii} YM by IIF (R.R. Freeman, unpublished data).

2.09.4 \textbf{Anti \textit{P.yoelii} 230kd (PMMSA) IgG purification.}

Five millilitres of anti \textit{P.yoelii} 230kd (PMMSA) rabbit polyclonal antiserum was added to an equal volume of saturated ammonium sulphate and mixed at room temperature for 1 hour. After centrifugation in an SS34 rotor at 12,000 rpm for 15 minutes the pellet was resuspended in 20mM bis-tris propane pH6.4 (Sigma Cat.No.B-6755) and dialysed against the same buffer overnight. The dialysed sample was applied to the ion exchange resin CL-4B (Pharmacia Cat.No. 17-0150-01) on a Pharmacia fast protein liquid chromatography (FPLC) system in 20mM bis-tris propane with a flow rate of 1ml.min$^{-1}$. Column fractions were collected and those containing material absorbing at 280nm were assayed for the presence of antibody by analytical SDS PAGE under reducing and non-reducing conditions (Laemmli and Favre, 1973). Fractions containing antibody were pooled and concentrated using a Minicon concentrator (Amicon Macrosolute Concentrators Cat.No. B-15). Protein concentration was measured using a Pierce BCA protein assay kit (see section 2.11.1).
2.09.5 Antisera against isoelectrically focussed *P. yoelii* proteins.

Antisera against isoelectrically focussed (IEF) *P. yoelii* proteins were prepared by Mr. J. B. De Souza, Middlesex Hospital. Rabbits were immunised with 500µg of isoelectrically focussed protein in Freund's complete adjuvant and boosted five times with the same concentration of protein in Freund's incomplete adjuvant. Mouse antisera were prepared in the same way using 50µg antigen per immunisation. Where the protein concentration of IEF fractions was low, animals were immunised with more than one fraction, eg. pH4.2-4.4, or a pool of fractions eg. pH6 pool pH6.0-6.8.

2.10. IMMUNOCHEMICAL ANALYSIS.

2.10.1 Indirect Immunofluorescence.

*P. yoelii* antigen slides were prepared by resuspending mouse erythrocytes, parasitised to 50%, in 200 volumes of PBS. The diluted cells were dropped into preformed wells on a microscope slide, air dried and stored at -20° in sealed bags containing silica gel. Premade antigen slides were thawed at room temperature in a humid box containing tissues soaked in PBS, air dried, dipped in acetone and allowed to dry. Ten microlitres of each selected antibody solution was dropped into a well and the slide incubated in the humid box for 30 minutes. After washing in PBS, a species specific FITC (fluorescein isothiocyanate conjugated) second antibody (Sigma) was added to each well and
incubation in the humid box continued for a further 30 minutes. Slides were washed in PBS, air dried and dipped in acetone before mounting in 50% PBS/glycerol (Voller and O'Neill, 1971. Freeman et al, 1980a). Slides were examined using a Leitz Ortholux 2 microscope with incident light fluorescence.

2.10.2 Immune precipitation.

P yoelii proteins were labelled with $^{35}$S methionine and precipitated with antiserum followed by staphylococcal Protein-A linked to sepharose (Kessler, 1975. Holder and Freeman, 1984b). Mouse erythrocytes parasitised to 50% were washed in PBS followed by 1x RPMI 1640 (Flow Labs. Cat.No.12-604-54) containing all amino acids except methionine. Glutamine was added immediately prior to use. (Selectamine Kit RPMI 1640 Flow Labs. Cat.No. 300-7402). Packed cells were resuspended to 1.5ml in 1x RPMI 1640 containing all amino acids except methionine and 5% foetal calf serum (Flow Labs. Cat.No. 29-101-54). Glutamine was added immediately prior to use. One hundred and fifty microCuries of $^{35}$S methionine (Amersham International plc. Cat.No. SJ204) was added to the cell suspension in a 50ml tissue culture flask which was incubated in 5% CO$_2$ in air at 37$^\circ$. Five microlitre aliquots were removed hourly, treated with a bleach mixture (1N NaOH, 1mg.ml$^{-1}$ cold methionine, 5% w/v 100 vol H$_2$O$_2$) to bleach any haemoglobin and proteins precipitated using 25% trichloracetic acid (TCA). $^{35}$S methionine incorporated into precipitated parasite proteins was measured by scintillation
counting. When $^{35}$S methionine incorporation had ceased, the cells were washed with 1x RPMI 1640 containing 5x concentration cold methionine, aliquoted, quick frozen in a dry ice/methanol bath and stored at -80°. For immuneprecipitations the preparations were thawed on ice and 200µl of solubilising solution added (50mM TrisHcl pH8.0, 5mM EDTA, 5mM EGTA, 5mM iodoacetamide, 1mM PMSF, 0.1mM TLCK, 1% NP40). The cells were pipetted up and down to break up the pellet, incubated on ice for 5 minutes and centrifuged at 15,000 rpm in an Eppendorf centrifuge for 5 minutes at 4°. The supernatant was aliquoted, made up to 200µl with buffer A (50mM TrisHcl pH8.0, 0.5M NaCl, 5mM EDTA, 0.5% NP40, 1mg.ml$^{-1}$ BSA) and 5µl antiserum added. The samples were rotated at 4° for 30 minutes before adding 50 µl Protein-A Sepharose (Fermentech Ltd. Cat.No. CL-4B) equilibrated in buffer A. Mixing was continued for a further 30 minutes at 4° followed by centrifugation at 15,000 rpm in an Eppendorf centrifuge for 3 minutes. The sepharose pellets were washed 3 times in buffer A and twice in buffer B (50mM TrisHcl pH8.0, 0.5mM EDTA, 0.5% NP40). Precipitated, $^{35}$S labelled parasite proteins were released from the sepharose beads by addition of 50µl PAGE loading buffer and heating samples to 70° for five minutes. Samples were centrifuged at 15,000 rpm in an Eppendorf centrifuge for three minutes and the supernatants electrophoresed on 10% and 5% polyacrylamide gels in the presence of SDS (Laemmli and Favre,1973). Proteins were fixed in destain solution (7% glacial acetic acid, 20% methanol) for 30 minutes and treated with Enhance (Dupont Biotechnology Systems Cat.No. NEF-981) for 1
hour. Gels were rocked in water at room temperature for 30 minutes, dried under vacuum on a slab gel dryer at 70° and exposed to film at -80°.

For depletion experiments, immuneprecipitation of the 230kd PMMSA was carried out as described above using rabbit polyclonal anti 230kd IgG. The supernatant was retained following centrifugation of the protein-A sepharose and a second immuneprecipitation carried out using anti IEF fraction antiserum.

2.10.3 Western Blot Analysis.

Western blots of pGex-3xS6 fusion proteins were carried out by electrophoresing E.coli lysates of 12 recombinants plus one lysate of religated pGex-3x vector, expressing the Sj26 peptide, on 10% SDS gels and blotting onto nitrocellulose using a Sartoblot Semidry Electroblotter (Sartorius GmbH Model SM 175 56) at 4mA.cm⁻² for 15 minutes, using buffers recommended by the manufacturer. The filters were blocked in 3% BSA in PBS for 30 minutes and rocked in either a 1 : 500 dilution of P.yoelii hyperimmune serum or a 1 : 100 dilution of monoclonal antibody 25.77. Filters were washed in wash buffer (1xPBS, 0.5% Tween 20) and rocked in second antibody, in both cases sheep anti-mouse IgG alkaline phosphatase conjugate, (Sigma Cat.No.F6257) for 1 hour. After rocking in wash buffer, antibody binding was visualised with NBT/BCIP colour reaction solution (4mM MgCl₂, 0.1mg.ml⁻¹ nitro blue tetrazolium (Sigma Cat.No.N6876), 0.05mg.ml⁻¹

For western blots on total P.yoelii parasite lysates, samples were separated on 5% SDS polyacrylamide gels and blotted onto nitrocellulose using a semidry electroblotter as above. The TX-100 soluble lysate, a gift from Mr J.B. De Souza, was prepared as described in section 2.11.1 (De Souza and Playfair 1988). Total parasite lysate was a crude preparation of P.yoelii parasitised erythrocytes pelleted and quick frozen immediately after collection. A cocktail of protease inhibitors was dripped onto each lysate as it thawed on ice (8mM PMSF in isopropanol, 20mM EDTA, 10μg aprotinin, 10μg pepstatin, 10μg chymostatin, 20μg leupeptin). A crude mouse erythrocyte lysate was prepared as a control. Western blots were blocked with 3% BSA in PBS and rocked in a 1 : 50 dilution of either antibody selected from P.yoelii hyperimmune serum using the pGex-3xS6 fusion protein, or antibody selected from rabbit anti PI peptide antiserum as described in section 2.10.4. Antibody binding was visualised, as detailed above, by an alkaline phosphatase conjugated second antibody and NBT/BCIP colour reagents.

2.10.4 Immunescreen of P.yoelii genomic DNA library in lambda gt11.

One hundred thousand recombinant phage, estimated to contain inserts of approximately 1kb, representing 5 P.yoelii genome
equivalents, were plated on LB amp plates in Y1088 plating cells and top agarose containing 10mM MgCl$_2$ and incubated overnight at 30°. Plates were overlaid with dry nitrocellulose circles (Schleicher and Schuell BA85) previously soaked in 10mM IPTG and incubated for 2 hours at 37°. Filters were washed in wash buffer (10mM TrisHcl pH9.6, 150mM NaCl, 0.05% Tween 20) for 10 minutes at room temperature, followed by equilibration in antibody diluent (10mM TrisHcl pH9.6, 150mM NaCl, 3% BSA) for 30 minutes at room temperature. Filters were then rocked in a 1:2500 dilution of _P.yoelii_ hyperimmune serum for 1 hour at room temperature and washed in three changes of wash buffer at room temperature. Colonies recognised by immune serum were visualised by rocking filters in wash buffer containing 10$^6$ dpm $^{125}$I protein A (Amersham International plc. Cat.No. IM 144), followed by three washes and exposure to film overnight at -80° (Young and Davis, 1983).

2.10.5 Antibody selection.

Following the method of Ozaki et al (1986), five purified bacteriophage clones were grown and induced as described in section 2.03.1 except that filters were flipped and plates incubated for a further 2 hours at 37°. Individual filters were washed for 10 minutes in wash buffer (10mM TrisHcl pH9.6, 150mM NaCl, 0.05% Tween 20), followed by a 10 minute wash in antibody diluent (10mM TrisHcl pH9.6, 150mM NaCl, 3% BSA, 0.01% Na azide). Filters were rocked for 1 hour in _P.yoelii_ hyperimmune serum
diluted 1:50 in antibody diluent. After 3 washes, specific antibodies were eluted from each filter in 10ml elution buffer (0.2M glycine, 0.15M NaCl, 0.05% Tween 20 pH2.8). Each eluate was immediately neutralised with 8mg Tris.ml\(^{-1}\). Solutions were concentrated to 1ml volumes in a Minicon concentrator (Amicon Macrosolute Concentrators Cat. No B-15), Na azide added to 0.01% and the solutions stored at 4°.

Antibody selection using the pGex-3xS6 fusion protein was carried out by electrophoresing the fusion protein on 10% preparative SDS polyacrylamide gels and blotting onto nitrocellulose using a semidry electroblotter. Filters were blocked in 3% BSA in PBS and rocked in either a 1 : 50 dilution of \textit{P.yoelii} hyperimmune serum or a 1 : 50 dilution of anti P1 peptide antiserum, washed in wash buffer and specific antibodies eluted as described in the previous section.

2.11 ANALYSIS OF ISOELECTRICALLY FOCUSED BLOOD STAGE \textit{P.YOELII} PROTEINS.

All isoelectrically focussed (IEF) protein fractions were prepared by Mr. J. B. De Souza, Middlesex Hospital. Lysates of \textit{P.yoelii} YM proteins were obtained by saponin lysis of parasitised erythrocytes and separated by isoelectric focussing on a broad range ampholine column, pH 3.5-10.0 (De Souza and Playfair 1988).
2.11.1 Calculation of protein concentration of IEF fractions.

The protein concentration of IEF fractions was calculated using a Pierce BCA protein assay kit (Pierce, (UK) Ltd. Cat.No. 23225) using the "micro" protocol detailed by the manufacturer.

2.11.2 Preparation of TritonX-100 soluble $^{35}$S methionine labelled P.yoelii proteins.

Erythrocytes from 10 P.yoelii YM infected mice with parasitaemias of approximately 65% with 50% schizonts were washed in PBS and parasite proteins labelled with $^{35}$S methionine as described in section 2.10.2 and a lysate was prepared by the method of Playfair and De Souza (Playfair and De Souza 1986. De Souza and Playfair 1988). Following metabolic labelling, parasitised erythrocytes were lysed in 0.01% saponin (BDH Cat.No. 44092) in PBS at 37° for 30 minutes and washed in PBS until the supernatant was clear. The pellet was resuspended in 5 volumes of extraction buffer (0.5% TritonX-100, 50mM Tris-Hcl pH8.0, 5mM EDTA, 20mM iodoacetamide, 5mM PMSF, 1µg.ml$^{-1}$ pepstatin, 50µg.ml$^{-1}$ trasylol, 20µg.ml$^{-1}$ leupeptin) and incubated at 4° for 3 hours. Debris was removed by centrifugation at 8000g for 10 minutes and the supernatant dialysed overnight against PBS.

2.11.3 IEF fractionation of $^{35}$S methionine labelled TX-100 soluble P.yoelii lysate.
The $^{35}$S methionine labelled TX-100 soluble proteins were fractionated by Mr. J. B. De Souza on a broad range ampholine column with a gradient of pH3.5-10.0. Twenty four fractions were recovered for analysis by SDS PAGE and immuneprecipitation. Fraction numbers and their corresponding pH values are listed below.

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2.11.4 Fast protein liquid chromatography (FPLC) separation of IEF fractions.

A Superose 6 gel filtration column (Pharmacia Cat.No. 17-0537-01), on a Pharmacia FPLC system, was used to further separate pH4.2-4.4 IEF fraction IF18-7. The cross linked, agarose based medium has an exclusion limit for globular proteins of
approximately $4 \times 10^7$ MW with an optimal separation range of 500-5$x10^6$ MW. The sample was freeze dried, resuspended in 0.1M ammonium bicarbonate and applied to the column which had been equilibrated in the same buffer. The flow rate through the column was 0.5ml 0.1M ammonium bicarbonate per minute. One millilitre fractions were collected, freeze dried and resuspended in PBS for injection into mice.

IEF fraction IF18.8, pH4.4-4.6, was analysed by FPLC using Superose 12 (Pharmacia Cat.No. 17-0538-01) which has an exclusion limit for globular proteins of $2 \times 10^6$ MW and an optimal separation range of 1000-3$x10^5$ MW. The sample was vacuum dried, resuspended in 0.1M ammonium bicarbonate and filtered through a 0.22 micron cellulose acetate filter (Costar SpinX centrifuge filter unit, Costar, Cambridge, Mass. 02139) and the ammonium bicarbonate soluble material run on a Superose 12 column with a flow rate of 0.5ml 0.1M ammonium bicarbonate per minute. The insoluble material was washed in PBS, dissolved in 70% formic acid and separated on a Superose 12 column with a flow rate of 0.5ml 70% formic acid per minute. One millilitre fractions were collected from both columns, freeze dried and resuspended in PBS for injection into mice.

2.11.5 High pressure liquid chromatography (HPLC) separation of IEF fractions.

A pool of pH4 IEF fractions (pH4.0-4.8) was analysed by reverse phase HPLC on a C4 column using a WISP 710B Milipore system. The sample was dissolved in 0.1% trifluoracetic acid and
separated in a gradient of acetonitrile. Fractions corresponding to protein peaks were collected, vacuum dried and resuspended in PBS for PAGE which was carried out as described in section 2.04.3.

2.11.6 T helper cell assay.

The T helper cell assay was performed by Mr. J. B. De Souza, Middlesex Hospital. Balb/c x C57Bl F1 hybrid mice were immunised with half the material recovered from the Superose 6 separation of IEF fraction IF18-7 and boosted with the remaining material after 14 days. Three weeks later mice were injected intravenously with $10^5$ P. yoelii parasitised erythrocytes coated with the hapten trinitrophenyl (TNP) or with TNP coated normal mouse erythrocytes. Four days later, the spleens of immunised mice were assayed for direct anti TNP plaque forming cells as described by Playfair et al (Playfair et al, 1977. De Souza and Playfair, 1988).
RESULTS AND DISCUSSION.
CHAPTER THREE.
ISOLATION OF A SMALL FRAGMENT OF *P. yoelii* DNA CODING FOR A PORTION OF AN APICAL COMPLEX ANTIGEN.

A number of *Plasmodium* antigens have been identified using immune serum to screen recombinant bacteriophage induced to express fragments of parasite antigens as fusion proteins (eg. Kemp *et al*, 1983. Ozaki *et al*, 1986. Kemp *et al*, 1987b. Coppel *et al*, 1987. Smythe *et al*, 1988. Galinski and Barnwell, 1989). Freeman and Parish demonstrated that hyperimmune serum, prepared from mice that had been challenged after recovery from *P. yoelii* infection, was highly protective and that protective antibody appeared to act against schizont and/or merozoite stages of the parasite (Freeman and Parish, 1981). In order to study antigens targeted by the murine immune response to *P. yoelii* infection, a bacteriophage expression library containing fragments of blood stage *P. yoelii* genomic DNA was immunochemically screened with this *P. yoelii* hyperimmune serum.

3.01 *P. yoelii* genomic DNA preparation.

Using the alkaline lysis method of Martin *et al*, (1971), DNA was prepared from *P. yoelii* YM infected mouse erythrocytes. The average parasitaemia of five randomly selected, infected mice was 36% on day three when blood was collected for DNA preparation. The total erythrocyte count of the pooled blood from thirty mice was calculated to be $9.6 \times 10^{11}$ using a haemocytometer. From a Giemsa stained cytocentrifuge preparation the total lymphocyte count was calculated to be $1.2 \times 10^6$. The total parasite
count was $1.08 \times 10^{12}$ and the parasite to erythrocyte ratio was $9.5 \times 10^5$ parasites for every mouse lymphocyte. Total DNA yield was approximately 0.8mg and was diluted to 0.275mg.ml$^{-1}$.

3.02 Estimation of mouse lymphocyte DNA contamination of P. yoelii DNA.

Although mice were treated with cyclophosphamide to minimise lymphocyte numbers, the P. yoelii DNA preparation was monitored for mouse DNA contamination. The signal density from dot blot hybridisation of nick translated genomic mouse DNA to dilutions of homologous DNA, or the newly prepared P. yoelii DNA, was compared. Estimations of autoradiograph signal either by eye or by densitometer scan suggested mouse DNA contamination of between 3% and 6% as shown in figure 3.1.

These results were confirmed by scintillation counting of radioactive signal from the nitrocellulose filters carrying immobilised, hybridised DNA.

3.03 Lambda gt11 library preparation.

P. yoelii genomic DNA was digested with EcoR1 under EcoR1* conditions to produce fragments suitable for cloning into lambda gt11. Timed digestion showed a 120 minute incubation to produce DNA fragments covering a wide molecular weight range which were ligated into lambda gt11. A Rheo test insert, supplied with the lambda gt11 kit was used as a control. Following packaging,
Figure 3.1 Dot blot to estimate mouse DNA contamination of \textit{P.yoelii} genomic DNA.
recombinant bacteriophage were used to infect *E.coli* strain Y1088. Packaging efficiencies for the Rheo test insert were 1.5x10^3 plaque forming units (pfu) per microgram of DNA and for EcoR1 *P.yoelii* DNA fragments, 7.2x10^7 pfu.µg⁻¹ DNA.

The titre of the lambda gt11 library containing *P.yoelii* EcoR1 inserts was calculated at 5.4x10^5 pfu.ml⁻¹.

3.04 **Immunescreen of a *P.yoelii* genomic DNA library in lambda gt11**.

Approximately 20,000 recombinant lambda gt11 bacteriophage containing *P.yoelii* genomic DNA inserts were immunochemically screened with *P.yoelii* hyperimmune serum and clones expressing polypeptides recognised by the antiserum were visualised by ^125^I protein-A. The immunescreen produced 121 recombinant bacteriophage clones which were plaque purified three times, rescreening each time with *P.yoelii* hyperimmune serum. Phage stocks were prepared and titred for all 121 clones. Figure 3.2 is an example of the variable signal produced by different clones when immunescreened with *P.yoelii* hyperimmune serum. The variation may reflect quantities of recombinant protein produced by bacteriophage due to many factors such as messenger RNA or protein stability, or the toxicity of recombinant proteins resulting in restricted growth of the *E.coli* host or the bacteriophage. Alternatively, the differential sequence intensity observed could be due to high concentrations of specific antibody in immune serum related to the presence of highly antigenic
repeated amino acid sequences which are found in many malaria proteins (Weber, 1988).

One hundred and fourteen high titre stocks gave a positive signal when rescreened with hyperimmune serum and all gave a negative result when immunescreened with normal mouse serum as shown in figures 3.3 and 3.4.

3.05 Analysis of five recombinant bacteriophage clones.

Five high titre recombinant lambda gt11 clones containing EcoR1* P.yoelii inserts were selected for further study and recombinant DNA was prepared from each stock by the infection method (Maniatis, 1982, p.77).

The exact recognition site of EcoR1 used under EcoR1* conditions is unknown but insert DNA can rarely be recovered by EcoR1 digestion of recombinants. The P.yoelii EcoR1* fragments could be separated from bacteriophage DNA by BanII and PvuII digestion but the complicated restriction pattern produced by digestion of DNA with these enzymes made recognition and recovery of insert DNA difficult. Prior digestion with the enzymes KpnI and SstI, electroelution and purification of DNA followed by BanII and PvuII digestion was used to identify P.yoelii insert fragments, figures 3.5, 3.6a and 3.6b. The recognition sites of KpnI and SstI flank the EcoR1 insert site in lambda gt11, producing a 2.08kb fragment when used to digest non-recombinant vector. The increase in size of this fragment, following
Figure 3.2 Example of the variable signal intensity produced when purified lambda gt11 clones were rescreened with *P. yoelii* hyperimmune serum.
Figure 3.3 Purified lambda gt11 clones rescreened with P. yoelii hyperimmune serum.
a serum dilution 1:50
1 week exposure to film

Figure 3.4 Purified lambda gt11 clones screened with normal mouse serum.
Figure 3.5 Schematic to show the position of recognition sites of KpnI, SstI, BanII and PvuII enzymes used to excise *P. yoelii* DNA fragments from lambda gt11 recombinants.
Figure 3.6  KpnI SstI and BanII PvuII digests of lambda gt11 recombinants.
digestion of recombinant DNA with the same enzymes, represents the size of inserted DNA.

The second double digest using BanII and PvuII was intended to release the *P. yoelii* DNA with the addition of 6bp of *lacZ* DNA to the 5' end and 19bp to the 3' end of each insert. However, following these two double digests, the actual size of recovered insert fragments was always larger than had been predicted from KpnI SstI digestion. This anomaly was later shown to be due to the failure of the BanII enzyme to digest the DNA to completion. The consistent appearance of bands of 0.7kb and 0.9kb, following BanII, PvuII digestion of each KpnI, SstI fragment suggested they were vector derived, figure 3.7. The remaining bands, approximately 300bp in clone E15, 900bp in F8, 2.3kb in I7, 500bp in J7 and 450bp in Z20 were recovered by electroelution of digested DNA, nick translated and used to probe Rsal digested *P. yoelii* genomic DNA that had been transferred to nylon hybridisation membrane.

All *P. yoelii* inserts tested, except that from clone Z20, hybridised to genomic DNA digested with Rsal as shown in figures 3.8 and 3.9. The nick translated insert recovered from clone J7 hybridised to seven *P. yoelii* Rsal fragments of 4.5kb, 3.3kb, 2.5kb, 1.71kb, 1.35kb, 1.21kb and 1.15kb, figure 3.8.

Insert DNA from clones E15 and I7 recognised a band of 1.175kb and clone F8, fragments of 4.6kb, 2.5kb and 1.375kb, figure 3.9.

The reason for the multiple hybridisation patterns on Southern blots of genomic *P. yoelii* DNA probed with J7 and F8
Figure 3.7  
Kpn1 SstI, BanII PvuII preparative digests of lambda gt11 recombinant J7.
Figure 3.8  Southern blot of Rsal digested P.yoelii genomic DNA probed with nick translated J7 insert DNA.
Southern blot of Rsal digested P. yoelii genomic DNA probed with nick translated E15, F8 and I7 insert DNA.

1% agarose gel
Filters washed at high stringency
Overnight exposure to film
inserts is unknown. A possibility is that Rsal digestion of
*P. yoelii* genomic DNA was not complete, although the ethidium
bromide stained DNA, examined after electrophoresis, appeared to
have been digested to completion. Other alternatives are that the
sequences used as probes contain repeat motifs; or that the gene
containing the DNA fragments used as probes are present as more
than one copy in the *P. yoelii* genome.

Further study of clone Z20 was abandoned after the
failure of insert DNA to hybridise to *P. yoelii* genomic DNA on
Southern blots.

3.06 Indirect immunofluorescence (IIF) using antibodies
selected from *P. yoelii* hyperimmune serum.

To determine the location of antigens encoded by the
cloned fragments of *P. yoelii* DNA, monospecific antibodies were
immunoaffinity selected from hyperimmune serum by the induced
bacteriophage clones E15, F8, I7 and J7 (Ozaki et al, 1986).
Selected antibodies were used in indirect immunofluorescence
assays on acetone fixed preparations of *P. yoelii* parasitised

Antibodies selected from hyperimmune serum by clone J7
produced an IIF pattern suggestive of binding to an antigen
located in or around rhoptries as shown in figure 3.10a. This
characteristic, punctate pattern of fluorescence has identified
numerous *P. falciparum* apical complex antigens (Howard et al, 1984.
Figure 3.10a  Indirect immunofluorescence on an acetone fixed P.yoelii preparation using antibodies selected from P.yoelii hyperimmune serum by lambda gt11 recombinant J7.

Figure 3.10b  Indirect immunofluorescence on an acetone fixed P.yoelii preparation using monoclonal antibody 25.77.

3.07 Immune precipitation using antibodies selected from \textit{P. yoelii} hyperimmune serum.

To determine the size of the \textit{P. yoelii} antigen recognised by antibodies selected from hyperimmune serum by clone J7, the antibodies were used to immune precipitate \textsuperscript{35}S methionine labelled \textit{P. yoelii} proteins. An autoradiograph of immune precipitates separated by SDS PAGE shows the antibodies to recognise a 235kd protein, possibly a doublet, figure 3.11. The size and appearance of the material recognised by the J7 selected antibodies resembles closely the protein immune precipitated by McAb 25.77, (Freeman et al, 1980a).

3.08 Subcloning and sequencing of J7.\textsuperscript{144}

In order to prepare J7 DNA on a large scale and to enable plasmid primed sequencing, the 500bp insert from lambda gt11 clone J7 was subcloned into pUC9. Enzyme digests of the recombinant plasmid pPyJ7, revealed the presence of two EcoR1
Figure 3.11  Immune precipitation of $^{35}$S methionine labelled *P. yoelii* proteins.
fragments of approximately 360bp and 150bp, shown in figures 3.12a and 3.12b. Sequence data showed the 360bp fragment to be lacZ derived. Consistent failure of the BanII enzyme to cut had resulted in excision of a fragment of 500bp containing P. yoelii DNA but extending to an upstream PvuII site 360bp into the lacZ gene. The failure of BanII to cut successfully explains why apparent insert sizes were always larger than those estimated following Kpnl SstI digestion of lambda clones, figure 3.13. In the case of lambda gt11 clone J7, EcoR1 sites were regenerated after insertion of the EcoR1 fragment, the P. yoelii insert could, therefore, have been excised from lambda gt11 with a single EcoR1 digest.

The plasmid clone, pPyJ7, contained 144bp of P. yoelii sequence with an AT content of 75%. There is a stop codon at position 104 and from the derived amino acid sequence it was deduced that the recombinant polypeptide expressed in the original lambda gt11 clone, J7, contained 35 amino acids encoded by P. yoelii DNA, figure 3.14.

3.09 Discussion.

An immunochemical screen of a lambda gt11 library containing EcoR1 fragments of P. yoelii genomic DNA produced a number of clones one of which, J7, was selected for further study. The sequence of the 144bp DNA fragment from the plasmid subclone pPyJ7, has an AT content typical of P. yoelii DNA and the peptide encoded by this fragment appears to represent an
Figure 3.12  Excision of *P. yoelii* insert DNA from pUC9 plasmid clone pPyJ7.
Figure 3.13 Map of pUC9 plasmid clone pPyJ7.
Figure 3.14 DNA sequence of *P. yoelii* insert from plasmid clone pPyJ7.

A 235kd rhoptry protein was isolated from *P. yoelii* YM infected erythrocytes by Holder and Freeman using monoclonal antibody 25.77 Holder and Freeman, 1981. Oka *et al.*, 1984). The 235kd protein and the monoclonal antibodies raised against it protected mice against *P. yoelii* YM challenge infection (Freeman
et al, 1980a. Holder and Freeman, 1981). Immunisation of mice with protein or antibody apparently altered the cell preference of merozoites, which normally invade mature erythrocytes after day 4, causing their restriction to reticulocytes after which infections were cleared with parasitaemia following a course similar to the non-lethal 17X strain of P. yoelii (Knowles and Walliker, 1980. Freeman et al, 1980a. Holder and Freeman, 1981). It can therefore be assumed that the 235kd rhptry protein is in some way involved in erythrocyte invasion by P. yoelii merozoites but it is not known why immunisation of mice with the 235kd protein or passive transfer of monoclonal antibody 25.77 causes P. yoelii YM infection to be restricted to reticulocytes. Holder and Freeman speculated that antibodies against the 235kd rhptry protein may reduce the invasiveness of merozoites below that required for successful penetration of mature erythrocytes, assuming that invasion of reticulocytes by P. yoelii is a more efficient process than invasion of mature erythrocytes, perhaps dependent on the membrane properties of target cells (Holder and Freeman, 1984c). Studies by Fahey and Spitalny (1984) on the host cell preference of P. yoelii YM and 17X NL, showed that both strains are capable of invasion of reticulocytes or mature erythrocytes although 17X preferentially invades reticulocytes. Fahey and Spitalny suggested that the adaptive mechanism may be due to selection of a pre-existing subpopulation of parasites capable of invading mature erythrocytes or may be the result of a change in one or several genes that, under certain conditions, enable parasites to invade an alternative host cell type. The
primary and secondary structure of the 235kd protein derived from the gene sequence, together with analysis of epitopes recognised by protective antibody, may help to identify regions of the molecule involved in host cell recognition. Freeman and Holder (1984c) have detected a protein in *P. yoelii* 17X which is homologous to the 235kd protein isolated from *P. yoelii* YM. It is possible that comparison of the gene sequences encoding the 235kd protein in both strains, may reveal variations between 17X and YM which could provide clues to the host cell preferences displayed by the two strains.

The pattern of hybridisation observed when the pPyJ7 insert was used to probe Southern blots of Rsal digested *P. yoelii* genomic DNA suggested the presence of repeated sequences or multiple gene copies. The isolation of the 144bp DNA sequence from clone pPyJ7 provided a probe for identification and cloning of larger portions of the gene sequence(s) encoding the 235kd protein.
ISOLATION AND ANALYSIS OF FURTHER SEQUENCES FROM THE P. YOELII 235KD PROTEIN GENE.

To obtain larger fragments of P. yoelii DNA encoding the 235kd protein, two plasmid libraries, one containing Dral digested P. yoelii genomic DNA and the second containing EcoRl digested DNA, were screened using the 144bp pPyJ7 insert or an oligonucleotide as a probe. The DNA fragments isolated were sequenced and their arrangement in the P. yoelii genome investigated by Southern blotting.

4.01 Screen of a Dral P. yoelii DNA library in pUC9.

Using the J7 144 fragment as a probe, four clones were isolated from a library of Dral digested P. yoelii genomic DNA in the plasmid vector pUC9. Clones pPyS6, pPyS7, pPyS8 and pPyS12 were rescreened several times until pure and DNA was prepared from each isolate. Preliminary single enzyme digests of DNA prepared from the four clones produced unexpected restriction patterns. The patterns were much more complicated than anticipated but certain fragments, indistinguishable from vector fragments on a 1% gel, hybridised to nick translated J7 144 insert sequence, figures 4.1a and 4.1b. Double digests of DNA from each clone using EcoRl and HincII revealed the presence of more than one foreign DNA insert but in each case a low molecular weight fragment hybridised to nick translated J7 144 on Southern blots as shown in figures 4.2a and 4.2b.
Figure 4.1a  Single enzyme digests of pUC9 plasmid clones pPyS6, pPyS7, pPyS8 and pPyS12.  

m = markers

Figure 4.1b  Single enzyme digests of pUC9 plasmid clones pPyS6, pPyS7, pPyS8 and pPyS12 Southern blotted and probed with nick translated pPyJ7 insert.
Figure 4.2a Double enzyme digests of pUC9 plasmid clones pPyS6, pPyS7, pPyS8 and pPyS12.

Figure 4.2b Double enzyme digests of pUC9 plasmid clones pPyS6, pPyS7, pPyS8 and pPyS12 Southern blotted and probed with nick translated pPyJ7 insert.
From the restriction enzyme data, plasmid maps were constructed, Figures 4.3, 4.4, 4.5 and 4.6. The enzyme Dral recognises the sequence TTTAAA and produces blunt ended fragments. In all four cases concatameric molecules had been formed by the ligation of more than one Dral fragment. In pPyS6, pPyS8 and pPyS12 multiple copies of the pUC9 vector were also present.

4.02 Polymerase chain reaction (PCR) amplification of pPyS6, pPyS7 and pPyS8 inserts.

Excision of insert DNA from pPyS6, pPyS7 and pPyS8 was hampered by the presence of multiple copies of pUC and unrelated Dral fragments. For use as probes and for subcloning into an expression vector, PCR amplification of inserts was carried out using oligonucleotides based on conserved sequences present at the 5' and 3' ends of each Dral fragment. Figure 4.7 shows 10μl of each total reaction mix electrophoresed on a 1% agarose gel.

4.03 Screen of an EcoRI P. yoelii DNA library in pUC9.

A 21mer oligonucleotide representing two copies of the repeated sequence present in the inserts of Dral clones pPyS6, pPyS7, pPyS8 and pPyS12 (see section 4.04) was used to screen an EcoRI P. yoelii genomic DNA library in the plasmid vector pUC9.
Figure 4.3 Map of pUC9 plasmid clone pPyS6.
Figure 4.4 Map of pUC9 plasmid clone pPyS7.
Figure 4.5  Map of pUC9 plasmid clone pPyS8.
Figure 4.6  Map of pUC9 plasmid clone pPyS12.
Figure 4.7  PCR amplification of *P. yoelii* inserts in pPyS6, pPyS7 and pPyS8.
The oligonucleotide sequence is shown below,

\[
\text{amino acids} \quad D \quad I \quad N \quad D \quad I \quad N
\]

\[
\text{Oligo IV} \quad 5' \quad -T \quad GAT \quad ATT \quad AAT \quad GAC \quad ATT \quad AAT \quad GA- \quad 3'
\]

When used to probe Southern blots of EcoRl digested \textit{P. yoelii} DNA, Oligo IV hybridised to a fragment of approximately 6kb and to higher MW material as shown in figure 4.8a. The hybridisation at >6kb may be due to partial digestion of DNA, however genomic Southern blots using pPyE8, pPyS6 inserts (which contain the sequence represented by oligo 1V) or pPyJ7 insert as probes suggest hybridisation to \textit{P. yoelii} DNA fragments of >7kb, section 4.05.

The pUC9 EcoRl library screen produced two clones, pPyE3 and pPyE8. A gel of EcoRl digested QPP DNA shows pPyE3 to contain an insert of approximately 5.7kb and pPyE8 an insert of approximately 5.0kb, figure 4.9. Insert fragments hybridised strongly to Oligo IV on Southern blots, figure 4.8b.

4.04 Sequence analysis of inserts from plasmid clones.

Since more than one copy of pUC9 was present in three of the Dral clones it was impossible to sequence inserts by plasmid priming using universal primers. Initial sequence data was obtained using oligonucleotides based on the known J7\textsuperscript{144} sequence and additional oligonucleotides were synthesised as the sequence progressed until Dral sites were reached at the 3' and 5' end of each insert, figures 4.10, 4.11, 4.12 (pPyS12 not shown). The size of each Dral fragment identified by hybridisation with J7\textsuperscript{144}
Figure 4.8a Southern blot of EcoR1 digested *P. yoelii* DNA probed with end labelled oligo IV.

Figure 4.8b Southern blot of EcoR1 digested pPyE3 and pPyE8 QPP DNA probed with end labelled oligo IV.
Figure 4.9  EcoR I digest of pPyE3 and pPyE8 DNA.
Figure 4.10 DNA sequence of P.yoelii Dral insert fragment in pPyS6.
Figure 4.11 DNA sequence of *P. yoelii* Dral insert fragment in *pPyS7.*
Figure 4.12 DNA sequence of *P. yoelii* Dral insert fragment in pPyS8.

**Figure 4.12 DNA sequence of *P. yoelii* Dral insert fragment in pPyS8.**
was 533bp in pPyS6 and pPyS12, 499bp in pPyS7 and 526bp in pPyS8. The DNA sequences of pPyS6 and pPyS12 inserts were identical.

The AT content of each fragment was 75% and each fragment contained the entire J7 sequence. To the 5' end of the pPyS6, pPyS8 and pPyS12 inserts is a small repeated sequence, the major constituent of which codes for the trimer DIN (Asp Ile Asn). When compared to pPyS6, a truncated form of this sequence, missing 36bp, is present in pPyS7 and pPyS8 lacks a DVI trimer which immediately precedes the repeated sequence in pPyS6. The amino acid sequence derived from the pPyS7 insert contains 21 amino acids which differ from those found in pPyS6 with the variant amino acids scattered throughout the sequence. The P. yoelii insert in pPyS8 contains 17 scattered amino acid differences when compared to pPyS6, 8 of which are substitutions found in pPyS7. The reading frame in clones pPyS6 and pPyS8 is open until a TAA stop codon, present in the original J7 sequence, 107bp from the 3' end of both inserts is reached. pPyS7 contains a TGA stop codon 117bp from its 3' end. Figure 4.13 shows a comparison of the derived amino acid sequences for each DNA fragment.

Plasmid primed sequencing of insert fragments in both EcoR1 pUC9 clones is underway. Approximately 700bp of pPyE8 sequence has been determined. The 3' end of the fragment corresponds to the 5' EcoR1 site present in the original J7 sequence. The sequence overlaps with that obtained from pPyS6 providing 987bp of continuous sequence as shown in figure 4.14. Figure 4.15 shows the relationship of cloned fragments. Limited sequence determination of the pPyE3 insert suggests that the DNA
Figure 4.13.
Comparison of the derived amino acid sequences of inserts from clones pPyS6, pPyS7 and pPyS8.
Figure 4.14 DNA sequence of a part of the P. yoelii EcoR1 insert fragment in pPyE8 and overlap with pPyS6 and pPyJ7 insert DNA.
Figure 4.15 Diagram to show the relationship of *P. yoelii* insert fragments from lambda gt11 clone J7 and plasmid clones pPyS6, pPyS7, pPyS8, pPyS12, pPyE3 and pPyE8.
contains a region with a DIN repeat motif analogous to that found in pPyS8.

4.05 Southern blots of P.yoelii genomic DNA.

Southern blots of restriction enzyme digested, genomic P.yoelii DNA were hybridised with nick translated insert DNA from pPyJ7, pPyS6 and pPyE8. As shown in figure 4.16 and in table 4.1, the Southern blots produced complex hybridisation patterns with each probe recognising several fragments of P.yoelii genomic DNA.

The multiple hybridisation pattern observed on Southern blots is difficult to interpret. Bands totalling >14kb hybridised to the 5kb EcoR1 insert from pPyE8 when it was used to probe EcoR1 digested P.yoelii DNA. It is possible that the probe recognises sequences that are repeated in the genome or that multiple copies of the gene containing the pPyE8 sequence exist but with restriction enzyme site polymorphisms that alter the size of the DNA fragments recognised by the cloned DNA. Sequence data from Dral clones pPyS6, pPyS7 and pPyS8 has shown that variations exist within their sequences, some of which alter restriction enzyme recognition sites.

When the pPyS6 Dral fragment was used to probe Dral digested P.yoelii genomic DNA two bands of hybridisation were seen. The pPyS6 sequence must, therefore be present more than once in the P.yoelii genome, either as a repeated element within one gene, or as an element represented in one or more genes.
Probed with nick translated
6Kb E8 insert  100bp J7 insert  500bp S6 insert

Figure 4.16 Southern blots of \textit{P.yoelii} genomic DNA.
<table>
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<tr>
<th>Rsal digest</th>
<th>Dral digest</th>
<th>EcoRl digest</th>
</tr>
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<tr>
<td></td>
<td>kb</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E8</td>
<td>J7</td>
</tr>
<tr>
<td>3.80</td>
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Table 4.1.
Sizes of *P. yoelii* genomic DNA fragments hybridising to pPyE8, pPyJ7 and pPyS6 inserts.
To rule out the possibility that partial digests were responsible for the hybridisation patterns produced when pPyE8 insert was used to probe EcoR1 digested \textit{P.yoelii} DNA, a control hybridisation using a 6kb fragment of the \textit{P.yoelii} 230kd (PMMSA) gene was carried out. The gene encoding the 230kd protein is known to be present in the \textit{P.yoelii} genome as a single copy. The autoradiograph showed one band of hybridisation at 6kb (not shown). A simultaneous repeat hybridisation using pPyE8 insert as a probe produced the same hybridisation pattern as shown in figure 4.16. It is possible that the presence of \textit{P.yoelii} genomic DNA sequences from a multiple parasite population could produce complex hybridisation patterns due to restriction enzyme site polymorphisms. However, the \textit{P.yoelii} genomic DNA used in this experiment was prepared from a YM line originally cloned by Dr. D. Walliker, Edinburgh University and recloned by Mr. J. B. De Souza, Middlesex Hospital.

4.06 Cross species Southern blots.

To establish whether the gene sequences coding for the 235kd rhoptry protein of \textit{P.yoelii} cross hybridised with other \textit{Plasmodia} species, insert fragments from pPyS6 and pPyE8 were used to probe Southern blots of \textit{P.falciparum}, \textit{P.berghei} and \textit{P.chabaudi} genomic DNA.

Figure 4.17 shows cross hybridisation with \textit{P.chabaudi} and \textit{P.berghei} DNA and table 4.2 gives the approximate sizes of DNA
Figure 4.17  Cross species Southern blots.
<table>
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<th>S6 500 bp PROBE</th>
<th>E8 5kb PROBE</th>
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<td>P. c</td>
</tr>
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<td>0.53</td>
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<td></td>
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</tbody>
</table>

wash  wash  wash  wash  wash  wash

temp  temp  temp  temp  temp  temp

65°   42°   65°   65°   42°   65°

P. b = P. berghei  P. c = P. chabaudi  P. y = P. yoelii

Table 4.2.
Sizes of genomic DNA fragments hybridising to pPyS6 and pPyE8 inserts on cross species Southern blots.
fragments recognised by the probes. Wash buffer (2xSSC 1% SDS) temperatures are shown in the table.

Both pPyE8 and pPyS6 inserts hybridised very strongly to P.berghei DNA and the probes were not removed when the filters were washed under stringent conditions at 65°. P.chabaudi DNA hybridised with pPyS6 insert but the signal was lost when the wash temperature was increased to 42° but, when hybridised with the larger pPyE8 probe, P.chabaudi DNA retained the signal when washed at 42°. A faint signal can still be seen on autoradiographs exposed overnight to filters washed at 65°. This result suggests that more DNA sequence homology may exist between the two rodent Plasmodia species, upstream of the pPyS6 sequence.

No hybridisation was seen, using either probe, to Southern blots of P.falciparum DNA.

4.07 PCR amplification of P.yoelii genomic DNA.

To try to establish whether the Dral fragments, represented by pPyS6, pPyS7 and pPyS8 were present as three, individual copies in the genome, PCR amplification of P.yoelii genomic DNA was carried out using oligonucleotide primers previously used to amplify the plasmid inserts. The primers represented 3' and 5' sequences common to the 3 Dral fragments.

As shown in figure 4.18, two or more bands of similar size were visible when amplified DNA was electrophoresed on a 1% agarose gel. The pPyS7 Dral fragment is 34bp smaller than pPyS6 and 27bp smaller than pPyS8. The amplified pPyS6 and pPyS8

146
Lane 1 PCR amplified genomic DNA.
2 DNA size markers.

Figure 4.18 PCR amplification of *P. yoelii* genomic DNA using oligonucleotides based on conserved sequences in pPyS6, pPyS7 and pPyS8 inserts.
fragments differ only by 7bp and are therefore, unlikely to be
distinguishable. There was no evidence of double bands of DNA
when the same oligonucleotides were used to amplify the Dral
fragments in pUC9, section 4.02. As a control, PCR amplification
of a portion of the \textit{P.yoelii} CSP gene, known to be present in the
genome as a single copy, produced a single band of DNA (not
shown).

4.08 \textbf{Northern blot analysis.}

To establish whether the cloned DNA is translated and to
assess the size of the resulting mRNA, the insert from pPyE8 was
used to probe a northern blot of \textit{P.yoelii} total RNA. Vaidya \textit{et al}
(1984) published evidence to suggest that some mRNA's coding for
\textit{P.yoelii} protective antigens purify as polyA-, therefore total
\textit{P.yoelii} RNA was used at high concentration in northern blot
analyses.

The insert from pPyE8 hybridised to a message of
approximately 8.8kb, figure 4.19. Further hybridisation to
material of >10kb was also apparent. It is not known whether this
represents binding to other high molecular weight species or is
an artefact due to excess RNA or the size of the probe. As a
control a 500bp fragment of the \textit{P.yoelii} PMMSA (230kd protein)
was used to probe a northern blot containing the same
concentration of RNA. As expected, hybridisation to a message of
7.4kb was observed (not shown).
Figure 4.19 Northern blot of *P. yoelii* total RNA probed with nick translated pPyE8.
4.09 Discussion.

Sequence analysis of three \textit{P. yoelii} Dral DNA fragments from plasmid clones pPyS6, pPyS7 and pPyS8 has shown that each contains the original J7 fragment isolated from a \textit{P. yoelii} genomic DNA library in lambda gt11 screened with \textit{P. yoelii} hyperimmune serum. The larger, EcoR1 inserts from \textit{P. yoelii} genomic DNA clones pPyE3 and pPyE8 contain sequences analogous to those found in pPyS8 and pPyS6 respectively, see figure 4.15, section 4.04.

There are several lines of evidence to suggest that more than one copy of the gene encoding the 235kd protein may be present in the \textit{P. yoelii} genome:

1. Three separate Dral DNA fragments were isolated from a \textit{P. yoelii} genomic library in clones pPyS6, pPyS7 and pPyS8. The sequence of each fragment was broadly similar with regions of homology at 5' and 3' ends but with variations in the size of a small repeated sequence and scattered differences throughout the derived amino acid sequence. A fourth Dral fragment isolated from the same plasmid library contained insert DNA identical in sequence to that in pPyS6.

2. Two larger DNA fragments recovered from a \textit{P. yoelii} EcoR1 library contained Dral fragments corresponding exactly to two of the Dral clones previously isolated.
3. The 144bp EcoR1* insert from lambda gt11 clone J7 hybridises to 7 Rsal P. yoelii genomic DNA fragments on Southern blots, totalling >15kb.

4. Hybridisation of the EcoR1 insert from pPyE8 to EcoR1 digested P. yoelii DNA produces a complex hybridisation pattern of fragments totalling more than the size of the probe. In addition, the 533bp Dral fragment of P. yoelii DNA from pPyS6 hybridises to Dral genomic DNA fragments of 0.5kb and 0.48kb on Southern blots.

5. An oligonucleotide corresponding to a short, repeated sequence found in pPyS6 and pPyS8 inserts hybridised to EcoR1 fragments of approximately 6kb and >7kb on Southern blots of P. yoelii genomic DNA.

6. Using oligonucleotides corresponding to sequences common to the 5' and 3' ends of the three Dral fragments from pPyS6, pPyS7 and pPyS8 to PCR amplify P. yoelii genomic DNA, multiple bands can be seen on ethidium bromide stained agarose gels.

Further sequencing of pPyE8 and pPyE3 is necessary to discover if the differences between the two sequences are restricted to certain variable regions or whether upstream sequences diverge. A screen to try to isolate a larger DNA fragment analogous to the pPyS7 insert is required for comparison to pPyE8 and pPyE3 sequences and isolation of the DNA fragments containing the ends of the genes(s) is also necessary to provide information on 5' and 3' untranslated regions. Southern blots of
P. yoelii chromosomes separated by pulsed field gel electrophoresis and probed with the cloned DNA sequences may indicate whether the genes are present on one or more chromosomes. Long range mapping could also be used to provide information on copy number and the relative position of the genes within the P. yoelii genome.

Homology between the P. yoelii cloned DNA fragments and DNA from P. berghei and P. chabaudi was observed. P. yoelii is evolutionarily related to P. berghei and may be expected to contain homologous sequences whereas P. chabaudi is a morphologically distinct species (Killick-Kendrick, 1978). When pPyE8 insert was used as a probe, the homology observed with P. chabaudi was greater than when the smaller pPyS6 insert fragment was used. This suggests that regions of stronger homology lie upstream of the Dral fragment contained in pPyS6.

Cloning and sequencing of analogous P. berghei and P. chabaudi genes would reveal the extent of the homology and whether it is retained at the amino acid level. In IIF experiments using protective McAb 25.77, which recognises a 235kd P. yoelii, rhoptry protein, Holder and Freeman reported cross reaction with P. yoelii sub-species, P. vinckei petterei, P. chabaudi chabaudi, and P. chabaudi adami. A second protective McAb, 25.37, which also recognises a 235kd protein, reacted only with P. yoelii sub-species (Holder and Freeman, 1984c). It is not known whether the epitopes recognised by these antibodies are encoded by the larger DNA fragments in pPyE8 and pPyE3. Similar IIF studies using antiserum to recombinant proteins may indicate whether
these gene fragments encode proteins targeted by the previously identified protective monoclonal antibodies.

Disregarding post translational modifications that could affect the apparent molecular weight on polyacrylamide gels, a protein of 235kd may be expected to be translated from a mRNA of approximately 6.5kb. A band of approximately 8.8kb and hybridisation to higher MW material was observed on a northern blot of P. yoelii total RNA probed with pPyE8 insert DNA. It is not known whether the signal on the northern blot represents hybridisation to more than one mRNA species. Analysis of complete sequences and in vitro translation of cloned sequences would indicate whether the three DNA sequences are translated and if some difference in calculated and observed mRNA size can be accounted for by 5' and 3' untranslated regions. If upstream sequences from pPyE3 and pPyE8 diverge, northern blotting with probes from regions of diversity may clarify whether both sequences are translated.

No striking homology was recorded when the E8S6 deduced amino acid sequence was compared to known Plasmodium sequences held in a database but a comparison of the E8S6 sequence with protein coding sequences held in the PIR general database (Protein identification resource of the National Biological Research Facility, U.S.A.), revealed a small area of homology with mammalian calpactin heavy chain. In mammals, calpactin is a protein found at high levels in certain epithelial and endothelial cells and has been shown to be involved in exocytosis (Saris et al, 1986. Kretsinger and Creutz, 1986. Geisow et
Calpactin heavy chain associates with a light chain and has a C-terminal domain containing two Ca++/phospholipid binding sites. Thirty-four per cent identity between calpactin heavy chain and the derived E8S6 sequence was noted within a 44 amino acid overlap defined by a consensus sequence (Kretsinger and Creutz, 1986). Although the homology between E8S6 and the mammalian calpactin sequence is minimal it is feasible that a protein (such as the P. yoelii 235kd protein) present in rhoptries may belong to a class characterised by calcium and phospholipid binding. The apical complexes of Plasmodia are thought to contain lipid, highlighted by tannic acid fixation (Bannister et al, 1986) and EM studies have revealed the presence of membranous whorls associated with rhoptries (Stewart et al, 1986). Concentrations of calmodulin, a vital, calcium dependent protein have been demonstrated in the apical complex of P. falciparum merozoites suggesting that erythrocyte penetration is a calcium/calmodulin dependent process (Scheibel et al, 1987). Further sequencing of pPyE8 and pPyE3 may reveal more homology with the calpactin consensus sequence and facilitate secondary structure predictions. The conformation of the calpactin protein is known to be important in calcium/phospholipid binding (Saris et al, 1986). Using purified 235kd or recombinant protein fragments it should be possible to carry out calcium binding studies and to test for the ability of the protein to bind phospholipid.
CHAPTER FIVE.
IMMUNOCHEMICAL ANALYSIS WITH ANTISERA RAISED AGAINST PROTEINS ENCODED BY CLONED DNA SEQUENCES.

The 533bp, Dral fragment of \textit{P.yoelii} DNA in pPyS6 was known to contain the 144bp of sequence present in the original lambda gt11 clone J7. Antibodies selected from hyperimmune serum by clone J7 appeared to recognise rhoptries by IIF and immunoprecipitated a \textit{P.yoelii} protein of 235kd. To produce a polyclonal antiserum, the insert from pPyS6 was subcloned into the expression vector pGex-3x (Smith and Johnson,1988. Johnson et al,1989) and the recombinant protein used to immunise mice. Peptides based on the small, repeated sequence from pPyS6 and pPyS8 were also prepared to provide rabbit antiserum. The antisera were used in indirect immunofluorescence, immunoprecipitation and western blot studies with \textit{P.yoelii} proteins.

5.01 Subcloning into pGex-3x.

The pPyS6 Dral fragment contains a single Hpal enzyme site at nucleotide 88 and a single Ssp1 site at position 455. A double digest using these two enzymes produced a 367bp, blunt ended DNA fragment suitable for cloning into the Smal site of the pGex-3x vector, this version of the expression vector was required to maintain the open reading frame of the \textit{P.yoelii} DNA fragment. The pPyS6 sequence contains a TAA stop codon at position 426, therefore the predicted size of the recombinant
fusion protein was 38.4kd, of which 26kd was represented by Sj26 and 12.4kd encoded by P.yoelii DNA.

Following initial identification and rescreening using nick translated pPyS6 insert as a probe, 50 colonies were picked and screened for expression of fusion proteins. Twelve clones were selected for further analysis; all appeared to be producing a recombinant protein of approximately 45kd, 6.6kd larger than anticipated. EcoR1/BamH1 double digestion of QPP DNA from the 12 isolates showed each to contain inserts corresponding to a Hpal/EcoR1 fragment of 235bp and an EcoR1/Ssp1 fragment of 132bp.

5.02 Purification of the pGex-3xS6 fusion protein.

A single, purified clone was selected for large scale production of the fusion protein. Uninduced cell cultures of the pGex-3xS6 fusion did not produce recombinant protein indicating that expression was not constitutive. Timed incubations were carried out to determine optimal pre and post IPTG induction incubation times required for maximum expression of recombinant protein. One hour pre and post induction incubations produced similar amounts of protein when compared to 4 hour pre induction / 1 hour post induction or 1 hour pre induction / 5 hour post induction incubations. Microscopic examination of induced cultures of pGex-3xS6 showed no evidence of inclusion bodies and the expressed protein was therefore assumed to be soluble.

The majority of the pGex-3xS6 fusion protein was recovered from the TX-100 soluble fraction following glutathione
agarose affinity chromatography with approximately one tenth of the protein remaining in the TX-100 insoluble fraction. The total protein concentration recovered from a 400ml culture was estimated at approximately 14mg, figure 5.1.

5.03 Western blot of the pGex-3xS6 fusion protein.

A Coomassie blue stained polyacrylamide gel and a western blot of lysates expressing the pGex-3xS6 fusion protein probed with \textit{P. yoelii} hyperimmune serum are shown in figure 5.2.

On the western blot, \textit{P. yoelii} hyperimmune serum recognised a dense band at approximately 45kd and several lower MW bands, presumed to be fusion protein breakdown products. No reaction was seen in lane 9 containing induced, religated pGex-3x vector. Monoclonal antibody 25.77, did not recognise the recombinant fusion protein.

5.04 IIF using antisera raised against the pGex-3xS6 fusion protein.

Sera from mice immunised with the pGex-3xS6 fusion protein, using saponin or Freund's adjuvant, were tested by IIF on acetone fixed preparations of blood stage \textit{P. yoelii}. As shown in figure 5.3 the antisera produced a punctate IIF pattern suggestive of antibody binding to rhoptry complexes. The type of adjuvant used did not appear to affect antibody titre which was low, 1:10 for serum from mice immunised with 1.5\textmu g of antigen and
Figure 5.1 PAGE of purified pGex-3xS6 fusion protein.
Figure 5.2  PAGE of lysates from timed incubations of pGex-3xS6 and a western blot probed with *P. yoelii* hyperimmune serum.

Figure 5.2  PAGE of lysates from timed incubations of pGex-3xS6 and a western blot probed with *P. yoelii* hyperimmune serum.
1:10 dilution of antiserum from mouse immunised with 1.5\(\mu\)g pGex-3xS6 fusion protein.

1:20 dilution of antiserum from mouse immunised with 10\(\mu\)g pGex-3xS6 fusion protein.

Figure 5.3 Indirect immunofluorescence on acetone fixed P.yoelii preparations using anti pGex-3xS6 fusion protein antiserum.
1:20 from those immunised with 10µg. Antiserum to a pGex-2T HPV (human Papilloma virus) fusion, used as a control for the Sj26 portion of the recombinant protein, did not react with the P.yoelii preparations and there was no reaction with serum from mice immunised with marker proteins.

5.05 IIF using anti peptide antisera.

Some Plasmodia antigens containing repeated sequences are known to be immunodominant for antibody induction (Nussenzweig and Nussenzweig, 1985. Kemp et al, 1987b). Peptides corresponding to the repeated sequences found in pPyS6 and pPyS8, differing by one amino acid, were used to immunise rabbits and the serum was monitored for antibody by IIF on acetone fixed P.yoelii blood stage preparations. The sequences of the peptides P1 and P2, were DIN DIN DVI DIN DIN DTN DIN derived from the pPyS6 insert sequence and DIN DIN DIN DIN DIN DTN DIN derived from pPyS8.

On day 63, 2 weeks after boosting, serum from the rabbit immunised with the pPyS6 peptide showed a punctate pattern by IIF which was barely detectable above background. No specific IIF could be detected with the serum from the rabbit immunised with the pPyS8 peptide. On day 90, 21 days after a 5 site sub-cutaneous boosting immunisation, a punctate IIF pattern was observed using serum from the rabbit immunised with the pPyS6 peptide. Background reactivity in serum from the rabbit immunised with the pPyS8 peptide made detection of any specific signal
impossible. Three weeks after a second 5 site sub cutaneous immunisation, a punctate IIF pattern could be detected using a 1:200 dilution of anti pPyS6 peptide antiserum as shown in figure 5.4.

5.06 Western blots using anti fusion protein and anti peptide antisera.

Western blots on lysates of *P. yoelii* proteins using anti pGex-3xS6 fusion protein antiserum were inconclusive, probably due to the extremely low titre of the antiserum. Purified pGex-3xS6 fusion protein was used to affinity select specific antibodies from *P. yoelii* hyperimmune serum. These antibodies recognised a protein of 235kd on western blots of TX-100 soluble *P. yoelii* lysates together with a protein of 110kd. The lower MW species was also recognised in preparations of parasitised erythrocytes, especially in those not treated with protease inhibitors, figure 5.5.

Antiserum raised in rabbits against the pPyS6 peptide appeared to recognise proteins of 235kd and 110kd on western blots of *P. yoelii* TX-100 soluble lysates. As with IIF studies using this antiserum, the signal to background ratio was low. A protein of 235kd was also faintly evident on western blots of *P. yoelii* TX-100 soluble lysates probed with monoclonal antibody 25.77, data not shown.
Figure 5.4 Indirect immunofluorescence on an acetone fixed *P. yoelii* preparation using anti peptide antiserum.
1. TX-100 soluble lysate + protease inhibitors – non reduced
2. Total parasite lysate + protease inhibitors – reduced
3. TX-100 soluble lysate + protease inhibitors – reduced
4. Mouse erythrocyte lysate + protease inhibitors
5. TX-100 soluble lysate no protease inhibitors – reduced

2nd antibody = rabbit anti-mouse IgG (whole molecule).

Figure 5.5 Western blot of a *P. yoelii* TX-100 lysate probed with antibody selected from hyperimmune serum by the pGex-3xS6 fusion protein.
5.07 **Immune precipitation using anti fusion protein and anti peptide antisera.**

To identify proteins recognised by anti fusion and anti peptide antiserum, \(^{35}\)S methionine labelled *P. yoelii* proteins were immune precipitated with the anti pGex-3xS6 fusion protein antiserum, anti pPyS6 repeat peptide antiserum and antibodies selected from the anti peptide antiserum by the pGex-3xS6 fusion protein. Normal mouse serum, normal rabbit serum, monoclonal antibody 25.77 and antiserum raised against a pGex-2tHPV (human Papilloma virus) fusion protein were used as controls.

Figure 5.6 shows the immune precipitation of a 235kd protein by monoclonal antibody 25.77. Anti pGex-3xS6 fusion protein antiserum and anti pPyS6 repeat peptide antiserum appear to immune precipitate a protein of similar size. On the autoradiograph, a faint signal, which has not reproduced on the photograph, corresponding to the immune precipitation of a 235kd protein by antibodies selected from *P. yoelii* hyperimmune serum by lambda gt11 clone J7 and pGex-3xS6 fusion protein can be seen. The same faint band is also seen using antibodies selected by the pGex-3xS6 fusion protein from anti pPyS6 repeat peptide antiserum.

5.08 **Discussion.**

The pGex-3xS6 fusion protein is not recognised by McAb 25.77 on western blots. It is possible that the epitope of the 235kd protein recognised by McAb 25.77 is not represented in the
Lanes:
1 pGex-2t HPV (human papilloma virus) fusion protein
2 normal rabbit serum
3 normal mouse serum
4 monoclonal antibody 25.77 (a 235 Kd)
5 antibodies selected from P. yoelii hyperimmune serum by λgt11 clone J7
6 antibodies selected from P. yoelii hyperimmune serum by pGex-3xS6 fusion protein
7 a pGex-3xS6 fusion protein
8 antibodies selected from anti pPyS6 repeat peptide antiserum by pGex-3xS6 fusion protein
9 a pPyS6 repeat peptide

7.5% PAGE treated with Enhance.
3 week exposure to film
polypeptide sequence of the pGex-3xS6 protein. The P. yoelii DNA inserted into the pGex vector was calculated to code for a polypeptide of approximately 12.5kd; 5% of the total 235kd protein. Larger fusion proteins encoded by DNA sequences in pPyE3 and pPyE8 may react with the monoclonal antibody.

Antibodies selected from hyperimmune serum by the pGex-3xS6 fusion protein recognise proteins of 235kd and 110kd on western blots. It is not known whether the 110kd species is related to the 235kd protein or if the antiserum cross reacts with the 110kd protein. Anti pGex-3xS6 antiserum does not immunoprecipitate a protein of 110kd from preparations of $^{35}S$ methionine labelled P. yoelii proteins.

The low titres of antisera raised against the pGex-3xS6 fusion may be indicative of the poor immunogenicity of the recombinant protein. This factor may also account for the slow appearance of specific antibodies in serum of rabbits immunised with peptides representing the pPyS6 and pPyS8 repeated sequences. It is not known whether the single amino acid difference between the peptide sequences could affect their immunogenicity. A five site sub cutaneous immunisation protocol using low antigen concentrations, intended to induce high affinity antibodies, did not dramatically improve antibody titres. High background, possibly due to the use of Freund's adjuvant, was a problem in IIF and western blot experiments.

Larger recombinant proteins encoded by DNA sequences present in clones pPyE3 and pPyE8 may be more immunogenic than the pGex-3xS6 fusion protein and may be recognised by monoclonal
antibody 25.77. The tertiary structure of the protein may affect its immunogenicity and expression of cloned sequences in a eukaryotic system such as Baculovirus which has been shown to appropriately modify and fold recombinant proteins into a biologically active form (Jarvis and Summers, 1989) may be required for immunogenicity. A second protective monoclonal antibody, 25.37, recognises rhoptries by IIF and also immunoprecipitates a P. yoelii protein of 235kd (Freeman et al, 1980a. Holder and Freeman, 1984c). It is not clear whether monoclonal specificities 25.37 and 25.77 recognise distinct merozoite antigens or different determinants on the same antigen. However, the observation from IIF tests, that monoclonal antibody 25.77 cross reacted with several rodent Plasmodia species whereas 25.37 reacted only with P. yoelii subspecies, may indicate that this antibody possesses a different specificity from monoclonal antibody 25.77 (Holder and Freeman, 1984c). It is not known if monoclonal antibody 25.37 recognises the pGex-3xS6 fusion protein. It is possible that the monoclonal antibodies will recognise proteins encoded by the P. yoelii DNA sequences from pPyS7 or pPyS8 and pPyE3.

The two remaining mice from each group immunised with the pGex-3x6 fusion protein were challenged with P. yoelii parasitised erythrocytes. In this preliminary experiment, the 12.5kd fragment of the fusion protein encoded by P. yoelii DNA was not protective and parasitaemias in immunised mice developed at the same rate as unimmunised controls. However, the antibody titres induced by the fusion protein were extremely low and the challenge dose of
parasites may have been too high for elimination by antibody.
Immunisation experiments using 2 mice per group cannot be
considered statistically significant, therefore these experiments
should be repeated using groups of at least 7 mice and a suitable
challenge infection.
A TritonX-100 (TX-100) soluble lysate of *P. yoelii* proteins prepared from parasitised erythrocytes was shown by Playfair and De Souza to protect mice against lethal challenge infection (Playfair and De Souza, 1986). In order to identify the antigenic components of the lysate responsible for protection, De Souza and Playfair separated the TritonX-100 soluble material by isoelectric focussing (IEF) and tested the recovered fractions for their ability to protect mice against *P. yoelii* challenge infection. Individual fractions were also assayed for their capacity to stimulate T cell help measured by the anti TNP response of spleen cells from protected mice to TNP coated parasites (De Souza and Playfair, 1988). When the TX-100 soluble material was separated by IEF, the majority of parasite protein focussed in the pH4.1-4.6 range with pH4.2-4.4 fractions consistently conferring 100% protection to mice. Two other peaks of protection were observed, with fractions of pH6.4-6.6 and pH7.8-8.0 providing >75% protection. The ability to stimulate T cell help correlated well with protection for fractions in the pH4 range but was less significant for fractions in the pH6 and pH8 range (De Souza and Playfair, 1988).

The experiments described here were intended to further separate and identify parasite antigens present in protective IEF fractions with a view to testing individual proteins, and
combinations of proteins for their ability to protect mice against *P. yoelii* challenge infection.

6.01 Analysis of stages of a TritonX-100 soluble lysate by western blotting.

To ensure that intact *P. yoelii* proteins were present in the TX-100 soluble lysate used as the starting material for isoelectric focussing, samples from each stage of preparation were collected by Mr. J. B. De Souza. Following saponin lysis of heavily parasitised erythrocytes containing >90% schizonts, pelleted material was resuspended in water. Water insoluble proteins were pelleted, TritonX-100 was added to 0.5% and the TX-100 soluble material recovered after centrifugation to remove insoluble material. A sample from each stage of lysate preparation was analysed by SDS PAGE, and probed with *P. yoelii* hyperimmune serum and anti 230kd PMMSA antiserum.

Figure 6.1 shows a western blot of the stages of lysate preparation probed with *P. yoelii* hyperimmune serum and figure 6.2 shows a similar western blot probed with polyclonal anti *P. yoelii* 230kd (PMMSA) antiserum. The use of hyperimmune serum confirmed that the TX-100 soluble fraction contained large amounts of *P. yoelii* proteins covering a broad molecular weight range. Hyperimmune serum also recognised proteins in the water soluble fraction and in the TX-100 insoluble fraction. Erythrocyte cytoskeleton and *P. yoelii* proteins associated with the
Lanes:
1  Parasitised erythrocytes
2  Saponin lysed erythrocytes
3  Pellet following saponin lysis
4  Pellet + dH₂O = H₂O soluble parasite proteins
5  Insoluble parasite proteins
6  Insoluble parasite proteins + TX-100
7  TX-100 insoluble proteins
8  Total parasite lysate (different batch)
9  Mouse erythrocyte lysate

10% PAGE probed with mouse hyperimmune serum 1:200
2nd Ab: goat to mouse alkaline phosphatase conjugate

Figure 6.1 Western blot of the stages of *P. yoelii* TX-100 lysate preparation probed with hyperimmune serum.
Figure 6.2 Western blot of the stages of *P. yoelii* TX-100 lysate preparation probed with anti 230kd PMMSA antiserum.
cytoskeleton may be expected to fractionate with the TX-100 insoluble material. The western blot probed with mouse polyclonal anti 230kd (PMMSA) antiserum demonstrates that the \textit{P. yoelii} 230kd PMMSA and its processed fragments are recovered in the TX-100 soluble fraction. Lane 8 contains a previously prepared \textit{P. yoelii} lysate stored at -20°. In the western blot probed with anti 230kd antiserum, some breakdown of the 230kd protein is apparent; this may have resulted from proteases active during storage or may be due to freezing and thawing.

6.02 Analysis of the protein content of IEF fractions.

Polyacrylamide gel electrophoresis of isoelectrically focussed \textit{P. yoelii} TX-100 soluble lysates suggested that the protein concentration of fractions was considerably lower than estimated by absorbance at 280nm (assuming 1 $\text{A}_{280}$ unit = 1mg.ml$^{-1}$ protein). Using a Pierce BCA micro assay, protein concentrations of IEF fractions were calculated to be approximately half of the values obtained from spectrophotometric estimations. The average protein concentration of pH4.2-4.4 material was approximately 0.5mg.ml$^{-1}$. It was established that residual ampholines did not interfere with protein concentration measurements using the Pierce assay.

Coomassie blue staining of fractions separated by SDS PAGE produced a smear of protein possibly consistent with protein degradation during storage and manipulation. Proteolytic degradation may have been responsible for the reduced protein
concentrations observed when absorbance estimations were compared with protein concentrations by the Pierce BCA method. Alternatively, TritonX-100 is known to absorb strongly at 280nm and the presence of this detergent in the IEF fractions may have been responsible for the elevated protein concentrations estimated by absorbance at 280nm.

6.03 Chromatographic analysis of pH4 IEF fractions.

Two preparations of IEF fractions from the pH4 range, previously shown by De Souza and Playfair to be highly protective, IF18-7 (pH4.2-4.4) and IF18-8 (pH4.4-4.6), were subjected to gel filtration by FPLC to separate component proteins. IF18-7 (concentration 0.41mg.ml\textsuperscript{-1}) was analysed using a Superose 6 gel filtration column. Approximately 1.2mg of IF18-7 was freeze dried and resuspended in 0.1M ammonium bicarbonate; insoluble material was removed before the sample was applied to the column. Peaks of material absorbing at 280nm were detected with an apparent MW below 20,000, as shown in figure 6.3. Twenty two 1ml fractions containing absorbance peaks were recovered, freeze dried and resuspended in PBS. Individual fractions were injected into mice by Mr. J. B. De Souza and analysed for their ability to stimulate T cell help (Playfair et al,1977). No evidence for the induction of T helper cell activity in the spleens of immunised mice was obtained. The concentration of material recovered in FPLC fractions was very low as judged by
### IF18-7 Superose 6

#### Supernatant in 0.1M NH₄CO₃

<table>
<thead>
<tr>
<th>Standards</th>
<th>MW D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat IgG</td>
<td>120,000</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>12,384</td>
</tr>
</tbody>
</table>

**Figure 6.3** Ammonium bicarbonate soluble IEF material recovered from a Superose 6 column.
absorbance measurements at 280nm and therefore the quantity of protein used may have been too low for successful immunisation.

Gel filtration of preparation IF18-7 suggested that component proteins were of low MW, therefore 1mg of the pH4.4-4.6 material, IF18-8 (concentration 0.40mg.ml\(^{-1}\)) was separated by gel filtration using Superose 12. The sample was freeze dried, resuspended in 0.1M ammonium bicarbonate and the insoluble material was dissolved in 70% formic acid. Both preparations were separated by FPLC. Peaks of absorption at 280nm were seen with apparent molecular weights below 20,000 for the ammonium bicarbonate soluble material and at 220,000, 36,000 and 2,000 for the formic acid soluble material, figures 6.4 and 6.5. Fractions containing absorbance peaks from both separations were recovered, freeze dried and resuspended in PBS for injection into mice by Mr. J.B. De Souza. None of the immunised were protected when subsequently challenged with *P. yoelii* parasitised erythrocytes.

6.04 **Analysis of a \(^{35}\)S methionine labelled TX-100 soluble *P. yoelii* lysate.**

In order to follow the fate of *P. yoelii* proteins during lysate preparation and IEF fractionation, \(^{35}\)S methionine was incorporated into *P. yoelii* proteins before erythrocyte lysis with saponin and preparation of a TX-100 soluble parasite lysate as described by De Souza and Playfair (1988). Labelled *P. yoelii*
Figure 6.4 Ammonium bicarbonate soluble IEP material recovered from a Superose 12 column.
Figure 6.5 Formic acid soluble material recovered from a Superose 12 column.
proteins were separated by SDS PAGE or immunoprecipitated with anti 230kd PMMSA antiserum.

To assess the effect of storage temperature on parasite proteins, aliquots of the TX-100 soluble fraction were stored for two days at room temperature, 4°, -20° or -80° before SDS PAGE. Figure 6.6 shows that storage temperature had little effect on the integrity of labelled parasite proteins.

The remaining $^{35}$S methionine labelled TX-100 soluble parasite material was fractionated on an IEF column by Mr. J. B. De Souza. Individual IEF fractions were then electrophoresed on SDS polyacrylamide gels. Figure 6.7 shows the presence of protein in the first nine fractions, ie. pH2.7-4.9, with the majority of labelled protein focussing between pH2.7 and pH3.6. The strongest signal as compared to unfractionated lysate, was seen at the gel front on 10% gels, suggesting that degradation of protein had taken place or that the majority of protein was not focussed. Figure 6.8 shows autoradiographs of immunoprecipitates of the $^{35}$S methionine labelled IEF fractions using polyclonal anti P. yoelii 230kd (PMMSA) antiserum. A faint band at approximately 230kd corresponding to immunoprecipitation of labelled protein from fraction 6, pH4.0, was evident. A signal at the gel front on 10% gels suggested the immunoprecipitation of very low molecular weight material. Lane 14 (top), figure 6.8 shows that the 230kd PMMSA and its processed products were immunoprecipitated from the $^{35}$S methionine labelled TX-100 soluble starting material by the anti 230kd antiserum. Lane 15 (top) contains a 2µl aliquot of
Lanes:
1  Total lysate stored at room temperature
2  Total lysate stored at 4°
3  Total lysate stored at -20°
4  Total lysate stored at -80°
6  Parasitised erythrocytes before saponin lysis stored at 4°
7  Parasitised erythrocytes before saponin lysis stored at -80°

7.5% PAGE treated with En3hance. Overnight exposure to film.

Figure 6.6  Effect of storage temperature on $^{35}$S methionine labelled TX-100 soluble P. yoelii proteins.
I.E.F.
1 - 9
pH 2.7 - 4.9

I.E.F.
10 - 18
pH 5.1 - 7.6

I.E.F.
19 - 24
pH 7.9 - 10.5

10% PAGE treated with Enhance. 3 week exposure to film.

m = markers

Figure 6.7 PAGE of $^{35}$S methionine labelled IEF fractions.
IEF fractions 6-18 = pH4.0-7.6
lane 14 TX-100 soluble lysate immuneprecipitated with mouse polyclonal anti 230kd antiserum.

IEF fractions 1-5 = pH2.7-3.9  19-24 = pH7.9-10.5
lane 15 total labelled TX-100 soluble lysate.

Figure 6.8  Immuneprecipitation of $^{35}$S methionine labelled IEF fractions with anti 230kd PMMSA antiserum.
labelled \textit{P.yoelii} proteins, removed before TX-100 lysate preparation.

The appearance of the majority of $^{35}$S methionine labelled \textit{P.yoelii} material in pH2.7-3.6 IEF fractions represents a shift in pH when compared to fractions previously prepared where peak protein concentration is normally observed between pH4.2 and pH4.8. The reasons for this pH shift are not known.

\textbf{6.05 Discussion.}

Western blots of the stages of TritonX-100 lysate preparation suggest that a broad molecular range of \textit{P.yoelii} proteins are present in the starting material used for isoelectric focussing and the presence of the 230kd PMMSA indicates that high molecular weight proteins may be expected to be intact. Recognition of proteins by hyperimmune serum in the water soluble fraction and in the TX-100 insoluble fraction shows that certain \textit{P.yoelii} proteins, such as proteins that may be associated with the erythrocyte cytoskeleton, are not included in the lysate applied to the IEF column. It may be possible to solubilise \textit{P.yoelii} proteins in a stronger detergent such as octyl-\beta-D-glucopyranoside (octyl glucoside), which is neutral, dialysable and does not absorb strongly at 280nm, however, lysates prepared in this detergent have been found to be slightly less protective than those prepared with TX-100 (Playfair and De Souza, 1986).
Chromatographic analysis of protective IEF fractions suggested that ammonium bicarbonate soluble component proteins were below 20,000 MW. Gel filtration of ammonium bicarbonate insoluble material produced peaks of protein at higher molecular weights when separated in 70% formic acid. SDS PAGE analysis of a pool of pH4 fractions (pH4.0-4.8) and of material recovered from reverse phase chromatography separation of a pool of pH4 fractions did not indicate the presence of high molecular weight proteins (data not shown). However, any multimeric structures present in the material separated by gel filtration would be dissociated during preparation of the sample for electrophoresis. In recent unpublished experiments, Mr. J. B. De Souza has further separated protective IEF fractions on a sephacryl S-200 column. S-200 separation of protective pH8 IEF fractions produced material with an apparent MW of approximately 150kd which appeared as a 50kd species when electrophoresed on a polyacrylamide gel under reducing conditions. The S-200 separated material conferred "early" type protection to mice. Protective pH6 material from the S-200 column of approximately 60kd produced bands of 45kd and 50kd on polyacrylamide gels run under reducing conditions. Material from the pH4 region with a MW of 250kd when separated by S-200, resolved to bands covering a MW range from 200kd to 29kd. These results suggest that the material recovered from the IEF column may contain multimeric structures that are dissociated under reducing conditions.

Immuneprecipitation of IEF fractions with P. yoelii anti 230kd (PMMSA) antiserum suggested that the 230kd protein was
present in the pH4.0 fraction. The concentration of 230kd protein precipitated from this fraction by the antibody was extremely low when compared to the concentration of 230kd protein precipitated by the same antibody from the $^{35}$S methionine labelled \textit{P.yoelii} lysate used as starting material for the IEF column. Figure 6.8 shows that $^{35}$S methionine had been incorporated into \textit{P.yoelii} proteins used to prepare the TX-100 soluble lysate and figure 6.6 demonstrates that labelled proteins were present in the lysate applied to the IEF column. It is not known whether the low concentration of labelled material recovered in IEF fractions was due to proteolytic degradation or failure of the proteins to focus.
CHAPTER SEVEN.
ANALYSIS OF ANTIBODIES RAISED AGAINST IEF FRACTIONS.

Antisera raised against IEF fractions provided an alternative approach to the identification of *P. yoelii* proteins responsible for the protective properties of the fractions. Proteins identified by antisera could be isolated from a *P. yoelii* TX-100 lysate by immunoaffinity chromatography, further purified by, for example, gel filtration or SDS PAGE and tested for their ability to induce protection against *P. yoelii* infection in mice. Antibodies raised against the protective IEF fractions were used on western blots of *P. yoelii* lysates and IEF fractions separated by PAGE. Anti IEF antisera were also used to identify $^{35}$S methionine labelled *P. yoelii* proteins by immunoprecipitation.

7.01. Western blots using anti IEF antisera.

Figure 7.1 shows a western blot probed with mouse antiserum raised against a pool of pH4 IEF fractions (pH4.0-4.8). The antiserum does not appear to recognise any discrete proteins in the lane containing reduced, pooled pH4 IEF fractions. It does, however, recognise a doublet at approximately 68kd and material at the gel front in the pooled pH4 IEF fractions electrophoresed in the absence of DTT. The anti pH4 pool antiserum recognises a protein of approximately 39kd in the TX-100 soluble lysate and three proteins of 150kd, 90kd and 39kd in a preparation of parasitised erythrocytes. A number of high molecular weight proteins were recognised in a non-reduced preparation of the TX-100 soluble lysate, including a protein of
Western blot of *P. yoelii* preparations probed with mouse anti pH4 fraction antiserum.
approximately 230kd. Normal mouse serum recognised a protein of 26kd in non-reduced, parasitised erythrocytes, figure 7.2.

As shown in figure 7.3, rabbit antiserum raised against pH4.2-4.4 IEF fractions appeared to be more reactive than the mouse antiserum raised against pooled pH4 fractions. The rabbit antiserum recognised a ladder of polypeptides in both reduced and non-reduced preparations of pooled pH4 IEF fractions. This ladder pattern may be indicative of proteolytic degradation. Two distinct proteins of approximately 80kd and 100kd were recognised in reduced and non-reduced TX-100 soluble parasite material and a 100kd protein was recognised in reduced, parasitised erythrocytes. The rabbit anti pH4.2-4.4 antiserum recognised several proteins in a mouse erythrocyte lysate preparation which were not seen on western blots probed with normal rabbit serum, figure 7.4, suggesting that certain mouse erythrocyte antigens were present in the IEF fractions used to immunise rabbits.

A rabbit antiserum raised against pH4.6-4.8 IEF fractions appeared unreactive with a pool of pH4 IEF fractions reduced with DTT, figure 7.5, but a faint band of approximately 33kd was visible in the lane containing the same protein pool electrophoresed in the absence of DTT. Discrete polypeptides of 109kd, 97kd, 84kd and 59kd were recognised in the preparation of TX-100 soluble parasite material. Proteins of 109kd, 105kd and 84kd were also recognised in the lane containing parasitised erythrocytes. The antiserum recognised proteins of 93kd, 84kd and 59kd in a total P. yoelii lysate and proteins of 105kd and 84kd in mouse erythrocytes. High molecular weight proteins were seen by
Figure 7.2  Western blot of *P. yoelii* preparations probed with normal mouse serum.
Figure 7.3 Western blot of *P. yoelii* preparations probed with rabbit anti pH 4.2-4.4 antiserum.
Figure 7.4 Western blot of *P. yoelii* preparations probed with normal rabbit serum.
Figure 7.5  Western blot of *P. yoelii* preparations probed with rabbit anti pH4.6-4.8 antiserum.
this antiserum in the non-reduced TX-100 soluble preparation, including a protein of 230kd and a doublet of 116/120kd, the antiserum also recognised proteins of 109kd, 97kd and 84kd.

As shown in figure 7.6, mouse antiserum raised against a pool of pH6 IEF fractions (pH6.2-6.8) recognised a 33kd polypeptide in a non-reduced preparation of pH4 pooled antigens. A 33kd protein was also recognised by rabbit anti pH4.6-4.8 antiserum in a preparation of pH4 fractions electrophoresed in the absence of DTT, figure 7.5. The mouse anti pH6 antiserum did not recognise any proteins, reduced or non-reduced, in a pool of pH6 IEF fractions. The anti pH6 antiserum recognised several polypeptides in a non-reduced TX-100 soluble lysate, reacting strongly with a 75kd species, with proteins of 112kd, 116kd and 230kd and material of approximately 33kd.

Mouse polyclonal anti P. yoelii 230kd (PMMSA) antiserum was unreactive with pooled pH4 IEF fractions. It recognised the 230kd protein and its processed products in reduced and non-reduced total P. yoelii lysate, parasitised erythrocytes and TX-100 soluble lysate, figure 7.7.

7.02 Immune precipitation using anti IEF antisera.

Figure 7.8 shows an autoradiograph of $^{35}$S methionine labelled P. yoelii proteins immuneprecipitated with anti IEF antisera. Rabbit anti pH4.2-4.4 and pH4.6-4.8 antisera immuneprecipitated numerous parasite proteins spanning a wide molecular weight range similar to those precipitated by P. yoelii
Figure 7.6 Western blot of P.yoelii preparations probed with mouse anti pH6 antiserum.
Figure 7.7 Western blot of *P. yoelii* preparations probed with mouse anti 230kd PMMSA antiserum.
Figure 7.8 Immune precipitation of $^{35}$S methionine labelled $P$.yoelii proteins with anti IEF antisera.
hyperimmune serum. All mouse anti pH4 antisera immuneprecipitated a protein of 230kd and mouse anti pH4.1-4.3 and mouse anti pH4 pool (pH4.0-4.8) also immuneprecipitated proteins of 170kd and 95kd. Similar proteins were immuneprecipitated by a mouse monoclonal anti *P. yoelii* 230kd (PMMSA) antibody. All mouse anti pH4 antisera precipitated a 28kd protein and anti pH4.1-4.3 and antiserum raised against a pool of pH4 fractions (pH4.0-4.8) precipitated a protein of approximately 15kd. Mouse antiserum to a pool of pH6 IEF fractions (pH6.0-6.8) immuneprecipitated a parasite protein of 230kd plus a 29kd species.

A comparison of 35S methionine labelled *P. yoelii* proteins immuneprecipitated with rabbit and mouse anti IEF antisera with proteins immuneprecipitated by a panel of monoclonal antibodies recognising *P. yoelii* proteins by IIF showed no striking similarities, figures 7.9 and 7.10. Figure 7.9 confirms that rabbit anti pH4.2-4.4 antisera effectively immuneprecipitates a 230kd protein, as do antisera raised against a pH6 pool (pH6.0-6.8) and a pH8 pool (pH8.0-8.8). The rabbit anti pH8 antiserum also strongly immuneprecipitates proteins of approximately 90kd and 30kd that are not recognised by the anti pH4.2-4.4 antiserum or the anti pH6 antiserum and also appears to recognise a large protein of >235kd. A 26kd protein is strongly immuneprecipitated by both anti pH6 and anti pH8 antisera.

A comparison of proteins immuneprecipitated by mouse anti IEF antisera with those recognised by the monoclonal antibodies confirmed that anti pH4 and anti pH6 immuneprecipitated a
Figure 7.9 Comparison of $^{35}$S methionine labelled P. yoelii proteins immuneprecipitated by a panel of monoclonal antibodies and by rabbit anti IEF fraction antisera.
Figure 7.10 Comparison of $^{35}$S methionine labelled *P. yoelii* proteins immunoprecipitated by a panel of monoclonal antibodies and by mouse anti IEF fraction antisera.
**P.yoelii** protein of 230kd plus several lower MW species, figure 7.10.

### 7.03 Anti IEF antiserum immuneprecipitation of **P.yoelii** proteins depleted of the 230kd PMMSA.

Figure 7.11 shows that although anti IEF antisera contain antibodies directed against the **P.yoelii** 230kd PMMSA, the antisera also immuneprecipitate other proteins apparently unrelated to the PMMSA. Depletion of the PMMSA with rabbit polyclonal anti 230 IgG from a labelled **P.yoelii** lysate followed by immuneprecipitation of proteins from the same sample using anti pH4.1-4.3 antiserum (lane 6), suggests that proteins of approximately 95kd, 75kd, 40kd, 28kd and 15kd were not depleted by the anti 230kd antisera. Following PMMSA depletion of an aliquot of labelled **P.yoelii** lysate, the anti pH8 antiserum immuneprecipitates lower MW proteins including those of approximately 95kd, 32kd and 26kd, lanes 7 and 8. These proteins may correspond to those identified by Mr. J. B. De Souza (personal communication) by PAGE of sephacryl S-200 separated, protective IEF fractions. A western blot of S-200 separated proteins probed with anti IEF antisera may indicate which proteins are similar. Lanes 11 and 12 also show the selective depletion of the 230kd PMMSA and immuneprecipitation of numerous lower MW proteins from the previously depleted sample by rabbit anti pH4.6-4.8 antisera.
### Table

<table>
<thead>
<tr>
<th>Sample</th>
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<tbody>
<tr>
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<tr>
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<tr>
<td>12</td>
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<td>13</td>
<td>Recovered 230kd PMMSA</td>
</tr>
<tr>
<td>14</td>
<td>Depleted of 230kd PMMSA</td>
</tr>
</tbody>
</table>

*Depleted of 230kd PMMSA*

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**Figure 7.11** Immunoprecipitation of $^{35}$S methionine labelled *P. yoelii* proteins with anti IEF fraction antisera from samples previously depleted of the 230kd PMMSA.
Western blots probed with anti fraction antiserum provided very little information about the proteins present in protective IEF fractions. The low reactivity of all antisera with IEF fractions of the same pH as those against which they were raised, made it difficult to identify discrete proteins that may be contributing to the protective activity of the focussed fractions. IEF antisera did however, react with a preparation of TX-100 soluble \textit{P. yoelii} proteins indicating that parasite proteins, or fragments of proteins were present in the IEF fractionated material used to immunise animals.

The rabbit anti pH4.2-4.4 antisera reacted with a pool of pH4 fractions (pH4.0-4.8) but produced a ladder effect which could be due to proteolytic degradation of the sample. The rabbit anti pH4.6-4.8 antiserum recognised a protein of 33kd in a non reduced preparation of pH4 pooled fractions and a protein of similar size was recognised in a sample from the same preparation by a mouse anti pH6 antiserum. This result may be explained by the fractionation of fragments of a protein across the pH gradient from pH4 to pH6 which were recovered in IEF fractions and used to immunise animals for antiserum production.

Immuneprecipitation results suggest that protective IEF fractions in the pH4 and pH6 range contain immunogenic \textit{P. yoelii} material that induces an anti 230kd response in mice. However, the presence of intact 230kd protein or its processed fragments could not be demonstrated in pH4 IEF fractions or pH6 IEF fractions by western blotting. Immuneprecipitation of proteins
from an IEF fractionated $^{35}$S methionine labelled *P. yoelii*
preparation with polyclonal anti 230kd PMMSA IgG suggested that small amounts of the 230kd protein were present in the pH4.0 fraction. Material immunoprecipitated from several IEF fractions was also seen at the gel front. It is possible that the 230kd antigen is degraded on the isoelectric focussing column. Resulting peptides may be expected to have varying isoelectric points and could therefore focus across a broad pH range.

Although the 230kd PMMSA has been shown to undergo proteolytic processing during schizogony (Holder and Freeman, 1984a), depletion experiments suggest that other proteins immunoprecipitated by mouse anti pH4.1-4.3, mouse anti pH6 and mouse anti pH8 antisera are not PMMSA derived since they are not depleted by anti 230kd polyclonal IgG. It is possible that these proteins, or fragments resulting from IEF fractionation are contributing to the protective effect of the IEF fractions.

Western blots of protective IEF material separated by sephacryl S-200 (De Souza, personal communication) probed with anti 230kd PMMSA antiserum and antiserum to protective IEF fractions may help to clarify which polypeptides are PMMSA derived. It is possible that S-200 separated proteins not recognised by anti IEF antiserum may represent antigens that are stimulatory to T cells. It may be possible to isolate proteins by PAGE of freshly prepared IEF fractions and electroelution of both proteins recognised by anti IEF antisera and those which are not recognised by antisera. Comparisons of the capacity of individual antigens to stimulate T cells or to induce antibody production
could be carried out. Monospecific antiserum could be used to
immunoaffinity purify single antigens from *P. yoelii* lysates for
use in immunisation experiments. Alternatively, antisera to
protective IEF fractions could be used to screen recombinant DNA
libraries induced to produce *P. yoelii* polypeptides. Preparations
of recombinant proteins could be tested for their capacity to
protect mice against challenge and monospecific antisera raised
against recombinant proteins could be compared to anti IEF
antisera by western blotting and immuneprecipitation to identify
protective antigens.
SUMMARY.

The clinical symptoms of malaria are associated with the erythrocytic cycle of the disease and since the blood stage merozoite is accessible to the host immune system, much effort has been concentrated on the isolation and characterisation of blood stage Plasmodium antigens and the analysis of their role in protective immunity. Once isolated, individual antigens can be tested for their ability to stimulate T cells and their capacity to induce the production of protective antibody.

Several blood stage antigens have been isolated by screening DNA libraries with immune serum, (Kemp et al, 1983. Ozaki et al, 1986. Coppel et al, 1987. Smythe et al, 1988. Galinski and Barnwell, 1989). In the experiments described above an immunoscreen of a P. yoelii genomic DNA library in lambda gt11 with serum from mice hyperimmune to P. yoelii, led to the isolation of a clone, J7, containing 144bp of P. yoelii DNA which appeared to encode all or part of an epitope from a 235kd rhoptry.
protein. Antibodies selected from P. yoelii hyperimmune serum by the polypeptide encoded by clone J7, immunoprecipitated a 235kd protein from a preparation of labelled P. yoelii proteins; the protein appeared identical to that precipitated by protective McAb 25.77 (Freeman et al, 1980a). Holder and Freeman (1981) purified the 235kd antigen from P. yoelii infected erythrocytes and showed it to protect mice from YM challenge infection. Both the monoclonal antibody and the purified protein appeared to alter the course of lethal P. yoelii YM infection causing restriction of parasites to reticulocytes, as seen in non lethal 17X infections.

The 144bp fragment of DNA from lambda gt11 clone J7 was used as a probe to screen two P. yoelii genomic DNA libraries in the plasmid vector pUC9 and several clones were isolated. Four Dral clones containing P. yoelii DNA fragments of approximately 500bp were identified; the DNA sequence of three of the inserts was broadly similar but each contained differences scattered throughout the sequence, see figure 4.13. The fourth clone contained DNA with an identical sequence to one other clone obtained from the same screen. Two larger EcoR1 clones were identified and preliminary sequence analysis has shown them to contain Dral fragments identical to the inserts from two of the previously isolated Dral clones.

A 367bp DNA fragment represented in one of the Dral clones was expressed in E. coli as a fusion protein which was used to immunise mice for antiserum. In IIF studies the anti fusion protein antiserum produced a punctate pattern on acetone fixed
preparations of *P. yoelii* infected erythrocytes, similar to the pattern seen with McAb 25.77 and the antibodies selected from hyperimmune serum by lambda gt11 clone J7. The anti fusion protein antiserum immuneprecipitated a labelled *P. yoelii* blood stage protein of 235kd and antibodies selected from hyperimmune serum by the fusion protein also recognised a protein of 235kd on western blots. The fusion protein was recognised by *P. yoelii* hyperimmune serum on western blots but not by McAb 25.77.

Southern blots of *P. yoelii* genomic DNA probed with the original 144bp insert from lambda gt11 clone J7, one of the 500bp DraI DNA fragments or a 5kb EcoRl DNA fragment suggest that sequences encoding the 235kd protein may represented several times in the *P. yoelii* genome. PCR amplification of *P. yoelii* genomic DNA using oligonucleotides based on cloned DNA sequences supports this finding but it is not known whether the gene contains repeated sequences or if the cloned sequences are present in more than one gene. The 5kb EcoRl DNA fragment hybridised to a species of 8.8kb on northern blots of *P. yoelii* total RNA. The cloned DNA sequences cross hybridise, on Southern blots, with *P. chabaudi* and *P. berghei* DNA. In IIF studies by Holder and Freeman (1984c), McAb 25.77 was shown to cross hybridise with these species.

A second approach to the identification of blood stage *P. yoelii* antigens involved in immunity was to try to further separate the components of IEF fractions previously shown by De Souza and Playfair (1988) to protect mice against lethal challenge and to stimulate T cell help. Following FPLC separation
of a pool of pH4 fractions on the basis of size, material of MW 220,000, 36,000 and <20,000 was recovered but fractions did not protect mice against challenge infection nor did they stimulate T cell help.

PAGE analysis of isoelectrically focussed $^{35}$S methionine labelled P. yoelii proteins suggested either that degradation of material was taking place on the IEF column or that the labelled proteins failed to focus. Immuneprecipitation of labelled IEF fractions with anti serum to the 230kd P. yoelii PMMSA suggested the presence of this protein in the pH4 fraction, a fraction De Souza and Playfair have consistently found to confer 100% protection.

Antisera to pH4, pH6 and pH8 fractions immuneprecipitated a protein of 230kd from preparations of labelled blood stage P. yoelii proteins suggesting that the PMMSA may be broken down on the IEF column with the resulting fragments focussing over a broad pH range. Anti IEF antiserum immuneprecipitation of labelled P. yoelii proteins previously depleted of the 230kd PMMSA suggested that other lower MW proteins (or fragments of lower MW proteins) were present in the IEF fractions used to immunise animals for antiserum. Comparison of the size of these proteins with those recovered from sephacryl S-200 separation of protective IEF fractions showed some similarities (De Souza, personal communication). These proteins may contribute to the protective effect of IEF fractions; in recent experiments by Mr. J. B. De Souza have shown sephacryl S-200 separated IEF material to protect mice against P. yoelii challenge infection.
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